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Foreword

"Extraordinary claims require extraordinary evidence."
— Carl Sagan

Indeed scientific evidence is a main requirement for technological advancement, and in 2012 Foreign Animal Disease Research Unit (FADRU) scientists made significant contributions to the knowledge of foreign animal diseases. This knowledge was translated in some cases into new technologies and approaches for diagnostics and vaccines. During 2012, the FADRU had a very significant year in Technology Transfer activities with multiple material transfers to Department of Homeland Security (DHS), Animal and Plant Health Inspection Service (APHIS) and industry partners developing diagnostics and vaccines. Through three currently active CRADAs (Collaborative Research and Development Agreement) we continue the development of novel vaccines against Foot-and-Mouth Disease (FMD) and Classical Swine Fever (CSF). These are important steps toward the full development and deployment of these vaccines that are a direct product of ARS innovation. During 2012, a novel FMD molecular vaccine based on a defective human adenovirus vector (Ad5-FMD-A24) was awarded conditional licensing for use in cattle in the United States. This is the first molecular FMD vaccine licensed and one of the most significant innovations on FMD vaccinology in the last 50 years. The development of this vaccine was done in an inter-agency effort between ARS and DHS, and was recognized by the Federal Laboratory Consortium Mid-Atlantic Region with their 2012 inter-agency partnership award and the 2012 DHS- Under Secretary's Award for Science and Technology. Additionally, during 2012 ARS-scientists in close collaboration with industry (Zoetis – former Pfizer Animal Health) continue development of a safer platform for inactivated FMD vaccine production. ARS scientists will continue to carry out research to improve these first-generation molecular vaccines and also to provide new scientific evidence toward novel vaccine technologies appropriate for the global control and progressive eradication of FMD.

In this 2012 collection of peer-reviewed manuscripts and patents, FADRU scientists and collaborators both in the US and overseas put forward some of the best scientific evidence that will play a critical role in the development of better countermeasures to combat the devastating effects of FADs. Furthermore, some of their findings have wider implications not only to animal health but also to human health and well-being.

FADRU OVERVIEW

Foreign animal diseases such as foot-and-mouth disease, classical swine fever and African swine fever pose a major burden on the livelihoods of millions of people around the world, mostly in impoverished areas without adequate means to control them. Further, when these diseases incur into disease-free countries their economic and social impacts are immense and their control and eradication often involves the destruction of millions of animals, most of which may not even be infected. The mission of the USDA-ARS Foreign Animal Disease Research Unit (FADRU) at Plum Island is to carry out the research needed to understand the pathogenesis and the host response to foreign animal disease agents, and to translate this knowledge into useful interventions such as new diagnostic tools and vaccines for effective outbreak response, control and eradication.

This mission is accomplished by carrying out basic and applied research directed toward:

- understanding the genomic structure, viral factors determining virulence
- determining the pathogenesis and mechanism of defense and host resistance in livestock
- understanding the evolution and field epidemiology of FAD agents
- developing effective disease control strategies including novel agent detection methods, better vaccines, antiviral drugs and biotherapeutics

The collection of scientific publications presented here represents the product of FADRU scientist's research efforts over the past year (2012). This scientific knowledge is needed to develop better and more effective tools to prevent and control FAD. Other products include patents for inventions and funded research grants obtained by several FADRU researchers. Over the last year the FADRU also had a strong presence in international meetings and expert forums on FAD, vaccines and animal health. Our international presence is also strong with ongoing research collaborations with over a dozen countries including: Argentina, Australia, Cameroon, India, Israel, Mexico, Pakistan, R. Georgia, Russia, Spain, South Africa, Uganda, UK, and Vietnam. This is the result of increased effort to connect our scientific program to the rest of the world, increase our international presence, leverage our resources and increase opportunities to apply our research to provide solutions to real-world situations.

2012 Highlights

- **An Alternate Delivery System Improves Vaccine Performance against Foot-and-Mouth Disease Virus (FMDV):** In the event of an outbreak of foot-and-mouth disease in the United States, it is anticipated that vaccination of susceptible livestock will be an important part of the control strategy. Deployment of vaccine will be challenging, as the virus spreads very rapidly when there are naïve populations, like the livestock herds in the US. Vaccination of thousands of animals will present large logistical challenges and availability of enough doses of vaccine could be a limiting factor in the effectiveness of the outbreak control measures. In this manuscript we address both of these issues. We have tested an automated vaccine delivery device, the DermVac, for efficacy in controlled vaccine trials with the presently available vaccine. The intradermal delivery of vaccine leads to effective protection at early times (7 days following vaccination). The device can rapidly deliver vaccine to animals being processed through standard means. Most importantly, the potency of the vaccine is enhanced when delivered with the DermaVac. These data provide those tasked with responding to outbreaks of FMDV in the US with necessary information to make informed decisions about responding to FMD.

- **The Nuclear Protein Sam68 is Cleaved by the FMDV 3C Protease Redistributing Sam68 to the Cytoplasm during FMDV Infection of Host Cells**: In this study we demonstrate that the cellular host factor Sam68 is critical to the life cycle of Foot-and-Mouth Disease Virus (FMDV), and is re-localized from the cell nucleus to the cytoplasm during the course of FMDV infection. As infection progresses, increasing amounts of a truncated form of Sam68 is detected lacking the C-terminal nuclear localization signal (NLS), thus providing a mechanism for its nuclear egress distinct from what we previously reported for the cytoplasmic shift of RNA Helicase A (RHA). Interestingly, we discovered that Sam68 interacts with the FMDV 3C proteinase, which is potentially responsible for the C-terminal cleavage of Sam68. Additionally, we show that Sam68 binds the internal ribosomal entry site (IRES) within the 5' non-translated region (NTR) of the FMDV genome, which suggests that Sam68 plays a role in the translation of virus gene products. To the best of our knowledge, this is the first reported instance of virus-induced proteolysis of Sam68 in any virus system.

- **Bovine Type III Interferon Significantly Delays and Reduces the Severity of Foot-and-Mouth Disease in Cattle**: Interferons (IFNs) are the first line of defense against viral infections. In previous work we demonstrated that type I IFN is effective to control foot-and-mouth disease virus (FMDV) infection in swine, however a similar approach only had limited success in cattle. More recently we have identified a member of the bovine type III IFN family (boIFN -lambda3) and demonstrated that cattle inoculated with a replication defective human adenovirus type 5 (Ad5) expressing boIFN -lambda3 have systemic antiviral activity and a localized induction of IFN stimulated genes in epithelial tissues that are known targets for FMDV growth. In the current study we evaluated if treatment of cattle with Ad5-boIFNlambda3 can prevent or limit FMD. We demonstrated that cattle treated with Ad5-boIFNlambda3 can be protected from disease for 6 days when challenged 24 hours post treatment by direct inoculation in the tongue

with FMDV A24 Cruzeiro. Such protection was significantly improved –no disease until day 9- when treated cattle were challenged by an aerosol route with FMDV O1 Manisa. These results suggest that, in
cattle, boIFNlambda3 plays a critical role in the innate immune response against infection with FMDV.

- **Foot-and-Mouth Disease: Global Status and Indian Perspective:** Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals (cattle, swine, buffalo) that affects a large portion of animals in endemic regions in Asia, Africa and South America. The disease is seldom lethal but causes major losses in milk and meat production and is perhaps the main disease limiting trade of animals and animal products. The disease is a major threat to cattle, buffalo (both milk and meat) and pig production in endemic countries and therefore considered to cause food insecurity, both locally and globally. Control is complicated by the fact that the FMD virus is highly transmissible and exists in 6 different serotypes and multiple subtypes that require specific vaccines to induce protection. The emergence of new FMD viruses that are not covered by current vaccines occurs regularly and require a constant surveillance and testing for vaccine coverage. One of the serotypes, A viruses have been continuously emerging in nature requiring frequent replacement of the vaccine strains. The present review summarizes and updates the situation of FMD viruses and vaccines with particular emphasis on current control programs in India.

- **Inoculation of Swine with Foot-and-Mouth Disease SAP-Mutant Virus induces Early Protection against Disease**: Foot-and-Mouth Disease Virus (FMDV) is one of the most contagious agents of cloven-hoofed species. Current vaccines are efficacious but require at least 7 days to induce protection against disease. Therefore, there is a need to develop countermeasure strategies that effectively work prior to this elapsed time. We have previously reported that mutation of FMDV leader protein SAP motif [SAP-mutant] results in attenuated growth in laboratory cell culture. Here, we studied the ability of the SAP-mutant to cause disease in swine. In a first experiment, we evaluated the virulence of this virus by inoculating different doses of SAP-mutant or wild type (WT) virus and looking at the response for 21 days. Animals inoculated with the SAP-mutant did not develop clinical signs, did not have virus infection in the blood stream or shedding from nasal and oral cavities even when the inoculation dose was 100-fold higher than that needed to cause disease by the wild-type virus. Since the induction of neutralizing antibodies was equivalent for both viruses, we evaluated the SAP mutant as a candidate vaccine against challenge with WT virus. Animals vaccinated with the SAP mutant were completely protected after inoculated with WT FMDV at 21 days post vaccination. We also performed a second experiment, challenging with WT FMDV at different times post vaccination (2, 4, 7 and 14 days). Remarkably, animals inoculated with the SAP-mutant virus were protected against challenge with WT-FMDV as early as 2 days post-vaccination before antibodies were detectable. In addition, animals inoculated with SAP-mutant developed a memory cellular immune response resembling infection with WT-FMDV. Our results suggest that SAP-mutant, or similar viruses containing mutations in the leader protein, may serve as the basis for effective live attenuated FMD vaccines.

- **Early Detection and Visualization of Human Adenovirus Serotype 5-Viral Vectors carrying Foot-and-Mouth Disease Virus or Luciferase Transgenes in Cell Lines and Bovine Tissues**: The purpose of this study was to characterize critical events that occur subsequent to the administration of a recombinant vaccine constructs in cattle. Several novel techniques were used to analyze the effects of the vaccine including: real-time PCR, luminometry, and microscopy. The analysis allow us to pinpoint the sequence of events after the administration. The results will serve as a valuable guide for the vaccine development.

- **Role of the Arg56 of the Structural Protein VP3 of Foot-and-Mouth Disease Virus (FMDV) 01 Campos in Virus Virulence.** Foot-and-mouth disease virus (FMDV) is an extremely infectious virus that causes economically important disease in cattle and swine. Several reports indicated that FMDV O1 subtype strains undergo antigenic variation under diverse growth conditions. Of particular interest is the amino acid variation observed at position 56 within the structural protein VP3 (one of the four proteins that form the virus particle). Based upon the amino acid residue at that position (arginine or histidine) is present at this position the virus will have different growth characteristics in cell cutures and, more important different virulence during the infection in susceptible animals. We have constructed two different FMDV type O1 Campos variants differing only at VP3 position 56, possessing either an histidine (H) or arginine (R) (O1Ca-VP3-56H and O1 Ca-VP3-56R, respectively), and characterized their in vitro phenotype and virulence in the natural host. We demonstrated here that virus O1Ca-VP3-56H and O1Ca-VP3-56R had similar growth kinetics in different cell cultures but, in contrast, the viruses displayed significantly different pathogenicities in cattle and swine. The O1Ca-VP3-56H viruses induced typical clinical signs of FMD, whereas O1Ca-VP3-56R viruses were completely nonpathogenic unless they reverted in vivo from the R residue to either. These results indicate that the presence of R at position 56 of the FMDV VP3 protein plays a role in virus attenuation in the natural host.

- **Classical Swine Fever Virus p7 Protein is a Vioporin Involved in Virulence in Swine**: Classical swine fever virus (CSFV) causes severe and often lethal disease in swine. The disease has been eradicated in the US but it is still present in many countries around the world including areas of Central America and the Caribbean representing an important risk to the US swine industry. As part of our continuing effort to develop better vaccines through a better understanding of the function of CSFV viral proteins, we studied the viral proteins p7. The role of p7 in virus replication and virulence is not well understood. Here we demonstrated that p7 is essential for virus replication in cell cultures and that small areas (5-7 amino acid residue stretches) of p7 that were critical for virus growth in cell cultures. Importantly, we also created recombinant CSFV having mutated small areas of p7 and showed that some of these mutations produced attenuation of virus virulence. This is the first report indicating that p7 is involved in the disease process in swine. Interestingly, some of these mutated viruses, although being significantly attenuated, were able to immunize animals and protect them against the challenge with a virulent strain of viruses, showing some promise as potential live attenuated vaccine candidates. Finally, we were able to clearly demonstrate that p7 belongs to a group of proteins called viroporines. Viroporines are viral proteins that have the role of producing

pores in the internal membranes of the cell where the virus replicates. It is known that the formation of those pores is a critical step in the process of virus replication.

- **Disinfection of Foot-and-Mouth Disease and African Swine Fever Viruses with Citric Acid and Sodium Hypochlorite on Birch Wood Carriers**: Transboundary disease viruses such as foot-and-mouth disease virus (FMDV) and African swine fever virus (ASFV) are highly contagious and cause severe morbidity and mortality in livestock. Accidental or intentional introduction of these viruses into non-endemic countries can result in the culling of a large proportion of resident livestock, causing enormous economic losses. Proper disinfection during an outbreak can help prevent virus spread and will shorten the time for contaminated agriculture facilities to return to food production. Porous surfaces present a challenge because disinfectants must penetrate into the surface to effectively inactivate the viruses. To model porous surface disinfection in the laboratory, we modified our nonporous surface disinfection assay to work with small squares of birch veneer. We found that 2 percent citric acid was effective at a minimum of 99.99 percent disinfection of both viruses dried on the wood surface after a thirty-minute contact time. While 2000 ppm sodium hypochlorite was capable of inactivating ASFV on wood, this chemical did not meet the 99.99 percent threshold for FMDV disinfection. Thus, we recommend 2 percent citric acid for porous surface disinfection of FMDV and ASFV.

- **Designing Bovine T-Cell Vaccines via Reverse Immunology**: T-cell responses contribute to immunity against many intra-cellular infections. There is, for example, strong evidence that major histocompatibility complex (MHC) class I restricted cytotoxic T lymphocytes (CTLs) play an essential role in mediating immunity to East Coast fever (ECF), a fatal lymphoproliferative disease of cattle prevalent in sub-Saharan Africa and caused by Theileria parva. To complement the more traditional approaches to CTL antigen identification and vaccine development that we have previously undertaken we propose a use of immunoinformatics to predict CTL peptide epitopes followed by experimental verification of T cell specificity to candidate epitopes using peptide-MHC (pMHC) tetramers. This system, adapted from human and rodent studies, is in the process of being developed for cattle. Briefly, we have used an artificial neural network called NetMHCpan, which has been trained mainly on existing human, mouse and non-human primate MHC-peptide binding data in an attempt to predict the peptide binding specificity of bovine MHC class I molecules. Our data indicate that this algorithm needs to be further optimized by incorporation of bovine MHC-peptide binding data. When re-trained, NetMHCpan may be used to predict parasite peptide epitopes by scanning the predicted T.parva proteome and known parasite CTL antigens. A range of pMHC tetramers, made “on demand”, will then be used to assay cattle that are immune to ECF or in vaccine trials to determine if CTLs of the predicted epitope specificity are present or not. Thus, pMHC tetramers can be used in one step to identify candidate CTL antigens and to map CTL epitopes. Our current research focuses on 9 different BoLA class I molecules. By expanding this repertoire to include the most common bovine MHCs these methods could be used as generic assays to predict and measure bovine T cell immune responses to any pathogen.

- **Homology Modeling and Analysis of Structure Predictions of the Bovine Rhinitis B Virus RNA-dependent RNA Polymerase (RdRp):** In this study we have built a homology model for the 3D polymerase, the enzyme responsible for the replication of Bovine rhinitis B virus (BRBV). This is the closest relative to foot-and-mouth disease virus (FMDV), which causes devastating disease in livestock. The aim of this study was to examine relevant functional domains to broaden the understanding of the function of the 3D polymerase. This information will be useful in identifying targets for antiviral drugs and in predicting regions of the 3D polymerase that could be changed in order to attenuate BRBV. This information will ultimately be applied to the development antivirals and also attenuated FMDV strains that could be used as candidate vaccines and against this devastating disease.

- **Full Length Genome Analysis of Vesicular Stomatitis New Jersey Virus Strains Representing the Phylogenetic and Geographic Diversity of the Virus**: Vesicular Stomatitis (VS) is a disease of cattle, horses and pigs that causes economic losses throughout North, Central and South America. The disease in cattle and pigs is markedly similar to foot-and-mouth disease, a devastating reportable disease, making a rapid differential diagnosis crucial. VS is caused by viruses classified into two different types: New Jersey (VSNJV) and Indiana (VSIV). VSNJV accounts for the majority of clinical cases of vesicular disease in livestock throughout the Americas. In the United States outbreaks of VS occur every 8-10 years, causing significant economic losses to the cattle and horse industries. Despite the fact that VSNJV accounts for the vast majority of VS cases in the US, very little is known about the genetic sequence of this economically important pathogen. Here we report for the first time the complete genetic sequences of nine natural isolates of VSNJV, representing the main virus groups and comprising the known geographic distribution of the virus. These complete sequences, the first for VSNJV natural isolates, should prove useful in the design of better control measures and molecular detection tests.

- **Foot-and-Mouth Disease Virus Serotype O Phylodynamics: Genetic Variability Associated with Epidemiological Factors in Pakistan:** Foot-and-mouth disease (FMD) is a highly contagious viral disease of livestock, producing losses that are insidious in endemic settings and devastating in disease-free countries. The causing virus (FMDV) is highly variable with 7 different serotypes (A, O, C, Asia, Sat 1, Sat 2, and Sat 3). Vaccines do not confer cross protection between serotypes and in many cases not even among different strains within a given serotype. In Pakistan, FMDV causes numerous outbreaks and associated economic losses to the livestock industry. Little information is available regarding the epidemiology, viral strains circulating in Pakistan and their coverage by currently available vaccines. Additionally, the role of Asian buffalo in the ecology of FMDV has not been fully characterized even though they are known to remain asymptomatically infected for prolonged periods. Little is known about FMDV infections in the absence of clinical signs (subclinical infections and carrier state), which are believed to be key to the maintenance of FMDV in mixed animal populations. The importance of Asian buffalo as reservoirs of FMDV in persistent infection therefore remains as a critical knowledge gap in understanding FMD ecology in Asia. This knowledge is necessary to guide the implementation of effective and sensible control

programs. Currently, only 15% of animals are vaccinated each year and at this time no government organized vaccination plan for the country has been implemented. A pilot control program sponsored by the United States Agency for International Development (USAID) is being implemented by the Pakistani government, with support from the Food and Agriculture Organization of the United Nations (FAO). The information developed with this proposed research project will provide critical information regarding the effectiveness of FMD vaccines and the most suitable vaccination strategies leading to successful control of the disease.

- **Direct Contact Transmission of Three Different Foot-and-Mouth Disease Virus Strains in Swine Demonstrates Important Strain-Specific Differences**: Foot-and-mouth disease (FMD) is a highly contagious viral disease affecting domestic and wild cloven-hoofed animals. Pigs are excellent viral amplifiers of foot-and-mouth disease virus (FMDV) and play an important role in the airborne transmission and spread of FMDV to other species and among different premises. Thus, protection of pigs either by vaccination or antiviral therapy could be essential to control FMD outbreaks. Currently the international standard challenge method for pigs consists of direct virus inoculation in the foot. This method results in clinical disease in most cases, but it bypasses many of the important defense mechanisms during the viral infection and could also overwhelm vaccine-induced immune responses. A direct contact transmission method would be a more natural way of FMD transmission to pigs. However, vaccine trials using direct contact often fail due to either overwhelming or not enough exposure to challenge virus. In this study, we have established a consistent contact exposure methodology in pigs for two different strains of FMDV. Each strain required a different exposure time, but an 18 hour exposure time was sufficient for both strains. This controlled virus challenge methodology will help in the development of better vaccines, antiviral therapies, and other control measures in pigs.

- **Genus Vesiculoviruses:** The vesiculovirus genus of the family Rhabdoviridae contains a numbers of viruses that have been taxonomically classified using a combination of serological relatedness, host range, genome organization, pathobiology and phylogenetic analysis of sequence data. There are 11 viruses assigned to the genus vesiculovirus and a number of serologically related viruses that have been tentatively assigned to the genus. The best known members of the genus are vesicular stomatitis viruses which cause vesicular disease in livestock. This book chapter reviews the state-of-the-art knowledge about vesiculoviruses as well as some related viruses tentatively assigned to the genus.

- **Foot-and-Mouth Disease Virus (FMDV) with a Stable FLAG Epitope in the VP1 G-H Loop as a New Tool for Studying FMDV Pathogenesis:** In this study, we designed, produced, and characterized a Foot-and-Mouth Disease Virus (FMDV) containing a marker protein tag (FLAG tag) embedded in an immunogenic region of the virus structure. The FLAG-tagged virus replicated to similar titers as the parental progenitor virus (A24 Cruzeiro) in cell culture and the mutant FMDV was detected with antibodies specific to the FLAG-tag. Moreover, anti FLAG antibodies could effectively neutralize the mutant virus. When inoculated in cattle, they developed disease similar to the original parental virus, but the animals failed to generate antibodies that react with FLAG peptide.

Virus particles could be screened with anti-FLAG antibodies making this variant virus a useful tool in the study of the mechanisms of disease for FMDV.

- **Use of Replication-Defective Adenoviruses to Develop Vaccines and Biotherapeutics against Foot-and-Mouth Disease**: Foot-and-mouth disease virus (FMDV) is an antigenically variable virus that causes an economically devastating disease of cloven-hoofed animals. Vaccines produced by chemical inactivation of virus are available, but there are concerns about their safety and about the ability to serologically distinguish vaccinated animals from infected animals.
  We initiated a project which uses a replication-defective human adenovirus to deliver an FMD subunit vaccine (Ad5-FMD). Production of this vaccine does not require expensive high-containment manufacturing facilities, can be made in the U.S., which currently prohibits work with infectious FMDV on the mainland, and allows distinction of infected from vaccinated animals. This Ad5-FMD vaccine can protect both swine and bovines after one inoculation. In collaboration with the Department of Homeland Security and the biotechnology company GenVec, Inc. this vaccine format is currently under regulatory licensure approval for inclusion in the U.S. Veterinary Vaccine Stockpile. More recently we have developed second generation Ad5-FMD vaccines that when delivered subcutaneously have enhanced efficacy as compared to our first generation vaccine inoculated intramuscularly. In addition, since FMDV replicates and spreads very rapidly we have developed a biotherapeutic approach to induce protection prior to the vaccine induced adaptive immunity. We constructed Ad5 vectors containing the genes for porcine and bovine interferons (IFN). Inoculation of swine with Ad5 vectors containing IFN induces protection in 1 day. We obtained similar results in cattle. We propose that a combination of Ad5-IFN and Ad5-FMD can induce both rapid and long-lasting protection.

- **A Safe Foot-and-Mouth Disease Platform with Two Negative Markers for Differentiating Infected from Vaccinated Animals (DIVA):** Vaccination of domestic animals with chemically inactivated Foot-and-Mouth Disease Virus (FMDV) is widely practiced to control FMD. Current vaccine production technology requires; growing large volumes of virulent virus that is then chemically inactivated to remove infectivity and purification to remove undesirable proteins. This process poses the risk of release of infectious virus from production plants, as illustrated by the accidental FMDV outbreak in the United Kingdom in 2007. In this study, we describe the development of a safe production platform for inactivated vaccines that consists of a genetically engineered FMDV lacking a protein (L) that renders the virus not virulent and not transmissible in cattle and swine. This attenuated virus platform has built in markers that allow easy differentiation of infected from vaccinated animals (DIVA) using a companion ELISA test, a critical feature during eradication campaigns. Inactivated vaccine prepared with this platform provides full protection to vaccinated animals from challenge with homologous virulent FMDV. The platform also includes a feature that allows for rapid development specific vaccines against newly emerging field strains addressing the great genetic diversity of FMDV. This new platform represents one of the most important innovations in FMD vaccine production in decades and will allow safe production of vaccines to help in the global control and eventual eradication of this devastating disease.

- **Novel Antiviral Therapeutics to Control Foot-and-Mouth Disease:** Foot-and-mouth disease virus (FMDV) is an antigenically variable virus consisting of 7 serotypes and multiple subtypes within each serotype, and it causes an economically devastating disease of cloven-hoofed animals. Vaccines produced by chemical inactivation of virus are available, but there are concerns about their safety and they do not induce protection prior to about 7 days post vaccination. In the event of an FMD outbreak in a disease-free country such as the U.S., it is necessary to induce immediate protection in order to limit or inhibit disease spread prior to induction of vaccine induced immunity. We have previously shown that in cell culture all 7 serotypes of FMDV are inhibited by type I interferon. Furthermore, we have constructed an adenovirus vector containing the gene for type I interferon (Ad5-pIFN alpha) and demonstrated that swine inoculated with this vector are protected from viral infection when challenged, by intradermal inoculation, 1 day later with FMDV serotypes A24, O1 Manisa, or Asia-1. We also demonstrated that Ad5-pIFN alpha protects against infection in swine that are challenged by direct contact to infected animals, which is a natural route of FMDV transmission. In this study we examined the potential of other inducers of the innate response to rapidly protect swine. We found that poly ICLC, a synthetic double-stranded RNA, given alone or in combination with Ad5-pIFN alpha can protect swine challenged one day after administration against infection with FMDV. Furthermore, the combination treatment induces a broader array of innate responses. These results suggest that a combination of poly ICLC and IFN plus FMD vaccine may represent a more economical approach to provide rapid and long-lasting protection to animals against FMD in an emergency outbreak situation.

- **Foot-and-Mouth Disease Virus Non-Structural Protein 2C Interacts with Beclin1 Modulating Virus Replication:** Foot-and-mouth disease virus (FMDV) is the causative agent of foot-and-mouth disease. Replication of the virus is a highly multifarious process that occurs in association with host cell membrane in specific areas called replication complex. The largest viral protein found in these in the replication complex, 2C, is thought to have multiple roles during virus replication. However, studies examining the function of FMDV 2C have been rather limited. To better understand the role of 2C in the process of virus replication we used a yeast two-hybrid approach to identify host proteins that interact with 2C. We report here that cellular Beclin1 is a specific host binding partner for 2C. Beclin1 is an important regulator of a cellular metabolic pathway named autophagy, which is required for efficient FMDV replication. We present evidence that confirmed that 2C-Beclin1 interaction initially identified by the yeast two-hybrid approach was further confirmed by co-immunoprecipitation and confocal microscopy to actually occur in FMDV infected cells. Interestingly, the induction of over-expression of Beclin1 strongly reduces virus yield in cell culture. Further work demonstrated that this excess of Beclin1estimulates the enzymatic destruction of virus inside the infected cells, a process that is normally absent during the infection. It is proposed as a hypothesis that during the infection 2C binds to Beclin1 to prevent the enzymatic destruction of the virus. These results suggest that interaction between FMDV 2C and host protein Beclin1 could be essential for virus replication and perhaps a potential target to develop anti-viral strategies.

- **Identification of Peptides from Foot-and-Mouth Disease Virus Structural Proteins Bound by Class I Swine Leucocyte Antigen (SLA) Alleles, SLA-1*0401 and SLA-2*0401**: The adaptive immune response of mammals is mediated by lymphocytes that can bind foreign antigens. Both the B lymphocytes, that have antibodies as receptors and T lymphocytes expressing a T cell receptor, exquisitely differentiate a normal cell from a cell infected with virus. Where antibodies on B cells can directly bind foreign antigens, T cells see small peptides bound by major histocompatibility complex (MHC) proteins. These highly polymorphic, cell surface proteins are the "transplantation" antigens that have to be matched between donors and recipients to ensure successful organ transplants. Class I MHC proteins scan the intracellular environment and class II MHC proteins scan the extracellular space for antigenic peptides. When a peptide is derived from normal proteins of the cell and bound by a MHC protein, the cell looks like self to the immune system. However, when the protein is derived from a virus or other foreign entity, the cell looks foreign and triggers a cellular immune response. The responding, antigen specific T cell activates adaptive immune responses by proliferating and signaling B lymphocytes to make antibody and cytotoxic T lymphocytes to kill virus infected cells.
  Previously, we described the first analysis of a swine MHC protein (SLA-1) for binding of peptide antigens, thus defining what peptides are capable of stimulating T cells. Here we now analyze a second swine MHC protein, SLA-2, and apply the peptide antigen binding data to an analysis of the structural proteins of foot-and-mouth disease virus (FMDV). These data provide the first biochemical evidence of what T cell antigens of FMDV look like in the context of a genetically defined swine population.

- **Time-dependent Biodistribution and Transgene Expression of a Recombinant Human Adenovirus Serotype 5-Luciferase Vector as a Surrogate Agent for rAd5-FMDV Vaccines in Cattle**: The purpose of this study was to characterize biodistribution and gene expression of a recombinant adenovirus vaccine vector in tissues of vaccinated cattle. In preliminary work, adenovirus infection and gene expression were characterized during time-course experiments in cell culture. This preliminary data was used to conduct subsequent experiments in which steers were injected with the same adenovirus construct and euthanized at various time-points for the purpose of tissue collection and analysis. Several novel techniques were used to analyze the harvested tissue samples including: real-time PCR, luminometry, and microscopy. The substantial findings were that 1) subsequent to inoculation with the recombinant vector, a peak of virus protein and gene expression occurred at 24 hours post injection (hpi) at the injection site, 2) cells containing adenovirus and/or transgenic proteins were mostly consistent with macrophages and dendritic cells, and 3) adenovirus DNA, RNA, and proteins were detected (by PCR and microscopy respectively) at local lymph nodes at 24hpi. This information is critical to determine the ideal route of inoculation for Ad5-based vaccines.

- **Early Adaptive Immune Responses in the Respiratory Tract of Foot and Mouth Disease-Infected Cattle:** Foot and mouth disease (FMD) is a highly contagious viral disease affecting a wide range of cloven hooved domestic and wild animals. The FMD virus (FMDV) acts very rapidly and causes disease 48-72 hours after infection. It is believed that the early local immune response by the infected animal defines the outcome

of the infection. However, little is known about this phase of the disease. Here we analyzed the role of the early antibody responses induced infected cattle for their ability to limit or eliminate the infection. Animals infected using an aerosol exposure method developed previously in out laboratory had detectable FMDV-specific antibody producing immune cells in the respiratory tract and draining lymphatic organs as early as 5 days post infection. FMDV-specific antibodies were detected at the same time. The increase in antibodies coincided with a sharp decrease in virus in the blood, emphasizing the important role that antibody responses plays in clearing viral infection. The information generated here will be utilized to improve the immune response induced by FMD vaccines.

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Role of arginine-56 within the structural protein VP3 of foot-and-mouth disease virus (FMDV) O1 Campos in virus virulence

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ABSTRACT

FMDV O1 subtype undergoes antigenic variation under diverse growth conditions. Of particular interest is the amino acid variation observed at position 56 within the structural protein VP3. Selective pressures influence whether histidine (H) or arginine (R) is present at this position, ultimately influencing *in vitro* plaque morphology and *in vivo* pathogenesis in cattle. Using reverse genetics techniques, we have constructed FMDV type O1 Campos variants differing only at VP3 position 56, possessing either an H or R (O1Ca-VP3-56H and O1Ca-VP3-56R, respectively), and characterized their *in vitro* phenotype and virulence in the natural host. Both viruses showed similar growth kinetics *in vitro*. Conversely, they had distinct temperature-sensitivity (ts) and displayed significantly different pathogenic profiles in cattle and swine. O1Ca-VP3-56H was thermo stable and induced typical clinical signs of FMD, whereas O1Ca-VP3-56R presented a ts phenotype and was nonpathogenic unless VP3 position 56 reverted *in vivo* to either H or cysteine (C).

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Introduction

Foot-and-mouth disease (FMD) is an infectious viral disease that affects cloven-hoofed animals, including cattle, sheep, pigs, goats, camelids and deer. Its wide host range and rapid spread make FMD an international animal health concern, since all countries are vulnerable to accidental or intentional trans-boundary introduction (Grubman and Baxt, 2004; Rowlands, 2003). The disease is caused by foot-and-mouth disease virus (FMDV), an *Aphthovirus* within the viral family *Picornaviridae* that exists as seven immunologically distinct serotypes: O, A, C, Asia 1, and South African Territories (SAT) type 1, SAT2, and SAT3. The viral genome consists of a single-stranded, positive sense RNA of about 8200 nucleotides (nt). The open reading frame (ORF) encodes a long polyprotein that is processed by virus-encoded proteases into four structural proteins (VP1 through 4) and eight non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C and 3D) (Belsham, 1993; Ryan et al., 1989). Due to high error rates inherent in RNA replication, viral RNA genomes display a high level of nucleotide variation (Drake and Holland, 1999; Figlerowicz et al., 2003) that can affect replication speed (Jarousse et al., 1994; Nunez et al., 2001; Pelletier et al., 1998; Zhang et al., 1995), virulence (Conenello et al., 2007; Zurbriggen et al., 1989), antigenicity or biochemical properties (Li et al., 2010; Martin-Acebes et al., 2010; Mateu et al., 1990; Szepanski et al., 1992). The availability of infectious cDNA picornavirus clones provides an opportunity to manipulate specific genomic mutations and examine their effects on viral infection.

The genetic and antigenic variation has been extensively studied for FMDV both in field virus samples and laboratory cell-passed isolates (Carrillo et al., 2007; Cottam et al., 2009; Haydon et al., 2001; Meyer et al., 1994; Piatti et al., 1995; Sevilla et al., 1996). For type O viruses selective pressures influence whether a histidine (H) or arginine (R) residue is present at position 56 of VP3. It has been reported that an R amino acid (aa) in position 56 of VP3 (R56) is acquired after serial passage in BHK-21 cells (Kitson et al., 1990; Marquardt et al., 1991) and that this mutation correlates with a small plaque phenotype, a marker indicative of virus attenuation in mice (Jensen and Moore, 1993). Indeed, Sa-Carvalho et al. (Sa-Carvalho et al., 1997) constructed a type A chimera virus expressing the O1 Campos structural proteins carrying, among others aa substitutions, R56 in VP3 and demonstrated that this virus exhibited a small plaque phenotype in cells and was highly attenuated in bovines. These authors suggested that adaptation of type O1 viruses to cell culture results in the ability of the virus to utilize heparin sulfate (Jackson et al., 1996) as a receptor with a concomitant loss in virulence. In view of this data, Rodriguez-Pulido et al. (Rodriguez Pulido et al., 2009) introduced an R56H substitution in VP3 of an O1 Kaufbueren infectious cDNA clone (pO1K) to induce reversion of an attenuated phenotype. However, RNA transcripts derived from pO1K containing H56 did not exhibit

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differences in virulence in suckling mice compared to RNA transcripts carrying R56, indicating that the substitution itself was not sufficient to revert to the attenuated phenotype (Baranowski et al., 2003). It should be noted that no complete genomic sequence was determined for the viruses constructed in these previous works, so it is possible that additional residue substitutions may have affected these results.

Among the FMDV subtype O1 genomic sequences reported to GenBank, only the amino acids R or H are found in position 56 of VP3; however, the passage history of these viruses is unclear. Furthermore, there are no pathogenicity studies using viruses containing these sequences. The O/JPN/2000 strain isolated in the 2000 Japan outbreak exhibited a mild pathogenicity in bovines, and two different viral plaque phenotypes of this strain appeared after two passages in primary bovine cells. Genomic sequencing of the structural proteins revealed two aa substitutions (VP2 D133N and VP3 H56R) in the mildly pathogenic small plaque virus when compared to the large plaque virus in suckling mice (Kanno et al., 2002; Morioka et al., 2008). Nevertheless, these two mutations did not account for the characteristics of another PanAsia strain isolated from China (Bai et al., 2010). This study demonstrated that the BHK-21 cell-adapted O/Fujian/CHA/5/99 strain had high virulence in suckling mice and formed larger plaques than the bovine-isolated O/Tibet/CHA/1/99, while sequence analysis revealed that both strains carried a D residue in position 133 of VP2 and an H in position 56 of VP3.

To clarify the role of these amino acids in FMDV virulence, we mutated the aa at position 56 of VP3 in a full-length infectious cDNA clone derived from the FMDV O1 Campos/Bra/58 strain and evaluated the biological properties and virulence of these viruses in swine and cattle. Our results demonstrate that identical viruses differing only in aa residue 56 of VP3, H56 (O1Ca-VP3-56H) or R56 (O1Ca-VP3-56R), had similar growth kinetics in BHK-21 and LF-BK cells and slight differences in plaque size in BHK-21, LF-BK, IBRS-2 and LK cells. In contrast, the viruses displayed very different pathogenicities in two natural host species. Virus O1Ca-VP3-56H induced typical clinical signs of FMD in swine and cattle, whereas virus O1Ca-VP3-56R was innocuous unless it reverted to either H or cysteine (C) at VP3-56 during the animal infection. These results clearly demonstrate that the presence of R56 in VP3 is associated with virus attenuation during infections in natural host animals.

Results

Generation and characterization of pO1Ca

The full-length cDNA clone pO1Ca was constructed by overlapping PCR fragments flanked by unique restriction enzymes as described in Materials and methods and shown schematically in Fig. 1A. The full-length cDNA was preceded by the T7 polymerase promoter and two additional G residues to improve transcription efficiency (Rieder et al., 1993) and followed by a polyA tail of 15 residues and a unique EcoRV site. Plasmid containing the substitution H56R in VP3 derived from the infectious clone pO1Ca was constructed as described in Materials and methods.

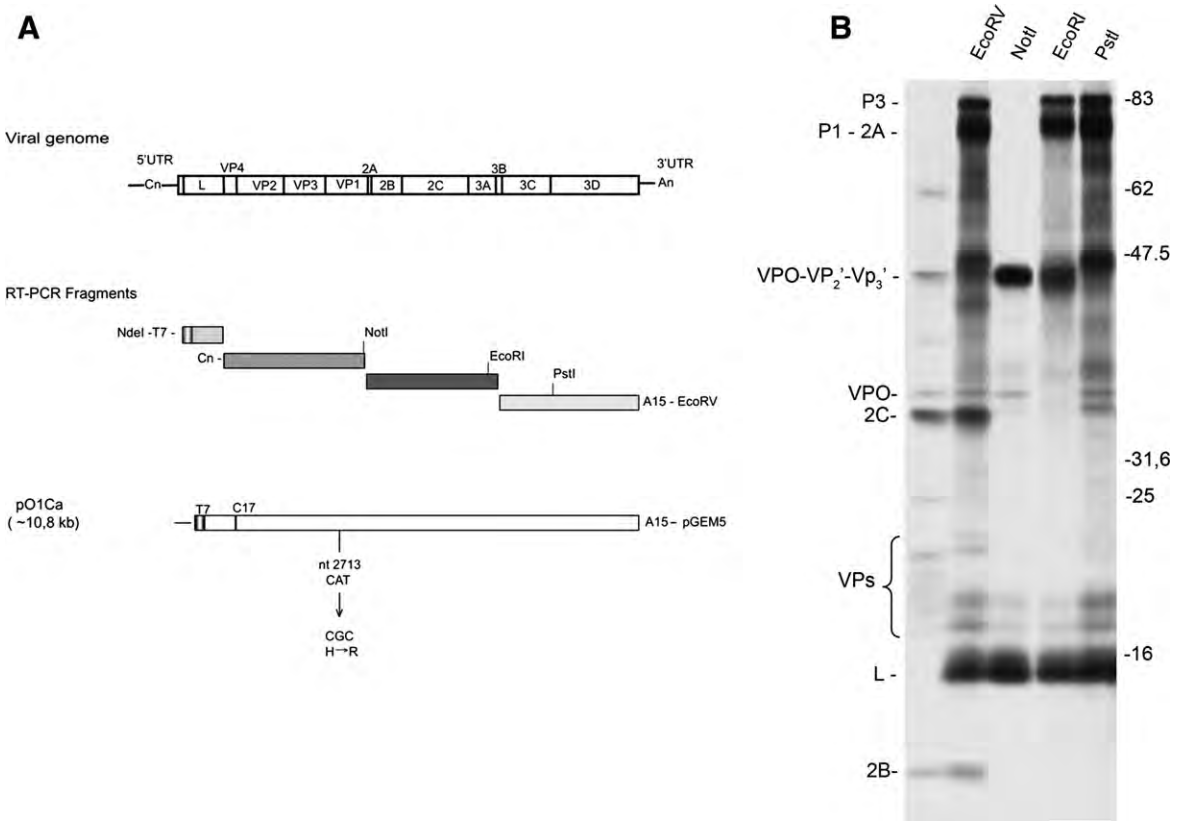

Fig. 1. Construction of the full-length cDNA clone of FMDV O1Campos/Bra/58 strain and *in vitro* translation of derivative RNA transcripts. (A) At the top of the figure is a schematic diagram of the FMDV genome (8168 nucleotides, (Pereda et al., 2002)) showing the polyC tail (Cn) and the polyA (An) tail at the end of the 3′ untranslated region (UTR). The boxes below the genome represent the RT-PCR fragments used for the generation of the full-length cDNA clone (named pO1Ca) including the restriction sites used in the cloning strategy. A T7 RNA polymerase promoter was added at the 5′ end of the viral cDNA as well as a unique EcoRV site at the 3′ end to allow the synthesis of RNA transcripts. Restriction site EcoRI is also indicated. The mutations at position 2713 CAT → CGC resulting in the substitution of aa H by R at position 56 of VP3 are indicated. (B) RNAs derived from *in vitro* transcription of pO1Ca digested with selected enzymes were translated in a rabbit reticulocyte system and the products were then analyzed by PAGE on a 15% gel. Truncated proteins are indicated by a prime. Molecular weight markers and cleavage products are indicated on the right and left, respectively. O1 Campos infected BHK-21 cell extract labeled with (^{35}S) methionine as previously described (Piccone et al., 2010) was used as marker (lane 1).

To check the ability of pO1Ca to function as a template for protein synthesis, RNA transcripts derived from EcoRV-linearized plasmid were translated *in vitro* using reticulocyte lysates as described by Piccone et al. (1995). To facilitate the identification of the viral proteins, pO1Ca was digested with selected restriction enzymes that remove the downstream portion of the viral genome from the plasmid facilitating the interpretation of the viral protein pattern using SDS-PAGE. Fig. 1B shows that the polyprotein derived from transcripts of pO1Ca was processed into the virus precursors and mature proteins, indicating its ability to undergo viral proteolytic processing.

In vitro characterization of the infectious clone-derived viruses

RNA transcript derived from pO1Ca-VP3-56H or pO1Ca-VP3-56R was used to transfect BHK-21 cells by electroporation, and then supernatant from transfected cells was subjected to serial passage in LF-BK αVβ6 cells to amplify the clone-derived viruses. Sequencing of the entire genome of recovered viruses (O1Ca-VP3-56H and O1Ca-VP3-56R) verified that they were identical to the parental DNAs, confirming that only the predicted mutations were incorporated into the viruses. Sequencing results also revealed that nt changes in the third position of codons 55 and 57 (flanking position 56) of VP3 introduced during the construction of pO1Ca-VP3-56R were retained (data not shown), excluding the possibility of contamination by the parental virus.

The *in vitro* growth characteristics of viruses O1Ca-VP3-56H and O1Ca-VP3-56R were comparatively evaluated in multi-step growth curves in BHK-21 and LF-BK cells. Cells were infected at a low (0.01) or high (10) MOI and samples were collected at times post-infection through 24 h. The results shown in Fig. 2A demonstrate that both viruses exhibited similar growth characteristics and reached comparable titers in these cell lines. Additionally, the plaque size and morphology of these viruses was analyzed in a standard plaque assay in cells from different origins (Fig. 2B). Both viruses produced a similar plaque size in BHK-21 cells, while virus O1Ca-VP3-56R produced smaller plaques compared to O1Ca-VP3-56H in IBRS-2 and LF-BK cells, but clearly larger plaques compared to O1Ca-VP3-56H in LK cells.

Virulence assessment of FMDV O1Ca-VP3-56H and O1Ca-VP3-56R in cattle

Virulence of both viruses in cattle was assessed utilizing a well established intradermolingual inoculation method (Henderson, 1949). Two cows were inoculated with 10^4 PFU/animal of either O1Ca-VP3-56H or O1Ca-VP3-56R, and infection and clinical signs were monitored daily as described by Pacheco and Mason (2010). Results demonstrated that the two animals inoculated with the O1Ca-VP3-56H strain (#42 and #43) developed clinical FMD and reached the maximum clinical score (4 feet affected with vesicles) by 2 or 3 days post-inoculation (dpi). Both animals displayed fever (>40 °C) from 2 to 4 or to 6 dpi. Viremia lasted 4 to 5 days, starting at 1 dpi. Virus shedding was detected in nasal swabs from both animals beginning at 1 dpi to the end of the collection period (7 dpi) (Fig. 3). Sequence analysis of the P1 polyprotein confirmed that viruses collected from secondary replication sites exhibited no differences from the inoculated virus (data not shown). Interestingly, cows inoculated with O1Ca-VP3-56R (#44 and #45) showed no clinical signs, no fever, no viremia and no virus shedding (Fig. 3). Additionally, no serum neutralizing antibodies were detected 2 months after inoculation, no virus was isolated from probangs and no transmission occurred to two cows that were in direct contact since 1 dpi (results not shown). These results suggest that O1Ca-VP3-56R does not undergo any detectable replication when inoculated into animals.

Virulence assessment of FMDV O1Ca-VP3-56H and O1Ca-VP3-56R in swine

To examine the effect of H and R amino acids at position 56 of VP3 on virus virulence in pigs, six animals were intradermally inoculated with approximately 10^4 PFU/animal of either O1Ca-VP3-56H or O1Ca-VP3-56R (Figs. 4 and 5, respectively) and monitored for clinical disease. All animals infected with O1Ca-VP3-56H (#01 through #06) developed classic FMD disease. Animals became febrile, depressed, lame and developed lesions in the non-inoculated feet and mouth. By the second week post-inoculation, the disease had subsided and

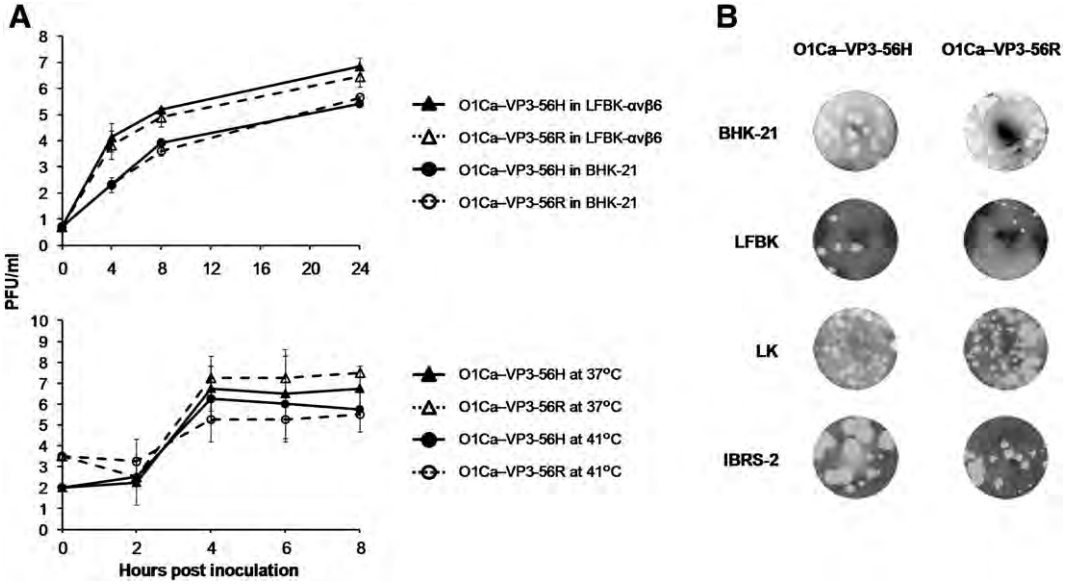

Fig. 2. *In vitro* characterization of the FMDV O1Ca-VP3-56H and O1Ca-VP3-56R recovered viruses. (A) *In vitro* growth curves at 37 °C of O1Ca-VP3-56H and O1Ca-VP3-56R viruses at MOI 0.01 in BHK-21 and LF-BK (upper panel), or MOI 10 at 37 °C and 41 °C in LF-BK (bottom panel). Cells were inoculated with the indicated MOI, and incubated at the described temperature. Virus yield was determined by $TCID_{50}$/ml at the time points indicated. The figure is representative of two independent experiments. (B) Plaques corresponding to O1Ca-VP3-56H and O1Ca-VP3-56R viruses in BHK-21, LF-BK, LK and IBRS-2 cells. Infected cells were incubated at 37 °C under a tragacanth overlay and stained with crystal violet at 48 h post infection. Plaques from appropriate dilutions are shown.

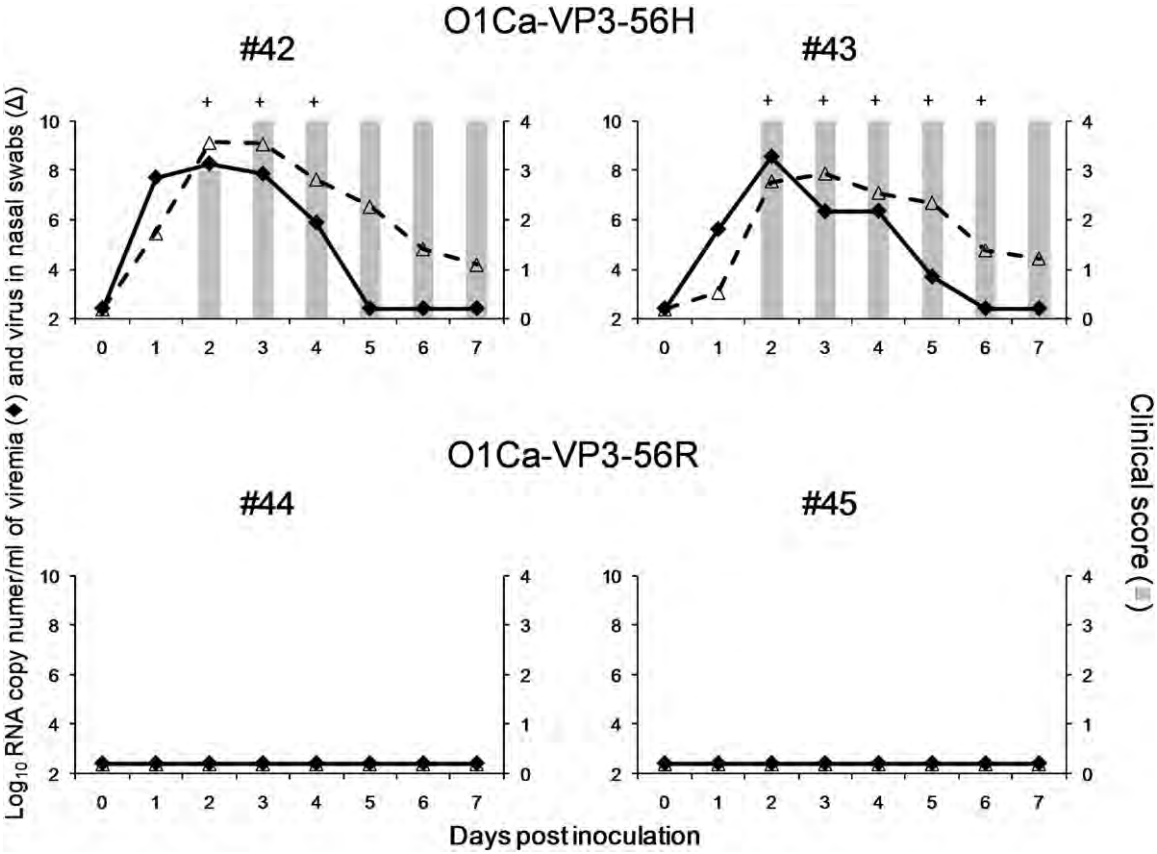

Fig. 3. Assessment of O1Ca-VP3-56H and O1Ca-VP3-56R virus virulence in cattle. Steers were intradermolingual (IDL) inoculated with 10^4 PFU of either O1Ca-VP3-56H (animal #42 and #43) or O1Ca-VP3-56R SK6 (animal #44 and #45). Presence of clinical signs (clinical scores), rise in body temperature over 40 °C (+), and virus yield (quantified as $\log_{10}$ of viral RNA copies/ml of sample) in blood and nasal swabs were daily determined during a seven day observational period.

animals were returning to their normal physiological state. Presence of viremia, detected by real-time RT-PCR, demonstrated the presence of virus in blood starting on the first day of disease peaking by day 2 to 3. Virus was also detected by rRT-PCR in nasal swabs of all 6 animals starting as early as 2 dpi and peaking at 2 to 4 dpi. Conversely, the six animals inoculated with O1Ca-VP3-56R (#07 through #12) presented a heterogeneous phenotype. Two of the animals (#11 and #12) did not display any FMD-related symptoms, presenting as clinically normal throughout the entire experimental period. Accordingly, no virus could be detected in the blood or nasal swabs of these animals at any test time points. Conversely, the other four animals inoculated with O1Ca-VP3-56R presented a disease that was nearly indistinguishable in intensity compared to that observed in animals infected with O1Ca-VP3-56H, differing only by a slight delay in the course of the disease (approximately by 1–3 days). Consequently, in these four animals viremia kinetics was also delayed by a similar lag of time in relation to the appearance of clinical symptoms (Fig. 5).

Partial RNA sequencing of the viruses isolated from animals was performed to evaluate the genetic stability of the inoculated virus during the infection *in vivo*. All virus isolates from local lesions in animals inoculated with O1Ca-VP3-56H presented an H at position 56 of VP3. As mentioned earlier, no virus was isolated from animals infected with O1Ca-VP3-56R that did not develop disease. Interestingly, all viruses isolated from the four symptomatic animals infected with O1Ca-VP3-56R possessed a mutation at position 56 of VP3; in three of them R was substituted by H and in the fourth case by C (data not shown). The above results indicate that animals inoculated with O1Ca-VP3-56R remain asymptomatic unless R at position 56 mutates to either an H or a C.

Effect of R at position 56 of VP3 on thermal stability

Since temperature sensitivity has been linked with attenuation phenotype for FMDV (Butrapet et al., 2000; Omata et al., 1986), viruses O1Ca-VP3-56H and O1Ca-VP3-56R were analyzed for their ability to produce plaques at 41 °C, a non-permissive temperature. Using ten-fold dilutions of the virus stocks, plaque assays were performed at low MOI and, after adsorption at 37 °C, cells were incubated at either 37 °C or 41 °C for 48 h. While virus O1Ca-VP3-56H displayed a slight reduction in the number of plaques at 41 °C regarding those detected at 37 °C, virus O1Ca-VP3-56R failed to produce plaques in LF-BK cells at all dilutions tested (between 10^{-2} and 10^{-6}) (data not shown). To further characterize the effect of temperature on the viruses' growth, LF-BK cells were infected at a high MOI (MOI = 10) and incubated at 41 °C. Both viruses achieved lower titers at all tested time points at 41 °C compared to 37 °C (Fig. 2A). In addition, virus O1Ca-VP3-56R grew slower than virus O1Ca-VP3-56H but reached similar final yield, indicating that the substitution H56R had no significant effect on virus production.

To further test the temperature sensitivity of these viruses, samples were heated up to 24 h at either 37 °C or 41 °C and aliquots were taken at different time points during the heat treatment and titrated on LF-BK cells. Infectivity titers of FMDV O1Ca-VP3-56R virus were strongly reduced at both temperatures when compared with O1Ca-VP3-56H. An approximately 4 logs reduction titer was observed after heating for 8 h at 37 °C or after 4 h at 41 °C (Fig. 6). Moreover, to determine if H56 confers a better fitness to the viral population at a higher temperature, a serial passage experiment was performed in LF-BK cells at 41 °C with these viruses. Sequencing analysis revealed that after

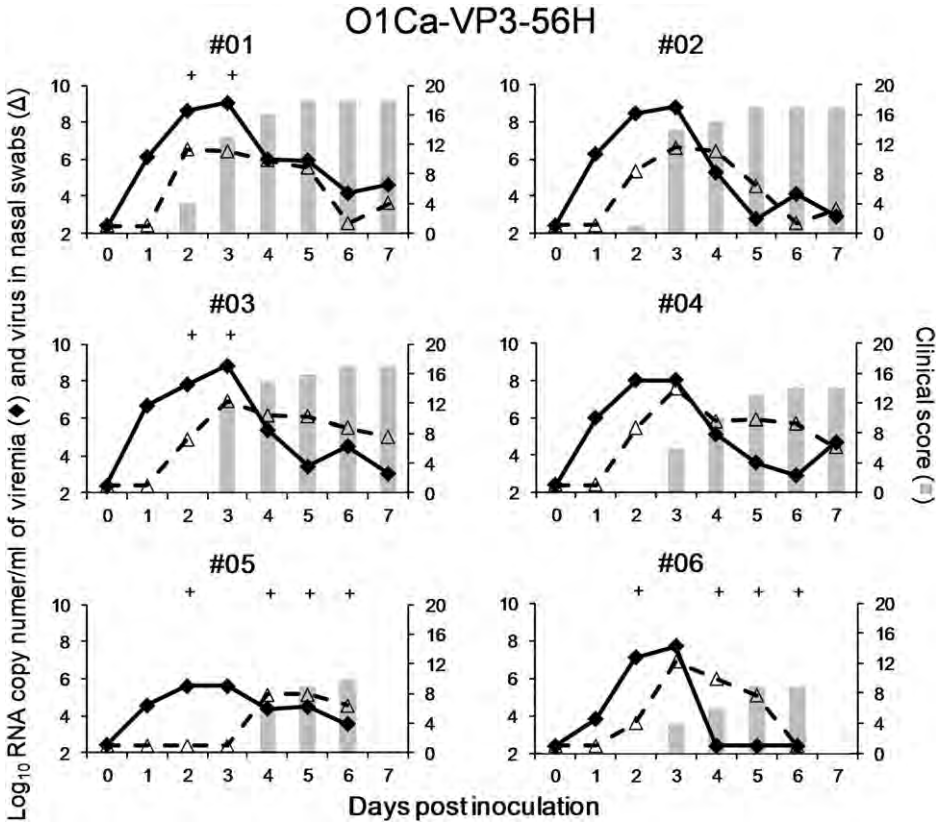

Fig. 4. Assessment of O1Ca-VP3-56H virus virulence in swine. Six pigs (animal #01 through #06) were intradermically inoculated into the foot bulb with 10^4 PFU of O1Ca-VP3-56H. Presence of clinical signs (clinical scores), rise in body temperature over 40 °C (+), and virus yield (quantified as $\log_{10}$ of viral RNA copies/ml of sample) in blood and nasal swabs were daily determined during a seven day observational period.

three passages virus O1Ca-VP3-56R acquired an H codon at position 56, whereas virus O1Ca-VP3-56H conserved the original sequence (Table 1). The presented results indicate that the H56R substitution in VP3 drastically reduces the thermal stability of the virus.

Discussion

Previous works have suggested that the H56R substitution is acquired during the process of virus adaptation to cell cultures, conferring an attenuated phenotype in mice and bovines with an increased affinity for heparin (Baranowski et al., 2003; Jackson et al., 1996; Jensen and Moore, 1993; Rodriguez Pulido et al., 2009; Sa-Carvalho et al., 1997). In fact, R56 of VP3 is the critical viral residue used for recognition of the cellular heparin sulfate (HS) co-receptor by different strains of FMDV, as seen in the structure of the serotype O1BFS in complex with various glycosaminoglycans (Fry et al., 1999). Nevertheless, none of these studies have assayed the infectivity of type O transcripts or viruses exclusively differing in the aa residue at position 56 of VP3 (either H or R) in the natural host. The purpose of this study was to experimentally demonstrate the role of each of these amino acids in this particular position on viral virulence in the natural host. To this end, recombinant viruses were created by modifying a full-length infectious cDNA clone of the O1 Campos/Bra/58 strain to introduce either H or R at VP3-56. Viruses O1Ca-VP3-56H and O1Ca-VP3-56R recovered after electroporation of BHK-21 cells showed similar growth kinetics and small differences in plaque sizes in cell culture. In contrast, these viruses displayed a marked difference in thermal inactivation at 41 °C. *In vitro* heating of the viruses caused a pronounced loss of infectivity for virus O1Ca-VP3-56R compared to O1Ca-VP3-56H, suggesting a role for the aa at VP3-56 in the thermal stability of the virion. At this temperature, plaque assays also showed significant differences between both viruses at higher dilutions, although at MOI 10 they replicate to similar titers. These results suggest that the substitution H56R may affect the capsid assembly or stability of the virion. Amino acid 56 of VP3 is a surface partially exposed residue, which also participates in interactions with VP1 and VP2 residues that stabilize the FMDV protomer (Fig. 7A). Hence, having an H instead of an R at this position may affect such interactions (Fig. 7B, C). Further experiments are necessary to validate this assumption. In any case, H56 likely increased the thermal stability of the virus, since after two passages of O1Ca-VP3-56R in cell culture at 41 °C VP3-R56 changed to VP3-H56. On the other hand, the identification of aa that increase the thermal stability of the virion without disrupting its infectivity could support the rational engineering of FMD vaccines (Mateo et al., 2003, 2008).

Another major difference between O1Ca-VP3-56H and O1Ca-VP3-56R relates to their ability to cause disease in the natural host. Principally, cattle inoculated with O1Ca-VP3-56H showed typical clinical signs of FMD, while cattle inoculated with O1Ca-VP3-56R did not display any clinical signs of disease. Furthermore, no neutralizing antibodies or virus in blood or saliva was detected in animals inoculated with O1Ca-VP3-56R. Since both viruses differ only in the aa at position 56 of VP3, this data clearly indicates that the presence of R56 is responsible for the attenuation of O1Ca-VP3-56R in cattle. Additionally, the virulence of both viruses was tested in swine. All animals inoculated with O1Ca-VP3-56H exhibited clinical signs of FMD while those inoculated with O1Ca-VP3-56R remained clinically normal with the exception of some animals that developed delayed FMD. Interestingly, the virus recovered from lesions in these animals had a R56C or R56H mutation and no other mutations in their protein coding region, confirming the involvement of R56 in viral attenuation. Similar VP3 R to H or C substitutions were previously reported by Sa-Carvalho et al. (1997) in A24/O1 Campos chimeric viruses recovered from infected cattle, although additional substitutions accompanied that at position 56 of VP3.

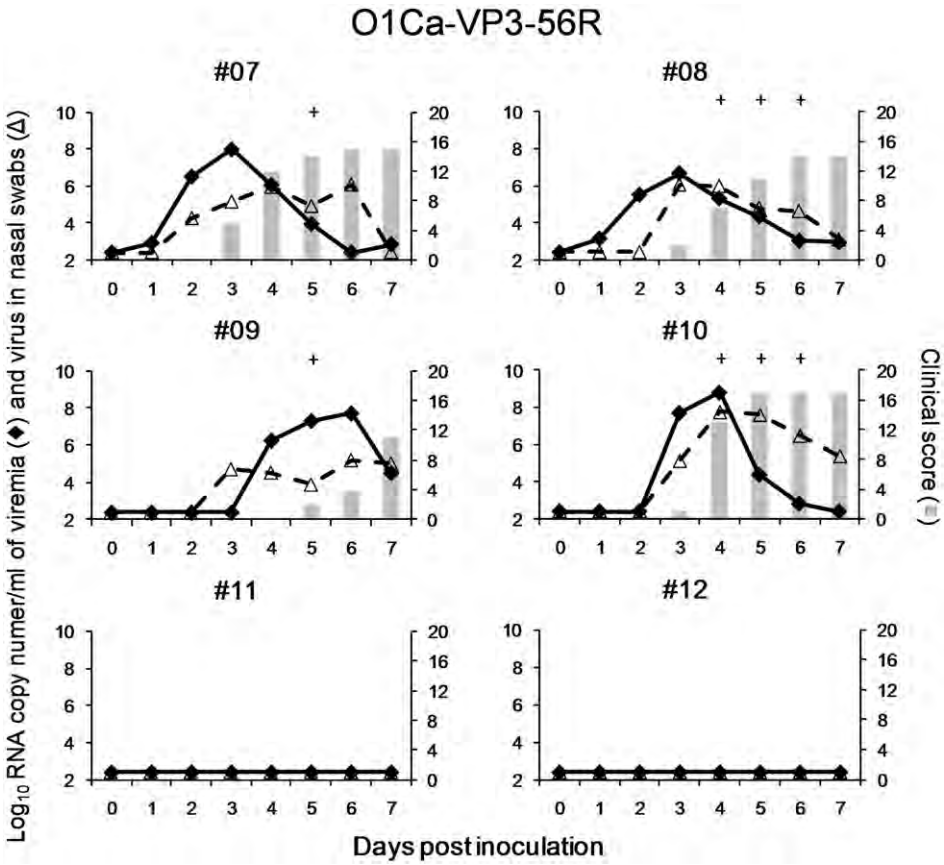

Fig. 5. Assessment of O1Ca-VP3-56R virus virulence in swine. Six pigs (animal #07 through #12) were intradermically inoculated into the foot bulb with 10^4 PFU of O1Ca-VP3-56R. Presence of clinical signs (clinical scores), rise in body temperature over 40 °C (+), and virus yield (quantified as $\log_{10}$ of viral RNA copies/ml of sample) in blood and nasal swabs were daily determined during a seven day observational period.

As seen in Fig. 7, residue 56 is located at the interface between VP2 and VP3 proteins, in close contact with the C-terminus of VP1, near the icosahedral three-fold axis. Structural comparisons between O1BFS and O1K, having R and H in position 56 of VP3, respectively, demonstrated important changes at the capsid surface between the two strains (Lea et al., 1995). These changes may explain the different properties exhibited by viruses carrying R or H in VP3-56 such as reactivity with MAbs (Kitson et al., 1990; Sa-Carvalho et al., 1997) or thermal stability properties. Additional studies with subtype O1 viruses have shown that R56 of VP3 increases the positive charge of the viral surface at this site and strongly enhances binding to negatively charged HS (Fry et al., 1999; Jackson et al., 1996). Fry et al. (1999) hypothesized that the high affinity to HS presumably hampers the spread of infection by sequestering viruses from the target cells/tissues. Consistent with this hypothesis, a virus carrying R56 cannot develop virulent infections *in vivo* unless it undergoes a reversion to H or C, reducing HS affinity.

Materials and methods

Cell lines and virus

Baby hamster kidney (BHK-21) cells, secondary lamb kidney (LK) cells, bovine kidney (LF-BK) cells (Swaney, 1988) and a derivative LF-BK cell line expressing the bovine αVβ6 integrin (LF-BK αVβ6) (LaRocco et al., unpublished data) were maintained as previously described (Piccone et al., 2010). Growth kinetics and plaque assays

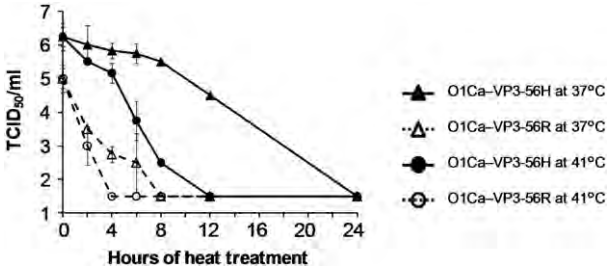

Fig. 6. Temperature sensitivity of O1Ca-VP3-56H and O1Ca-VP3-56R viruses. Virus stocks were heated up to 24 h at either 37 °C or 41 °C and aliquots were taken at different times during the heat treatment and titrated on LF-BK cells. Infectivity titers are expressed in $TCID_{50}$/ml. Threshold of detection is $\log_{10}1.80$ $TCID_{50}$/ml.

Table 1
Sequence comparison around the position VP3-56 between O1Ca-VP3-56H and O1Ca-VP3-viruses amplified at different temperatures.

	O1Ca-VP3-56H			O1Ca-VP3-56R		
Passage 0	ctA	cAT	ttT	ctg	cgc	ttc
	L	H	F	L	R	F
Passage 3 at 37 °C	ctA	cAT	ttT	ctg	cgc	ttc
	L	H	F	L	R	F
Passage 3 at 41 °C	ctA	cAT	ttT	ctg	cAc	ttc
	L	H	F	L	H	F

Virus stocks were passaged three times in LF-BK cells at MOI 10 and the temperature indicated. Changes in nucleotides and amino acid sequence are compared with original virus (Passage 0).

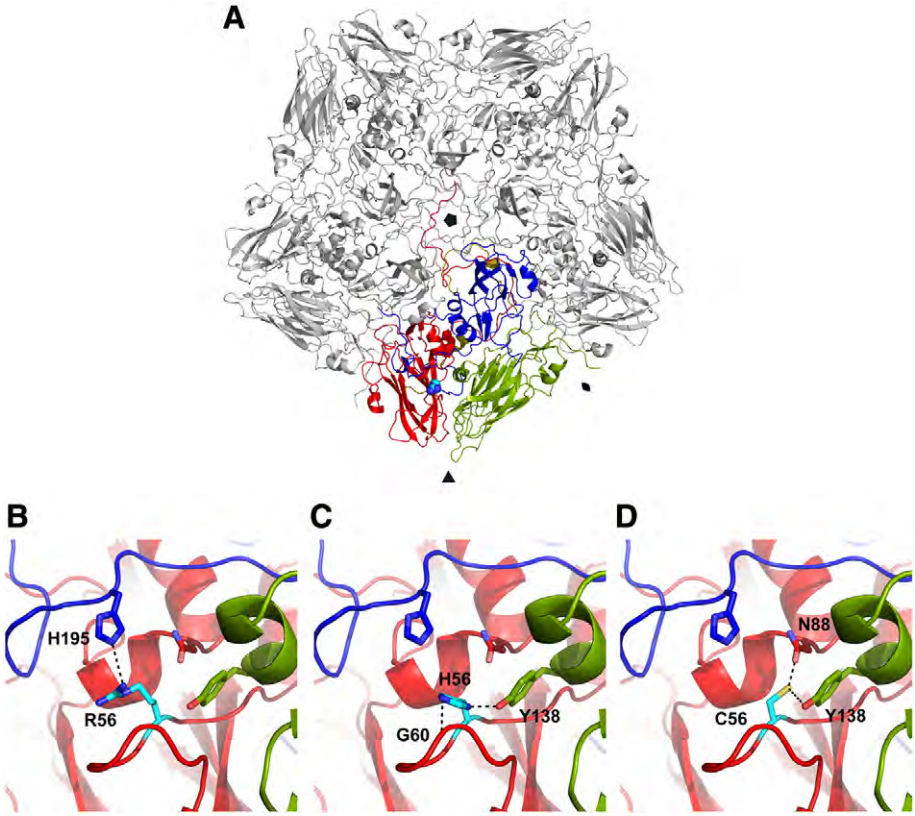

Fig. 7. (A) Ribbon diagram showing the structure of a FMDV pentamer with the position of the five-fold, three-fold and two-fold icoshedral axes explicitly indicated. The biological protomer is shown with standard coloring for the proteins VP1 (blue), VP2 (green), VP3 (red) and VP4 (yellow). The rest of the pentamer is shown in gray. Residue 56 of VP3 is shown as a space filling surface in cyan. (B, C, D) Close up of the interactions involving the position 56 of VP3, when the residue is R (B), H (C) and C (D). Hydrogen bonds are depicted as discontinuous lines and the residues involved are explicitly labeled. Coordinates are taken from O1BFS (PDB id 1FOD). H and C were modeled using the program coot (Emsley and Cowtan, 2004) with the preferred side chain rotamer conformation.

were performed also as described previously (Piccone et al., 1995). The FMDV strain O1Campos/Bra/58 was obtained from the Institute of Virology at the National Agricultural Technology Institute, Argentina.

Construction of the FMDV O1 Ca full-length cDNA clone and derivative

Total RNA from FMDV-infected BHK-21 cells was isolated with the TRIzol® system (Life-Technologies), and reverse transcribed by standard methods using random hexamers (PROMEGA) and SuperScript II reverse transcriptase (Life-Technologies). cDNA fragments were amplified with the Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, IN) using primers on unique restriction sites naturally found in the FMDV O1Campos/Bra/58 sequence. All nucleotide numbering included in this study refers to the sequence deposited in GenBank under accession number AJ320488.

A total of 4 fragments covering the complete viral genome were amplified (Fig. 1A). The 3′ and 5′ ends of the viral genome as well as the introduction of the polyC tail were obtained by a RACE-PCR technique. For the 3′ end, a 40mer primer (5′GAATTCCATATGgatatcT$_{15}$GATTAAG3′) complementary to the 3′ end of the viral genome, including 15 adenine residues, followed by an adaptor sequence containing an EcoRV site (lower case) was used for priming the cDNA synthesis. Thereafter, PCR was performed using sense primer nt 5416-5′ GGCAGCAATTgaattcTTT3′ on a unique EcoRI site (lower case) and antisense primer 5′GAATTCCATATGgatatc3′ complementary to the adaptor sequence.

For the 5′ end, PCR was performed using sense primer 5′ GGAATTCcatagTAATACGACTCACTATA**GG**TTGAAAGGGGCGCTAGGC3′, which contained the sequences of a NdeI site (lower case), T7 promoter (underlined), two additional G nucleotides (bold) and 19 nucleotides of the 5′ end of the viral genome; and antisense primer nt 350-5′TTggatccTGAAAGGCGGGCGTCGGG3′ which incorporated a StyI site (lower case). To add the polyC tail, the PCR reaction was carried out using sense primer 5′ TtcctaggC$_{17}$TAGGTTTTACCGTCATTCCC which contained a StyI site (lower case) and antisense primer nt 3019-5′CAGTGCgcggccgcCTCAGGTGTC3′ on a unique NotI site (lower case).

All purified PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced using selected primers. The assembly of the full-length cDNA clone was done using a multi-step strategy that implemented the unique restriction sites NdeI, StyI, NotI, EcoRI and EcoRV on pGEM vectors. Each step of the construction was monitored by restriction enzyme analysis to ensure the generation of fragments of predicted sizes. The final plasmid containing the genome-length cDNA of FMDV O1Campos/Bra/58 was designated as pO1Ca and completely sequenced using an Applied Biosystems 3730 xl automated DNA sequencer (Applied Biosystems, Foster City, CA).

Substitution of aa H56 in the structural protein VP3 was carried out by site-directed mutagenesis in plasmid pO1Ca using the QuickChange system (Stratagene) and forward primer nt 2695-5′ GGCATGCCCGACGTTTCT**GC**GCTT**C**GAGGGT GGCGTACCGTAC3′ containing the desired change (in bold), following the manufacturer's protocol. The full-length mutant clone was completely sequenced to confirm the presence of expected modifications and absence of unwanted substitutions.

Transfection and recovery of the infectious clone-derived viruses

Plasmid pO1Ca or its mutant version was linearized at the EcoRV site following the poly(A) tract and used as a template for RNA synthesis using the MegaScript T7 kit (Ambion, Austin, TX), according

to the manufacturer's protocols. BHK-21 cells were transfected with these synthetic RNAs by electroporation (Electrocell Manipulator 600; BTX, San Diego, CA) as previously described (Mason et al., 1994). Briefly, 0.5 ml of BHK-21 cells at a concentration of 1.5×10^7 cells/ml in phosphate-buffered saline (PBS) were mixed with 10 μg of RNA in a 4-mm-gap BTX cuvette. The cells were then pulsed once at 330 V, infinite resistance, and a capacitance of 1000 μF; diluted in cell growth medium and allowed to attach to a T-25 flask. After 4 h, the medium was removed, fresh medium was added and the cultures were incubated at 37 °C for up to 24 h.

The supernatants from transfected cells were passed in LF-BK αVβ6 cells until a cytopathic effect (CPE) appeared. After successive passages in these cells, virus stocks were prepared and the viral genome completely sequenced using the Prism 3730xl automated DNA sequencer (Applied Biosystems), as previously described (Piccone et al., 2010).

Viruses derived from plasmid pO1Ca and the derived mutant plasmid encoding the aa substitution H56R in VP3 are referred to in this work as O1Ca-VP3-56H and O1Ca-VP3-56R, respectively.

In vitro processing

Plasmid pO1Ca containing the complete genome sequence was linearized with EcoRV and *in vitro* transcribed using the T7-based MegaScript kit (Ambion, Inc., Austin, Tex.). RNA transcripts were translated *in vitro* by using rabbit reticulocyte lysates (Promega, Fitchburg, WI) in the presence of [^{35}S] methionine and translation reaction mixtures were resolved by electrophoresis on 15% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) and fluorographed, following previously described procedures (Piccone et al., 1995). Additionally, pO1Ca was digested with selected restriction enzymes that eliminate part of the viral open reading frame, and used in *in vitro* transcription/translation assays

Virulence of O1Ca-VP3-56H or O1Ca-VP3-56R viruses in cattle and swine

Animal experiments were performed under biosafety level 3 conditions in the animal facilities at PIADC following a protocol approved by the Institutional Animal Care and Use Committee. Two steers (300–400 kg) were infected per the intradermolingual inoculation method (Henderson, 1949) with approximately 10^4 PFU/cattle of FMDV O1Ca-VP3-56H or O1Ca-VP3-56R, diluted in MEM. Six pigs (20 kg) were infected intradermally (Pacheco and Mason, 2010) with 10^4 PFU/pig of either FMDV O1Ca-VP3-56H or O1Ca-VP3-56R, diluted in MEM. For 7 days after inoculation, animals were clinically examined, including rectal temperature recordings and serum and nasal swabs collection. After collection, clinical samples were aliquotted and frozen at −70 °C. One serum and one swab aliquot were used to perform viral titration by rRT-PCR as described previously (Pacheco et al., 2010). For cattle, clinical scoring was made based on lesions present in each foot, with a maximum of 4. For swine, the clinical score was based on severity of affected digits and/or snout, as previously described (Pacheco and Mason, 2010), with a maximum of 20.

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The nuclear protein Sam68 is cleaved by the FMDV 3C protease redistributing Sam68 to the cytoplasm during FMDV infection of host cells

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ABSTRACT

Picornavirus infection can lead to disruption of nuclear pore traffic, shut-off of cell translation machinery, and cleavage of proteins involved in cellular signal transduction and the innate response to infection. Here, we demonstrated that the FMDV $3C^{pro}$ induced the cleavage of nuclear RNA-binding protein Sam68 C-terminus containing the nuclear localization sequence (NLS). Consequently, it stimulated the redistribution of Sam68 to the cytoplasm. The siRNA knockdown of Sam68 resulted in a 1000-fold reduction in viral titers, which prompted us to study the effect of Sam68 on FMDV post-entry events. Interestingly, Sam68 interacts with the internal ribosomal entry site within the 5′ non-translated region of the FMDV genome, and Sam68 knockdown decreased FMDV IRES-driven activity in vitro suggesting that it could modulate translation of the viral genome. The results uncover a novel role for Sam68 in the context of picornaviruses and the proteolysis of a new cellular target of the FMDV $3C^{pro}$.

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Introduction

Foot-and-mouth disease virus (FMDV), the prototypic member of the *Aphthovirus* genus of the family *Picornaviridae* is a highly contagious viral pathogen of cloven-hoofed animals (Domingo et al., 2002; Grubman and Baxt, 2004; Mason et al., 2003; Saiz et al., 2002; Sobrino et al., 2001). Previous studies have shown that infection with many picornaviruses belonging to the Enterovirus and Rhinovirus genus leads to the efflux of specific nuclear proteins into the host cell cytoplasm (Gustin and Sarnow, 2002; Huber et al., 1999; Lawrence and Rieder, 2009; McBride et al., 1996). This is an intriguing finding given that picornaviruses are single-stranded positive-sense RNA viruses, whose entire replication cycle is believed to be confined to the cytoplasm. It is well established that the efflux of nuclear host factors to the cytoplasm during the course of viral infections is not a generalized process, rather only a certain subset of proteins is relocalized. Many of the redistributed proteins described thus far for picornaviruses are RNA-binding proteins known to participate in virus replication in cells, including: La autoantigen, nucleolin, poly-C binding protein (PCBP2), RNA helicase A (RHA), and the 68 kDa Src-associated substrate during mitosis (Sam68) (Gustin and Sarnow, 2002; Lawrence and Rieder, 2009; McBride et al., 1996; Niepmann et al., 1997). Currently, very little is known about the effect of *Aphthovirus* infection on host cells.

Our previous studies have shown that FMDV infection in cell culture induces the nuclear-to-cytoplasmic redistribution of a predominantly demethylated form RNA-binding protein: RHA, which was determined to be critical to the life cycle of the virus (Lawrence and Rieder, 2009). It has also been demonstrated with HIV infection, that RHA partners with Sam68, another nuclear RNA-binding protein (Reddy et al., 2000a, 2000b). Early reports suggested that Sam68 mimics the activity of the HIV Rev protein and Rev-like proteins of other complex retroviruses facilitating the export of unspliced viral RNA transcripts (Bolinger and Boris-Lawrie, 2009; Li et al., 2002; Modem et al., 2005; Reddy et al., 2000b). However, more recent studies have shown that Sam68 can significantly enhance the translation of retrovirus genes by "marking" the viral RNA transcripts (Bolinger and Boris-Lawrie, 2009; Cochrane, 2009; Coyle et al., 2003; He et al., 2009; Henao-Mejia et al., 2009). The significance of Sam68 to the life cycle of HIV has been highlighted by the discovery that prior transfection of host cells with a dominant negative mutant of Sam68 with a C-terminal deletion (Sam68DeltaC) rendered the cells refractory to subsequent HIV infection (Cochrane, 2009; Marsh et al., 2008; Modem et al., 2005; Reddy et al., 1999; Soros et al., 2001).

Unlike RHA and other nuclear RNA-binding proteins, which are redistributed during picornavirus infections, Sam68 is not a shuttling protein and is confined to the nucleus as a result of specialized domains discussed below (Ishidate et al., 1997; McBride et al., 1998; Soros et al., 2001). It has been implicated in many cellular processes

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including downstream signaling from T-cell receptors, cellular responses to insulin stimulation, male fertility, and the progression of cancer (Hong et al., 2002; Lukong and Richard, 2007; Richard et al., 2008; Sette et al., 2010; Taylor et al., 2004). It is a member of the signal transduction and activation of RNA (STAR) superfamily of proteins, and the prototypic member of a unique group of RNA-binding proteins possessing a single heteronuclear ribonucleoprotein (hRNP) K homology (KH) domain embedded within a larger GSG (GRP33 Sam68 GLD-1) domain (Lukong and Richard, 2003). Downstream of the GSG domain, there are several proline rich regions, which allow Sam68 to interact with other proteins bearing Src-homology 3 (SH3) domains. The protein also possesses both an RGG box within the N-terminus as well as several RG repeats proximal to the C-terminus, and is a known substrate of the arginine methyltransferase, PRMT1 (protein-arginine N-methyltransferase) (Cote et al., 2003). The C-terminal portion of the protein consists of a non-canonical nuclear localization sequence (NLS) and a tyrosine-rich region (Ishidate et al., 1997). Phosphorylation of the tyrosine residues within this cluster has been described to both promote association with SH2 domains and reduce interaction with RNA (Coyle et al., 2003). In addition to the aforementioned post-translational modifications, Sam68 is also a substrate for acetylation and sumoylation on specific lysine residues (Babic et al., 2004; Babic et al., 2006). Increasing the diversity of its potential cellular functions, Sam68 is also capable of homo-dimerization and hetero-dimerization with closely related single KH domain STAR proteins: Sam68-like mammalian protein 1 and 2 (SLM-1 and SLM-2) as well as GRP-333, GLD-1 and Qk1 (Chen et al., 1997; Di Fruscio et al., 1999).

Apart from *Retroviridae*, the only other virus family with which Sam68 has been implicated is the *Picornaviridae*. Infection with poliovirus or rhinovirus was shown to induce the redistribution of Sam68 from the nucleus to the cytoplasm (Gustin and Sarnow, 2002; McBride et al., 1996). In poliovirus (PV) infected cells, the relocalized Sam68 bound the PV RNA-dependent RNA polymerase, and co-localized with the PV 2C protein on viral-induced membranous structures (McBride et al., 1996). However, the functional significance of these interactions in PV replication has remained obscure. Specific mechanisms have been reported that explain how infection by different picornaviruses perturbs the normal nuclear-cytoplasmic trafficking of the host cell (Gustin and Sarnow, 2002). For instance, the rhinovirus 2A protease ($2A^{pro}$) has been shown to cleave components (nucleoporins or Nup proteins) of the nuclear pore complex (NPC): the channel that regulates active import and export across the nuclear membrane. Alternatively, the leader protein of cardioviruses phosphorylates surface residues of the NPC components leading to the disruption of normal trafficking and also inhibits RNA nuclear export mediated by the Ran GTPase (Park et al., 2008, 2010). Specifically, Nup proteins migrating at 62 and 98 kDa (Nup62 and Nup98) are rapidly degraded, while Nup153 is degraded very slowly during poliovirus and rhinovirus infection (Park et al., 2008, 2010).

Here, we examined the cleavage and subsequent relocalization of Sam68 within wild-type FMDV-infected BHK and LFBK cells induced by viral $3C^{pro}$. As infection progressed, we observed increasing amounts of truncated Sam68 products apparently lacking the C-terminal NLS (Ishidate et al., 1997), thus suggesting a mechanism for its nuclear egress distinct from what we previously reported for RHA (Lawrence and Rieder, 2009). In FMDV-infected cells, the cellular Sam68 protein was shown to co-precipitate and be sensitive to cleavage by FMDV $3C^{pro}$, but not by L^{pro}. Interestingly, we showed that Sam68 also binds the internal ribosomal entry site (IRES) within the 5′ non-translated region (NTR) of the FMDV genome. This was further supported by experiments using a targeted reporter assay driven by the FMDV IRES that exhibited reduced reporter gene expression following treatment with Sam68-specific siRNAs suggesting that Sam68 augments the translation of the viral RNA.

Results

FMDV infection alters Sam68 sub-cellular localization

Sam68 has been described as an exclusively nuclear protein, which can associate with specific nuclear and sub-nuclear structures depending on the physiological state of the cell (Chen et al., 1999; Soros et al., 2001). However, it has been demonstrated that the exclusive nuclear localization of Sam68 can be altered as a result of infection with picornaviruses including: poliovirus and rhinovirus (Gustin and Sarnow, 2002; McBride et al., 1996). To determine if infection with FMDV could stimulate a similar redistribution, we used immunofluorescent microscopy (IFM) to examine the sub-cellular localization of Sam68 at different stages of infection probing with an antibody directed against the first 50 amino acids of Sam68. As shown in Fig. 1A, Sam68 remained sequestered in the nucleus of uninfected cells as well as FMDV-infected cells examined at early time points (approximately 1 h post infection, hpi). In contrast, at 3 and 5 hpi, FMDV-infected cells demonstrated a gradual increase in punctate Sam68-specific cytoplasmic fluorescence, which was later seen distributed throughout the cytoplasm (Fig. 1A, upper panel). This pattern was confirmed using three different serotypes of FMDV: A24-Cruzeiro (Fig. 1A, upper panel), O1-Campos and C3-Resende (data not shown). Notably, the complete nuclear efflux of Sam68 previously shown with poliovirus was not observed here (McBride et al., 1996). To determine if such a pronounced relocalization would only be achieved with an enterovirus, we performed the IFM analysis of Sam68 using bovine enterovirus 1 (BEV-1). Infection with BEV-1 resulted in a more rapid redistribution of Sam68 with cytoplasmic puncta evident early in infection with the complete redistribution of Sam68 from the nucleus to the cytoplasm by 5 hpi (Fig. 1A, lower panel). Importantly, both FMDV and BEV-1 share similar replication timelines with respect to the appearance of viral proteins and viral RNA synthesis (Garcia-Briones et al., 2006; Taylor et al., 1974). These findings indicate that infection with FMDV results in the nuclear egress of Sam68 as infection progresses.

To further confirm these observations, we utilized a plasmid encoding green fluorescent protein (GFP)-tagged Sam68 (Chen et al., 1999) in transient expression experiments. Expression of the GFP-Sam68 was detected in the lysates of transfected cells on a Western blot probing with anti-Sam68, which revealed both the endogenous and ectopically expressed forms of the protein (Fig. 1B). In a separate experiment, cells transfected with the GFP-Sam68 plasmid were mock-infected or infected with FMDV and examined at 4 hpi by IFM with a GFP filter. As seen in Fig. 1C, in the absence of virus, cells transfected with GFP-Sam68 exhibit exclusively nuclear GFP-associated fluorescence. However, after infection with FMDV for 4 h, the GFP-associated fluorescence is split between the nucleus and the cytoplasm (Fig. 1C), with similar Sam68-specific punctate fluorescence observed in the cytoplasm as was previously observed in the context of endogenous Sam68 (Fig. 1A). This suggests that, similar to what has been observed for enteroviruses and rhinoviruses, infection with FMDV induces the redistribution of both ectopically expressed and endogenous Sam68 from the nucleus to the cytoplasm.

Examination of nuclear pore proteins in FMDV-infected cells

Structural alterations of the nuclear pore complex result in changes in nucleo-cytoplasmic traffic during infection with some picornaviruses. We carried out experiments to evaluate mock and FMDV-infected cell lysates for evidence of Nup degradation by Western blot probing with an antibody specific to the Nup proteins. FMDV infection resulted in no detectable Nup98 degraded product with some proteolysis of Nup62 and Nup153 by 5 hpi (Figs. 2A, B). Three FMDV serotypes failed to degrade Nup98 and exhibited some minor

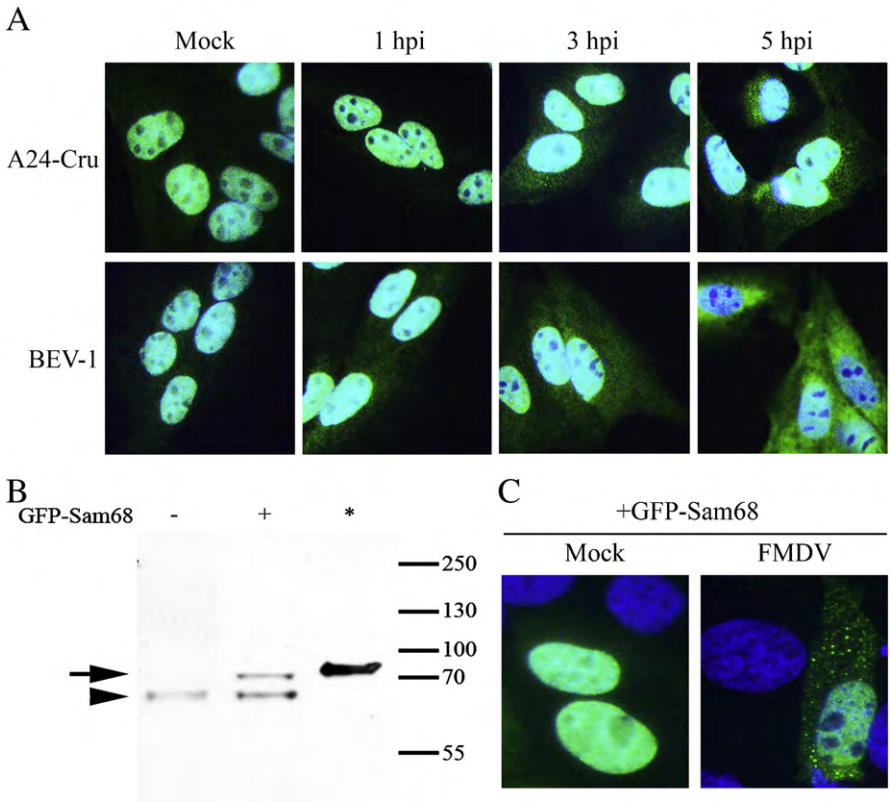

Fig. 1. Sub-cellular distribution of Sam68 is altered by FMDV infection. A. LFBK cells were infected with FMDV or mock-infected (top panel) or BEV-1 (bottom panel) at a MOI of 10 and incubated at 37 °C for 1, 3, and 5 h. Virus-infected cells were probed with rabbit anti-Sam68 (N-terminal) followed by goat-anti-rabbit-AF488 (green). Nuclear material was stained with DAPI (blue). B. LFBK cells were transfected with a plasmid encoding GFP-tagged full-length Sam68, and 2 days post-transfection, the transfected cell lysates were examined by Western blot probing with anti-Sam68 (C-terminal). Arrowhead indicates endogenous Sam68. Arrow indicates ectopically expressed Sam68. The lane marked with an asterisk contains a purified preparation of GST-tagged Sam68 expected to have a similar approximate molecular weight as GFP-tagged Sam68. C. LFBK cells were transfected with pGFP-Sam68, subsequently either mock-infected or FMDV-infected for 4 h, and then were examined by IFM using a GFP filter.

degradation of Nup62 at later time points including: A24-Cruzeiro (Fig. 2), C3-Resende and SAT2 (not shown). Since the gradual redistribution of Sam68 to the cytoplasm was observed earlier than 5 hpi, we concluded that the mechanism of Sam68 redistribution likely does not involve FMDV-induced proteolysis of NPC components, though it may contribute later in infection.

The C-terminus of Sam68 is degraded during FMDV infection

Having established that FMDV infection did not alter nucleo-cytoplasmic trafficking patterns by targeted degradation of components of the NPC, we explored whether Sam68 was modified during FMDV infection in such a way as to catalyze its redistribution. Sam68 is a substrate for multiple post-translational modifications, thus we evaluated Sam68 for such changes during the progression of FMDV infection. Specifically, lysine acetylation, tyrosine phosphorylation, and arginine methylation were investigated. We were able to detect gradually reduced levels of all three modified forms of the protein (data not shown). Two possible scenarios could account for these findings. First, the reduction in the level of lysine-acetylated, tyrosine-phosphorylated, and arginine-methylated Sam68 could be the result of enzymatic de-acetylation, de-phosphorylation, and de-methylation.

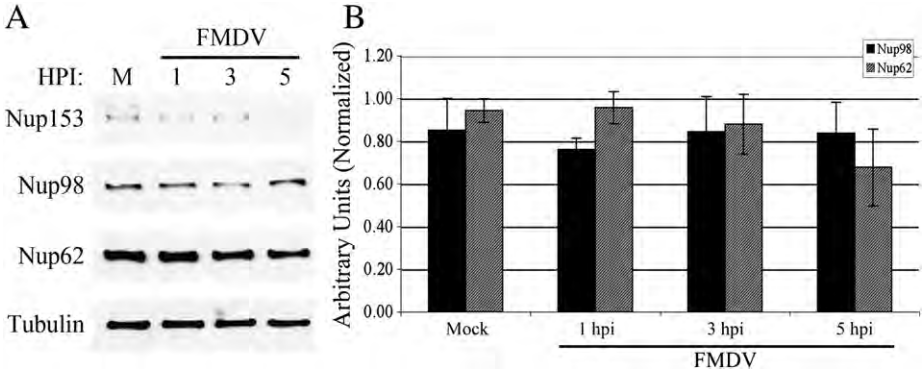

Fig. 2. FMDV-induced cytoplasmic accumulation of Sam68 is not triggered by Nup degradation. A. LFBK cells were mock-infected or infected with FMDV at a MOI of 10 and the infected cells were harvested at 1, 3, and 5 hpi. Virus-infected cell lysates were examined by Western blot probing with anti-nucleoporin protein or anti-tubulin (loading control). B. Densitometry scans were conducted using ImageJ software (NIH), and Nup62 (gray bars) and Nup98 (black bars) values, normalized against tubulin, obtained over 3 experiments were plotted.

Alternatively, it was also possible that the reduction detected was due to degradation of Sam68 as infection progressed. Therefore, we assayed for the cleavage of the C-terminus of Sam68. Mock and FMDV-infected cells were examined by Western blot probing with an antibody raised against the C-terminus of Sam68 (amino acids 331–443) (Fig. 3A). As shown in Fig. 3B (right panel), the amount of Sam68 began to decrease at 3 hpi, and was almost undetectable at 5 hpi. This was detected with 3 different FMDV serotypes including: A24-Cruzeiro (Fig. 3), C3-Resende and SAT2 (not shown). However, a parallel Western blot using an antibody directed against the N-terminus of Sam68 (amino acids 1–50) (Fig. 3A) detected a faster-migrating band, potentially representing a Sam68 degradation product (Fig. 3B, left panel). The failure of the first Sam68 antibody to react with one of the faster-migrating bands suggests that the C-terminus of Sam68 had been proteolyzed. Additionally, the lower molecular weight Sam68 degradation product was less than 50 kDa, and as such should passively diffuse through the nuclear pore complex (Belov et al., 2000; Talcott and Moore, 1999). Degradation of Sam68 was not detected when these experiments were performed with BEV-1 (Fig. 3C), suggesting that this was an FMDV-specific phenomenon. RHA stability was also examined in the context of FMDV and BEV-1 infection, and no degradation was detected (Fig. 3C). These results indicate that not all nuclear RNA-binding proteins are being modified in the same manner by FMDV, which is also consistent with our previous report of RHA exiting the nucleus due to an alteration in post-translational modification (Lawrence and Rieder, 2009).

Sam68 is not cleaved by the FMDV leader protease

Next, we wanted to determine if one of the viral proteases was responsible for the C-terminal degradation of Sam68. FMDV encodes two functional proteases: the leader protease (L^{pro}) and the 3C protease ($3C^{pro}$). In an effort to determine if either of these proteases was responsible for the observed degradation of Sam68 (Fig. 3), we mixed cell lysates from different cell lines expressing endogenous Sam68 with either purified wild-type L^{pro} or an inactive mutant of L^{pro}. The mixtures were incubated at 37 °C for 5 h, and evaluated by Western blot for evidence of degradation. No degradation of Sam68 was detected in Western blots of lysates incubated with wild-type L^{pro} (Fig. 4A). Consistent with what has been previously described, the activity of the recombinant wild-type L^{pro} was confirmed by the induced cleavage of eIF4G observed during incubation with the

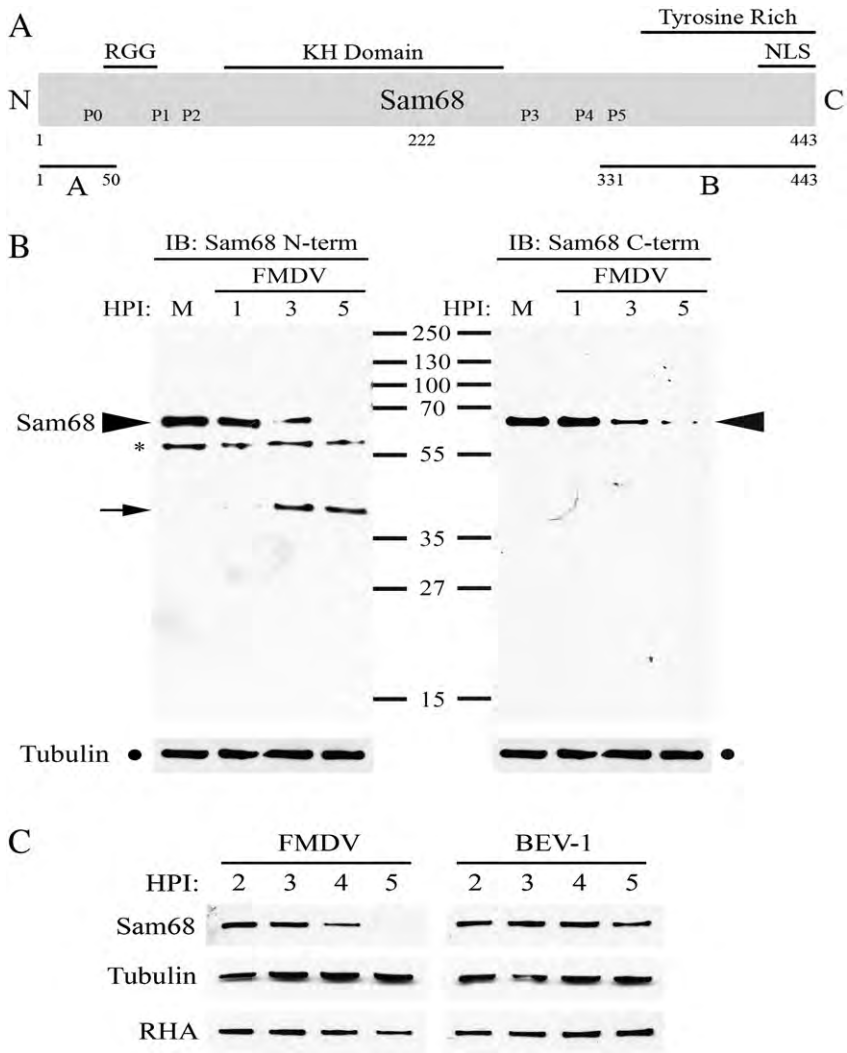

Fig. 3. Sam68 is degraded during FMDV infection. A. Schematic representation of Sam68 with specific domains and regions indicated. The region recognized by the N-terminal anti-Sam68 antibody (A) and C-terminal anti-Sam68 antibody (B) are shown. B. LFBK cells were mock-infected or infected with FMDV at a MOI of 10 and incubated at 37 °C for 1, 3, or 5 hpi. Lysates were examined on side-by-side Western blots probing with antibodies directed against the N-terminus (left panel) or C-terminus (right panel) of Sam68. Arrowheads indicate full-length Sam68. Arrow indicates a stable Sam68-reactive degradation product. Asterisk (*) indicates a protein cross-reacting with the N-terminal anti-Sam68 antibody that might correspond to SLM-1. Shaded circle indicates the tubulin loading control. C. LFBK cells were mock-infected (not shown) and infected with either FMDV or BEV-1 at a MOI of 10 and incubated at 37 °C for 2, 3, 4, or 5 h. Lysates were examined by Western blot probing with anti-Sam68 (C-terminal), anti-RHA, or anti-tubulin (loading control).

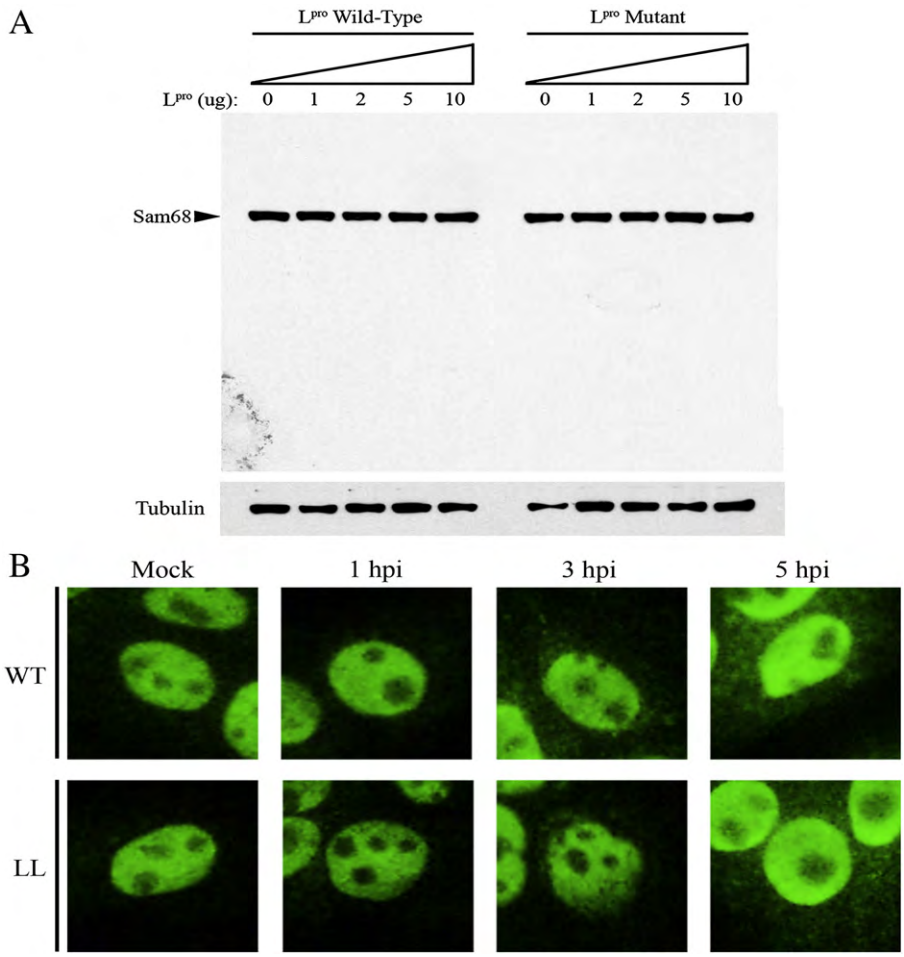

Fig. 4. Sam68 degradation is not due to the FMDV leader protease. A. Recombinant full-length Sam68 was mixed with increasing concentrations of recombinant wild-type or mutant L^{pro}. The in vitro reactions were incubated at 37 °C for 5 h, and subsequently analyzed by Western blot probing with the N-terminal Sam68 antibody. B. LFBK cells grown on coverslips were mock-infected or infected with wild-type (WT) FMDV A24-Cruzeiro or leaderless (LL) FMDV A24-Cruzeiro at a MOI 10 and incubated at 37 °C for 1, 3 or 5 hpi. Mock and virus-infected cells were probed successively with rabbit anti-Sam68 then goat-anti-rabbit-AF488 (green).

wild-type L^{pro}, but not the inactivated mutant (data not shown) (Devaney et al., 1988; Piccone et al., 1995a, 1995b).

To corroborate these findings, we repeated our previous IFM analysis (Fig. 1) to compare cells infected with either wild-type FMDV A24-Cruzeiro (WT) or a mutant lacking the leader protease (LL). The progressive accumulation of Sam68-specific fluorescent puncta in the cytoplasm of infected cells was identical for both the WT and LL viruses (Fig. 4B). We concluded that L^{pro} was neither responsible for the nuclear efflux of Sam68 nor the proteolysis of Sam68 detected during FMDV infection.

Sam68 is cleaved by the FMDV 3C protease

Next, we sought to ascertain if the FMDV $3C^{pro}$ was responsible for the cleavage of Sam68. Several possible FMDV $3C^{pro}$ cut sites were identified within Sam68 (Fig. 5A), and the projected N-terminal fragments were evaluated for their theoretical molecular weights (Table 1) to see if they corresponded with the banding pattern detected in Fig. 3. We encountered difficulty in the purification of large quantities of active recombinant $3C^{pro}$ for in vitro studies, which is consistent with past reports (Marquardt, 1993). Instead, we were able to use crude and partially purified extracts containing active wild-type $3C^{pro}$ from bacterial cells transformed with a plasmid encoding the 3ABC segment of the FMDV genome. Induced cultures were lysed and separated into pellet and supernatant fractions. To evaluate the activity of $3C^{pro}$, these lysate fractions were examined by Western blot probing with a monoclonal antibody against $3C^{pro}$ (Fig. 5B), which detected a band migrating at the approximate molecular weight of $3C^{pro}$, thus indicating that it successfully cleaved itself from the 3ABC precursor polypeptide. Purified recombinant full-length Sam68 was then mixed with increasing amounts of crude lysates of either the 3ABC or an inactive $3C^{pro}$ mutant ($3C^{pro}$-46) (Bablanian and Grubman, 1993; Grubman et al., 1995). The recombinant Sam68 was degraded during incubation with increasing amounts of the $3C^{pro}$-WT, but not by the $3C^{pro}$-46 mutant (Fig. 5C). Next, we examined if endogenous Sam68 would be equally susceptible to degradation by the recombinant WT 3C protease. Partially purified WT and the inactive $3C^{pro}$ mutant were mixed individually with cellular lysates and incubated at 37 °C for 5 h, after which they were probed with anti-Sam68 on a Western blot. Degradation of Sam68 was only observed in lanes where the cell lysates were mixed with the $3C^{pro}$-WT, while no difference in Sam68 levels was detected in lanes containing mock-treated and $3C^{pro}$ mutant treated lysates (Fig. 5D, upper panel). To confirm that active $3C^{pro}$ had not been depleted during the partial purification, we looked for evidence of histone H3 degradation in the lysates incubated with $3C^{pro}$-WT, which is degraded by FMDV $3C^{pro}$ during the course of infection (Capozzo et al., 2002; Falk et al., 1990; Grigera and Tisminetzky, 1984; Tesar and Marquardt, 1990). The Western blot was stripped and re-probed with anti-histone H3, which revealed histone H3 degradation only in the lane containing lysates treated with $3C^{pro}$-WT, thus confirming that the partially purified $3C^{pro}$-WT was indeed active (Fig. 5D, middle panel). We concluded that the C-terminal truncation of Sam68 observed as FMDV infection progressed was due to $3C^{pro}$ cleavage.

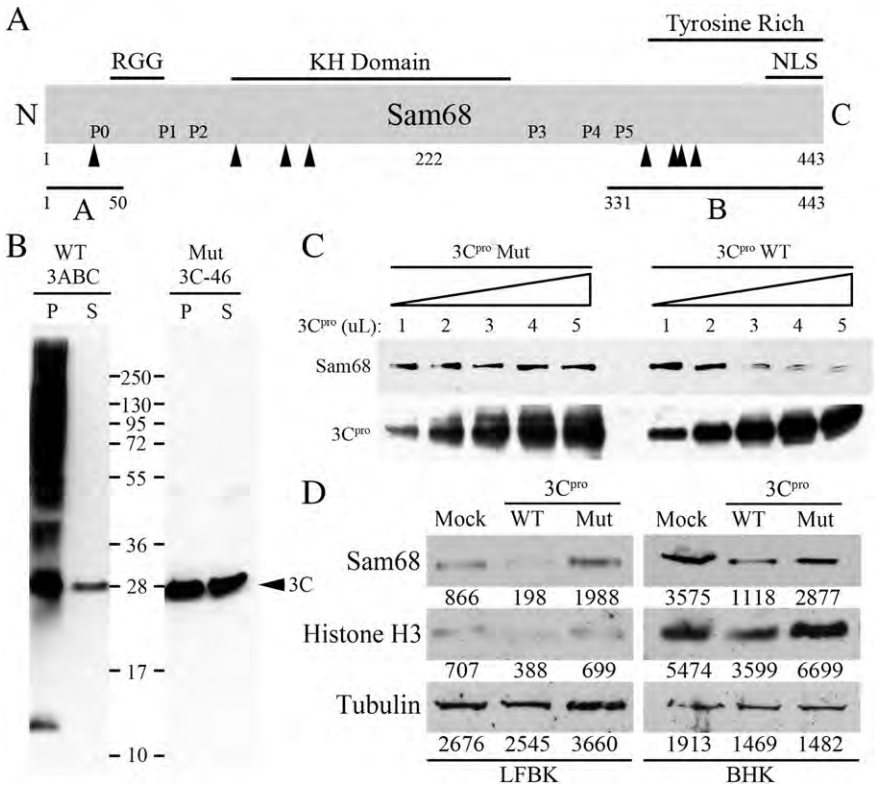

Fig. 5. Sam68 is cleaved by the FMDV 3C protease. A. Diagram of Sam68 with the RGG box, KH domain, tyrosine rich region, nuclear localization sequence (NLS) and polyproline regions (P0, P1, P2, P3, P4, P5) indicated. The anti-Sam68 antibody targeted to the N-terminus reacts with the region labeled as "A", and the C-terminal anti-Sam68 antibody reacts with the region labeled as "B". Arrowheads mark potential FMDV 3C protease cut sites. B. An active WT 3C protease was expressed in bacteria from a plasmid encoding the FMDV 3ABC polypeptide. Transformed cultures were induced, lysed, and fractionated into soluble (S) and insoluble (P) portions, which were examined on a Western blot probing with a mouse monoclonal anti-$3C^{pro}$ antibody followed by goat-anti-mouse-HRP. C. Recombinant purified Sam68 was incubated 5 h at 30 °C with increasing amounts of bacterial lysates containing the active wild-type $3C^{pro}$ or an inactive mutant. The reactions were analyzed on a Western blot probing first with rabbit anti-Sam68, then stripped and probed with mouse anti-$3C^{pro}$. D. Partially purified WT and inactive $3C^{pro}$ were mixed with LFBK or BHK cellular lysates and incubated at 37 °C for 5 h. The solutions were then examined by Western blot probing with the C-terminal anti-Sam68. Afterwards, the blot was stripped and re-probed first with anti-histone H3 to confirm the enzyme activity and second with anti-tubulin to confirm equal loading between the lanes. Densitometry scanning was used to assign arbitrary values to relative changes in protein concentration on the Western blots.

A direct interaction between Sam68 and the FMDV IRES is revealed by biochemical assays

Given that Sam68 is a RNA binding protein, we were interested in evaluating whether this protein interacts with viral RNA structural elements within the 5′ and 3′ NTRs of the FMDV genome. To assay these interactions, we produced biotinylated RNA transcripts corresponding to the FMDV S-fragment, IRES, cis-acting replication element (cre), and the 3′ NTR and carried out filter-binding assays. The biotinylated RNA transcripts were mixed with increasing amounts of recombinant full-length Sam68 in the presence of excess tRNA, to control for non-specific RNA-protein interactions, and the mixtures were analyzed by the RNA filter-binding assay (see Materials and methods) probing with streptavidin-HRP. As shown in Fig. 6, a specific protein-RNA interaction was detected between Sam68 and the FMDV IRES. Increasing the concentration of Sam68 retained greater quantities of the biotinylated FMDV IRES. In contrast, Sam68 was unable to retain the S-fragment, cre, or 3′NTR RNA elements at any of the concentrations tested (Figs. 6A, B).

To provide further evidence for the direct interaction between Sam68 and the IRES, we performed biotin-RNA pull-down assays. Biotinylated RNA transcripts were bound to streptavidin paramagnetic beads as described in Materials and methods section and subsequently, the biotinylated RNA-beads mixtures or bead particles alone were incubated with recombinant full-length Sam68 protein in the presence of (100×) excess tRNA to control for non-specific RNA-protein interactions (Fig. 6C). The treated beads were washed several times, and any bound protein was eluted and analyzed by Western blot probing for Sam68. Sam68 was specifically detected when the IRES transcript was present in the reaction mixtures (Fig. 6C). This data suggested that the IRES recruited Sam68 specifically.

FMDV replication is affected by knockdown of Sam68 expression

Since Sam68 relocalized to the cytoplasm during FMDV infection and bound both the viral $3C^{pro}$ and IRES, we examined the role of Sam68 in the FMDV life cycle. Four short-interfering RNA (siRNA) molecules targeted to different regions of the Sam68 gene (numbered 6, 7, 8, and 9) were employed to examine whether a reduction in the cellular level of Sam68 would be inhibitory to viral production. Cells were transfected with individual or combinations of the designated Sam68 siRNA constructs or nonsense negative control (NC) siRNA constructs at equivalent siRNA concentrations and incubated at 37 °C for 72 h. Following siRNA treatment, cell lysates were prepared and analyzed by Western blot probing with anti-Sam68. Cells transfected with siRNA constructs targeted to the Sam68 gene effectively reduced the cellular concentration of Sam68, while NC siRNA constructs had no effect (Fig. 7B). The transfection conditions were evaluated in parallel for relative cytotoxicity by the XTT assay (Fig. 7A). None of the transfection conditions resulted in elevated cytotoxicity, thus allowing us to conclude that results obtained from downstream experiments could not be attributed to interference with cell processes. Of the various combinations tested, a combination of siRNA constructs #7 and #9 and a cocktail of all 4 Sam68 siRNA constructs were the most effective at reducing the concentration of the endogenous protein (Fig. 7B). A combined mixture of all 4 Sam68 siRNA constructs was selected for all subsequent Sam68-targeted siRNA experiments.

Table 1
Predicted FMDV $3C^{pro}$ cut sites within Sam68. The amino acid sequence of Sam68 was displayed in single letter code with the predicted $3C^{pro}$ cut sites in bold and underlined. The NLS at the C-terminus of Sam68 was shaded and underlined. The predicted N-terminal fragments of Sam68 from each cut site were tabulated below showing the position of the cut site, the sequence of the N-terminal fragment, and the estimated molecular weight of the N-terminal Sam68 fragment.

MQRRDDPAARMSRSSGRSGSMDPSGAHPSVR**QT**PSRQPPLPHRSRGGGGGSRGGARASPATQPPPLLPPSATG
PDATVGGPAPTPLLPPSATASVKMEPENKYLPELMAEKDSLDPSFTHAM**QL**LTAEIEKIQKGDSKKDDEENYL
DLFSHKNMKLKERVLIPVKQYPKFNFVGKILGP**QG**NTIKRLQE**ET**GAKISVLGKGSMRDKAKEEELRKGGDP
KYAHLNMDLHVFIEVFGPPCEAYALMAHAMEEVKKFLVPDMMDDICQEQFLELSYLNGVPEPSRGRGVPVR
GRGAAPPPPPVPRGRGVGPPRGALVRGTPVRGAITRGATVTRGVPPPPTVRGAPAPRARTAGIQRIPLPPPPAP**E**
TYEEYGYDDTYAEQSY**EG**Y**EG**YYSQS**QG**DSEYYDYGHGEVQDSYEAYGQDDWNGTRPSLKAPPARPVKG
AYREHPYGRY

$3C^{pro}$ Cut Site	Predicted Sam68 Cuts*	Predicted N-terminal Sam68 Fragment(s)	Predicted Molecular Weight
E/G	2 (380, 383)	MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGGSRGGARASPATQPPPLLPPSATGPDATVGGPAPTPLLPPSATASVKMEPENKYLPELMAEKDSLDPSFTHAMQLLTAEIEKIQKGDSKKDDEENYLDLFSHKNMKLKERVLIPVKQYPKFNFVGKILGPQGNTIKRLQEETGAKISVLGKGSMRDKAKEEELRKGGDPKYAHLNMDLHVFIEVFGPPCEAYALMAHAMEEVKKFLVPDMMDDICQEQFLELSYLNGVPEPSRGRGVPVRGRGAAPPPPPVPRGRGVGPPRGALVRGTPVRGAITRGATVTRGVPPPPTVRGAPAPRARTAGIQRIPLPPPPAPETYEEYGYDDTYAEQSYE-	41 kDa
		MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGGSRGGARASPATQPPPLLPPSATGPDATVGGPAPTPLLPPSATASVKMEPENKYLPELMAEKDSLDPSFTHAMQLLTAEIEKIQKGDSKKDDEENYLDLFSHKNMKLKERVLIPVKQYPKFNFVGKILGPQGNTIKRLQEETGAKISVLGKGSMRDKAKEEELRKGGDPKYAHLNMDLHVFIEVFGPPCEAYALMAHAMEEVKKFLVPDMMDDICQEQFLELSYLNGVPEPSRGRGVPVRGRGAAPPPPPVPRGRGVGPPRGALVRGTPVRGAITRGATVTRGVPPPPTVRGAPAPRARTAGIQRIPLPPPPAPETYEEYGYDDTYAEQSYEGYE-	41 kDa
E/T	2 (189, 363)	MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGGSRGGARASPATQPPPLLPPSATGPDATVGGPAPTPLLPPSATASVKMEPENKYLPELMAEKDSLDPSFTHAMQLLTAEIEKIQKGDSKKDDEENYLDLFSHKNMKLKERVLIPVKQYPKFNFVGKILGPQGNTIKRLQEE-	21 kDa
		MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGGSRGGARASPATQPPPLLPPSATGPDATVGGPAPTPLLPPSATASVKMEPENKYLPELMAEKDSLDPSFTHAMQLLTAEIEKIQKGDSKKDDEENYLDLFSHKNMKLKERVLIPVKQYPKFNFVGKILGPQGNTIKRLQEETGAKISVLGKGSMRDKAKEEELRKGGDPKYAHLNMDLHVFIEVFGPPCEAYALMAHAMEEVKKFLVPDMMDDICQEQFLELSYLNGVPEPSRGRGVPVRGRGAAPPPPPVPRGRGVGPPRGALVRGTPVRGAITRGATVTRGVPPPPTVRGAPAPRARTAGIQRIPLPPPPAPE-	39 kDa
Q/G	2 (179, 390)	MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGGSRGGARASPATQPPPLLPPSATGPDATVGGPAPTPLLPPSATASVKMEPENKYLPELMAEKDSLDPSFTHAMQLLTAEIEKIQKGDSKKDDEENYLDLFSHKNMKLKERVLIPVKQYPKFNFVGKILGPQ-	19 kDa
		MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGGSRGGARASPATQPPPLLPPSATGPDATVGGPAPTPLLPPSATASVKMEPENKYLPELMAEKDSLDPSFTHAMQLLTAEIEKIQKGDSKKDDEENYLDLFSHKNMKLKERVLIPVKQYPKFNFVGKILGPQGNTIKRLQEETGAKISVLGKGSMRDKAKEEELRKGGDPKYAHLNMDLHVFIEVFGPPCEAYALMAHAMEEVKKFLVPDMMDDICQEQFLELSYLNGVPEPSRGRGVPVRGRGAAPPPPPVPRGRGVGPPRGALVRGTPVRGAITRGATVTRGVPPPPTVRGAPAPRARTAGIQRIPLPPPPAPETYEEYGYDDTYAEQSYEGYEGYYSQSQ-	42 kDa
Q/I	0	----------	----------
Q/L	1 (122)	MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGGSRGGARASPATQPPPLLPPSATGPDATVGGPAPTPLLPPSATASVKMEPENKYLPELMAEKDSLDPSFTHAMQ-	13 kDa
Q/M	0	----------	----------
Q/T	1 (32)	MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQ-	3 kDa

[a] Numbers in parentheses represent the amino acid position starting at the N-terminus.

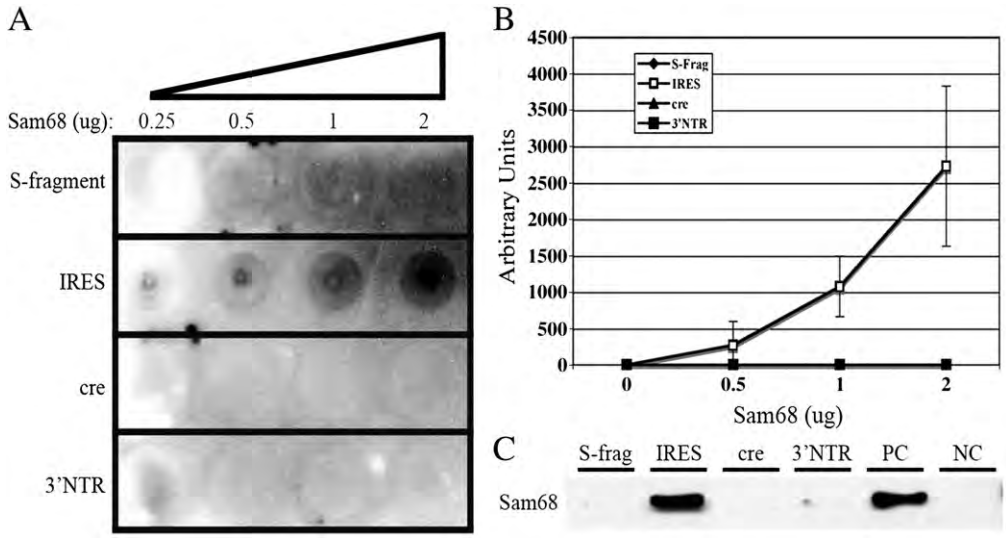

Fig. 6. Sam68 interacts with the FMDV IRES. A. Full-length Sam68 was mixed with biotinylated single-stranded RNA transcripts corresponding to the FMDV S-fragment, IRES, cre, or 3′NTR. Following the previously described RNA filter-binding assay (see Materials and methods), protein-RNA interaction was evaluated by probing the nitrocellulose membrane with SA-HRP. B. Three of the RNA filter binding assays were quantified by densitometry scanning using ImageJ software (NIH) and the values were plotted using Microsoft Excel. C. Biotinylated RNA transcripts of the FMDV S-fragment, IRES, cre, and 3′NTR were separately mixed with SA-coated magnetic beads. Next, they were incubated with full-length Sam68, washed, and the eluates examined by Western blot probing with anti-Sam68. NC, negative control; PC, positive control.

After incubation with Sam68-specific siRNAs, the cells were infected with FMDV and harvested after incubation at 37 °C for 24 h. The levels of endogenous Sam68 were evaluated by Western blot. Again, the combination of 4 Sam68-targeted siRNA constructs effectively reduced the levels of Sam68 relative to NC siRNAs (Fig. 7C). Densitometry scanning was used to quantify the reduction in Sam68 concentration in cells transfected with Sam68-specific siRNAs relative to negative controls, which an approximate 86% drop in Sam68 levels (arbitrary units shown below Western blots in Fig. 7C). The resulting virus titers were evaluated as described in the Materials and methods section. The transfection of Sam68-targeted siRNA molecules reduced the virus titer 1000-fold relative to cells transfected with NC siRNAs (Fig. 7D). These results led us to conclude that Sam68 plays one or more important roles in the life cycle of FMDV, evidenced from the strong effect perturbations in Sam68 expression had on virus titer.

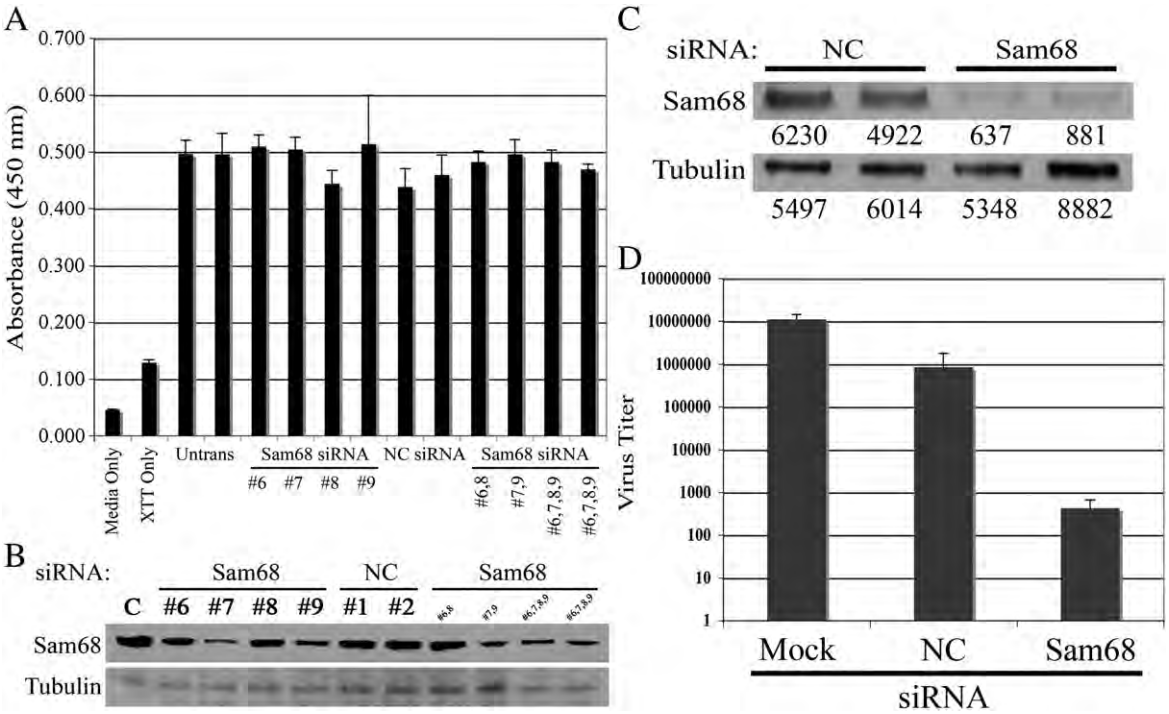

Fig. 7. Reduced Sam68 expression negatively affects progression of FMDV infection. LFBK cells were untransfected (C), transfected with nonsense negative control siRNA (NC), or individual and pooled Sam68-targeted siRNA molecules (#6, 7, 8, and 9) and incubated 72 h at 37°C. Post-incubation, the transfected cells were analyzed by XTT cytotoxicity assay (A) and Western blot probing with anti-Sam68 antibodies (B). Equal loading was confirmed by re-probing with anti-tubulin antibodies. C–D. LFBK cells were transfected with nonsense or all 4 Sam68-specific siRNA molecules, and the cells incubated for 72 h at 37 °C. Post-incubation, the transfected cells were infected with FMDV at an MOI of 0.001 and incubated at 37 °C for 24 h. The resulting samples were evaluated for Sam68 concentration by Western blot (the blot in C represents one replicate experiment with values obtained from densitometry scanning beneath the Western blot bands) and virus growth by plaque assay as previously described (D). The assay was conducted in triplicate.

Reducing Sam68 concentration negatively impacts the activity of the FMDV IRES

Given its interaction with the FMDV IRES and the negative impact of diminished cellular concentration of Sam68 on the outcome of FMDV infection, we investigated if the interaction between Sam68 and the FMDV IRES confers a functional benefit to virus translation. To examine the contribution of Sam68 to IRES-directed translation, we tested the effect of siRNA-induced reduction in Sam68 on the activity of the firefly luciferase (FFLuc) reporter gene under the control of the FMDV IRES within a bicistronic reporter construct (Martinez-Salas et al., 1993) that also contained the chloramphenicol acetyltransferase (CAT) gene under the control of CAP-dependent translation (Fig. 8A). The effect of Sam68 knockdown on the reporter gene expression was evaluated 48 h after co-transfection of the siRNA constructs and the reporter plasmid. By this method, after normalizing against the results of the CAT assay, FFLuc levels dropped approximately 40% when the cellular concentration of Sam68 dropped by two-thirds relative to negative control samples (Figs. 8B, C). The co-transfection approach exhibited negligible cytotoxicity as evaluated via trypan blue exclusion assay and the XTT assay (data not shown). However, given the negative impact observed on virus titer following a reduction in the cellular concentration of Sam68, if Sam68 was exclusively facilitating IRES-driven translation, we expected a greater deficit in reporter gene expression as a result of Sam68 siRNA co-transfection. We suspected that the two nucleic acids (siRNA versus plasmid DNA) may compete for complex formation with the cationic liposome transfection reagent, possibly impairing the effectiveness of the Sam68-specific siRNAs in reducing cellular levels of Sam68.

In an effort to eliminate the hypothesized antagonism between the siRNA constructs and the plasmid DNA and optimize the evaluation of the contribution of Sam68 to FMDV IRES-driven translation, a step-wise triple transfection strategy was devised (Fig. 8D). Cells were transfected with NC siRNA constructs or siRNA constructs targeted to Sam68 and incubated at 37 °C for 72 h. The cells received a siRNA booster transfection to maintain the subversion of Sam68 expression and 8 h later were transfected with the bicistronic reporter plasmid and incubated at 37 °C for 24 h. As evaluated by densitometry scanning, this modified transfection scheme resulted in an approximate 90% reduction in Sam68 concentration regardless of which reporter plasmid was used (Fig. 8E). Consequently, there was an approximate 50% decrease in FMDV IRES activity, as shown from reporter gene expression (Fig. 8F). Given that similar results were

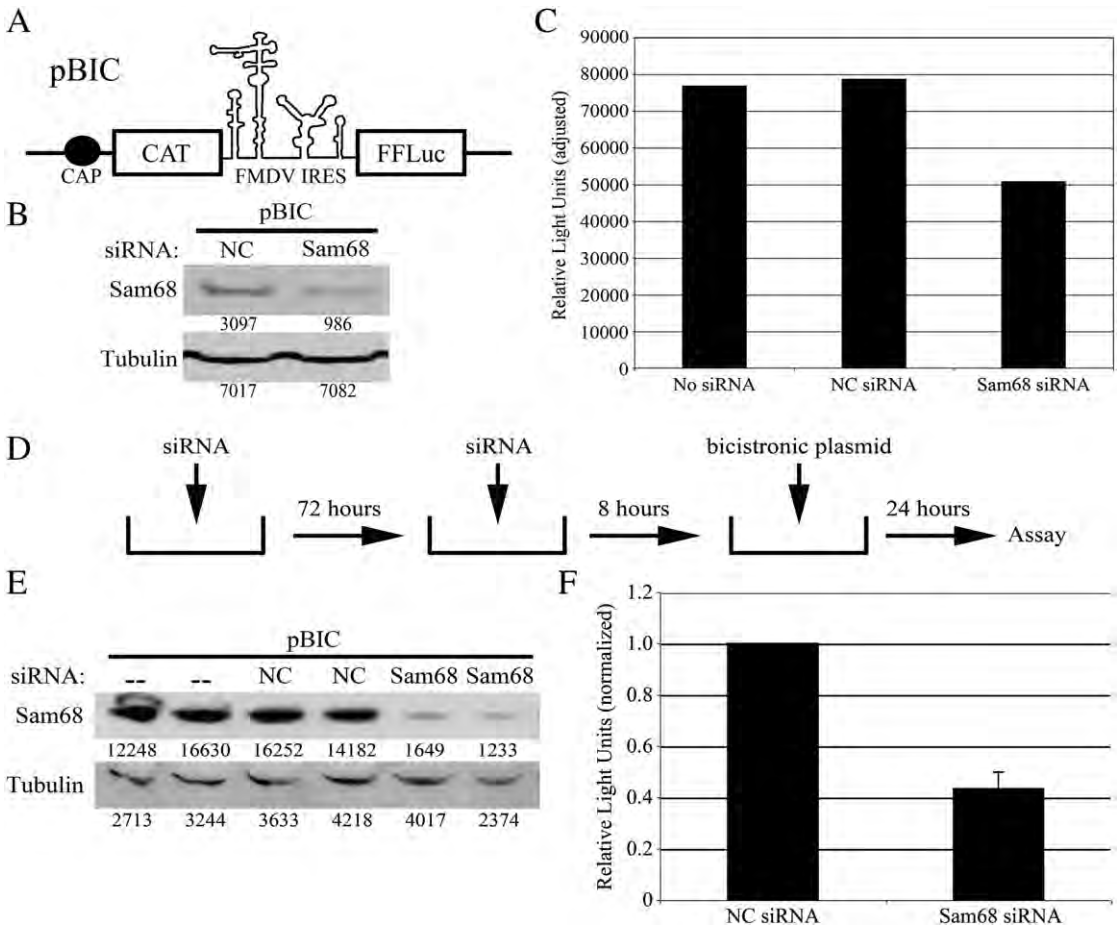

Fig. 8. The cellular concentration of Sam68 affects translation from the FMDV IRES. A. Schematic of the bicistronic reporter plasmid, pBIC with the chloramphenicol acetyltransferase (CAT) gene under control of CAP-dependent translation and the firefly luciferase (FFLuc) gene under control of CAP-independent translation driven by the FMDV IRES. B. LFBK cells were transfected with pBIC simultaneously with Sam68-targeted siRNAs (Sam68 siRNA), nonsense negative control siRNAs (NC siRNA) or with no siRNA (No siRNA) and incubated for 48 h. Harvested cells were evaluated for reduction in Sam68 by Western blot probing with anti-Sam68 followed by anti-tubulin (loading control). C. Harvested cell lysates were also evaluated for FFLuc and CAT activity. Values obtained from the FFLuc assay were normalized against those obtained from the CAT assay, and the adjusted relative light units were plotted using Microsoft Excel. The graph is representative of 1 of 3 independent experiments. D. Overview of the step-wise triple transfection protocol employed to overcome the competition between siRNAs and plasmid DNA during co-transfection. E. Western blot of LFBK cells following the step-wise triple transfection depicted in D, probing with anti-Sam68 followed by anti-tubulin (loading control). F. Harvested cell lysates from the step-wise triple transfection were also evaluated for FFLuc and CAT activity. The values obtained for the FFLuc assay were normalized against the CAT assay values, and the normalized RLUs were plotted. Values shown are the average of 3 separate experiments. Densitometry scanning was used to assign arbitrary values to relative changes in protein concentration on the Western blots (B and E).

generated by both assays, we concluded that Sam68 is making some contribution to the translation of the viral RNA.

Discussion

Several picornaviruses have been shown to encode factors that alter nucleo-cytoplasmic transport to block/inhibit cellular defense mechanisms and promote viral replication. With limited coding capacity, picornaviruses co-opt a variety of cellular proteins during the course of infection, frequently altering their sub-cellular localization (Gustin and Sarnow, 2002; Lin et al., 2009). Consistent with what has been previously observed for enteroviruses and rhinoviruses (Gustin and Sarnow, 2002; McBride et al., 1996), FMDV infection induced the redistribution of Sam68 to the cytoplasm (Fig. 1), though there were a number of differences. Unlike the pronounced degradation that has been observed during enterovirus infections, the subset of Nup proteins, Nup62, Nup98, and Nup153, is not significantly degraded in early stages of FMDV infection when the re-localization of Sam68 is first detected, thus reducing the likelihood of their involvement (Fig. 2) (Park et al., 2008). During PV infection, Sam68 interacts with the viral RNA-dependent RNA polymerase and appears to survive infection intact (McBride et al., 1996), which is consistent with what we detected using another enterovirus: BEV-1. In contrast, we detected the FMDV 3C protease cleavage of the C-terminal NLS of Sam68 (Figs. 3 and 5). This provides a possible mechanism for the shift from the nucleus to the cytoplasm, since the 3CD precursor has been detected in the nucleus and in a juxtanuclear position in FMDV infected cells (Garcia-Briones et al., 2006; O'Donnell et al., 2006).

The C-terminally cleaved Sam68 exhibits a molecular weight below 50 kDa (Fig. 3), presumably allowing it to freely diffuse across the NPC (Talcott and Moore, 1999; Tartakoff and Tao, 2010). Additionally, the enzymatic removal of the NLS prevents the re-importation of cleaved Sam68 to the nucleus, cumulatively shunting Sam68 to the cytoplasm during FMDV infection. This represents a relocalization strategy distinct from what we reported for another nuclear RNA-binding protein during FMDV infection RHA. In this case, a reduction in the level of methylated arginine residues in the C-terminus of RHA initiates the redistribution (Lawrence and Rieder, 2009). The precise $3C^{pro}$ cleavage site or sites within Sam68 have yet to be determined, though several candidates have been identified (Table 1), which is currently being examined. Furthermore, Sam68 represents a previously unidentified cellular substrate of the FMDV $3C^{pro}$, which has been reported to target histone protein H3 for proteolysis (Falk et al., 1990; Grigera and Tisminetzky, 1984; Marquardt, 1993; Tesar and Marquardt, 1990). A C-terminally truncated isoform of Sam68 (Sam68DeltaC) reportedly represses translation of HIV genes by several mechanisms including sequestration of HIV transcripts to perinuclear bundles (PBs) and stress granules (SGs) as well as blocking the association of PABP1 with the 3′ end of the viral transcripts (Henao-Mejia et al., 2009; Marsh et al., 2008; McLaren et al., 2004; Soros et al., 2001). Although, the significance of the FMDV-induced formation of a similar Sam68DeltaC isoform still remains to be determined, the evidence presented indicates that, in addition to binding the FMDV IRES (Fig. 6), Sam68 exerts a positive effect on FMDV translation (Fig. 8). While we have shown that the full-length Sam68 interacts with the FMDV IRES, we are also investigating whether the truncated isoform detected during FMDV infection also interacts with the IRES.

Sam68 can now be added to the list of non-canonical host factors already described to interact with the IRES of various picornaviruses (Belsham and Sonenberg, 2000; Lin et al., 2009). Importantly, the Sam68-FMDV IRES interaction makes some contribution to IRES activity. This can be inferred from the diminished IRES-driven reporter gene expression in cells with reduced cellular levels of Sam68, which was observed by two different methodologies. While the findings from the in vitro assay suggest that Sam68 may enhance the translation of the viral RNA, the significant reduction in virus titer resulting from the decrease in available Sam68 hints that the role of Sam68 in the FMDV life cycle may not be limited to augmentation of viral translation.

Based on the current data, we hypothesize a general model for the "hijacking" of Sam68 by FMDV. Our results are consistent with evidence showing that the FMDV 3CD precursor protein enters the host cell nucleus (Garcia-Briones et al., 2006; O'Donnell et al., 2006) via a putative NLS site in the N-terminus of the $3D^{pol}$ protein (Aminev et al., 2003; Amineva et al., 2004). The 3CD precursor undergoes proteolytic maturation, thus depositing an active FMDV 3C protease in the nuclear compartment. The results showed that $3C^{pro}$ cleaves a C-terminal portion of Sam68 encompassing the NLS. Truncated Sam68 begins to diffuse into the cell cytoplasm, where it eventually encounters FMD viral RNA, and attaches to the IRES triggering an augmentation in the translation of the viral RNA. Many questions remain unanswered, and the model will certainly undergo further refinement. The theoretical cleavage sites within Sam68 (Table 1) have yet to be confirmed, though production of new cDNAs and protein expression systems is currently underway to test this further. The proposed interaction site between Sam68 and the FMDV IRES and the precise mechanism by which Sam68 enhances the FMDV RNA translation both need to be delineated. Furthermore, given the diverse cellular functions attributed to Sam68 as well as the significant reduction in virus amplification in cells previously treated with Sam68 siRNA constructs, it is likely that "hijacked" Sam68 may fulfill multiple distinct roles in the life cycle of FMDV. It is very likely that the FMDV-induced redistribution of Sam68 to the cytoplasm subverts the multiple distinct cellular functions attributed to the protein (see the Introduction). Among these roles, the altered sub-cellular localization might support the inhibition of Sam68 promoted apoptosis (Taylor et al., 2004) would benefit the virus life cycle. These items are all currently being investigated.

In conclusion, we have identified the host protein Sam68 as a new substrate for the FMDV $3C^{pro}$ proteolysis of the C-terminal NLS of Sam68 by $3C^{pro}$ (without simultaneous Nup degradation) that appears to be the predominant mechanism by which FMDV affects its redistribution to the cytoplasm. The data suggest that Sam68 binding to the FMDV IRES during infection could potentially enhance translation of the viral RNA. However, given the multiple distinct cellular roles for Sam68 and the effect that Sam68 knockdown had on FMDV replication, it is plausible that Sam68 might be supportive of more than one aspect of the virus life cycle. Interestingly, this is the first report of Sam68 degradation being attributed to a viral infection and provides a potentially valuable new target for FMD therapy.

Materials and methods

Materials

Qiagen HiPerfect siRNA transfection reagent was purchased from Qiagen (Valencia, CA). Lipofectamine 2000 DNA transfection reagent and streptavidin-coupled magnetic beads were purchased from Invitrogen (Carlsbad, CA). Seize X Protein G coupled beads were purchased from Pierce Biotechnology (Rockford, IL). GST-tagged full length Sam68 was purchased from Abnova (Walnut, CA).

Antibodies

Polyclonal rabbit anti-Sam68 directed against the C-terminus of Sam68 was purchased from both Upstate Laboratories (Billerica, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-Sam68 directed against the N-terminus of Sam68 was purchased from Novus Biologicals (Littleton, CO). Monoclonal mouse anti-Sam68 and polyclonal rabbit anti-histone H3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit

anti-RHA antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Monoclonal mouse anti-Nup antibody was purchased from Covance (Princeton, NJ). Polyclonal rabbit antibodies directed against FMDV non-structural proteins: 2C, 3A, 3B, $3C^{pro}$, and $3D^{pol}$ were generously provided by Dr. Peter Mason and Dr. Marvin Grubman. Goat-anti-rabbit antibodies conjugated to AlexaFluor-488 (AF488) and goat-anti-mouse antibodies conjugated to AlexaFluor-568 (AF568) were purchased from Molecular Probes (Carlsbad, CA).

Viruses, cell lines, and plasmids

FMDV types A_{12} and A_{24} Cruzeiro/Brazil 1955 (A24-Cruzeiro, Gb AY593768) were derived from the infectious cDNA clones pRMC35 and pA_{24}Cru (Rieder et al., 1993, 2005). Isolates of FMDV C3 Resende, SAT2 viruses and bovine enterovirus (BEV1, Gb D00214) were obtained from Drs. Marvin Grubman or Peter Mason, Agricultural Research Service, Plum Island. A24-Cruzeiro carrying a deletion of the leader gene was produced by site directed mutagenesis of full-length genome copy plasmid pA_{24}Cru to produce LL-pA_{24}Cru. Baby hamster kidney strain 21, clone 13 cell line (BHK-21) was maintained in Eagle's basal medium (BME) (Life Technologies, Gaithersburg, MD) supplemented with 10% bovine calf serum (BCS) (Hyclone, South Logan, UT), 10% tryptose phosphate broth, and antibiotic/antimycotic. The LFBK cell line was cultured in 10% fetal calf serum (FCS) in Dulbecco's minimal essential media (DMEM) supplemented with antibiotic/antimycotic (Swaney, 1988). Cells were grown at 37 °C in a humidified 5% CO_2 atmosphere. Expression plasmids pET26b-Ub/$3C^{pro}$ and pET26b-Ub/$3D^{pol}$ encoding FMDV A12 $3C^{pro}$ and $3D^{pol}$ sequences were amplified by PCR primers 5′-GCGGAATTCCCGCGGTGGAAGTGGT GCCCCACCGACC-3′ (plus-strand sequence) and 5′-GCGGAATTCGGAT CCCTCGTGTTGTGGTTCAGGGTC-3′ (minus-strand sequence) and primers 5′-GCGGAATTCCCGCGGTGGAGGGTTAATCGTTGATAC-3′ (plus-strand sequence) and 5′-GCGGAATTCGGATCCTGCGTCACCGCACACGGCGTTCACC C-3′ (minus-strand sequence), respectively. The fragments were digested with *Sac*I and *Bam*HI and then ligated into the same sites of pET26b-Ub (kindly provided by Dr. Craig E. Cameron) (Gohara et al., 1999). The 3ABC segment was amplified from the P3 region of a plasmid encoding wild-type A24-Cruzeiro using primers 5′-GGGCATATGATCTCAATT CCTTCCCAAAAATCCGTG-3′ (plus-strand) and 5′-TGCTCGAGGTGGTGTG GTTCGGGGTCAACGTGTGCCT-3′ (minus-strand), respectively. The 3ABC segment was digested with *Nde*I and *Xho*I and then ligated into pET21b to produce the expression plasmid for 3ABC. The plasmid encoding the $3C^{pro}$-46 inactive mutant was kindly provided by Dr. Marvin Grubman (Bablanian and Grubman, 1993; Grubman et al., 1995). The plasmid encoding the GFP-tagged full-length human Sam68 was kindly provided by Dr. Stephane Richard (Chen et al., 1999).

Plasmid transfection

Mammalian cells were transfected with plasmid DNA using the Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer's protocol. Briefly, cells were grown to approximately 30–40% confluence. The cell culture medium was replaced with serum-free media containing the desired plasmid and Lipofectamine 2000. Cells were incubated with the mixture for 24 or 48 h at 37°C. After which time, they were harvested for examination by Western blot to confirm expression of the desired construct.

Immunofluorescent microscopy

Cells were grown on glass coverslips in 12-well plates until approximately 40% confluence. Control uninfected cells were fixed immediately with 4% paraformaldehyde (PF) in PBS for 10 min at room-temperature (RT) prior to the introduction of virus into adjacent wells. After fixation of the control cells, the remaining wells containing cells on coverslips were infected with wild-type FMDV A24-Cruzeiro at a specified MOI and incubated at 37°C for 1 h. Excess virus was removed by acid washing the cells briefly followed by several rinses with virus growth media (VGM). Designated cells were then fixed with 4% PF, representing a 1 h post-infection (hpi) time point. Remaining cells were provided fresh VGM and incubated for 2, 3, 4, or 5 hpi at 37°C, after which, they were fixed with 4% PF. Cells were washed in PBS, permeabilized with 0.1% Triton-X100 in PBS for 20 min on ice, and blocked with 3% BSA for 30 min at RT. The cells were probed with primary antibodies overnight at 4 °C. The following day the cells were incubated for 1 h at RT with goat-anti-rabbit and goat-anti-mouse conjugated with AF488 or AF568 (Molecular Probes), respectively. The coverslips were washed 3 consecutive times with PBS after each antibody treatment. After the final antibody treatment, the coverslips were air-dried and mounted onto glass slides with ProLong antifade medium supplemented with DAPI stain (Molecular Probes). Cells were examined and images captured using a 100× oil immersion objective on an Olympus fluorescent microscope (Center Valley, PA). Images were refined and figures generated using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). When examining cells transfected with GFP-tagged Sam68, the permeabilization and subsequent antibody steps were omitted. Following fixation and PBS washes, the coverslips of the transfected cells were mounted using ProLong antifade containing DAPI and examined as described above.

Western blot

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out using a 12% Nu-PAGE® pre-cast gel system (Invitrogen). Subsequently the separated proteins were electroblotted onto a nitrocellulose membrane (Sigma). After blocking with 5% milk in PBS-T, specific proteins were detected with primary antibodies at indicated dilutions followed by goat-anti-rabbit or goat-anti-mouse antibodies conjugated with HRP (Sigma). Cellular tubulin, employed as an internal loading control protein, was detected with an HRP-conjugated monoclonal antibody (Tubulin-α, Abcam, Cambridge, MA). The bound HRP conjugate antibodies were reacted with the WestDura SuperSignal chemiluminescent reagent (Pierce) according to the manufacturer's instructions and visualized on X-ray film (X-Omat; Kodak, N.Y., USA).

In vitro protease assay

Recombinant full-length Sam68 (Abnova) was mixed with increasing amounts (1, 2, 5, and 10 μL) of wild-type leader protease (L^{pro}-WT) or an inactive mutant (L^{pro}-Mut). The purified active and inactive recombinant leader protease preparations were kindly provided by Dr. Marvin Grubman. The activity of L^{pro}-WT was confirmed by eIF4G cleavage as previously described (Devaney et al., 1988; Piccone et al., 1995a, 1995b). The mixtures were incubated at 30 °C for 5 h, and then analyzed by Western blot probing with the antibody directed against the C-terminus of Sam68 (Santa Cruz Biotechnology). Equal loading between lanes was confirmed by reprobing the blot with anti-tubulin-HRP (Abcam). The aforementioned assay was repeated using fractionated bacterial cell lysates containing over-expressed wild-type 3C protease ($3C^{pro}$-WT) or an inactive mutant ($3C^{pro}$-46), which were mixed with recombinant full-length Sam68 (Abnova) in increasing amounts (1, 2, 3, 4, and 5 μL). The transformed bacterial cell cultures were kindly provided by Dr. Marvin Grubman. Alternatively, partially purified $3C^{pro}$-WT and $3C^{pro}$-Mut were incubated with LFBK cell lysates for 5 h at 37 °C. Subsequently, the extracts were examined on a Western blot probing with anti-Sam68. The activity of the $3C^{pro}$-WT was confirmed by stripping the blot and re-probing with anti-histone H3 (Santa Cruz Biotechnology). Equal loading was confirmed by probing with anti-tubulin-HRP.

RNA filter binding assay

Viral cDNA corresponding to the sense S-fragment, the internal ribosomal entry site (IRES), the 3′ non-translated region (NTR) and *cis*-acting replication elements (*cre*) (Mason et al., 2002) of the FMDV genome were amplified from plasmid $pA_{24}Cru$ (see above) by using standard PCR with specific oligonucleotides containing the T7 promoter at the 5′end. Biotinylated RNA transcripts of these different regions of the virus genome were produced using biotinylated rNTPs (Roche) and a T7 transcription kit (Ambion, Austin, TX). Various concentrations of recombinant full-length Sam68 (Abnova) was separately mixed with positive-sense fragment RNAs labeled with biotinylated-CTP in binding buffer (5 mM MOPS, 25 mM KCl, 2 mM $MgCl_2$, 5 mM DTT). After mixing at RT for 15 min in the presence of an excess of nonspecific RNA (30 μg tRNA), the protein-RNA complexes were applied to pre-wetted nitrocellulose membranes (0.45 μm pore size, Sigma) overlying a nylon membrane (hybond™-N+, Amersham) as described previously (Yin et al., 2003). Filters were washed twice with buffer (20 mM HEPES-KOH [pH 6.8], 1 mM $Mg(OAc)_2$, 5 mM 2-mercaptoethanol), dried, probed with streptavidin-HRP (SA-HRP) (Pierce), reacted with the SuperSignal WestDura chemiluminescent reagent (Pierce), and detected by Kodak X-Omat films. Prior to the mixing step in this assay, the starting protein concentration of Sam68 was confirmed using the BCA assay (Pierce).

Biotin RNA pull-down assay

Biotinylated FMDV S-fragment, IRES, cre, and 3′NTR RNAs were prepared by in vitro transcription using the Megascript T7 kit (Ambion) with rNTPs supplemented with biotin-11-CTP (Roche). Cell lysates for the pull-down assay were prepared using 1×10^8 LFBK cells suspended in 1 mL of ice cold hypotonic buffer (1 mM HEPES, 10 mM KOAc, and 1.5 mM $Mg(OAc)_2$), which were then lysed by Dounce homogenization. The lysates were centrifuged at 12,000×*g* for 20 min at 4 °C and the recovered supernatant was stored at −80 °C supplemented with 10% glycerol. In the pull-down experiments, 2 μg of biotinylated FMDV RNA was mixed with 30 μL of streptavidin magnetic beads (Invitrogen) resuspended in 1× binding and washing buffer (5 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, and 1 mM NaCl), and incubated at RT for 30 min. Then, 250 μg of cell lysate (25 μL) and 100 μg of tRNA (10 μL) were added to the beads and incubated on ice for 30 min. The beads were pulled-down by a magnet, and the supernatant was removed. The beads were washed three times with 120 μL of B&W, resuspended in 1× SDS sample buffer, heated at 98 °C for 10 min, and the supernatant collected after centrifugation was loaded onto a 12% SDS-PAGE gel followed by Western blot analysis probing with anti-Sam68 (Santa Cruz Biotechnology).

RNA interference

Four validated siRNA constructs targeted to different regions of the Sam68 gene designated Sam68-6, Sam68-7, Sam68-8, and Sam68-9 were purchased from Qiagen (Valencia, CA). These 4 constructs were transfected individually or in specified combinations into LFBK cells using the Qiagen HiPerfect siRNA transfection reagent (Valencia, CA). Briefly, the Sam68-specific siRNA constructs were diluted to a concentration of 10 μM in a mixture of DMEM (Invitrogen) and HiPerfect (Qiagen). Cells grown to 60% confluence in 6-well plates were incubated with specified Sam68 siRNA dilutions for 72 h at 37 °C.

Viral growth, virus-yield inhibition and plaque assays

To assess the effects of the siRNA treatments on virus replication in LFBK cells, monolayers transfected with siRNA cocktails as described above were infected with the specified virus at a MOI of 10^{-3}. Following virus absorption for 1 h, the inoculum was removed and VGM was added. After 24 h at 37 °C, virus-infected cells were harvested and viral titers were determined by plaque assay as previously described (Rieder et al., 1993). Plates were fixed, stained with crystal violet (0.3% in Histochoice; Amresco, Solon, OH), and the plaques counted. The values calculated for the number of plaque-forming units (PFUs) per mL were plotted using Microsoft Excel (Microsoft Corporation, Redmond, WA). All assays were performed in triplicate.

Bicistronic reporter assay

A bicistronic reporter plasmid (pBIC) was generously provided by Dr. Encarnacion Martinez-Salas and is described in Martinez-Salas et al. (1993). Briefly, the plasmid contains two reporter genes: chloramphenicol acetyltransferase (CAT) and firefly luciferase (FFLuc). The CAT gene is downstream of a CAP-dependent promoter, and the FFLuc gene is downstream of the FMDV IRES. *Step-wise triple transfection*: To assess the effects of Sam68 on FMDV IRES activity, LFBK cells were transfected with Sam68-specific siRNA constructs (Qiagen) as described above. Seventy-two hours post-transfection, the cells were delivered a "booster" transfection with the siRNA cocktails as described in the first transfection step and incubated for 8 h. Afterwards, the cells were transfected with pBIC using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Twenty-four hours post-transfection with pBIC, the triple transfected cells were harvested by cell scraping. *Single step co-transfection*: Alternatively, the reporter plasmid (pBIC) and the siRNA constructs were transfected simultaneously using the Lipofectamine 2000 reagent (Invitrogen). Briefly, LFBK cells were grown to a confluency of 80%. Prior to transfection, 1 μg of plasmid DNA with or without siRNA were incubated together at RT in the presence of 5 μL Lipofectamine 2000 (Invitrogen) in OptiMEM (Gibco) per reaction for 20 min. Post-incubation, the reaction mixture was applied dropwise onto 500 μL of cell culture media without antibiotics and OptiMEM (Gibco) was added to a final volume of 1 mL. The final concentration of the siRNA was 1 nM. Forty eight hours post-transfection, the cells were harvested, lysed, and examined for cytotoxicity, reduction in Sam68, CAT activity, and FFLuc activity. Reduction in Sam68 concentration was evaluated as described above. The activity of the CAT reporter gene was quantified by a CAT ELISA assay (Roche) following the manufacturer's protocol. The activity of the FFLuc reporter gene was quantified using the Promega (Madison, WI) dual-luciferase kit in combination with a Veritas luminometer (Promega). The values obtained from the FFLuc assays were normalized against protein concentration (BCA assay, Pierce) and activity scored from the CAT assay. The adjusted relative light units (RLUs) were plotted using Microsoft Excel (Microsoft).

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Bovine Type III Interferon Significantly Delays and Reduces the Severity of Foot-and-Mouth Disease in Cattle

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Interferons (IFNs) are the first line of defense against viral infections. Although type I and II IFNs have proven effective to inhibit foot-and-mouth disease virus (FMDV) replication in swine, a similar approach had only limited efficacy in cattle. Recently, a new family of IFNs, type III IFN or IFN-λ, has been identified in human, mouse, chicken, and swine. We have identified bovine IFN-λ3 (boIFN-λ3), also known as interleukin 28B (IL-28B), and demonstrated that expression of this molecule using a recombinant replication-defective human adenovirus type 5 (Ad5) vector, Ad5-boIFN-λ3, exhibited antiviral activity against FMDV in bovine cell culture. Furthermore, inoculation of cattle with Ad5-boIFN-λ3 induced systemic antiviral activity and upregulation of IFN-stimulated gene expression in the upper respiratory airways and skin. In the present study, we demonstrated that disease could be delayed for at least 6 days when cattle were inoculated with Ad5-boIFN-λ3 and challenged 24 h later by intradermolingual inoculation with FMDV. Furthermore, the delay in the appearance of disease was significantly prolonged when treated cattle were challenged by aerosolization of FMDV, using a method that resembles the natural route of infection. No clinical signs of FMD, viremia, or viral shedding in nasal swabs was found in the Ad5-boIFN-λ3-treated animals for at least 9 days postchallenge. Our results indicate that boIFN-λ3 plays a critical role in the innate immune response of cattle against FMDV. To this end, this work represents the most successful biotherapeutic strategy so far tested to control FMDV in cattle.

The synthesis of antiviral cytokines such as type I interferons (IFN-α/β) is the first cellular response to a virus infection. Recently, a new family of IFN, type III (IFN-λ), has been described in several species, including humans, mice, swine, and chickens (27, 28, 44, 45, 46), and our group has identified, expressed, and characterized a member of the type III IFN family in bovines (boIFN-λ3) (15).

Similarly to IFN-α/β, IFN-λ is rapidly produced within a cell after infection with viruses or intracellular bacteria (25). Compared with type I IFN, type III IFN induces similar innate antiviral responses but signals through very different receptors. IFN-λs mediate their biological activity through a heterodimeric cellular receptor composed of two subunits, interleukin 28B receptor alpha (IL-28Rα) and IL-10Rβ, the last shared by the IL-10 family of cytokines (28, 45). The binding of IFN-λ to its receptor results in activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT), a signal transduction cascade that ultimately induces IFN-stimulated gene (ISG) expression (55). Most cell types express IFN-α/β receptors as well as the IL-10R subunit of the IFN-λ receptor; however, only a limited range of cells express the IL-28Rα receptor subunit, which is specific for signaling only by IFN-λ. In fact, IL-28Rα is significantly expressed in epithelial cells, and as a consequence, cells of epithelial origin in the skin and mucosae respond to treatment with this cytokine (2, 11, 46, 50, 51).

The main biological function of IFN-λ, as well as of the other IFNs, is to inhibit virus replication in virus-infected cells and to protect uninfected cells from virus infection. Such IFN-λ-induced antiviral activity has been demonstrated against many different viruses, most of them replicating primarily in epithelial cells from the respiratory tract, including influenza virus, respiratory syncytial virus, human metapneumovirus, and coronavirus (33). Other viruses, such as hepatitis B and C viruses, HIV, and herpes simplex virus 2, are also sensitive to the activity of IFN-λ (3, 23, 41). Foot-and-mouth disease (FMD) is an acute viral disease of domestic cloven-hoofed animals, including swine, cattle, goats, and sheep, and many wild animals. The etiological agent is the FMD virus (FMDV), a positive-sense single-stranded RNA virus belonging to the *Aphthovirus* genus of the *Picornaviridae* family. FMDV is characterized by a high replication rate, short incubation times, a high level of virus excretion via aerosol, and a high level of contagiousness within susceptible animals. Moreover, FMDV has a high antigenic variation, as displayed by the presence of 7 serotypes (A, O, C, Asia 1, and South African Territories 1, 2, and 3) and multiple subtypes (17, 20). The main natural route of FMDV infection is via aerosol through the upper respiratory tract (1). Experimental infection of cattle by aerosol has shown that the nasopharynx region is the primary site of viral replication, with subsequent spread to pneumocytes in the lungs (4, 37). Moreover, in ruminants, the virus can persist by unknown mechanisms in the pharyngeal region of the upper respiratory tract for many years after the resolution of the acute infection (carrier state), increasing the threat of new FMD outbreaks under certain conditions (47).

In areas where it is enzootic, FMD control is achieved by vaccination with chemically inactivated vaccines formulated with adjuvants (16, 42). Although they are effective, the use of these vaccines presents limitations that have led the World Organization of

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Animal Health (OIE) to impose more severe trade restrictions on countries that opt to vaccinate instead of slaughtering animals (20). These restrictions make FMD-free countries reluctant to use the inactivated vaccine. An alternative vaccine that overcomes some of the limitations of the commercially available inactivated vaccine has been recently developed and is in the pipeline to be approved for commercial use (21). This vaccine uses a replication-defective human adenovirus type 5 (Ad5) to deliver empty FMDV capsids (21, 31). However, neither this live vectored nor the inactivated FMD vaccine is able to confer complete protection prior to 7 days postvaccination (dpv). In an effort to address this limitation, we have previously shown that treatment of swine with Ad5s that express porcine type I IFN (Ad5-poIFN-α/β) resulted in complete protection against FMDV infection when challenge was performed at 1 day postinoculation, and protection lasted for 3 to 5 days (8, 12, 32). Unfortunately a similar approach in cattle only delayed disease by 1 to 2 days and resulted in the appearance of milder clinical signs (52). Our recent studies with boIFN-λ3 demonstrated antiviral activity against FMDV and vesicular stomatitis virus (VSV) in bovine cell culture. Moreover, we showed that treatment of cattle with Ad5-boIFN-λ3 induced systemic antiviral activity and upregulation of ISGs in multiple tissues, including those of the upper respiratory tract and skin (15).

In the current work, we evaluated the efficacy of treatment of bovines with Ad5-boIFN-λ3 against FMDV challenge at 24 h after Ad5 inoculation. Our results demonstrated that treatment with Ad5-boIFN-λ3 alone or in combination with Ad5-poIFN-α resulted in significant delay (6 days) and reduced severity of FMD when animals were challenged by intradermolingual (IDL) inoculation with FMDV. Interestingly, treatment with Ad5-boIFN-λ3 alone resulted in milder and delayed disease for at least 9 days when the challenge was performed by aerosolization of FMDV. These results suggest that type III IFN is a potent biotherapeutic against FMDV in cattle and that its use in prevention and treatment of this feared disease deserves further consideration.

MATERIALS AND METHODS

Cell and viruses. Human 293 cells (ATCC CRL-1573) were used to generate and propagate recombinant Ad5s. Embryonic bovine kidney (EBK) and porcine kidney (IB-RS2) cells were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at Plum Island Animal Disease Center (PIADC), Greenport, NY. Baby hamster kidney cells (BHK-21, clone 13, ATCC CL-10) were used for propagation and titration of FMDV serotype A24. Bovine kidney LF-BK cells (48) were used for titration of FMDV serotype O1 Manisa. MDBK-t2 cells (Madin-Darby bovine kidney cells transfected with plasmid expressing the human MxA promoter linked to a chloramphenicol acetyltransferase [CAT] reporter and with resistance to blasticidin [18]) were kindly provided by B. Charleston (Institute for Animal Health, Pirbright, United Kingdom). All cells were maintained in Eagle's minimal essential medium (EMEM) containing either 10% calf serum or 10% fetal bovine serum (FBS) and supplemented with antibiotics, glutamine, and nonessential amino acids. MDBK-t2 medium was also supplemented with 10 μg/ml blasticidin (Invitrogen, Carlsbad, CA). Replication-defective recombinant Ad5s containing type I IFN, bovine and porcine IFN-α, boIFN-λ3, and vector control Ad5-Blue (empty vector, negative control) were produced as previously described (15, 32, 52). For cattle challenge, FMDV A24 (strain Cruzeiro, Brazil, 1995), a gift from A. Tanuri, Federal University of Rio de Janeiro, Brazil, was obtained from vesicular lesions of the coronary band of an FMDV A24-infected steer. The challenge virus FMDV O1 Manisa was obtained from macerated tongue epithelia of two experimentally infected cattle (37). The 50% bovine infectious doses (BID_{50}) of both FMDV strains were determined in bovines by inoculation of multiple dilutions intradermally in the tongue (22). FMDV titers were determined by standard plaque assay on BHK-21 cells for A24 and on LF-BK cells for O1 Manisa. Alternatively, FMDV RNA levels were determined by quantitative real-time PCR (6).

Determination of boIFN-λ3 biological activity against FMDV serotype O1 Manisa *in vitro*. The biological activity of recombinant boIFN-λ3 was measured in EBK cells as previously described (15). Briefly, EBK cells were treated with different dilutions of supernatants from IB-RS2 cells infected with Ad5-boIFN-λ3 which had been centrifuged to remove virus particles. Twenty-four hours later, treated cells were washed with phosphate-buffered saline (PBS) and infected with FMDV O1 Manisa at a multiplicity of infection (MOI) of 1. One hour after the infection, cells were washed with 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6) for 5 min, followed by an incubation in cell culture medium for an additional 7 h. Supernatants were then collected, and virus yield was measured on LF-BK cells by standard plaque assay (9). Results were expressed as $\log_{10}$ PFU/ml of supernatant.

Animal experiments. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Plum Island Disease Center. Animals were held for at least 1 week for acclimatization before the start of experiments and were kept under strict controlled conditions at the PIADC biosafety level 3 animal facilities. Two different experiments using a total of 26 Holstein steer calves, of about 400 lb each, were performed to evaluate the efficacy of boIFN-λ3 *in vivo*.

Experiment I: efficacy of Ad5-boIFN-λ3 and Ad5-poIFN-α against FMDV intradermolingual challenge. Twelve animals divided in 4 groups of 3 animals each were intramuscularly (i.m.) immunized in the neck with 2 ml of recombinant adenoviruses as follows: one group was inoculated with 10^{11} PFU/animal of Ad5-poIFN-α, one group was treated with 10^{11} PFU/animal of Ad5-boIFN-λ3, and two groups were treated with a combination of Ad5-boIFN-λ3 and Ad5-poIFN-α at a dose of 10^{11} PFU/animal ("high-dose combination") or 5×10^{10} PFU/animal ("low-dose combination") of each Ad5. Two other animals were inoculated with 2×10^{11} PFU Ad5-Blue as control group. One day after the Ad5 inoculation (1 day postinoculation [dpi]), all animals were challenged with 10^4 BID_{50} FMDV A24 Cruzeiro by IDL inoculation at 4 sites (100 μl/each). Rectal temperatures were monitored daily. Clinical signs of FMD were evaluated at 0, 2, 4, 6, and 8 days postchallenge (dpc), equivalent to 1, 3, 5, 7, and 9 dpi, respectively. A clinical score of 1 to 5 was assigned to describe the severity of the disease, with a maximal score of 5 (a score of 1 was assigned when one or more vesicular lesions were detected in one foot, a score of 2 was assigned if two feet had vesicular lesions, a score of 3 was assigned if three feet had vesicular lesions, etc., and an additional score of 1 was assigned if lesions were found in the mouth at other than the inoculation site). Animals were considered protected when no clinical signs of disease were detected during the course of the experiment. Animals were bled daily from the day of the Ad5 inoculation, −1 dpc, until 7 dpc and at 14 and 21 dpc for viremia, serology analysis, and antiviral activity determination. Nasal swabs were obtained daily from 0 until 7 dpc for measuring FMD viral loads and antiviral activity.

Experiment II: efficacy of Ad5-boIFN-λ3 and Ad5-boIFN-α against FMDV aerosol challenge. Twelve animals divided in 4 groups of 3 animals each were subcutaneously (s.c.) inoculated with 4 ml of recombinant Ad5s at 2 sites in the neck as follows: two groups were inoculated with either 1.5×10^{11} PFU/animal of Ad5-boIFN-α or Ad5-boIFN-λ3, a third group was inoculated with a combination of 7.5×10^{10} PFU/animal each of Ad5-boIFN-λ3 and Ad5-boIFN-α, and the fourth group, a control group, was inoculated with 4 ml of PBS/animal. The control group was inoculated with PBS based on previous experiments where this treatment gave the same results as treatment with the control vector Ad5-Blue or Ad5-Null (12, 13). Twenty-four hours later, all animals were challenged with 10^7 BID_{50} of FMDV O1 Manisa in a total volume of 2 ml by aerosol exposure for 12 min as previously described by Pacheco et al. (37). Ani-

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mals were monitored for clinical signs at 0, 3, 5, 7, 9, and 12 dpc, and blood and nasal swabs were collected for analyses as described for experiment I. The clinical score was evaluated as in experiment I, but detection of one or more lesions in the mouth was always scored as 1. The complete blood count (CBC) was analyzed in a Hemavet 950 analyzer (Drew Scientific, Waterbury, CT). Animals were kept for 3 weeks postchallenge and humanely euthanized according to IACUC guidelines.

Determination of antiviral activity in sera and nasal swabs. MDBK-t2 cells were seeded into 24-well tissue culture plates at 2×10^5 cells/well, and after 24 h of incubation at 37°C and 5% CO_2, the culture medium was replaced with 0.25 ml of medium containing 0.1 ml of either the serum samples or the nasal swab secretions. Known amounts (from 1.95 to 1,000 U/ml) of recombinant human IFN-α2A were used as a standard (PBL Interferon Source, Piscataway, NJ). At 24 h after incubation, cells were lysed for 30 min in lysis buffer and CAT expression was determined from the cell extracts using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Sciences, Indianapolis, IN) in accordance with the manufacturer's protocol. This method has been previously validated by Fray et al. (18). Units of antiviral activity per ml of the samples were calculated from a standard curve using recombinant human IFN-α2A.

Detection of anti-FMDV neutralizing antibodies. Serum samples from 0, 4, 7, 14, and 21 dpc were heat inactivated (30 min, 56°C) and used in a microtiter neutralization assay on LF-BK cells. Serial dilutions of serum were incubated with 100 50% tissue culture infective doses ($TCID_{50}$) of FMDV O1 Manisa for 1 h at 37°C and 5% CO_2, followed by infection of monolayers of LF-BK cells in 96-well plates for 72 h. Titers were calculated as the reciprocal of the highest dilution of serum that neutralized the virus in 50% of the wells.

Analysis of gene expression by qRT-PCR. The expression of IFN and ISGs was determined in purified peripheral blood mononuclear cells (PBMCs). PBMCs from −1, 0, 1, and 2 dpc were purified by density gradient centrifugation on Ficoll-Hypaque (density, 1.007 g/liter) and lysed in RLT buffer (Qiagen, Valencia, CA). Total RNA was isolated using an RNeasy kit (Qiagen) following the manufacturer's protocol, and gene expression was measured by quantitative real-time PCR (qRT-PCR) in an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA) using primers and probes previously described (15). Relative mRNA levels were determined by comparative threshold cycle (C_T)analysis (User Bulletin 2; Applied Biosystems), utilizing as a reference the samples at 0 dpi and GADPH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression as internal control.

Statistical analysis. Differences in antiviral activities, neutralizing antibodies, and percentages of lymphocytes in blood were analyzed by the Student *t* test using GraphPad Prism software version V5.04 (GraphPad Software, Inc., San Diego, CA).

RESULTS

boIFN-λ3 displays antiviral activity against FMDV O1 Manisa *in vitro*. We had previously demonstrated that boIFN-λ3 displays antiviral activity against VSV and FMDV A24 in cell culture. In both cases the antiviral activity of the IFN-λ3 was dose dependent, and when it was combined with IFN-α, an additive effect was observed (15). In the current study, we tested the bioactivity of boIFN-λ3 alone or in combination with bovine type I IFN, boIFN-α, in EBK cells but using FMDV O1 Manisa. We tested this serotype because we anticipated that we would perform an *in vivo* experiment using the aerosol inoculation model recently optimized at Plum Island Animal Disease Center, which had given very consistent results, particularly with FMDV O1 Manisa (37). Similarly to our previous studies, treatment of EBK cells with boIFN-λ3 or boIFN-α alone (from 0 to 4 U) reduced the FMDV O1 Manisa titer in a dose-dependent manner from 5.5×10^5 to 2.0×10^4 PFU/ml, with statistically significant differences among

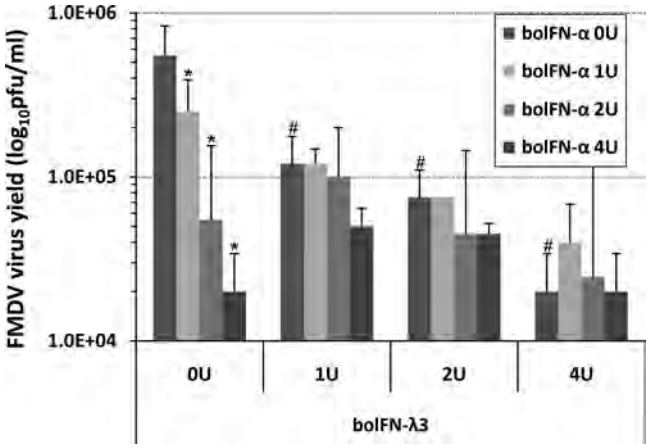

FIG 1 Antiviral activities of boIFN-λ3 and boIFN-α proteins. EBK cells were incubated with acid-treated or filtered supernatants of Ad5-boIFN-α- or Ad5-boIFN-λ3-infected IB-RS2 cells. After 24 h, cells were challenged with FMDV serotype O1 Manisa at an MOI of 1. The virus yield at 24 h postinfection was titrated in LF-BK cells. Each data point represents the mean and standard error of a representative assay performed in triplicate. * and #, statistically significant difference for cells treated with 1, 2, or 4 U of boIFN-α (*) or 1, 2 or 4 U of boIFN-λ3 (#) with respect to no treatment ($P < 0.05$).

the tested concentrations ($P < 0.05$) (Fig. 1). In contrast, the combination of both IFNs did not show a clear additive effect at the tested concentrations. No statistically significant differences were detected when the effects of the various combinations of IFNs were compared to that of each IFN alone. We concluded that as for FMDV A24, boIFN-λ3 and boIFN-α display antiviral activity against FMDV O1 Manisa in bovine cell culture.

Inoculation of cattle with Ad5-boIFN-λ3 results in delayed disease after IDL challenge with FMDV A24. In our previous studies, we demonstrated that inoculation of cattle with Ad5-boIFN-λ3 results in systemic antiviral activity and induction of ISGs. We therefore examined the protective effect of Ad5-boIFN-λ3 treatment, alone or in combination with Ad5-IFN-α, against FMDV infection in cattle. We decided to use Ad5-poIFN-α because in earlier experiments, a slightly higher antiviral activity had been detected when steer calves were inoculated with Ad5-poIFN-α than when inoculation was with Ad5-boIFN-α (52), despite the similar antiviral activities measured in bovine and porcine cultured cells (7). The experimental design is described in Materials and Methods. As shown in Fig. 2, FMD clinical signs appeared the earliest in control animals (Ad5-Blue group), by 2 dpc, and reached the maximum clinical score of 5 by 6 dpc. Elevated temperature (≥40°C) was observed in both animals by 1 dpc and continued intermittently until 4 to 5 dpc, while viremia and nasal shedding were detected between 1 and 4 dpc. All animals of the Ad5-poIFN-α group showed a 2-day delay in the appearance of FMD clinical signs compared to the control group, but by 4 dpc they had high scores (4 or 5), with elevated temperatures after 2 dpc. High viremia was detected between 2 and 4 dpc. Viral shedding in nasal swabs was detected in 2 out of 3 animals between days 3 and 5, although one animal remained negative throughout the experiment. Similar results for viremia and nasal shedding were found in the "low-dose combination" group. However, 2 out of 3 animals showed a delay in the appearance of FMD compared to the control and the Ad5-poIFN-α groups. Moreover, the severity of the disease in the "low-dose combination" group

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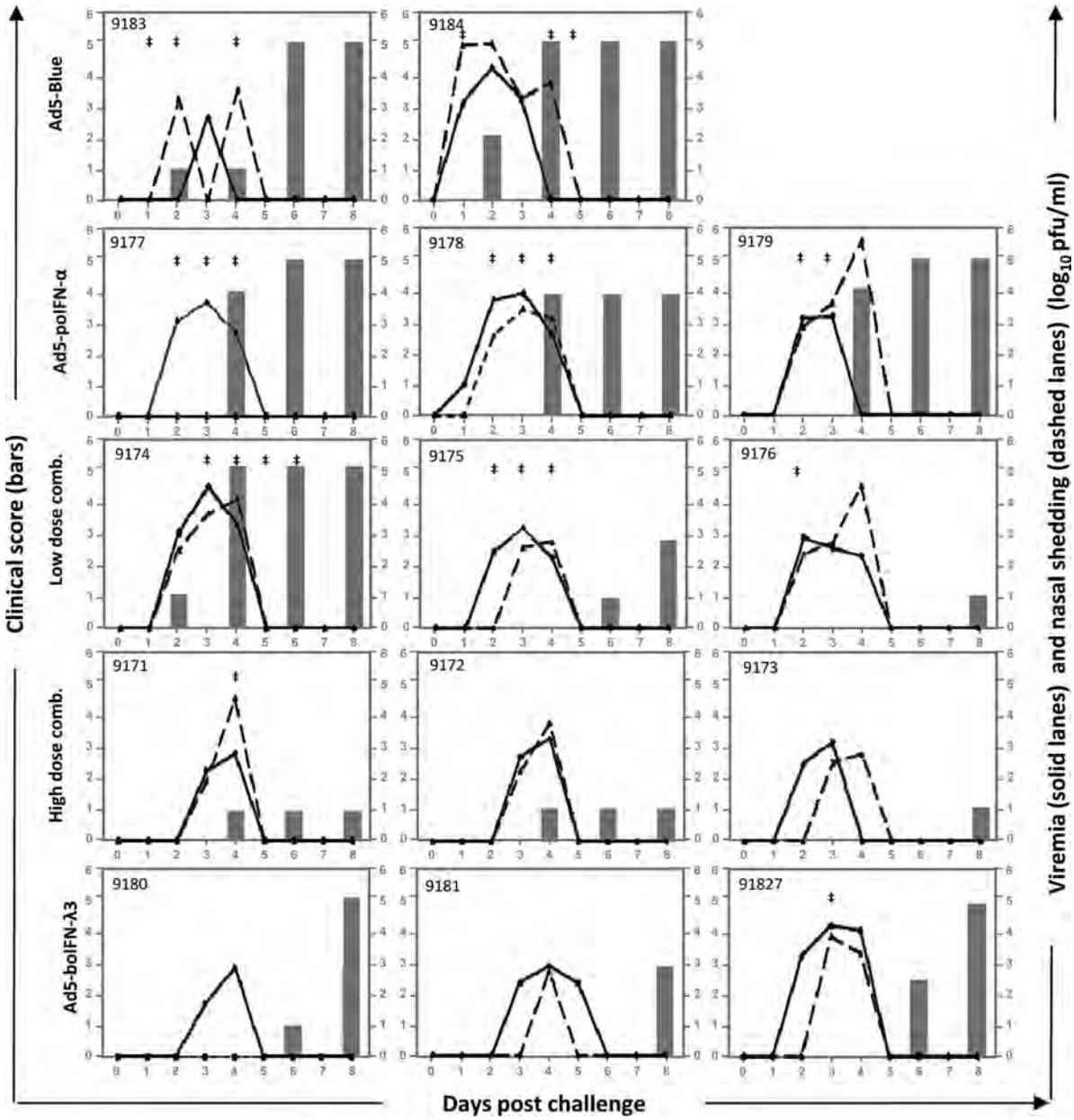

FIG 2 Experiment I, clinical outcome. The efficacy of Ad5-boIFN-λ3 and Ad5-poIFN-α against IDL challenge with FMDV was evaluated. Cattle treated with Ad5-Blue (2×10^{11} PFU), Ad5-poIFN-α (1×10^{11} PFU), Ad5-boIFN-λ3 (1×10^{11} PFU), the "low-dose combination" (5×10^{10} PFU Ad5-poIFN-α and 5×10^{10} PFU Ad5-boIFN-λ3), or the "high-dose combination" (1×10^{11} PFU Ad5-poIFN-α and 1×10^{11} PFU Ad5-boIFN-λ3) were IDL challenged with 10^4 BID_{50} of FMDV A24 Cruzeiro at 24 h posttreatment. The maximum clinical score (bars) is 5. Viremia (solid lines) and nasal shedding (dashed lines) are expressed as log_{10} PFU/ml of serum or nasal secretion. ‡, detection of high temperature (rectal temperature of ≥40°C).

was reduced; 2 out of 3 animals had clinical scores of only 1 and 3 by 8 dpc. In the "high-dose combination" group, 2 out of 3 animals showed a very low clinical score (1) at 4 dpc, and one animal remained free of FMD until 8 dpc. By 8 dpc, all 3 animals had vesicles only on the tongue, at other than the challenge site. Remarkably, all animals inoculated with only Ad5-boIFN-λ3 had a considerable delay in the appearance of FMD compared with the control animals and the other vaccinated groups. Similar to the "high-dose combination" and "low-dose combination" groups, one animal did not show clinical signs until day 8. In general, viremia was delayed until 2 to 3 dpc, and viral shedding was delayed until 3 to 4 dpc, continuing for only one additional day in the Ad5-boIFN-λ3 and "high-dose combination" groups. One animal from the Ad5-boIFN-λ3 group remained negative for viremia throughout the experiment. Overall, most animals in the Ad5-boIFN-λ3 and "high-dose combination" groups developed less severe clinical signs, viremia, and nasal shedding than those in the other three groups. Elevated rectal temperatures were observed in all animals in the Ad5-poIFN-α and "high-dose combination" groups at a time when these animals presented FMD clinical signs (between 2 and 4 dpc) and lasted for approximately 2 to 3 days. However, only one animal in each of the Ad5-boIFN-λ3 and

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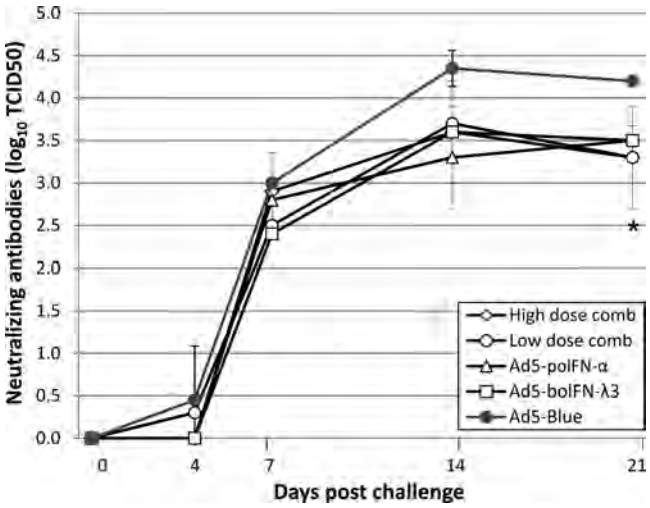

FIG 3 Experiment I, neutralizing antibody titer. The presence of FMDV-neutralizing antibodies was detected by a microtiter neutralization assay on LF-BK cells in sera of animals treated as described for Fig. 2 at 0, 4, 7, 14, and 21 days after IDL challenge with FMDV A24 Cruzeiro. Titers are reported as the log_{10} of the reciprocal of the highest dilution of serum that neutralized the virus in 50% of the wells. Each data point represents the mean ± standard deviation (SD) of each group. *, statistically significant difference between groups ($P < 0.05$).

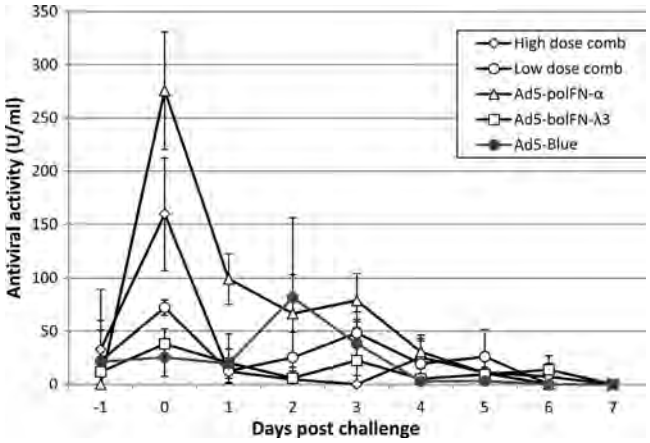

FIG 4 Experiment I, antiviral activity. The relative antiviral activity in sera of animals treated as described for Fig. 2 was measured by an Mx-CAT ELISA from −1 to 7 dpc. MDBK-t2 cells were treated for 24 h at 37°C with 0.1 ml of sera from all animals. CAT enzymatic activity was determined in cell lysates using a commercially available kit. Results are expressed as relative units of antiviral activity/ml of serum with respect to purified standard type I IFN. Each data point represents the mean (± SD) for each group.

"high-dose combination" groups had elevated temperature for 1 day.

All animals developed neutralizing antibodies against FMDV by 7 dpc, with values that increased by 14 dpc. The neutralizing-antibody titers at 21 dpc were statistically significantly lower ($P < 0.05$) in all the IFN-treated animals than in the Ad5-Blue-inoculated animals, consistent with reduced FMDV infection (Fig. 3).

With respect to antiviral activity, all IFN-treated animals showed measurable levels of antiviral activity in serum 24 h after the Ad5 inoculation (0 dpc) which declined substantially by 1 dpc (Fig. 4). Animals inoculated with Ad5-poIFN-α, alone or in combination, showed the highest levels of antiviral activity, in a dose-responsive manner. However, we could not detect any direct correlation between the levels of systemic antiviral activity and the lag in appearance of disease. Although low levels of systemic antiviral activity were detected in the animals treated with Ad5-boIFN-λ3, disease was delayed until 6 to 8 dpc. In contrast, Ad5-poIFN-α-inoculated animals, which showed the highest levels of antiviral activity in serum by 1 dpi, developed disease by 4 dpc, with high clinical scores. The control group showed some levels of systemic antiviral activity at 2 and 3 dpc, concurrent with the peak of viremia. No measurable antiviral activity was observed in nasal swabs at any time point (data not shown).

These results suggested that treatment of cattle with Ad5-boIFN-λ3 alone or in combination with Ad5-poIFN-α caused delayed and reduced severity of disease after IDL challenge with FMDV.

Inoculation of cattle with Ad5-boIFN-λ3 significantly delays disease after aerosol challenge with FMDV. Given that several reports have demonstrated that IFN-λ renders epithelial cells of the respiratory tract resistant to viral infections (33) and that FMDV replicates primarily in epithelial cells of the upper respiratory tract after natural infection or aerosol exposure (4, 37, 39), we decided to test the efficacy of Ad5-boIFN-λ3 in cattle after an aerosol challenge with FMDV. As mentioned above, we used the O1 Manisa strain of FMDV because the aerosol method has been well standardized with this serotype (37). Since no protection was found in the previous experiment using Ad5-poIFN-α, in this experiment we used Ad5-boIFN-α, whose potency in cattle had also been tested earlier (52). One day after the inoculation, all groups were challenged with 10^7 BID_{50} of FMDV O1 Manisa by the aerosol route.

(i) Clinical outcome. A summary of the clinical data is displayed in Fig. 5. All animals from the control group showed the maximum clinical score of 5 by 3 dpc. Similarly, all animals inoculated with Ad5-boIFN-α developed vesicles at 3 dpc, reaching a maximum score at 5 dpc. In contrast, animals treated with Ad5-boIFN-λ3, either alone or in combination with Ad5-boIFN-α, showed a clear delay in the appearance of FMD. Within the combination group, 2 out of 3 animals were negative for vesicular lesions at 5 dpc and 1 animal remained negative until 12 dpc. Most interestingly, animals inoculated with Ad5-boIFN-λ3 alone did not develop clinical signs until 9 dpc, and 1 out of the 3 animals in the group remained free of FMD throughout the experiment (12 dpc). Consistent with the appearance of clinical signs, animals of the control and Ad5-boIFN-α groups were positive for FMDV in blood and nasal swabs by virus isolation at 2 and 3 dpc, respectively, and most of them remained positive for additional days. Two of the three steer calves from the combination group were positive for viral shedding in nasal swabs, although at later times postchallenge (4 to 8 dpc). Two of these animals had no detectable viremia. Interestingly, none of the animals in the Ad5-boIFN-λ3 group was positive for viremia or viral shedding in nasal swabs at any time point after the challenge as determined by virus isolation. In order to confirm these results, we measured the levels of viral RNA in serum and nasal swabs by a more sensitive method, qRT-PCR (Fig. 6A). Threshold cycle (C_T) values of 40 or less were considered positive. Animals from the control and Ad5-boIFN-α groups were viral RNA positive as early as 2 dpc and continued to be positive for 3 to 4 consecutive days. Animals from the combination group were positive for FMDV in serum from 3 to 5 dpc.

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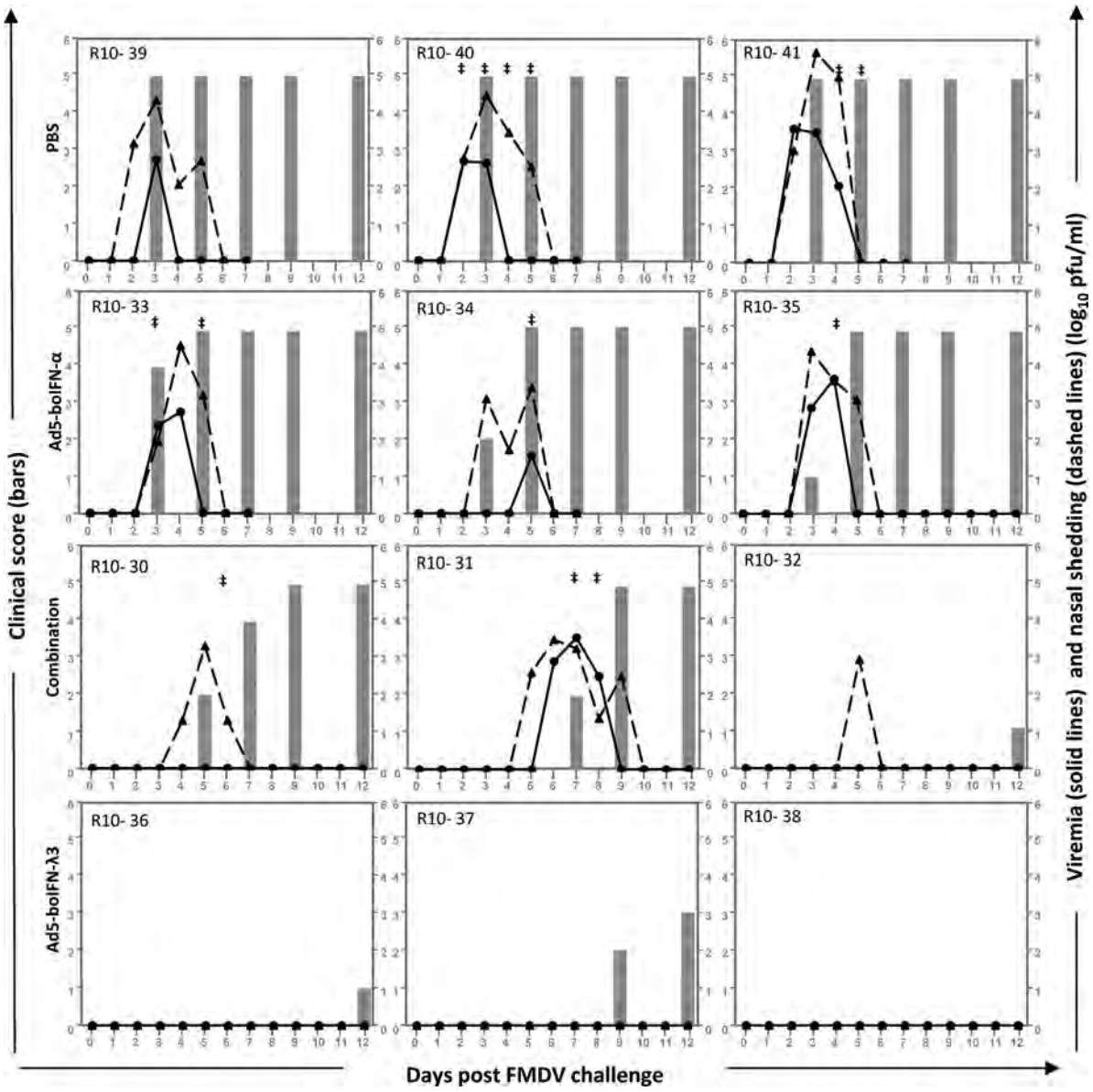

FIG 5 Experiment II, clinical outcome. The efficacy of Ad5-boIFN-λ3 and Ad5-boIFN-α against aerosol challenge with FMDV was evaluated. Animals treated with PBS, Ad5-boIFN-α (1.5×10^{11} PFU), Ad5-boIFN-λ3 (1.5×10^{11} PFU), or a combination (7.5×10^{10} PFU Ad5-boIFN-α and 7.5×10^{10} PFU Ad5-boIFN-λ3) were aerosol challenged with 10^7 PFU of FMDV O1 Manisa at 24 h posttreatment. The maximum clinical score (bars) is 5. Viremia (solid lines) and nasal shedding (dashed lines) are expressed as $\log_{10}$ PFU/ml of serum or nasal secretion. ‡, detection of high temperature (rectal temperature of ≥40°C).

Animals inoculated with Ad5-boIFN-λ3 only had detectable viral RNA by 5 dpc, which disappeared by day 6 in 2 out of the 3 animals of the group.

The percentage of lymphocytes in total white blood cells was calculated. As shown in Fig. 6B, the percentage of lymphocytes decreased by 3 dpc in animals of the control and Ad5-boIFN-α groups. Despite the high individual variation, this decrease was statistically significant ($P < 0.05$) in the PBS group at 3 and 4 dpc relative to −1 dpc. The percentage of lymphocytes recovered by 6 dpc.

(ii) Neutralizing-antibody response. The levels of neutralizing antibodies were consistent with the delay in the appearance of clinical signs and viremia. As seen in Fig. 6C, the Ad5-boIFN-λ3 group, which showed the longest delay in disease onset or no disease during the course of the experiment, had lower levels of anti-FMDV neutralizing antibodies than the control, Ad5-boIFN-α, and combination groups, with statistically significant differences by 21 dpc ($P < 0.05$). Consistently, the combination group, which showed clinical signs earlier than the Ad5-boIFN-λ3 group, displayed intermediate levels of neutralizing antibodies by 7 dpc; however, by 14 and 21 dpc these animals reached levels similar to those for the control and Ad5-boIFN-α groups.

(iii) Antiviral activity in sera and nasal swabs. The levels of systemic antiviral activity in all animals were measured using an Mx1-CAT ELISA (18). All Ad5-IFN-treated animals showed the highest levels of antiviral bioactivity in sera before challenge (1 dpi

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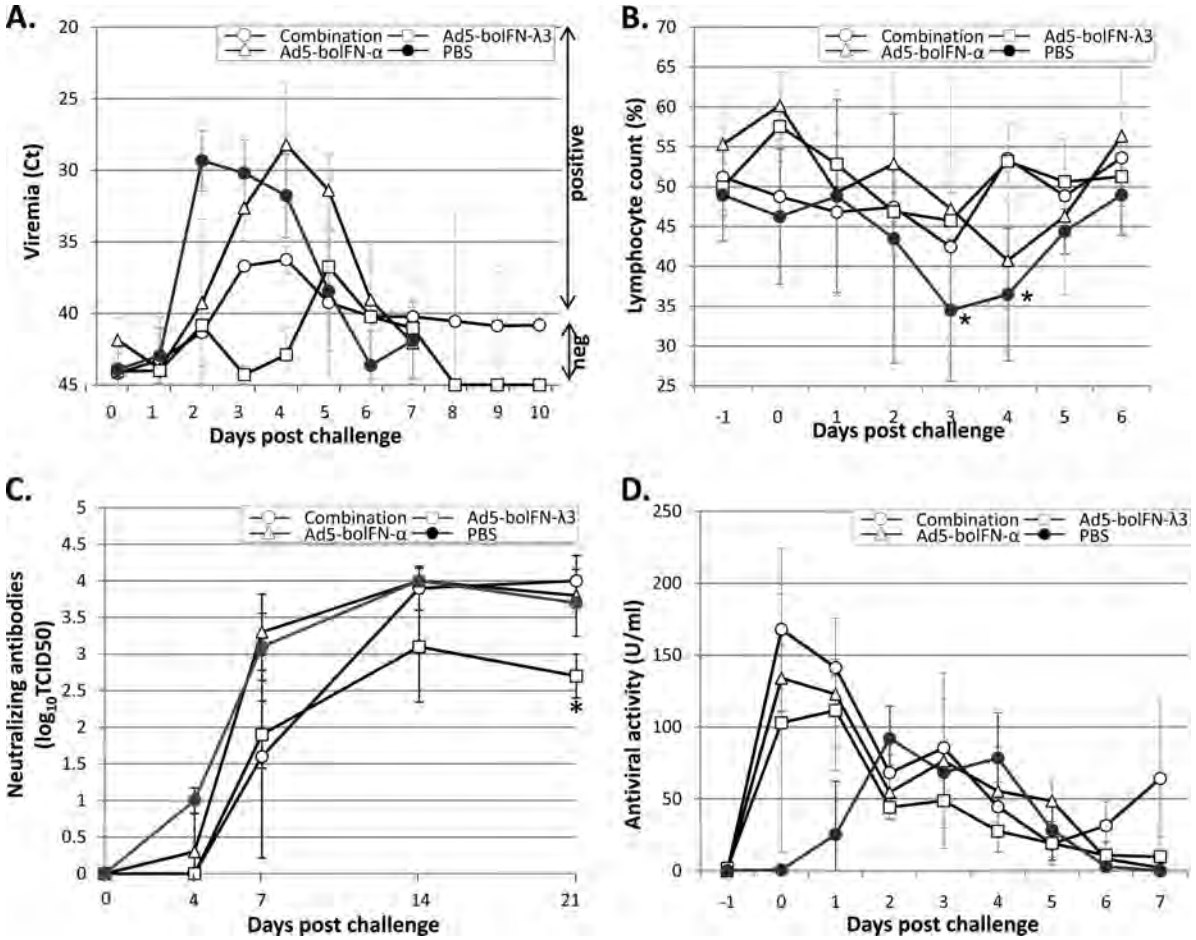

FIG 6 Experiment II. (A) Viremia determined by qRT-PCR. FMDV RNA was detected by RT-PCR in all animals daily from 0 to 10 dpc. C_T values of 40 or lower were considered positive. (B) Lymphocyte count. The percentage of lymphocytes in whole blood was determined. Cells were counted in a Hemavet 950 analyzer and expressed as the percentage of lymphocytes with respect to the total number of white cells. *, statistical significance within a group relative to −1 dpc. (C) Neutralizing antibody titer. The presence of FMDV-neutralizing antibodies was detected by a neutralization assay on LF-BK cells. Titers are reported as $\log_{10}$ of the reciprocal of the highest dilution of serum that neutralized the virus in 50% of the wells. *, statistically significant differences between groups ($P < 0.05$). (D) Antiviral activity. The relative antiviral activity in serum was measured by an Mx-CAT ELISA. MDBK-t2 cells were treated with sera from all animals, and after 24 h at 37°C, CAT enzymatic activity in cell lysates was determined. Results are expressed as relative units of antiviral activity/ml of serum with respect to purified standard type I IFN. In all panels, each data point represent the mean (± SD) of each group.

or 0 dpc) or at 1 dpc (Fig. 6D). These levels declined with time and mostly disappeared by 6 dpc. However, different dynamics were observed in the group inoculated with PBS. Control animals developed antiviral bioactivity levels by 1 dpc with a peak between 2 and 4 dpc, correlating with the levels of viremia. By 6 dpc, the levels of antiviral activity returned to baseline values (−1 dpc). As observed in experiment I, no correlation between the levels of systemic antiviral activity before challenge and the delay of disease observed thereafter was detected. In fact, animals treated with Ad5-boIFN-λ3, which displayed the longest delay of FMD onset, had the lowest levels of systemic antiviral activity at 0 dpc, 24 h after Ad5 inoculation In contrast, all animals in the Ad5-boIFN-α-treated group, which had slightly higher levels of antiviral activity at 0 dpc, developed disease by 3 dpc, with the highest clinical scores (4 or 5). The combination group also showed higher levels of antiviral activity than the control group, but again no correlation with the delay or severity of disease was observed. We did not detect any antiviral activity in nasal swabs at any time point (data not shown).

(iv) Expression of ISGs in PBMCs. It is known that both type I and type III IFNs induce the expression of ISGs, resulting in antiviral functions (3, 55). Some of these ISGs include those for chemokines (such as CCL2, CCL3, CCL20, and CXCL10), those for activators of the IFN pathway (IRF7, MDA5, and RIGI) and genes with known antiviral activity (ISG15, Mx1, OAS1, and PKR). We measured the levels of these ISGs along with IFN-β and IFN-λ3 by qRT-PCR using mRNA extracted from PBMCs at −1, 0, 1, and 2 dpc. We also examined the expression of the type III IFN receptor subunits, IL-28Rα and IL-10Rβ. Results were expressed as the group average relative fold induction for each gene at 0, 1, and 2 dpc with respect to the values obtained at −1 dpc, the inoculation day. Values of 2-fold induction or higher were arbitrarily considered upregulation. Table 1 shows that at 1 dpi (0 dpc), only the Ad5-IFN-treated groups had upregulation of most of the analyzed ISGs. Overall, the Ad5-boIFN-λ3 group showed the highest values. Meanwhile, control animals showed upregulation of the same genes only after FMDV challenge (1 and 2 dpc). Control animals showed upregulation of IFN-λ3 and its receptors

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TABLE 1 Analysis of gene expression by qRT-PCR

	Relative fold induction (avg ± SD) for the indicated treatment and dpc[a]											
	Combination			Ad5-boIFN-α			Ad5-boIFN-λ3			PBS		
Gene	0	1	2	0	1	2	0	1	2	0	1	2
IFN-β	1.5 ± 0.7	1.5 ± 0.6	1.3 ± 0.6	1.5 ± 0.5	1.3 ± 0.6	1.7 ± 0.6	1.8 ± 0.5	1.2 ± 0.2	1.7 ± 0.4	1.6 ± 0.4	2.1 ± 0.1	1.1 ± 0.1
IFN-λ3	1.4 ± 0.8	1.4 ± 0.6	1.2 ± 0.7	1.1 ± 0.9	1.3 ± 0.9	1.8 ± 1.1	0.4 ± 0.1	0.4 ± 0.1	1.6 ± 2.2	3.1 ± 1.6	9.6 ± 2.0	3.6 ± 0.8
IL-28 Rα	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.6	1.4 ± 0.5	1.6 ± 0.5	1.7 ± 0.1	1.0 ± 0.1	0.7 ± 0.0	1.7 ± 0.9	1.6 ± 0.4	2.5 ± 0.6	1.7 ± 0.4
IL-10 Rβ	1.6 ± 0.3	1.1 ± 0.3	1.1 ± 0.2	1.0 ± 0.0	1.4 ± 0.2	1.9 ± 0.2	1.4 ± 0.2	1.3 ± 0.3	1.7 ± 0.3	1.2 ± 0.1	2.2 ± 0.1	1.9 ± 0.2
CCL2	5.3 ± 4.4	4.9 ± 5.2	1.3 ± 1.0	1.9 ± 1.7	1.9 ± 1.2	2.2 ± 1.4	4.0 ± 0.9	1.4 ± 0.3	4.2 ± 0.9	1.5 ± 0.5	8.4 ± 6.7	10.8 ± 3.2
CCL3	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0.3	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.3	0.8 ± 0.3	1.1 ± 0.7	1.2 ± 0.4	1.2 ± 0.2	1.5 ± 0.3
CCL20	1.0 ± 0.3	1.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.1	1.4 ± 0.6	1.6 ± 1.2	1.3 ± 0. 5	1.4 ± 0.8	2.0 ± 1.3	2.2 ± 0.8	3.9 ± 1.0	1.8 ± 0.9
CXCL10	14.9 ± 10.7	9.3 ± 3.3	11.2 ± 12.1	10.0 ± 2.2	6.7 ± 2.0	16.1 ± 9.5	55.2 ± 52.0	7.1 ± 2.4	10.0 ± 3.2	1.4 ± 0.1	24.1 ± 23.7	31.9 ± 5.2
IRF7	7.2 ± 4.3	2.6 ± 1.0	2.7 ± 1.3	4.0 ± 1.1	5.3 ± 5.0	6.1 ± 4.5	11.3 ± 11 0.4	1.3 ± 0.4	2.4 ± 1.8	1.2 ± 0.7	10.5 ± 9.7	5.1 ± 3.0
ISG15	55.6 ± 31.6	12.3 ± 3.3	16.5 ± 2.0	22.7 ± 11.6	10.2 ± 4.8	21.4 ± 1.1	40.8 ± 18.6	7.0 ± 2.1	10.0 ± 2.4	0.5 ± 0.3	90.0 ± 133	25.9 ± 16.5
MDA5	6.6 ± 0.8	5.4 ± 2.1	4.6 ± 2.2	3.7 ± 0.7	2.4 ± 0.9	4.6 ± 1.6	7.0 ± 1.1	2.8 ± 0.8	3.5 ± 1.0	1.3 ± 0.3	8.6 ± 7.7	7.9 ± 0.9
MX1	9.2 ± 1.9	5.4 ± 3.1	7.0 ± 4.5	9.8 ± 1.5	6.9 ± 2.1	13.1 ± 3.6	5.8 ± 1.5	5.4 ± 1.3	6.3 ± 1.6	1.5 ± 0.3	17.1 ± 14.7	14.2 ± 1.0
OAS1	12.6 ± 3.1	10.0 ± 2.0	9.1 ± 2.9	14.9 ± 3.4	9.1 ± 3.1	15.3 ± 4.4	22.9 ± 10.4	7.9 ± 1.6	10.7 ± 4.3	1.7 ± 0.8	26.0 ± 30.3	15.6 ± 8.4
PKR	6.4 ± 0.8	4.4 ± 1.9	3.8 ± 2.3	6.7 ± 1.2	4.9 ± 2.0	8.3 ± 2.9	7.9 ± 2.3	3.3 ± 0.5	4.0 ± 1.2	0.8 ± 0.1	9.3 ± 8.4	6.8 ± 0.9
RIGI	17.0 ± 1.6	11.4 ± 4.7	9.6 ± 5.0	13.4 ± 3.3	10.3 ± 4.7	14.5 ± 6.9	27.4 ± 15.1	8.8 ± 0.9	14.0 ± 3.7	1.9 ± 0.5	27.0 ± 31.1	20.6 ± 4.7

[a] RNA was extracted from PBMCs of the animals from experiment II at 0, 1, and 2 days postchallenge with FMDV by aerosol (equivalent to −1, 0, and 1 days postinoculation with PBS or Ad5s). Gene expression was measured by RT-PCR and expressed as the group average relative fold induction compared to the values before treatment (−1 dpi). Shading denotes >2-fold induction.

peaking at 1 dpc. In contrast, no or little induction of type III IFN or its receptors was observed in Ad5-IFN-treated animals either before or after the challenge. Altogether, these results indicated that the expression of similar ISGs is induced in animals treated with type I and/or type III IFN before FMDV challenge, while control animals showed similar patterns but only after challenge.

DISCUSSION

The role of type III IFN in conferring resistance to viral infections has been previously demonstrated in several species, including human, avian, porcine, and murine, mainly using *in vitro* models (3, 41, 44, 49). Moreover, the role of IFN-λ in protection has been demonstrated *ex vivo* in primary cells of pteroid bats (54) and, more importantly, *in vivo* against human viruses, including influenza A virus, metapneumovirus, and herpesvirus (2, 33), but using animal models of viral infection. In a previous study we had shown that boIFN-λ3 displays antiviral activity against FMDV (serotype A24) in bovine cell culture; furthermore, cattle treated with an Ad5-boIFN-λ3 had upregulation of ISGs in multiple tissues susceptible to FMDV infection (15).

Here we demonstrate that type III IFN (boIFN-λ3) significantly delays and reduces the severity of disease caused by two different FMDV serotypes (A and O) in cattle when the animals are exposed to the virus by either direct inoculation in the epithelia of the tongue, as recommended by OIE in efficacy testing (51a), or by controlled aerosolization, a viral exposure method that best resembles the natural route of infection (37). In general, there was a good correlation, within the groups of animals included in both experiments, between the delay in the appearance and the levels of viremia and viral shedding as well as in the titers of neutralizing antibodies after FMDV challenge. Upregulation of ISGs was detected in PBMCs. Interestingly animals treated with boIFN-λ3 displayed the longest delay of disease in both experiments, and one animal was protected for the whole course of the experiment. Animals treated with poIFN-α alone, but not with boIFN-α, had a slight delay of the disease and in the appearance of viremia and nasal shedding compared to the control animals, which is in agreement with a previous study (52). However, porcine or bovine IFN-α treatment seemed to have little or no effect in protection against FMD when combined with boIFN-λ3. In fact, at comparable doses, combination treatment resulted in a more severe clinical outcome than boIFN-λ3 treatment alone (Fig. 5), results that were consistent with the data obtained with cultured EBK cells (Fig. 1). Previous studies utilizing other viruses, encephalomyocarditis virus and herpes simplex virus 2, have shown that complex interactions might occur when a combination of type I and type III IFN is used (3). A cooperative effect was seen in human HepG2 cells treated with IFN-λ1 or -λ2 and IFN-α only under a limited range of IFN dose combinations. However, a dose-dependent response was detected when either IFN was used alone (3). Further biochemical studies are required to understand cooperative, redundant, or synergistic effects in the antiviral properties of these cytokines. In our previous cattle study (15), we tested the activities induced by both IFNs by examining the expression of multiple ISGs in tissues by qRT-PCR. These results demonstrated that treatment with both IFNs induced enhanced expression of many ISGs compared to the levels observed after treatment with either IFN alone. In the present study, we observed that protection against or a significant delay in the appearance of FMD is clearly dependent on type III IFN. However, we could not detect a correlation between this delay and the levels of antiviral activity detected in serum on the day of challenge (0 dpc) and 1 day after (1dpc). In the first experiment, the groups of animals treated with Ad5-poIFN-α alone or in combination with Ad5-boIFN-λ3, displayed the highest levels of antiviral activity at 0 dpc, in a dose-dependent manner. The high levels detected in the animals inoculated with Ad5-poIFN-α decreased rapidly by 1 dpc. These results suggested that detection of levels of antiviral activity in serum is not a good predictor of protection against or delay in the appearance of clinical signs of FMD in cattle. It is reasonable to hypothesize either that the method of detection of antiviral activity used in these studies is not sensitive or accurate enough to evaluate the systemic activity induced by type III IFN or that this family of IFNs preferentially induces an antiviral response locally,

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in epithelial tissues that are also susceptible to FMDV infection. We therefore tested the antiviral activities in serum by a classical bioassay, treating MDBK cells that were subsequently infected with vesicular stomatitis virus (32). Consistent with the results of the Mx-CAT ELISA, animals treated with Ad5-type I IFN showed the highest detectable levels of systemic antiviral activity at 0 dpc, in a dose-dependent manner, reaching values of 200 to 400 units/ml, while animals treated with Ad5-boIFN-λ3 alone had lower levels (50 units/ml). We had previously observed in swine studies that despite the systemic antiviral activity induced by inoculation with an Ad5-type I IFN, controlling FMD requires a local, tissue-specific response (13). Consistently, several studies have shown that IFN-λ plays a key role in the defense against pathogens that infect mucosal surfaces of the respiratory tract and intestine (3, 33, 36, 38, 49). Indeed, in our second animal experiment we decided to study the effect of bovine type III IFN against FMD when the animals were exposed to aerosolized FMDV. This method of challenge with FMDV uses a nebulizer to administer a definite amount of virus in the upper respiratory tract, thus resembling FMDV transmission in nature or in an animal production facility (37). Furthermore, by using FMDV aerosol exposure, Arzt et al. (4) identified the nasopharynx as the initial site of virus replication. Induction of an antiviral state in this tissue should correlate with better protection against FMD. The results from our second animal experiment (aerosol challenge) supported this hypothesis and correlated with previous reports in the literature showing in other species that the antiviral activity of type III IFN protects the mucosa of the upper respiratory tract from viral infection (24, 33, 38, 50). The delay of disease in cattle challenged by aerosol with FMDV O1 Manisa lasted for at least 9 days, and one out of three animals was free of disease throughout the experiment. In contrast, treatment with boIFN-α had little or no effect on protection, delaying viremia and nasal shedding for only 1 day.

Treatment with boIFN-λ3 not only delayed clinical signs but inhibited viremia and viral shedding in nasal swabs as demonstrated by virus isolation. Utilizing a more sensitive method, qRT-PCR, we did detect viral RNA but at much lower levels than in the animals that developed clinical signs (C_T values of 37 for the boIFN-λ3-treated groups versus 27 to 29 for the control or boIFN-α groups) and lasting for a shorter period of time (2 versus 4 to 5 days). Consistently, the titer of neutralizing antibodies developed in animals treated with Ad5-boIFN-λ3 was lower than that detected in the control or the Ad5-boIFN-α groups. We do not know whether the viremia detected in the animals treated with Ad5-boIFN-λ3 originated from the initial exposure to FMDV at day 0 or if the steer calves were reinfected with residual virus present in the environment after challenge. Nevertheless, it seemed that Ad5-boIFN-λ3 treatment prevented FMDV replication for several days, probably until the effect of the IFN weakened. As reported for type I IFNs (41), type III IFN may have a rapid but short antiviral activity. Therefore, treatment of cattle with a combination of type III IFN and FMD vaccine should be evaluated as a method to induce a rapid and complete protection against FMDV. Correlating with viremia and clinical disease, we observed a significant decrease in the percentage of lymphocytes in control animals by 3 and 4 dpc. Lymphopenia has also been reported in earlier studies in cattle (30) and swine (5, 14, 35) infected with FMDV. However, the mechanism involved in the induced lymphopenia in bovines and swine still remains unclear. It has been proposed that either infection of lymphocytes (14) or the presence of IFN-α in serum after FMDV infection (35) could account for the observed lymphopenia in swine. Recently, Reid et al. (40) have shown that bovine plasmacytoid dendritic cells are the major producers of IFN in response to FMDV infection and that despite the relative high levels of IFN, no lymphopenia is observed in cattle. Perhaps the lymphopenia observed in our experiment is FMDV serotype specific, as previously reported for swine (34). Alternatively, some other, unknown mechanisms related to the pathogenesis of FMDV could be involved.

Despite the lack of correlation between the delay in the appearance of disease and the systemic levels of antiviral activity, we analyzed the pattern of expression of several ISGs in PBMCs. A similar pattern of gene expression was detected in animals treated with Ad5-boIFN-α and/or Ad5-boIFN-λ3 as previously reported in bovines or other species (3, 13, 43, 53). Basically, no differences in gene expression that supported the differences in the clinical response were detected for the analyzed genes. These results suggest that type III IFN might be selectively inducing ISGs that were not measured in our experiments and that could be responsible for the observed delay of FMD. Alternatively, type III IFN could induce the localized expression of ISGs in tissues directly affected by FMDV. The expression of ISGs in PBMCs was similarly upregulated in the control (PBS) group at 1 to 2 dpc compared to the IFN-treated groups at 0 dpc (1 dpi). Interestingly, in contrast to the case for the Ad5-boIFN-α/λ-treated animals, the mRNAs for IFN-λ3 and its receptor subunits (IL-28BRα and IL-10Rβ) were also upregulated in the control group. These results are consistent with previous reports showing that IFN-λ is the most predominant IFN induced by respiratory viruses in nasal epithelial cells (36), including in influenza A virus infection *in vivo* (26). Moreover, these results corroborate our previous results with primary bovine cell culture showing that FMDV infection induces the expression of IFN-λ3 (56).

Altogether, our results indicate that type III IFN is able to limit FMDV replication and spreading in cattle, one of the economically most important susceptible livestock species, especially when the virus infects using its natural route. No other biotherapeutic approach has shown such an effective activity against FMD in cattle. Although the mechanism that mediates the observed significant delay in disease onset is still unknown, type III IFN has a strong potential to be developed as a biotherapeutic strategy against FMD and possibly against other bovine viral respiratory infections.

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Foot-and-mouth disease: Global status and Indian perspective

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious and transboundary viral disease of domesticated and wild cloven-hoofed animals. Wide prevalence of the disease in Asia and Africa associated with huge economic loss to the livestock farming and industry has increased the concern worldwide. The disease is a major threat to cattle, buffalo (both milk and meat) and pig production in endemic countries and therefore considered to cause food insecurity, both locally and globally. Currently, 6 serotypes of FMD virus (O, A, Asia-1, SAT-1,-2, and -3) are circulating globally, and serotype C has not been recorded since 1995. In India, the disease is caused by serotypes O, A and Asia-1, of which serotype O is responsible for most of the outbreaks. Emergence and re-emergence of FMD virus genotypes/lineages has been detected in serotypes. Serotype A viruses have been continuously emerging in the nature necessitating frequent replacement of the vaccine strains. The knowledge generated in epidemiology, diagnosis and surveillance of the disease in the country has been instrumental in formulation and implementation of FMD Control Programme through regular 6 monthly vaccination with the aim to create disease free zones in India. The control programme, in operation since X Plan, has resulted in progressive and substantial reduction in occurrence of the disease and DIVA reactors/converters in vaccinated areas. The present review summarizes the disease, the causative agent, and epidemiology of FMD in India and the world.

Key words: DIVA reactors, FMD control programme, FMD surveillance, FMDV serotypes, Global transmission

Foot-and-mouth disease (FMD), a clinically acute, contagious viral disease of domesticated ruminants, pigs, camelids and more than 70 wildlife species including elephant, is of transboundary nature posing threat to global food security, and causes severe economic loss to livestock farmers and industry (Fenner 1993). The causative agent, FMD virus (FMDV) is a member of the genus *Aphthovirus*, in the family *Picornaviridae*. The virus exists in 7 immunologically distinct serotypes: A, O, C, Southern African territories (SAT)-1, 2, 3 and Asia-1, and within each serotype there are a substantial number of strains showing variable degree of genetic and antigenic diversities. Clinically, the disease is characterised by fever, lameness and vesicular lesions on the mouth, tongue, feet, snout and teats of infected animals (Alexandersen *et al.* 2003b). Unvaccinated FMDV-infected cattle and pigs usually develop obvious signs of the disease but in sheep and goats, diagnosis is more difficult because the clinical manifestation of the disease is often mild (Callens *et al.* 1998, Barnett and Cox 1999, and Viuff *et al.* 2002). High mortality is sometimes observed among young animals and is often attributed to affect the myocardium of heart (Barker *et al.* 1993). Though the disease causes low mortality in adult animals, morbidity is very high, causing weight loss, decrease in milk production and loss of draught power resulting in huge economic loss. An asymptomatic FMDV persistent infection (carrier state) can be established in ruminants beyond 28 days post-infection to several years, irrespective of vaccination status (Salt 1993) and such animals act as nucleus of infection in herd(s). Due

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to the difficulties in effectively controlling the disease, extreme contagiousness with wide host range, and its economic impact; FMD ranks first among 'Risk Group 4' animal pathogen.

FMDV genome organisation: The positive sense viral RNA consists of a single open reading frame (ORF), flanked by 2 highly structured un-translated regions (UTRs) at the 5' and 3' ends of the genome (Belsham 1993, Lin and Flint 2000). A small viral protein VPg is covalently linked to 5' end of the RNA molecule (Grubman 1980, Sangar *et al.* 1977). 5' UTR at its most 5' end contains a highly structured 'S' fragment of about 360 nucleotides residues and is predicted to form hairpin structure (Witwer *et al.* 2001). 'S' fragment prevents the digestion of viral genome in infected cells by host cell exonuclease (Mason *et al.* 2003). 'S' fragment is followed by an internal poly-ribocytidylate (poly C) tract of variable length (usually 100-400 residues) (Costa-Giomi *et al.* 1984). Following the 3' end of poly C tract there is a series of RNA pseudoknot structures of unknown function. Downstream of the pseudoknots (PK) there is a short hairpin loop structure termed as *cis*-acting replication element (*cre*) and this element includes a conserved motif, AAACA in the loop region. The *cre* region precedes the internal ribosome entry site (IRES), a complex highly structured element of about 440 residues, which is responsible for the internal initiation of protein synthesis in a cap-independent fashion (Martinez-Salas 1999).

The 3' UTR of the FMDV genome is also highly structured, consisting of 100 nucleotides that contains 2 stem loop structures (SLI and SLII) and a genetically encoded poly A tract (Chatterjee *et al.* 1976). There is extensive evidence of interaction between the 3´ UTR of picornaviruses and several viral and host proteins (Agol *et al.* 1999).

The viral ORF is divided into 4 regions (L, P1, P2 and P3) and encodes for a single polyprotein, which is cleaved by viral proteases (L,2A and 3C) (Ryan *et al.* 1989) to yield 4 structural and 8 non-structural proteins (NSPs). Each of these NSPs, as well as some of the precursor polypeptides, are involved in functions relevant to the virus life cycle in infected cells (Belsham 1993 and Porter 1993).

The L region contains two in-frame functional AUG initiation codons, which result in the generation of 2 overlapping L proteins, Lab and Lb (Beck *et al.* 1983, Rieder *et al.* 1993). The L protease (L^{pro}) is a member of the papain-like cystein proteases, which acts both intra and intermolecularly (Guarne *et al.* 1998). Both Lab and Lb catalyse their proteolytic excision at L/P1 junction of the polyprotein (Strebel and Beck 1986) and also initiate the cleavage of host translation initiation factor eIF-4G, which results in the shut-off of host cap-dependent mRNA translation (Devaney *et al.* 1988). It has been reported that L^{pro} blocks the innate immune response to FMDV infection in susceptible animals by inhibition of host IFN-α/β mRNA translation (de Los Santos *et al.* 2006).

The P1 region encodes the structural proteins VP1 (1D), VP2 (1B), VP3 (1C), and VP4 (1A). Although the 2A region was considered as a part of the P2 region, the 16-amino-acid peptide 2A catalyses in *cis* the excision of P1-2A from 2B (Ryan *et al.* 1991). It has also been proposed that 2A peptide prevents the formation of the peptide bond at the 2A/2B junction through the ribosome slippage mechanism during the polyprotein synthesis (Donnelly *et al.* 2001). The processing of the FMDV P1-2A precursor to 1AB, 1C and 1D is achieved by the 3C protease (Vakharia *et al.* 1987 and Ryan *et al.* 1989).

The P2 region encodes 2 viral non-structural (NS) proteins (2B and 2C). The function of P2 region of FMDV is poorly understood. The polypeptide 2C and its precursor 2BC are associated with cell membranes and induce vesicle proliferation (Bienz *et al.* 1990). Transport of proteins from endoplasmic reticulum to Golgi apparatus is reportedly blocked by the FMDV 2BC protein (Moffat *et al.* 2007). P3 is composed of 4 non-structural proteins, 3A, 3B, $3C^{pro}$ and $3D^{pol}$. 3A is the membrane anchored protein and plays a role in the pathogenesis of FMDV (Pacheco *et al.* 2003), due to changes of the amino acids in 3A in various strains and serotypes of FMDV produced attenuated viruses in cattle (Sagedahl *et al.* 1987, Beard and Mason 2000). FMDV contains 3 tandem, non-identical copies of the 3B protein (VPg) (Falk *et al.* 1992). The VPg protein is covalently linked to the 5' end of the viral RNA and participates in the initiation of viral RNA synthesis (Nayak *et al.* 2005,2006). 3C protein is a serine-protease responsible for most of the cleavage of individual proteins from the viral polyprotein (Ryan *et al.* 1989). The 3D protein is the viral RNA-dependent RNA polymerase and thought to recognise and interact with both positive and negative strands of FMDV RNA (Belsham 2005).

With respect to the Indian FMDV strains, a few changes were detected in the genome. Two different lineages (V-A and VI) of serotype Asia-1 virus were detected with 3 and 4 pseudoknots (PK) respectively (Mohapatra *et al.* 2008). The discrepancy in the number of PKs is due to deletion of 43 nucleotides in 5'-UTR large fragment of lineage V-A viruses. Mutagenesis studies carried out with infectious clone of FMDV O1K showed that the pyrimidine rich sequence preceding the FMDV start codon was most sensitive in the conversion of single pyrimidine residue to purine decreased the protein translation efficiency drastically. However, lineage VI-B Asia-1 viruses exhibit a group-specific transversion ($U_{695} \rightarrow G$) within the pyrimidine tract without any impact on viral multiplication (Biswas *et al.* 2005, Mohapatra *et al.* 2008). Similar to Asia-1 viruses, a block deletion of at least 45 nucleotides was observed in 5'-UTR large fragment of serotype A viruses isolated in India. However, unlike serotype Asia-1, deletion in type A isolates were not genotype/lineage specific (Subramaniam *et al.* 2011). With respect to the number of PKs, most of the

serotype A field isolates showed to have at least 2 PKs (Subramaniam *et al.* 2011). In type A Indian viruses, a lineage specific (lineage VIIg) transversion ($T_{712} \rightarrow A$) similar to Asia-1 FMDV was detected within the pyrimidine tract (Mohapatra *et al.* 2009, Subramaniam *et al.* 2011). However, as in Asia-1 serotype, this substitution had no role on the infectivity titre of type A viruses. Therefore, it was speculated that either a minimum numbers of pyrimidine residues or a core motif (CTTT) rather than a complete pyrimidine is essential for efficient viral translation (Mohapatra *et al.* 2009).

In serotype O Indian isolates, the majority of recent isolates of dominant lineages circulating in India were with either 1 or 2 PKs, so the role of lesser number of PKs in giving fitness advantage to the viruses cannot be ruled out. During a recent study, a serotype O isolate with no PK was found. The number of PKs was predicted by using software (pknotsRG tool), if the prediction limits of the software is considered totally genuine, probably the FMDV natural isolate can exist without a pseudoknot (unpublished data). Therefore, the role of PK on FMDV replication needs to be determined.

Due to circulation/co-circulation of multiple lineages of different FMDV serotypes, genetic recombination events may be possible between the genome of related strains of FMDV. Evidence of recombination in capsid coding region was reported earlier in Indian serotype A isolate (Tosh *et al.* 2002a). However, for Asia-1 viruses recombination events was reported in the non-capsid protein coding region (Mohapatra *et al.* 2008). Recently, recombination events on both structural and non-structural protein coding regions were detected in serotype O viruses (unpublished data).

Cell recognition by FMDV: The first requirement for FMDV infection is the interaction of the virus with the host cell receptors. Integrin was the first molecule identified as a primary receptor for FMDV (Berinstein *et al.* 1995, Jackson *et al.* 1997). The integrin receptor recognition site includes a highly conserved arg-gly-asp (RGD) triplet on the G-H loop of the VP1 capsid protein (McKenna *et al.* 1995, Rieder *et al.* 1996). Despite being the part of important antigenic site, the RGD triplet is highly conserved amongst all the strains of FMDV, which probably reflects its requirements for the *in vivo* interaction with the cell receptors (Duque and Baxt 2003, Neff *et al.* 1998).

Infectious FMDV can enter the cultured cells using $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$ and $\alpha v\beta 8$, as receptors (Berinstein *et al.* 1995, Jackson *et al.* 2000, 2002, 2004). The $\alpha v\beta 6$ integrin receptor is expressed constitutively at high levels on the surfaces of epithelial cells at sites where infectious lesions occur during a natural infection in the cattle, but not at the sites where lesions are not normally formed (Monaghan *et al.* 2005). In infected animal tissues, $\alpha v\beta 6$ integrin receptor is distributed on the surface of those epithelial cells also expressing FMDV antigen both in the tongue and coronary bands (O'Donnell *et al.* 2009). Thus $\alpha v\beta 6$ is the major receptor that determines the tropism of FMDV. During cell culture, FMDV can acquire the capacity to initiate infection via heparan sulphate (Jackson *et al.* 1996), by replacement of amino acid at a shallow depression on the virion surface, at the junction of 3 major capsid proteins VP1, VP2 and VP3. The replacement of histidine residue to arginine at position 56 in VP3 protein of cell culture adapted virus enhances the interaction with heparan sulphate (HS) (Jackson *et al.* 1996, Fry *et al.* 1999, Fry *et al.* 2005). The ability of the cell culture adapted virus to bind HS was also reported for serotypes C (Baranowski *et al.* 2000), serotype A (Fry *et al.* 2005) and SAT-1 viruses (Maree *et al.* 2010). However, both field and cell culture adapted Indian viruses belonging to Asia-1 serotypes have amino acid arginine residue at position 56 in VP3 protein (unpublished data). It was reported that upon multiple passages of FMDV in cell culture, the RGD motif can become dispensable, this is associated with the use of alternative receptors for cell entry (Baranowski *et al.* 2001a, Baranowski *et al.* 2001b). Antibody-complexed virus can infect cells via Fc receptor-mediated adsorption (Baxt and Mason 1995). An alternative cell-binding site was also reported for genetically engineered virus harbouring lys-gly-glu (KGE) sequence instead of the RGD triplet in swine (Zhao *et al.* 2003).

FMD transmission and pathogenesis: The most important features of the epidemiology of the disease are extremely rapid replication and transmission of the virus. The common mechanism of spread of the disease is by direct contact which may occur by mechanical transfer of virus from infected to susceptible animals through damaged skin or intact mucosae or by deposit of droplets or droplet nuclei (aerosols) in the respiratory tract of recipient animals (Alexandersen *et al.* 2003a). Contact with virus from infected animals can also occur via fomites, for example by people contaminated at the time of shearing, de-worming, lambing and blood sampling during FMD epidemics. By air-borne exposure a minimum 10 $TCID^{50}$ of virus is sufficient to infect a ruminant experimentally. However, pigs are relatively resistant to aerosol exposure (Alexandersen *et al.* 2002). Infected pigs are a significant source of FMDV for long distance aerosol spread , as pigs release largest quantities of air-borne virus (Alexandersen *et al.* 2002). Ruminants excrete lower titres of virus in their breath but are highly susceptible to infection by the respiratory route through air-borne transmission. Pigs usually become infected either by eating FMDV-contaminated food or by direct contact with infected animals. Sheep and goat are highly susceptible to virus infection by aerosol. As clinical diagnosis is often difficult in sheep, the infection can be unnoticed and therefore sheep can play a major role in the spread of disease as has been widely implicated in the prolific spread during the 2001 outbreak in the United Kingdom.

The incubation period of FMD is highly variable, 2-14

days, and depends on the strain and dose of virus, the route of transmission, the animal species and the husbandry conditions (Alexandersen and Mowat 2005). Charleston *et al.* (2011) found that period of infectiousness in cattle is only 1.7 days and animals are not infectious until 0.5 days after the appearance of clinical signs.

The epithelial cells of the dorsal soft palate, the roof of the pharynx and part of the tonsil are thought to play a special role in the primary infection (Alexandersen *et al.* 2003b). This concept was described for cattle, but is only indirectly suggested in other host species. Recent studies reported that subsequent to aerosol inoculation, the FMDV infection is initiated at the epithelia of mucosal associated lymphoid tissue of the nasopharynx in cattle (Pacheco *et al.* 2010a, Arzt *et al.* 2010, 2011b). Shortly after initial infection ('pre-viremic' phase), virus replication is detected within pulmonary alveolar septa and as viraemia approached, FMDV is replicated more prominently within the pulmonary pneumocytes with a significant decrease in virus load within pharyngeal tissues (Arzt *et al.* 2011b). Other studies have similarly implicated roles for nasopharynx and lungs as the primary sites of natural infection in cattle (Burrows *et al.* 1981, Brown *et al.* 1996). In pigs, palatine tonsil or lungs are shown to be the primary site of infection following oral or aerosol inoculation, respectively (Terpstra 1972).

Early replication is followed by a viraemic phase of 3-5 days and during this time, the virus spreads to epithelial tissues of secondary sites of replication via the bloodstream. The secondary sites of replication include cornified stratified sqaumous epithelia of the oral cavity and skin (hairy and non-hairy parts) including the feet and mammary teats (Alexandersen *et al*. 2003b). Less commonly affected epithelial lesion sites include external genitalia and rumen. During this secondary stage, lesions which are observed initially as a blanched area subsequently develop into vesicles that cause lesions at the mouth, feet and teats (Seibold 1963, Alexandersen and Mowat 2005, Arzt *et al.* 2009). There is often secondary bacterial infection which delays healing of the lesions. Myocardial infection, when it does occur, is typically during the viremic phase in young pigs, small ruminants, and wildlife (Arzt *et al.* 2011a). Clearance of viraemia and viral tissue load is achieved by the induction of an effective immune response, and is characterised by the generation of virus specific antibody and may be dependent on the interaction of virus-antibody complex with phagocytic cells of reticuloendothelial system (McCullough *et al*. 1986,1988, 1992).

Carrier state in FMD: In ruminants, an asymptomatic, persistent infection can be established following clinical recovery, irrespective of vaccination status. This state has a significant impact on control and eradication programs (Perry and Rich 2007). Any animal from which FMDV can be recovered in oropharyngeal scrapings for a period greater than 28 days post-challenge is considered as a carrier animal (Sutmoller *et al.* 1968). The nasopharynx, particularly the dorsal soft palate was suggested to be the site of predilection for FMDV persistence in cattle (Burrows 1966, Donn *et al.* 1994, Zhang and Kitching 2001). Juleff *et al*. (2008) demonstrated depots of FMDV antigens in lymph nodes of cattle during persistent infection which are likely involved in maintaining long term immunity. However, the potential role of these depots in long term shedding of infectious virus remains uncertain. In sheep, tonsil is shown as the site of persistence of the virus (Burrows 1968, Ryan *et al.* 2008).

More than 50% of FMD-recovered ruminants may become carriers of the virus. However, pigs usually clear the virus within 3 to 4 weeks of infection and do not become carriers (Alexandersen *et al*. 2003b, Parida *et al*. 2007) with an exception of a single report which showed pigs as carriers (Mezencio *et al.* 1999). The duration of carrier status varies between the species, the maximum reported duration of the carrier state in African buffalo, cattle, sheep and goats are 5 years, 3.5 years, 9 months and 4 months, respectively (Burrows 1968, Hedger 1968, Brooksby 1982, Condy *et al.* 1985, Salt 1993, Alexandersen *et al.* 2003b). However, the majority of cattle and sheep appear to lose their carrier status within a relatively short period of time. A meta-analysis of persistence studies indicated that carrier cattle cleared infection @0.115/month (Tenzin *et al*. 2008).The mechanisms for the establishment and maintenance of the carrier state are not well understood, since persistence can occur in both vaccinated and non-vaccinated cattle (Doel *et al.* 1994). The risk of carrier cattle or sheep transmitting virus to uninfected animals is generally believed to be extremely low (Kitching 2002). The only direct evidence of transmission of virus from a carrier to a susceptible animal is that of transmission from African buffalo to cattle during the outbreaks in Zimbabwe in 1989 and 1991 (Dawe *et al.* 1994a). In addition, there is experimental evidence of transmission of the virus by sexual contact from infected buffalo carrying a small amount of virus in oesophageal-pharyngeal (OP) fluids to uninfected cattle (Dawe *et al.* 1994b).

Immune response against FMDV: Upon infection, FMDV elicits rapid humoral and cellular immune responses in susceptible animals, which induce an efficient protection against re-infection with homologous and antigenically related viruses (McCullough *et al*. 1992, Salt 1993). Protection against FMDV is generally correlated with high levels of neutralizing antibodies in serum. The neutralizing antibody response is directed to the well characterised B cell epitopes located on the 3 external capsid proteins (Sobrino *et al.* 2001). IgM is the first serum neutralizing antibody that appears as early as 3-4 days following infection or vaccination, with a peak response at approximately 10-14 days post-infection, and then the response declines (Sobrino *et al.* 2001, Golde *et al.* 2008). The IgM response is followed by IgA and then IgG, which is first detectable 4-7 days after

infection or vaccination and then becomes the major neutralizing antibody by 2 weeks following vaccination (Salt *et al.* 1996). In both vaccinated and infected cattle, the IgG1 antibody response was reported to be higher than IgG2 (Salt 1993). Although neutralizing antibody often provide protection against FMD, it was also seen that hyper immune sera raised to mutant FMDV antigen with minimal neutralizing activity *in vitro* offered passive protection against wild type FMDV challenge (Dunn *et al.* 1998). Therefore, it is purposed that, non-neutralizing antibody may also play a preposed role in the clearance of FMDV from infected animal by macrophage mediated phagocytosis of opsonised virus-antibody complex (McCullough *et al.* 1986, 1988, 1992).

In contrast to the serotype restricted B-cell mediated humoral immunity, the T-cell response to FMDV in animals is shown to be cross reactive between FMDV serotypes, which is of interest for vaccine design (Collen *et al.* 1998, Blanco *et al.* 2001, Parida *et al.* 2006b and Cox *et al.* 2010). In cattle and pig, B-cell activation and antibody production are associated with a lymphoproliferative response mainly mediated by CD4+ T-cells (Sobrino *et al.* 2001). These helper T-cells recognise a number of epitopes located both in structural and non-structural proteins of FMDV (Collen *et al.* 1989 and Blanco *et al.* 2001). For the production of anti-FMDV antibodies, CD4+ T-cells assists in the maintenance of appropriate microenvironment required for a synergistic immune response (Sobrino *et al.* 2001). The role of CD8+ T cell mediated cytotoxic T-cell response to FMDV infection is unclear and largely remains unexplored. This may be partly due to the unavailability of a reliable assay of cell killing for highly cytolytic FMDV or due to down regulation of MHC class-I molecules on the surface of infected epithelial cells (Sanz-Parra *et al.* 1998). There is a single report of FMDV specific MHC-I restricted proliferation response in the CD8+ enriched fraction collected from the blood of FMDV infected cattle (Childerstone *et al.* 1999). Nevertheless, the levels of proliferation were low, variable and completely absent in a significant proportion of the animals tested. However, using a sensitive IFN-γ restimulation ELISpot assay, the FMDV specific MHC-I restricted CD8+ T cells response was detected in cattle (Guzman *et al.* 2008).

Diagnosis: The accurate diagnosis of infection with FMDV is of great importance for both control and eradication campaigns in FMD endemic areas and as a supportive measure to maintain disease free zones. It is also important to differentially diagnose the disease from other vesicular diseases such as, swine vesicular disease, vesicular stomatitis, and vesicular exanthema of swine. In addition, FMDV infected sheep and goats are difficult to diagnose clinically. The suspected cases showing symptoms are usually confirmed by laboratory diagnosis either by detecting the viral antigen or antibody.

Virus detection: The OIE Terrestrial Manual (OIE 2009) describes 4 methods for the detection of FMDV. These tests are virus isolation (VI), antigen detection ELISA (Ag-ELISA), complement fixation test (CFT) and nucleic acid recognition (NAR) methods.

Virus isolation is currently the only *in vitro* method for the detection of live virus in the clinical samples. FMDV can be grown on a variety of cell culture systems of bovine (Snowdon 1966), ovine (Hess *et al.* 1963) and porcine (Bachrach *et al.* 1955) origin. The cytopathic effect (CPE) usually develops within 48 h. If no CPE is detected, the cells are frozen and thawed and the lysate is used to inoculate fresh cultures and examined for CPE after 48 h. The earlier used CFT has been substituted by more sensitive antigen capture ELISA (Ag-ELISA) for antigen detection and serotyping of the virus (Roeder and Le Blanc Smith 1987, Ferris and Dawson 1988, Bhattacharya *et al.* 1996). A pen side test based on the principle of Ag-ELISA and chromatography technology was developed for diagnosis of FMD (Reid *et al.* 2001, Ferris *et al.* 2001,2009 and 2010).

Various reverse transcription-polymerase chain reaction (RT-PCR) procedures were developed for detection of FMDV RNA: conventional RT-PCR (Rodriguez *et al.* 1992, Pattnaik *et al.* 1997), RT-PCR ELISA (Callens *et al.* 1998), nested RT-PCR (Moss and Haas 1999), real-time RT-PCR (Reid *et al.* 2002, Moonen *et al.* 2003), portable real time RT-PCR (Donaldson *et al.* 2001, Hearps *et al.* 2002) and automated RT-PCR (Reid *et al.* 2003). Other nucleic acid detection methods were also developed for FMDV detection, the nucleic acid sequence based amplification (NASBA) test (Collins *et al.* 2002, Lau *et al.* 2008) and the RT loop-mediated amplification (LAMP) test (Dukes *et al.* 2006). Lineage differentiating RT-PCR for serotype A (Mohapatra *et al.* 2007) and Asia-1(Mohapatra *et al.* 2006) and multiplex-PCR (Mohapatra *et al.* 2011b) were also developed, and are in use in India for detection of circulating Indian strains. In India, a double antibody sandwich ELISA (Bhattacharya *et al.* 1996) is routinely used for identification of FMDV serotypes. RT-PCR (Pattnaik *et al.* 1997, Mohapatra *et al.* 2006, Mohapatra *et al.* 2007) is applied on ELISA negative samples. If the disease is reported late, diagnosis is done by a liquid-phase blocking ELISA.

Antibody detection: Detection of antibody against structural protein of FMDV: Virus neutralization test (VNT), liquid-phase blocking ELISA (LPBE) and solid phase competitive ELISA (SPCE) are 3 serological tests prescribed (OIE 2009), for detection of antibody against the structural proteins of FMDV. These tests are used as serotype specific serological tests. VNT is labour intensive, requires sensitive cell lines, live FMDV and containment laboratory facility. The advantages of ELISA over VNT are that the test is rapid, can use inactivated antigens, and requires smaller volumes of post-vaccination sera, which are often available in limited quantities (Paton *et al.* 2005). LPBE based antibody detection system is quicker, more reproducible, less variable and the result correlates well with VNT (Hamblin *et al.* 1986,

Hamblin *et al.* 1987, Pattnaik and Venkataramanan 1994). However, the LPBE was criticised for the specificity and variable stability of inactivated antigen used in the test (Mackay *et al.* 2001). The SPCE was been developed to overcome this problem, which has higher specificity than LPBE (Mackay *et al.* 2001, Paiba *et al.* 2004). In India an indigenously developed LPBE is used extensively to monitor post vaccination antibody response.

Detection of antibody against non-structural protein of FMDV: Detection of FMD-specific structural antibody can be useful for diagnosis, but this requires the absence of any history of vaccination, as vaccination with purified vaccine elicits antibodies only against structural proteins (SPs). FMDV infection elicits antibodies against both SP and NSPs. Therefore, an ELISA that measure antibodies to FMDV NSPs can be used for differentiating infection from vaccinated animals (DIVA). The detection of antibodies to the NSP 3ABC of FMDV was shown to be a sensitive and specific DIVA test when combined with the confirmatory immunoblotting test (EITB) against 5 bioengineered FMDV NSPs 3A, 3B, 2C, 3D and 3ABC (Bergmann *et al.* 2000, OIE 2009). However, this OIE-index test for NSP serology is only available from PANAFTOSA, Brazil to the South American laboratories. A number of 3ABC ELISA test kits have recently become available and their sensitivity and specificity were compared to one another and to the OIE-index screening method at an international workshop in Brescia in 2004 (Brocchi *et al.* 2006). It was concluded in the workshop that 2 tests performed comparably to the OIE-index method of which the Ceditest (currently known as PrioCHECK® FMDV NS) is the only one available as a commercial kit (Paton *et al.* 2006). In addition to 3ABC ELISAs, a variety of NSP tests based on the detection of antibodies to the recombinant 2B (Inoue *et al.* 2006), 2C (Mezencio *et al.* 1998), 3AB (Mohapatra *et al.* 2011a), 3D (Sorensen *et al.* 1998) were developed. Moreover, multiplex profiling assays were also evaluated (Mackay *et al.* 1998, Perkins *et al.* 2006, Perkins *et al.* 2007). In India, an indigenously produced 3AB/3ABC-ELISA kit is extensively used to monitor virus circulation and clearance in vaccinated areas. A multiple NSP ELISA using FMDV 2C, 3AB, 3ABC and 3D antigens was also developed in the country for monitoring of disease free zones.

Mucosal antibody detection: Parida *et al.* (2006a) studied the oropharyngeal IgA responses in FMDV-vaccinated and infected cattle and demonstrated that parenteral administration of conventional FMD vaccine does not elicit any IgA antibody in saliva. In contrast, challenge with live virus elicits a strong local IgA response, including vaccinated animals that have become persistently infected. Pacheco *et al.* (2010b) reported that parenterally vaccinated pigs do not elicit mucosal antibody whereas vaccinated and subsequently infected pigs produce high levels of mucosal antibody, as seen in cattle. Thus the IgA test has considerable potential for the detection of persistently infected cattle following the application of a vaccinate-to-live policy (Parida *et al.* 2006a, Biswal *et al.* 2008).

FMD vaccine and protection: Currently the FMD vaccine is produced by infecting baby hamster kidney-21 (BHK-21) cells with virulent FMD virus, followed by chemical inactivation by binary ethylenimine (BEI) and purification by ultrafiltration. Some vaccine manufacturers use industrial scale chromatography to purify whole virus particles from NSPs. As inactivated FMDV is poorly immunogenic, effective formulation of FMD inactivated vaccines requires the use of adjuvants to enhance the antigenicity of the vaccine. Two types of adjuvants are used in general, aluminium hydroxide gel ($Al(OH)_3$) supplemented with saponin and oil emulsion adjuvant (Doel 1999). Oil-emulsion vaccines are widely used for the immunization of pigs, sheep, goats and cattle, whereas $Al(OH)_3$/saponin adjuvant is mainly used for cattle. In endemic countries, FMD vaccine formulation contains more than 1 serotype of virus depending upon the epidemiological situation of the particular country. The inactivated FMD vaccine induces a relatively short duration of protection which lasts for 4-6 months. Boosting of the immune response by repeated vaccination, as done in endemic countries, dramatically increases both the magnitude and duration of neutralising antibody responses (Doel 2003, Parida 2009). Emergency vaccines, which are normally used in FMD free countries during the incidence of an outbreak, are of higher potency ($\geq$ 6 protective dose 50 (PD_{50})) than the conventional vaccines ($\geq$ 3 PD_{50}). Higher antigen dose ensures rapid protective immunity and better cross production within serotype (Cox and Barnett 2009). There has been good documentary evidence that emergency vaccines, as either an oil or aqueous formulation confer rapid and protective immunity in appropriate target species within 4 to 5 days of vaccination (Barnett and Carabin 2002). This is attributed to induction of combined innate and early adaptive immune responses (Barnett *et al.* 2002, Rigden *et al.* 2003, Barnard *et al.* 2005).

Efficacy of vaccination against FMD is affected by the lack of cross protection between serotypes, as well as incomplete protection between subtypes (Mattion *et al.* 2004). Therefore, it is necessary to match the vaccine strains with the circulating filed strains (Doel 1999). Currently, the methods of vaccine strain selection are mainly based on the serological approaches (Paton *et al.* 2005). The relationship between field isolates of FMDV and vaccine strains are usually expressed as 'r' values (Doel 2003), calculated between the viruses using pools of antisera prepared against vaccine strains and other candidate strains to be matched. The antigenic similarities between the vaccine strain and field isolates can either be calculated by using a neutralization test (Rweyemamu *et al.* 1978) or an ELISA based approach (Kitching *et al.* 1988). The advantages of ELISA over a virus neutralisation test (VNT) are that the test is rapid, can use

inactivated antigens and requires smaller volumes of post-vaccination sera, which are available in limited quantities (Paton *et al.* 2005). If VNT is used to determine the antigenic relationship an 'r' value of 0.3 or higher indicates a close antigenic match between vaccine strain and the field isolate (Rweyemamu 1984). While in ELISA based approach (LPBE), an 'r' value of 0.4-1.0 indicated a close match between vaccine strain and field isolate (Kitching *et al.* 1988). If the r value is less than the prescribed value, the field isolates need to be examined against alternative vaccine candidates and there may be a need to identify and select another vaccine strain (Paton *et al.* 2005). Another approach for determining the antigenic relationship and selection of vaccine strain could be by comparison of capsid coding gene (P1) sequences of both vaccine strain and field isolates, which would provide the genetic information regarding the epitopes playing a role in vaccine-induced protection (Parida *et al.* 2009). Presently, this approach is being investigated in various FMD laboratories (Reeve *et al.* 2010). However, caution must be taken when extrapolating between nucleotide or deduced amino acid differences and antigenic homology, since the impacts of specific amino acid changes on antigenicity are completely not well determined (Paton *et al.* 2005).

There are various concerns associated with the use of conventional FMD vaccine such as duration of immunity, thermal stability, spectrum of protection, bio-containment and efficient distinction between infected and vaccinated animals. Therefore, many approaches were made for development of an alternative safe and effective vaccine (Rodriguez and Grubman 2009). Based on the information concerning FMDV capsid structure and knowledge about the immunological importance of G-H loop, several strategies were used to develop subunit vaccines. These include use of VP1 protein produced by recombinant DNA technology (Kleid *et al.* 1981), VP1 derived peptides (Strohmaier *et al.* 1982, Venkataramanan *et al.* 1994), use of live vector expressing VP1 fusion proteins (Kit *et al.* 1991, Kitson *et al.* 1991), DNA vaccine expressing VP1 epitopes alone (Wong *et al.* 2000), and co-administration of DNA vaccine expressing VP1 with DNA encoding IL-2 (Wong *et al.* 2002). However, till now none of these vaccines elicit the same level of protection as the current, conventional inactivated whole virus vaccine (Doel 2005). The lesser amount of protection offered by the subunit vaccine may be due to the limited number of antigenic sites and/or T-cell epitopes, which are unable to induce significant protection (Rodriguez and Grubman 2009). An alternative approach to protein and peptide vaccines is the production of the empty capsid which involves expression of the regions of FMDV genome (P1-2A-3C) that are essential for synthesis, processing and assembly of viral structural proteins to form the empty capsid. The empty capsid based vaccines which are highly immunogenic could easily be used for DIVA purposes (Grubman and Mason 2002). FMDV empty capsid based vaccines were produced using baculovirus (Li *et al.* 2008), adenovirus (Mayr *et al.* 1999, Mayr *et al.* 2001), herpesvirus (D'Antuono *et al.* 2010) some of them were tested in targeted species (Mayr *et al.* 2001, Pena *et al.* 2008, Li *et al.* 2011) . One of these molecular vaccines, based on an adenovirus vector has undergone advanced development and licensure process (Rodriguez and Gay 2011). In India, a trivalent (O, A and Asia-1) inactivated and oil adjuvanted vaccine at 3 PD_{50}/dose is being used for control of FMD. There are 4 FMD vaccine manufacturers in the country to meet domestic demand.

Global distribution of FMDV serotypes and topotypes

Various FMDV serotypes are not uniformly distributed across the world, the virus infection is primarily maintained within 3 continental epidemiological clusters Asia, Africa

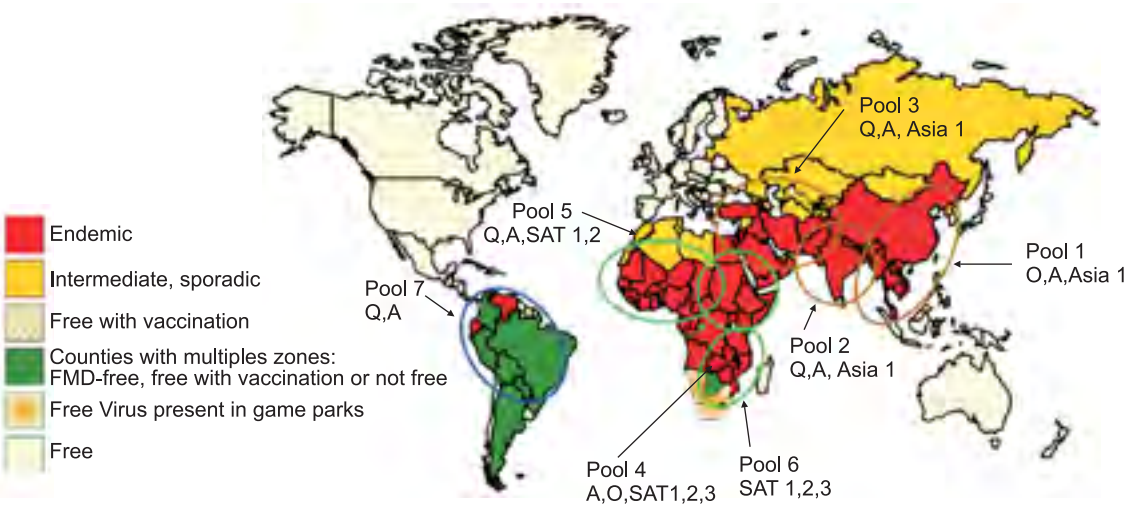

Fig. 1 Conjectured foot and mouth disease status in 2010 with regional foot and mouth disease virus pools and predominant virus serotypes (Modified by Paton *et al.* 2009)

and South America, which can be further grouped into seven major virus pools (Paton *et al.* 2009) (Fig. 1). Each of these virus pool contains at least 3 serotypes of virus, and because virus circulation is mainly within these regional reservoirs, strains have evolved which are specific to the region and that often (in type A and SAT viruses) require tailored vaccines (Paton *et al.* 2009). Only 4 serotypes (O, A, C and Asia-1) have been recorded in Asia and Middle East, while 6 out of the 7 serotypes (O, A, C, SAT-1,-2 and -3) have been circulating in Africa and only 3 serotypes (O, A and C) in South America. Globally last outbreak due to FMDV type C was recorded in 1996. With the advent of molecular biology techniques, it is now possible to divide FMDV into different genotypes/lineages/sub-lineages based on the genetic distances in the VP-1/P1 coding region (Pattnaik *et al.* 1998, Hemadri *et al.* 2000, Samuel and Knowles 2001, Tosh *et al.* 2002b and Knowles and Samuel 2003). Geographically restricted genotypes were named as topotypes, as they usually circulate in the defined geographic region (Knowles and Samuel 2003). Eleven topotypes were assigned for serotype 'O', while 3 topotypes were defined for serotypes A and C, and all the Asia-1 viruses were considered into a single unnamed topotype. In Africa, 9, 14 and 5 topotypes were defined for SAT-1, SAT-2 and SAT-3 viruses respectively (Knowles *et al.* 2010).

Europe: With respect to the epidemiological patterns of FMD, entire Europe is grouped into 2 categories, countries recognised by OIE as free of FMD without vaccination, and countries not recognised by OIE as free of FMD without vaccination because of being at risk of incursion of FMD from neighbouring regions. Almost all European countries, west of Russian federation and the Balkan countries of Bosnia, Macedonia and Serbia-Montenegro are free of FMD in Europe. Although the trans-caucasus countries are mainly free of FMD but are bordering endemically affected parts of Iran and Turkey. In Turkey, FMD is being reported throughout the year in the east and south-east of Anataolia, while western Anataolia only experience periodic incursion of disease due to animal movement from the east and south-eastern parts of the country (Leforban and Gerbier 2002, Rweyemamu *et al.* 2008 and Valarcher *et al.* 2008).

North America, Central America and Caribbean: North America is free from FMD and last reported outbreak was seen during 1952 and 1953 (Bachrach 1968). North America was able to control FMD due to 'stamping out' policy (Sutmoller *et al.* 2003). Central America and the Caribbean have never reported outbreaks of FMD and have remained free of the disease up to now.

South America: FMD was first recognised in South America in 1870, in the Province of Buenos Aries, Argentina, in the central region of Chile, in Uruguay and southern Brazilian states, due to importation of livestock from Europe (Correa Melo *et al.* 2002, Sutmoller and Casas Olascoaga 2003). FMD spread further into central western Brazilian States and was recorded in Peru, Bolivia and Paraguay during the first half of 20th Century and in Columbia during 1950s and in Ecuador during 1961. Since then, FMD has become endemic in South America. Three distinct epidemiological situations could be observed in South America: the FMD free countries/zones (Chile, Uraba in Colombia, the Guyanas, eastern and western Brazil states and the Atlantic coast in Columbia), the FMD endemic regions (the Andean areas, northern and north-eastern Brazil), and epidemic area in the Southern Cone (Saraiva 2004).

In South America, FMD spreads mainly with the trade of bovines. The knowledge of time-space behaviour of the disease, also known in South America as FMD ecosystems is useful in planning and executing the control/eradication strategy. The concept of ecosystems (primary endemic, secondary and sporadic) is based on a series of variables such as cow/calf ratio, number of milking cows per herd, existence of other susceptible species and average size of herd. Besides, the Omega 1 index, which is based on time/space distribution of disease for each country/region on a 10-year time series is also a variable for the consideration of FMD ecosystems (Saraiva 2004). The Hemispheric Foot-and-Mouth Disease Eradication Plan (PHEFA), launched in late 1980s, was instrumental in the application of the FMD ecosystems concept (Rweyemamu *et al.* 2008).

FMDV serotypes A, O and C were recorded in South America. FMDV type C was prevalent in Argentina, Bolivia, Brazil, Paraguay, Peru and Uruguay between 1972 and 1995. While type C is rare in the Andean region, type O and A are quite common. Since 2003, type O has occurred frequently in Ecuador. In Venezuela, type A FMD virus is widespread in the country, especially close to the border with Colombia. In Venezuela, serotype O virus is also present along with type A (Saraiva 2003).

To control the disease, authority in Argentina, Uruguay and Brazil decided to implement measures such as vaccination of the whole population in three consecutive rounds, stringent animal movement control, controlled culling of in-contact herds and serological sampling to evaluate the presence of viral activity. As a part of the project to achieve freedom from FMD, serological studies by 3D (VIA) and 3ABC-ELISA/EITB were conducted by several countries in South America. This served as a support to South American countries in designing their FMD control/freedom strategies (Correa Melo *et al.* 2002). As of now, Chile, Guyana, Patagonia, Southern and Central Western zones in Peru are free from FMD without vaccination (Correa Melo and Lopez 2002).

Africa: Depending on the distribution of FMDV serotypes, topotypes, disease prevalence data and various factors such as animal movement patterns, impact of wildlife and farming system, various epidemiological clusters were proposed for FMDV distribution in Africa. These epidemiological clusters are Southern African Development Community (SADC),

Angola, East African Community, Coastal West Africa, Soudan/Sahel, North Africa/Meghreb, and Inter-governmental Authority on Development (IGAD) (Rweyemamu *et al*. 2008). South-SADC countries (Swaziland, Lesotho, South Africa, Botswana and Nambia), meet the conditions of the OIE for zonal/country freedom from FMD without vaccination. However, in some of these countries there are isolated wildlife areas, where the African buffaloes are known to be asymptomatically infected with FMD virus serotypes SAT-1, -2 and -3. Therefore, the wildlife areas are separated from livestock through a system of game-proof fencing and vigorous surveillance. The north-SADC cluster, consists of Zimbabwe, Zambia, Mozambique, Malawi and southern Tanzania. Though, in these countries FMD was in control through intensive vaccination and animal movement control during the 1970s and 80s, currently serotypes SAT-1,-2 and -3 are circulating in the north SADC cluster (Rweyemamu *et al.* 2008). The primary source of infection seems to be from African wild buffaloes, which are capable of maintaining silent infection of SAT serotypes (Vosloo *et al.* 2002b). In Angola, serotypes exotic to Southern Africa occurred regularly up to 1975 when official reporting seems to have ceased. Therefore, there is little information about the true incidence of FMD in this cluster (Rweyemamu *et al.* 2008).

The epidemiology of FMD in the West and Central Africa has not been deeply studied. In the Central Africa, serotypes O, A, SAT-1 and SAT-2 were responsible for most outbreaks of FMD. In West Africa most outbreaks were caused by serotype A, followed by serotypes SAT-2, SAT-1 and O respectively. East African Community (EAC) consists of the countries Tanzania, Uganda, Kenya, Rwanda and Burundi. These countries perhaps promote the most complicated FMD situation in the world. Serotypes O, A, C, SAT-1 and SAT-2 are endemic in the EAC cluster. This appears to be the only region in the world where serotype C was found in recent times (Vosloo *et al.* 2002a).

The Soudan/Sahel cluster consists of Western Sudan, Niger, Chad, Burkina Faso, Mali, Northern Nigeria and Senegal. This cluster serves as an important disease corridor, linking the IGAD cluster with West Africa and West Africa with North Africa. In North Africa, FMD has not been reported since 1999, through routine preventive vaccination and other measures. However, in Libya and Egypt sporadic cases of FMD were reported byKnowles *et al.* (2007) and Rweyemamu *et al.* (2008). FMD is endemic in the IGAD cluster comprising Sudan, Eritrea, Ethiopia, Somalia, Northern Kenya and Northern Uganda. Habiela *et al.* (2010) reported that long-distance animal movement and within country circulation are the main factors for the prevalence of O, A and SAT-2 serotypes in Sudan. Molecular characterization of viruses recovered from FMD outbreaks in Ethiopia during 1981-2007, revealed the emergence and re-emergence of serotypes O and SAT-1 (Ayelet *et al.* 2009). Therefore, continuing emergence of FMD viruses in Ethiopia may affect the spread and consequent control strategy of FMD in Africa continent.

Asia: Based on the distribution of FMD virus serotypes, disease prevalence and proximity to FMD endemic neighbouring country, the following epidemiological clusters can be described for prevalence of FMD in Asia.

Middle East: FMD was recorded in almost all countries of the Middle East on numerous occasions in the past; Iran, Iraq and Syria can now endemic to FMD. Molecular characterisation of FMD viruses at World Reference Laboratory for FMD, revealed a link between the virus strains from Afghanistan, Pakistan, Saudi Arabia, Iran and Turkey, suggesting that FMD possibly spreads from South-Central Asia westward through the trade of ruminants. Therefore, viruses of serotype Asia-I are now constantly present, together with the serotypes O and A. From Africa, both SAT-1 and SAT-2 serotypes invaded from Ethiopia, Somalia and Sudan into Middle East primarily by trade. Therefore, sustainable FMD control in the Middle East depends on the success of control programme in Far East, Central and South Asia and also in Africa (Aidaros 2002).

South-East and East Asia: With respect to South-east Asia, FMD is endemic in Cambodia, Laos, Malaysia, Myanmar, the Philippines, Thailand and Vietnam. However, three countries (Brunei, Indonesia and Singapore) are recognised as free from FMD without vaccination by OIE. East Malaysia and parts of the Philippines (Mindanao, Visayas, Palawan and Masbate) are also recognized internationally as being free of FMD without vaccination. Serotypes O, A and Asia-1 are the only 3 serotypes endemic in the region and type O is the most common circulating serotype with 3 distinct topotypes, South-east Asia (SEA), Middle east-South Asia (ME-SA) and Cathay (pig-adapted) (Gleeson 2002). Phylogenetic analysis revealed how different introductions of viruses may be sourced from different countries and similar viruses can consequently be found in various countries in South-East Asia. A phylogenetic analysis showed that Malaysian isolates belonging to the O/Mya-98 lineage were interleaved with those from neighbouring countries in close groups (Abdul-Hamid *et al.* 2011).

In East Asia, Japan, Republic of Korea and Taiwan Province of China are recognised as countries free of FMD without vaccination. The last outbreaks of FMD in Japan and Republic of Korea were during 2000 and 2002 respectively (Sakamoto and Yoshida 2002). However, in 2010 FMD virus type O (lineage Mya-98) outbreaks were reported in Japan and Republic of Korea (Akashi 2010, Yoon *et al.* 2011).

In China, the FMD virus serotypes O and Asia-1 and A are prevalent. The Cathay topotype of FMD virus serotype O appears to be endemic in Swine production systems of Southern China. FMD viruses regularly move into China from Myanmar and Laos through cattle trade into Yunnan

(Rweyemamu *et al.* 2008).

Control and eradication of the disease was facilitated in the regions through inter-country participation, as the disease is of transboundary nature. South-east Asia—Foot and Mouth Disease (SEA-FMD) campaign was launched in 1997 to coordinate sub-regional control of FMD. In 2010, China joined the campaign and it was renamed as South-East Asia and China Foot and Mouth Disease (SEAC-FMD) campaign. The main strategy is the implementation of a progressive zoning approach to ensure effective use of limited resources from the donors and national governments. The campaign has established Epidemiology Network (EpiNet) and a Laboratory Network (LabNet) to provide technical support to enhance member countries' capacity for effective surveillance and diagnosis.

Central Asia: With respect to Central Asia, countries which form the core are Kazakhstan, Kyrgystan, Tajikistan, Turkmenistan and Uzbekistan that were kept FMD free in the past by virtue of the USSR border disease control programme. However, since the transition to independence, FMDV serotype O and Asia-1 have become widespread due to increased cross border trade and contiguous livestock farming system. Most of the times the source of FMD infection to Central Asia, is from Afghanistan and China. In the absence of information and molecular phylogenetic study, little epidemiological information can be deduced from unsystematic reporting in Central Asia (Rweyemamu *et al.* 2008).

South Asia: India and Pakistan are the key countries for the progressive control of FMD in South Asia (Rweyemamu *et al.* 2008). Jamal *et al.* (2010) found that serotype O is most prevalent followed by Asia-1 and A in Pakistan. The authors also reported that higher number of outbreaks of the disease was noted between January and March, due to the movement of livestock during the religious festival *Eidul Azha*. With respect to FMD distribution in Sri Lanka, Bhutan, Nepal, Bangladesh and Maldives, very little is known.

Epidemiology of FMD and its control in India

Livestock sector is an important contributor in Indian economy–its overall contribution to the agricultural GDP is 28-32% and to national GDP is 4 to 6%; and it employs 8-10% country's labour force. India has largest population in the world with 528 million domesticated animals; highest buffalo population in the world (105.3 million), second highest in cattle (199 million) and goats (140.5 million), and third in sheep (71.5 million). Such a big population is under great risk of FMD due to unrestricted movements of animals throughout the country, limited systematic vaccinations, and inapparent infection in small ruminants which probably act as reservoir of the virus. Direct loss of ₹ 20,000 crore/annum was reported by Venkataramanan *et al.* (2006). Eighty per cent of the total direct loss caused by FMD is due to drop in milk production (Mathew and and Menon 2008). The other economic losses caused by the disease are due to massive expenditures by the government sector on FMD control, added cost on treatment, low productivity (meat, wool etc.) and loss of draught power.

In India, FMD remains endemic and was first officially documented in 1864 during extensive outbreaks in many parts of the country (Government of India 1868). However, research on FMD by Indian Council of Agricultural Research (ICAR) dates back to 1929. Epidemiological studies on FMD in India was initiated by ICAR in the form of an "All India Co-ordinated Research Project (AICRP) for virus typing" in 1968 with a central laboratory at Mukteshwar and 3 regional centres. Subsequently, the scope of the project was expanded in 1971 to "AICRP for Epidemiological studies on FMD" with addition of 4 more regional centres. The AICRP was expanded to Project Directorate on Foot and Mouth Disease (PDFMD), which is the premier institute for FMD research in the country under ICAR, in 2001. Currently the project directorate has 8 regional centres and 15 network units covering the length and breadth of the country. The PDFMD has developed scientific and technical expertise in both conventional and cutting edge areas of FMD research for use in the country. Further, PDFMD also acts as the FAO Reference Centre for South-Asia, in the field of FMD diagnosis, epidemiology and research. O, A and Asia-1serotypes of the FMD virus are currently prevalent in India and the disease is reported throughout the year. Among the serotypes, type O is the most prevalent one and accounts for 83-93% of the outbreaks followed by Asia 1 (3-10%) and A (3-6.5%). Serotype C has not been reported in the country since 1995. Different genotypes and lineages of the 3 serotypes were detected in the country. Serotype O, FMD virus dominates the FMD outbreak scenario in India, and on molecular studies all the serotype O isolates were grouped into 7 sub-lineages namely Branch A, B and C-I, C-II, Pan Asia I, Pan Asia II and 'Ind2001' under Middle East-South Asia (ME-SA) topotype (Fig. 2). Currently 3 lineages, viz. 'Ind2001', Pan Asia I and Pan Asia II, are responsible for type O outbreaks in the country. The Pan Asia II lineage emerged in 2003 and since then it is causing outbreaks along with parent Pan Asia I viruses. Lineage 'Ind2001' first identified in the year 2001, re-merged in 2008 and is co-circulating along with Pan Asia lineages since then. Though the current serotype O vaccine strain (IND R2/1975) is not genetically identical to the circulating field strains, it continues to antigenically cover the outbreak strains. Serotype A is the most diverse among all the 7 serotypes of FMDV. Throughout the world, 26 serotypes have been identified till now, however in India, 4 genotypes [genotypes I (2), IV (10), VI (16) and VII (18)] were identified (Fig.3) (Mohapatra *et al.* 2011c). Genotypes 2 (Euro–South Asian topotype) and 10 (Asia topotype) of serotype A were recorded before 1990 and no longer exist in India. Endemic co-circulation of 2 genotypes (16 and 18) with dominance of

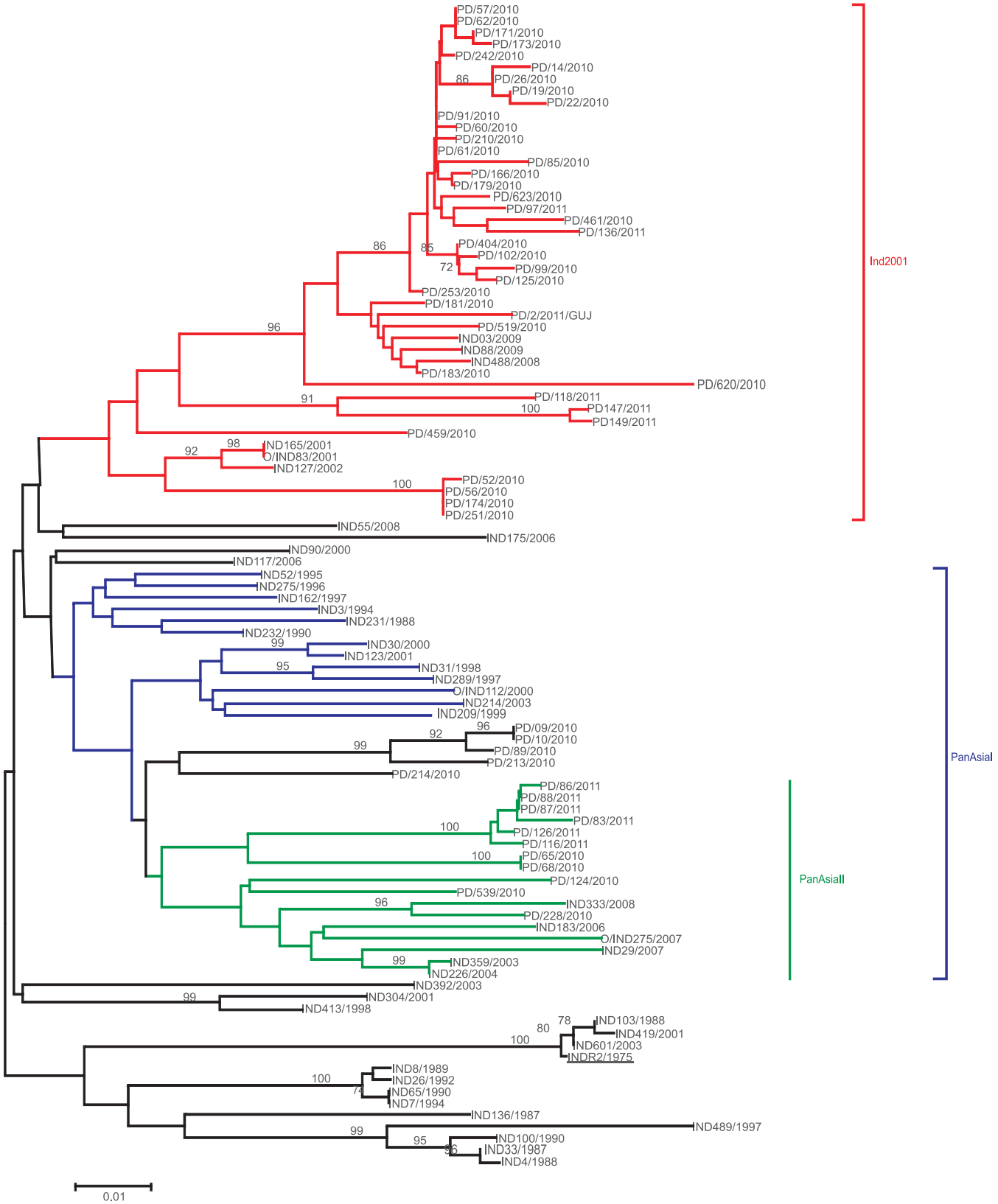

Fig. 2 Phylogenetic tree depicting genetic relationship among serotype O isolates at VP1 coding region.

Currently used vaccine strain is underlined. The isolates from different states could be placed in 3 major lineages designated 'Ind2001' and Pan Asia. Within parent Pan Asia lineage, a divergent Pan Asia II sub-lineage emerged in 2003.

genotype 18 was observed in the country, while other 2 (2 and 10) genotypes have not been detected since 1990. The isolates belonging to genotypes 16 and 18 are divergent both genetically and antigenically. The current serotype A vaccine strain is from genotype 18 and is most appropriate in covering the circulating filed strains. The earlier vaccine strains were from genotype IV (10) and VI (16). In serotype Asia-1 three prominent lineages (lineages B, C and D) are in circulation in India (Sanyal *et al.* 2010) (Fig. 4). The lineage B which includes the vaccine strain IND 63/1972 has 210 amino acids in VP1 and this lineage never appeared after the year 2000. The lineage C, which was prominently circulating in India during the period 1993 to 2001, has an extra amino acid at position 44 of VP1. Lineage D, within the lineage C appeared in 2001 and it outnumbered the parent lineage in terms of field outbreaks. Lineage C has been responsible for all Asia-1 outbreaks in the country since 2005. The serotype Asia1 vaccine strain IND 63/1972 currently in use for vaccine production is continuing to provide optimum antigenic coverage for the circulating field strains.

Control of FMD is significant for protecting the livestock industries and for improving livelihoods and income generation in the developing countries, where FMD is endemic. Progressive risk reduction of FMD can help in progressive market access of livestock commodities from developing countries. In India, progressive control pathway (PCP) for the control and eradication of the disease was adopted (Rweyemamu *et al*. 2008). The FMD control programme (FMDCP) was launched in India in X Plan (based on epidemiological data acquired over more than 35 years) in 54 districts selected in 8 states of the country covering 30 million cattle and buffalo. In these regularly vaccinated areas, there has been progressive build up of herd immunity and substantial decline in the occurrence of the disease, severity of clinical disease and NSP reactors/converters since 2006-07 (Table 1) and 2003-04 (Table 2). With example of the states like Punjab and Haryana where only a few sporadic cases of FMD could be recorded due to impact of regular vaccination and building up of herd immunity. With the implementation of FMDCP, surveillance was intensified for antibody against structural and non structural proteins of FMDV to a clear picture of the disease in terms of herd immunity, virus circulation and clearance from time to time. For differentiation of infected animals from vaccinated ones, a panel of recombinant NSP of FMDV were utilized for development of profiling immunoassay. Some of the vaccines used in India are having very low levels of NSP that do not elicit prolonged immune response even after multiple vaccinations (Mohapatra *et al.* 2011a), however for exclusion of occasional false positive cases, a multiple DIVA ELISA using 3AB3, 3ABC, 2C and 3D has been developed. Detailed investigation in Haryana (FMDCP area) using serum samples collected periodically has revealed decline in 3AB3 DIVA reactors from 31.94% before implementation of regular vaccinations to 12% after eight rounds of vaccinations, indicating reduction in DIVA converters. Due to success of the FMDCP in 54 districts, additional 167 districts (another 80-90 million cattle and buffalo) have been included under

Table 1. Number of confirmed FMD outbreaks/cases in different geographical region of the country during 2006-07 to 2010-11

Year	South Z	North Z	Central Z	West Z	East Z	North-East Z	Total
2006-07	224	7	23	29	431	64	778
2007-08	445	20	35	31	258	88	877
2008-09	64	18	33	16	66	43	240
2009 -10	59	55	20	24	365	75	598
2010-11	51	9	29	18	29	40	176
Total	843	109	140	118	1149	310	2669

Z, Zone

Table 2. FMD outbreaks/cases in FMDCP districts

Year	Uttar Pradesh	Haryana	Punjab	Maharashtra	Gujarat	Andhra Pradesh	Tamil Nadu	Kerala	Andaman Nicobar	Lakshadweep	Dadra and Nagar Haveli
2003-04	42	60	4	29	12	37	….	63	….	….	….
2004-05	3	1	….	1	1	….	….	1	….	….	….
2005-06	2	….	….	3	1	….	….	….	1	….	….
2006-07	1	1	….	….	1	….	….	….	….	….	….
2007-08	….	….	….	1	1	….	….	….	….	….	….
2008-09	….	….	….	…..	2	….	….	….	….	….	….
2009-10	15	1	…..	6	0	3	….	….	….	….	….

…., Disease not reported/ Data not available

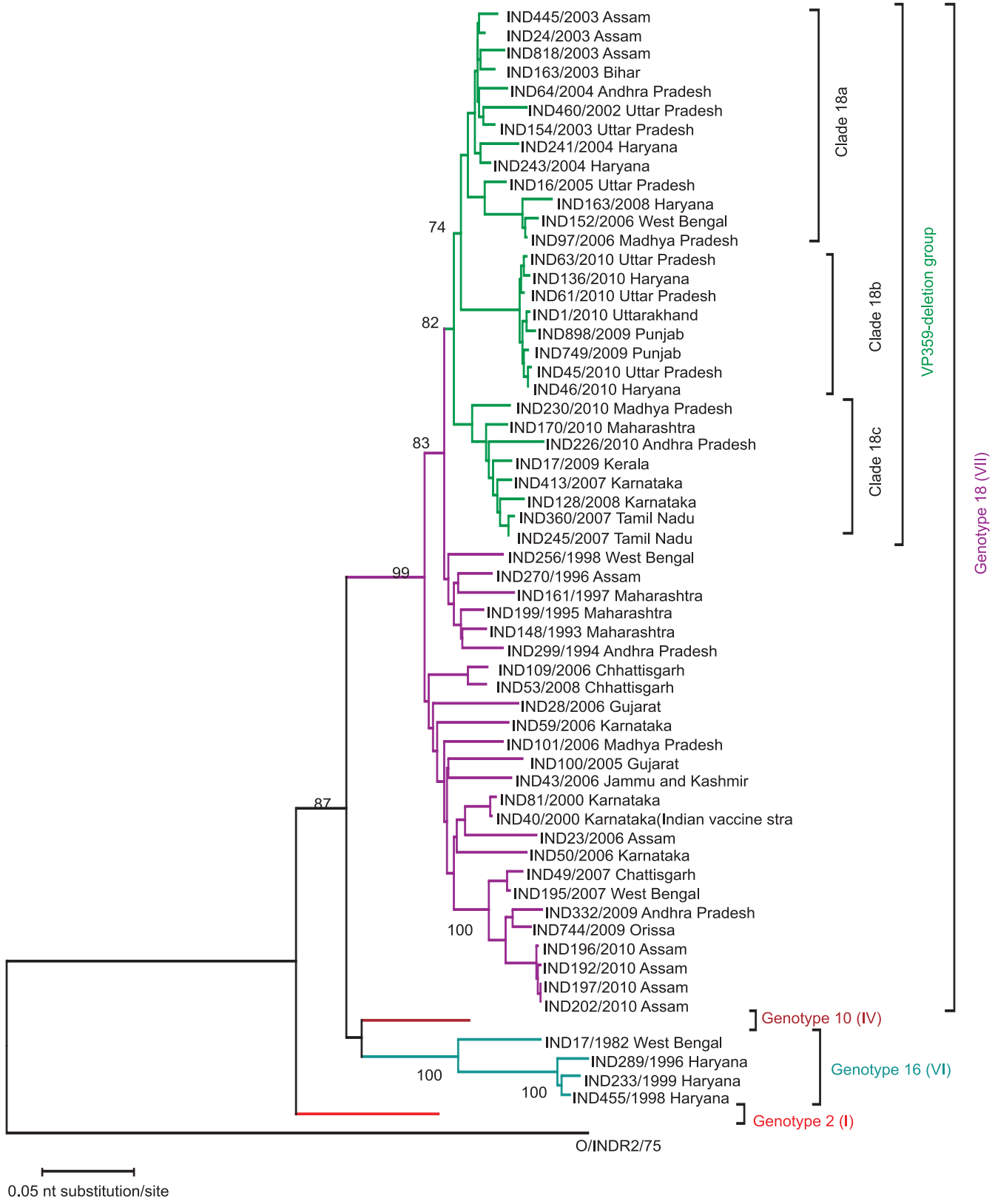

Fig. 3 Phylogenetic tree depicting genetic relationship among serotype A isolates at VP1 coding region.

The isolates from India were placed in four genotypes (2, 10, 16 and 18) showing more than 15% nt. divergence among them. Isolates of genotype 18 exclusively dominates type A outbreaks in the country since 2001 and currently used vaccine strain belonged to this genotype.

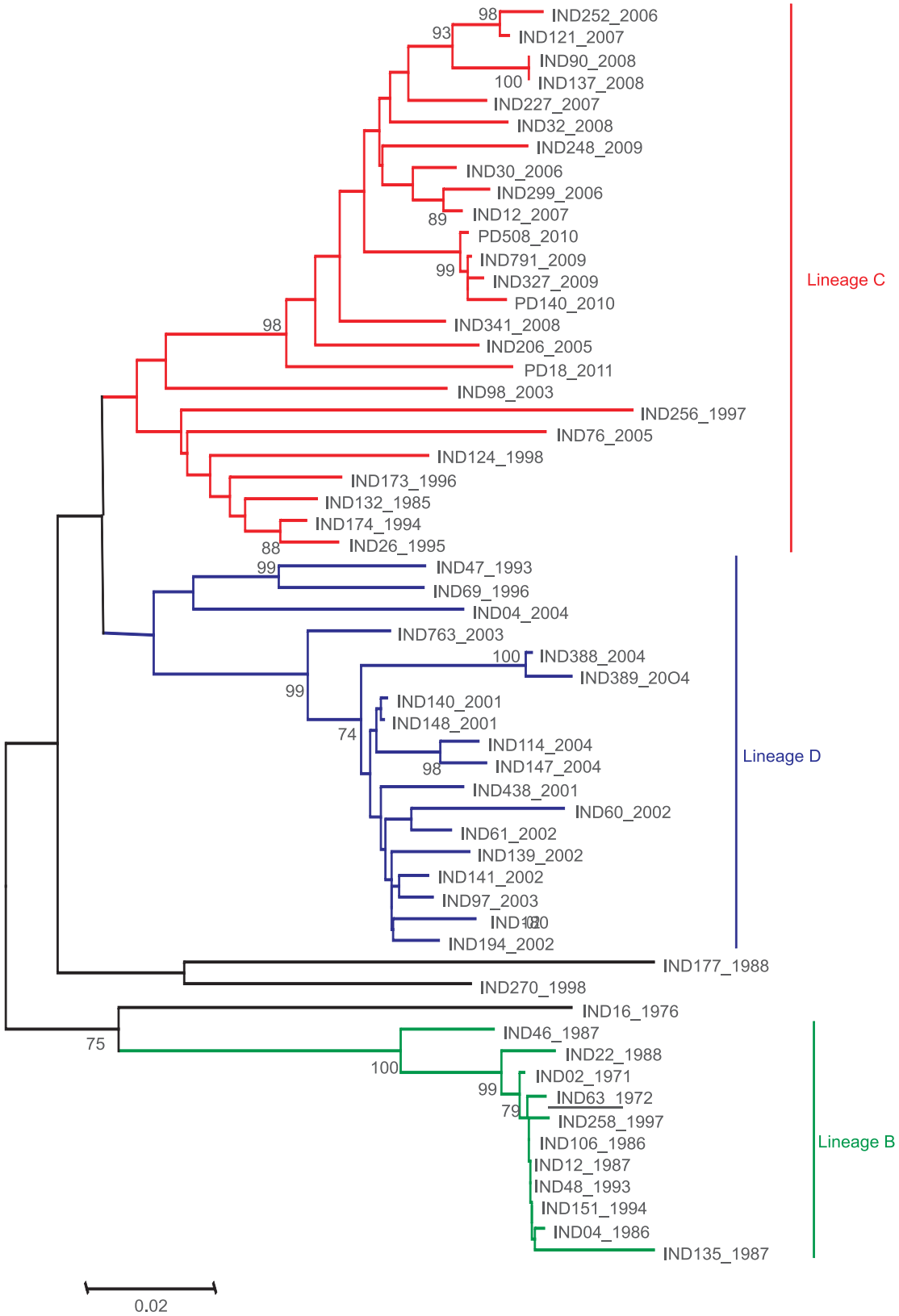

Fig. 4 Phylogenetic tree depicting genetic relationship among serotype Asia1 isolates at VP1 coding region.

The isolates from India were placed in three lineages designated B, C and D. Indian vaccine strain (IND63/1972) belonged to lineage B. Lineage C is exclusively responsible for all type Asia1 outbreaks in the country since 2005.

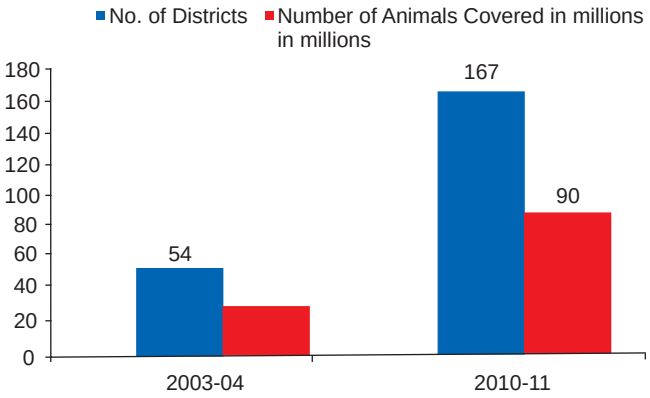

Fig. 5 Coverage of FMD-CP

the programme in 2010-11 (Fig.5) bringing total districts under FMD-CP to 221 covering states of Southern peninsula (Kerala, Tamilnadu, Puducherry, Karnataka and Andhra Pradesh), Maharashtra, Goa, Daman and Diu, Gujarat, Punjab, Haryana, Delhi, Dadra and Nagar Haveli, Andaman & Nicobar Islands, Lakshadweep and 16 districts in Uttar Pradesh (Fig. 5 and Fig. 6). Required doses of trivalent vaccine and companion diagnostics (LPBE and DIVA) are available locally. Recently, establishment of a National FMD Commission was proposed to coordinate all activities associated with FMD control programme including quality assurance of vaccines in order to effectively implement and monitor the control programme and achieve zoning of the disease (initially with vaccination) in the country in a definite time frame. Now great emphasis is given on improvements in vaccination strategies so that vaccination density and timing is made uniform to obtain the best outcome. In addition, the availability of quality vaccine as per the requirement is being ensured with public-private partnership. Research and development in the country is oriented towards obtaining quality vaccine with high potency which will cover wide antigenic spectrum and elicit longer duration of immunity (9-12 months). Use of bivalent and monovalent vaccines based on epidemiological data is under discussion for judicial use of limited resources for maximum outcome. Use of monovalent vaccines (where a particular serotype is prevalent for a long time) will reduce the cost of the operation. Additionally, due importance has been diverted to study the role of small ruminants in FMD epidemiology in the country in recent years (Ranabijuli *et al.* 2010) and inclusion of small ruminants in vaccination campaign is under consideration.

As per the current scenario, it is expected that by 2018, the disease situation in entire Southern peninsula will be under control (stage 3 of PCP) with a herd immunity of >85%. By 2025, it is anticipated that the southern peninsula will be disease free with vaccination (stage 4) when it will be monitored for the next five years. It is expected that by 2025, most parts of the country will be in stage 3, southern peninsula will be in stage 4 (free with vaccination), and parts of northern India will enter stage 5 of PCP.

Fig. 6 Districts covered under FMD-CP.
Districts covered: Red, 2003-04; Green, 2010-11

From the above account of global distribution of FMD, it can be proposed that any coordinated regional/global strategy for FMD control should be based on the efficient epidemiological assessment of incidences and distributions of FMD. Identification of primary endemic areas (virus maintenance areas), secondary endemic areas (areas of virus propagation) and epidemic areas (areas of explosive outbreak) are equally important for the development of a sustainable programme for the progressive control of FMD in endemic settings. Ecosystem-based description of FMD in South America can be a guiding principle for the progressive control of FMD in India and other endemic countries. It is relevant to apply landscape genetics approach to elucidate mechanisms, understanding basic ecological process driving infectious disease dynamics and to understand the linkage between spatially dependent population process and the geographical distribution both within host and parasite (Biek and Real 2010).

Future researches in FMD

1. Considering the extreme contagiousness of the disease and high antigenic diversity of the virus, the research would be directed towards thorough understanding of the origin and maintenance of nuclei of virus infection, spread of the virus and differential distribution of virus serotypes in the country. An important research requirement is to establish how the disease, ecosystem condition, livestock rearing pattern and other

demographic meta-data modulate the antigenic diversity of FMDV. It is essential to study the spatial and temporal dynamics of FMDV by means of Bayesian phylogeography.

2. Early warning and immediate epidemic response require rapid diagnosis of FMDV isolates. Research work should be directed to develop animal-side diagnostics and pre-clinical diagnosis of FMD for an early and confirmatory detection of FMDV.
3. Current serological methods to select vaccine strains are cumbersome, difficult to standardize and require the generation of monovalent (vaccine-specific) bovine antisera. In future, these tests shall be replaced by rapid, gene sequence-based prediction of antigenicity and diversity/similarity. The combined application of virus gene sequence, virus structure and virus neutralization titre data would be helpful for the prediction of antigenicity of the vaccine strain.
4. Control of FMD in India through vaccination could be enhanced, if vaccines are available with improved thermal stability and longer duration of immunity. Molecular biology and biotechnological intervention to replace the unstable amino acids with a stable version in order to achieve better stability of the viral capsid structure that could result in an improved thermal stability of the existing vaccine. The stabilised capsid antigen would also able to produce a faster and stronger immune response *in vivo*.
5. By using reverse genetics techniques, quick substitution of vaccine viral capsid gene sequence with that of the field virus, would lead to the generation of robust, efficacious and customised vaccine as per the FMD outbreak situation.
6. There are also research gaps with respect to the ability to predict the performance of vaccine against FMDV infection without conducting *in-vivo* vaccine potency tests in cattle. Research work would be directed towards the development of alternate assay systems for the assessment of FMD vaccine potency to replace costly cattle challenge-protection experiments.
7. An understanding of host and viral factors for establishing FMD carrier status may lead to a strategy to cure or limit the persistent infection. Stimulation of local mucosal immune response by means of mucosal vaccination or by modulation of various cytokines may be useful in eliminating the virus. Alternatively if persistent infection cannot be cured or prevented, future research must be directed towards absolute reliable means for carrier identification.
8. Though considerable progress has been made in developing and determining the performance characteristics of different DIVA tests, in future assays based on a panel of various non-structural proteins would be required in estimating the likely prevalence of infection following the use of prophylactic vaccination. The DIVA panel test would help in post-outbreak serosurveillance strategies to facilitate rapid recovery of FMD-free status.

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Inoculation of Swine with Foot-and-Mouth Disease SAP-Mutant Virus Induces Early Protection against Disease

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Foot-and-mouth disease virus (FMDV) leader proteinase (L^{pro}) cleaves itself from the viral polyprotein and cleaves the translation initiation factor eIF4G. As a result, host cell translation is inhibited, affecting the host innate immune response. We have demonstrated that L^{pro} is also associated with degradation of nuclear factor κB (NF-κB), a process that requires L^{pro} nuclear localization. Additionally, we reported that disruption of a conserved protein domain within the L^{pro} coding sequence, SAP mutation, prevented L^{pro} nuclear retention and degradation of NF-κB, resulting in *in vitro* attenuation. Here we report that inoculation of swine with this SAP-mutant virus does not cause clinical signs of disease, viremia, or virus shedding even when inoculated at doses 100-fold higher than those required to cause disease with wild-type (WT) virus. Remarkably, SAP-mutant virus-inoculated animals developed a strong neutralizing antibody response and were completely protected against challenge with WT FMDV as early as 2 days postinoculation and for at least 21 days postinoculation. Early protection correlated with a distinct pattern in the serum levels of proinflammatory cytokines in comparison to the levels detected in animals inoculated with WT FMDV that developed disease. In addition, animals inoculated with the FMDV SAP mutant displayed a memory T cell response that resembled infection with WT virus. Our results suggest that L^{pro} plays a pivotal role in modulating several pathways of the immune response. Furthermore, manipulation of the L^{pro} coding region may serve as a viable strategy to derive live attenuated strains with potential for development as effective vaccines against foot-and-mouth disease.

Foot-and-mouth disease (FMD) is one of the most contagious diseases of livestock animals. The etiologic agent, FMD virus (FMDV), infects cloven-hoofed animals, including cattle and swine, causing a devastating disease that can significantly impact the economy of affected countries (33). The virus is the prototype member of the *Aphthovirus* genus of the *Picornaviridae* family and consists of a positive-sense single-stranded RNA genome of about 8,000 nucleotides surrounded by an icosahedral capsid containing 60 copies each of four structural proteins. Upon infection, the viral RNA is translated as a single polyprotein which is concurrently processed by three virus-encoded proteins, leader (L^{pro}), 2A, and $3C^{pro}$, into precursors and mature structural (VP1, VP2, VP3, and VP4) and nonstructural (NS) (L^{pro}, 2A, 2B, 2C, 3A, $3B_{1,2,3}$, $3C^{pro}$, and $3D^{pol}$) proteins (67).

Control of FMD is achieved by vaccination, inhibition of movement of susceptible animals, slaughter of infected and FMD-susceptible contact animals, and decontamination. The current commercial FMD vaccine, a chemically inactivated whole-virus preparation emulsified with adjuvant, is most commonly used in enzootic areas, and it has been very successful in reducing the number of outbreaks worldwide (33). However, this vaccine platform has some deficiencies: (i) the vaccine manufacturing requires a biosafety level 3 (BSL3) containment facility, (ii) unless highly purified, the vaccine does not allow differentiation between infected and vaccinated animals (DIVAs), (iii) there is a potential risk of developing asymptomatic disease carriers upon exposure of vaccinated animals to infectious virus, and (iv) affected countries need more time to regain FMD-free status and resume trading if vaccination rather than slaughter is used. To address some of the disadvantages of the inactivated vaccine, we have developed a new approach using a replication-defective adenovirus subunit vaccine expressing empty viral capsids that has been very successful in swine and cattle (36, 51, 63). Nevertheless, both the inactivated and the subunit vaccines require approximately 7 days to induce protection. It has been reported that rapid and long-lasting protection against viral infection is usually best achieved by vaccination with attenuated viral vaccines. Indeed, some viral diseases, including smallpox and rinderpest, have been eradicated using such vaccines (30, 56). So far, no attenuated vaccine has been successfully used against FMDV. Among others, a candidate attenuated vaccine was previously developed by deletion of the NS viral L^{pro} coding region (leaderless virus) (64). Despite the reduced pathogenicity of this virus in swine and cattle, vaccinated animals were not completely protected against homologous wild-type (WT) virus challenge, probably due to the slow and limited viral replication of the mutant strain.

FMDV has evolved several mechanisms to evade the host immune response, and L^{pro} plays a central role in pathogenesis (35). L^{pro} is a papain-like proteinase that autocatalytically removes itself from the growing polypeptide chain (74) and cleaves the host translation initiation factor eIF4G, resulting in the shutoff of host cap-dependent mRNA translation (22), a characteristic of most picornavirus infections (29). As mentioned above, it has been demonstrated that a virus lacking the L^{pro} coding region, leaderless virus, is highly attenuated in cattle and swine (12, 48, 64). Apparently, the reason for this attenuation is the inability of the virus to block type I interferon (alpha/beta interferon [IFN-α/β])

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translation (14) and transcription of IFN-β (19), similar to other picornaviruses (5, 18, 46). Inhibition of IFN-β transcription is associated with L^{pro} translocation to the nucleus of the infected cell and subsequent degradation of p65/RelA, a subunit of transcription factor nuclear factor kappa B (NF-κB) (20). We have recently identified a conserved protein domain within the L^{pro} coding region known as the SAF-A/B, acinus, and PIAS (SAP) domain (21). SAP domains which are present in some DNA binding proteins usually involved in transcriptional control mediate protein-protein interactions between activators and repressors (1, 41). Mutations of two conserved amino acid residues in the SAP domain of FMDV L^{pro} altered the protein subcellular localization during the course of infection, making the virus (SAP-mutant virus [A12-SAP]) unable to induce degradation of NF-κB and thus resulting in upregulation of expression of several cytokines, chemokines, and interferon-stimulated genes (ISGs) (21).

Upon viral infection, NF-κB translocates to the nucleus of the cell, where it binds to its cognate promoter sites to activate transcription of an array of genes, including proinflammatory cytokines, chemokines, and adhesion molecules, most of them involved in the natural response against pathogens (50). Apart from a role in the adaptive immune response, NF-κB also has a very important function in innate immunity by the activation of the Janus kinase (JAK)/signal transducer and activators of transcription (STAT) antiviral pathway (39). Furthermore, proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor alpha (TFN-α), directly induced by NF-κB, are also involved in the establishment of an antiviral state against RNA viruses (7).

Inflammatory cytokines are divided into two major groups, depending on their action in the organism: proinflammatory cytokines (IL-1, IL-6, TNF-α, and IFNs) and anti-inflammatory cytokines (IL-10). A dynamic balance exists between pro- and anti-inflammatory components (57) that could be disrupted upon viral infection. Previous studies have shown that during acute FMDV infection in swine, the virus induces an immunosuppressive stage, characterized by T cell unresponsiveness and transient lymphopenia affecting all T cell subsets and correlating with the appearance of viremia (3, 26). One possible mechanism by which the virus induces immunosuppression might be related to the production of IL-10 (25), an immunosuppressive cytokine that plays an important stimulatory role in the function of B lymphocytes and the production of antibodies by B1 lymphocytes (2). In addition, FMDV interferes with expression of type I IFNs, which are effective in inhibiting virus replication *in vitro* and *in vivo* (13, 19, 23, 24, 52, 53). Little is known about the role of FMDV in modulating the expression of other cytokines; however, it has been reported that NS proteins of other picornaviruses inhibit secretion of IL-6 in tissue culture (15).

In the present study, we demonstrate that A12-SAP FMDV is attenuated not only *in vitro* but also *in vivo*. Intradermal (i.d.) inoculation of swine with high doses (10^5 to 10^7 PFU/animal) of A12-SAP did not result in detectable clinical signs, viremia, or virus shedding. A strong adaptive immune response comparable to that induced in animals infected with WT virus (A12-WT) was elicited, and animals inoculated with A12-SAP were completely protected when challenged with A12-WT virus 21 days after inoculation. Interestingly, inoculated animals were also completely protected when challenged as early as 2 days after vaccination, a time when the adaptive immune response could not be detected. Analysis of cytokines in sera and peripheral blood mononuclear cells (PBMCs) of FMDV WT-infected animals showed a decrease in the levels of IL-1β, IL-6, and TNF-α and an increase in IL-10. In contrast, FMDV SAP-vaccinated animals showed upregulation in the levels of TNF-α, IL-1β, and IL-6 as well as IL-10. These results suggest that *in vivo*, L^{pro}-dependent inhibition of a proinflammatory response in combination with virus-induced lymphopenia may play an important role in allowing successful FMDV infection and spread within the host.

MATERIALS AND METHODS

Cells and viruses. Porcine kidney (IBRS-2) cell lines were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the Plum Island Animal Disease Center. These cells were maintained in minimal essential medium (MEM; Gibco BRL, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) and supplemented with 1% antibiotics and nonessential amino acids. BHK-21 cells (baby hamster kidney cells, strain 21, clone 13, ATCC CL10), obtained from the American Type Culture Collection (ATCC; Manassas, VA), were used to propagate virus stocks and to measure virus titers. BHK-21 cells were maintained in MEM containing 10% calf serum and 10% tryptose phosphate broth supplemented with 1% antibiotics and nonessential amino acids. Cell cultures were incubated at 37°C in 5% CO_2.

FMDV A12-WT was generated from the full-length serotype A12 infectious clone pRMC35 (66). A12-SAP-mutant virus, a derivative of A12-WT containing mutations I55A and L58A in the L^{pro} region, was constructed by site-directed mutagenesis (21). All viruses were propagated in BHK-21 cells, concentrated by polyethylene glycol precipitation, titrated on the same cells, and stored at −70°C. Viruses of passage 6 for A12-WT and passage 5 for A12-SAP were used for all experiments, and the full-length sequences were confirmed by DNA sequencing of derived viral cDNA using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA).

Animal experiments. Animal experiments were performed in the high-containment facilities of the Plum Island Animal Disease Center following a protocol approved by the Institutional Animal Use and Care Committee. In a first experiment, 15 Yorkshire guilts (age, 5 weeks; weight, approximately 40 lb each) were divided into 5 groups of 3 animals each. Animals were inoculated i.d. in the heel bulb of the right hind foot with different doses of FMDV A12-WT (1×10^5 or 1×10^6 PFU/animal) or A12-SAP (1×10^5, 1×10^6, or 1×10^7 PFU/animal). Rectal temperatures and clinical signs, including lameness and vesicular lesions, were monitored daily during the first week postinoculation, and samples of serum and nasal swabs were collected on a daily basis. Serum samples were also collected at 14 and 21 days postinoculation (dpi). Clinical scores were determined by the number of toes presenting FMD lesions plus the presence of lesions in the snout and/or mouth. The maximum score was 17, and lesions restricted to the site of inoculation were not counted. Those pigs inoculated with A12-SAP were challenged 21 days later with 1×10^5 PFU/pig of FMDV A12-WT i.d. in the heel bulb of the left hind limb. Clinical signs and samples were collected on a daily basis for 7 days, and serum samples were also collected at 14 and 21 days postchallenge (dpc).

In a second experiment, 4 groups of 3 Yorkshire guilts each (age, 5 weeks; weight, approximately 40 lb) were subcutaneously (s.c.) vaccinated with attenuated FMDV A12-SAP (1×10^6 PFU/animal) followed by challenge at different times postvaccination (2, 4, 7, and 14 days postvaccination [dpv]) with 5×10^5 PFU/animal of virulent FMDV A12-WT i.d. in the heel bulb. One extra group of 3 pigs was inoculated with phosphate-buffered saline (PBS) and challenged 14 days later (control group). Serum samples were collected at 2, 4, 7, and 14 dpv. After the challenge, clinical signs were monitored daily during the first week, and samples were collected as described for the first experiment.

Virus titration in serum and nasal swabs. Serum and nasal swabs were assayed for the presence of virus by plaque titration on BHK-21 cells (passage levels 60 to 70). Serial 10-fold dilutions of the samples were

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allowed to adsorb on monolayers of BHK-21 cells grown in 6-well plates. Following 1 h adsorption, the inoculum was removed and 2 ml of MEM containing antibiotics, essential amino acids, and 0.6% gum tragacanth was added to each well. The plates were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 and then stained with a crystal violet-formalin solution to visualize the plaques. Virus titers were expressed as $\log_{10}$ PFU per ml of serum or nasal swab. The detection level of this assay is 5 PFU/ml.

Detection of FMDV RNA by rRT-PCR. At 1 to 7 dpc, frozen serum samples from animals that had no detectable clinical disease were thawed and processed for RNA extraction and measurement of specific FMDV RNA by real-time reverse transcription-PCR (rRT-PCR) as previously described (58). Samples were considered positive when threshold cycle (C_T) values were <40.

Determination of neutralizing antibody titer. Serum samples were tested for the presence of FMDV-specific neutralizing antibodies by a plaque reduction neutralization assay as previously described (48). Neutralizing titers were reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) induced by FMDV A12-WT in BHK-21 cells.

RIP of [^{35}S]methionine/[^{35}S]cysteine-labeled FMDV A12-infected cell lysates with swine serum samples. Radiolabeled lysates of FMDV A12-infected BHK-21 cells were incubated with individual swine serum samples from 0 and 21 dpc and examined for the presence of antibodies specific to FMDV structural and NS polypeptides by radioimmunoprecipitation (RIP) (34). Convalescent-phase serum from an FMDV-infected bovine was used as a positive control. After 60 min incubation at room temperature, antibodies were precipitated with *Staphylococcus aureus* protein A. Proteins were resolved by SDS-PAGE on a 15% gel and visualized by autoradiography.

Quantification of antibody isotypes by enzyme-linked immunosorbent assay (ELISA). The presence of FMDV-specific immunoglobulin M (IgM), IgG1, and IgG2 antibodies was detected by an indirect double-antibody sandwich assay as described previously (17), with some modifications. Briefly, Costar enzyme immunoassay/radioimmunoassay high-binding 96-well flat-bottom plates (Corning, NY) were coated with anti-FMDV antibody and incubated with an optimal dilution of either positive or negative FMDV antigen (17) prior to addition of test sera. Positive-control sera for IgM or for IgG1 and IgG2 were obtained from a swine inoculated with virulent FMDV A24 at 7 or 21 dpc, respectively. Positive-control sera were chosen for their ability to generate a definitive signal in their respective isotype-specific assays. Negative-control sera for each assay were preimmune sera from the same animals.

Analysis of cytokines in serum. IFN-α, IL-1β, IL-6, IL-10, and TNF-α protein concentrations in sera from infected animals were determined using an ELISA. IFN-α was detected using monoclonal antibodies (MAbs) K9 and F17 (PBL Interferon Source, Piscataway, NJ) as previously described (52). IL-10 Cytoset ELISA (Biosource-Invitrogen, Carlsbad, CA) and IL-1β, IL-6, and TNF-α Duo Set ELISAs (R&D Systems, Minneapolis, MN) were performed following the manufacturers' directions. All ELISAs were developed with 3,3′,5,5′-tetramethylbenzidine (TMB) from KPL (Gaithersburg, MD). The absorbance at 450 nm was measured in an ELISA reader (VersaMax; Molecular Devices, Sunnyvale, CA). Cytokine concentrations were calculated on the basis of the optical densities obtained with the standards and are expressed in relative levels for each individual at different times postinfection with respect to its own level at day 0.

Detection of cytokines in PBMCs by real-time PCR. Expression of several cytokines in PBMCs was analyzed. RNA was extracted from purified PBMCs, approximately 10^7 cells, by utilizing an RNeasy miniprep kit (Qiagen, Valencia, CA). A quantitative rRT-PCR method was used to evaluate the mRNA levels of several cytokines: for IFN-α, primers 236FW (5′ TGGTGCATGAGATGCTCCA) and 290RW (5′ GCCGAGCCCTCT GTGCT) and probe FAM-CAGACCTTCCAGCTCT (where FAM is 6-carboxyfluorescein); for IL-1β, primers 737FW (5′ TTGAATTCGAGT CTGCCCTGT) and 812RW (5′ CCCAGGAAGACGGGCTTT) and probe FAM-CAACTGGTACATCAGCACCTCTCAAGCAGAA; for IL-6, primers 478FW (5′ AATGTCGAGGCTGTGCAGATT) and 559RW (5′ TGGTGGCTTTGTCTGGATTCT) and probe FAM-AGCACTGATCCA GACCCTGAGGCAAA; for IL-10, primers 138FW (5′ TGAGAACAGCT GCATCCACTTC) and 241RW (5′ TCTGGTCCTTCGTTTGAAAG AAA) and probe FAM-CAACCAGCCTGCCCCACATGC; and for TNF-α, primers 338FW (5′TGGCCCCTTGAGCATCA) and 405RW (5′ CGGGCTTATCTGAGGTTTGAGA) and probe FAM-CCCTCTGGCCC AAGGACTCAGATCA. Porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to normalize the values for each sample (primers 327FW [5′ CGTCCCTGAGACACGATGGT] and 380RW [5′ CCCGATGCGGCCAAAT] and probe FAM-AAGGTCGGAG TGAACG). Reactions were performed in an ABI Prism 7500 sequence detection system (Applied Biosystems). Relative mRNA levels were determined by comparative cycle threshold analysis (user bulletin 2; Applied Biosystems) utilizing as a reference the samples at 0 dpi.

Intracellular cytokine staining (ICCS). $CD8^+$ IFN-γ-producing cells in total PBMCs were analyzed by flow cytometry after specific stimulation with FMDV A12-WT or with a nonspecific stimulator (phorbol myristate acetate plus Ca ionophore) for 18 h at 37°C in 5% CO_2. Nonspecific stimulus was used as a negative control. Cells were thereafter incubated with GolgiStop protein transport inhibitor (BD Bioscience, Franklin Lakes, NJ), according to the manufacturer's recommendations, followed by pelleting and resuspension in staining buffer (10% FBS in PBS). To analyze the expression of cell surface molecules, biotinylated mouse anti-porcine CD8 (Southern Biotech, Birmingham, AL) detected by streptavidin conjugated with peridinin chlorophyll protein (BD-Pharmingen) was used. After staining, cells were fixed and permeabilized with BD Cytoperm/Cytofix (BD Biosciences) for 30 min at 4°C, washed in BD Perm/wash buffer three times, and finally stained intracellularly with mouse anti-porcine IFN-γ conjugated with R-phycoerythrin (BD-Pharmingen). Cells were acquired using a FACSCalibur flow cytometer (BD Bioscience). Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed with CellQuest software (BD Bioscience).

Statistical analyses. Data handling and analysis and graphic representation were performed using Prism (version 5.0) software (GraphPad Software, San Diego, CA) or the Microsoft Excel program. Statistical differences were determined using a Student *t* test ($P < 0.05$, $P < 0.01$, $P < 0.005$).

RESULTS

FMDV SAP mutant is attenuated in swine. To compare the virulence of the FMDV WT, A12-WT, with that of an FMDV mutant containing mutations in the SAP domain of L^{pro} (A12-SAP) (21), groups of three pigs were inoculated i.d. in the rear heel bulb with different doses of either FMDV. We inoculated animals with 10^5 or 10^6 PFU/animal of A12-WT, doses that we had previously shown caused clinical disease in swine (12), and with 10^5, 10^6, and 10^7 PFU/animal of A12-SAP. In animals inoculated with WT virus, disease was detectable as early as 2 dpc, but only the group inoculated with 10^6 PFU/animal had temperatures of 40°C or higher. By 7 dpc, all animals inoculated with WT virus showed clinical signs of disease, with no statistically significant differences between the two groups (Fig. 1A). However, all animals inoculated with A12-SAP, even those inoculated with a 10-fold higher dose than WT (10^7 PFU/animal), never showed clinical signs or elevated temperatures throughout the experiment (Fig. 1A).

Animals inoculated with A12-WT developed viremia on the day prior to (group inoculated with 10^6 PFU) or concomitantly with (group inoculated with 10^5 PFU) the appearance of clinical signs (Fig. 1B; C_T values = 33 to 37) and lymphopenia (data not shown). Interestingly, none of the animals inoculated with A12-

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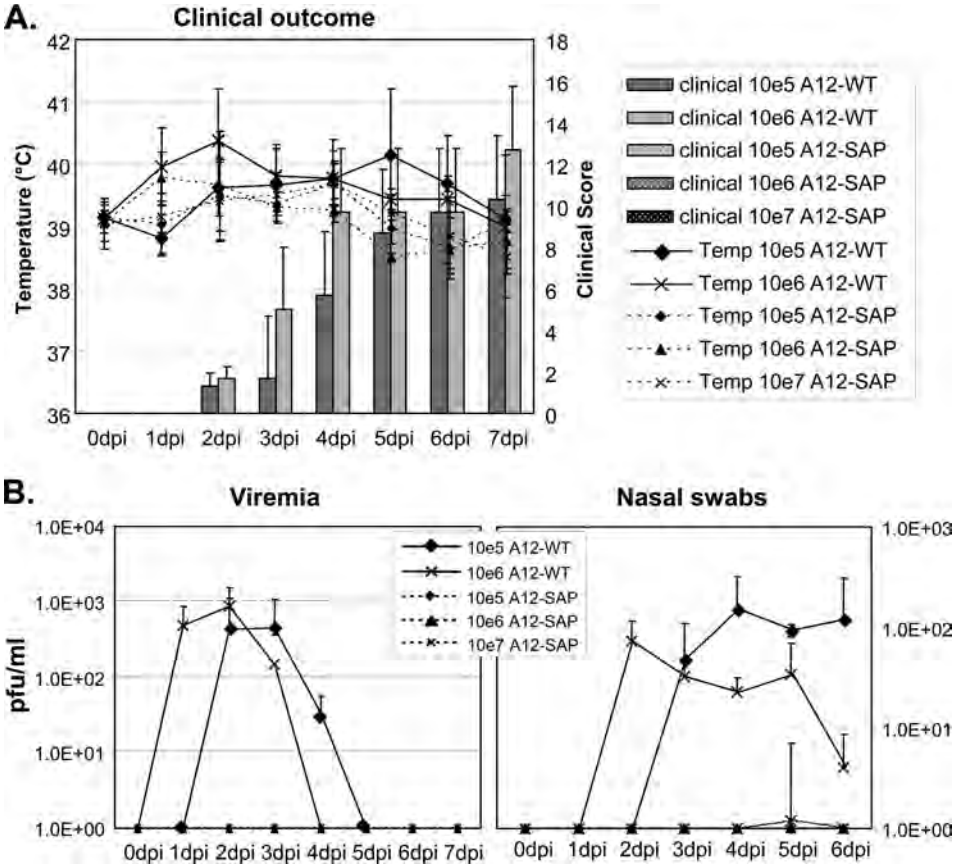

FIG 1 Clinical outcome after FMDV A12-WT and A12-SAP inoculation. Groups of three pigs were i.d. inoculated with different doses of A12-WT (10^5 or 10^6 PFU/animal) or A12-SAP (10^5, 10^6, or 10^7 PFU/animal), and temperature and clinical signs (A) and the presence of virus in serum and nasal swabs (B) were monitored daily for 7 dpi. Clinical score is expressed as the number of toes showing lesions plus one more point scored when lesions were present in either the mouth or snout, or both (the maximum score is 17). Virus levels are expressed as the number of PFU per ml in plasma or medium (in which the swabs were collected). Each data point represents the mean (±SD) of each group.

SAP had detectable viremia either by virus isolation (Fig. 1B) or by rRT-PCR (C_T values ≥ 40 [58]), nor did they develop lymphopenia. In parallel to viremia, animals inoculated with WT virus had detectable virus in nasal swabs starting at 2 to 3 dpi, and only one out of three animals inoculated with 10^7 PFU of A12-SAP showed virus with a very low titer (<10 PFU/ml) in nasal swabs at 5 dpi (Fig. 1B). These data indicate that A12-SAP FMDV displays significantly reduced virulence in swine compared to A12-WT.

FMDV A12-SAP and A12-WT elicit equivalent adaptive immune responses. It has previously been demonstrated that animals inoculated with an attenuated strain of FMDV lacking L^{pro} (leaderless FMDV) developed significant antibody titers against viral proteins in serum after 14 dpi (12, 48). In the current experiment, we observed that despite the absence of viremia, all the animals inoculated with A12-SAP developed significant levels of FMDV-specific neutralizing antibodies starting at 7 dpi, with a peak occurring at 14 dpi (Fig. 2A). No statistically significant differences between animals inoculated with A12-WT and A12-SAP were detected when all groups were considered. In order to test for the presence of total antibodies, including those raised against structural and NS viral proteins, we performed RIPs using the serum of inoculated animals at 21 dpi and a radiolabeled extract of cells infected with A12-WT. All A12-WT-inoculated animals developed antibodies against structural (VP0, VP1, and VP3) and NS ($3D^{pol}$) viral proteins, indicative of productive viral replication. Interestingly, all A12-SAP-inoculated animals, with the exception of animal 96, also had significant levels of antibodies against $3D^{pol}$, despite the absence of detectable viremia or virus shedding (Fig. 2B). All the serum samples from the animals were negative for the presence of antibodies at 0 dpi (data not shown).

In order to characterize the FMDV antibody response, the specific Ig isotype present in swine sera after inoculation was determined. The presence of IgM was detected by 7 dpi in all inoculated animals, and the level of IgM peaked at 14 days and declined by 21 days, while the levels of IgG1 and IgG2 increased in all inoculated animals (Fig. 2C). Together, these data indicate that A12-SAP replicates in the animal, eliciting a strong adaptive immune response comparable to that of WT virus, but does not cause vesicular lesions, viremia, or fever.

Animals inoculated with FMDV A12-SAP are completely protected when challenged with FMDV WT. In our previous studies, animals inoculated with the attenuated leaderless virus never showed clinical signs but were only partially protected when challenged with WT FMDV (12, 48). To test whether or not the animals inoculated with the FMDV SAP mutant were protected against FMD, we challenged the three groups of A12-SAP-inoculated swine with A12-WT (10^5 PFU/animal i.d. in the heel

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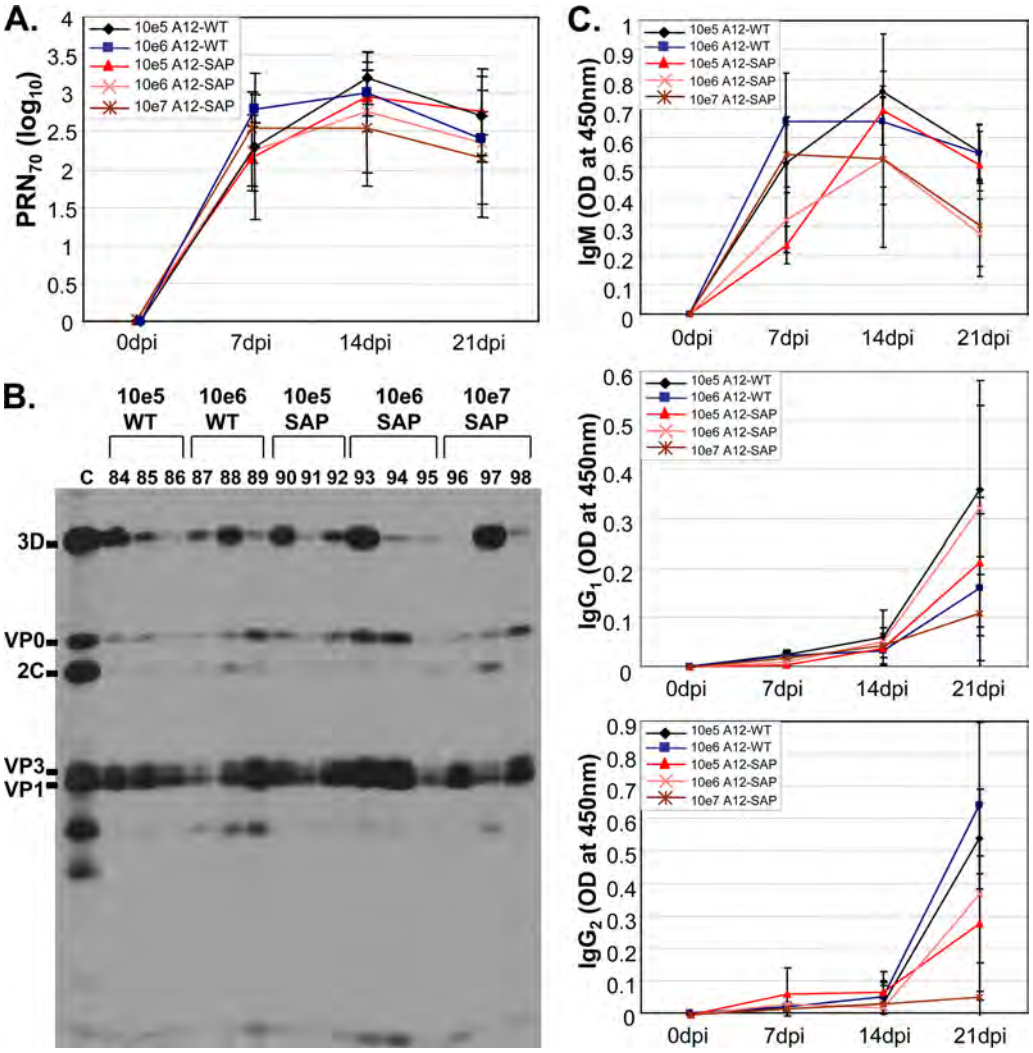

FIG 2 Presence of antibodies in serum of inoculated animals. (A) Serum neutralization titers. The neutralizing antibodies of swine inoculated with different doses of A12-WT (10^5 or 10^6 PFU/animal) or A12-SAP (10^5, 10^6, or 10^7 PFU/animal) were determined at 0, 7, 14, and 21 dpi. Titers are expressed as the $\log_{10}$ of the inverse dilution of serum yielding a 70% reduction in the number of plaques (PRN_{70}). (B) Immunoprecipitation of structural and NS viral proteins with serum of animals inoculated with different doses of FMDV A12-WT (10^5 PFU/animal or 10^6 PFU/animal) or A12-SAP (10^5 PFU/animal, 10^6 PFU/animal, or 10^7 PFU/animal). Cytoplasmic extracts of FMDV A12-WT-infected BHK-21 cells labeled with [^{35}S]methionine/[^{35}S]cysteine were immunoprecipitated with serum from infected animals after 21 dpi and with convalescent-phase serum as a positive control (lane C). The products were examined by SDS-PAGE on a 15% gel. (C) Antibody isotype profiles in swine sera after infection. FMDV-specific IgM, IgG1, and IgG2 were detected by sandwich ELISA at 7, 14, and 21 dpi. Each data point represents the mean (±SD) of each group. OD, optical density.

bulb) at 21 days. All challenged animals were protected, and none of the animals showed clinical signs (fever or vesicles) or the presence of virus in blood or nasal secretions (Table 1). As expected, the challenge acted as a boost, and the animals had increased neutralizing antibody titers by 7 dpc (Table 1).

FMDV L^{pro} is involved in reducing the expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α in swine. Previous *in vitro* studies demonstrated that FMDV L^{pro} antagonizes the innate immune response by limiting the expression of IFN and ISGs (11, 14, 19, 20, 74). Furthermore, WT infection induces production of the anti-inflammatory cytokine IL-10, impairing T-cell proliferation (25). These data suggest that L^{pro} might play a role in the induction of an anti-inflammatory state, thereby impairing rapid virus clearance. In order to test this hypothesis, we analyzed the expression of pro- and anti-inflammatory cytokine protein levels in the sera of animals inoculated with A12-WT and A12-SAP for 4 days after infection. Figures 3 and 4 show the levels of IFN-α, IL-10, IL-1β, IL-6, and TNF-α in the serum of A12-WT- or A12-SAP-inoculated swine. Relative values were plotted with respect to the basal levels of each cytokine at 0 dpi. In the case of IFN-α, animals infected with the highest dose of A12-WT showed a slight increase peaking at 2 dpi, although high variation within the group was observed. Similarly, there were not statistically significant differences in the groups inoculated with A12-SAP-mutant virus (Fig. 3A). In contrast, pigs inoculated with A12-WT or A12-SAP showed an increase of the anti-inflammatory cytokine IL-10, with a peak at 2 dpi (Fig. 3B). Although the variation of the levels of expression between individuals was high, all the animals had increased expression of IL-10 by 2 dpi compared with the levels observed at 0 dpi, and this difference was statistically signif-

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TABLE 1 Clinical outcome and presence of neutralizing antibodies in animals challenged with FMDV A12-WT 21 days after FMDV A12-SAP mutant inoculation[a]

A12-SAP dose (no. of PFU/animal)	Animal no.	Challenge result[b]		Neutralizing antibody PRN_{70}[c]	
		Viremia	Nasal swabs	0 dpc	7 dpc
1×10^5	90	Neg.	Neg.	2.4	>3.1
	91	Neg.	Neg.	3.3	>3.1
	92	Neg.	Neg.	2.7	>3.1
1×10^6	93	Neg.	Neg.	3	>3.1
	94	Neg.	Neg.	2.7	>3.1
	95	Neg.	Neg.	1.8	>3.1
1×10^7	96	Neg.	Neg.	1.5	3.0
	97	Neg.	Neg.	2.7	3.1
	98	Neg.	Neg.	2.1	>3.1

[a] The dose of the A12-WT challenge virus was 1×10^5 PFU per animal.
[b] The animals were tested for 7 days after the challenge. Neg., negative (less than 5 PFU/ml).
[c] The neutralizing antibody titer is reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}).

icant ($P < 0.05$). Interestingly, the serum levels of the cytokines IL-1β, IL-6, and TNF-α dropped by 2 dpi only in the animals inoculated with A12-WT, independently of the inoculation dose and coinciding with the peak of viremia (Fig. 4). The difference at 2 dpi was statistically significant compared to the levels observed at day 0 ($P < 0.01$). In contrast, two of the three groups inoculated with A12-SAP (1×10^5 and 1×10^6 PFU/animal) showed an increase in the levels of IL-1β and IL-6 compared to the levels observed at 0 dpi, and the differences with respect to the groups inoculated with A12-WT were also statistically significant by 2 dpi ($P < 0.01$) (Fig. 4). Surprisingly, this effect was not seen in the group of swine inoculated with the highest dose of A12-SAP (1×10^7 PFU/animal). In the case of TNF-α, all A12-SAP-inoculated animals showed an increase by 2 to 3 dpi that was statistically significant ($P < 0.01$) for the low- and medium-dose groups (1×10^5 and 1×10^6 PFU/animal, respectively). At 2 dpi, the relative levels of TNF-α were statistically significantly higher ($P < 0.01$) for the three A12-SAP groups than the A12-WT-inoculated groups (Fig. 4C).

Vaccination with attenuated FMDV A12-SAP confers protection against challenge with FMDV A12-WT as early as 2 dpv. Inoculation of animals with live attenuated viral vaccines can induce early and long-lasting protection. As mentioned above, the FMDV SAP mutant not only was attenuated *in vivo* but also induced a robust adaptive immune response that conferred protection at 21 dpi. In order to determine if inoculation with this mutant virus could induce rapid protection, groups of three swine were s.c. vaccinated with 1×10^6 PFU/animal A12-SAP, followed by challenge with A12-WT virus at different times postvaccination (2, 4, 7, and 14 dpv). Another group inoculated with PBS was used as a control. We decided to use s.c. vaccination to determine if this route of inoculation would induce the same level of protection as that obtained by i.d. inoculation. s.c. vaccination is a practical approach in the field and is commonly used for live vaccines. Control animals developed clinical signs of disease as early as 2 dpc, and by 7 dpc they had a maximum lesion score of 14 (Fig. 5), with fever starting at 3 dpc. In parallel to clinical signs, control animals showed the presence of virus in serum and nasal swabs with a peak at 3 dpc (Fig. 5). However, vaccination with A12-SAP-mutant virus conferred full protection, even in the group of animals vaccinated just 2 days prior to WT virus challenge. Only one animal in the group vaccinated 14 days before challenge showed one lesion, which was first apparent at 5 dpc, and virus was detected in nasal swabs but not in blood (Fig. 5).

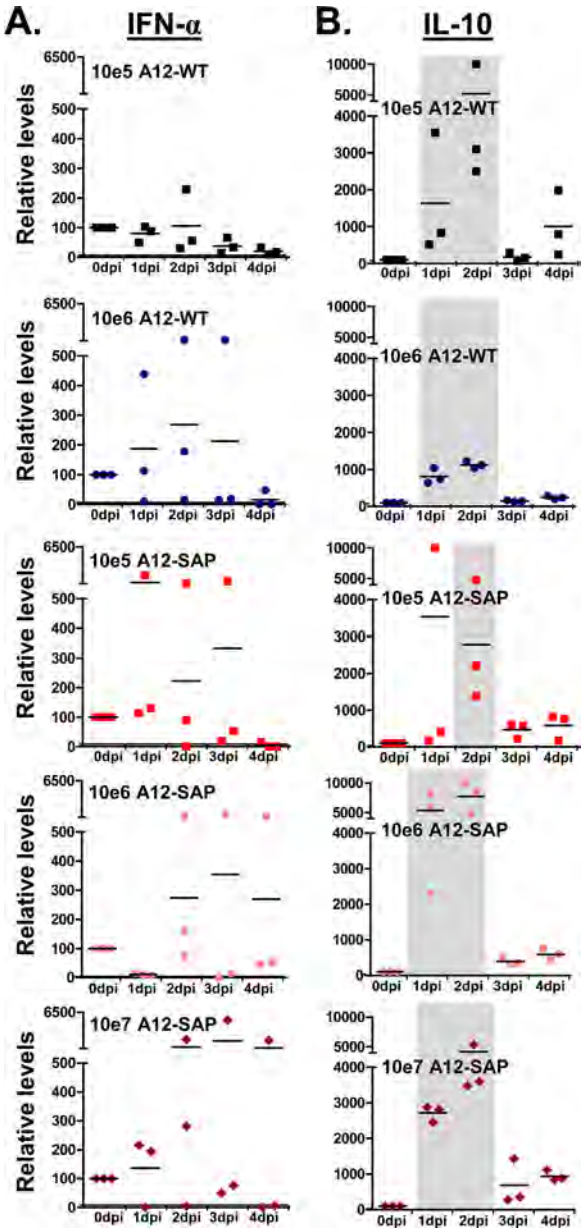

FIG 3 Cytokine protein profiles in serum after FMDV infection. Levels of IFN-α (A) and IL-10 (B) in the serum of animals inoculated with FMDV A12-WT (10^5 or 10^6 PFU/animal) or A12-SAP (10^5, 10^6, or 10^7 PFU/animal) during the first 4 days after infection were detected by sandwich ELISA. Amount of protein is expressed in relative levels for each individual animal at different times postinfection with respect to its own level at day 0. The gray areas represent time points at which a statistically significant difference from the amount at 0 dpi was observed ($P < 0.05$).

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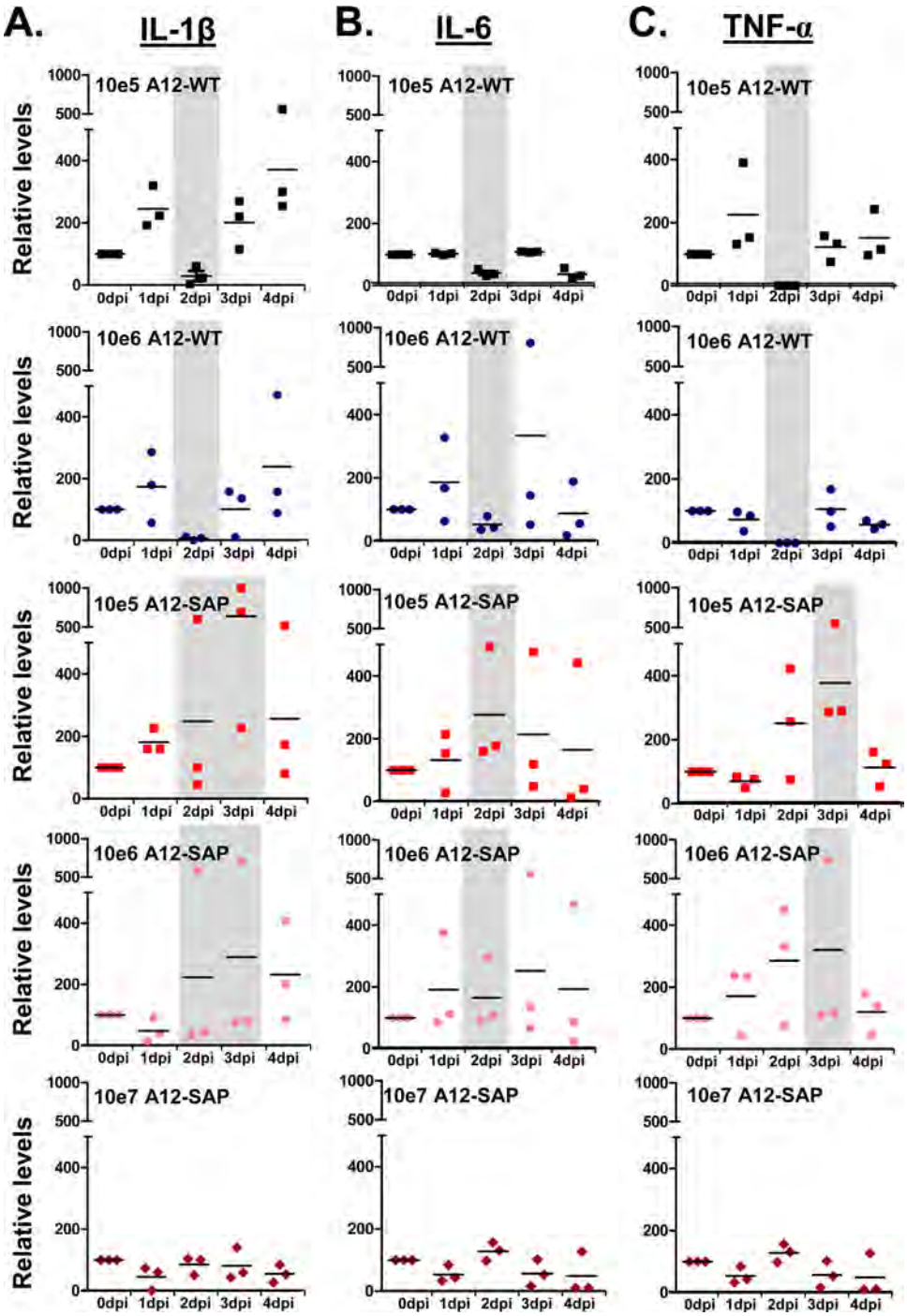

FIG 4 Cytokine protein profiles in serum after FMDV infection. Levels of IL-1β (A), IL-6 (B), and TNF-α (C) in the serum of animals inoculated with FMDV A12-WT (10^5 or 10^6 PFU/animal) or A12-SAP (10^5, 10^6, or 10^7 PFU/animal) during the first 4 days after infection were detected by sandwich ELISA. Amount of protein is expressed in relative levels for each individual animal at different times postinfection with respect to its own level at day 0. The gray areas represent time points at which a statistically significant difference from the amount at 0 dpi was observed ($P < 0.01$).

Detectable levels of humoral or cellular immunity do not directly correlate with early protection. It is widely established that in the primary adaptive immune response, several days are required for the clonal expansion and differentiation of lymphocytes into effector T cells and antibody-secreting B cells. However, it has been reported that different levels of antibodies can develop as early as 3 days after FMDV infection (27). We evaluated the titers of neutralizing antibodies and stimulation of $CD8^+$ T cells in all vaccinated groups before and after challenge. With the exception of the group challenged at 2 dpv, all the other vaccinated animals (4, 7, and 14 dpv) had detectable levels of neutralizing antibodies in serum prior to challenge (Fig. 6). All but one animal

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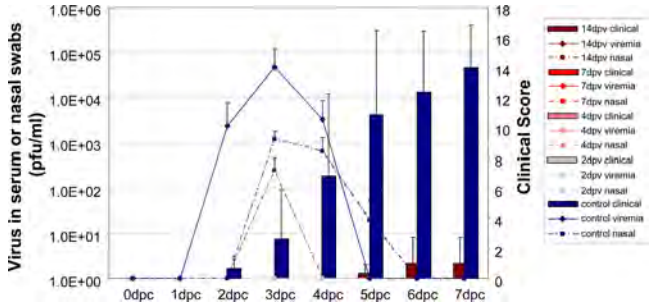

FIG 5 Clinical outcome of animals vaccinated with attenuated A12-SAP after FMDV A12-WT challenge. Groups of three pigs were s.c. vaccinated with A12-SAP (10^6 PFU/animal) and i.d. challenged at different times postvaccination (2, 4, 7, and 14 dpv) with 5×10^5 PFU/animal of A12-WT, and clinical signs (bars) and the presence of virus in serum (solid lines) and nasal swabs (dashed lines) were monitored daily during various dpc. Clinical score and virus levels are expressed as described in the Fig. 1 legend. The error bars represent the variation within the three animals from each group.

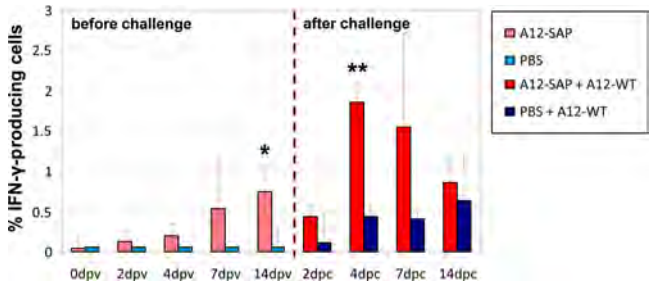

FIG 7 Cell-mediated immunity induced by A12-SAP vaccination. Specific cellular response was measured by intracellular cytokine staining (ICCS). PBMCs from A12-SAP-vaccinated and control animals were extracted at different times before (dpv) and after (dpc) challenge with A12-WT and stimulated with homologous FMDV A12-WT, and the capacity of $CD8^+$ T cells to produce IFN-γ was evaluated by ICCS. The percentage of $CD8^+$ T cells that produce IFN-γ is shown. A vertical dashed line separates the data between vaccination and challenge. The error bars represent the variation within the three animals from each group. *, $P < 0.05$; **, $P < 0.01$.

belonging to the group challenged at 14 dpv were completely protected against disease. Moreover, by 4 dpc, animals challenged at 2 dpv showed levels of neutralizing antibodies equivalent to those for the control group, even when viremia or virus in nasal swabs was never detected (Fig. 6). As expected, FMDV challenge acted as a boost in all groups which showed higher neutralizing antibodies by 4 dpc.

Analysis of a specific T cell response revealed a modest induction at 7 and 14 dpv. As shown in Fig. 7, before the challenge, there were statistically significant differences in the levels of $CD8^+$ IFN-γ-producing T cells at 14 dpv ($P < 0.05$). After the challenge, control animals developed specific cell-mediated immunity, detected between 4 and 7 dpc. Interestingly, at 4 dpc vaccinated animals showed a clear increase in the number of $CD8^+$ IFN-γ-producing T cells compared to the number on the day of challenge and to the number for control animals ($P < 0.01$), showing the capacity for a rapid response in primed individuals compared with control animals.

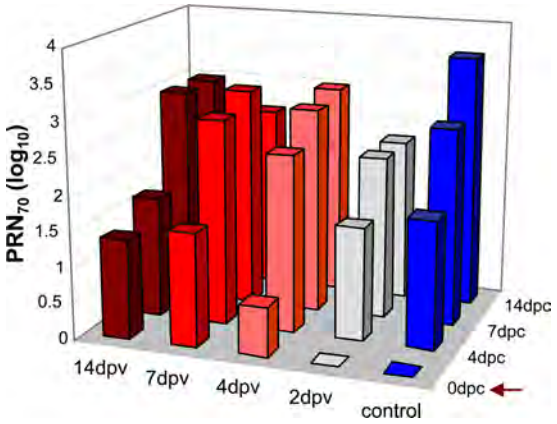

FIG 6 Serum neutralization titers of vaccinated and control animals at the day of challenge and up to 14 dpc. The neutralizing antibodies of swine vaccinated at different time points (2, 4, 7, and 14 dpv) with attenuated A12-SAP (10^6 PFU/animal) were measured at the day of challenge (arrow) with A12-WT (5×10^5 PFU/animal) and at 4, 7, and 14 dpc. Titers are expressed as described in the Fig. 2 legend.

A12-SAP vaccination induced the expression of TNF-α in serum and PBMCs. As expected, animals that were challenged early after vaccination (2 dpv) did not show detectable levels of antibodies or cell-mediated immunity by the time of challenge. On the basis of these observations and results from the first animal experiment in which animals inoculated with WT FMDV showed a decrease in the level of some cytokines in serum 2 days after infection, while those inoculated with the attenuated SAP-mutant virus did not (Fig. 3 and 4), we decided to analyze the systemic levels of some pro- and anti-inflammatory cytokines by ELISA in serum and by real-time RT-PCR in PBMCs (Fig. 8). Control animals challenged with A12-WT showed a statistically significant increase in the levels of IL-10 by 2 to 3 days postinfection and a significant decrease in the levels of IL-1β, IL-6, and TNF-α starting at 1 dpi by ELISA (Fig. 8A). Similar results were observed when we analyzed the relative levels of cytokine mRNAs in PBMCs, in which we could detect an increase only of the relative levels of IL-10 in control A12-WT-infected animals (Fig. 8B). In the case of IFN-α, animals inoculated with A12-WT showed a decrease in the relative levels of protein by 3 dpi compared with the levels at 0 dpi (Fig. 8A), while no variation in the relative levels of mRNA was detected (data not shown). On the other hand, animals vaccinated with the SAP mutant showed a statistically significant increase in the levels of IL-10 by ELISA, as was observed in the first experiment (Fig. 3). For the other cytokines, there was an overall tendency toward increased protein levels at 2 to 3 dpi, but the variation was statistically significant only for TNF-α, which showed a peak at 3 dpi (Fig. 8A). IL-1β, IL-6, and TNF-α were upregulated, as detected by rRT-PCR starting at 2 dpi (Fig. 8B).

DISCUSSION

The FMDV NS protein L^{pro} plays a key role in antagonizing the innate immune response (35). We have previously reported that FMDV lacking L^{pro} is attenuated in swine and cattle; however, this virus is unable to completely protect animals against challenge with virulent FMDV (9, 12, 48). Recently, we constructed an FMDV mutant containing amino acid substitutions in a conserved domain of the L^{pro} coding region, A12-SAP, which is attenuated *in vitro* (21). Here we show that A12-SAP-mutant FMDV is also attenuated *in vivo*. Remarkably, mutation of just two amino

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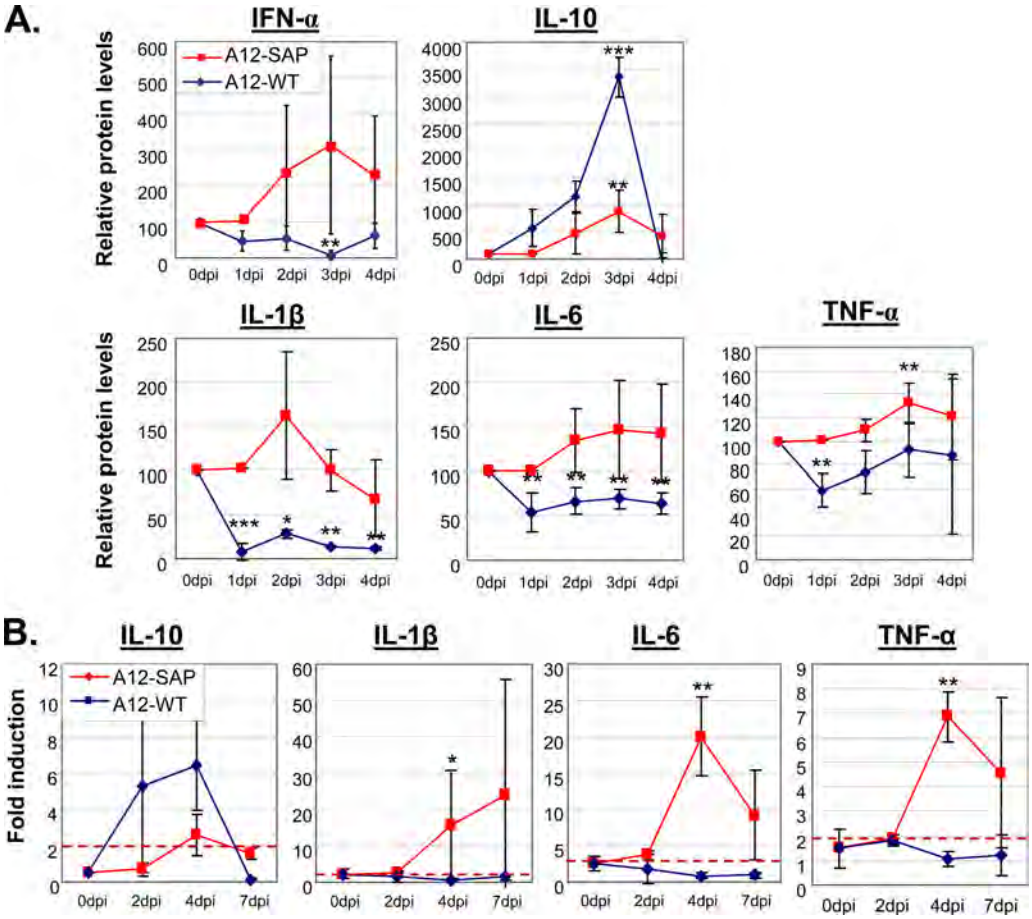

FIG 8 Cytokine profile in animals inoculated with FMDV A12-SAP or FMDV A12-WT. Pro- and anti-inflammatory cytokines were detected in serum by ELISA (A) or in PBMCs by rRT-PCR (B). (A) Levels of IFN-α, IL-10, IL-1β, IL-6, and TNF-α are expressed relative to the amount detected at day 0. (B) Relative mRNA levels of IL-10, IL-1β, IL-6, and TNF-α were determined by comparative cycle threshold analysis utilizing as a reference the samples at 0 dpi. Only values of ≥ 2 are considered upregulated. The error bars represent the variation within the three animals from each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

acid residues contained within the L^{pro} SAP domain prevented virus spread and disease but was sufficient to induce complete protection against WT challenge. We show that inoculation with the FMDV A12-SAP mutant induces humoral and cellular immunity at levels equivalent to the levels found during infection with WT FMDV. More importantly, our study reveals that while animals inoculated with the SAP mutant significantly increase proinflammatory cytokines at early times postinoculation (2 to 3 dpi), WT FMDV infection results in suppression of this response, as reflected by lower levels of these cytokines in serum. This, in turn, correlates with complete protection against WT FMDV challenge early after vaccination with SAP-mutant virus (2 dpv). The most important question arising from these observations relates to the early activation of cytokine networks by the SAP mutant and the integration of these findings into the host capacity to mount an effective anti-FMDV immune response.

Dendritic cells (DCs) are the professional antigen-presenting cells responsible for mounting an effective adaptive immune response (31). Although FMDV can infect DC precursors *in vitro*, interfering with proper maturation, infection *in vivo* is abortive in swine (25, 55) and does not affect the capability of at least skin DCs and monocyte-derived DCs (moDCs) to present antigen (25, 55). Similar to infection with WT FMDV, the high levels of neutralizing antibodies and the induction of cell-mediated immunity in the animals inoculated i.d. with A12-SAP, even in the absence of detectable viremia or virus shedding, suggest that local skin DCs might have taken up antigen or become infected with FMDV, followed by lymphatic migration to the draining lymph nodes, thus eliciting a significant adaptive immune response. It is well characterized that during natural infection FMDV induces a strong neutralizing antibody response that ultimately clears the infection (16, 69). However, in our vaccine experiment one animal inoculated with the FMDV A12-SAP mutant developed mild disease after challenge with WT virus, despite the presence of significant levels of neutralizing antibodies. We believe that this animal was a nonresponder. Previous studies have shown that protection against FMD does not always rely on the levels of neutralizing antibodies, since resistance to challenge has been observed in animals showing low levels of antibodies and disease has been detected even in the presence of significant antibody titers (49). Recently, a cytotoxic T cell lymphocyte (CTL) response during natural FMDV infection has been reported (37). The idea that a good vaccine against FMDV should combine stimulation of both humoral and cellular responses has been considered for a long time (4), and several attempts to include T cell stimulation in FMDV vaccine strategies have been pursued (6, 32, 38, 62). How-

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ever, none of the vaccine platforms evaluated to date are able to induce the same immune response as the natural infection. It is expected that use of a live attenuated vaccine platform could offer protection that better resembles the protection afforded by natural infection. One of the main concerns of attenuated vaccines is the possibility of reversion to wild type, especially for FMDV, given the high error rate of viral RNA replication and its quasispecies nature (10, 28). Tissue culture passage of SAP-mutant virus displayed remarkable stability of the SAP mutation for at least 12 passages (data not shown), suggesting that this mutant could potentially be developed as a live attenuated vaccine candidate. Inclusion of markers for DIVAs and additional mutations that stabilize the attenuated phenotype, decreasing the probability of reversion to WT, should be considered.

The transcription factor NF-κB can be activated by a variety of stimuli, including infection with picornaviruses (20, 60, 61). Activated NF-κB promotes the expression of over 150 target genes, most of which participate in the host immune response (59), and among them there are several cytokines, such as TNF-α, IL-1, and IL-6 (40, 45). In the case of FMDV, NF-κB activation and translocation to the nucleus occur at a relatively early stage of infection, but at later times the p65/RelA subunit of NF-κB disappears from infected cells (20). Previously, we demonstrated that FMDV L^{pro} is necessary and sufficient for degradation of p65/RelA and that mutations in the L^{pro} SAP domain abolished this function (20, 21). Little is known about the influence of natural FMDV infection on the profile of proinflammatory cytokines *in vivo*. Increased mRNA levels of TNF-α and IL-1α have been reported in nasal tissue-associated lymphoid tissue of infected cattle at 7 days after infection and later (73). In our study, a consistent decrease in the levels of TNF-α, IL-1, and IL-6 in blood was detected concurrently with the peak of viremia in animals inoculated with FMDV WT. We observed similar effects in swine infected with FMDV serotypes Asia 1 and O1 Manisa (data not shown). Some viruses, such as cytomegalovirus (42), or, more specifically, some viral proteins, such as paramyxovirus V (47) and poliovirus 3A (15), can inhibit the secretion of IL-1 or IL-6. However, animals inoculated with SAP-mutant virus showed significant induction of TNF-α and maintained the levels of IL-1 and IL-6, in contrast to animals inoculated with FMDV WT. These cytokines play an important role in the acute inflammatory response to infection and in tissue repair (43). Several roles involved in the regulation of the adaptive immune response have also been described for these cytokines. For FMDV, TNF-α and IL-6 have been reported to be molecular adjuvants involved in the maturation of DCs (71). It is possible that increased expression of TNF-α results from the inability of A12-SAP to cause degradation of NF-κB, which ultimately induces an innate immune response sufficient to neutralize the virus, preventing the appearance of disease while improving the development of the adaptive immune response. Furthermore, IL-1 has been demonstrated to have antiviral activity against RNA viruses, including vesicular stomatitis virus (VSV) (68). Although IL-6 does not have any known antiviral activity (47), its involvement in viral pathogenesis of vaccinia virus (44) or herpes simplex virus type 1 (54) has been demonstrated. Similarly, it is possible that IL-1 and IL-6 play a role in controlling FMDV replication and spread *in vivo*.

Another important molecule modulated by NF-κB that could be involved in early protection against challenge is IFN. It has been demonstrated that FMDV is sensitive to the action of IFN (11, 14, 53). We have previously demonstrated that, *in vitro*, WT FMDV interferes with full induction of transcription of IFN-β (19). However, *in vivo*, IFN-α mRNA or protein has been detected in WT FMDV-infected bovine and swine (8, 65, 72). We did not detect significant differences in the amount of IFN-α protein in the serum of animals inoculated with A12-WT or A12-SAP. Since there are 17 different types of IFN-α in pigs (70), it is possible that differences in other IFN-α subtypes might exist but were not detected. Alternatively, differences in the IFN levels might be detectable only in specific tissues, correlating with the number of virus particles present at the specific site of infection (8). Nevertheless, we have previously observed that a relatively large amount of IFN ($\geq$1,000 pg/ml serum) induces protection against FMDV, but in some cases, protection has been observed even when no systemic IFN was detected (23). Therefore, at this point there is no evidence that the levels of systemic IFN induced by infection with different strains of FMDV play a clear role in pathogenesis.

The other cytokine analyzed in our study, IL-10, showed an increase in both animals inoculated with WT virus and animals inoculated with SAP-mutant virus. It has been demonstrated that FMDV infection causes the induction of IL-10, a molecule that modulates DC function early after infection, possibly favoring a Th2 cell/cytokine-like environment, thus inducing FMDV-specific neutralizing antibodies (25). Infection of animals with the SAP mutant triggers the expression of proinflammatory cytokines (IL-1, TNF-α, IL-6), presumably through stronger activation of the NF-κB pathway. Therefore, IL-10 expression may blunt the proinflammatory cytokine response to avoid an exaggerated cytokine production that could lead to inflammation-mediated disease. Our results expand the concept that IL-10 is a key regulatory cytokine.

In summary, our results suggest that FMDV L^{pro} plays a pivotal role in modulating the innate and adaptive immune response to viral infection, affecting multiple overlapping pathways. Manipulation of the L^{pro} coding region has allowed us to derive a viable attenuated mutant virus that, when used as a vaccine, was able to induce complete protection from challenge as early as 2 days post-vaccination. This observation highlights the potential of using live attenuated vaccine candidates to fight FMDV and deserves further consideration. Moreover, a comprehensive study of viral pathogenesis with WT and FMDV L^{pro} mutant strains should help to provide a better understanding of virus-host interactions and, it is hoped, facilitate the development of improved FMD countermeasures.

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Early detection and visualization of human adenovirus serotype 5-viral vectors carrying foot-and-mouth disease virus or luciferase transgenes in cell lines and bovine tissues

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ABSTRACT

Recombinant replication-defective human adenovirus type 5 (Ad5) vaccines containing capsid-coding regions from foot-and-mouth disease virus (FMDV) have been demonstrated to induce effective immune responses and provide homologous protective immunity against FMDV in cattle. However, basic mechanisms of Ad5-FMDV vaccine function including virus tropism, transgene expression, and antigen presentation, remain incompletely understood. The current study characterized the dynamics of Ad5 viral vector (Ad5-FMDV-A24 and Ad5-luciferase) infection in cell lines and early post-inoculation vector–host interactions in cattle. Adenovirus dissemination was described utilizing novel rPCR, rRT-PCR, luminometry, and immunomicroscopy techniques. *In vitro* infection of human and bovine cells with both Ad5 vectors resulted in dose-dependent detection of vector DNA, mature mRNA transcripts, and transgene-encoded proteins. Subsequent to intramuscular inoculation of cattle, Ad5 and transgene products were detected at the injection sites of all animals at all time-points examined (6, 24, and 48 hpi). Microscopically, injection sites were characterized by marked infiltrates of interstitium consisting of predominantly large mononuclear cells. Immunomicroscopy indicated these cells infrequently contained adenovirus and/or transgenic proteins and were phenotypically consistent with antigen-presenting cells (macrophages and dendritic cells). Vector DNA and mature mRNA transcripts were first detected at the draining and local lymph nodes as early as 6 hpi and systemically at 24 hpi. These results provide novel insights for understanding Ad5-mediated immunity against FMDV using novel techniques that will contribute to ongoing efforts for the improvement of future Ad-FMDV vaccine platforms.

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1. Introduction

Foot-and-mouth disease virus (FMDV) [1,2] causes an economically important vesicular disease in cloven-hoofed animals and is one of the most infectious known animal viruses. FMDV spreads quickly among susceptible animals and is endemic in many regions of the world lacking of resources for adequate control strategies [3,4]. Vaccination approaches currently utilized for the control of FMD consist of partially purified inactivated virus products that are effective in controlling the clinical signs of disease and limiting virus shedding, but have several important limitations. Because these products do not prevent infection, a substantial proportion of vaccinated animals that are subsequently exposed to FMDV become persistently infected carriers. Also, the presence of FMDV non-structural proteins (NSP) contaminants confound differentiation of vaccinated from infected animals [5,6].

One of the most promising novel vaccination approaches has been the development of a vaccine platform based on the use of replication-defective human adenoviruses serotype 5 (Ad5) for the delivery of FMDV-derived transgenes [4,7]. The Ad5-A24 vaccine, which is the lead experimental vaccine candidate for advanced development and manufacturing in the US [8], contains the sequences for FMDV P1 (coding for the capsid proteins), NSP 2A, 2B, nearly all 3B, and the 3C protease. The construct lacks the 3D (polymerase) NSP which allows the potential for differentiation of infected from vaccinated animals (DIVA). The Ad5-A24 candidate is a modified proprietary version of the original Ad5-FMDV-A24 prototype [7] which has conferred 100% protection to both swine and cattle challenged with homologous FMDV as early as 7 dpi [9,10]. Similarly, over 150 cattle have been recently tested with the new Ad5-A24 experimental vaccine and demonstrated to provide protection against generalized disease and viremia following a single immunization and intra-dermal lingual or contact challenge at 7 or 21 dpi [8,11]. Although the prototype and the new vaccine

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candidate have consistently demonstrated effective immunogenicity and protection against FMD, the pathogenesis underlying induction of immunity is poorly understood.

Vector–host interactions associated with replication-defective adenovirus-based vaccines have characterized biodistribution, permissiveness, and intrinsic biology in several animal models [12–20]. However, to our knowledge, there is no published account of Ad5 vector biodistribution and early transgene expression in cells and tissues of immunized cattle. Similarly, functional correlations between presence of adenovirus and adenovirus encoded transgenes, infection of specific cell subsets, and visualization of vector–host interactions by immunomicroscopy in immunized animals have not been documented. Recently, pilot studies using a replication-defective Ad5-luciferase reporter system [21–24] have demonstrated transgene expression in porcine and bovine tissues subsequent to intramuscular and transdermal inoculation (Schutta and Gregg, personal communication). In those studies, luciferase activity and FMDV antigens were primarily detected at the interstitium of injection sites and secondarily at the draining lymph nodes.

The purpose of the current study was to characterize the immunopathogenesis of replication-defective Ad5 vectors in cattle by developing and applying novel molecular and cell-based techniques for the detection of Ad5 vectors and transgene products (mRNA, protein). Vector kinetics and biodistribution were characterized using three different techniques: real-time polymerase chain reaction (rPCR and rRT-PCR), luciferase activity bioassay, and immunomicroscopy (immunohistochemistry (IHC) and immunofluorescence (IF)). The novel techniques and data herein will enable further characterization of biodistribution and mechanisms of action of replication-defective Ad5 vectors in livestock.

2. Materials and methods

2.1. Cells and viruses

The replication-defective Ad5 vectors used in this study have deletions in the E1, E3, and E4 regions that render them incapable of generating progeny virus in non-complementary cell lines. Neither LFBK (continuous bovine kidney) [25] nor human 293 [26] cells utilized in this study constitutively express E4 proteins and therefore cannot support vector replication. The vectors contain foreign coding regions under the control of a cytomegalovirus promoter. The first vector, Ad5FMDV strain A24 Cruzeiro (Ad5-A24; Adt.A24.11D), contains the FMDV P1-2A and $3C^{pro}$-coding regions [8]. The second (*i.e.* surrogate) vector, Ad5-luciferase (Ad5-Luc; AdL.11D), contains the firefly (*Photinus pyralis*) luciferase gene. *In vitro* infections were carried out as previously described with minor variation [27]. Briefly, cells were infected with adenovirus vectors at increasing m.o.i. (10, 50, 100, 300, 500, and 1000) and incubated in 6-well plates and 1-well tissue culture slides (cat# 154453, Lab-Tek®II Chamber Slide™ System; Nalgene Nunc, International; Naperville, IL) for 6 or 24 h at 37 °C. Following incubation of chamber slides, the monolayers were rinsed twice with media and the upper structure removed. The slides were then fixed in acetone at room temperature for 10 min and stored at −70 °C until further use.

2.2. Generation of a specific calibration curve for quantification of adenovirus

Adenovirus vectors (Ad5-A24, 8.49×10^{10} particle units (PU)/ml (PU/FFU ratio 14.8) and Ad5-Luc, 6×10^{11} PU/ml (PU/FFU ratio 9.5) were produced by GenVec (GenVec, Inc., Gaithersburg, MD), and provided by DHS S&T PIADC. Viral DNA was extracted and amplified by rPCR (described below in Section 2.7). In order to convert cycle threshold (Ct) values to genome copy numbers, specific calibration curves were generated using the original Ad5 stocks of known virus concentrations and total DNA content (ng/μl). For each Ad5 vector, serial ten-fold dilutions (10^{11}–10^{-1} PU/ml) were made in DMEM medium (Invitrogen, Carlsbad, CA). Ct values were converted to genome copy numbers (per cell or tissue weight) based on the molecular weight (MW) and base pairs content (C-G and A-T) of each vector. Duplicates of each dilution were tested to generate a standard curve by plotting the log of the vector copy number against the measured Ct values. The Ct value reflects the linear phase in the amplification curve that can be used for quantitation of the template input.

2.3. Extraction of viral nucleic acids and transgenic product from cell lines

DNA and RNA, and total protein were extracted from cell cultures by adding 1× reporter lysis buffer (cat# E397A; Promega, Madison, WI), AL buffer (Qiagen), and RLT buffer (Qiagen), respectively, followed by a single freeze–thaw cycle at −70 °C to ensure complete monolayer lysis. Total content of each well was centrifuged at $10{,}000 \times g$ for 15 s, and supernatants were frozen at −70 °C until nucleic acid and luciferase extractions were performed. Total DNA and RNA were extracted from cell monolayers using the Gentra Puregene Cell Kit (cat# 158722, Qiagen) and the RNeasy Mini Kit (cat# 74106, Qiagen) as previously described [28] and stored at −70 °C until assayed. Luciferase content was extracted following the manufacturer's protocol for cell lysates (Promega) and stored at −70 °C until luciferase assays were performed.

2.4. Experimental animals and inoculation system

Five 6- to 9-month-old 300 kg Holstein steers were obtained from an experimental-livestock provider accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (Thomas-Morris Inc., Reistertown, MD). All studies were conducted under an approved Institutional Animal Care and Use Committee protocol and animals were individually housed in a BSL-3Ag animal facility from time of inoculation until time of euthanasia. Three experimental subjects, received a total dose of 1.5×10^{11} PU of Ad5-A24 (steers #2 and #3) or Ad5-Luc (steer #4) in a total volume of 9 ml of Final Formulation Buffer (FFB, cat# B5003; Lonza, Walkersville, MD, USA). To ensure maximum recovery of virus inoculums, inoculation proceeded as three cervical 3-ml intramuscular injections forming the shape of an equilateral triangle separated 2.5 cm from each other. One animal (steer #1) received the same dose of Ad5-A24 divided in three 3-ml injections separated by 2-h intervals. One subject was mock-inoculated with FFB and used as a negative control in various experimental techniques (data not shown).

2.5. Sample collection

In vivo experiments were terminated by euthanasia *via* intravenous barbiturate overdose at predetermined end-points at 6, 24, and 48 h post-inoculation (hpi). Necropsies were performed immediately thereafter. In order to maximize retrieval of the injection depot from the IS area, 8 specimens of the regional muscle were dissected based on anatomic location and visualization of focal hemorrhage and/or edema associated with deposit of the inoculum. Splenius muscle specimens of the contralateral side were also obtained for control purposes. Non-IS specimens collected from each animal were spleen, thymus, liver, lung, pancreas, adrenal, kidney, and local and distant lymph nodes (Table 3). Tissue

specimens were collected and preserved as previously described [29] until assayed.

2.6. Extraction of viral nucleic acids and luciferase from tissues

Tissue samples obtained post-mortem were homogenized in 1 ml of TRI Reagent (cat# AM9738; Applied Biosystems/Ambion, Austin, TX) using a tissue homogenizer (cat# 15338420, Tissue Miser; Fisher Scientific, Ottawa, Ontario, Canada) prior to nucleic acid extraction. Nucleic acids were extracted from tissues using the RiboPure™ Kit (cat# AM1924; Applied Biosystems/Ambion) according to the manufacturer's recommendations. Total DNA and RNA content was eluted in 40 μl of elution buffer and stored at −70 °C until assayed.

For luciferase assays, frozen tissues were individually transferred to aluminum foil pouches, snap-frozen in liquid nitrogen for 5 min, and pulverized into a fine powder by the use of a hammer. Frozen powders were resuspended in 500 μl of 1× reporter lysis buffer (Promega) followed by luciferase extraction as previously described [21]. Luciferase-containing supernatants were stored at −70 °C until assayed.

2.7. Real-time PCR (rPCR) and real-time reverse transcriptase PCR (rRT-PCR)

The strategy to characterize Ad5 infection utilized two distinct systems for detection and differentiation of vector nucleic acids *in vitro* and *ex vivo*. Systems used consisted of an "Ad5-PIX" rPCR system (PIX) for detection of Ad5 DNA and an "Ad5-splice" rRT-PCR system (splice) for detection of Ad5 mature mRNA transcripts (representing transgene expression in infected cells). The PIX system (primers – PIX *fwd*, 5′-CGCGGGATTGTGACTGACT-3′, PIX *rev*, 5′-GCCAAAAGAGCCGTCAACTT-3′; probe – PIX, 6FAM-AGCAGTGCAGCTTCCCGTTCATCC-MGBNFQ) was utilized to detect the input adenovirus vector genome and to characterize biodistribution. In the splice system (primers – splice *fwd*, 5′-TGCCAAGAGTGACGTGTCCA-3′, splice *rev*, 5′-CAGGGATCCGTATAGTGAGTCGT-3′; probe – 6FAM-CTGCAGCCGGTACC-MGBNFQ), the forward primer spans an intron in the vector genome and immature mRNA transcript. Thus, target amplification from the splice system could only occur subsequent to intron removal by RNA splicing. Therefore, the splice system detects the processed, vector-encoded, transgene-containing mRNA (mature mRNA transcript) but not the input vector DNA. Extracted nucleic acids were analyzed as previously described [30]. Results are expressed as cycle threshold (Ct) values with cutoff values for positive samples set at Ct < 40.

2.8. Luciferase assays

Luciferase activity was evaluated in 20 μl of tissue extract or cell lysate using an automated Veritas™ microplate luminometer (Promega) and Lumitrac-200 plates (cat# 655075; Greiner Bio-One, Monroe, NC) as previously reported [21]. The luciferase content of each sample was recorded as relative light units (RLU) and normalized by the respective total protein concentration of the samples (RLU/mg total protein) obtained by the bicinchoninic acid assay (BCA) [31]. The total protein concentration (mg/ml) and weights (mg) of cell cultures and tissue sample replicates were not statistically significantly different ($p > 0.05$) between groups included in statistical analyses.

2.9. Immunomicroscopy

Localization of Ad5 and transgene products (FMDV-A24 protein and luciferase) in cell lines and bovine tissues was similarly determined by immunohistochemistry (IHC) and multichannel immunofluorescence (mIF) techniques as previously described [32] with minor variation. Specific antibodies used in this study are shown in Table 1. Primary antibodies were used individually or in combination for mIF assays. Detection was performed using goat-anti-rabbit and isotype-specific, anti-mouse secondary antibodies labeled with Alexa Fluor dyes (Invitrogen) diluted in Fluorescence Antibody Diluent (Biocare Medical). For IHC, immunoreactivity was detected with a micropolymer alkaline phosphatase kit (Biocare Medical, Concord, CA). Isotype controls were included with each experiment. Slides were evaluated with a wide-field epifluorescence microscope and images captured with a Nikon DS-Qi1 digital camera.

2.10. Statistical analysis

Data analyses and graphic representations were performed by using Microsoft Office Excel 2007. Statistical differences were determined by using a Student *t* test, when applicable. A cutoff value of $p \leq 0.05$ was considered significant.

3. Results

3.1. Detection and quantitation of Ad5 nucleic acids in LFBK cells

To determine the efficacy of novel rPCR and rRT-PCR systems for the assessment of Ad5 infection in bovine-derived cells, LFBK cells were infected with either Ad5-A24 or Ad5-Luc at increasing m.o.i. and assayed by PIX and splice systems at 24 hpi (Table 2). As expected, for both constructs and detection assay systems there was a dose-dependent inverse correlation between increasing m.o.i. and Ct value. High coefficient of determination (R^2) values indicated a high degree of correlation between the m.o.i. utilized and the Ct values obtained: [splice Ad5-A24 (0.82), splice Ad5-Luc (0.75)] and [PIX Ad5-A24 (0.91), PIX Ad5-Luc (0.72)] (Table 2). Ct values obtained using splice were statistically significantly higher ($p = 0.0001$) in LFBK cells infected with the Ad5-Luc vector than in LFBK cells infected with Ad5-A24. In contrast, average Ct values obtained using PIX were not statistically significantly different between constructs.

Adenovirus DNA quantitation followed a similar inverse correlation pattern between the corresponding Ct values (PIX) and calculated genome copies (GC), ranging from 0.1 to 15.5 GC/cell for Ad5-A24 ($R^2 = 0.70$) and from 0.1 to 9.3 GC/cell for Ad5-Luc ($R^2 = 0.76$), with no statistically significant differences between groups ($p > 0.05$).

3.2. Determination of luciferase activity in Ad5-luc infected cell lines

To characterize and compare *in vitro* luciferase activity and expression levels in cell lines, bovine LFBK and human 293 cells were infected with Ad5-Luc at increasing m.o.i. and assayed at 24 hpi (Fig. 1). With all data values included, luciferase expression in LFBK cells followed a direct correlation between normalized luciferase activity and increasing m.o.i. ($R^2 = 0.98$). In 293 cells, luciferase expression followed the same linear pattern as for LFBK cells $R^2 = 0.96$. At low m.o.i. (10, 50, and 100) the luciferase content in 293 cells was not statistically significantly different from the content in LFBK cells. However, luciferase activity was found elevated with statistically significant differences at m.o.i. 300 ($p = 0.03$), 500 ($p = 0.04$), and 1000 ($p = 0.05$) in LFBK cells when compared to the corresponding experimental groups in 293 cells. Overall, the analysis of luciferase activity in infected cells complemented rPCR and rRT-PCR data in determining optimal infectious levels (m.o.i.) *in vitro*. These results demonstrate expression of luciferase

Table 1
Antibodies used in this study.

Antibody	Clone	Specificity	Isotype	Manufacturer
Anti-adenovirus	2Q1850	Hexon protein	IgG_1	cat# A0977; USBiological, Swampscott, MA
	8C4	Hexon protein	IgG_{2a}	cat# ab8249; Abcam, Cambridge, MA
Anti-FMDV	6HC4	VP1 capsid protein	IgG_{2b}	Hybridoma-A12 VP1
Anti-luciferase	LUC-1	Firefly luciferase	IgG_1	cat# L2164; Sigma–Aldrich, Saint Louis, MO
CD11c	BAQ153A	Mono,[a] MΦ,[b] sub T, NK, sub B, DC[c]	IgM	cat# BAQ153A; VMRD, Inc., Pullman, WA
MHCII (HLA-DR alpha)	TH14B	MHC class II	IgG_{2a}	cat# TH14B; VMRD
CD14 (M-M9)	CAM36A	Mono, MΦ, DC subset	IgG_1	cat# CAM36A; VMRD
CD172a	CC149	Mono, granulo	IgG_{2b}	cat# MCA2041G; AbD Serotec
MAC387	MAC387	Granulo,[d] mono, histiocytes	IgG_1	cat# M0747; Dako, Carpinteria, CA
Von Willebrand factor (vWf)	Polyclonal	Endothelial cells	Rabbit IgG	cat# A0082; Dako, Carpinteria, CA
Anti-collagen I	COL-1	Extracellular matrix (ECM)	IgG_1	cat# ab90395; Abcam
ACTA-1	Polyclonal	Actin (N-terminus)	Rabbit IgG	cat# LS-B3339; LSBio, Seattle, WA

[a] Mono = monocytes.
[b] MΦ = macrophages.
[c] DC = dendritic cells.
[d] Granulo = granulocytes.

Table 2
Direct comparison of nucleic acid sequences detected by rPCR and rRT-PCR and quantification of adenovirus genomes in LFBK cells inoculated with two adenovirus vectors at different multiplicities of infection (m.o.i).

Construct	Multiplicity of infection (m.o.i.)	PCR target sequence		Adenovirus genome copies	
		Processed mRNA Ct (splice)	DNA Ct (PIX)	Genome copies in elution vol[a]	Genome copies/cell
Ad5-A24	10	22.12	29.56	19,770	0.1
	50	19.61	27.40	85,705	0.4
	100	18.90	27.10	104,563	0.5
	300	17.16	25.12	401,212	2.0
	500	15.76	24.22	740,223	3.7
	1000	14.79	22.11	3,094,962	15.5
		$R^2 = 0.82$	$R^2 = 0.91$		$R^2 = 0.70$
Ad5-Luc	10	24.01	29.52	18,266	0.1
	50	21.22	27.14	98,263	0.5
	100	20.36	26.02	215,411	1.1
	300	19.43	24.43	663,944	3.3
	500	18.29	23.65	1,147,967	5.7
	1000	17.35	22.96	1,862,823	9.3
		$R^2 = 0.75$	$R^2 = 0.72$		$R^2 = 0.76$

Ct values corresponding to detection of processed mRNA were obtained using rRT-PCR splice system and Ct values for DNA detection were obtained using rPCR PIX system.
[a] Final elution volume (200 μl) contains DNA from approximately 200,000 LFBK cells. Genome copies were calculated based on the molecular weight of each adenovirus construct and known concentration of DNA standards.

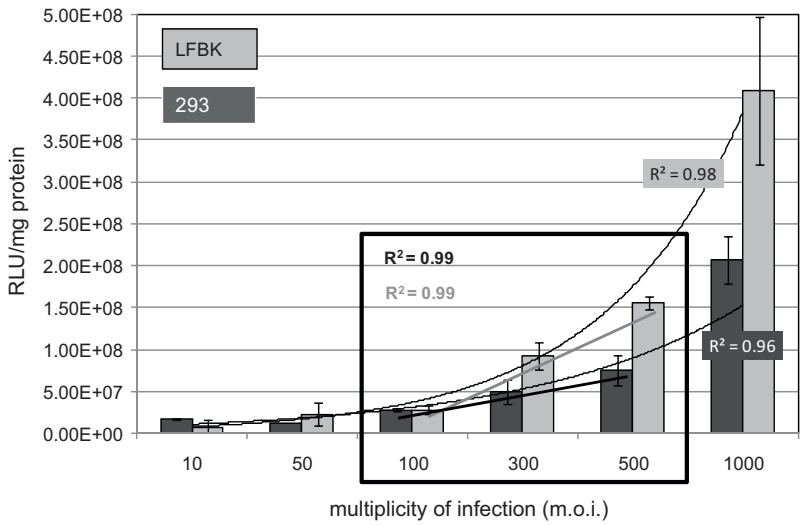

Fig. 1. Luciferase activity in 293 (dark grey) and LFBK (light grey) cell lines infected with an Ad5-Luc vector at different multiplicities of infection (m.o.i.) at 24 hpi. Each column represents mean luciferase activity (relative light units (RLU) per mg of total protein) ± SD of 3 replicates assayed simultaneously. Trendlines were associated with coefficients of determination (R^2) specific for each cell line, an indicator of variability and predictor of fitness of selected data between m.o.i. groups. The rectangle encompasses optimal m.o.i. as determined based on high linearity and R^2 values, and low statistical variation (SD) of data.

in bovine (and human) cell lines infected with Ad5-Luc, and the suitability of using these cells lines for both further characterization of virus-cell interactions and transgene expression *in vitro* and direct applications in vaccination studies of cattle.

3.3. Immunomicroscopy of Ad5-infected cell lines

Anti-hexon monoclonal antibodies were used to localize residual adenoviral structural protein of both Ad5 vectors (supplemental Fig. 1A and B). Ad5 hexon was detected at moderate levels in infected cells. Overall, hexon protein had substantially more limited distribution than transgene products, and followed a punctate pattern in both cytoplasm and nucleus. Transgene expression was visualized in both LFBK and 293 cells infected with Ad5-A24 or Ad5-Luc. In Ad5-A24 infected LFBK cells at 24 hpi, staining of the FMDV-A24 protein was punctate and diffuse throughout the cytoplasm with mildly increased perinuclear intensity (supplemental Fig. 1A). In Ad5-Luc infected LFBK cells at 24 hpi, luciferase was distributed homogenously in the cytoplasm with a more accentuated perinuclear distribution than FMDV-A24 proteins (supplemental Fig. 1B). In Ad5-A24 infected cells (Fig. 2C), FMDV-A24 protein was only detectable in cultures infected at m.o.i. 50 and above. At m.o.i. 300–1000, approximately 20–30% of the cells contained detectable FMDV-A24 protein, with substantial cytopathic effects (CPE) observed in the form of massive detachment of cells and presence of many autofluorescent degenerated cells (supplemental Fig. 1C, encircled). In Ad5-Luc-infected cultures (supplemental Fig. 1D) few luc^+ cells were observed at the lowest m.o.i. (10 and 50). Optimal luc^+:luc^- cells ratios (1:1 of the monolayers) were visualized at m.o.i. 100 and 300. Cultures infected at m.o.i. 500 and 1000 were predominantly luc^+ (70–80%) with highly variable signal intensity, likely corresponding to different stages of infection/gene expression. Morphology was preserved without signs of CPE.

3.4. Detection and quantitation of Ad5 nucleic acids in bovine tissues

Tissues from cattle inoculated with Ad5 vectors were processed, nucleic acids extracted, and target sequences detected and amplified by rPCR (PIX) and rRT-PCR (splice) (Table 3). For all four experimental subjects, the greatest quantities of Ad5 (GC/50 mg tissue) were detected in IS; however there was substantial variability across the distinct IS site samples of each animal which was indicative of the multifocal (*i.e.* not diffuse) nature of Ad5 genomic distribution. Additionally, there was a trend of decreasing Ad5 GC with increasing time post-inoculation. Average GC quantities in IS sites were: steer #1 (Ad5-A24, 6 hpi) = 29,796 GC, steer #2 (Ad5-A24, 24 hpi) = 9823 GC, and steer #3 (Ad5-A24, 48 hpi) = 111 GC. The GC numbers in IS of steer #4 (Ad5-Luc, 24 hpi) was 4.8 ± 4.6-fold higher than the comparable subject inoculated with Ad5-A24 (steer #2) (Table 3).

At 6 hpi, steer #1 had substantial quantities of Ad5 DNA and mature mRNA transcript at all IS sites examined (mean PIX Ct = 31.9; mean splice Ct = 33.1). Ad5-A24 DNA was also detected in most of the ipsilateral lymph nodes and in all visceral organs examined. Ad5 DNA was undetectable in tissues of the contralateral side. Excluding IS, vector quantities in rPCR positive tissues ipsilateral to the IS side ranged from 31 to 185 GC/50 mg tissue, whereas in the contralateral side, only the dorsal portion of the prescapular LN was positive, containing 14 GC/50 mg tissue. Among non-IS tissues, mature mRNA transcripts were only detected in the dorsal area of the prescapular LN at relatively low levels (Ct = 38.5).

Steer #2 (24 hpi) had an overall pattern of nucleic acid detection that reflected increased dissemination (local and systemic) of Ad5 DNA and greater expression of transgene mRNA compared to steer #1. The IS of steer #2 had less Ad5 DNA (mean PIX Ct = 34 and 31.9, respectively) and more mature mRNA transcripts (mean splice Ct = 31.4 and 33.1, respectively) relative to steer #1. Non-IS tissues of steer #2 had higher vector (Ad5 DNA and GC) quantities than non-IS tissues of steer #1. Similar to steer #1, most ipsilateral lymph nodes and visceral organs contained Ad5 DNA. Other than IS tissues, maximum Ad5 detection occurred in the lateral retropharyngeal LN (40,426 GC/50 mg). It is noteworthy that although Ad5 distributed systemically to multiple visceral organs in steer #2, vector DNA was not found in liver, which is a well known primary target for Ad5 in other species [33]. Abundant vector DNA was also detected in muscle and lymph nodes of the contralateral side, likely due to hematogenous dissemination. Unlike steer #1, mature mRNA transcripts were also found in multiple non-IS tissues including LN. It is noteworthy that the lateral retropharyngeal LN had the strongest splice signal (Ct = 25.0) of all non-IS tissue examined within this study.

Steer #3 had the lowest quantity of Ad5 DNA in tissue specimens of the IS of the 3 animals inoculated with Ad5-A24 (mean PIX Ct = 37.7; mean GC = 111.7). Excluding IS, only the axillary LN had detectable DNA (Ct = 37.8; 73.1 GC). Mature mRNA signal was detected in two IS specimens (mean splice Ct = 34.8) and in the ventral section of the prescapular LN (Ct = 36.7).

One animal (steer #4) was inoculated with Ad5-Luc and evaluated at 24 hpi in order to provide direct comparison of Ad5-A24 distribution and host responses to steer #2 (Ad5-A24, 24 hpi). Overall, steer #4 had a different pattern of vector biodistribution and transgene detection levels relative to steer #2 with relatively higher Ad5 DNA quantities in IS tissues. However, despite having more Ad5-Luc DNA, steer #4 had fewer mature mRNA positive IS tissues and markedly less systemically disseminated RNA than steer #2. Beyond the IS region, Ad5 DNA was only present at the ventral area of the prescapular (Ct = 38.9; 23.9 GC), axillary (Ct = 38.2; 40.3 GC), and popliteal LN (Ct = 39.1; 21.5 GC), and mature transgene mRNA transcripts were absent from LN and organs. In contrast, the overall pattern of detection for Ad5-Luc at 24 hpi was more similar to Ad5-A24 at 48 hpi than to other time-points included in the study.

3.5. Determination of luciferase activity in tissues from an Ad5-Luc inoculated steer

Steer #4 was intramuscularly inoculated with Ad5-Luc and tissues evaluated at 24 hpi for luciferase activity. Luciferase activity was largely confined to the IS sites (supplemental Fig. 2). Similar to Luc mature mRNA expression, luciferase expression at the IS was not found uniformly distributed. Three of eight IS examined had substantial luciferase activity; IS 7 had the highest activity with levels approximately 100-fold higher than the lowest activity of the grid found in IS site 4 (supplemental Fig. 2, inset). Luciferase activity was not detected in any tissues beyond IS from this animal.

3.6. Microscopic characterization of Ad5-inoculated bovine tissues

3.6.1. Brightfield microscopy of injection sites

In order to characterize histological changes associated with vaccination, tissues from up to 8 IS sites of each steer inoculated intramuscularly with Ad5 vectors were examined by routine brightfield microscopy (Fig. 2A). Normal muscle architecture was disrupted by extensive edema and cellular infiltrates consisting of predominantly large mononuclear cells with fewer neutrophils and small mononuclear cells. Infiltrates predominantly dissected myofibers and fascicles following collagenous interstitial connective tissue (endomysium and perimysium). Although there was disruption of contiguity of myocytes by edema, there was minimal cellular disruption or myonecrosis.

Table 3
Detection of adenovirus vector and transgene target sequences in bovine tissues from animals inoculated with either Ad5-A24 or Ad5-Luc constructs at 6, 24, and 48 hpi.

	Steer #1 (6 hpi)			Steer #2 (24 hpi)			Steer #3 (48 hpi)			Steer #4 (24 hpi)		
	Mature mRNA Ct (splice)	DNA Ct (PIX)	Genome copies/50 mg tissue[a]	Mature mRNA Ct (splice)	DNA Ct (PIX)	Genome copies/50 mg tissue[a]	Mature mRNA Ct (splice)	DNA Ct (PIX)	Genome copies/50 mg tissue[a]	Mature mRNA Ct (splice)	DNA Ct (PIX)	Genome copies/50 mg tissue[a]
Injection sites (IS)												
IS1	N/E	N/E		32.5	37.6	82.9	33.5	36.4	191.4	31.7	31.9	3419.5
IS2	33.3	27.4	85,511.5	34.1	37.8	74.1	Neg	Neg	0	31.5	34.2	675.7
IS3	N/E	N/E		24.3	27.7	71,934.9	Neg	Neg	0	34.4	29.8	15,350.5
IS4	38.2	32.0	3679.3	33.2	33.5	1400.2	Neg	Neg	0	36.6	34.7	475.0
IS5	N/E	N/E		33.4	34.6	648.6	36.0	Neg	0	Neg	36.3	159.3
IS6	27.7	36.3	199.4	33.2	34.5	696.5	Neg	39.0	32.0	Neg	39.3	18.5
IS7	N/E	N/E		28.1	32.6	2603.7	Neg	Neg	0	27.9	25.5	309,342.2
IS8	N/E	N/E		32.4	33.8	1146.3	Neg	Neg	0	N/E	N/E	
Mean (IS)	33.1	31.9	29,796.7	31.4	34.0	9823.4	34.8	37.7	111.7	32.4	33.1	47,063.0
SD	5.3	4.5	48,281.77	3.4	3.2	25,110.06	1.8	1.9	112.75	3.3	4.5	115,784.41
Local lymph nodes												
Prescapular-Dor[b]	38.5	39.1	31.5	Neg	38.7	39.4	Neg	Neg	0	Neg	Neg	0
Prescapular-Med[b]	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Prescapular-Ven[b]	Neg	37.7	77.7	Neg	Neg	0	36.7	Neg	0	Neg	38.9	23.9
Submandibular[c]	Neg	37.8	72.6	Neg	36.8	145.9	Neg	Neg	0	N/E	Neg	0
Lat. retropharyngeal[c]	Neg	Neg	0	25.0	28.5	40,425.5	Neg	Neg	0	Neg	Neg	0
Med. retropharyngeal[c]	Neg	38.2	58.0	35.6	36.2	218.5	Neg	Neg	0	Neg	Neg	0
Axillary[c]	Neg	38.9	35.1	38.2	38.2	56.9	Neg	37.8	73.1	Neg	38.2	40.3
Parotid[c]	N/E	N/E	N/E	Neg	37.7	77.2	Neg	Neg	0	N/E	N/E	
Distant lymph nodes												
Popliteal (ipsilateral)	Neg	39.0	34.0	35.6	37.2	108.3	Neg	Neg	0	Neg	39.1	21.5
Popliteal (contralateral)	Neg	Neg	17.1	35.7	Neg	0	Neg	Neg	0	Neg	Neg	0
Organs												
Liver	Neg	38.0	66.9	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0
Spleen	Neg	38.8	38.1	36.7	37.2	113.9	Neg	Neg	0	Neg	Neg	0
Lung	N/E	N/E	N/E	Neg	37.7	77.7	Neg	Neg	0	Neg	Neg	0
Pancreas	N/E	N/E	N/E	37.0	37.8	75.9	Neg	Neg	0	Neg	Neg	0
Adrenal gland	N/E	N/E	N/E	Neg	38.2	56.5	Neg	Neg	0	Neg	Neg	0
Kidney	N/E	N/E	N/E	37.2	37.0	131.8	Neg	Neg	0	Neg	Neg	0
Thymus	Neg	36.5	185.0	36.0	36.8	146.9	Neg	Neg	0	Neg	Neg	10.6
Mean (LN and organs)	38.5	38.2	61.6	35.2	36.8	3,205.72	36.7	37.8	73.1	Neg	38.7	24.1
SD		0.8	47.79	3.9	2.6	11,183.26					0.48	12.26
Contralateral side tissues												
Splenius muscle	Neg	Neg	0	Neg	36.8	145.9	Neg	Neg	0	Neg	38.2	40.3
Prescapular-Dor	Neg	Neg	14.3	Neg	36.7	156.2	Neg	Neg	0	Neg	39.3	18.5
Prescapular-Med	Neg	Neg	0	Neg	37.6	84.3	Neg	Neg	0	Neg	Neg	0
Prescapular-Ven	Neg	Neg	0	Neg	Neg	0	Neg	Neg	0	Neg	39.2	19.9
Mean (contralateral)	Neg	Neg	14.3	Neg	37.0	128.8	Neg	Neg	0.0	Neg	38.9	26.2
SD					0.5	38.9			0.00		0.61	12.17

N/E, not examined.

[a] Quantification of adenovirus genomes was performed in 50 mg of tissue specimens (GC/50 mg) based on the molecular weight of each adenovirus construct and known concentration of DNA standards.

[b] Expected draining lymph node of the injection site.

[c] Lymph nodes in regional proximity to the injection site.

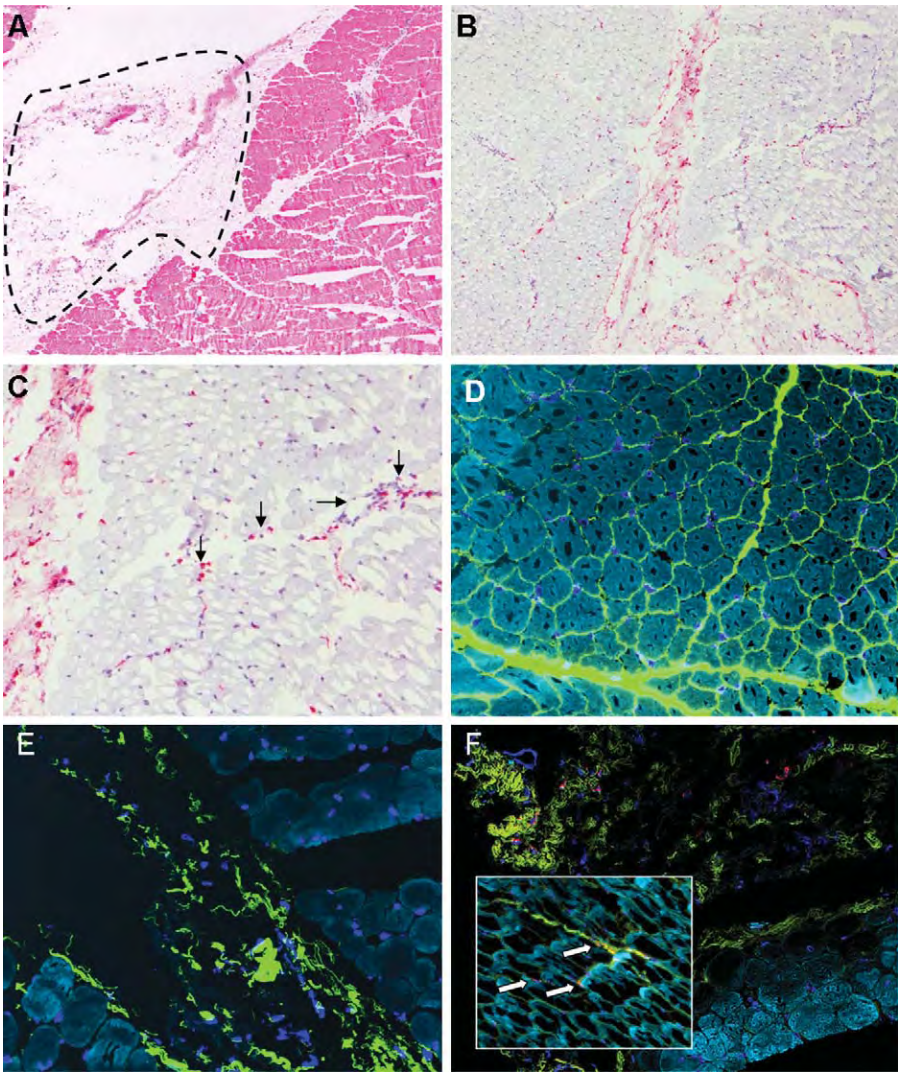

Fig. 2. Microscopy of injection sites (IS) of Ad5-inoculated steers. (A) Steer #4, IS. Dashed line indicates infiltrated and rarefied interstitial connective tissue of the IS. Eosinophilic (pink) corresponds to muscle. H&E stain. (B and C) Steer #4, IS. Immunohistochemical localization of hexon antigens (red) to interstitial tissue of perimysium (B) and endomysium (C, black arrows) of the IS of steer #4. (D) Steer #1, unaffected muscle adjacent to IS. Immunofluorescent visualization of collagen (green) and actin (light blue) in regular pattern. (E) Steer #1, IS. Infiltrated and edematous regions of intermuscular connective tissue showing separation of myocytes (actin; light blue) with marked disruption of collagen fibers (green). (F) Interstitial connective tissue of the IS of steer #1 showing myocytes (light blue), collagen (green), and presence of Ad5 hexon antigens (red). Higher magnification of IS showing co-localization of hexon (red) and collagen (green) to endomysium (F, inset).

Ad5 hexon antigens were localized within IS tissues (Fig. 2B and C). There was a gradual, time-dependent decline in immunoreactivity to Ad5 hexon antigens in IS of animals euthanized at 6 (steer #1), 24 (steer #2 and steer #4), and 48 (steer #3) hpi (data not shown). Immunohistochemical localization of hexon antigens was largely confined to large mononuclear cells within disrupted regions of perimysium (Fig. 2B), endomysium, often associated with small vessels (Fig. 2C), and, rarely, within adipose tissue. FMDV antigens were not successfully localized at injection sites of any Ad5-A24 inoculated animal. However small clusters of luc$^+$ cells were observed in IS specimens from steer #4 (data not shown).

3.6.2. *Immunofluorescent microscopy of injection sites*

In order to characterize the phenotypic environment of Ad5 cellular infection, immunofluorescent labeling of Ad5 hexon, FMDV A24 protein, and luciferase were performed in various combinations with one or more cell markers. Myocytes were defined by specific staining of skeletal muscle actin, whereas perimysium and endomysium of the interstitium were identified by presence of collagen and absence of actin (Fig. 2D and E). Regions of muscle tissue unaffected by injection had an orderly and regular pattern of myocytes invested with endomysium (Fig. 2D). By contrast, infiltrated and edematous regions had marked rarefaction of collagen fibers and separation of myocytes (Fig. 2E). Ad5 hexon protein, when detectable, was visualized in a punctate pattern generally associated with rarefied collagen of the perimysium (Fig. 2F) or within intact collagen of the endomysium in contact with myocytes (Fig. 2F, inset). Presence of hexon decreased with time post-inoculation. The greatest quantities of hexon protein were observed in steer #1 at 6 hpi followed by a substantial reduction of hexon signal at 24 and 48 hpi (steers #2 and #3). There were also differences between animals that were inoculated with the 2 vectors; moderately higher quantities of hexon$^+$ cells were identified in IS of steer #2 (Ad5-A24) *versus* in IS of steer #4 (Ad5-Luc) (data not shown).

Ad5-associated tissue disruption and presence of infiltrates were important pathological hallmarks of this study. These regions had, consistently, greater quantities of MHCII and CD11c single and double positive cells (antigen-presenting cells (APC)) compared to normal areas of the IS (Fig. 3A–C). Ad5 hexon signal was found

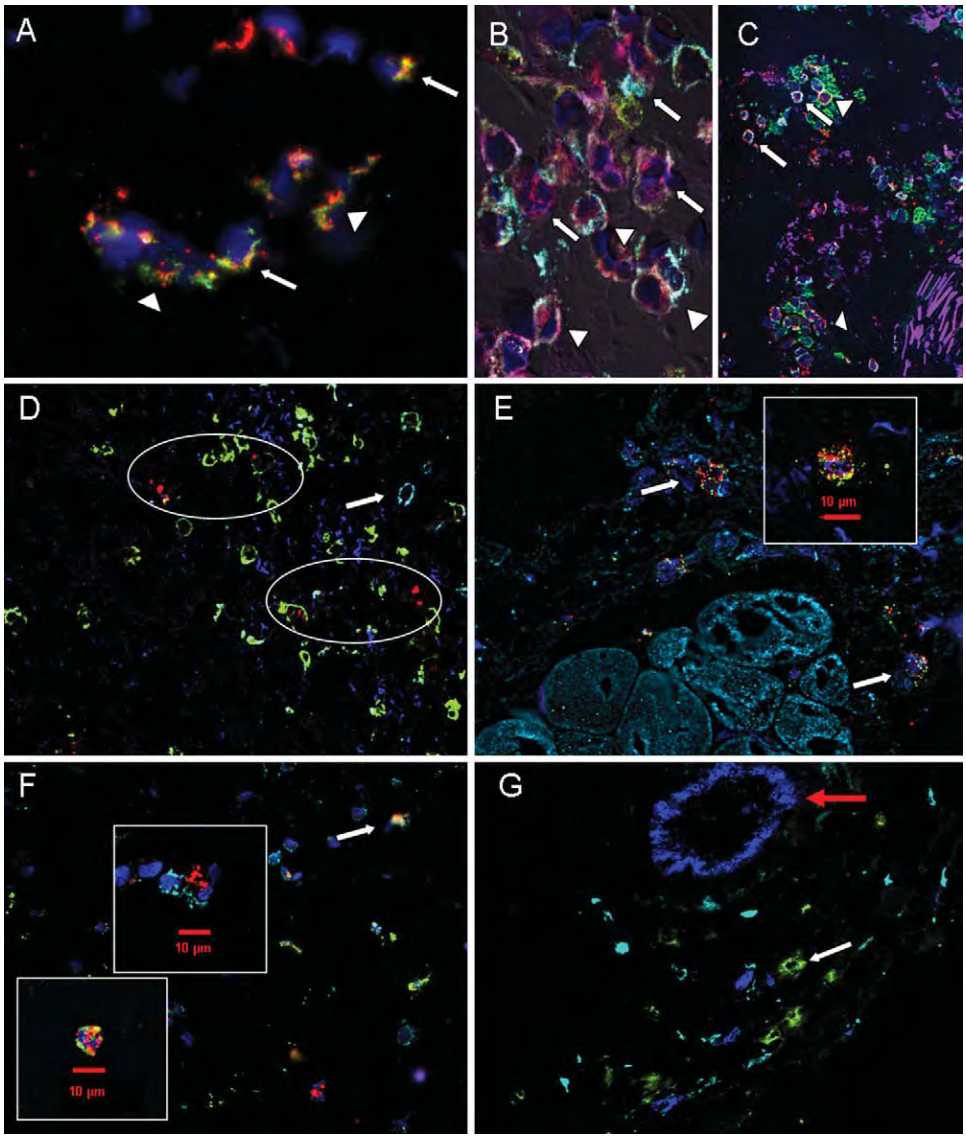

Fig. 3. Immunofluorescent characterization of tissues from Ad5-inoculated cattle. (A) Steer #1, IS. Ad5 hexon (red) colocalizes with either MHCII (green, arrow heads), or MHCII and CD11c (light blue, arrows). (B) Steer #4, IS. Mixed mononuclear cell infiltrates expressing MHCII (green), CD11c (light blue), CD172a (red), and CD14 (pink) antigens. CD172a predominates and co-localizes with CD14 ($CD172^+CD14^+$, arrows) and CD11c ($CD172^+CD11c^+$, arrow heads). $CD14^+CD11c^+$ cells (green + light blue) also present. (C) Steer #2, IS. Inflammatory aggregates expressing MAC387 (green), MHCII (red), and CD11c (light blue). Presumptive dendritic cells are $MHCII^+$ $CD11c^+$ (arrows) whereas most macrophages stain only for $MAC387^+$ (green) in proximity to $CD11c^+MHCII^+$ cells. Few double-positive $MHCII^+MAC387^+$ present (arrow heads). Capillary endothelial cells expressing vWf (purple). Nuclei blue. (D) Steer #4, IS. Abundant $CD14^+$ cells (green) in close proximity to Ad5 hexon (red, ovals) with few $CD11c^+$ cells (light blue, arrow). (E) Steer #2, IS. Co-localization of hexon (red) and FMDV antigens (green) to interstitial cells (arrows and inset). Myocytes (actin, light blue). (F) Steer #4, IS. Co-localization of hexon (red) and luciferase (green) to interstitial cells (arrow and lower inset). Rare $hexon^+$ (red) $CD11c^+$ (light blue) double positive presumptive dendritic cells (upper inset). (G) Steer #4, dorsal prescapular LN. Cells expressing luciferase (green, white arrow) in close proximity to $CD11c^+$ cells (light blue) and blood vessel (vWf+, blue stain, red arrow).

primarily co-localized to CD11c and/or MHCII antigens (double or triple co-localization) (Fig. 3A). Hexon was also found associated within cells lacking CD11c or MHCII markers (Fig. 3A). The greatest abundance of APC-like phenotypes (CD11c and MHCII) was found at 6 hpi (steer #1) within, or adjacent to, areas with extensive cellular infiltrates. Both steers examined at 24 hpi (steers #2 and #4) had more mild infiltrates and similarly less reactivity against APC antigens. Further decrease in infiltrates and APC phenotype cells was seen at 48 hpi (steer #3) (data not shown).

At 6 and 48 hpi, FMDV A24 protein was absent from any tissue specimen. However at 24 hpi, mild to moderate immunoreactivity to FMDV A24 protein and luciferase was observed in the IS of steers #2 and #4, respectively (Fig. 3E and F). Cells that expressed FMDV antigens (Fig. 3E) or luciferase (Fig. 3F), often co-expressed Ad hexon proteins and/or CD11c. These cells were primarily visualized at the interstitial connective tissue of muscle fascicles and less frequently between myofibers.

Detailed phenotypic characterization of the injection site inflammatory infiltrates in steer #4 indicated various combinations of reactivity for CD11c, MHCII, MAC387, CD172A, and CD14. The predominant inflammatory cell phenotype was co-expression of CD172a and CD14 with less abundance of MHCII-positive cells. This phenotype ($CD172^+/CD14^+$) was also found in close proximity to $CD11c^+/CD14^-$ and $CD11c^+/CD14^+$ cells (Fig. 3B) in areas where Ad5 $hexon^+/CD11c^+$ and Ad5 $hexon^+/CD11c^-$ cells were present in lesser quantities (Fig. 3D). In this animal, co-localization of MHCII with CD11c antigens was common with, or without, co-localization with Ad5 hexon, whereas expression of MAC387 occurred with

minimal, or without, co-localization to other APC antigens (Fig. 3C). Overall, the phenotypic pattern observed across Ad5-A24 and Ad5-Luc-vaccinated subjects at injection sites was consistent with the presence of cells of monocyte/macrophage, dendritic, and granulocytic lineages.

3.6.3. Microscopy of non-injection site tissues

No morphologic lesions were identified in non-injection sites tissues of any animal used in this study. Ad5 hexon antigens were detected as early as 6 hpi in the prescapular LN where immunoreactivity was cell-associated and located at cortical trabecular sinuses and corticomedullary junctions (Fig. 4A), and within the capsule and subcapsular sinuses (Fig. 4B). Rarely, hexon$^+$ cells were identified in perivascular connective tissue surrounding blood vessels (Fig. 4B, inset), suggesting hematogenous dissemination. At 24 and 48 hpi (steers #2 and #3), the dorsal portion of the prescapular LN had a similar distribution of hexon proteins but with reduced intensity and quantity of immunoreactive cells, compared to 6 hpi. Similarly, hexon signal was detected in the ventral portion of the prescapular LN at 24 hpi; however, the quantity of hexon antigen was substantially less than at 6 hpi.

FMDV A24-capsid antigens were detected by IHC in only one tissue of one animal. Within the dorsal portion of the prescapular LN of steer #1 (6 hpi) there was a single small cluster of FMDV A24$^+$ cells located in cortical sub-trabecular lymphoid tissue (Fig. 4C). In contrast to FMDV antigens, the Ad5-Luc transgene product (luciferase) was detected in somewhat greater abundance in the axillary LN of steer #4 (Fig. 4D). Immunofluorescent microscopy of capsule and subcapsular sinus of the prescapular LN of steer #4 confirmed the presence of luciferase-positive cells in close proximity, but not reactive to CD11c$^+$ APCs, CD14, or vWf$^+$ endothelium (Fig. 3G).

4. Discussion

The current study provides novel insights into vector–host interactions occurring within the first 48 h after intramuscular administration of cattle with a recombinant replication-defective human adenovirus serotype 5 vaccine carrying FMDV transgenes (Ad5-A24). In addition, an Ad5-luciferase vector was demonstrated as a useful surrogate screening tool for Ad5 vector distribution and studies on vector–host interactions in inoculated cattle.

Ad5-A24 and Ad5-Luc infection dynamics were first assessed in cell culture using human 293 and bovine LFBK cell lines infected at various multiplicities of infection (m.o.i.). At 24 hpi, the Ad5-A24 vector induced higher mature mRNA transcript synthesis than Ad5-Luc. However, despite higher levels of mature mRNA transcripts detected from Ad5-A24 infected LFBK cultures, transgene protein detection, as determined by immunomicroscopy, was substantially higher in Ad5-Luc infected cells. This distinction may indicate differences in efficiency of post-transcriptional gene expression or decreased sensitivity of the anti-FMDV antibody compared to anti-luciferase antibody. Results from Ad5-Luc (surrogate) experiments demonstrated a dose-dependent relationship between m.o.i. and (1) detection of Ad5 DNA, (2) presence of mature mRNA transcripts, (3) luciferase bioactivity, and (4) microscopic visualization of luciferase positive cells. Therefore, similarities in RNA and DNA detection between the two adenovirus vectors together with high coefficients of determination (statistical predictor value) obtained by the luciferase assays indicated concurrence of the detection techniques and suggested that the Ad5-Luc was an appropriate surrogate for Ad5-FMDV (Ad5-A24). Thus, characterization of transgene expression in relation to infection levels in bovine cells *in vitro* supported the use of Ad5 vectors in subsequent vaccination studies of cattle.

In order to characterize adenovirus infection and biodistribution *in vivo*, we inoculated cattle with both Ad5-A24 and Ad5-Luc vectors. Time-course data using Ad5-FMDV indicate that subsequent to intramuscular inoculation, adenovirus DNA is consistently present in greatest abundance at the IS, rapidly traffics to local LN and organs as early as 6 hpi, and commences to clear from tissues between 24 and 48 hpi. Detection of transgene mRNA was interpreted as either (1) expression in specific cells and tissues to which the vector had distributed and/or (2) migration of cells that were infected at other sites (*e.g.* from IS to local LN). As expected, in the Ad-FMDV time-course study, transgene mRNA detection lagged behind Ad5 hexon biodistribution with peak detection at 24 hpi in both IS and non-IS tissues. Throughout the time-course, mature mRNA transcripts were detected at the prescapular, retropharyngeal, and axillary LN suggesting these groups of LN as the primary sites of lymphatic drainage of the inoculum.

Comparison of *in vivo* inoculation of the two adenovirus constructs resulted in similar detection levels of Ad5 DNA and transgene mRNA at the IS when compared at 24 hpi (steer #2; Ad5-A24 and steer #4; Ad5-Luc). However there were substantial differences in Ad5 distribution and expression at non-IS tissues between the vectors. While Ad5-A24 was widely disseminated to local LN and systemically, Ad5-Luc had more limited distribution. Detection of Ad5-Luc DNA at contralateral sites, suggested that systemic, hematogenous, dissemination was in progress. However, presence of vector or vector products in blood was not confirmed. Notably, the contrast between wide distribution of Ad5-A24 mature mRNA and complete absence of dissemination of Ad5-Luc mature mRNA indicates a pathophysiological distinction between the vectors. Overall, the pattern of distribution of Ad5-Luc at 24 hpi is similar to that of Ad5-A24 at either 6 hpi or 48 hpi. Ongoing work in our laboratory is directed towards establishing the time-course of events subsequent to Ad5-Luc inoculation of cattle.

The current results indicate that subsequent to inoculation of cattle, Ad5-derived transgene products are only detectable within a relatively narrow time-frame with concomitant biodistribution and expression peaking at 24 hpi. These findings are in agreement with previous work suggesting 24 hpi as a critical time-point for initiation of immune responses to Ad5 vectors [34,35] in mice. However, characterization of inflammatory responses and expression of proinflammatory mediators early after administration of Ad5 vectors have not been extensively studied in cattle.

In this study, inflammatory infiltrates recruited to the sites of inoculation in response to replication-defective adenovirus were characterized by multichannel immunofluorescence microscopy. Infiltrates were distributed multifocally and were generally limited to interstitial regions of muscle with minimal disruption of myocytes. The factors determining this inflammatory distribution were not apparent, but were, likely, related to diffusion and/or intravascular movement of virus-laden inoculum. Infiltrates varied in severity and consisted of a mixture of large mononuclear cells with fewer neutrophils and small mononuclear cells. Ad5 hexon proteins were abundant in inflamed regions early post-inoculation but decreased substantially by 24 hpi. Contrarily, FMDV A24 protein and luciferase were detected, although minimally, as early as 6 hpi at the IS and primary draining LN, and remained at similar low levels of detection through 24 hpi. It is possible that phagocytically active macrophages were part of the inflammatory response evoked in IS tissues in this study and that some portion of transgene products were phagocytosed, degraded, and eliminated, thereby decreasing immunomicroscopy detection. This notion is supported by results of previous studies in animal models for tissue damage, showing that during the first 24 h after muscle injury, phagocytic macrophages are attracted to the inflamed areas to degrade damaged muscle fibers [36,37]. This

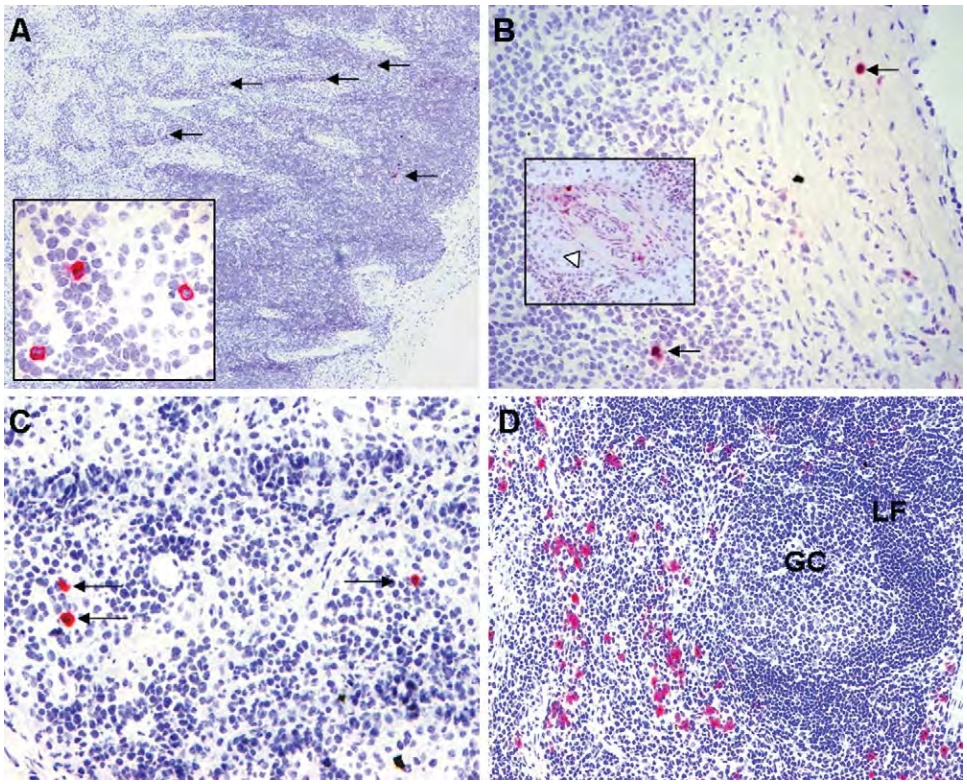

Fig. 4. Microscopy of non-injection site tissues of Ad5-inoculated cattle. (A and B) Steer #1, prescapular LN. Cells containing hexon antigen (arrows) within cortex (A) and capsule/subcapsular sinus (B). Ad5 hexon localizes to cytoplasm of mononuclear cells (A, inset) and rarely associated with blood vessels in medulla (B, inset, white arrow head). (C) Steer #1, prescapular LN. Rare FMDV$^+$ cells in the deep cortex. (D) Steer #4, axillary LN. Abundant luciferase$^+$ cells in perifollicular cortex. Lymphoid follicle (LF); germinal center (GC). Micropolymer alkaline phosphatase technique.

could explain the observed discrepancies between presence of high luciferase activity as determined by luciferase bioassays and low detection levels of luciferase epitopes by microscopy at the IS. Overall, in agreement with our *in vitro* data, luciferase activity was more successfully localized microscopically compared to FMDV A24 protein expression. As with the *in vitro* results, this distinction may be due to either difference in quantities of the proteins which are present or differential sensitivity between the anti-FMDV and anti-luciferase primary antibodies.

In agreement with previous descriptions of the involvement of innate immune system responses to infections of viral origin [38–41], IS tissues of cattle inoculated with replication-defective Ad5 contained abundant cells expressing MAC387, CD14, MHC class II, and CD11c antigens consistent with the phenotype of antigen-presenting cells (macrophages and dendritic cells). Cells that were immunopositive for MAC387 were interpreted as macrophages, whereas presence of combinations of the other markers was interpreted as suggestive of dendritic cell phenotypes. The demonstration of vector and transgenic protein products at IS tissues, within small quantities of presumptive dendritic cells represents a critical, early event in the function of these Ad5 vectors. This observation combined with the subsequent finding of Ad5 vector and transgene product localization to afferent regions of local lymph nodes is generally consistent with classical pathways of antigen presentation and induction of protective immunity to pathogens. It is noteworthy that previous work has demonstrated lack of preinoculation reactivity to Ad5 vectors in cattle (M. Grubman, personal communication); therefore we consider it likely that the immunopathogenesis processes described herein are indicative of the naïve host response to the Ad5 vectors.

Comparison of the Ad5 vectors by the three detection systems utilized in this study reveals consistencies but also important discrepancies. The overall pattern of nucleic acid detection was only similar between the two Ad5 vectors at 24 hpi; however, based on the rPCR and rRT-PCR data, it is plausible to speculate that the Ad5-Luc construct could have been at an earlier or later pathophysiological state as compared to Ad5-FMDV. This observation deserved further investigation. The apparent relatively low utility of luminometry compared to rPCR, rRT-PCR, and immunomicroscopy could be explained by either (1) detectable quantities of bioactive luciferase were only translated in a subset of specimens that contained transgene mRNA or (2) detection sensitivity of luminometry is markedly inferior to that of the splice rRT-PCR system and in some cases immunomicroscopy. The rarity of microscopic detection of luciferase or FMDV A24 antigen immunoreactivity as compared with detection of Ad5 hexon supports the former hypothesis; however, the finding of luciferase immunoreactivity in the axillary lymph node of steer #4 (luminometry negative) supports the latter. Although the reason for suboptimal performance of luminometry in non-IS tissues was not determined, the most likely explanation is the dilution effect created by inoculation of a 200–300 kg steer. It is anticipated that in future studies using Ad5-Luc as a surrogate vector, luminometry would be a more effective screening tool if applied to a smaller research subjects such as swine.

In summary, the results of this study improve understanding of the early immunopathogenesis subsequent to vaccination of cattle with replication-defective Ad5 vectors. The techniques used herein provided a robust system capable of simultaneously characterizing adenovirus vector biodistribution, transgene transcription, and

transgene expression in cell culture and in tissues of inoculated cattle. Because improvements in Ad-FMDV vaccine potency and a reduction in the minimum protective dose remain an important goal [8], further application of these techniques in future studies will facilitate more thorough elucidation of the critical events required for adenoviral vaccine function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.12.082.

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An alternate delivery system improves vaccine performance against foot-and-mouth disease virus (FMDV)

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Immune response

ABSTRACT

Foot-and-mouth disease virus (FMDV) causes vesicular disease of cloven-hoofed animals with severe agricultural and economic implications. One of the most highly infectious and contagious livestock pathogens known, the disease spreads rapidly in naïve populations making it critical to have rapidly acting vaccines. Needle inoculation of killed virus vaccine is an efficient method of swiftly vaccinating large numbers of animals, either in eradication efforts or in outbreak situations in disease free countries, although, to be efficient, this requires utilizing the same needle with multiple animals. Here we present studies using a needle free system for vaccination with killed virus vaccine, FMDV strain O1 Manisa, as a rapid and consistent delivery platform. Cattle were vaccinated using a commercially available vaccine formulation at the manufacturer's recommended dose as well as four and sixteen fold less antigen load per dose. Animals were challenged intradermalingually (IDL) with live, virulent virus, homologous strain O1 Manisa, at various times following vaccination. All non-vaccinated control cattle exhibited clinical disease, including fever, viremia and lesions, specifically vesicle formation. Cattle vaccinated with the 1/16× and 1/4× doses using the needle free device were protected when challenged at both 7 and 28 days after vaccination. These data suggest that effective protection against disease can be achieved with 1/16 of the recommended vaccine dose when delivered using the needle free, intradermal delivery system, indicating the current vaccine stockpile that can be extended by many fold using this system.

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1. Introduction

Foot-and-mouth disease virus (FMDV) causes a high impact disease of cattle and other cloven-hoofed animals, with severe agricultural and economic implications [1,2]. Fortunately, foot and mouth disease vaccines confer rapid protection of susceptible animals in a single dose, thus allowing a quick response to control the spread of disease in outbreak situations [3,4]. Although the standard needle inoculation method is an efficient system of swiftly vaccinating large numbers of animals at once, this requires utilizing the same needle for all the animals. A needle free vaccine delivery system allows for rapid vaccination of large numbers of animals more efficiently and with increased safety than needle delivery [3,5]. Besides eliminating the cumbersome needle inoculation process, intradermal (ID) vaccination has advantages compared to intramuscular (IM) vaccination, the traditional method for FMD vaccines [3,5].

Prior studies by Eble and coworkers show ID vaccination of pigs against FMD with 1/10 dose confers comparable vaccine efficacy as IM vaccination with a full dose [3]. These results suggest that current vaccine stocks can be extended many fold, using ID inoculation. This is supported by other studies with hepatitis B, rabies and influenza virus vaccines suggesting that ID vaccination results in enhanced immunogenicity [5,6]. Reports indicate that such enhancement can be attributed to the skin being heavily populated with dendritic cells [6,7]. These cells are efficient and potent antigen-presenting cells that are required for the initiation of an adaptive immune response eventually leading to protective immunity [3,5,6].

We have developed a new vaccination system termed the Dermavac®, which uses compressed gas to deliver 0.5 ml doses of vaccine formulation either intradermally or subcutaneously by adjusting pressure for differential delivery. The Dermavac® takes up to a 25 ml syringe, allowing delivery of 50 doses of vaccine without changing syringes and with no requirement for needles. A clear

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Table 1
Challenge at 28 days postvaccination.

Vaccination formulation		Dose (volume)	Animal #	Clinical assessment				Fever
Antigen	Adjuvant			Day 0	Day 4	Day 7	Day 10	
1×	1×	0.5 ml	689	0	0	0	0	No
			690	0	0	0	0	No
			691	0	0	0	0	No
1×	1/4×	0.5 ml	686	0	0	0	0	No
			687	0	0	0	0	No
			688	0	0	0	0	No
1/4×	1/4×	0.5 ml	683	0	0	0	0	No
			684	0	0	0	0	No
			685	0	1	1	1	No
PBS	PBS	0.5 ml	692	0	4	4	4	Yes
			693	0	4	4	4	Yes

advantage of ID delivery of the vaccine is that it is less painful for the animal compared to the IM route. Importantly, there is minimal site reaction after vaccination when using appropriately formulated vaccines.

The Office International des Epizooties (OIE) standards for all registered FMD vaccines are based on inactivated viral particles in an adjuvant [8]. Different adjuvants confer different performance characteristics of the vaccine and further, some adjuvants are limited in the route of inoculation, such as ID, IM or subcutaneous [9]. In the present study, we evaluated the response to ID inoculation of killed virus vaccine in aluminum hydroxide and saponin adjuvant using this needle free system. We tested for protection against live virus challenge at the standard time of 4 weeks following vaccination as well as induction of rapid protection by challenging 7 days after vaccination. Results presented here provide data to support the use of this needle free device in vaccination for rapid protection, such as during outbreaks of FMD in disease free countries as well as for standard vaccination utility in eradication programs.

2. Materials and methods

2.1. Animals

All cattle experiments were performed in a secure biosafety level three laboratory at Plum Island Animal Disease Center following the protocol approved Institutional Animal Use and Care Committee. One week prior to the start of testing, castrated male Holstein cattle, ranging from 200 to 250 kg, were acquired from Thomas Morris Inc., Reiserstown, MD. Cattle were held for one week to allow for acclimation and recovery from shipping. Baseline temperatures, serum, and nasal swabs were taken before any inoculations.

2.2. Vaccine

The vaccine was provided following standard manufacturing protocols by Merial Animal Health Limited, Pirbright, UK, using inactivated, purified O1 Manisa strain of FMDV. The killed virus antigen was formulated with aluminum hydroxide and saponin as adjuvant and formulated according to OIE standards as described [10]. A placebo vaccine, containing no antigen in the aluminum hydroxide and saponin mixture, was also prepared by Merial Animal Health. The vaccines were administered at various doses in 0.5 ml volume per animal, using the needle free device intradermally in the neck.

2.3. Vaccine trials

To confirm that ID delivery of vaccine protects cattle as well as subcutaneous, needle delivery already established for this vaccine, the standard dose of killed virus vaccine was adjusted to a 0.5 ml volume for delivery with the Dermavac®. Further we tested 1/4 dose of both antigen and adjuvant as well as full dose of antigen with quarter dose of adjuvant. We tested at both doses of adjuvant as we were concerned that a full dose of saponin adjuvant in a 1/4 of the normal, prescribed volume would induce an injection site reaction. Groups of three animals each were vaccinated with each different formulation and animals were challenged 28 days later (Trial 1, Table 1).

In the second trial, we tested delivery 1×, 1/4× and 1/16× doses of killed virus antigen in the 1/4 dose of adjuvant by ID delivery with the Dermavac®. Groups of 7 animals were tested at each dose, and challenged 7 days later (Table 2).

In the final trial, three groups of three cattle were vaccinated at 31 days and three more groups of three cattle were vaccinated at 7 days prior to challenge. The cattle in the three different groups were vaccinated with either 1/4×, 1/16×, or a placebo (adjuvant but no antigen) vaccine (Table 3). Each experiment included at least two or three naïve animals used as controls. The naïve animals co-mingled with the vaccinated animals until the end of each experiment. In the third experiment, six cattle vaccinated with the placebo vaccine were removed to a separate room prior to challenge to avoid overwhelming vaccinated animals with shedding virus from infected animals if, as expected, the placebo failed to protect cattle against disease.

2.4. Challenge

As previously described, the challenge virus was isolated from tongue epithelium macerate harvested from two cattle infected with FMDV stain O1 Manisa [4,11]. Virus aliquots were maintained and stored at −70 °C until use. The challenge virus was titrated in the tongue of a cow to determine 50% bovine tongue infectious doses ($BTID_{50}$). Animals were IDL challenged with 10^4 $BTID_{50}$ [4].

2.5. Clinical assessment of cattle

Cattle were monitored for clinical signs of FMD during the vaccination and challenge periods. Temperatures were recorded daily for each experiment. Animals were examined with sedation for clinical lesions on days 0, 4, 7, and 10 post-challenge. A clinical score was determined based on the number of affected feet [4]. The

Table 2
Challenge at 7 days postvaccination.

Vaccination formulation		Dose (volume)	Animal #	Clinical assessment				Fever
Antigen	Adjuvant			Day 0	Day 4	Day 7	Day 10	
1×	1/4×	0.5 ml	978	0	0	0	0	No
			979	0	0	0	0	No
			980	0	2	2	2	No
			981	0	0	0	0	No
			982	0	1	1	1	No
			983	0	0	0	0	No
			984	0	0	0	0	No
1/4×	1/4×	0.5 ml	985	0	0	0	0	No
			986	0	0	0	0	No
			987	0	0	0	0	No
			988	0	0	0	0	No
			989	0	0	0	0	No
			990	0	0	0	0	No
			991	0	0	0	0	No
1/16×	1/4×	0.5 ml	992	0	0	0	0	No
			993	0	0	0	0	No
			994	0	3	3	4	No
			995	0	0	0	0	No
			996	0	0	0	0	No
			997	0	0	0	0	No
			998	0	0	0	0	No
PBS	PBS	0.5 ml	999	0	4	4	4	Yes
			1000	0	4	4	4	Yes
			1001	0	4	4	4	Yes

maximum clinical score is 4. Vesicles in the tongue and mouth were not part of the clinical scoring system due to IDL challenge.

2.6. FMDV neutralizing antibodies from serum

Serum samples were tested for the presence of neutralizing antibodies against FMDV by a standard protocol [4]. Serum samples were heat inactivated at 56 °C for 30 min. Serial dilutions were incubated with 100 $TCID_{50}$ of FMDV-O1 Manisa for 1 h at 37 °C. These samples were then transferred to preformed monolayers of BHK-21 cells and incubated at 37 °C for 72 h. Cytopathic effect (CPE) was determined microscopically where end-point titers were the reciprocal of the last serum dilution to neutralize virus in 50% of the wells (4 wells for each titration).

2.7. Virus titration

Virus titers in serum were established by determining the tissue culture infectious dose 50 ($TCID_{50}$). Briefly, 10-fold serial dilutions of serum were added to pre-formed monolayers of LFBK cells transfected with αVβ6 (obtained from M. LaRocco and L. Rodriguez, USDA, ARS) in a 96-well microtiter plate, four replicates per

Table 3
Challenge at 7 or 31 days postvaccination.

Vaccination formulation		Dose (volume)	Animal #	Day of challenge (after vaccination)	Clinical assessment				Fever
Antigen	Adjuvant				Day 0	Day 4	Day 7	Day 10	
1/4×	1/4×	0.5 ml	9119	31	0	0	0	0	No
			9120		0	0	0	0	No
			9121		0	0	0	0	No
1/16×	1/4×	0.5 ml	9116	31	0	0	0	0	No
			9117		0	0	0	0	No
			9118		0	0	0	0	No
Placebo	1/4×	0.5 ml	9122	31	0	4	4	4	Yes
			9123		0	4	4	4	Yes
			9124		0	4	4	4	Yes
1/4×	1/4×	0.5 ml	9128	7	0	0	0	0	No
			9129		0	0	0	0	No
			9130		0	0	0	0	No
1/16×	1/4×	0.5 ml	9125	7	0	0	1	1	No
			9126		0	0	0	0	No
			9127		0	4	4	4	No
Placebo	1/4×	0.5 ml	9131	7	0	4	4	4	Yes
			9132		0	4	4	4	Yes
			9133		0	4	4	4	Yes
PBS	PBS	0.5 ml	9134	–	0	4	4	4	Yes
			9135		0	4	4	4	Yes
			9136		0	4	4	4	Yes

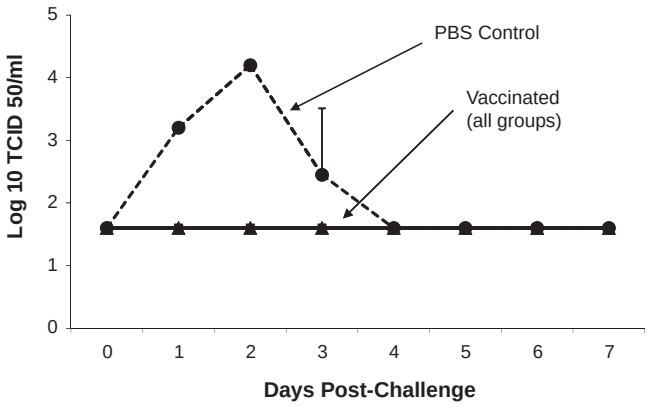

Fig. 1. Virus isolation from serum samples from zero to seven days post challenge is shown for Trial 1. PBS control animals (solid circle) showed viremia peaking on day 2 and no virus was detected by day 4 post challenge. Vaccinated animals are shown as solid squares Virus titers were established by determining the tissue culture infectious dose 50 ($TCID_{50}$). Averages +1 standard deviation (SD) are shown.

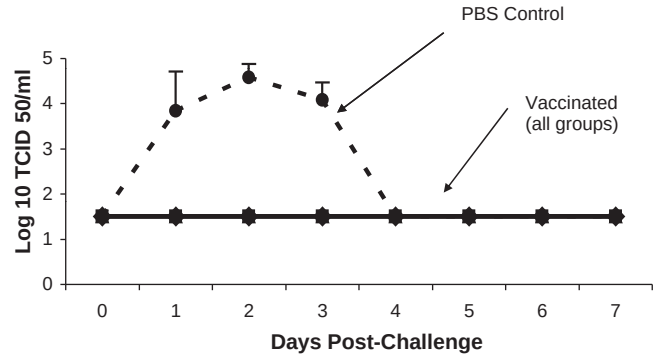

Fig. 2. Virus isolation from serum samples from zero to seven days post challenge is shown for Trial 2. PBS control animals (solid circle) showed viremia peaking on day 2 and no virus was detected by day 4 post challenge. Vaccinated animals are shown as solid squares, triangles and diamonds Virus titers were established by determining the tissue culture infectious dose 50 ($TCID_{50}$). Averages +1SD are shown.

dilution. Tissue culture plates were incubated at 37 °C for 48–52 h and monitored for CPE in order to calculate $TCID_{50}$ [12].

3. Results

3.1. Clinical assessments

In the first trial, all animals were housed in the same room and challenged on the same day, including two naïve cattle. All vaccinated animals were protected from infection after challenge at 28 days following vaccination with the exception of one cow. This animal received a 1/4 dose of viral antigen with a 1/4 dose of adjuvant and showed vesicles on 1 foot (#685, Table 1). There was no fever (Table 1) and no viremia (Fig. 1) exhibited by any of the vaccinated animals. Naïve cows showed fever 2–3 days following challenge and viremia that peaked on day 2, as expected (Fig. 1). Viremia and fever resolved by 5 days after challenge and vesicular lesions started to heal by day 10. It is noteworthy that Dermavac® delivery of FMDV vaccine with saponin adjuvant showed very mild adverse site reactions, and this site reaction was similar regardless of the dose tested. Any observed site inflammation resolved in 24–48 h.

A second trial was performed to determine if protection from disease can be induced as rapidly as the same vaccine in double oil emulsion as we previously reported [4]. In this trial, 3 groups of 7 cattle each were vaccinated 7 days prior to challenge with the needle free device. All animals were housed in the same room, and challenged the same day, including three naïve cattle. All of the cattle vaccinated with the quarter dose of antigen (of the recommended PD_{50}) were completely protected from clinical disease when assessed for clinical signs on day 4, 7 and 10 after challenge (Table 2). Two of the seven cattle in the 1X antigen group had vesicles after challenge, one with vesicles on a single hoof and one with vesicles on two feet, at day 4 post challenge. The rest of this cohort showed no clinical signs. These two protection failures could be a result of animal-to-animal variation in quality of antibody response, variations in the rate of decline of antibody for individual animals [13] or other variables in the humoral response of outbred cattle. Like many vaccines, this vaccine does not induce sterilizing immunity and commonly viral replication occurs, including at epithelial surfaces, causing lesions on the feet.

In the group of cattle vaccinated with 1/16× antigen load, one animal had vesicles on three feet by day 4 post challenge, and all four feet by day 10 post challenge. Again, the balance of this cohort, 6 cattle, showed no clinical signs. No vaccinated animals had fever or detectable viremia over the course of the trial, regardless of whether an animal had vesicles. As in the previous trial, naïve animals had vesicle formation on all 4 feet by day 4 post challenge. Fever was detected 1–2 days post challenge in all naïve animals (Table 2). As in the first trial, these naïve animals had detectable viremia, again peaking on day 2 and resolving by day 5 (Fig. 2).

The results in the second trial raised the possibility that protection at the early challenge time (day 7) may be mediated by a nonspecific innate response to the saponin adjuvant. Further, pathogen associated molecular patterns (PAMPs) [14] inherent in the virus could result in nonspecific stimulation via toll-like receptors (TLRs) or Nod-like receptors (NLRs). If so, such a response would have waned by weeks post-vaccination leaving the animals susceptible to infection. To test these possibilities, we conducted a third trial using 6 groups of 3 cattle each vaccinated with either 1/4× or 1/16× dose of viral antigen or with the saponin, aluminum hydroxide adjuvant alone (placebo vaccine). In addition, we challenged at both the early (day 7) or later (day 31) times following vaccination.

Cattle vaccinated 31 days prior to challenge with 1/16×, and 1/4× dose showed no signs of clinical disease. Cattle vaccinated at 7 days prior to challenge with 1/4× dose also showed no signs of clinical disease. However, 2 out of 3 cattle vaccinated with 1/16× dose 7 days prior to challenge, developed lesions on at least one foot by day 10 post challenge, i.e. reduced and delayed disease (Table 3). All antigen-vaccinated animals were free of fever regardless of antigen lode or day of challenge. As in the first two trials, no virus was detected in the serum on any day following challenge with analysis daily through day 10. Placebo groups challenged at either time point, as well as naïve cattle, had vesicle formation on all 4 feet by day 4 post challenge with elevated temperatures 1–2 days post challenge. All control and placebo animals also showed viremia that peaked on day 2 and resolved by day 5 following challenge (Fig. 3).

3.2. Neutralizing antibody responses

Virus neutralizing antibody titers are an indicator of protection from challenge [2,15]. All animals vaccinated with killed virus antigen showed measurable levels of anti-FMDV antibody detected by 7 days following vaccination. Titers of neutralizing antibody in serum were predictive of protection against FMDV (Fig. 4). Only a few animals developed signs of reduced and delayed disease and these all had equivalent titers to those animals protected from disease. As has been previously reported, there was increase in neutralizing antibody titer following challenge in all groups, including all vaccinated animals [3,4]. These data suggest that vaccinated animals were protected from disease but were not protected from infection. The boost in titer indicates the virus is likely to be systemic even

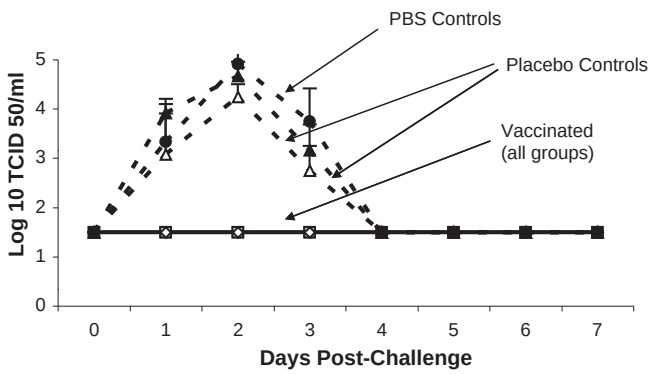

Fig. 3. Virus isolation from serum samples from zero to seven days post challenge is shown for Trial 3. PBS control animals (solid circle) and Placebo control animals (open and shaded triangles) showed viremia peaking on day 2 and no virus was detected by day 4 post challenge. Vaccinated animals are shown as solid squares and diamonds. Virus titers were established by determining the tissue culture infectious dose 50 ($TCID_{50}$). Averages +1SD are shown.

in vaccinated animals that had no detectable virus in blood samples taken daily after challenge. The lack of detection of viremia in these samples may be a result of vaccination reducing the duration of viremia from more than 24–48 h in naïve animals to less than 12–14 h. We do not anticipate it is a matter of sensitivity of the assay as real-time reverse transcriptase polymerase chain reaction (RT-PCR) for FMDV can detect 10 genome copies in a sample. When this assay was compared to the $TCID_{50}$ determination used here, Callahan, et al., found nearly 100% concordance between positive results of both assays [16].

4. Discussion

Outbreaks of FMDV in disease free countries continue to cause significant problems for livestock farmers and the economies of the effected regions. Developing tools to rapidly control such outbreaks, where the herds are completely susceptible to infection, is critical to minimizing slaughter of livestock and hardship to farmers. In addition, valuable breeding stock caught in an effected zone could be lost, as occurred in Japan in 2010. The spread of FMDV in naïve herds is remarkably rapid and a challenge to control [17].

In these studies we have tested the performance of the killed virus vaccine for FMDV using a rapid, needle free delivery system, the Dermavac®. The vaccine, in an aqueous preparation of aluminum hydroxide with the adjuvant saponin, was shown to be compatible with this delivery system, as cattle show minimal injection site reaction. This device can be loaded with a 25 ml syringe providing 50, 0.5 ml doses per refill of vaccine allowing animals to be vaccinated with much greater efficiency than using needle delivery. One important factor in the decision of whether or not to vaccinate in response to an outbreak of FMDV is the daunting logistics of deploying vaccine. Besides matching the vaccine strain to the strain of virus causing the present outbreak, recruiting professionals to administer vaccine and developing appropriate monitoring systems for tracking vaccinated animals is critical. The efficacy of a

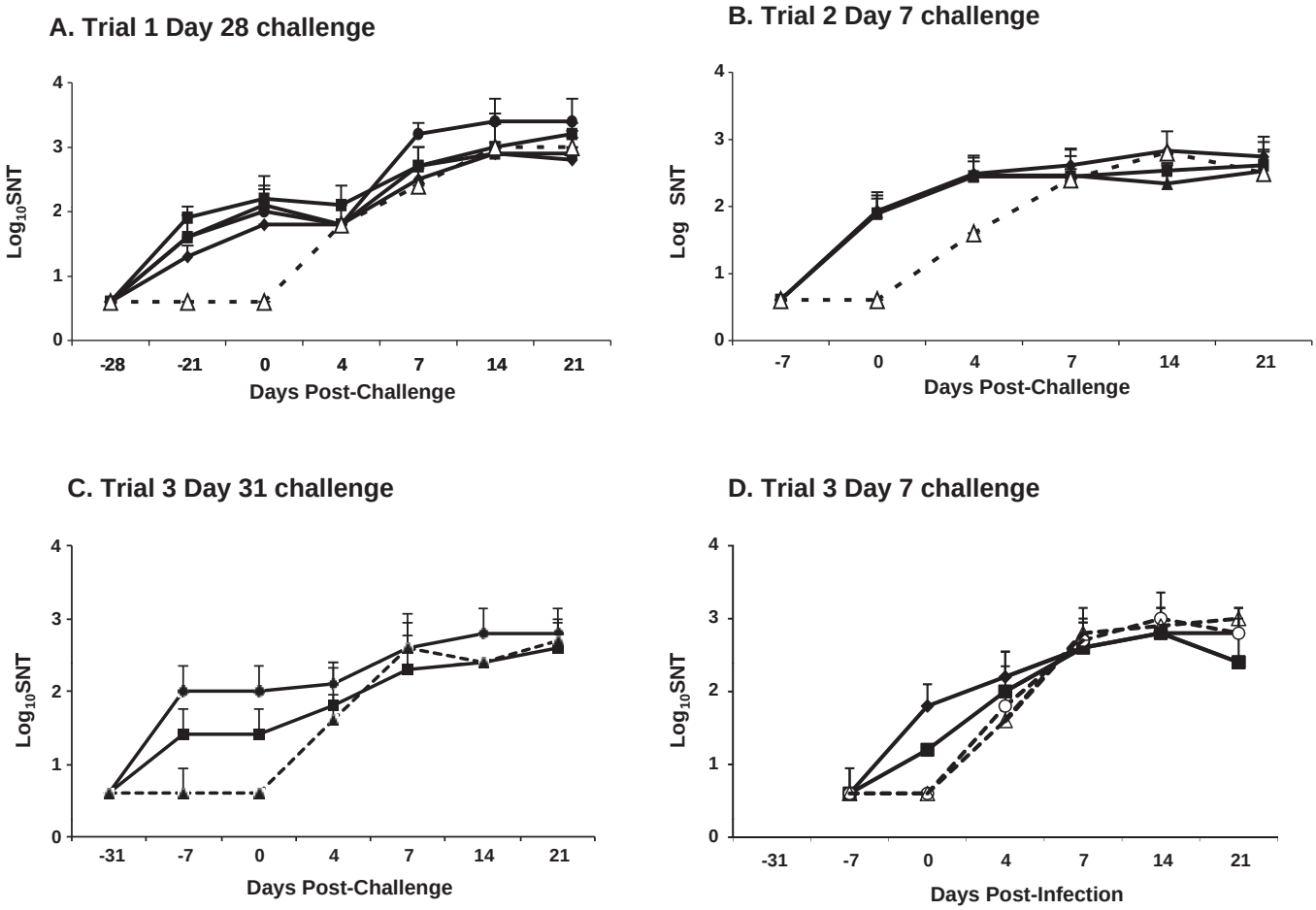

Fig. 4. Mean serum neutralizing antibody titers (SNT) +1SD are shown in cattle following vaccination and challenge with FMDV O Manisa. Groups of cattle were vaccinated at 28 (panel A), 7 (panels B and D), and 31 (panel C) days prior to challenge (solid lines), or not vaccinated (dashed lines).

rapidly applied, needle free delivery system such as the one tested here will enhance vaccination for FMDV.

Remarkably, the data we report here show that the vaccine, delivered intradermally by the device, has enhanced performance over the standard needle delivery of vaccine [4]. In all of the trials we report here, the vaccine protected against disease. The concentration of viral antigen can be reduced to 1/16 of the standard dose and still provide protection. Concentrations of anti-FMDV antibody that can neutralize virus in vitro were significant (more than 1 log) as early as 7 days following vaccination. This result was observed regardless of the concentration of viral antigen used in the vaccine. Further, efficacy was not dependent on concentration of the saponin adjuvant. The adjuvant alone (placebo with no viral antigen) conferred no protective effects, as placebo vaccinated animals were identical in disease assessment to naïve control animals. Fever and viremia were only detected in the placebo and naïve control animals following live virus challenge.

In the past two decades, formally FMD free countries have seen outbreaks that have raised awareness of the susceptibility of livestock and how rapidly the disease can spread [17,18]. In 1997, Taiwan suffered an outbreak that led to the island depopulating all pigs in order to re-attain FMDV free status [19]. The virus spread too quickly for vaccination to even be considered. In addition, the strain of FMDV causing the outbreak had a unique tropism for swine, with little infectivity in cattle [20]. In 2000 there were outbreaks in both South Korea and Japan for the first time in many years [21,22]. Again, slaughter was the method chosen to eliminate the disease and regain disease free status [21,23]. Then, in 2001, the United Kingdom suffered a large outbreak encompassing England, Scotland, Wales and even Northern Ireland [24]. Animals shipped from England before detecting the outbreak, were a source of disease in Greece, Italy, Ireland, France and the Netherlands. With the exception of the Netherlands, all countries slaughtered infected animals and all susceptible animals that were in contact with the virus. This process was very difficult as millions of animals were slaughtered and quarantine zones had a severe effect on economic activity far beyond the livestock industry. The Netherlands vaccinated and removed all vaccinated animals over time.

The decision to slaughter infected and exposed livestock was the policy of most of the governments involved for economic reasons. The OIE rules in place called for a period of 3 months of no new disease detected before export of animal products was allowed after quarantine and slaughter. If animals were vaccinated, that period was longer, 6 months. The UK outbreak led to a modification of OIE recommendations making the export waiting period 3 months after the last known case of FMDV even if animals were vaccinated to control disease [8]. This change still requires the eventual slaughter of all vaccinated animals. New parameters are being discussed following new outbreaks of FMDV in Japan in 2010 and South Korea in 2010 and 2011. In both cases, vaccine was deployed to help control disease spread. If data from experimental studies and these new outbreaks can confirm newly available tests accurately distinguish infected from vaccinated animals (DIVA), there is support for vaccination strategies to allow vaccinated animals that do not become infected or come in contact with infected animals, to live and be processed normally [25,26].

Data from this study gives the responsible officials more support for using vaccination to control disease outbreaks. The device described here will allow for rapid and safe vaccination of many animals compared to needle inoculation. In addition, vaccine resources can be expanded as the effective dose for vaccination intradermally can be lowered compared doses required for IM application. More studies with much larger numbers are required to confirm these results, but the data presented here provide a clear indication of the potential advantage of the ID vaccination device.

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Classical Swine Fever Virus p7 Protein Is a Viroporin Involved in Virulence in Swine

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The nonstructural protein p7 of classical swine fever virus (CSFV) is a small hydrophobic polypeptide with an apparent molecular mass of 6 to 7 kDa. The protein contains two hydrophobic stretches of amino acids interrupted by a short charged segment that are predicted to form transmembrane helices and a cytosolic loop, respectively. Using reverse genetics, partial in-frame deletions of p7 were deleterious for virus growth, demonstrating that CSFV p7 function is critical for virus production in cell cultures. A panel of recombinant mutant CSFVs was created using alanine scanning mutagenesis of the p7 gene harboring sequential three- to six-amino-acid residue substitutions spanning the entire protein. These recombinant viruses allowed the identification of the regions within p7 that are critical for virus production *in vitro*. *In vivo*, some of these viruses were partially or completely attenuated in swine relative to the highly virulent parental CSFV Brescia strain, indicating a significant role of p7 in CSFV virulence. Structure-function analyses in model membranes emulating the endoplasmic reticulum lipid composition confirmed that CSFV p7 is a pore-forming protein, and that pore-forming activity resides in the C-terminal transmembrane helix. Therefore, p7 is a viroporin which is clearly involved in the process of CSFV virulence in swine.

Classical swine fever (CSF) is a highly contagious often lethal disease of swine. The etiological agent, CSF virus (CSFV), is a small enveloped virus with a positive single-stranded RNA genome and, along with bovine viral diarrhea virus (BVDV) and border disease virus (BDV), is classified as a member of the genus *Pestivirus* within the family *Flaviviridae* (3). The 12.3-kb CSFV genome contains a single open reading frame that encodes a 3,898-amino-acid polyprotein that yields up to 12 final cleavage products (NH_2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) through co- and posttranslational processing of the polyprotein by cellular and viral proteases (20, 36).

In Pestiviruses the p7 gene encodes for a small hydrophobic polypeptide with an apparent molecular mass of 6 to 7 kDa (7) that has been regarded as an ion channel forming protein due to its similarities to hepatitis C virus (HCV) p7 protein. Initial reports suggest that in related BVDV p7 functions as an ion channel-forming protein (13). BVDV is sensitive to amantadine (13) and BIT225 (21), known inhibitors of HCV p7 ion channel function. Thus, it is hypothesized that CSFV p7, like HCV p7 and influenza virus M2 proteins, may function as a viroporin involved in pore formation and transport of ions across cell membranes (1, 11). Although there are similarities in the general protein structure between p7 from HCV and pestiviruses, the amino acid sequence identity between the proteins is <10% (Fig. 1). HCV p7 is also a small hydrophobic protein that consists of two putative transmembrane domains and a conserved basic cytosolic loop (5). HCV p7 has been shown to be essential for virus production but not for RNA replication (18) and possesses ion channel activity *in vitro* and establishes pores in liposomes (12, 34). The functions of p7 in the CSFV cycle are unknown, including its effect on virus replication or virulence in the natural host, and its role as a membrane channel-forming protein.

In the present study it was observed that the presence of p7 is essential for *in vitro* CSFV replication. *In vivo*, partial to complete attenuation of virulence was observed when pigs were inoculated with recombinant mutants, obtained by alanine scanning mutagenesis, harboring specific three to six amino acid residue substitutions within their native p7 amino acid sequence. The biochemical characterization of CSFV p7 using model membranes revealed that this protein possesses a robust membrane channel-forming capacity. Structure-function analyses of CSFV p7 using synthetic peptides showed that the pore-forming efficiency resides in the carboxyl half of the protein.

MATERIALS AND METHODS

Viruses and cells. Swine kidney cells (SK6) (35), free of BVDV, were cultured in Dulbecco minimal essential media (DMEM; Gibco, Grand Island, NY) with 10% fetal calf serum (FCS; Atlas Biologicals, Fort Collins, CO). CSFV Brescia strain was propagated in SK6 cells and used for the construction of an infectious cDNA clone (26). Growth kinetics were assessed using primary swine macrophage cell cultures prepared as described by Zsak et al. (40). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). After 4 days in culture, viral infectivity was assessed using an immunoperoxidase assay with the CSFV monoclonal antibody WH303 (MAb WH303) (6) and a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated according to the method of Reed and Muench

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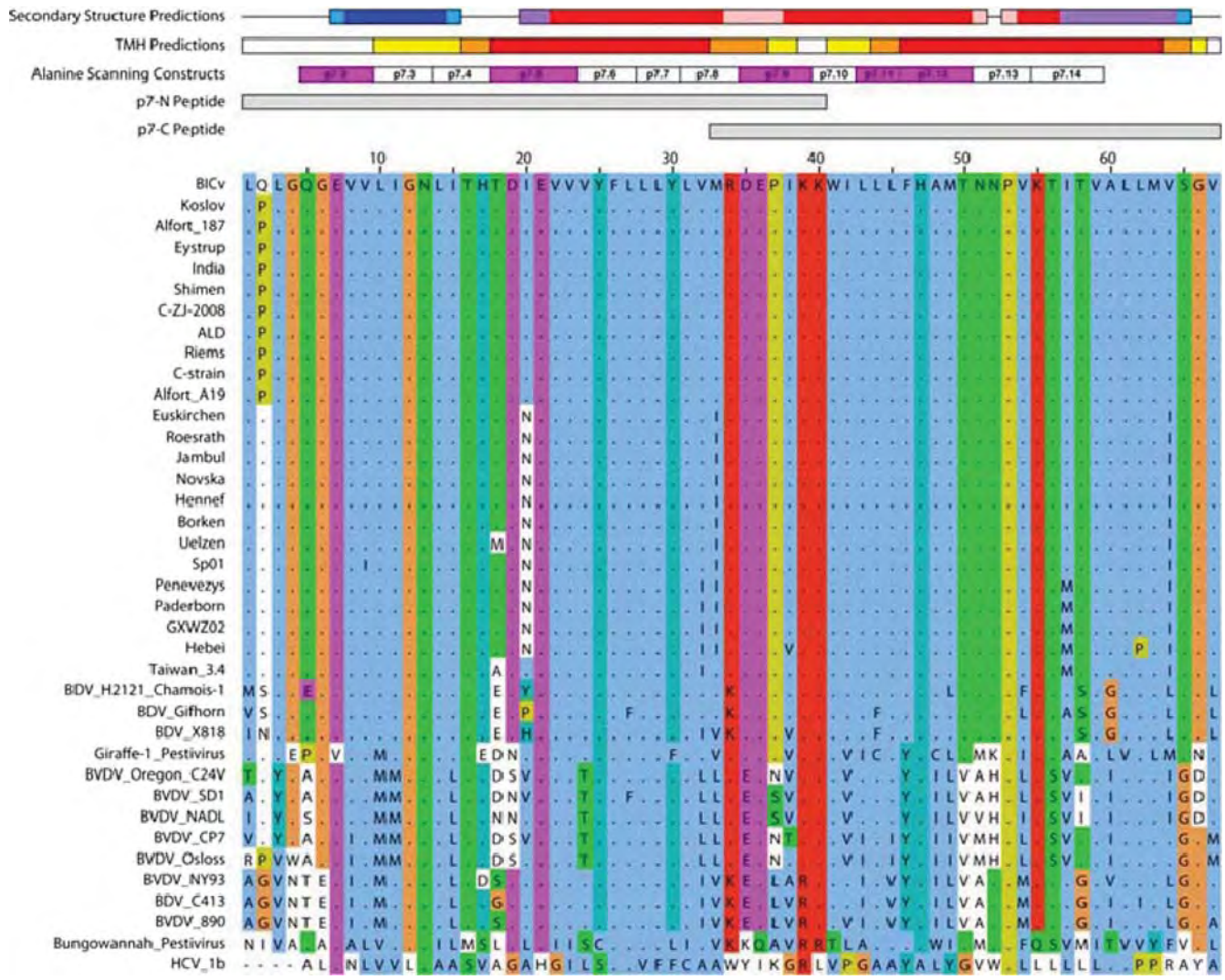

FIG 1 Annotated multiple sequence alignment of p7 homologs and predicted structural features. Residues conserved within CSFV isolates are shown as dots. The alignment is colored in the standard CLUSTAL coloring scheme. Above the multiple sequence alignment, the predicted secondary structure, the predicted transmembrane topology and alanine scanning data have been mapped to the alignment. (i) Secondary structure predictions. For areas in which PSI-PRED and SAM are in accord, helices are shown in red, strands are shown in dark blue, and coils are shown as lines. Where SAM and PSI-PRED secondary structure prediction conflict, light blue represents coil and strand predictions, light pink represents coil and helix predictions, and purple represents helix and sheet predictions. (ii) TMH predictions. A summary of the predictions from three transmembrane helix prediction algorithms—TMHMM, Spoctopus, and Memsat—is shown. Areas in which all three algorithms agree are shown in red, areas in which two agree are shown in orange, and areas in which only one algorithm predicts a TMH are shown in yellow. (iii) Alanine scanning constructs. Alanine constructs that resulted in nonviable viruses are indicated in violet.

(25) and expressed as 50% tissue culture infective dose(s) ($TCID_{50}$)/ml. The test sensitivity was $\geq \log_{10}$ 1.8 $TCID_{50}$/ml.

Construction of CSFV mutants. A full-length infectious clone (IC) of the virulent Brescia strain (pBIC) (4, 26) was used as a template to obtain all cDNA IC constructs described in this report. Constructs containing in-frame partial deletions of p7 were produced using a primer mutagenesis program (Stratagene, Cedar Creek, TX). Designed constructs of p7 have deleted regions encoding amino acids 15 to 51 (p7Δ15-51), 10 to 32 (p7Δ10-32), and 33 to 51 (p7Δ33-51). IC cDNA for p7Δ15-51, p7Δ10-32, and p7Δ33-51 constructs were obtained by using the QuikChange XL site-directed mutagenesis kit (Stratagene) performed according to the manufacturer's instructions with full-length pBIC as a template. The product was then digested with DpnI, leaving only the newly amplified plasmid, transformed into XL10-Gold ultracompetent cells, and grown on Terrific broth agar plates with ampicillin (Teknova). Positive colonies were selected for by sequence analysis of the p7 gene and grown for plasmid purification using a Maxiprep kit (Qiagen Sciences, MD). Each of the IC constructs was completely sequenced to verify that only site-directed mutagenesis-induced changes were present.

A library of mutated p7 genes was developed where codons encoding for three to six residue stretches of native p7 amino acid sequence were substituted with alanine codons (Fig. 1). Full-length pBIC was used as a template in which amino acids were substituted with alanine, introduced by site-directed mutagenesis using the QuikChange XL site-directed mutagenesis kit performed according to the manufacturer's instructions in the same manner as described above for the p7 deletion constructs. Primers were designed using the Stratagene primer mutagenesis program.

***In vitro* rescue of CSFV Brescia and p7 mutants.** Full-length genomic clones were linearized with SrfI and *in vitro* transcribed using the T7 Megascript system (Ambion, Austin, TX). RNA was precipitated with LiCl and transfected into SK6 cells by electroporation at 500 V, 720 Ω, and

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100 W with a 630 electroporator (BTX, San Diego, CA). Cells were seeded in 12-well plates and incubated for 4 days at 37°C in 5% CO_2. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at −70°C.

Amantadine and verapamil studies. SK6 cells were grown in a six-well plates up to a density of 80% confluence and treated with the indicated concentrations (see Fig. 4) of either drug for 30 min. Cells were then infected with BICv (multiplicity of infection [MOI] = 0.1) for 1 h, washed twice with phosphate-buffered saline (PBS), and then incubated in growth medium for 72 h. Viral progeny yields were determined in samples taken at 48 and 72 h postinfection. Each point represents the mean $\log_{10}$ $TCID_{50}$/ml and standard deviations from two independent experiments. The sensitivity of virus detection was $\geq\log_{10}$ 1.8 $TCID_{50}$/ml.

The toxic effect of amantadine or verapamil on treated SK6 cells was assessed with a colorimetric cell viability assay based on the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. SK6 cells were treated with different concentrations of amantadine or verapamil for 72 h and then exposed to MTT (5 mg/ml in PBS) (Promega, Madison, WI) for 1 h. The reduction of MTT was determined by measuring the absorbance as the optical density at 540 nm (OD_{540}). Cell viability was determined as follows: (OD_{540} untreated/OD_{540} treated) × 100.

Western blot analysis. Expression of glycoprotein E2 by BICv and the mutant viruses was analyzed in lysates of SK6-infected cells by Western immunoblotting. CSFV E2 was detected with MAb WH303. SK6 monolayers were infected (MOI = 1) with BICv or mutants, harvested at 48 h postinoculation (hpi) using the NuPAGE LDS sample buffer system (Invitrogen), and incubated at 70°C for 10 min. Samples were run under reducing conditions in precast NuPAGE 12% bis-Tris acrylamide gels (Invitrogen). Western immunoblots were performed using the Western-Breeze chemiluminescent immunodetection system (Invitrogen).

Detection of viral RNA. Total RNA was isolated from infected SK6 cell using RNeasy (Qiagen, Valencia, CA). The real-time PCR assay was designed as a probe hydrolysis (TaqMan)/reverse transcription-PCR (RT-PCR) single-tube assay (Tetracore, Rockville, MD). Specific oligonucleotide primers and the fluorogenic probe target a highly conserved region within the 5′ untranslated region of the CSFV genome (GenBank accession number NC_000294.1). The TaqMan probe was labeled with a 5′ reporter dye, 6-carboxyfluorescein, and a 3′ quencher, 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethyl rhodamine (TAMRA; PE Biosystems, Foster City, CA). RT-PCRs were performed as described by the manufacturer (Tetracore).

DNA sequencing and analysis. Full-length clones and *in vitro*-rescued viruses were completely sequenced with CSFV-specific primers by the dideoxynucleotide chain-termination method (30). Viruses recovered from infected animals were sequenced in the region of the genome that contained the desired mutations. Sequencing reactions were prepared with a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a Prism 3730xl automated DNA sequencer (Applied Biosystems). Sequence data were assembled using Sequencher 4.7 software (Genes Codes Corp., Ann Arbor, MI). The final DNA consensus sequence represented, on average, a 3- or 4-fold redundancy at each base position.

Animal infections. Virulence of p7 mutant viruses relative to BICv was initially assessed in 10 to 12 weeks old, forty-pound commercial-breed pigs inoculated intranasally (i.n.) with 10^5 $TCID_{50}$ of each virus. Pigs were randomly allocated into nine groups of four animals each and were inoculated with a p7 virus mutant or BICv. Clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea, and cough) and changes in body temperature were recorded daily throughout the experiment and scored as previously described (23). Blood was collected at various times postinfection from the anterior vena cava into EDTA-containing tubes (Vacutainer) for total and differential white blood cell counts (performed using a Beckman Coulter ACT [Beckman Coulter, CA]) and quantification of viremia by virus titration as described above.

For infection-challenge studies, 12 pigs were randomly allocated into two groups containing five animals each and a third group containing two animals. Pigs in groups 1 and 2 were i.n. inoculated with 10^5 $TCID_{50}$ of p7.10 virus, and pigs in group 3 were mock infected. At 3 days postinoculation (dpi) (group 1) or 28 dpi (group 2), animals were challenged with 10^5 $TCID_{50}$ of BICv along with animals in group 3. Clinical signs and body temperature were recorded daily throughout the experiment as described above. Blood samples were collected at times postchallenge for blood cell counts and quantification of viremia as described previously.

TABLE 1 Detection of viral genomic RNA using real-time RT-PCR in extracts from SK6 cells transfected with p7 mutant constructs that do not yield viral progeny

p7 mutant RNA construct	C_T value	Viral RNA detection
p7.2	21.53	+
p7.5	21.98	+
p7.9	17.97	+
p7.11	18.35	+
p7.12	22.98	+
p7Δ10-32	16.41	+
p7Δ15-51	0.00	–
p7Δ35-51	0.00	–
BICv	20.71	+

Synthetic peptides. The CSFV p7 Brescia strain protein and its derived p7-N and p7-C peptides (sequences displayed in Fig. 1) were commercially synthesized (Thermo Scientific). The purified peptides were dissolved in dimethyl sulfoxide (DMSO; spectroscopy grade), and their concentrations were determined by bicinchoninic acid microassay (Pierce, Rockford, IL). Small, diluted aliquots (typically, 20 μl [1 mg/ml]) were stored frozen and were thawed only once, upon use.

CD studies. Circular dichroism (CD) measurements were obtained from a thermally controlled Jasco J-810 CD spectropolarimeter calibrated routinely with (1*S*)-(+)-10-camphorsulfonic acid, ammonium salt. Samples consisted of lyophilized peptides dissolved at concentrations of 0.03 mM in 2 mM HEPES (pH 7.4) buffer containing 50% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). Spectra were measured in a 1-mm path-length quartz cell initially equilibrated at 25°C. The data were taken with a 1-nm bandwidth at 100 nm/min speed, and the results of 20 scans were averaged.

Pore formation in lipid vesicles. Large unilamellar vesicle(s) (LUV) were prepared according to the extrusion method (15). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS) were purchased from Avanti-Polar Lipids (Birmingham, AL). The 8-aminonaphthalene-1,3,6-trisulfonic acid sodium salt (ANTS) and *p*-xylenebis(pyridinium) bromide (DPX) were obtained from Molecular Probes (Junction City, OR). Lipid mixtures in organic solvent were dried under an N_2 stream. Traces of organic solvent were removed by vacuum pumping. Subsequently, dried lipid films were dispersed in buffer and subjected to 10 freeze-thaw cycles before extruding them 10 times through two stacked polycarbonate membranes with a nominal pore size of 0.1 μm (Nuclepore, Inc., Pleasanton, CA). Vesicle permeabilization was assayed by monitoring the release to the medium of encapsulated fluorescent ANTS (ANTS-DPX assay) (8). LUV containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, and 5 mM HEPES were obtained by separating the unencapsulated material by gel-filtration in a Sephadex G-75 column that was eluted with 5 mM HEPES and 100 mM NaCl (pH 7.4). Internal and external osmolarities were measured in a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany) and adjusted by adding NaCl. Fluorescence measurements (intensity versus time) were performed in an SLM Aminco 8100 spectrofluorimeter (Spectronic Instruments, Rochester, NY) by setting the ANTS emission at 520 nm and the excitation at 355 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. The baseline leakage (0%) corresponded to the fluores-

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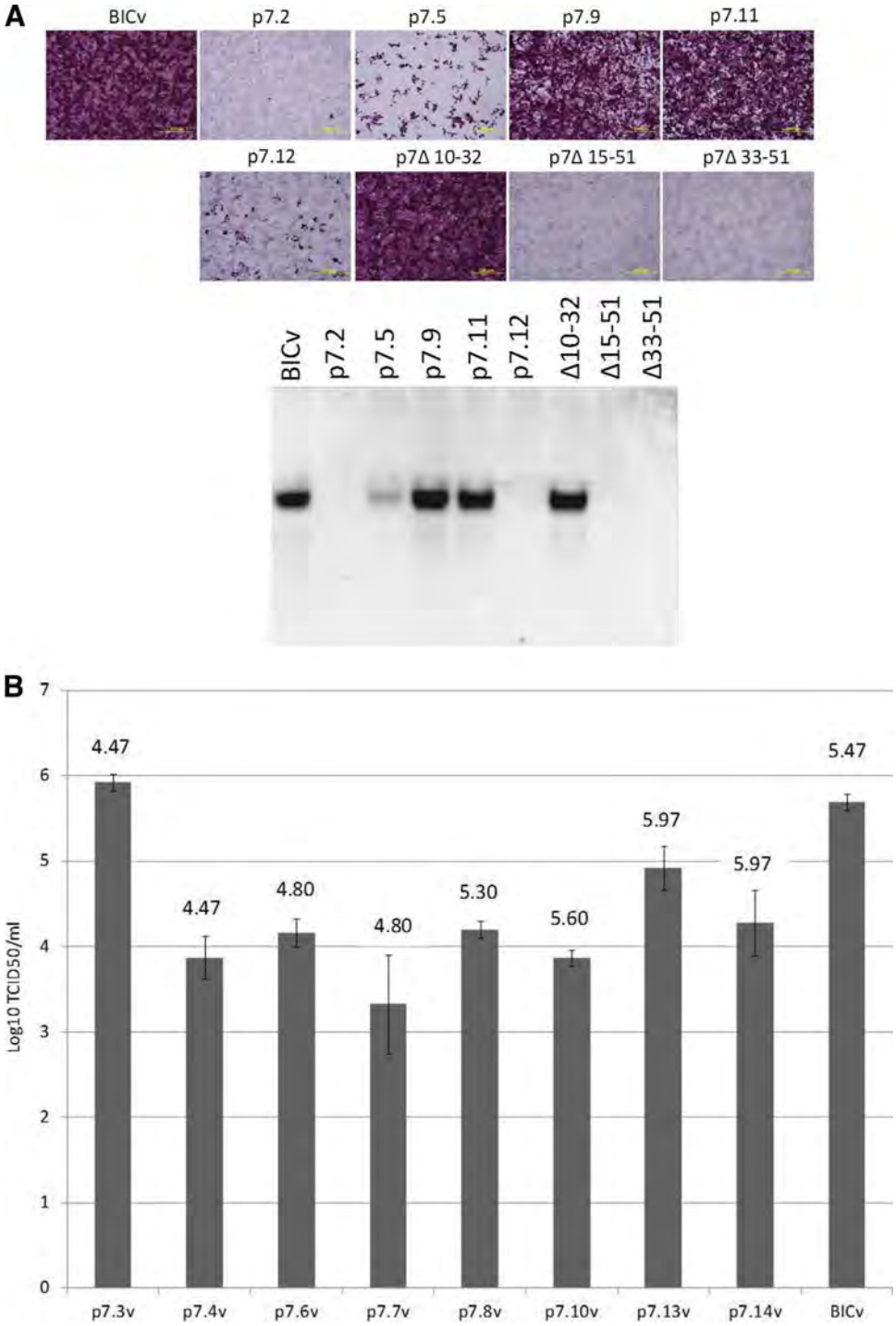

FIG 2 Virus protein expression after transfection and *in vitro* growth characteristics of p7 mutants and parental BICv. (A) Detection of CSFV E2 protein by immunocytochemistry (top panel) and Western blotting (bottom panel) in SK6 cells transfected with recombinant construct that do not yield virus progeny. (B) Primary swine macrophage cell cultures were infected (MOI = 0.01) with each of the mutants or parental BICv, and the virus yield was titrated at 72 h postinfection in SK6 cells. The data represent means and standard deviations from two independent experiments. The sensitivity of virus detection was $\geq \log_{10}$ 1.8 $TCID_{50}$/ml. Values shown above the bars represent the virus yield ($\log_{10}$ $TCID_{50}$) detected 4 days after transfection of SK6 cells with the corresponding *in vitro*-transcribed RNA.

cence of the vesicles at time zero, while 100% leakage was the fluorescence value obtained after addition of Triton X-100 (0.5% [vol/vol]).

Bioinformatic and structural predictions. Secondary structure predictions were made by SAM and PSI-PRED (19, 22). Transmembrane helix topology predictions were made using TMHMM, Memsat, and Spoctopus (22, 31, 39). Tertiary predictions were performed using RosettaMembrane (2). Multiple folding trajectories were performed for CSFV p7 of the Brescia strain (BICv), and homologs and the resulting models were clustered with the lowest energy BICv p7 member of the largest cluster selected for further analysis.

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RESULTS

Comparison of CSFV p7 amino acid sequence with those of other pestiviruses and HCV. CSFV p7 is a small hydrophobic protein comprised of 67 amino acid residues. Comparison of its amino acid sequence among different CSFV isolates reveals more than 89.5% identity among isolates (Fig. 1). Compared to other pestiviruses, the amino acid identity ranges between 50 and 55% relative to BVDV isolates and between 50 to 70% relative to BDV isolates. The protein homolog in HCV is also a small hydrophobic protein, although amino acid identities between CSFV and HCV p7 proteins are typically <10%. HCV p7 largely comprises of two predicted transmembrane alpha helices joined through a fully conserved basic loop (Fig. 1). Despite a low degree of amino acid sequence identity, CSFV p7 presents a general structure similar to that of HCV p7.

p7 is essential for CSFV production. To assess the importance of the integrity of p7 in the development of infectious particles, infectious clone cDNA constructs containing in-frame partial deletions of p7 were designed in the context of a full-length cDNA clone of CSFV Brescia strain (26). Three different in-frame partial deletions were designed involving central areas of p7 (Δ10-32, Δ15-51, and Δ33-51), while leaving the possible cleavage sites on both ends of the protein unaltered (residues 1 to 9 and residues 52 to 67, respectively). Infectious RNA was *in vitro* transcribed from each of the cDNA constructs and was subsequently used to transfect SK6 cells. Transfections using any of these p7 partial deletion constructs were invariably negative (after three independent events) in terms of recovering infectious particles (data not shown).

Construction of CSF p7 mutant viruses. To assess the importance of p7 for virus production, a series of recombinant CSFVs containing mutations in p7 were designed using the cDNA infectious clone of the Brescia strain (BICv) as a template. A total of 14 cDNA constructs containing sequential areas of three to six amino acid residues in the native p7 amino acid sequence substituted by alanine residues were constructed (Fig. 1). Infectious RNA was *in vitro* transcribed from each mutated full-length cDNA and used to transfect SK6 cells. Infectious virus was rescued from transfected cells by day 4 posttransfection using constructs p7.3, p7.4, p7.6, p7.7, p7.8, p7.10, p7.13, and p7.14 (depicted as white blocks in Fig. 1). In contrast, after three independent transfection procedures, p7.2, p7.5, p7.9, p7.11, and p7.12 constructs did not produce infectious viruses (depicted as violet blocks in Fig. 1). Real-time RT-PCR analysis of total RNA extracted from these cells and cells transfected with p7Δ10-32, p7Δ15-51, and p7Δ33-51 constructs revealed genomic RNA replication, except for the p7Δ15-51 and p7Δ33-51 constructs (Table 1). In addition, immunohistochemistry and Western blot analysis (Fig. 2A) of transfected cell extracts showed comparable results showing a differential expression of structural glycoprotein E2. As expected, p7Δ15-51 and p7Δ33-51 failed to produce any detectable levels of E2. p7.2-transfected cells present a very low level of expression of E2 only detectable by immunocytochemistry, whereas the levels of E2 in p7.5- and p7.12-transfected cells are intermediate relative to the levels observed in p7.9, p7.11, p7Δ10-32, and BICv cell extracts. Partial nucleotide sequencing of the rescued p7 mutated viruses was performed to ensure the presence of the predicted mutations.

Growth of the CSFV p7 mutants *in vitro*. *In vitro* growth characteristics of the p7 mutant viruses relative to parental BICv were evaluated in a single-step growth curve. Primary swine macrophage cell cultures were infected at an MOI of 0.01 $TCID_{50}$ per cell. Viruses were adsorbed for 1 h (time zero), and samples were collected at 72 h postinfection and titrated in SK6 cell cultures. With the exception of p7.3v, all mutant viruses exhibited a reduction (between 1 to 2.5 $\log_{10}$, depending on the virus considered) in their virus titers compared to that of the parental BICv (Fig. 2). This reduction varies among the mutants, being most drastic for viruses p7.4, p7.7, and p7.10. Each exhibited a reduction in their titers by approximately 2 to 2.5 $\log_{10}$ compared to the parental BICv.

TABLE 2 Swine survival and fever response after infection with p7 CSFV mutant viruses and parental BICv

Virus	No. of survivors (n = 4/virus)	Mean time to death (days ± SD)	Fever (days ± SD)	
			Time to onset	Duration
p7.3	0	13.67 ± 3.51	5 ± 0	9.32 ± 3.06
p7.4	4		5 ± 0	2 ± 0
p7.6	0	11.34 ± 4.95	5.5 ± 0.58	5.5 ± 4.95
p7.7	0	14 ± 0	5.67 ± 0	5 ± 0
p7.8	4		2 ± 1.72	5.67 ± 0.58
p7.10	4			
p7.13	0	12.67 ± 3.79	4 ± 0	7.32 ± 1.52
p7.14	3	11[a]	5 ± 0	3 ± 2.65
BICv	0	11.25 ± 0.96	3.75 ± 0.5	6.75 ± 0.96

[a] The data here correspond to the only animal that did not survive the infection.

Virulence of CSFV p7 mutants *in vivo*. To examine whether alterations of different regions of p7 affect virulence, different groups of pigs were i.n. inoculated with ~10^5 $TCID_{50}$ of each of the p7 mutant viruses (p7.3, p7.4, p7.6, p7.7, p7.8, p7.10, p7.13, and p7.14) and monitored for clinical disease, evaluated relative to parental BICv (Table 2). All animals infected with BICv presented clinical signs of CSF starting 3 to 5 dpi, developing classical symptoms of the disease and dying around 11 to 12 dpi. Total white blood cells, lymphocytes, and platelet counts dropped by 4 to 6 dpi in animals inoculated with BICv and continued declining until death (Fig. 3A, B, and C). Viruses p7.3, p7.6, p7.7, and p7.13 presented a virulence phenotype almost indistinguishable from that of the parental BICv (Table 1). All animals infected with these viruses presented clinical signs of CSF starting at 4 to 5 dpi, with clinical presentation and severity similar to those observed in animals inoculated with BICv. White blood cells, lymphocytes, and platelet counts dropped by 4 dpi and continued declining until death (Fig. 3A, B, and C) at 11 to 14 dpi. Animals infected with viruses p7.4 or p7.8 presented a transient (during 2 and 5 days, respectively) rise in body temperature accompanied by mild depression and anorexia, returning to clinical norms by 9 to 10 dpi (Table 2). A drop in lymphocytes and platelet counts was also transient accompanying the presence of clinical signs (Fig. 3A, B, and C). Animals inoculated with p7.14 virus presented disease-related symptoms by 5 dpi. Interestingly, disease progressed in one of the animals dying at 11 dpi, while the others clinically recuperated after a 2- to 4-day period of transient rise in body temperature, depression, and anorexia (Table 2). White blood cells, lymphocytes, and platelet counts decreased in all animals in this group, remaining low in the animal dying at 11 dpi and reaching intermediate values in the surviving animals by the end of the

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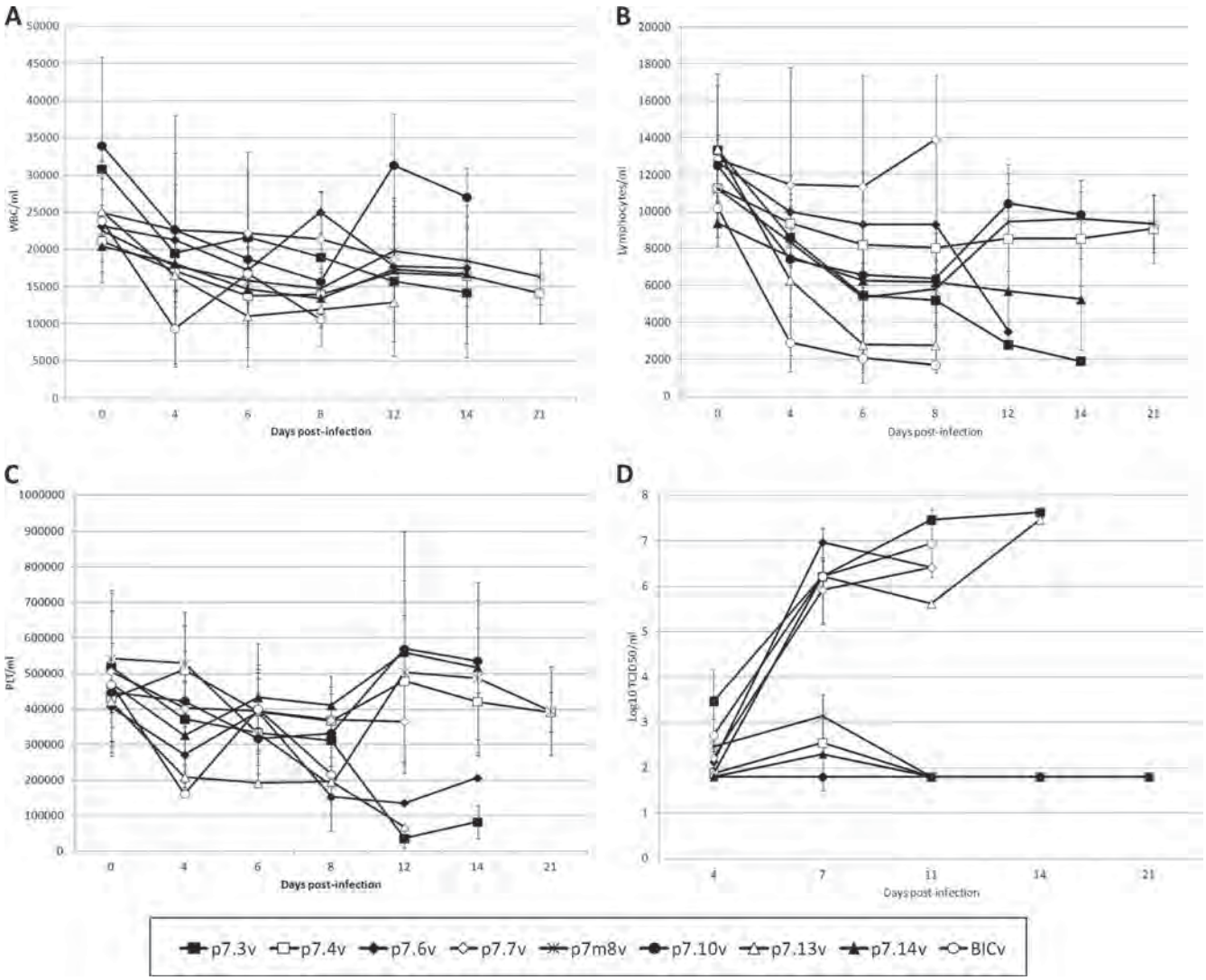

FIG 3 Hematologic changes (A, B, and C) and viremia titers (D) detected in pigs inoculated with p7 mutant viruses or parental BICv. Animals were intranasally infected with 10^5 $TCID_{50}$ of each of the p7 mutant viruses or BICv. Each point represents the mean $\log_{10}$ $TCID_{50}$/ml and standard deviations from at least three animals. The sensitivity of virus detection was $\geq\log_{10}$ 1.8 $TCID_{50}$/ml. White blood cell (WBC [A]), lymphocyte (lymph [B]), and platelet (PLT [C]) counts were determined during the infection.

experimental period (Fig. 3A, B, and C). Finally, animals inoculated with virus p7.10 survived the infection and remained clinically normal throughout the observation period (21 days). In these animals there was a transient decrease in all of the hematological values by 6 dpi, with values returning to normal by 12 dpi (Table 2 and Fig. 3A).

Viremia in animals inoculated with p7 mutants varied and in general accompanied the evolution of the clinical disease (Fig. 3D). Animals inoculated with virulent viruses p7.3, p7.6, p7.7, or p7.13 exhibited viremia kinetics almost undistinguishable from that induced by parental BIC virus, presenting high titers that remained until death of the animal. Viruses p7.4, p7.8, and p7.14 induced a mild disease and transient viremia, with virus titers significantly lower than those found in animals inoculated with parental BIC virus. Finally, no virus could be detected at any sample point in blood from animals infected with the p7.10 virus,

TABLE 3 Swine survival, fever response, and viremia in pigs infected with p7.10v and challenged with parental BICv

Challenge, time administered	No. of survivors/ total no.	Mean time to death (days ± SD)	Fever (days ± SD)		Viremia (avg titer at 5 dpc ($TCID_{50}$/ml ± SD)
			Time to onset	Duration	
p7.10v, 3 dpi	3/5	11 ± 1.41; –[a]	3.2 ± 0.45; 3 ± 0[a]	7.5 ± 1.41; 2.67 ± 0.58[a]	5.89 ± 1.2; 2.85 ± 0.95[a]
p7.10v, 28 dpi	5/5				≤1.8
Mock	0/2	10 ± 2.12	3.5 ± 1.41	7.5 ± 0.71	5.97

[a] The first value corresponds to the two animals that did not survive challenge; the second value corresponds to the three animals that survived challenge.

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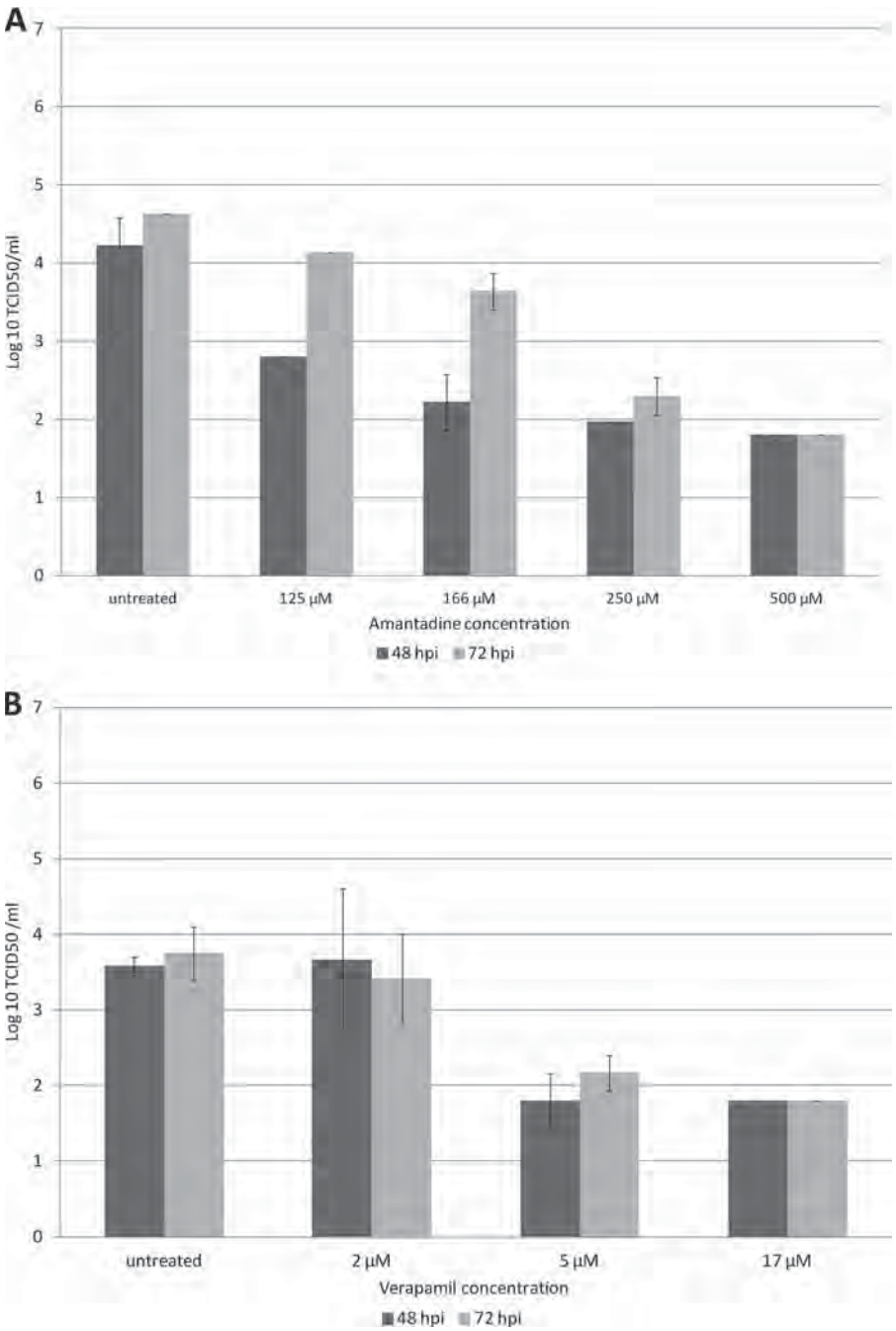

FIG 4 Effect of amantadine (A) and verapamil (B) treatment on SK6 cell cultures infected with CSFV. SK6 cell cultures were treated with either drug for 30 min and then infected with BICv (MOI = 0.01), and the virus yield was assessed at 48 and 72 h postinfection. Each point represents the mean $\log_{10}$ $TCID_{50}$/ml and standard deviations from two independent experiments. The sensitivity of virus detection was $\geq\log_{10}$ 1.8 $TCID_{50}$/ml.

indicating that this virus developed a very mild infection when inoculated i.n.

p7.10 mutant virus infection protects pigs against lethal CSFV challenge. The limited *in vivo* replication kinetics of p7.10 virus is similar to that observed with CSICv, a CSFV vaccine strain (26). However, restricted *in vivo* replication could also impair protection against wild-type virus infection. Thus, the ability of p7.10 virus to induce protection against virulent BICv was assessed in early and late vaccination-exposure experiments. Groups of pigs (n = 5) were i.n. inoculated with 10^5 $TCID_{50}$ of p7.10 virus and i.n. challenged with 10^5 $TCID_{50}$ of parental BICv at either 3 or 28 dpi. Mock-vaccinated control pigs receiving BICv only (n = 2) developed anorexia, depression, and fever by 4 days postchallenge (dpc), as well as a marked reduction in circulating total leukocytes, lymphocytes, and platelets by 4 dpc (data not shown), and died or were euthanized *in extremis* by 10 dpc after showing high viremia titers since dpc 4 (Table 3). Animals challenged at 3 dpi presented a heterogeneous behavior: two of them showed a clinical disease,

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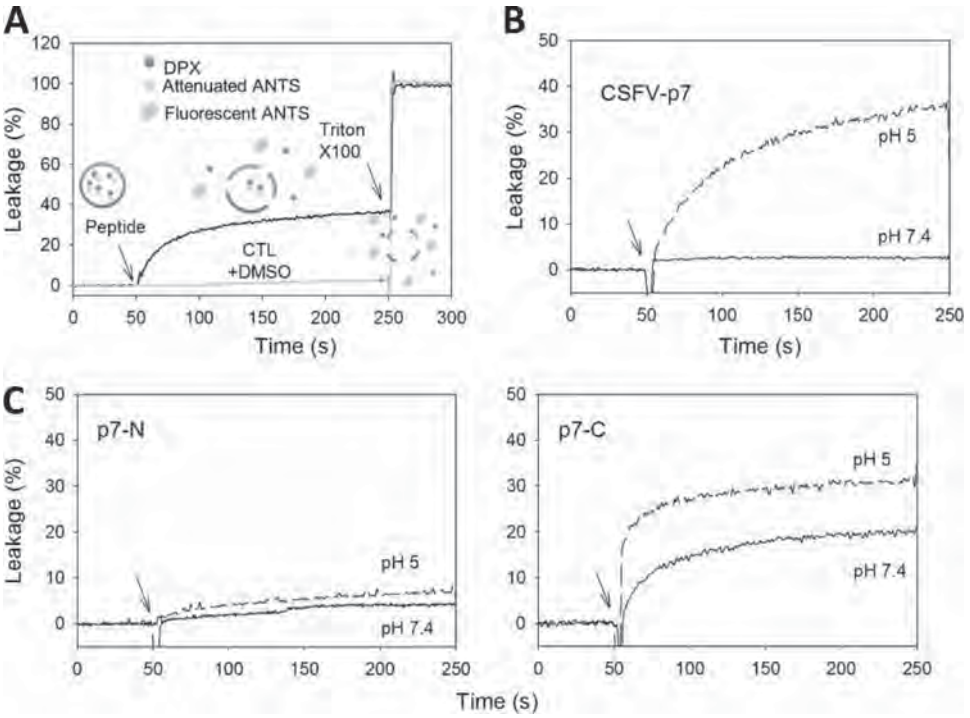

FIG 5 Pore-forming activity of BICv p7 protein and derived peptides as detected by the ANTS-DPX assay. (A) Schematics of the ANTS-DPX assay (see the text for the explanation). (B) Permeabilization induced by BICv p7 protein of large unilamellar vesicles composed of the main ER phospholipids PC-PE-PI (5:3:2 molar ratio). Leakage of vesicular internal aqueous contents is shown as a function of time. At the time indicated by the arrow, the protein was injected into a stirring cuvette. The protein/lipid molar ratio was 1:100. The lipid concentration was 100 μM. (C) Permeabilization induced by p7-N and p7-C (left and right panels, respectively) of ER LUV. Conditions were as described in the previous panel. Assays were performed at pH 7.4 or 5.0 as indicated in the panels.

hematological values, and viremia titers indistinguishable from mock-vaccinated animals (Table 3). Conversely, the other three animals in the group became clinically and hematologically normal after a transient disease by 5 dpc (Table 3). Infection with p7.10 virus induced complete protection by 28 dpi. All pigs survived the BICv challenge and remained clinically normal (Table 3) without significant changes in their hematological values (data not shown). Thus, even though p7.10 virus exhibited a limited *in vivo* growth, a solid protection was induced after vaccination.

Effect of treatment with amantadine or verapamil in cell cultures infected with CSFV. As demonstrated earlier, the integrity of p7 is critical to the process of CSFV replication. To gain insight into the possible mechanism mediating this role, the effects of amantadine and verapamil on CSFV replication were evaluated. These drugs have both demonstrated the ability to inhibit membrane permeation induced by HCV p7 viroporin (12, 13, 34). The effect of both drugs on the replication of BICv was analyzed using SK6 cell cultures. SK6 cell cultures were pretreated with 125 to 500 μM amantadine or 2 to 17 μM verapamil or were mock treated for 30 min before infection with BICv. The cells were incubated with CSFV Brescia strain at an MOI of 0.01 for 1 h at 37°C, washed with PBS, and incubated at 37°C in DMEM–10% FCS. Samples were taken for virus titration at 48 and 72 h. Treatment with either drug effectively decreased virus yields. Using the highest concentration of either drug produced an ~100-fold reduction in virus yield compared to the mock-treated cultures (Fig. 4).

In addition, it was determined that amantadine and verapamil had no significant cytotoxic effect on SK6 cells at the concentration used here. Toxicity was determined by reduction of MTT by SK6 cells exposed to these drugs for 72 h. The results showed that cells treated with amantadine (500 μM) or verapamil (17 μM) presented viabilities of 105% ± 10.7% and 87.02% ± 3.1%, respectively, relative to mock-treated cells. These results clearly indicate that the decrease in virus titers observed is not due to a cytotoxic effect mediated by these drugs.

Pore-forming activity and structural characterization of CSFV p7 and derived peptides p7-N and p7-C. Structure-function analyses of CSFV p7 as a prospective viroporin were next carried out using the synthetic BICv p7 protein sequence and its derived peptides, p7-N and p7-C. As displayed in the top diagrams of Fig. 1, p7-N and p7-C sequences spanned the potential N- and C-terminal transmembrane regions of BICv p7, respectively, and overlapped along the conserved polar loop sequence.

CSFV p7 is predicted to insert into the endoplasmic reticulum (ER) membrane (20). Therefore, the pore-forming activity of these synthetic sequences was assessed in lipid vesicles that emulated the ER membrane (Fig. 5). The lipid bilayer surrounding this organelle is essentially symmetric (both monolayers with equal lipid composition) and mostly devoid of sterols (37). Thus, the lipid composition of the liposomes was based on the main constituent phospholipids of the ER membrane, namely, zwitterionic PC and PE plus the anionic PI mixed in a roughly 5:3:2 molar ratio (37).

Permeabilization to low-molecular-weight markers was monitored by the ANTS-DPX assay (8, 17), which is based on the quenching of 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS; molecular weight, 427) fluorescence by *p*-xylenebis

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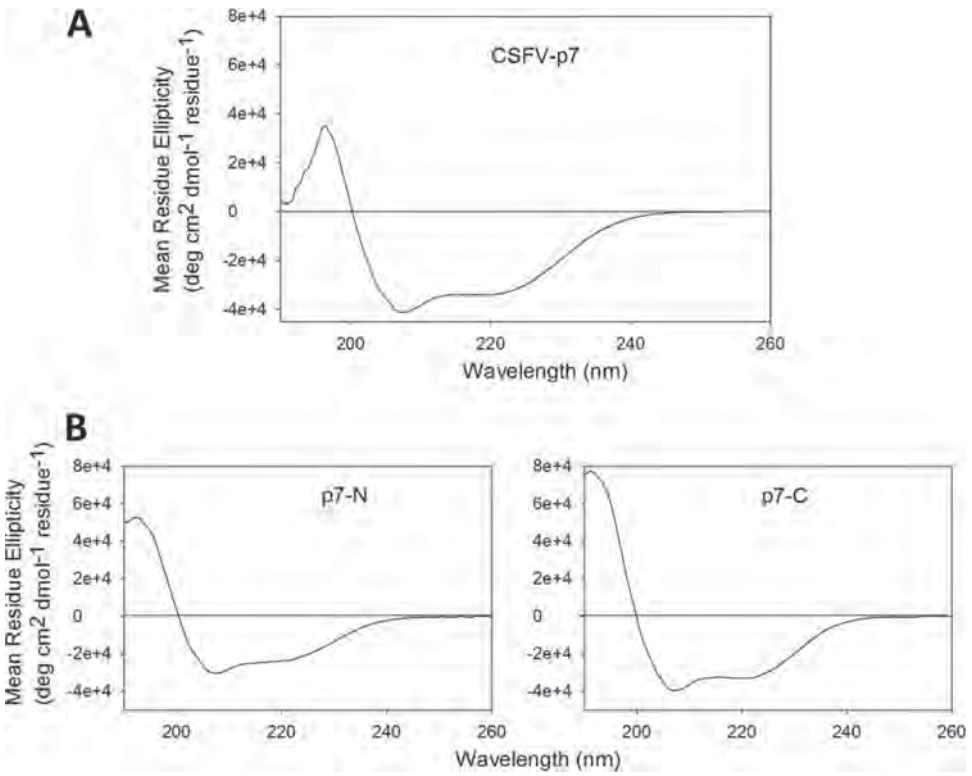

FIG 6 Structure of BICv p7 protein and derived peptides in 50% HFIP membrane-mimetic. (A) CD spectrum of BICv p7. (B) CD spectra of p7-N (left) and p7-C (right) peptides.

(pyridinium)bromide (DPX; molecular weight, 422). ANTS and DPX are true markers of the aqueous space of the liposomes and, when coencapsulated, DPX efficiently quenches ANTS fluorescence by collisional transfer (Fig. 5A). In addition, highly soluble ANTS and DPX remain trapped for extended periods, as reflected by the stability of the ANTS signal in negative-control samples treated with DMSO (Fig. 5A, CTL, gray trace), and allow permeability measurements at acidic pH (8). Upon addition of the permeabilizing agent (indicated by the arrow in Fig. 5A), both probes are released to the medium through membrane-permeating pores. Dilution of DPX prevents quenching outside the liposomes, and membrane permeabilization can be monitored as a function of time from the recovery of ANTS fluorescence (Fig. 5A, black trace). Maximal attainable ANTS fluorescence in the mixture (100% release) can be inferred after detergent solubilization of liposomes (indicated by the second arrow). In a typical experiment, 10 μl of peptide dissolved in DMSO was injected into a fluorimeter cell containing 1 ml of a stirring solution of liposomes. Previous works demonstrate that this experimental setup ensures fast and efficient partitioning of peptides into membrane surfaces (17).

The data displayed in Fig. 5B demonstrate that the complete p7 CSFV can efficiently permeabilize ER liposomes at pH 5.0 but not at pH 7.4. The lack of activity at neutral pH was probably due to limited solubility of the protein under those conditions (not shown). Liposome permeabilization and its elicitation at the lower pH could be reproduced by the p7-C peptide, but barely by the p7-N peptide (Fig. 5C, right and left panels, respectively). This observation would be consistent with a pore-forming activity residing in the carboxyl half of the protein represented by the p7-C peptide.

To test whether BICv-p7 could adopt a defined secondary structure in the nonpolar membrane medium, the synthetic sequences were next characterized by CD (Fig. 6). Consistent with their potential for adopting main α-helical conformations in nonpolar medium, the complete protein and the p7-N and p7-C peptides dissolved in 50% HFIP displayed spectra with absorption maximum located at 195 nm and minima at 208 and 222 nm (Fig. 6A and B, respectively). Deconvolution of these spectra using the CDPro software package (32) suggested a higher amount of helical conformers in p7-C than in p7-N. In contrast, this latter peptide disclosed a higher content of turns and disordered structures. Thus, the structural data support the capacity of the BICv-p7 hydrophobic domains for spanning lipid bilayers as transmembrane helices and suggest a higher conformational plasticity for the N-terminal sequence not bearing the pore-forming activity.

To further study the physiological relevance of the pore-forming activity of the p7-C sequence, we next characterized the lipid dependence of this phenomenon (Fig. 7 and 8) and the capacity of amantadine and verapamil for inhibiting the process (Fig. 9). The kinetic traces displayed in Fig. 7A demonstrate a PI-dependent pore-forming p7-C activity in vesicles emulating the ER membrane (right panel), which could not be detected in the case of p7-N (left panel). The permeabilization data also indicate that pore-formation by p7-C was even more efficient when PI was mixed with PC or PE. The data in Fig. 7B further show that anionic PS, a minor component of the ER membrane, could not substitute for PI. Interestingly, PG, an anionic phospholipid mostly present in bacterial membranes and mitochondria, could sustain p7-C

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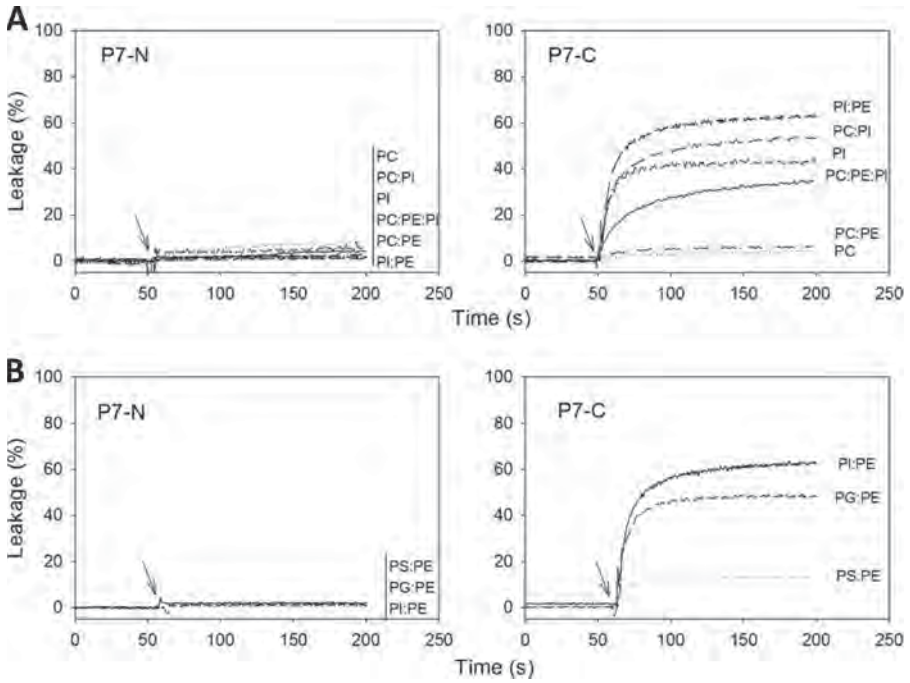

FIG 7 Effects of lipid composition on pore formation. (A) Permeabilization was induced by p7-N and p7-C (left and right panels, respectively) of large unilamellar vesicles composed of the main ER phospholipids mixed as follows: PC-PE-PI (5:3:2 molar ratio), PI-PE (1:1); PC-PI (1:1), PC-PE (1:1), PC alone, and PI alone. The peptide/lipid molar ratio was 1:50. The lipid concentration was 100 μM. (B) Permeabilization of vesicles made of equimolar mixtures of PE and anionic phospholipids. Conditions were as described in panel A.

pore-forming activity alone (data not shown) or upon mixing it with PE (Fig. 7B, right panel).

The dose dependency of p7-C-induced ER vesicle permeabilization is presented in Fig. 8. Both initial rates (proportional to the time required for the assembly of each pore) and final extents at the time of detergent addition (proportional to the total number of pores in the vesicle population) increased upon peptide dose increase (Fig. 8, left panel). Hence, both parameters, as inferred from this type of curve, reflected the capacity of p7-C peptide for efficient pore formation in the ER-mimicking lipid mixture (Fig. 8, right panel). Again, by comparison, p7-N activity could be barely detected under the same experimental conditions.

Contribution of the different ER mixture lipids to p7-C pore-forming efficiency was next evaluated (Fig. 8B to G). PE could not be tested in isolation because its nonlamellar nature precluded lipid bilayer formation when dispersed in buffer at pH 7.4. PC-based vesicles did not sustain pore formation (Fig. 8B). In contrast, pores were efficiently formed in vesicles made of pure PI (Fig. 8C). The probability of pore formation, but not the rate, increased when PI was mixed with PC (Fig. 8D). Moreover, compared to PI alone or a PC-PI mixture, pore-forming activity was maximal for the PI-PE mixture (Fig. 8E). This stimulatory effect of PE suggests that pore-opening may be regulated by bilayer structural properties such as spontaneous curvature or thickness, a common trend described for pore-forming proteins and channels (24). Finally, the data displayed in F and G panels demonstrate that PG, but not PS, could substitute for PI. Thus, although required, a negative net charge is not sufficient to sustain pore-forming activity, which appears to be also regulated by the chemical nature of the polar-head group moiety. Together, these data suggest that the C-terminal CSFV p7 hydrophobic domain is endowed with the capacity for effectively permeabilizing the ER membrane upon insertion.

The data displayed in Fig. 9 further demonstrate that p7-C pores can be inhibited by amantadine and verapamil (Fig. 9A and B, respectively). Preincubation with these compounds inhibited in a dose-dependent manner both the initial permeabilization rates and the final extents (right panels). Notably, in the liposome permeabilization assays verapamil was roughly an order of magnitude more potent than amantadine, following the same inhibitory pattern that was observed in the previously described viral replication assays (Fig. 4). In conjunction, the cell and liposome results suggest that pore formation by p7 constitutes a crucial event for the efficient replication of CSFV in cells.

Proposed model for CSFV p7. Secondary structure prediction algorithms predict a strand centered on residues Val8-Leu14 and two helices centered on residues Val22-Met33 and Ile38-Thr56 for BICv p7 (Fig. 1). The latter helix is predicted to have a disruption centered on residue Pro53. Transmembrane helix topology prediction algorithms predict two transmembrane helices centered on residues 18 to 32 and residues 46 to 63. Tertiary structure predictions place Glu21, His47, and Pro53 in close proximity to each other near the center of the membrane (Fig. 10). Gly12 and Asn13 are predicted to be at the luminal end of the first transmembrane helix; however, there is strong disagreement in the tertiary structure predictions surrounding these residues. The region from Arg34 to Lys40 is predicted to form a cytosolic loop by transmembrane helix and tertiary structure prediction algorithms.

DISCUSSION

This report focuses on the study of CSFV p7, a previously uncharacterized protein, to further understand its function in the process of

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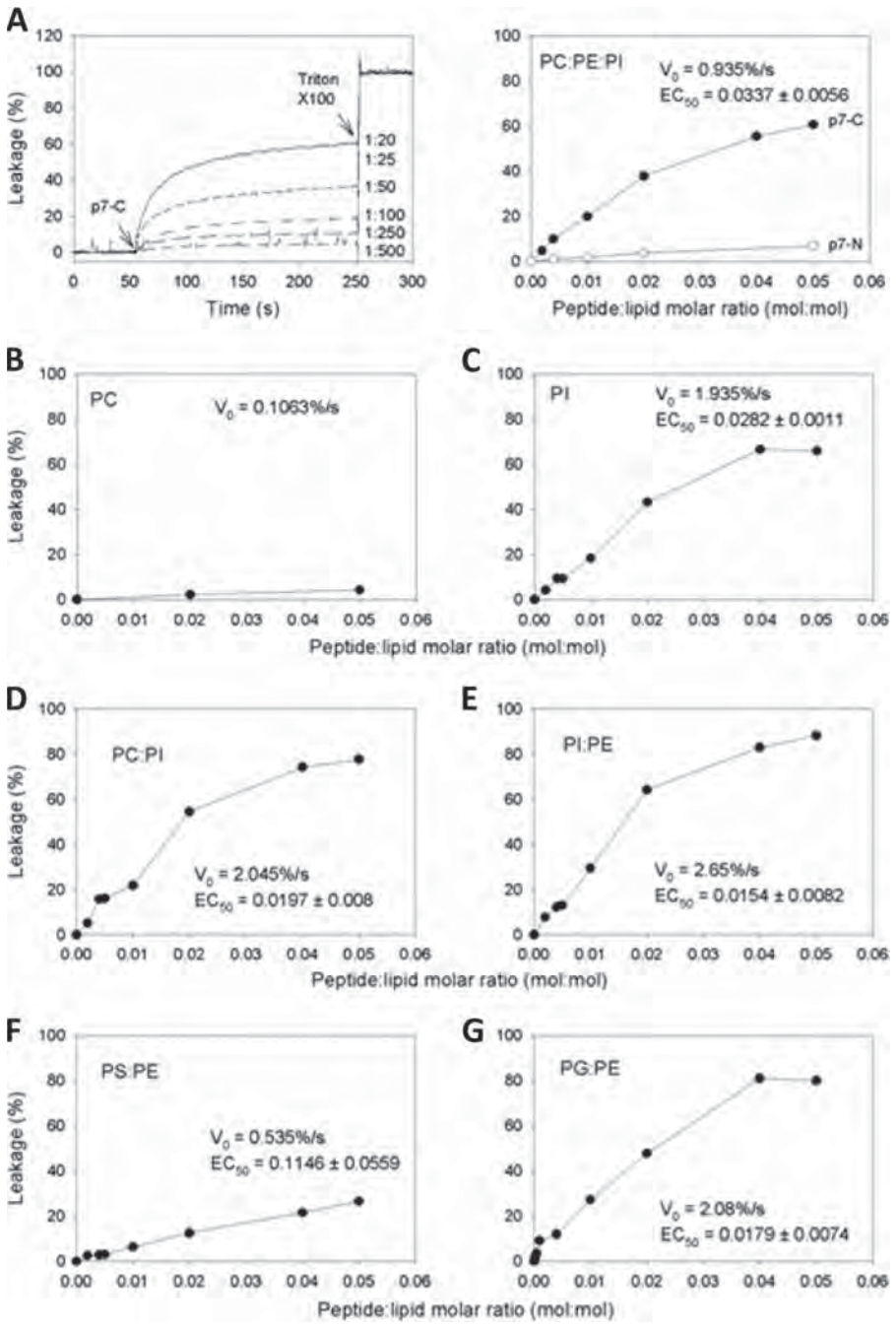

FIG 8 Contribution of the different ER mixture lipids to pore-forming efficiency. (A) The left panel shows the dose dependency of p7-C-induced permeabilization of PC-PE-PI (5:3:2) vesicles. The peptide/lipid molar ratios are indicated for each kinetic trace. The right panel shows the percentage of maximum leakage (measured at time = 200 s) as a function of the peptide/lipid molar ratio. ER-like vesicles (lipid concentration, 100 μM) were incubated with increasing doses of p7-C or p7-N (black and white circles, respectively). The V_0 value corresponds to the initial rate measured at a 1:50 peptide/lipid molar ratio. The peptide/lipid EC_{50} was inferred by fitting the experimental values of maximum leakage to a saturation curve. Depicted values are only applicable to the p7-C peptide. (B to E) Contribution of lipids in the ER-like mixture to pore-forming efficiency by p7-C peptide. (F and G) Effect of anionic phospholipids on p7-C pore-forming efficiency. In panels B to G experimental conditions were as in the right side of panel A. Mixtures of two lipids were equimolar.

virus replication and virulence. We demonstrated here that CSFV p7 possess an important pore formation activity, with maximal activity residing in the carboxyl half of the protein. We also show that the integrity of p7 is essential for the process of virus replication, and we mapped the specific areas of p7 critical for virus replication in cell cultures. In addition, we demonstrated that p7 plays a significant role in virulence during the infection in swine.

In accordance with results reported for BVDV (14), the integrity of p7 is decisive for CSFV production. Mutant CSFVs possessing three different partial in-frame deletions (two of them smaller

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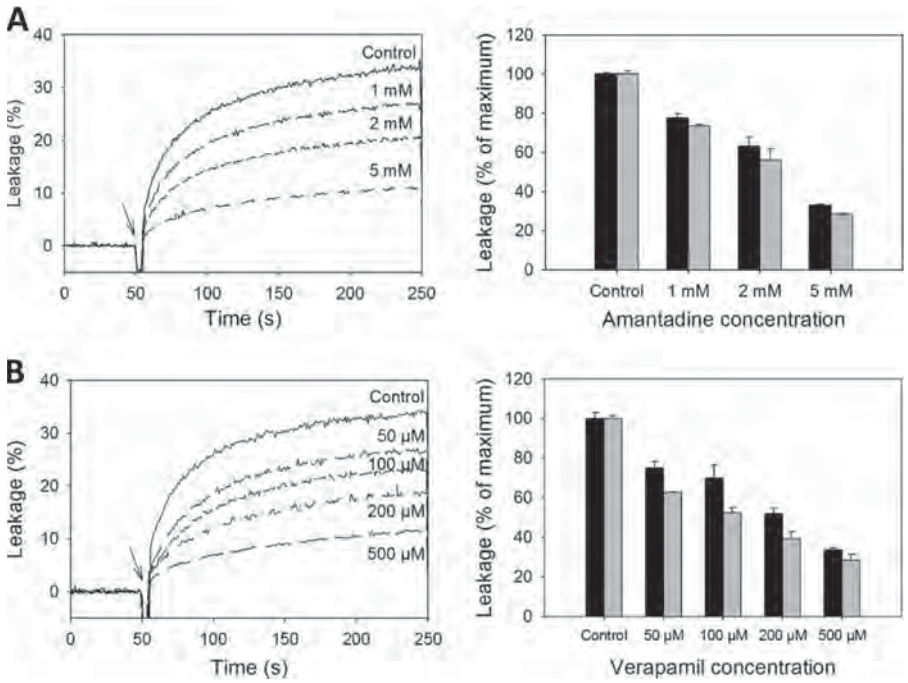

FIG 9 Effect of amantadine (A) and verapamil (B) on p7-C pore-forming activity. The left panels show the kinetic traces recorded after peptide addition to ER LUV (indicated by the arrow) in the presence of increasing amounts of the drugs. The peptide/lipid molar ratio was 1:50. The lipid concentration was 100 μM. The right panels show the percentage of maximum leakage and the initial rate (black and gray columns, respectively) as a function of the drug dose.

than that reported for BVDV) in the inner area of p7 were unable to grow in cell cultures. In contrast to what was observed with BVDV (14), CSFV mutant p7Δ15-51 failed to produce viral RNA after transfection, evidencing different roles for these residues between CSFV and BVDV. The importance of the integrity of p7 in the process of virus replication is demonstrated by the nonviability of CSFV constructs p7.2, p7.5, p7.9, p7.11, and p.7.12, where relatively small areas of the native sequence of the protein were substituted with alanine residues. Perhaps the role of CSFV p7 as a viroporin is critical in the process of virus replication, as has been shown for HCV p7 proteins (18, 29, 33). The significant sensitivity of CSFV to pore-forming inhibitors such as amantadine or verapamil supports this hypothesis.

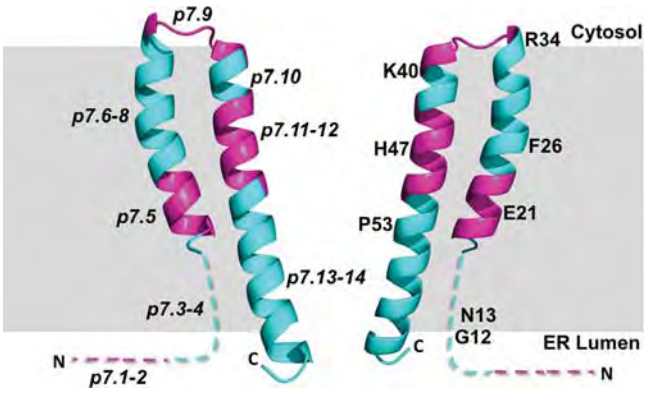

FIG 10 Predicted transmembrane topology of p7. The ER membrane is shown in gray, and the two predicted transmembrane helices are shown in ribbon diagrams with the N and C termini labeled. Two monomers are shown to indicate the predicted location of the pore; however, the model is not intended to show any oligomeric state. The extent of alanine constructs that resulted in nonviable viruses are shown in violet, and the remainder of the protein is shown in blue. The constructs are labeled p7.1–14 on the monomer on the left. The secondary, tertiary, and transmembrane helix prediction algorithms disagreed most strongly at the N-terminal 20 residues, so this region is represented as a dashed line. Residues discussed in the text are labeled on the p7 monomer shown on the left.

The role of CSFV p7 protein in the process of virus infection in the natural host has been largely ignored. This is the first report indicating a clear role for p7 in virulence in domestic swine. Mutations affecting areas p7.4, p7.8, p7.14, and, in particular, p7.10 significantly affect the ability of the viruses to produce disease. The mechanisms mediating this attenuation are not clear at this point. Perhaps it is the result of a decreased rate of replication, as demonstrated in primary swine macrophage cultures, which facilitates the host immune mechanisms that prevent the further dissemination of the virus. Although there is a correlation between the acquisition of attenuation in CSFV developed by reverse genetics and a certain degree of decreased ability to replicate in primary swine macrophage cultures (9, 26, 28), there are examples of attenuated viruses that replicate as efficiently as their virulent parental strain (10, 27). Further work will be necessary to assess other possible mechanisms producing virus attenuation.

Our experimental data (Fig. 5 to 9) and sequence-based secondary, tertiary, and topological predictions (Fig. 1 and 10) suggest that BICv p7 forms a channel, in which the C-terminal hydrophobic stretch is a pore lining transmembrane helix and the N-terminal hydrophobic stretch is of mixed secondary structure but containing a predominantly helical transmembrane segment with an unknown functional role in channel activity.

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In the simplest model of the channel portion, in which a cytosolic loop centered on residues Arg34-Lys40 connects two transmembrane helices, four of the six conserved residues (Glu21, Phe26, His47, and Pro53) are predicted to be well within the membrane (Fig. 10). Two (His47 and Glu21) of these conserved, putative membrane-embedded residues are hydrophilic, and the alanine scanning data show that mutating the blocks containing either residue results in nonviable viruses, indicating that these residues may be involved in conductance. The latter, His47, may line the predicted pore in a similar fashion as His37 in the influenza virus M2 ion channel (16, 38). One of the few other residues in HCV p7 shown to be essential for p7 function, Tyr42 (33, 34), is in a transmembrane-embedded region similar in position to His47 of CSFV p7. Tyr42 and His47 are among the few strictly conserved amino acids in HCV p7s and pestivirus p7s, respectively. In HCV p7, alanine mutations of the dibasic motif, Lys33 and Arg35, prevent HCV replication in chimpanzees (29). The corresponding CSFV alanine construct, p7.9, did not produce infectious viruses. In both proteins, this region is predicted to form a cytosolic loop. Although sequence identity between the pestivirus p7 proteins and HCV p7 is low, our results suggest that these proteins are functionally similar and may in fact be similar to some extent at the structural level. Residue level comparisons between CSFV p7 and HCV p7 are informative, given the similar predicted structures and the wealth of data available for HCV p7; however, these comparisons must be interpreted with caution since the low homology of CSFV p7 to HCV p7 prevents direct extrapolation of functionality ascribed to HCV p7 amino acid residues to CSFV p7. Further experimental studies are needed to validate the CSFV p7 model presented here and to elucidate the role of invariant residues in pore formation and channel function.

In summary, we presented here novel experimental evidence for the role of CSFV p7 in the process of virus replication, pore formation, and virulence in swine. Further research is needed in order to examine in more detail the areas of the protein specifically responsible for each of these functions.

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Disinfection of foot-and-mouth disease and African swine fever viruses with citric acid and sodium hypochlorite on birch wood carriers

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ABSTRACT

Transboundary animal disease viruses such as foot-and-mouth disease virus (FMDV) and African swine fever virus (ASFV) are highly contagious and cause severe morbidity and mortality in livestock. Proper disinfection during an outbreak can help prevent virus spread and will shorten the time for contaminated agriculture facilities to return to food production. Wood surfaces are prevalent at these locations, but there is no standardized method for porous surface disinfection; commercial disinfectants are only certified for use on hard, nonporous surfaces. To model porous surface disinfection in the laboratory, FMDV and ASFV stocks were dried on wood coupons and exposed to citric acid or sodium hypochlorite. We found that 2% citric acid was effective at inactivating both viruses dried on a wood surface by 30 min at 22 °C. While 2000 ppm sodium hypochlorite was capable of inactivating ASFV on wood under these conditions, this chemical did not meet the 4-log disinfection threshold for FMDV. Taken together, our data supports the use of chemical disinfectants containing at least 2% citric acid for porous surface disinfection of FMDV and ASFV.

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1. Introduction

The introduction of transboundary animal disease viruses (TADV) into non-endemic countries has severe economic consequences, including the mass culling of animals and the suspension of animal trade. The importance of fomites in the spread of disease has been widely reported (reviewed in Boone and Gerba, 2007). Among the TADV, foot and mouth disease virus (FMDV), a small, nonenveloped virus, is one of the most contagious viruses known (Grubman and Baxt, 2004) and this is partly due to its remarkable ability to persist in the environment, especially on fomites (Bartley et al., 2002). African swine fever virus (ASFV) and classical swine fever (CSFV), both highly pathogenic enveloped viruses, are also capable of spreading via fomites (Edwards, 2000; Kleiboeker, 2008).

Disinfection is crucial to prevent the spread of these highly transmissible livestock pathogens during outbreaks and to facilitate the repopulation of livestock at agricultural facilities. While there are many types of surfaces at these sites potentially contaminated with viruses, the disinfectants registered by the US Environmental Protection Agency are only recommended for hard, nonporous surfaces (USEPA, 2010). Currently, there is no standardized disinfection method for viruses on porous surfaces. Experiments describing the disinfection of TADV in suspension has been the basis for the use of disinfectants in the field, yet it is known that disinfectant efficacy is reduced when applied to dried viruses (reviewed in Springthorpe and Sattar, 2005).

Citric acid and sodium hypochlorite have been widely used for decades as disinfectants in home, health care and

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industrial settings. Sodium hypochlorite is extremely effective as a broad-spectrum microbial disinfectant (reviewed in Rutala and Weber, 1997) but due to its oxidation potential its repeated application to certain surfaces can cause corrosion (Zumelzu and Cabezas, 1996). Citric acid has also been shown to be efficacious against many microorganisms and as a natural chemical found in many food products, does not pose a significant biohazard to animals (reviewed in McDonnell, 2007). Citric acid has successfully been used against FMDV during outbreaks (Engvall and Sternberg, 2004) and is recommended by the World Organization for Animal Health for field use at a concentration of 0.2% (OIE Standards Commission, 2010).

While we have recently reported disinfection efficacy data for sodium hypochlorite and citric acid against FMDV, ASFV and CSFV dried on steel and plastic surfaces (Krug et al., 2011), there is little published information regarding the disinfection of these viruses on agriculturally relevant porous surfaces. Thus, virus disinfection on porous surfaces in the field presents a challenge due to the lack of available data and testing methodologies.

Wood construction is very common on farms thus modeling virus decontamination of wood fomites is pertinent to infection control. Other laboratories have successfully performed virus stability and disinfection assays on wood surfaces using viruses including bovine enterovirus and Newcastle disease virus (Yilmaz and Kaleta, 2003a,b), poxviruses (Hartnack et al., 2008) and avian influenza (Lombardi et al., 2008; Tiwari et al., 2006). In order to test the efficacy of disinfectants on porous surfaces, we modified the ASTM standard for virus disinfection on nonporous surfaces (ASTM, 1997) to use birch wood veneer as a porous surface model. The results presented here extend our previous nonporous surface disinfection data to the disinfection of FMDV and ASFV on a porous surface.

2. Methods

2.1. Cells and viruses

FMDV strain A24 stocks were generated in BHK-21 cells (ATCC# CCL-10). FMDV infection was identified by the presence of destructive cytopathic effects 2 or 3 days post infection. CSFV strain Brescia and the swine kidney cell line SK6 were obtained from Dr. Manuel Borca (PIADC). CSFV replication was detected by immunohistochemistry as described in Risatti et al. (2005). ASFV strain BA71/v was obtained from the PIADC virus repository and grown in Vero cells (ATCC# CCL-81). ASFV was identified by the formation of plaques after 5 to 7 days post infection. High titer virus stocks were produced as described previously (Krug et al., 2011). All virus work was conducted under biosafety level 3-Ag containment in accordance with the APHIS select agent regulations in title 9 part 121 of the code of United States federal regulations.

2.2. Disinfectants and neutralizers

1000 ppm sodium hypochlorite (Baker), mean pH 10.75 ± standard deviation 0.06, was neutralized with a 2× solution of Fluid Thioglycolate Medium (FTM, Difco) All other tested concentrations of sodium hypochlorite (1500 ppm, pH 10.94 ± 0.07 and 2000 ppm, pH 11.1 ± 0.02) were neutralized with a solution containing 1× FTM and 2× Fluid Thioglycolate Broth (FTB, Fluka Chemical). 2% citric acid, pH 1.8 (Acros Organics) was neutralized with 1.25 M sodium hydroxide (Ricca Chemical). The mean pH of the neutralized solutions was 7.16 ± 0.09. All disinfectants were diluted in 400 ppm calcium carbonate to simulate worst-case hard water conditions, and neutralizers were prepared in sterile distilled water. All disinfectants and neutralizers were made immediately before use.

2.3. Disinfection assay

This protocol is a modification of ASTM E1053: standard test method for efficacy of virucidal agents intended for inanimate environmental surfaces (ASTM, 1997) and the quantitative carrier test method described by Sattar et al. (2003). Briefly, 100 μl of high-titer virus stock, diluted in 1× phosphate-buffered saline, was pipetted directly onto 2 cm × 2 cm × 0.1 cm coupons of autoclaved birch veneer (Rockler Woodworking) in a stainless steel base mold (Fisher Scientific #15182505C). Each virus stock was diluted to equalize the final concentration of calf serum already present in the stocks to 1.0% (v/v); excess organic load was not added in these experiments. To dry the virus, the coupons were placed in the back of a biosafety cabinet with the laminar airflow on and the lights off at ambient temperature. After the virus suspension had dried (60–90 min, depending on ambient humidity), the birch coupons were exposed to 1 ml of sodium hypochlorite or citric acid in a static temperature incubator (Incufridge, Revolutionary Science) at 22 °C. At the end of the disinfectant contact time, 1 ml of neutralizer was added directly to the disinfectant, and each birch coupon with its associated disinfectant and neutralizer fluids was transferred to a conical tube containing an equal volume (2 ml) of cell culture medium. To release virus from the wood, the liquids were mixed for 1 min at maximum speed on a vortex shaker. The liquids were then diluted and used to infect susceptible cells to determine the 50% cell culture infectious dose ($CCID_{50}$) titer of residual virus using the Spearmann-Karber endpoint titration method (Hierholzer and Killington, 1996). Based on assay volumes and the amount of virus added to cells, the lower limit of virus detection in this assay is 1.1 $\log_{10}$ $CCID_{50}$.

2.4. Assay controls

To determine virus recovery in the absence of disinfection, birch veneer coupons with dried virus were exposed to a solution of premixed neutralizer and disinfectant for the longest contact time in each experiment, and then processed identically as the disinfection samples. This virus recovery control was used in each experiment as the 0 min contact time point, ensuring that the addition of the neutralizer to the disinfectant results in a solution that is not virucidal. No difference in virus recovery was observed when cell culture media or disinfectant/neutralizer mixtures were used, indicating the neutralization of the disinfectant was

complete (data not shown). In addition, two negative controls without virus, a surface control and a neutralization control, were included in each experiment: (1) to control for surface-induced cytotoxicity, uninoculated birch coupons were incubated with media alone and (2) to ensure effective neutralization, uninoculated birch coupons were incubated with a mixture of disinfectant and neutralizer. Both of these negative controls were processed in the same manner as the disinfection samples. Cell cultures were inoculated with these negative controls and compared to uninfected cell cultures included on the same plate. Experiments showing visual evidence of cytotoxicity were not included in the data analysis.

3. Results

To develop a method for disinfection of viruses on porous surfaces, the ASTM standard for nonporous surface disinfection was modified to use small squares of birch wood veneer as surface coupons. A common measure of efficacy in disinfection assays is the observation of a 4-log reduction in virus recovery (USEPA, 1981). Given the 1.1 $\log_{10}$ $CCID_{50}$/ml limit of detection in the described disinfection assay, recovering a minimum titer of 5.1 $\log_{10}$ $CCID_{50}$/ml after drying is required to be able to measure the EPA-recommended 4-log reduction in titer by disinfectant treatment. In order to ensure enough virus could be recovered in controls to achieve this standard, FMDV, ASFV and CSFV were dried on birch coupons and virus was extracted as described in the methods. Table 1 shows the effects of drying on virus recovery from birch surfaces. FMDV exhibited the least inactivation by drying alone (1.8 $\log_{10}$ reduction) followed by ASFV (2.5 $\log_{10}$ reduction) then CSFV (3.7 $\log_{10}$ reduction). The mean recovery of FMDV (7.0 $\log_{10}$ $CCID_{50}$) and ASFV (5.8 $\log_{10}$ $CCID_{50}$) in this experiment was sufficient to use these stocks for the disinfection assay. Since CSFV mean recovery was only 3.9 $\log_{10}$ $CCID_{50}$, this virus was not included in further wood surface disinfection experiments.

Next, assays were done to test disinfection efficacy on birch-dried FMDV and ASFV. Fig. 1 shows a time-course experiment using 2% citric acid (A) or 1000 ppm sodium hypochlorite (B) to disinfect FMDV and ASFV. Citric acid was able to reduce both ASFV and FMDV by greater than 4 $\log_{10}$ at 20 min, however 1000 ppm sodium hypochlorite was not able to do so by 30 min. With both disinfectants, a rapid decrease in virus recovery was observed in the first 5 to 10 min. After this time, citric acid continued the inactivation trend (Fig. 1A) and by 25 (FMDV) or 30 min (ASFV) the viruses were reduced to below the limit of detection. However, the ability for hypochlorite to extend the inactivation at the same rate was limited, as shown by the plateau between 10 and 20 min for both viruses (Fig. 1B). We interpret this difference in inactivation kinetics as both disinfectants rapidly inactivating the virus bound to the outer surface of the wood in the first 10 min but the hypochlorite was either inhibited or consumed by the wood itself in the next 20 min.

To address the possibility that increasing the hypochlorite concentration could overcome this inhibition and thereby enhance the ability to disinfect the virus in the pores of the wood, disinfection assays were performed using up to 2000 ppm sodium hypochlorite. Fig. 2 demonstrates that the increased hypochlorite concentration was able to reduce ASFV to the limit of detection by 30 min. In contrast, we observed only minor efficacy differences with the increased hypochlorite concentrations against FMDV, as treatment with 2000 ppm hypochlorite for 30 min did not result in a 4-log reduction for this virus. In a separate experiment, 2500 ppm sodium hypochlorite was not more effective at disinfecting birch-dried FMDV (data not shown).

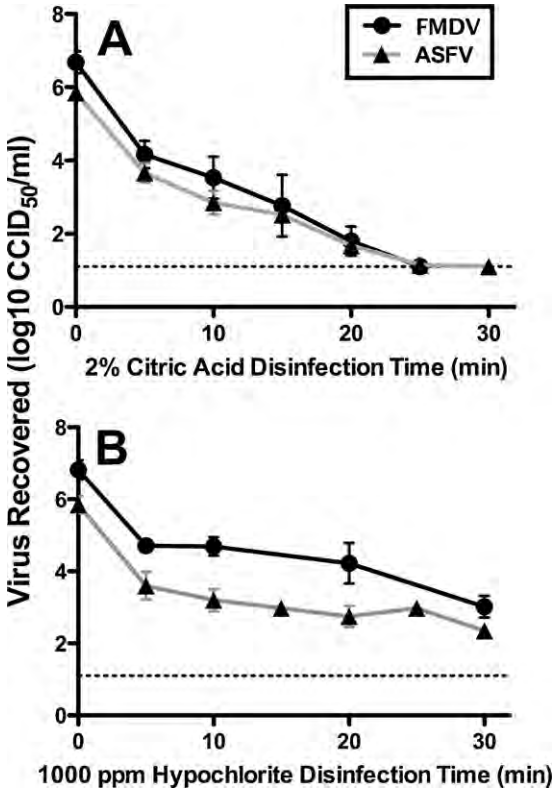

Fig. 1. Time course of TADV disinfection. FMDV or ASFV was dried on birch coupons and exposed to 2% citric acid (A) or 1000 ppm sodium hypochlorite (B). At the indicated time, the disinfectant was neutralized and the residual virus was extracted and quantified. Each point represents the mean of 3 or more experiments; error bars reflect the standard deviation. Dashed line indicates the lower limit of virus detection.

Table 1
Recovery of TAD viruses from birch wood carriers.[a].

Virus (strain)	Inoculum titer[b]	Birch recovery	$\log_{10}$ reduction[c]
FMDV (A24)	8.8 ± 0.63	7.0 ± 0.61	1.8
ASFV (BA71/v)	8.3 ± 0.56	5.8 ± 0.81	2.5
CSFV (Brescia)	7.6 ± 0.37	3.9 ± 0.37	3.7

[a] Titers are presented as $\log_{10}$ $CCID_{50}$/ml ± SD.
[b] Backtiter of the diluted virus stock used to inoculate the birch coupons.
[c] Determined by subtracting the birch recovery titer from the inoculum titer.

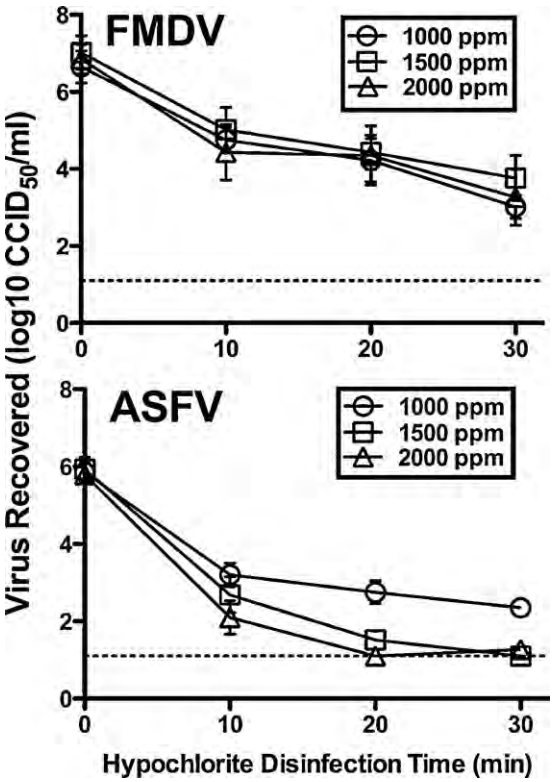

Fig. 2. Sodium hypochlorite dose–response. FMDV (top) or ASFV (bottom) was dried on birch coupons and exposed to 1000, 1500, or 2000 ppm sodium hypochlorite. At the indicated time, the disinfectant was neutralized and the residual virus was extracted and quantified. Each point represents the mean of 3 experiments; error bars reflect the standard deviation. Dashed line indicates the lower limit of virus detection.

To verify the efficacy of 2% citric acid and 2000 ppm hypochlorite for inactivation of these viruses, multiple assays with replicates were performed. The log reduction values by these chemicals are shown in Table 2. The results from the time course assays shown in Fig. 2 were confirmed as 2% citric acid was repeatedly more effective at virus disinfection than 2000 ppm hypochlorite. While most sample replicates treated with 2% citric acid had virus titers reduced to the limit of detection, this chemical was not able to completely eliminate the recovery of infectious virus, indicating that higher concentrations of disinfectant or longer contact times might be needed to achieve complete virus inactivation. In the case of hypochlorite disinfection, we recovered infectious virus in all of the FMDV replicates (11 of 11) and almost half of the ASFV replicates (7 of 15).

Table 2
Log_{10} reduction of TADV after 30 min disinfection on birch surfaces.[a].

Virus	2% Citric acid	Sodium Hypochlorite	
		1000 ppm	2000 ppm
FMDV	5.22 ± 0.78[b] $n = 20$ (4)	2.95 ± 0.57 $n = 10$ (10)	3.77 ± 0.44 $n = 11$ (11)
ASFV	4.72 ± 0.41 $n = 13$ (3)	3.75 ± 0.44 $n = 9$ (9)	4.43 ± 0.39 $n = 15$ (7)

[a] Log_{10} reduction values are determined by subtracting the mean residual virus titer from the mean recovery titer. n = total number of inoculated birch coupons disinfected (number of virus positive coupons). Each value reflects data from at least three individual experiments.
[b] Contact time was 25 min for FMDV/2% citric acid.

4. Discussion

Here we have demonstrated a methodology to test disinfectant efficacy against viruses dried on a porous surface, based on our adaptation (Krug et al., 2011) of the ASTM standard for virus disinfection on nonporous surfaces (ASTM, 1997). Initially we tested pine wood coupons but they were found to be unusable for cell culture-based assays due to considerable cytotoxicity (data not shown). Similar cell toxicity has been previously reported for pine extracts (Mark et al., 1995). Birch wood veneer did not induce cytotoxicity in pilot studies, thus it was utilized as the porous surface carrier in the experiments presented here. Furthermore, a drying time comparison between autoclaved birch and pine veneers demonstrated no difference (data not shown), suggesting these wood types had similar performance in our experiments.

Greater than 5 $\log_{10}$ $CCID_{50}$/ml of FMDV and ASFV was recovered after drying virus samples on birch coupons, allowing for the detection of at least a 4-log reduction after complete disinfection. Our results demonstrate the effectiveness of 2% citric acid for the inactivation of wood-dried FMDV and ASFV. We found that 2000 ppm sodium hypochlorite was unable to meet the 4-log inactivation threshold against FMDV. We postulate that the difference between citric acid and sodium hypochlorite may be due to consumption of the hypochlorite by the wood itself as the activity of hypochlorite can be inhibited in the presence of organic material (Terpstra et al., 2007; Weber et al., 1999). Another possibility might be that the ability of hypochlorite solutions to penetrate wood may be lower than that of acidic solutions. Indeed, it has been suggested that pH-amended hypochlorite solutions are more effective for microbial disinfection (Dychdala, 2001), however an experiment testing pH-neutral hypochlorite against wood-dried FMDV had no positive effect on efficacy in our hands (data not shown).

The highest concentration of sodium hypochlorite tested, 2000 ppm, was able to disinfect wood-dried ASFV (but not FMDV) to meet the 4-log reduction standard (Fig. 2, bottom panel), although under these disinfection conditions, approximately half of the ASFV replicates still had low levels of virus present (Table 2). The difference in inactivation by hypochlorite between FMDV and ASFV likely reflects the greater sensitivity of ASFV to hypochlorite previously reported (Krug et al., 2011). In that study, we found that sodium hypochlorite could completely disinfect ASFV at half the concentration required for complete FMDV disinfection with the same contact time on nonporous surfaces.

Disinfectant-induced cytotoxicity can introduce complications in virus disinfection assays that hamper efficacy determination. Because of the nature of cell-based assays to detect residual intact viruses, cytotoxicity can lower the detectable log reduction due to disinfectant by requiring further dilution of the disinfection samples. The US EPA guidelines for disinfectant testing take this issue into

account by accepting data demonstrating a 3-log reduction beyond the level of cytotoxicity instead of the usual 4-log reduction requirement (US EPA, 1981). It is possible, however, that a disinfectant could induce cytotoxicity but not be completely virucidal (e.g. a surfactant treatment of a non-enveloped virus). In this case, virus that potentially survived the disinfection process would not be able to replicate in the damaged cells, thereby demonstrating a false result and inflating the perceived efficacy of the disinfectant. While some disinfectant efficacy tests include a "cleanup" step utilizing column purification (ASTM, 2004), these procedures can increase disinfectant contact time and can result in a loss of virus in the resin. Therefore, in the experiments presented here, the disinfectant was completely neutralized in all assays and the virus recovery control was resuspended in a mixture of disinfectant and neutralizer to substantiate effective neutralization.

Overall, our results are in agreement with Lombardi et al. (2008) who demonstrated the failure of sodium hypochlorite and the effectiveness of citric acid for avian influenza disinfection on basswood surfaces. In that study, 1% citric acid was capable of disinfecting metal-, plastic- and wood-dried avian influenza to the limit of detection in the assay. Yilmaz and Kaleta (2003a) found that 2% formic acid was able to disinfect wood-dried picornavirus by 15 min; however, this concentration of formic acid did not inactivate reovirus or adenovirus in the same experiments.

In the current study we were not able to test CSFV disinfection on birch wood due to poor virus recovery. Other labs have also observed inefficient virus recovery from porous surfaces. We used sonication to help release CSFV from birch carriers in a manner similar to the method developed by Hartnack and colleagues (Hartnack et al., 2008) to extract dried vaccinia virus from poplar wood carriers, but that treatment failed to enhance CSFV recovery (data not shown). One reason for the low CSFV recovery in our experiments (Table 1) could be that the virus in the wood pores has been rendered noninfectious by the drying process; in this case the wood would no longer be considered a fomite since the virus is essentially inactivated. Alternatively, if residual infectious virus in the pores of the wood is trapped, the poor recovery could be due to inefficient elution as suggested by Tiwari et al. (2006). Either way, our inability to recover a sufficient titer of CSFV to perform experiments suggests that the porous surface disinfection of some enveloped viruses may require additional methodologies.

To our knowledge, this is the first report to describe the disinfection of FMDV or ASFV on a porous surface. Future investigations could adapt this method to other agriculturally relevant porous surfaces (e.g., concrete or soil) and to use infected animal fluids (e.g. vesicular fluid, saliva, and blood) as inocula to more closely simulate the virus in the field. We conclude that sodium hypochlorite-based disinfectants with 2500 ppm or less available chlorine should not be used for the disinfection of FMDV-contaminated wood surfaces. Our results are consistent with a recommendation for the use of acid-based disinfectants at pH 2 or below for the disinfection of wood surfaces contaminated with FMDV or ASFV. This report has direct implications to the selection of disinfectant products during outbreak control and recovery from two high-consequence transboundary diseases affecting livestock populations around the world.

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Mini review

Designing bovine T cell vaccines via reverse immunology☆

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Vaccine development

ABSTRACT

T cell responses contribute to immunity against many intracellular infections. There is, for example, strong evidence that major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTLs) play an essential role in mediating immunity to East Coast fever (ECF), a fatal lymphoproliferative disease of cattle prevalent in sub-Saharan Africa and caused by *Theileria parva*. To complement the more traditional approaches to CTL antigen identification and vaccine development that we have previously undertaken we propose a use of immunoinformatics to predict CTL peptide epitopes followed by experimental verification of T cell specificity to candidate epitopes using peptide–MHC (pMHC) tetramers. This system, adapted from human and rodent studies, is in the process of being developed for cattle. Briefly, we have used an artificial neural network called NetMHCpan, which has been trained mainly on existing human, mouse, and non-human primate MHC–peptide binding data in an attempt to predict the peptide-binding specificity of bovine MHC class I molecules. Our data indicate that this algorithm needs to be further optimized by incorporation of bovine MHC–peptide binding data. When retrained, NetMHCpan may be used to predict parasite peptide epitopes by scanning the predicted *T. parva* proteome and known parasite CTL antigens. A range of pMHC tetramers, made "on-demand", will then be used to assay cattle that are immune to ECF or in vaccine trials to determine if CTLs of the predicted epitope specificity are present or not. Thus, pMHC tetramers can be used in one step to identify candidate CTL antigens and to map CTL epitopes. Our current research focuses on 9 different BoLA class I molecules. By expanding this repertoire to include the most common bovine MHCs, these methods could be used as generic assays to predict and measure bovine T cell immune responses to any pathogen.

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Introduction

East Coast fever (ECF) is a major livestock disease in East, Central, and South Africa. The disease is caused by a protozoan parasite called *Theileria parva*. Transmitted by ticks, the parasite invades lymphocytes. Infected lymphocytes harbor the schizont life-cycle stage of the parasite and behave like cancer cells causing an acute lymphoproliferative disease, which is usually fatal. The disease kills over 1 million animals each year, significantly reducing the already limited economies and productivity of livestock farmers in at least 11 countries in sub-Saharan Africa. Although ECF is endemically stable in many countries, the disease is spreading and calf mortality rates are high [reviewed in Norval et al. (1992); Spielman (2008)]. This loss is particularly hard on pastoral people and farmers with small numbers of cattle since much of the family's wealth and food security resides in these animals.

Current entry points for disease control include acaricide treatment to prevent tick infestation, chemotherapy, and a live parasite vaccine. Chemical control of ticks is logistically difficult, and it is not a desirable long-term option as there is an increasing incidence of resistance and concern over environmental pollution and pesticide residues in the food chain. Drugs are available for treatment, but are expensive and need to be dispensed early during infection to be completely effective. Cattle that survive infection become immune, but if they are moved to new regions they may be reexposed to ECF risk due to parasite strain differences (Radley et al., 1975a) and to parasites present in buffalo, a wildlife reservoir of the disease (Norval et al., 1992).

This observation gave rise to a live parasite vaccine called the infection and treatment method of immunization (ITM), which depends on inoculation of parasites cryopreserved in liquid

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nitrogen together with a long-acting oxytetracycline and formulation of a broad-spectrum vaccine by combining 3 parasite isolates in a vaccine referred to as the Muguga cocktail (Radley et al., 1975b). The vaccine is difficult to manufacture, and quality control is problematic. A vaccine dose costs $8–12, and vaccinated cattle remain as a source of transmission of parasites present in the vaccine. Most farmers are unable to meet the high costs associated with the current types of ECF control, but there is a demand for it as farmers recognize the losses they incur due to the disease. Under a new initiative by the Global Alliance for Livestock Veterinary Medicines (GALVmed), the Muguga cocktail will be made more widely available in a few countries in sub-Saharan Africa (GALVmed, 2010). Nevertheless, improved products that are easier to produce and deploy are still required to enhance sustainable and cost-effective control of ECF. It is estimated that such products would reduce the risk of disease to ~25 million cattle to the benefit of resource-poor farmers and pastoralists by limiting their losses and increasing the productivity from the cattle sector in sub-Saharan Africa.

In developing disease control strategies, vaccines remain one of the most effective interventions deployed. An inactivated or subunit-based vaccine is preferable to a live vaccine, but both are challenging to design. In particular for control of ECF, the "gold standard" is the Muguga cocktail ITM vaccine. We have previously demonstrated that inoculation of cattle with a recombinant protein derived from the gene encoding the major surface coat protein called p67 of *T. parva* sporozoites, the cattle-infective life-cycle stage of the parasite induces immunity to ECF in about 50% of vaccinated cattle (Musoke et al., 1992, 1993, 2005; Nene et al., 1999). All cattle respond by developing antibodies to p67. However, with the immunoassays used to date (anti-p67 antibody titers, sporozoite neutralizing antibody titers, and linear epitope mapping), it has not been possible to determine a correlate between anti-p67 responses and immunity to ECF. Thus, improvement of the current antisporozoite vaccine relies on challenge experiments making it difficult to rapidly assess different adjuvant formulations.

Major histocompatibility complex (MHC) class I-restricted cytolytic $CD8^+$ lymphocytes (CTLs) that target schizont-infected lymphocytes, the pathogenic stage of *T. parva*, play a primary role in mediating immunity to ECF in cattle that have been vaccinated by ITM (reviewed in Morrison, 2009). Thus, *T. parva* antigens recognized by CTLs are likely to be essential components of a subunit vaccine targeting the schizont stage of *T. parva*. There is a strong correlation between immunity to reinfection and the antigen specificity of CTLs (Taracha et al., 1995a,b), and the immunity can be adoptively transferred by $CD8^+$, but not $CD4^+$ T cells from animals vaccinated by ITM to their naïve twin (McKeever et al., 1994). This immunity is, however, parasite strain-specific and is influenced by the antigenic type of the immunizing parasite and the MHC genotype of individual animals, which leads to skewing and immunodominance in the specificity of the CTL response to the live vaccine.

The antigen specificity of $CD4^+$ and $CD8^+$ T-cells is mediated by the αβ T cell receptor, which binds to peptides in association with MHC class II and class I molecules, respectively (Neefjes et al., 2011). As a component of a search for schizont CTL antigens, we have developed a high throughput random immunoscreen of cDNA libraries made from highly enriched *T. parva* schizonts and a targeted screen of candidate genes selected from *T. parva* genome sequence data. In brief, the screens involved transfection of DNA into antigen-presenting cells and coculture with CTL lines from cattle vaccinated by ITM and analysis of IFN-γ production by ELISPOT assays followed by validation using cytolytic assays. Antigen-presenting cells were either autologous skin fibroblasts that had been transformed using the large T antigen of SV40 or COS cells that were cotransfected with an appropriate bovine MHC class I gene. Using this method, a total of 6 genes encoding CTL antigens (labeled Tp) were identified, and CTL epitopes were mapped on the antigens using overlapping synthetic peptides (Graham et al., 2006, 2007) (see Table 1).

Five of these antigens were tested in a vaccine trial with 24 cattle using naked DNA or poxviral vector as antigen delivery systems. In a prime-boost strategy, 7 animals were found to be immune to a needle challenge with *T. parva* sporozoites (Graham et al., 2006). Nineteen cattle were positive in IFN-γ ELISPOTs among which 7 were positive in cytolytic assays and were immune to challenge. While the level of protection was disappointingly low, the results support the working hypothesis of the importance of $CD8^+$ T cell responses in immunity to ECF and that a cytolytic rather than IFN-γ assay is the critical immune response, which correlates with protection (Graham et al., 2006). Experiments are underway to assess different antigen delivery systems (Mwangi et al., 2011) in an effort to identify a consistent and robust method for priming CTL responses in cattle.

The approach we used to identify CTL antigens was facilitated by our ability to routinely generate and maintain *T. parva*-specific CTL lines and clones and their targets, namely schizont-infected cells, in sufficient number. The latter behave like cancer cells and can be derived by coculture of sporozoites with peripheral blood mononuclear cells (PBMCs) making it possible to derive relevant target cells for vaccine studies. This biology is unique to some *Theileria* species (Dobbelaere and Rottenberg, 2003; Shiels et al., 2006). While highly successful, the approach we used is labor intensive and would be difficult, if not impossible, to employ to study other pathogens because of the difficulties associated with generating parasite-specific CTL reagents. To facilitate and accelerate the study of T cell responses in livestock and their role in vaccine development, we have started to employ more modern methods that are being used to study human and rodent immune responses.

The human MHC project–implications for livestock vaccine research

The human MHC project proposed to map all human MHC peptide specificities (Buus, 1999). Under this initiative, methods have been developed for prediction and validation of peptide MHC interaction, the former through computational biology and the latter through positional scanning combinatorial peptide libraries (PSCPL) or peptide binding assays with recombinant MHC molecules and generation of peptide–MHC tetramers for T cell analyses. In particular, the construction and availability of a large bank of human MHC molecules and the ability to constitute peptide–MHC tetramers in a "one-pot, mix-and-read" system has greatly accelerated the ability to discover new epitopes and to determine the specificity of $CD8^+$ T cells (Leisner et al., 2008). Similar advances are being made to predict MHC class II peptide interactions and for the generation of peptide–MHC class II tetramers, but this is a challenge because of the more promiscuous nature of the MHC class II relative to peptide–MHC class I interactions and that the peptide binding grove on MHC class II molecules is formed by heterodimers.

We have recently demonstrated that concepts and methods derived from the human MHC project can be applied to support the study of livestock immune responses. For example, recombinant SLA-1*0401, a frequent swine leukocyte antigen (SLA) molecule, has been generated in *E. coli* and used to scan a 9-mer PSCPL to determine a peptide-binding motif for this swine MHC class I molecule. Peptide anchor positions included 3 sites of increasing importance, 9 > 3 > 2, with an aromatic residue being preferred at the major anchor position (Pedersen et al., 2011). NetMHCpan (Hoof et al., 2009) (see below) was used to predict binding of a peptide (MTAHITVPY) from the P1 capsid precursor of foot and mouth disease virus (FMDV) strain A24 Cruziero to SLA-1*0401.

Table 1
List of *T. parva* CTL epitopes, restricting BoLA MHC class I gene and NetMHCpan predictions.

Antigen	CTL epitope	BoLA allele	N	FP1	FP2	Alternative epitope	FP3
Tp1	^{214}VGYPKVKEEML$_{224}$	N*01301	2138	0.268	0.070	^{214}VGYPKVKEEML$_{224}$	0.070
Tp2	^{27}SHEELKKLGML$_{37}$	N*04101	662	0.169	0.133	**^{29}EELKKLGML$_{37}$**	0.009
Tp2	^{40}DGFDRDALF$_{48}$ [a]	*Bos indicus*	662	0.420	0.328	**^{37}LEGDGFDRDAL$_{47}$**	0.012
Tp2	^{49}KSSHGMGKVGK$_{59}$	N*01201	662	0.023	0.017	^{49}KSSHGMGKVGK$_{59}$	0.017
Tp2	^{96}FAQSLVCVL$_{104}$	BoLA-T2c	662	0.000	0.036	^{96}FAQSLVCVL$_{104}$	0.036
Tp2	^{98}QSLVCVLMK$_{106}$	N*01201	662	0.027	0.036	^{98}QSLVCVLMK$_{106}$	0.036
Tp2	^{138}KTSIPNPCKW$_{147}$	*Bos indicus*	662	0.080	0.104	**^{138}KTSIPNPCK$_{146}$**	0.018
Tp4	^{328}TGASIQTTL$_{336}$	N*00101	2282	0.089	0.086	**^{327}ATGASIQTTL$_{336}$**	0.023
Tp5	^{87}SKADVIAKY$_{95}$	BoLA-T5	586	0.003	0.017	^{87}SKADVIAKY$_{95}$	0.017
Tp7	^{206}EFISFPISL$_{214}$	BoLA-T7	2850	0.077	0.058	**^{207}FISFPISL$_{214}$**	0.002
Tp8	^{379}CGAELNHFL$_{387}$	N*00101	1726	0.135	0.168	**^{380}GAELNHFLTL$_{389}$**	0.009
Tp9	^{199}AKFPGMKKSK$_{228}$	N*02301	1302	0.089	0.113	^{199}AKFPGMKKSK$_{228}$	0.113

CTL antigens and epitopes were identified from the Muguga strain of *T. parva* as briefly reviewed in the text (Akoolo et al., 2008; Graham et al., 2006, 2007, 2008; MacHugh et al., 2011; a R. Pellé, personal communication). The columns list: the antigen; location and sequence of the experimentally verified peptide epitope in the antigen; the BoLA MHC class I restriction element; N, number of 8, 9 10, and 11-mer peptides contained within each antigen; FP1, the false-positive fraction score for NetMHCpan trained without any BoLA peptide binding data; FP2, the false-positive fraction score for NetMHCpan trained with BoLA peptide binding data; an alternative epitope sequence with optimal predicted binding score (those different to the original are shown in bold); PF3, the false-positive fraction score calculated using the alternative epitope sequence trained with BoLA peptide binding data. Peptide binding data from BoLA MHC molecules N*01301, T2c, and N*02301 were part of the training data set (data not shown).

Peptide–MHC tetramers with this specificity were made and tested positive on PBMC from swine that had been vaccinated with antigen delivered with an Ad-5 viral construct while control pMHC tetramers made to ATAAATEAY were negative (Patch et al., 2011). Although a role for CTL responses in FMD per se remains to be determined, these experiments provide a powerful demonstration of proof-of-concept and on the simplicity of the assays. It is this combined approach that we are now applying to bovine.

In silico identification of peptide–MHC interactions – NetMHCpan

Training conventional data-driven methods for prediction of peptide MHC binding relies on significant amounts of accurate and reliable data that characterize the peptide binding specificity of each MHC molecule of interest (Yu et al., 2002; Zhang et al., 2009). This makes prediction of a population-wide range of peptide–MHC specificities a daunting task due to the immense diversity of MHC class I sequences. We have recently started to generate a solution to this conundrum: a pan-specific MHC predictor (Hoof et al., 2009). Using almost 120,000 data points covering more than 120 different HLA and non-human equivalent MHC molecules, we have developed NetMHCpan, which can predict peptides that bind to all HLA-A, -B, and -C molecules, even for those where no experimental data exists, called query molecules. This predictor can even be extended to other species such as non-human primates, mice, and pigs. An analysis of the predictive performance of the NetMHCpan method revealed a direct and inverse relationship between the distance of the similarity between the amino acids defining the query MHC molecule and the MHC molecules with characterized binding specificity included in the training of the method. This relationship emphasizes the strong need to identify uncovered regions of the MHC–peptide specificity space in order to effectively and rationally improve the pan-specific predictor.

Given this observation and in order to effectively characterize bovine leukocyte antigen (BoLA) MHC class I-binding specificities, we characterized the peptide binding specificity of a set of 3 recombinant BoLA MHC class I molecules with peptide libraries as described above for swine. Next, the NetMHCpan method was retrained including this set of peptide binding data for each of the 3 BoLA molecules (N*01301, T2c, and N*02301, data not shown). The 2 versions of the NetMHCpan method (with and without the BoLA peptide binding data) were next evaluated on a set of 12 known *T. parva* CTL epitopes. For each epitope, the BoLA restriction element and source parasite protein antigen is known. An evaluation was made for each epitope by predicting the binding affinity for all 8 to 11mer peptides contained within the source protein, and next calculating the fraction of peptides with a predicted binding affinity stronger than the predicted affinity of the known CTL epitope (the fraction of false-positive predictions). The results of these analyses are shown in Table 1.

Two points are striking. First, the predictive performance of the NetMHCpan method in identifying most of the *T. parva* CTL epitopes was high. Depending on the prediction method used (with or without BoLA training) 58–67% of the CTL epitopes were predicted to fall within the top 10% of predicted peptides. The second point is that the defined CTL epitopes do not seem to be optimal peptide binders to the restricting BoLA class I molecules. For 7 of the 12 peptides, NetMHCpan predicts a stronger binding of an alternative sequence that overlaps the mapped epitopes; 92% of these fall within the top 10% of predicted peptides, and as many as 83% of the alternative peptides are predicted within the top 5% of peptide sequences. It remains to be determined whether the new predictions are valid, as in one example when mapping the epitope on Tp7, the alternative peptide, FISFPISL was tested and did not react in an IFN-γ ELISPOT assay relative to the original peptide EFISFPISL (Graham et al., 2008). However, it is also clear from the results that the inclusion of the 3 BoLA peptide binding data had limited impact on the performance of the NetMHCpan method for predicting BoLA CTL epitopes. The overall performance was only marginally improved when adding the BoLA peptide binding data (average false-positive score from 0.115 to 0.097, column FP1 and FP2 in Table 1), and was in fact decreased for 2 of the 3 BoLA alleles where peptide binding data are available (T2c and N*02301). This observation underlines the continued need for large peptide binding data sets covering multiple and diverse BoLA molecules for optimal training of in-silico pan-specific prediction methods (Hoof et al., 2009).

NetMHCpan – implications for predictions across large proteomes

Despite the relatively high performance of NetMHCpan, the approach of predicting CTL epitopes across a large pathogen proteome based on just prediction algorithms will remain challenging. As an example, we limit the analysis to just one BoLA MHC class I molecule (N*04101) where according to Table 1 (column FP3) we have one of the most accurate predictions. The nested epitope EELKKLGML, found within the mapped epitope SHEELKKLGML, has a predicted binding score of 0.57 (data not shown). If we now take a set of 200,000 random 9mer peptides and predict their binding to N*04101, we find that approximately 1% of them have a predicted

value greater than 0.57. If this were extrapolated across the whole proteome of *T. parva*, 1% of 1,811,309 unique 9mer peptides would be potential T cell epitopes (~18,000). So while this approach is good for the discovery of new epitopes on known CTL antigens, it is still a challenge in finding new antigens per se unless additional filters are added in terms of which fraction of the predicted parasite proteome is analyzed. For example, restricting the analyses to genes only expressed in the schizont stage of the parasite and those that are predicted to be secreted and can access the host cell MHC class I antigen processing and presentation pathway. A complementary method is to include comparative parasite genomics to search for genes that carry signatures of evolutionary pressure, which could contribute to immune evasion (Carlton et al., 2005).

BoLA MHC class I diversity

The Immuno Polymorphism Database MHC BoLA website is the definitive source on bovine MHC genes (Robinson et al., 2010) and currently contains 114 BoLA class I sequences. Unfortunately, the bovine genome project did not assemble a complete sequence of the bovine MHC locus (Elsik et al., 2009; Zimin et al., 2009). While there is evidence for at least 2 loci of MHC class I genes (Bensaid et al., 1991; Toye et al., 1990), it remains unclear how many other functional genes are encoded in this locus, and determining a "reference" sequence will be complicated by the high level of sequence polymorphism at this locus (L. Skow, personal communication). An additional complication is that unlike in humans there seems to be variation in the number of BoLA class I genes expressed by individual cattle (Ellis, 2004; Ellis and Codner, in press).

From the context of predicting BoLA peptide binding specificity knowledge of exon 2 and exon 3 sequences of the MHC class I gene would be sufficient as they encode the $\alpha 1$ and $\alpha 2$ domains, which together form the MHC peptide binding groove. However, to have a better understanding of true MHC diversity in cattle, sequences of full-length MHC class I genes are required in order to capture sequence polymorphisms in other exons (Robinson et al., 2010). In an effort to increase our knowledge on BoLA diversity, in particular on African cattle, we have started to apply next generation sequencing methods to determine the sequence of expressed BoLA MHC class I genes. Sequencing of full length ~1.1-kbp PCR products amplified using near universal primers is in our hands not efficient (N. Svitek, unpublished). It remains to be determined if an upgrade to the Roche 454 sequencing platform to generate longer read lengths will resolve this issue. In the interim, we are exploring sequencing overlapping amplicons of smaller size more suitable for the current sequencing platform.

Bovine peptide – MHC class I tetramers: a "one-pot system"

An efficient system for expression and purification of functional MHC class I molecules in *E. coli* followed by a rapid one step process for constituting peptide–MHC tetramers has been developed (Leisner et al., 2008; Pedersen et al., 2011). In brief, an *E. coli* codon-optimized gene is cloned and expressed in-frame with a factor Xa cleavage site, a biotinylation signal peptide, and a histidine affinity tag using pET-28-derived plasmids. Biotin addition occurs during expression in vivo, and recombinant MHC is purified using a combination of affinity, ion-exchange and size-exclusion chromatography under denaturing and non-reducing conditions. Recombinant human, swine, and bovine $\beta 2$-microglobulin ($\beta 2$m) have also been produced in *E. coli*. Methods have been developed for measuring peptide–MHC interaction using radioassay and ELISA of defined peptide sequences or when using peptide libraries (PSCPL). The latter was used to generate peptide binding data used to train NetMHCpan with data derived from 3 BoLA class I molecules (data not shown, Table 1). Peptide–MHC monomers are made at high efficiency by dilution of purified MHC class I molecules with an excess of peptide and $\beta 2$ m, followed by addition of streptavidin labeled with a fluorochrome to create peptide–MHC tetramers, which can then be used to rapidly assay T cells. At least 6 more BoLA MHC class I molecules will be expressed, and all will be available for the construction of peptide–MHC tetramers. These reagents should help in trying to predict the functional consequence of *T. parva* epitope variants that are being discovered through gene sequence analyses (Pellé et al., 2011).

Conclusions

The role of T cells in innate and acquired immune responses including the maturation of humoral immune responses to pathogens is understudied because of the complexities associated with the immunobiology of T cells. This represents a large gap in the field of vaccine development, but it is being addressed through increasing our basic knowledge of the immune system and by adding new methods to the repertoire of tools available for the identification of candidate vaccine antigens and formulating them into effective vaccination systems. Here, we describe the application of methods to predict and validate bovine MHC class I epitopes to facilitate the direct study of $CD8^+$ T cells. Efforts are underway within our group to extend these technologies to study $CD4^+$ T cells. Together, these technologies will facilitate the identification and study of the role of epitope-specific T cell immune responses in cattle to infection and during vaccine trials.

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Article

Homology Modeling and Analysis of Structure Predictions of the Bovine Rhinitis B Virus RNA Dependent RNA Polymerase (RdRp)

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Abstract: Bovine Rhinitis B Virus (BRBV) is a picornavirus responsible for mild respiratory infection of cattle. It is probably the least characterized among the aphthoviruses. BRBV is the closest relative known to Foot and Mouth Disease virus (FMDV) with a ~43% identical polyprotein sequence and as much as 67% identical sequence for the RNA dependent RNA polymerase (RdRp), which is also known as 3D polymerase ($3D^{pol}$). In the present study we carried out phylogenetic analysis, structure based sequence alignment and prediction of three-dimensional structure of BRBV $3D^{pol}$ using a combination of different computational tools. Model structures of BRBV $3D^{pol}$ were verified for their stereochemical quality and accuracy. The BRBV $3D^{pol}$ structure predicted by SWISS-MODEL exhibited highest scores in terms of stereochemical quality and accuracy, which were in the range of 2¡ resolution crystal structures. The active site, nucleic acid binding site and overall structure were observed to be in agreement with the crystal structure of unliganded as well as template/primer (T/P), nucleotide tri-phosphate (NTP) and pyrophosphate (PPi) bound FMDV $3D^{pol}$ (PDB, 1U09 and 2E9Z). The closest proximity of BRBV and FMDV $3D^{pol}$ as compared to human rhinovirus type 16 (HRV-16) and rabbit hemorrhagic disease virus (RHDV) $3D^{pols}$ is also substantiated by phylogeny analysis and root-mean square deviation (RMSD) between C-α traces of the polymerase structures. The absence of positively charged α-helix at C terminal, significant differences in non-covalent interactions especially salt bridges and CH -pi interactions around T/P

channel of BRBV 3D ^{pol} compared to FMDV 3D ^{pol}, indicate that despite a very high homology to FMDV 3D^{pol}, BRBV 3D^{pol} may adopt a different mechanism for handling its substrates and adapting to physiological requirements. Our findings will be valuable in the design of structure-function interventions and identification of molecular targets for drug design applicable to *Aphthovirus* RdRps.

Keywords: *Aphthovirus*; BRBV; homology modeling; 3D^{pol} structure predictions

1. Introduction

Viruses belonging to family *Picornaviridae*, cause a wide range of human and animal diseases. The family *Picornaviridae* consists of 12 distinct genera: *Enterovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Kobuvirus*, *Teschovirus*, *Sapelovirus*, *Senecavirus*, *Tremovirus* and *Avihepatovirus*. Picornaviruses are genetically diverse, small non-enveloped viruses with a single strand positive sense RNA genome of 7ï 8.9 Kb [1]. Their classification is based on physiochemical characteristics, genome and mode of replication.

Rhinoviruses were originally classified as separate genus of the family *Picornaviridae*. Based on available sequence and phylogenetic analysis, the International Committee on Taxonomy of Viruses (ICTV) has recently deleted the genus *Rhinovirus* and placed human rhinoviruses in the genus *Enterovirus* and bovine rhinoviruses in the genus *Aphthovirus* [2]. Bovine rhinitis viruses (BRV) are represented by two species, bovine rhinitis A virus (BRAV-1 and BRAV-2) and bovine rhinitis B virus (BRBV). The latter has only one serotype, bovine rhinitis B virus type-1 (BRBV-1). Initially a BRBV isolate EC-11, also known as BRV- 2, was isolated from the lung of a specific pathogen-free calf that unexpectedly developed a respiratory disease [3]. In a recent study the virus was reported to be genetically closer to FMDV than HRV on the basis of phylogenetic characterization and full genome analysis [2].

The BRBV genomic RNA encodes a large polyprotein that is processed into structural (capsid) and non-structural polypeptide products by viral proteases during the infection. Picornavirus replication is catalyzed by the RdRp enzyme. RdRp is encoded by the 3D region of the genome at the *C*-terminus of the polyprotein and hence termed as 3D^{pol}. The available crystal structures of a number of picornaviral 3D^{pols} show right hand structure with thumb, palm and finger domains common to all oligonucleotide polymerases [4ï 9]. 3D^{pols} have an additional fingertip region bridging finger and thumb domains [7]. In contrast to DNA polymerases that undergo conformational change to accommodate the substrate viral RdRps are not thought to involve conformational motions during the process of RNA replication. However, recent studies do show some evidence of conformational rearrangements in RdRps [10,11]. The crystal structure of FMDV RdRp in the unliganded form and in complex with nucleic acid and NTP revealed extensive similarities in terms of overall structural attributes with other RdRps [12]. The putative structural motifs involved in nucleotide recognition and binding (A and B), phosphoryl transfer (A and C), structural integrity of the palm domain (D) and priming nucleotide binding (E) [12,13] are conserved among polymerases [8,14ï 16]. Motif F in finger domain, a unique feature of RdRps, is involved in interactions with the incoming nucleotide [7].

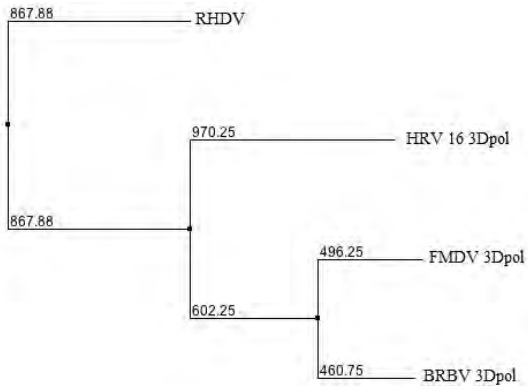
The availability of the sequence information of the bovine rhinitis virus genome and the high resolution crystal structure of related RdRps (picornaviruses such as FMDV, poliovirus, coxsackievirus, HRV and caliciviruses such as RHDV) allow us to model the structure of the BRBV 3D^{pol} protein, which can potentially reveal new information about the general feature as well as specific properties of viral RdRps. Therefore, in this study we examined the functional domains, sequence features and the three dimensional structure of the modeled BRBV 3D^{pol}, which could broaden our understanding of the structure-function relationship in RdRps among aphthoviruses.

2. Results and Discussion

2.1. Phylogenetic Analysis of BRBV RdRp

As shown in Figure 1, the distance map generated by BLOSUM62 scoring scoring matrix imbedded in Jalview platform [17,18] shows that BRBV 3D^{pol} is genetically closest to FMDV followed by HRV and RHDV 3D^{pol}, respectively. The result supports the earlier study reporting close proximity of BRBV (previously known as BRV-2) to FMDV [3].

Figure 1. Phylogenetic analysis of Bovine Rhinitis B Virus (BRBV) 3D^{pol}: An un-rooted tree showing the genetic relatedness of BRBV 3D^{pol} to other RdRps. The distance represents the difference from BRBV 3D^{pol} sequence conservation.

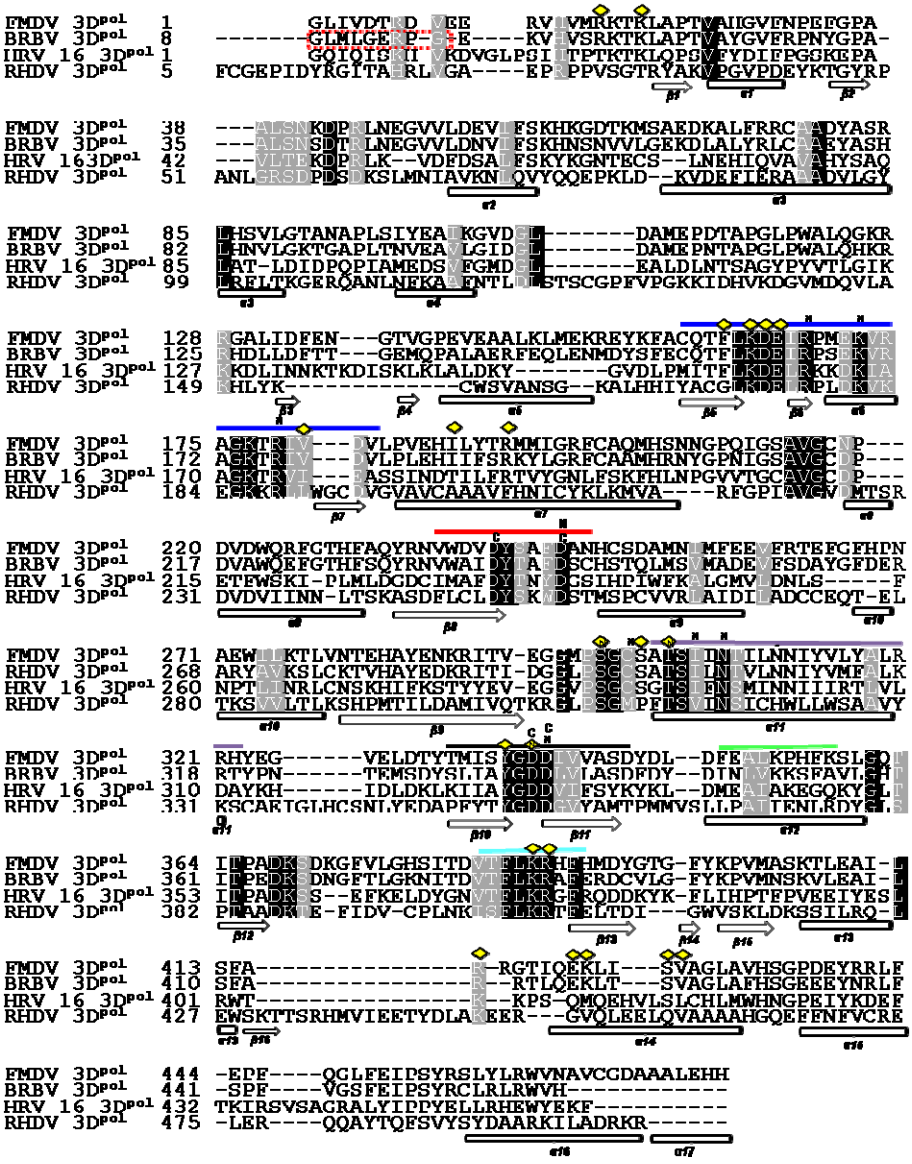


2.2. Structure Based Sequence Alignment

The structure based alignment of the sequences of 3D^{pol} proteins of BRBV, FMDV, HRV-16 and RHDV was carried out using magic fit algorithm followed by iterative magic fit refinement on Deep View platform [19]. The alignment was verified and curated manually by looking at the superposed structures in order to define the secondary structure elements (SSEs) and functional motifs of all RdRps accurately. The results indicated that the BRBV 3D^{pol} shares 64.03% sequence identity with the FMDV protein counterpart, followed by HRV-16 3D^{pol} (29.39%) and RHDV 3D^{pol} (20.21%) respectively (Figure 2). BRBV 3D^{pol} shows 76.9, 94.12, 85.7, 45, 100 and 95.8% conservation for motifs A, B, C, D, E and F, respectively. These motifs are conserved in HRV-16 3D^{pol} as well, though to a lesser extent. BRBV, HRV-16 and FMDV 3D^{pol} seem to utilize the same T/P binding, NTP binding and active site. RHDV 3D^{pol} has a noticeably different T/P binding interface with fewer positive residues (Figure 2). The catalytic site is absolutely conserved among all the polymerases

analyzed. The structure based sequence alignment suggests a close proximity between BRBV and FMDV 3D^{pol} followed by HRV-16 and RHDV 3D^{pol}.

Figure 2. Structure based alignment of BRBV 3D^{pol} with other related RdRPs: The conserved regions are shaded black and gray to mark identical and similar residues, respectively. Each polymerase sequence is labeled starting from the left margin and the numbers next to polymerase in each row represent the sequence position. The red broken rectangle shows the sequence of BRBV 3D^{pol} excluded while preparing the homology model. Functionally important motifs A, B, C, D, E and F are marked with red, purple, black, green, light blue and blue bars, respectively. α-helices and β-sheets are numbered starting from the N-terminus of protein and marked with open cylinders and open arrows. Functional motifs A, B, C, D, E and F are marked with red, purple, black, green, light blue and blue bars, respectively. Their putative function has been discussed in the introduction. The nucleic acid binding residues are marked with the yellow diamonds. The nucleotide binding and catalytic sites are marked with the letters N and C. The motifs and other key structural features are based on the structure of FMDV 3D^{pol} described earlier [7].



2.3. Search for Structural Homologues to BRBV 3D^{pol} via deconSTRUCT Web Server

The modeled structures of BRBV 3D ^{pol} prepared by Geno3D and SWISS-MODEL [20ï26] were submitted to the deconSTRUCT web server. deconSTRUCT finds homologous proteins via a totally different approach as discussed in the materials and methods section [27]. The details of the modeling protocol and analysis of structures are discussed in sections 2.4ï2.8. The results from the structure based homologue search generated by deconSTRUCT suggested that the BRBV 3D ^{pol} has the highest homology with the FMDV 3Dpol followed by Hepatitis C virus and RHDV polymerases (Table 1). However, as shown in Figure 2, when we superposed the structures of FMDV, BRBV, HRV-16 and RHDV RdRps and analyzed key structural motifs, BRBV 3D ^{pol} was found to be closest to FMDV followed by HRV-16 and RHDV 3D ^{pol}. This discrepancy of prediction could be attributed to the methodology adopted by deconSTRUCT. Since it compares parts of proteins with fewer SSEs, this could result in a stronger signal for a distantly related protein. The superposition of structures on the other hand provides better control over comparison of overall as well as in part structure of proteins.

Table 1. Alignment score from deconSTRUCT search.

PDB id	Aln. Score	Aln. Length	RMSD (Å)	Avg. dL	Geom. Z	Target Molecule
1U09A	330.50	370	1.42	1.88	−7.20	FMDV RdRp
2B43A	273.36	337	2.65	2.11	−4.04	Hepatitis C virus (HCV) RdRp
1KHWA	259.87	316	2.43	2.75	−3.44	RHDV RdRp
1S48A	182.59	231	2.87	2.50	0.51	Bovine viral diarrhea virus (BVDV Rna-dependent rna polymerase
2JL9A	116.57	148	2.94	1.67	1.58	Phi6 RNA polymerase

Aln. Score is the alignment value obtained from the web server after the search for the closely resembling protein structures available in PDB; Aln. Length is the length of the residues aligned to the query sequence.

2.4. Preparation of Homology Model of BRBV 3D^{pol} and the Assessment of Model Accuracy

BRBV 3D^{pol} was modeled on FMDV and FMDV+HRV-16 3D^{pol} via SWISS-MODEL and Geno3D web servers, respectively. The stereochemical quality and accuracy of the models were tested using PROCHECK [28]. Results from PROCHECK are reported as Ramachandran plots (Figure 3). The main and side chain parameters are summarized in (Tables 2A and 3A) and (Tables 2B and 3B), respectively and discussed in the context of accuracy of the structure prediction. Ramachandran plot is one of the oldest yet most reliable methods of determination of the quality of protein structure. A structure with ≥90% of its residues in the most favored regions A, B and L of Ramachandran plot is considered to be as accurate as a 2¡-resolution crystal structure [28]. Only 73.1% residues in the Geno3D generated model of BRBV 3D ^{pol} are in the most favorable regions of Ramachandran plot (Figure 3A, Table 1A). In contrast SWISS-MODEL generated structure has 90.15% of its residues in the most favorable region of Ramachandran plot (Figure 3B, Table 1B). Other main chain parameters such as omega angle standard deviation, bad contacts/100 residues, zeta angle standard deviation, hydrogen bond energy standard deviation and overall G factor, which underscores to structural

accuracy of a structure as determined by PROCHECK, also show that the SWISS-MODEL generated structure has better structural attributes (Tables 1A and 2A).

Figure 3. Ramachandran plot analysis of BRBV3D^{pol} structures: The Ramachandran plots of modeled BRBV structure generated by Geno3D (**A**) and SWISS-MODEL (**B**). The most favored regions are m arked as {A, B, L}. Th e additional allowed regions are m arked as {a, b, l, p}. All non-glycine and proline residues are shown as filled black squares, whereas, glycines (non-end) are shown as filled black triangles. Disallowed residues are colored red.

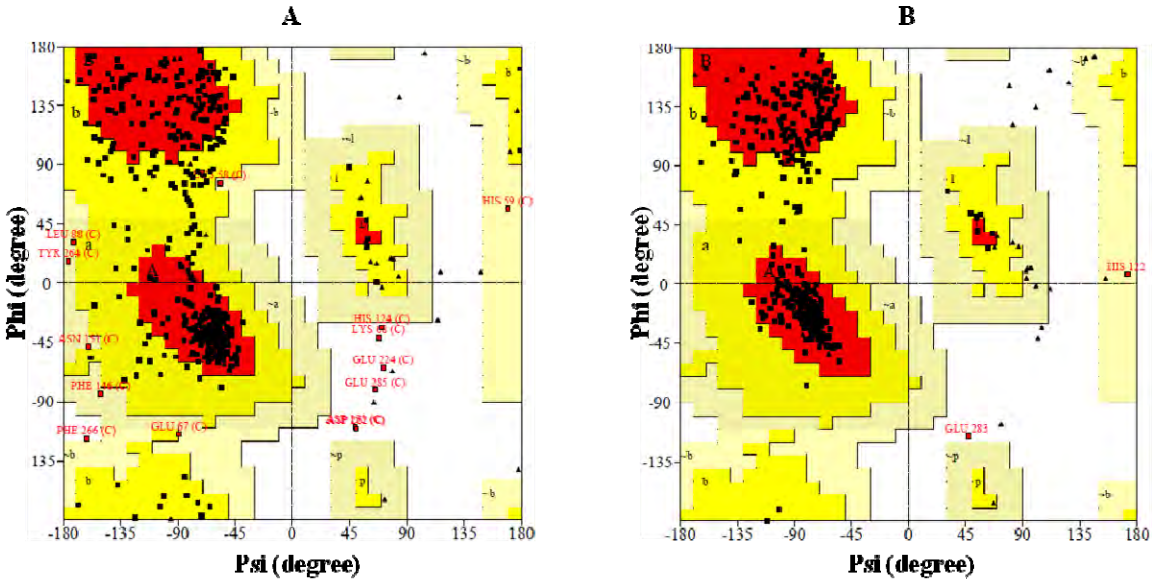


Table 2A. Summary of main chain parameters for the structure generated by Geno3D.

Stereochemical quality	No. of data points	Parameter value	Comparison typical value	Value band width	No. of band widths from mean	Interpretation
% residues in A,B,L	413	73.6	83.8	10	−1.0	WORSE
Omega angle St. Dev.	463	0.6	6.0	3.0	−1.8	BETTER
Bad Contact/100 Residue	0	0	4.2	10	−0.4	INSIDE
Zeta angle St. Dev.	433	1.1	3.1	1.6	−1.3	BETTER
H-bond Energy St.Dev.	283	0.7	0.8	0.2	−0.5	INSIDE
Overall G-Factor	465	0.2	−0.4	0.3	1.9	BETTER

St. Dev. denotes the stand ard deviation of the score obser ved. The accuracy of structure is depicted in th e order BETTER > INSIDE > WORSE each parameter.

Table 2B. Summary of main chain parameters for the structure generated by SWISS-MODEL.

Stereochemical quality	No of data points	Parameter value	Comparison typical value	Value band width	No. of band widths from mean	Interpretation
% residues in A,B,L	405	90.1	76.6	10	1.4	WORSE
Omega angle St. dev.	452	5.2	6.0	3.0	−0.3	BETTER
Bad Contact/100 Residue	0	0	10.5	10	−1.1	INSIDE
Zeta angle St. Dev.	425	1.0	3.1	1.6	−1.3	BETTER
H-bond Energy St. Dev.	320	0.7	0.9	0.2	−1.1	INSIDE
Overall G-Factor	454	0.1	−0.6	0.3	2.2	BETTER

St. Dev. denotes the stand ard deviation of the score obser ved. The accuracy of structure is depicted in th e order BETTER > INSIDE > WORSE each parameter.

Table 3A. Summary of side chain parameters for the structure generated by Geno3D.

Stereochemical quality	No. of data points	Parameter value	Comparison typical value	Value band width	No. of band widths from mean	Interpretation
Chi-1 gauche minus St. Dev.	52	10.0	22.7	6.5	−1.9	BETTER
Chi-1 trans St. Dev	118	10.1	22.7	5.3	−2.4	BETTER
Chi-1 gauche plus St. Dev.	200	10.1	21.3	4.9	−2.3	BETTER
Chi-1 pooled St. Dev.	370	10.2	22.0	4.8	−2.4	BETTER
Chi-2 transst dev	108	8.6	23.1	5.0	−2.9	BETTER

St Dev. denotes the standard deviation of the score observed. The accuracy of structure is depicted in the order BETTER > INSIDE > WORSE each parameter.

Table 3B. Summary of side chain parameters for the structure generated by SWISS-MODEL.

Stereochemical quality	No. of data points	Parameter value	Comparison typical value	Value band width	No. of band widths from mean	Interpretation
Chi-1 gauche minus St. Dev.	60	16.6	18.1	6.5	−0.2	INSIDE
Chi-1 trans St. Dev.	132	11.9	19	5.3	−1.3	BETTER
Chi-1 gauche plus St. Dev.	185	13.8	17.5	4.9	−0.8	INSIDE
Chi-1 pooled St. Dev.	377	14.2	18.2	4.8	−0.8	INSIDE
Chi-2 trans St. Dev.	86	14.7	20.4	5.0	−1.1	INSIDE

St. Dev. denotes the standard deviation of the score observed. The accuracy of structure is depicted in the order BETTER > INSIDE > WORSE each parameter.

Side chain parameters were shown as chi1-chi2 side chain torsion angle combinations for all residue types whose side chains are long enough to have both these angles. The chi1-chi2 plots which were presented as side chain parameters in Tables 2A and 2B indicate SWISS-MODEL generated structure exhibited higher score in terms of overall structural accuracy. In conclusion, the results from PROCHECK analysis clearly suggested that the modeled structure of BRBV 3D^{pol} generated by SWISS-MODEL program was superior in quality. Therefore, further characterization of the modeled structure of BRBV was therefore, carried out on SWISS-MODEL generated structure.

2.5. Comparison of BRBV 3D^{pol} and Other 3D^{pol} Crystal Structures

PDB coordinate file can be found as supplementary information. As shown in Figure 4, the modeled structure of BRBV (cyan) and crystal structures of HRV-16 (white) as well as RHDV 3D^{pol} (blue) were superimposed on FMDV 3D^{pol} (yellow) (PDB, 1U09). BRBV Model showed high degree of homology to FMDV 3D^{pol} followed by HRV-16 and RHDV counterparts. The thumb domain of RHDV 3D^{pol} was observed to be shifted inwards as compared to the FMDV and other RdRps. RHDV polymerase lacks critical positively charged loop (shown in red oval, Figure 4), which aligns the template-binding surface observed in FMDV. BRBV 3D^{pol} appears to lack *C*-terminal α-helix as compared to FMDV counterpart (marked with yellow oval). FMDV 3D^{pol} *C*-α trace RMSD were 0.758, 1.518, and 4.607 for BRBV 3D^{pol} model, HRV-16 and RHDV 3D^{pols}, respectively. It is important to note that the RMSD score of ≤1.0 ¡ indicates a very good agreement between the template and homology model. The comparison of three dimensional structures of the RdRps also

supports the close relatedness of BRBV 3D ^{pol} to its FMDV counterpart. As indicated with arrows in Figure 5 the modeled structure generated by Geno3D (pink color) has an important α-helix disordered in the finger domain. In addition, the loop aligning the template channel is pushed upwards in this model. In contrast the SWISS-MODEL generated structure (cyan color) has higher degree of conservation of these structural features in the context of FMDV 3D^{pol}.

Figure 4. Stereoview of superimposed RdRp structures: A stereoview of ribbon representation of BRBV, HRV-16 and rabbit hemorrhagic disease virus (RHDV) RdRp 3D^{pols} superimposed on to foot and Mouth Disease virus (FMDV) 3D ^{pol} (PDB, 1U09). Finger, palm and thumb domains are labeled. FMDV, BRBV, HRV-16 and RHDV 3D ^{pols} are colored yellow, cyan, white and blue, respectively. An important loop, which aligns the template channel, is not present in RHDV 3D ^{pol} (red oval). BRBV 3D ^{pol} lacks the *C*-terminal α-helix in comparison to FMDV 3D ^{pol} (yellow oval). The parts covering palm domain and the loop aligning T/P channel, have been removed to facilitate visualization of the regions with significant differences.

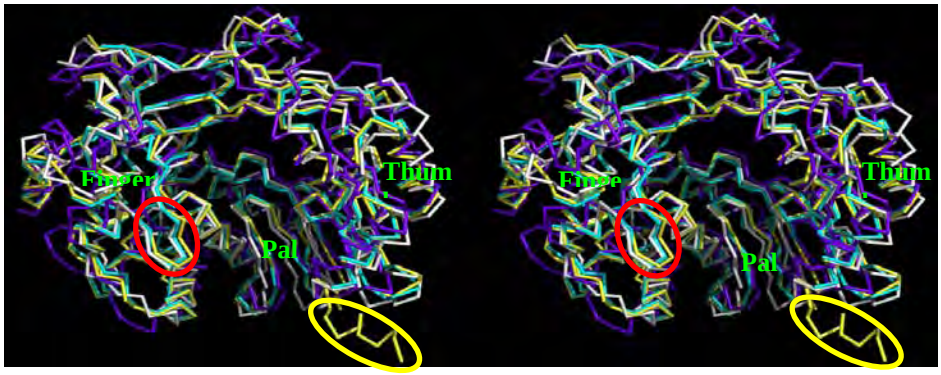
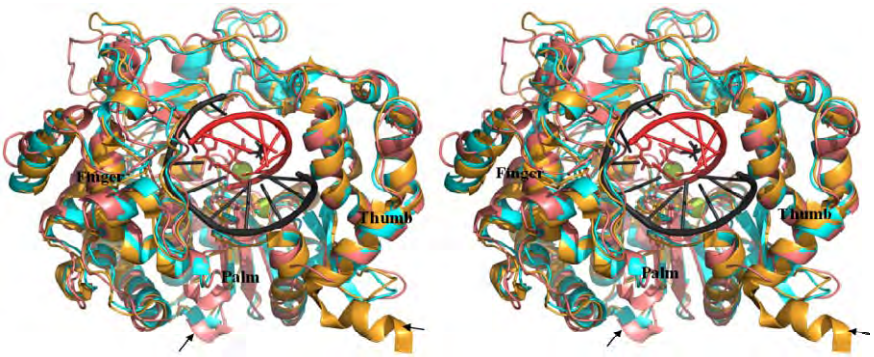


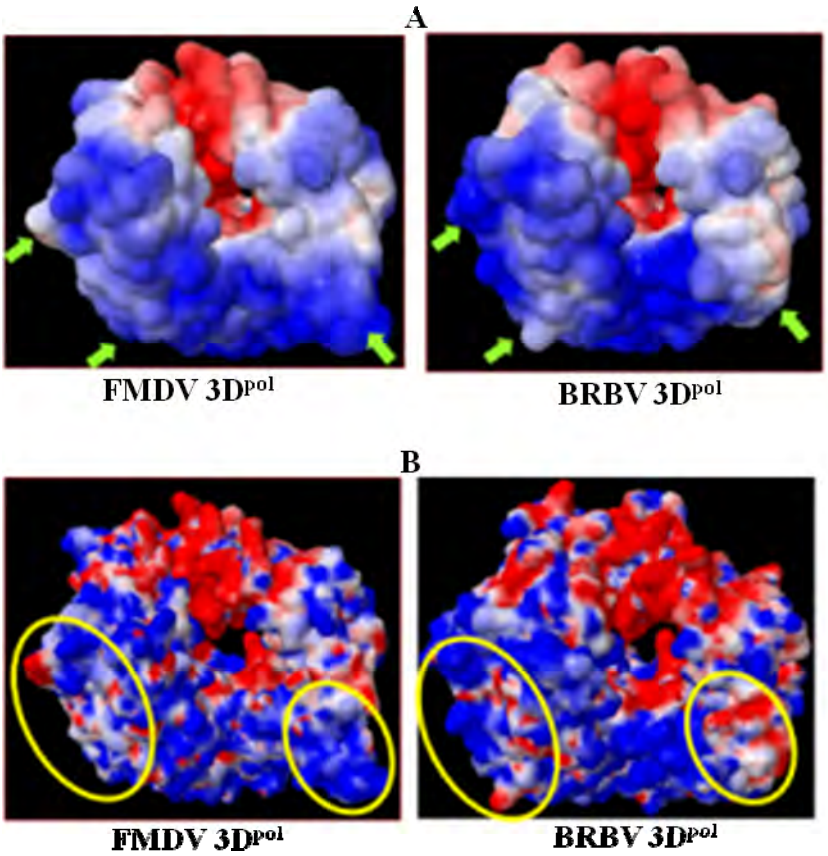
Figure 5. Stereoview of modeled BRBV 3D^{pol} superimposed on FMDV3D^{pol}: Polymerases and RNA template/primer are presented as cartoon structures. Uridine 5'-triphosphate (UTP) and pyrophosphate (PPi) are presented as sticks. Metals (Mg^{++}) are presented as green spheres. The modeled structures generated by Geno3D [A] and SWISS-MODEL [B] are colored pink and cyan, respectively. FMDV 3D pol protein is colored as bright orange. The template and primer RNA are colored as gray and red, respectively. UTP and PPi are colored red, and gray, respectively. Finger, palm and thumb domains of the polymerase are marked. Major structural differences are highlighted with black arrows and discussed in the text.



2.6. Analysis of Key Structural Features of FMDV 3Dpol and BRBV Homology Model

The electrostatic surface potentials of modeled BRBV $3D^{pol}$ and FMDV $3D^{pol}$ apo enzyme were calculated using the Adaptive Poison Boltzman Solver (APBS) via PDB2PQR web server [29,30]. Solvent exposed and solvent excluded electrostatic surface structures were prepared for both FMDV and BRBV $3D^{pol}$ (Figure 6A,B). Total electrostatic energies were recorded to be in total 4.15 Ĭ 10^5 and 4.29 Ĭ 10^5 kilo-Joule per mole (kJ/mol), respectively for FMDV and BRBV $3D^{pol}$. The similar distribution of the electrostatic potential energy also substantiates similar structural attributes of the two polymerases. However, as reflected in Figure 6A,B the BRBV $3D^{pol}$ lacks the C-terminal α-helix with positively charged surface observed in FMDV counterpart. Other key differences in the distribution of positively charged, neutral and negatively charged residues have been indicated by green arrows and yellow ovals in Figure 6A,B, respectively. It is important to note that BRBV $3D^{pol}$ has more positively charged residues at the base of finger subdomain as opposed to FMDV $3D^{pol}$, which has more neutral amino acids in this region (white). In contrast the base of thumb subdomain is positively charged in FMDV $3D^{pol}$, C terminus of BRBV $3D^{pol}$ has an uncharged surface. These differences could individually or collectively contribute to physiochemical behavior as well as the function of the two enzymes.

Figure 6. Electrostatic surface of BRBV $3D^{pol}$: Electrostatic surface of BRBV $3D^{pol}$ and FMDV $3D^{pol}$ are colored red to blue ranging from most negative (−5 kT/e) to most positively (+5 kT/e) charged regions. Neutral surface is shown in white color. Major differences in the distribution of solvent exposed and solvent excluded surface residues are indicated with green arrows (**A**) and yellow ovals (**B**).



2.7. Analysis on Non-Covalent Interactions in FMDV and BRBV 3D^{pol}

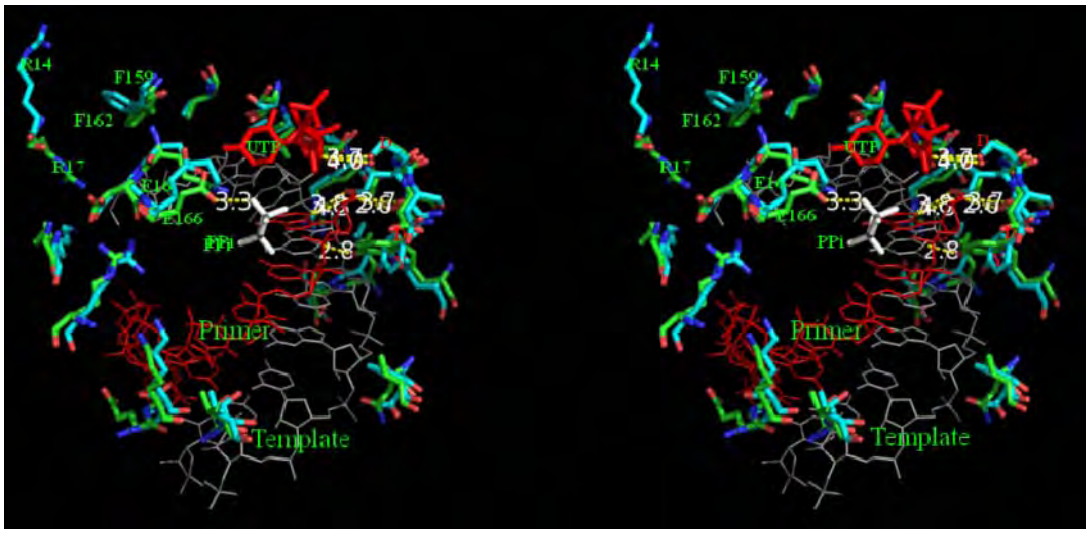
Non-covalent interactions such as hydrogen bonds, salt bridges, van der waals interactions, hydrophobic interactions, cation-pi and CH-pi bonds are individually weak but collectively they determine the structure and behavior of proteins. Hydrogen bonds and salt bridges were calculated using VMD [31]. BRBV 3D^{pol} was observed to forms an extensive network of hydrogen bonds (46 hydrogen bonds) exactly similar to FMDV 3D^{pol}. The distribution of these bonds was also very similar between FMDV and BRV 3D^{pol}. Since hydrogen bonds are major contributors of protein folding and function, it is conceivable that given other similar features BRBV 3D^{pol} is homologous to its FMDV counterpart. However, the number of salt bridges was strikingly different between BRBV FMDV 3D^{pol}, which form 1 and 17 salt bridges, respectively. Since salt bridges are critical for the thermal behavior of proteins it would be interesting to explore the temperature sensitivity of the protein and compare it to FMDV and other closely related proteins. The cation-pi interactions, formed between positively charged amino acids and aromatic amino acids, when they are in close proximity, contribute significantly to the protein structure [32]. Arginine (R) is more likely than lysine (K) to participate in a cation-pi interaction. The order of participation of aromatic side chains in cation-pi interactions is Tryptophan (W) > Tyrosine (Y)> Phenylalanine (F). We recorded 4 energetically significant cation-pi interactions in FMDV 3D^{pol}, which exist between R17-F162, R153-W273, R388-W223, and K400-W462. In contrast BRBV 3Dpol possesses only 1 energetically favorable cation-pi interaction, which is formed between R385 and W220.

2.8. Analysis of Template/Primer (T/P) Binding Interface and Active Site of BRBV 3D^{pol}

T/P binding interface of BRBV 3D^{pol} was mapped on to FMDV 3D^{pol} bound to decameric RNA, uridine tri-phosphate and pyrophosphate (PDB, 2E9Z). As shown in Figure 7, the template binding interface of both BRBV 3D^{pol} and FMDV 3D^{pol} are aligned with positively charged amino acids, which is an adaptation of the RdRp to accommodate negatively charged nucleic acid substrate. It is interesting to note that the side-chains of R14 and F159 of BRBV have been flipped out of the plane in comparison to FMDV 3D^{pol}. Their counterparts in FMDV 3D^{pol} form a strong cation-pi interaction. This might be a key stabilizing interaction, which holds the β-loop and keeps the T/P binding interface of the polymerase intact. The cation-pi interaction between W220 and R385 in BRBV 3D^{pol} could be an important determinant of its function. This bond is formed between β-13 strand and α-8 helix in the palm region that align the catalytic motif of the polymerase. Glutamate 163 is also moved out of the template channel losing an important hydrogen bond interaction with pyrophosphate in BRBV 3D^{pol} (Figure 7). Some of the differences in the bonding pattern could also be attributed to the use of apo structure of FMDV 3D^{pol} as a template for homology modeling. Since, it is an unliganded structure, there is more rotational freedom for the amino acids as compared to RNA and NTP bound polymerase. Such rotations may yield the altered conformations of amino acid side chains and hence alter the bonding pattern as well to some extent. Alternatively, it is also possible that in a replicating polymerase it is the alternate conformation of these amino acids that is predominant. Such structural rearrangements are gaining attention as a mechanism that RdRps adopt to facilitate the replication activity of their polymerases [10,11]. It is important to note that the available literature suggests that

growth of BRBV in tissue culture is difficult to achieve [33] and virus infected cells incubated at 31 or 33 ÁC display more pronounced CPE than when incubated at 37 ÁC [2]. These studies also support a different physiological and thermal behavior of BRBV 3D ^{pol} suggested herein on the basis of the structure and bonding pattern.

Figure 7. Stereoview of functionally important residues of BRBV 3D^{pol} and FMDV 3Dpol: Template/primer binding residues and active site of BRBV 3D ^{pol} (cyan) and FMDV 3D ^{pol} (green) are shown is stick representation. The nitrogen and oxygen atoms of the amino acids are colored blue and red, respectively. Template and primer are colored gray and red respectively and shown as lines. UTP and PPi are shown as sticks colored red and light gray, respectively. Amino acid residues are labeled as single letter amino acid codes with the identifiers. Catalytic tyrosine and glutamate residues are red colored one letter residue symbols. Arginine 14 and phenyl alanine 159 are flipped out of the nucleic acid binding interface in BRBV 3D^{pol}. Hydrogen bonds are shown with yellow dotted lines and marked with bond length.



3. Experimental Section

3.1. Sequence

BRBV polypeptide sequence (NCBI Reference Sequence: YP_003355055.1) was utilized to retrieve the putative 3D^{pol} region, which was later utilized for sequence comparisons and homology modeling.

3.2. Phylogenetic Analysis

Phylogeny tree was prepared in order to determine the relatedness of BRBV 3D ^{pol} to other related polymerases. The tree was calculated from distance matrices determined from percent identity or aggregate BLOSUM62 score using average distance as implemented in JalView [17,18]. In this method, pairwise distances used to cluster the sequences are represented as the percentage of mismatches between two sequences. The branch lengths are the percentage mismatch between two nodes; the leaves show the sequence IDs.

3.3. Structure based Alignment of the Polymerase Sequences

The structure based alig nment of BRBV 3Dpol with other Rd Rps was perform ed using iterative magic fit function in corporated in the Deep view -[19]. The aligned sequences were m anually curated to characterize the key structural motifs in each protein accurately. The key structural features such as functional motifs as well as T/P and nucle otide triphosphate interacting residues were a ssigned different color and symbols manually.

3.4. Preparation of Homology Model of BRBV 3D^{pol}

The homology models of the protein were prepared via:

(i) Geno3D web server

Crystal structures of FMDV (PDB, 1U09) a nd HRV-16 3D ^{pol} (PDB, 1XR7) were utilize d as templates for model generation. After the completion of run 10 hom ology models were prepared and their quality was assessed through PROCHECK [20,28]. Geno3D (http://geno3d- pbil.ibcp.fr) is an automated web server for protein molecular m odeling [20]. Starting with a query protein sequence, the server performs the homology modeling in six successive steps: first, it identifies homologous proteins with known structures by using PSI-BLAST [21]. This provides the user all potential tem plates for target selection. After the user defines the templa tes and subm its the job, the server perform s the alignment of both query and subject sequences and extr acts geometrical restraints (dihedral angles and distances) for corresponding atom s between the query and the tem plate. Lastly, it perform s the 3D construction of the protein by using a distance geometry approach.

(ii) SWISS-MODEL

The crystal structure of FMDV 3Dpol (PDB, 1U09) was identified as the closest match to the BRV counterpart and later utilized as template by SW ISS-MODEL workspace, which is an integ rated web-based modeling environm ent. For a given target protein it searches a library of experim ental protein structures to id entify suitable templates. On the basis of a sequence alig nment between the target protein and the template structure, a three-dimensional model for the target protein is generated. The alignment produced by SW ISS-MODEL was veri fied using another alignm ent algorithm (T-coffee). In hom ology modeling the most crucial steps is the evaluation and refinem ent of the raw model and SWISS-MODEL utilizes a set of unique analysis tools in addition to commonly tools such as PROCHECK and WHAT CHECK [22ï 26,28,34] to achieve this. These tools include atom ic empirical mean force p otential (ANOLEA) which performs energy calculations on a protein chain, evaluating the ñNon- Local Enviro nmentò (NLE) of each heavy atom in the m olecule and is used to assess packing quality o f the m odels; QMEAN whic h is a com posite scoring function for both the estimation of the global quality of the entir e model as well as f or the local per- residue analysis of different regions within a model; DFIRE, which is a n all-atom statistical potential based on a distance-scaled finite ideal-gas reference state and r eflects the quality of the m odel to indicate that a that a m odel is lower energy closer to the na tive conformation [35ï 37]. Finally, it minimizes the structure using GROMOS9639 performing 200 cycles by the steepest descent method and 300 cycles by the conjugate gradient method to m inimize the steric clashes [38]. With stringent quality controls SWISS-MODEL workspace usually generates models with a reasonably reliable quality.

3.5. DeconSTRUCT Analysis of the Homology Models of BRBV 3D^{pol}

In order to find the degree of homology of BRBV 3D^{pol} to other viral RdRps, the best scoring homology models of BRBV 3D^{pol} prepared by both SWISS-MODEL and Geno3D were submitted to the deconSTRUCT web server, which offers an interface to a protein database search engine that detects similar protein sub-structures. Firstly, it deconstructs the query structure into its SSEs and finds the match to the target by requiring a (tunable) degree of similarity in the direction and sequential order of SSEs. The search engine of deconSTRUCT utilizes the hierarchical organization and judicious use of the information about protein structures to achieve the sensitivity and specificity of the established search engines at orders of magnitude increased speed, without tying up irretrievably the substructure information in the form of a hash.

3.6. Calculation of Electrostatic Surface Potential

Electrostatic surface potential energies for BRBV and FMDV 3D^{pol} were calculated using APBS via PDB2PQR web portal (http://kryptonite.nbcr.net/pdb2pqr/) [29,30]. The input files were prepared from the PDB files of the two polymerases (BRBV and FMDV). Atomic charges and ionic radii were assigned according to Assisted Model Building with Energy Refinement (AMBER) force field. PROPKA was used to assign the protonation state to the proteins at pH 7.0. The proteins were assigned a low dielectric constant of 2.0 with a solvent dielectric constant of 78.54. Solvent accessible and solvent excluded surfaces of the two proteins were prepared. −5 and +5 kT/e (k is Boltzman Constant, T is temperature and e is the charge of electron) settings were used to color most positively charged and most negatively charged surface.

3.7. Calculation of Non-Covalent Interactions

The total number of hydrogen bonds was calculated in modeled as well as FMDV 3D^{pol} crystal structure, which was used as template to generate the model. The salt bridges were calculated using VMD [31]. Cation-pi interactions are formed when a cationic side-chain (Lys or Arg) is near an aromatic side-chain (Phe, Tyr, or Trp). Under such conditions, the geometry of aromatic ring is biased toward one that would experience a favorable cation-π interaction. Energetically favorable cation-pi interactions were alculated in both the modeled BRBV 3D^{pol} as well as FMDV 3D^{pol} structures via CaPTURE (http://capture.caltech.edu/) program developed by Justin Gallivan [32].

3.8. Preparation of Structures

Different representations of the structures of all the proteins were created Pymol version 1.3, wherever not mentioned otherwise [39].

4. Conclusions

In this study we built a high quality homology model of the 3D polymerase of bovine rhinitis B virus, which shares the highest homology to the FMDV counterpart. The modeled structures generated by different methods were characterized extensively for accuracy and relevance in terms of other

closely related RdRps. We found that BRBV 3D ^{pol} lacks *C*-terminal α-helix, which appears as an extension of thumb sub-domain in FMDV 3D ^{pol}. The active site and palm sub-domain show high conservation similar to most polymerases. The significant differences in the number and pattern of non-covalent interactions as compared to FMDV 3D ^{pol} could be an important determinant of the thermal enzyme behavior of BRBV 3D ^{pol}. Although, the template binding (97.5%) and active sites (100%) are highly conserved between FMDV and BRBV 3D^{pol}, the differences in adjacent residues, as well as bonding, could be important in determining the overall functionality of the protein. The finding of the present study would help broaden understanding of *Aphthovirus* RdRp, in particular, understanding the conformational changes in aphthoviral polymerases, which is as yet not an established phenomenon.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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ANNOTATED SEQUENCE RECORD

Full-length genome analysis of vesicular stomatitis New Jersey virus strains representing the phylogenetic and geographic diversity of the virus

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We describe the complete genomic sequence of nine isolates of vesicular stomatitis New Jersey virus (VSNJV) representing six distinct phylogenetic groups and spanning the known geographic range of the virus. The total genomic length (11,119-11,123 nt) and structure of these isolates were very similar to those of other vesicular stomatitis viruses. There were no differences in the number of amino acids in any of their viral proteins. As expected, the genomic termini, possessing initiation signals for viral transcription and replication, were highly conserved. These complete sequences, the first for natural isolates of VSNJV, should prove useful in the design of molecular detection tests and provide the basis for future functional genomics and pathogenesis studies.

Vesicular stomatitis (VS) virus causes disease in cattle, horses and pigs, and economic losses throughout North, Central and South America [16]. The clinical presentation of VS in cattle and pigs is markedly similar to foot-and-mouth disease, a devastating reportable disease, making a rapid differential diagnosis crucial. The etiologic agents of VS are viruses in the family *Rhabdoviridae*, genus *Vesiculovirus*, which have been classified into two serotypes, New Jersey (VSNJV) and Indiana (VSIV), both of which are endemic in northwest South America (Bolivia, Colombia, Ecuador, Peru, Venezuela), throughout Central America and in southern Mexico [8]. Throughout this work, virus nomenclature follows the Eight Report of the International Committee on Taxonomy of Viruses [4]. VSNJV accounts for the majority of clinical cases of vesicular disease in livestock throughout the Americas [5] [16]. In the United States, outbreaks of VS were reported in the southeastern states (Georgia, Florida, North Carolina, South Carolina) until the late 1970s. In the southwestern United States (Texas, New Mexico, Colorado, Wyoming, Utah), VS outbreaks are caused by cyclical incursions of VSV from endemic areas in Mexico every 8-10 years, causing significant economic losses to the cattle and horse industries [14, 16]. Although previous reports have documented overwintering of specific genetic lineages of VSNJV in the northwestern US for up to three years, disease outbreaks are usually associated with newly introduced viral lineages from endemic regions [13].

Multiple reports describing phylogenetic analysis of VSV field strains have shown that specific genetic lineages exist in different geographical areas [16]. Furthermore, studies suggest that ecological factors play an important role on the evolution of VSV [15]. In previous studies, we have described the complete genome sequences of VSIV strains from South, Central and North America [17] and the prototype viruses for the Indiana 2 and Indiana 3 subtypes [12]. However, despite the fact that VSNJV accounts for the vast majority of VS cases in the western hemisphere and that VSNJV was shown to be more pathogenic than VSIV in a natural host [10], very little work has been done on the full-length genome of this economically important virus. Current full-length genomic sequences deposited in GenBank are assembled from partial sequences of multiple VSNJV isolates of mixed and/or indeterminate passage history. Here, we report for the first time the characterization of the full-length genomic sequences of nine naturally occurring isolates of VSNJV, representing six

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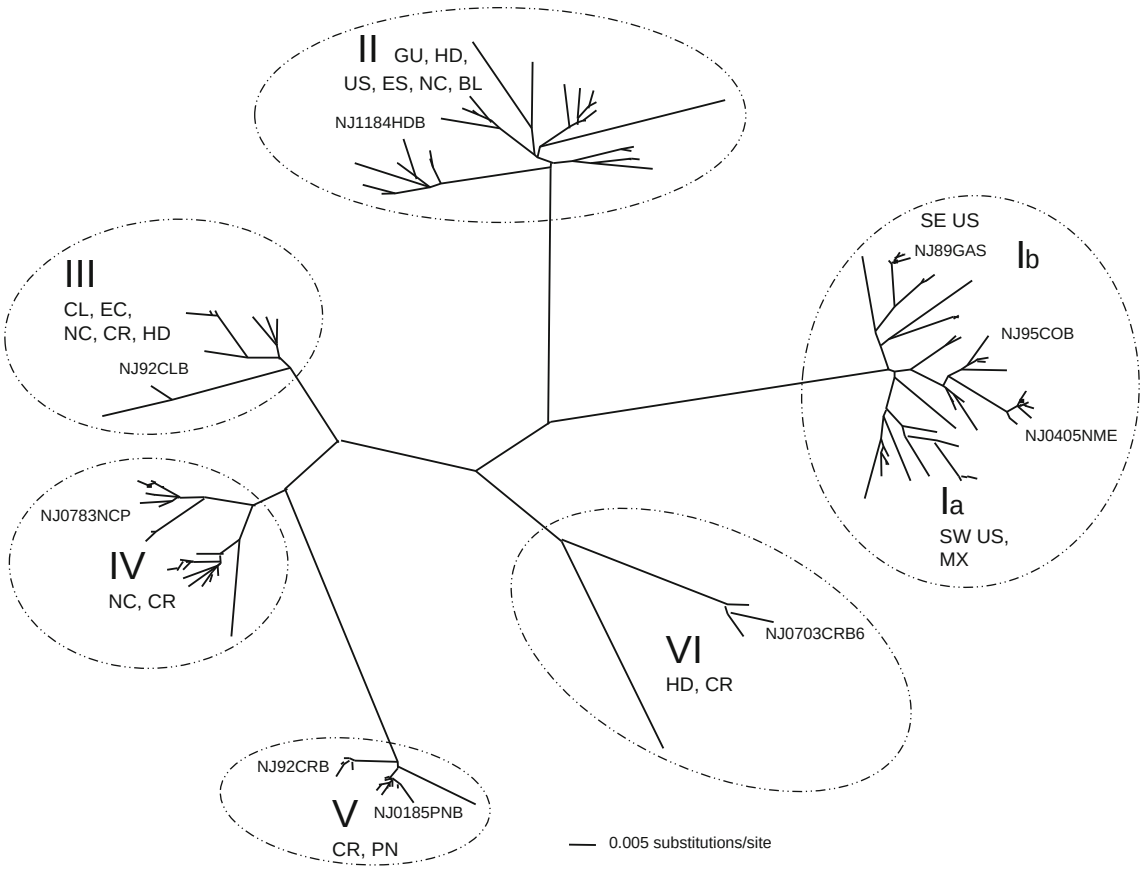

Fig. 1 Neighbor-joining tree of the phosphoprotein hypervariable region of the nine viruses analyzed in this study and all other available VSNJV sequences. Isolates were selected for sequencing from each of the six major clades, encircled by a broken line and labeled I-VI. For each clade, the country of origin of a viral isolate within the clade is indicated by a two-letter code as follows: BL, Belize; CL, Colombia; CR, Costa Rica; EC, Ecuador; ES, El Salvador; GU, Guatemala; HD, Honduras; MX, Mexico; NC, Nicaragua; PN, Panama; SE US, southeastern US; SW US, southwestern US. Additional isolate names were removed for clarity

phylogenetic groups and comprising the known geographic breadth of the virus.

To select the viruses for full-length genomic analysis, we aligned all available VSNJV phosphoprotein hypervariable region sequences using ClustalX [22] and reconstructed the phylogeny with PAUP* 4.0b10 [21]. The viruses formed six distinct genetic groups or clades (Fig. 1). Group I contains the viruses from the United States and Mexico. This group can be further divided into two subgroups: group Ia, containing viruses from the southeastern US, and group Ib, containing viruses from Mexico and the southwestern US. The greatest genetic diversity is observed in Central America, where viruses in group II principally originate from northern Central America, and groups IV, V and VI are mostly comprised of viruses from the middle and southern countries in Central America. Interestingly, group III contains viruses from the middle countries of Central America (Honduras, Nicaragua, and Costa Rica) and northern South America (Colombia, Ecuador) but not Panama.

At least one virus from each of the six groups in Fig. 1 was selected for full-length genomic sequencing to represent the known phylogeographic spectrum of the virus. Detailed isolate information is shown in Table 1. Virus stocks for full-length sequencing were made by infecting cells at a multiplicity of infection of 0.1 or less. Genomic sequences, including termini, were obtained and analyzed as described previously [12]. Primers for PCR and sequencing reactions were designed based on the published sequences of VSNJV or based on newly obtained sequences (primer sequences are available from the corresponding author upon request). Bioinformatic analysis was conducted using PAUP* 4.0b10 or MEGALIGN (DNASTAR).

The basic genomic structure of the VSNJV isolates was similar to that of other VS viruses, with a 47-nt 3' leader (Le) sequence followed by the five structural genes (Nucleoprotein [N], Phosphoprotein [P], Matrix [M], Glycoprotein [G], Polymerase [L]) and a 57-nt 5' trailer (Tr) sequence. Total genomic lengths were 11,119-11,123 nt which is shorter than previously seen in field isolates of

Table 1 Description of the viruses sequenced in this study

Virus	Year of Isolation	Area of isolation	Host Species	Pass History	Clade*
NJ89GAS	1989	Georgia, US	Sandflies	BHK p3	Ia
NJ95COB	1995	Colorado, US	Bovine	BHK p1	Ib
NJ0405NME	2005	New Mexico, US	Equine	Vero p1	Ib
NJ1184HDB	1984	Honduras	Bovine	BHK p3	II
NJ92CLB	1992	Colombia	Bovine	BHK p2	III
NJ0783NCP	1983	Nicaragua	Porcine	BHK p2	IV
NJ0185PNB	1985	Panama	Bovine	BHK p3	V
NJ92CRB	1992	Costa Rica	Bovine	BHK p3	V
NJ0703CRB6	2003	Costa Rica	Bovine	BHK p1	VI

Table 2 Bioinformatics analysis of the coding sequences of the sequenced viruses

Gene	CDS length	%GC	Ti/Tv ratio	Average, Minimum nt% similarity
N	1269	43 %	3.92	86.3, 82.0
P	825	41 %	3.13	85.0, 77.3
M	690	41 %	3.40	84.7, 79.3
G	1554	43 %	3.16	84.4, 78.6
L	6330	40 %	3.18	85.4, 80.6
Protein	**No. of invariant aa (%)**	**Positions under one alternate aa replacement (%)**	**Positions under multiple aa replacement (%)**	**Average, Minimum aa% similarity**
N	399 (95 %)	33 (8 %)	0 (0 %)	98.1, 96.7
P	201 (73 %)	53 (19 %)	20 (7 %)	89.2, 82.8
M	205 (90 %)	22 (9 %)	2 (1 %)	96.1, 91.7
G	441 (85 %)	67 (13 %)	9 (2 %)	95.0, 91.5
L	1831 (87 %)	219 (10 %)	59 (3 %)	94.9, 92.5

VSIV. Interestingly, there is significantly less variability in the overall lengths of VSNJV isolates compared to VSIV isolates (11,155-11,336 nt) [17]. There were no differences in length of the predicted aa sequence in any of the structural proteins of the VSNJV isolates (Table 2).

Elements governing viral replication have been mapped to the genomic termini of vesiculoviruses [11, 25]. For VSIV, the 15 terminal-most nucleotides of the 3' Le and the 5' Tr have been shown to be essential for replication of the positive- and negative-sense viral genome, respectively [25]. Despite the broad geographic and phylogenetic span of the isolates, the terminal 15 nucleotides were completely conserved in both the Le and Tr in all of the isolates (data not shown). Previous work has shown that increasing the complementarity between the 3' and 5' termini enhances the levels of positive- and negative-strand viral replication [24]. All of the isolates analyzed in this study showed 100 % complementarity through the 18 terminal-most nucleotides. While all vesiculoviruses possess a certain degree of terminal complementarity, the conservation of complementarity observed in the VSNJV isolates is interestingly high.

At each intergenic region, vesiculoviruses exhibit a conserved sequence that contains cis-acting signals for transcription termination, mRNA polyadenylation and downstream transcription reinitiation [18] [19]. This intergenic sequence is characteristically 23 nt in length (3'-AUAC$(U)_7$NNUUGUCNNNAG-5') with the $(U)_7$ polyadenylation signal followed by two non-transcribed nucleotides. These intergenic dinucleotides have a significant role in the cis-acting signals involved in the staggered termination and reinitiation of transcription in VSV [2, 20]. These dinucleotides were found to be quite heterogeneous in VSIV field isolates [17], with one virus even having an intergenic trinucleotide. However, for all of the viruses sequenced in this study, all intergenic dinucleotides were the same, (3'-GA-5'), even at the G-L junction, where variability was observed in VSIV. Interestingly, studies

have shown that the intergenic dinucleotide 3'-GA-5' is the most efficient sequence for transcription termination but not reinitiation [2].

Prior to this report, the solitary VSNJV G-L sequence available in GenBank was the Ogden strain, in which a unique variation of the conserved VS intergenic sequence was observed [9]. The Ogden sequence has a 19-nt insertion (with respect to VSIV) between the non-transcribed dinucleotides and the 3'-UUGUCNNAG-5' mRNA transcription start signal in L. Inserts of variable length seen in this region have been described previously for one specific Central American clade of VSIV [3]. Interestingly, this 19-nt sequence at the G-L junction of VSNJ isolates was not unique to one specific clade, but rather, it was present in all sequenced viruses. The length of the insert was quite conserved, as eight of the viruses also possessed a 19-nt insert, while NJ0703CRB6 had an 18-nt insert.

There was no difference in the predicted lengths of the five structural proteins, and the N and P proteins were the most and least conserved, respectively, at both the nucleotide and amino acid level (Table 2). The C'/C proteins, whose function remains elusive, were present in a second ORF in the P mRNA in all of the isolates analyzed here. There were no changes to predicted glycosylation sites in the glycoprotein.

Previous studies have shown that the multifunctional M protein has conserved methionines in the NH_2-terminal portion (positions 33 and 51 for VSIV, positions 33, 48 and 51 for VSNJV). Mutational analysis showed that these methionines are involved in cell rounding (position 33) [6] and inhibition of host-cell gene expression (position 51 and, to a lesser extent, position 48) [7]. We found that all three of these methionine positions (33, 48 and 51) were completely conserved in the viruses sequenced in this study.

The purpose of this work was to characterize the complete full-length sequence of natural isolates of VSNJV, the virus serotype responsible for the vast majority of outbreaks in the Americas. Isolates from every major geographic and evolutionary cluster, collected over a twenty-year span and from four different host species, were selected to give a comprehensive representation of the virus. Detailed knowledge of the primary structure of the VSNJV genome will aid in functional genomics studies using reverse genetics, allowing the identification of genetic determinants of viral virulence. This information should prove useful for the understanding of the VSNJV life cycle that could be applied to disease mitigation or prevention of disease in livestock. In addition, as VSV isolates find applications in the medical field as vaccine vectors or as therapeutic agents for cancer, more detailed knowledge of their primary structure and function should support the development of better vectors for medical use [1, 7, 23, 26].

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Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Foot-and-Mouth Disease Virus Serotype O Phylodynamics: Genetic Variability Associated with Epidemiological Factors in Pakistan

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Summary

One of the most challenging aspects of foot-and-mouth disease (FMD) control is the high genetic variability of the FMD virus (FMDV). In endemic settings such as the Indian subcontinent, this variability has resulted in the emergence of pandemic strains that have spread widely and caused devastating outbreaks in disease-free areas. In countries trying to control and eradicate FMD using vaccination strategies, the constantly evolving and wide diversity of field FMDV strains is an obstacle for identifying vaccine strains that are successful in conferring protection against infection with field viruses. Consequently, quantitative knowledge on the factors that are associated with variability of the FMDV is prerequisite for preventing and controlling FMD in the Indian subcontinent. A hierarchical linear model was used to assess the association between time, space, host species and the genetic variability of serotype O FMDV using viruses collected in Pakistan from 2005 to 2011. Significant ($P < 0.05$) amino acid and nucleotide variations were associated with spatial distance, but not with differences in host species, which is consistent with the frequent multi-species infection of this serotype O FMDV. Results from this study will contribute to the understanding of FMDV variability and to the design of FMD control strategies in Pakistan. Viruses sequenced here also provide the earliest reported isolate from the Pan Asia II^{ANT-10} sublineage, which has caused several outbreaks in the Middle East and spread into Europe (Bulgaria) and Africa (Libya).

Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of livestock, producing losses that are insidious in endemic settings and devastating in disease-free countries (James and Rushton, 2002; Thompson et al., 2002; Perry and Rich, 2007). FMD is caused by infection with a single-stranded, positive-sense RNA virus, from the *Picornaviridae* family, genus *Aphthovirus*, referred to as FMD virus (FMDV). The FMDV genome (ca. 8 400 nucleotides) includes a single large open reading frame (ORF), which is ~7 000 nucleotides long. It encodes a polyprotein that is processed into the leader (L) protein, four structural proteins: VP4 (1A), VP2 (1B), VP3 (1C) and VP1 (1D), plus the 2A peptide, as well as non-structural proteins from the P2 (2B and 2C) and P3 (3A, $3B_{1-3}$, 3C and 3D) precursors (Sobrino et al., 2001; Grubman and Baxt, 2004; Belsham, 2005; Carrillo et al., 2005). Seven serotypes, referred to as A, O, C, SAT 1, SAT 2, SAT 3 and Asia 1, have been described based on the antigenic

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characteristics of the virus (Brooksby, 1958). Serotypes O, A and Asia 1 are prevalent among the ruminant population of Pakistan (Klein et al., 2008; Valarcher et al., 2009; Jamal et al., 2010, 2011a,b,c). FMD control and eradication in endemic countries, such as Pakistan, would not only result in increased agricultural productivity, but also will allow for the international trading of animals and animal products. This could ultimately result in improved quality of life for the rural population (Perry and Rich, 2007). In Pakistan, vaccination against FMD is voluntary and performed by few livestock owners. Moreover, effectiveness of vaccines available in the country is uncertain (Jamal et al., 2010).

Many recent FMD outbreaks in FMD-free countries have been caused by viral strains that have originally emerged from the Indian subcontinent (Knowles and Samuel, 2003; Valarcher et al., 2008). This region offers favourable conditions for FMDV emergence, likely due to a combination of insufficient control measures, large susceptible animal populations, and frequent and unrestricted livestock movements. An endemic setting with such characteristics allow for the existence of a wide variety of genetically diverse viruses. In turn, the high mutation rate of FMDV allows the virus to adapt to different environments (Domingo et al., 2003). Genetic variation is the result of mutation and recombination events throughout the virus genome during its replication. When these mutations result in amino acid substitutions within the structural proteins, the antigenic characteristics of the virus may change and consequently allow the virus to evade the host's immune response (Haydon et al., 2001). FMDV antigenic variation has been attributed to amino acid substitutions in major antigenic sites located on exposed regions of the capsid proteins VP1, VP2 and VP3 (Acharya et al., 1989; Haydon et al., 2001; Yoon et al., 2011b). The most studied epitope is located in the G-H loop of the VP1 protein, although epitopes within VP2 and VP3 also have an important role in the antigenic characteristics of the virus (Sobrino et al., 2001). Antigenic variation is a major obstacle for FMD control, because it results in an absence of cross-protection between serotypes and, in many cases, even between strains of the same serotype. In this study, we have quantified the extent to which epidemiological factors contributed to accumulated nucleotide (nt) and amino acid (aa) variation in the serotype O FMDV (FMDV-O) field samples circulating in Pakistan over a period of 6 years (2005–2011). Results presented here will help to gain critical understanding of the epidemiological dynamics of FMD in Pakistan and, ultimately, to design and implement effective control measures in the country that might be mirrored at the global level.

Materials and Methods

Data source

A total of 82 nucleotide sequences encoding VP1 from FMDV-O isolates collected from 2005 to 2011 at 22 different locations in Sindh, Punjab and Khyber Pakhtunkhwa Provinces of Pakistan were included in the analysis. Sixty-eight sequences, processed at two laboratories, were obtained from published literature, 36 from the National Veterinary Institute (DTU-VET, Lindholm) in Denmark and 32 from the Institute for Animal Health in Pirbright in the U.K. Additionally, 14 samples from which FMDV was isolated were processed and sequenced (whole capsid coding region, P1) at the Plum Island Animal Disease Center (PIADC) in the USA. Furthermore, P1 sequences were generated from five FMDVs at DTU-VET, Lindholm in Denmark. One P1 sequence was taken from the published literature (Belsham et al., 2011). Thus, a total of 20 P1 sequences were used in the analysis (Table 1).

Epidemiological information associated with each isolate included species (cattle or buffalo), location (lower administrative division) and date of collection. The location was geocoded (latitude and longitude) using the centre of the polygon of the lower administrative division reported at a web-based library (available at http://www.heavens-above.com/). A standard nomenclature was given to all the sequences for a more convenient visualization of figures: the first three text characters correspond to the lower administrative location of sample collection, the following two numbers are the consecutive samples from a particular year, the next two numbers indicate the year and the last capital letter corresponds to the species from which the sample was collected (C = cattle, B = buffalo), that is, sequence KHI1706B is the 17th sample in 2006 collected in Karachi and from a buffalo. The corresponding virus name as previously published is indicated in Table 1.

Viral RNA detection and sequencing

At PIADC, viral RNA was extracted using a high-throughput method described elsewhere (Pacheco et al., 2010). RT-PCR products were generated using SuperScript®III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen, Grand Island, NY, USA). The amplification primers were designed to amplify the entire P1 region of any FMDV isolate and were obtained from the Animal Plant and Health Inspection Services at PIADC. The RT-PCR products were visualized, purified, sequenced and analysed following procedures described elsewhere (Pauszek et al., 2011). Additional internal sequencing primers specific to the Pakistan isolates were used to obtain the complete P1 and are available from

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Table 1. Foot-and-mouth disease virus serotype O used in the study.

Sequence name	Fig. 2 Nomenclature	Sample location	Species affected	Accession. number	Reference
O/PUN/PAK/L181/2005	SGD0305C	Sargodha	Cattle	HQ439224	Jamal et al. (2011a)
O/PUN/PAK/L149/2005	GUJ0405B	Kharian, Gujrat	Buffalo	HQ439222	Jamal et al. (2011a)
O/PUN/PAK/L150/2005	GUJ0505B	Kharian, Gujrat	Buffalo	HQ439223	Jamal et al. (2011a)
O/PUN/PAK/L133/2005	LHR0705C	Lahore	Cattle	HQ439221	Jamal et al. (2011a)
O/ISL/PAK/L285/2005	IBD1205B	Islamabad	Buffalo	HQ439226	Jamal et al. (2011a)
O/ISL/PAK/L286/2005	IBD105B	Islamabad	Buffalo	HQ439227	Jamal et al. (2011a)
O/ISL/PAK/L287/2005	IBD1305B	Islamabad	Buffalo	HQ439228	Jamal et al. (2011a)
O/PAK/68/2006	HBD1606B	Hafizabad	Buffalo	FJ798173	Waheed et al. (2010)
O/PUN/PAK/L1370/2009[a]	RWP0909B	Rawalpindi	Buffalo	HQ439215–JX171677[b]	Jamal et al. (2011a) and This study
O/PAK/1/2008	BKI0108C	Burki	Cattle	FJ798190	Waheed et al. (2011)
O/PAK/10/2006	KHI1106B	Landhi Cattle Colony, Karachi	Buffalo	EF494503	Klein et al. (2008)
O/PAK/11/2006	KHI1706B	Karachi	Buffalo	EF494504	Klein et al. (2008)
O/PAK/12/2006	KHI1806B	Nagori Cattle Colony, Karachi	Buffalo	EF494505	Klein et al. (2008)
O/PAK/14/2006	KHI1906B	Nagori Cattle Colony, Karachi	Buffalo	EF494506	Klein et al. (2008)
O/PAK/2/2006	KHI1206C	Quadriba, Karachi	Cattle	EF494499	Klein et al. (2008)
O/PAK/2/2008	PTK0208B	Patoki	Buffalo	FJ798191	Waheed et al. (2011)
O/PAK/3/2008	PTK0308C	Patoki	Cattle	FJ798192	Waheed et al. (2011)
O/PAK/38/2005[c]	KRK0605C	Karak	Cattle	FJ798167	Waheed et al. (2011)
O/PAK/4/2006	KHI1306C	Landhi Cattle Colony, Karachi	Cattle	EF494500	Klein et al. (2008)
O/PAK/53/2007	SGD0107C	Sargodha	Cattle	FJ798179	Waheed et al. (2011)
O/PAK/6/2006	KHI1406B	Landhi Cattle Colony, Karachi	Buffalo	EF494501	Klein et al. (2008)
O/PAK/6/2008	PWR0408C	Peshawar, KPK	Cattle	FJ798193	Waheed et al. (2011)
O/PAK/61/2006	SGD0506C	Sargodha	Cattle	FJ798169	Waheed et al. (2011)
O/PAK/66/2007 (B)	LHR0707B	Lahore	Buffalo	FJ798185	Waheed et al. (2011)
O/PAK/68/2007	TTS0607B	TT Singh	Buffalo	FJ798186	Waheed et al. (2011)
O/PAK/69/2007	LYH0807B	Layyah	Buffalo	FJ798187	Waheed et al. (2011)
O/PAK/70/2007	LYH0907B	Layyah	Buffalo	FJ798188	Waheed et al. (2011)
O/PAK/71/2007	LMT1107C	Lakki Marwat	Cattle	FJ798189	Waheed et al. (2011)
O/PAK/8/2006	KHI1506B	Landhi Cattle Colony, Karachi	Buffalo	EF494502	Klein et al. (2008)
Pak 23.1_8	KHI0106C	Karachi	Cattle	EF494490	Klein et al. (2008)
Pak 23.2_8	KHI0206C	Karachi	Cattle	EF494491	Klein et al. (2008)
Pak 24.1_8	KHI0306C	Karachi	Cattle	EF494493	Klein et al. (2008)
Pak 5.2_9	KHI0406B	Karachi	Buffalo	EF494498	Klein et al. (2008)
O/PUN/PAK/L282/2005[c]	LYH1405C	Layyah	Cattle	HQ439225	Jamal et al. (2011a)
O/ISL/PAK/L288/2005[c]	IBD1505C	Islamabad	Cattle	HQ439229	Jamal et al. (2011a)
O/NWF/PAK/L1414/2009	HAR0409C	CBF, Harichand	Cattle	HQ439212	Jamal et al. (2011a)
O/NWF/PAK/L1415/2009	HAR0509C	CBF, Harichand	Cattle	HQ439213	Jamal et al. (2011a)
O/NWF/PAK/L1416/2009	HAR0609C	CBF, Harichand	Cattle	HQ439214	Jamal et al. (2011a)
O/NWF/PAK/L1417/2009[a]	HAR0709C	CBF, Harichand	Cattle	HQ439215–JX171679[b]	Jamal et al. (2011a) and This study
O/NWF/PAK/L1418/2009[a]	HAR0809C	CBF, Harichand	Cattle	HQ439216–JX171680[b]	Jamal et al. (2011a) and This study
O/PAK/31/2005	HBD0105C	Hafizabad	Cattle	FJ798162	Waheed et al. (2011)
O/PAK/32/2005	HBD0205C	Hafizabad	Cattle	FJ798163	Waheed et al. (2011)
O/PAK/35/2005	LHR0805B	Lahore	Buffalo	FJ798166	Waheed et al. (2011)
O/PAK/33/2005	AWA0905C	Arifwala	Cattle	FJ798164	Waheed et al. (2011)
O/PAK/34/2005	AWA1005C	Arifwala	Cattle	FJ798165	Waheed et al. (2011)
O/PAK/60/2006	SGD0606B	Sargodha	Buffalo	FJ798168	Waheed et al. (2011)
O/PAK/63/2006	SKP0706B	Sheikhupura	Buffalo	FJ798170	Waheed et al. (2011)

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Table 1. Continued.

Sequence name	Fig. 2 Nomenclature	Sample location	Species affected	Accession. number	Reference
O/PAK/72/2006	GJW0806B	Gujranwala	Buffalo	FJ798176	Waheed et al. (2011)
O/PAK/73/2006	GJW0906B	Gujranwala	Buffalo	FJ798177	Waheed et al. (2011)
O/PAK/74/2006	OKA1006C	Okara	Cattle	FJ798178	Waheed et al. (2011)
O/PAK/66/2006	JHG2006C	Jhang	Cattle	FJ798171	Waheed et al. (2011)
O/PAK/67/2006	JHG2106B	Jhang	Buffalo	FJ798172	Waheed et al. (2011)
O/PAK/70/2006	AWA2206C	Arifwala	Cattle	FJ798174	Waheed et al. (2011)
O/PAK/71/2006	AWA2306C	Arifwala	Cattle	FJ798175	Waheed et al. (2011)
O/PAK/60/2007	JHG0207C	Jhang	Cattle	FJ798181	Waheed et al. (2011)
O/PAK/56/2007	SKP0307B	Sheikhupura	Buffalo	FJ798180	Waheed et al. (2011)
O/PAK/61/2007	HBD0407C	Hafizabad	Cattle	FJ798182	Waheed et al. (2011)
O/PAK/63/2007	GJW0507C	Gujranwala	Cattle	FJ798183	Waheed et al. (2011)
O/PAK/66/2007 (A)[c]	LYH1007B	Layyah	Buffalo	FJ798184	Waheed et al. (2011)
O/PUN/PAK/L1346/2008	FBD0508C	Faisalabad	Cattle	HQ439209	Jamal et al. (2011a)
O/PUN/PAK/L1347/2008	FBD0608C	Faisalabad	Cattle	HQ439206	Jamal et al. (2011a)
O/PUN/PAK/L1358/2008[a]	FBD0708C	Faisalabad	Cattle	HQ439208–JX171676[b]	Jamal et al. (2011a)
O/PUN/PAK/L1353/2008	FBD0808C	Faisalabad	Cattle	HQ439211	Jamal et al. (2011a)
O/PUN/PAK/L1360/2008	FBD0908C	Faisalabad	Cattle	HQ439210	Jamal et al. (2011a)
O/PUN/PAK/L1345/2008	FBD1008B	Faisalabad	Buffalo	HQ439213	Jamal et al. (2011a)
O/PUN/PAK/L1343/2008	FBD1108C	Faisalabad	Cattle	HQ439207	Jamal et al. (2011a)
O/ISL/PAK/L1412/2009[a]	IBD0109C	Islamabad	Cattle	HQ439214–JX171678[b]	Jamal et al. (2011a)
O/ISL/PAK/L1413/2009[a]	IBD0209C	Islamabad	Cattle	HQ439220–HQ113232[d]	Jamal et al. (2011a) and Belsham et al. (2011)
O/KHI/PAK/04/2009[a]	KHI1109C	Landhi, Karachi	Cattle	JX170744	This study
O/KHI/PAK/08/2009[a]	KHI1009B	Sargani, Karachi	Buffalo	JX170745	This study
O/SGD/PAK/17/2010[a]	SGD0110B	Sargodha	Buffalo	JX170746	This study
O/SGD/PAK/18/2010[a]	SGD0310C	Sargodha	Cattle	JX170747	This study
O/SWL/PAK/16/2010[a]	SWL0210B	Sahiwal	Buffalo	JX170748	This study
O/BWP/PAK/13/2010[a]	BWP0410B	Bahawalpur	Buffalo	JX170749	This study
O/SGD/PAK/09/2010[a]	SGD0510C	Sargodha	Cattle	JX170750	This study
O/SGD/PAK/10/2010[a]	IBD0610C	Islamabad	Cattle	JX170751	This study
O/BWL/PAK/12/2010[a]	BWP0710B	Bahawalpur	Buffalo	JX170752	This study
O/RWP/PAK/14/2010[a]	RWP0810B	Rawalpindi	Buffalo	JX170753	This study
O/RWP/PAK/15/2010[a]	RWP0910B	Rawalpindi	Buffalo	JX170754	This study
O/KHI/PAK/41/2011[a]	KRI0111B	Karachi	Buffalo	JX170755	This study
O/KHI/PAK/42/2011[a]	KRI0211B	Karachi	Buffalo	JX170756	This study
O/SGD/PAK/19/2011[a]	SGD0311C	Sargodha	Cattle	JX170757	This study

[a]All P1 sequence available.
[b]Accession number for P1 sequence.
[c]Not included in cluster analysis, sequences fell in cluster of $n < 3$ sequences.
[d]P1 sequence derived from rescued virus.

the authors upon request. The P1 sequences were submitted to GenBank and assigned accession numbers JX170744–JX170757. Additional P1 sequences generated at DTU-VET, Lindholm, were generated as described previously (Jamal et al., 2011a), and the sequences have been assigned accession numbers JX171676–JX171680 (Table 1).

VP1 and P1 nt and aa pair-wise difference correlation

To assess the hypothesis that VP1 alone (639 nt for the isolates listed in Table 1) contains information comparable to that provided by the entire P1 (2202 nt), we aligned the 20 P1 sequences using ClustalW algorithm (Larkin et al., 2007) implemented in BioEdit® (Hall, 1999). Subsequently, we computed all pair-wise nt and aa differences of P1 and their respective VP1. The nt and aa differences for VP1 between each pair relative to the whole P1 were computed as a percentage (#nt or aa differences in VP1/# nt or aa differences in P1). Additionally, Pearson's coefficient of correlation (r) for the nt and aa changes in VP1 and P1 was computed in PASW Statistics 18m release version 18.0.0 (SPSS, Inc., 2009, Chicago, IL, USA).

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Statistical model to assess the association of genetic variation and epidemiological factors

Genetic clusters of the 82 FMDV-O VP1 nucleotide sequences were identified by constructing a neighbour-joining tree using Phylip software package (Felsenstein, 2005) and visualized in Fig Tree v1.3.1 (Rambaut, 2006–2009). The F84 substitution model with gamma distributed rates across sites was used. Additionally, a phylogenetic tree using the predicted aa sequences was constructed to compare clustering of viruses based on aa and nt sequences.

A hierarchical linear model was formulated, where the nt and aa distance from each sequence to the earliest isolate in the cluster was the dependent variable. Host species, time difference and spatial distance between the earliest sample in the cluster and each of the remaining sequences in a cluster were assessed for fixed effects in the model. Time was calculated as the number of days between the sample collection, and spatial distance between the two sample point locations (latitude and longitude) was computed using the Haversine distance formula. Differences in species were categorized in samples collected from the same or different species (cattle and buffalo). The genetic cluster was used as a grouping variable (hierarchy). Models for prediction were selected based on the value of the squared correlation between predicted and observed values, to assess the fit of the mixed models, and by the Akaike information criterion (AIC). All statistical analyses were performed using PASW Statistics 18m release version 18.0.0 (SPSS, Inc.).

Results

VP1 and P1 nt and aa pair-wise difference correlation

The 20 whole P1 coding sequences were derived from samples collected from buffalo ($n = 11$) and cattle ($n = 9$) and originated in eight different locations. There were three pairs with identical nt sequences; interestingly, two of these identical samples were collected 1 day apart, from different species (buffalo and cattle) and in two different locations, Sargodha and Bahawalpur (Punjab Province), which are approximately 340 km apart from each other.

Pearson correlation coefficient of the distance measured in P1 and VP1 was high ($r = 0.99$, $P < 0.05$) for nucleotides and moderate ($r = 0.79$, $P < 0.05$) for the amino acid sequences (Fig. 1). The most divergent sequences had 50 and 137 nt differences for VP1 and P1, respectively, which resulted in five and 14 aa differences, respectively.

The mean proportion of VP1/P1 differences was 0.39 (SD = 0.12) and 0.68 (SD = 0.25) in nt and aa sequences, respectively.

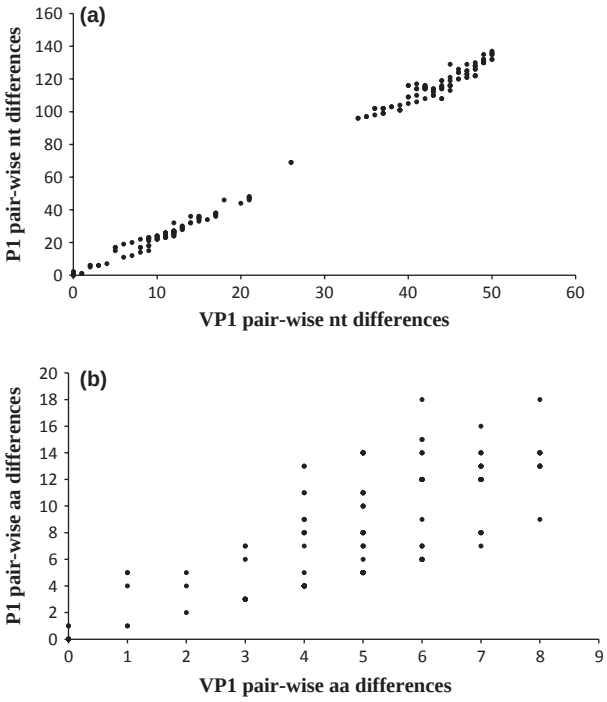

Fig. 1. Scatter plots of pair-wise differences between 20 VP1 and P1 nucleotide (a) and amino acid (b) sequences of FMDV serotype O collected from Pakistan.

Statistical model to assess the association of genetic variation with epidemiological factors

The neighbour-joining tree of the 82 VP1 nucleotide sequences showed seven major genetic clusters with $n = 24$, 14, 8, 5, 7, 9 and 11 sequences, respectively (Fig. 2). Phylogenetic trees constructed using predicted aa sequences yielded similar clusters (not shown), so initial genetic clusters provided by nucleotide sequence analysis was considered an appropriate grouping criterion for models assessing nt and aa changes. Sequence variability within each cluster was <5% (≤30 nt difference). Sequence O/PAK/66/2007 (A) did not fall into any cluster and one of the clusters included only three sequences (O/PAK/38/2005, O/PUN/PAK/L282/2005 and O/ISL/PAK/L288/2005); therefore, those four sequences were not included in the regression model because of insufficient data for contributing to intracluster variability. Consequently, 79 VP1 sequences (36 from buffalo and 43 from cattle) collected at 22 different locations were included in the regression model (Fig. 2). These viruses all belong within the O PanAsia lineage, and all the additional viruses (collected in 2009–2011) sequenced in this study belonged to the PanAsia II^{ANT-10} sublineage.

The model with the lowest AIC value included only spatial distance as a significant predictor for both the nt and aa

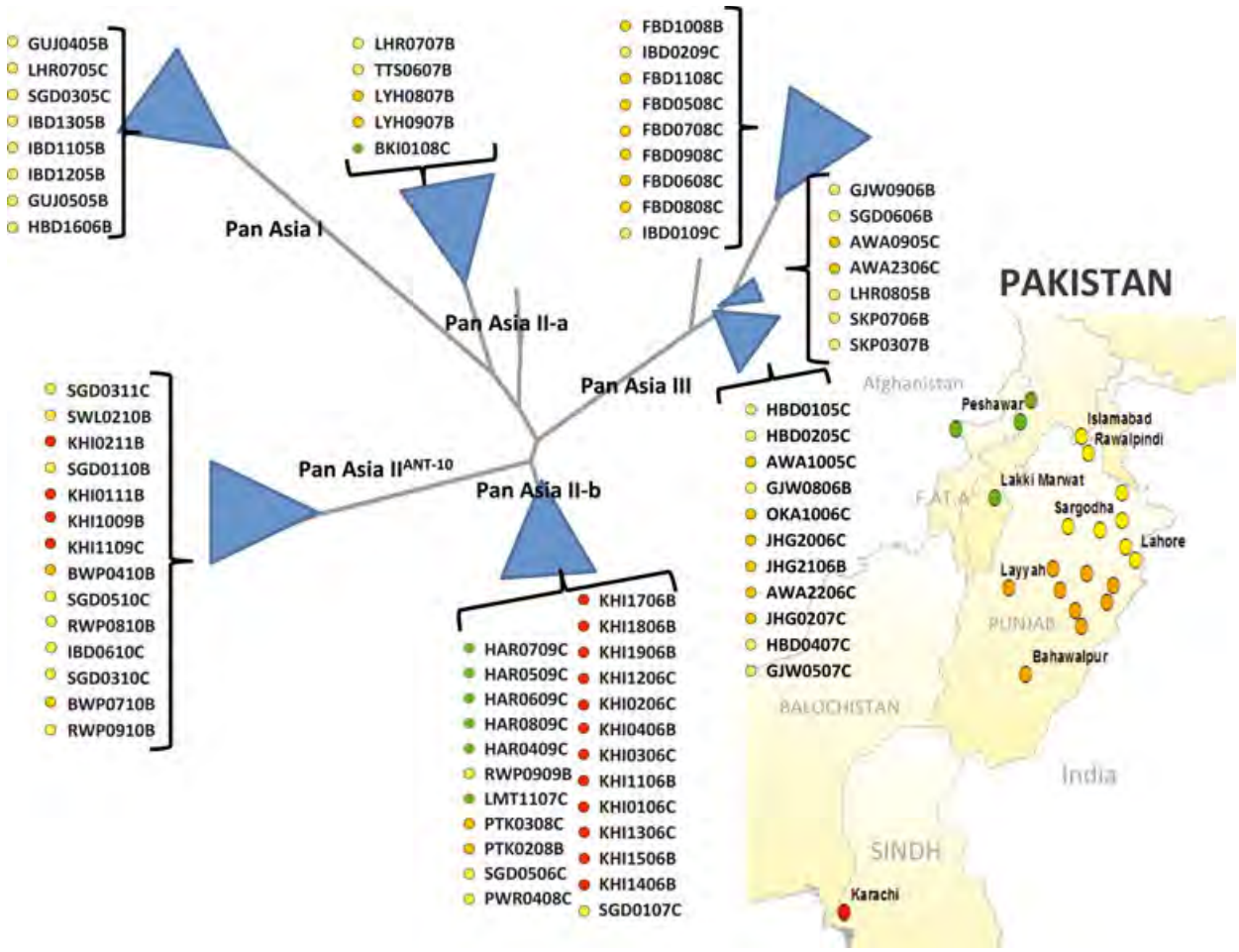

Fig. 2. Neighbour-joining tree of analysed VP1 nucleotide sequences. The topology of the tree shows seven major clusters with <5% difference within each cluster, while an eighth cluster was not included in additional analyses because of the low number of sequences within it (n = 3). Previously named lineages within the O PanAsia viruses (see Jamal et al., 2011a) are also indicated in the tree branches. The sequences were named as follows: the first three text characters correspond to the lower administrative location of sample collection, the following two numbers are the consecutive samples from a particular year, two numbers indicate the year and the last capital letter denotes the species from which the sample was collected (C = cattle, B = buffalo). The ranges of the difference variables assessed in the model are given for each cluster. Sampling location of the sequences is given by the coloured circle in front of the sequence name, corresponding to circle location in the map.

models. The squared correlation between the predicted and the observed values was 0.83 and 0.87 for the nt and aa sequences, respectively. The model predicted that the VP1 nt sequences in the same location differed by 2.5 (95% CI = 1.0–4.1) nucleotides and differences increased by 1.7 (95%CI = 1.4–1.9) nucleotides per 100 km. Amino acid sequences in the same location differed by 0.9 (95%CI = 0.5–1.3) aa and differences increased by 0.5 (95% CI = 0.4–0.6) aa every 100 km. Host species was not significantly associated with aa or nt differences. Geographical distance and time between sampling were highly correlated ($r = 0.72$, $P < 0.05$), and for that reason, inclusion of time did not improve the model fitness.

Discussion

Findings from this study provide information on the predictive nature of VP1, compared to P1, for assessing the nature and extent of nt and aa sequences variation of recent FMDV serotype O isolates from Pakistan. The 14 viruses sequenced for this analysis belonged to the PanAsia II^{ANT-10} sublineage, which is currently dominant in the West Eurasia region and has spread as far west as Libya and Bulgaria. Two of these viruses were sampled in 2009, in Karachi dairy colonies, and are the earliest reported PanAsia II^{ANT-10} viruses, suggesting that this sublineage may have originated in Pakistan.

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Compared to P1 sequences, VP1 was a good predictor of nt differences, but its ability to predict aa changes was moderate. This finding may be related with a higher synonymous substitution accumulation throughout P1, but increased non-synonymous substitution in VP1, arguably, because of differential changes driven by selective pressure and/or allowed by structural constraints (Sobrino et al., 2001). These results suggest that at least for serotype O in Pakistan and possibly other serotypes in endemic settings, the entire P1 sequences may be required to assess aa variation and immunological studies such as vaccine matching, whereas VP1 sequence alone may be sufficient when the objective of the study is to assess nt variations.

Few earlier studies have attempted to associate epidemiological factors with genetic variation in FMDV. Pairwise VP1 genetic variation for the epidemic that affected Argentina in 2001 was associated with longer distances and greater time intervals and with the interaction between incidence and duration of outbreaks (Perez et al., 2008). In that study, the regression model did not account for genetic clustering because all isolates included were from a single epidemic. In a different study, no association between nt substitution rate and host species or country of origin was found in O PanAsia FMDVs collected from 2005 to 2009 in Pakistan and Afghanistan (Jamal et al., 2011a). This lack of geographical association could be due to lack of specific point location information, because samples were only classified at the country level. In another study, in which FMDV-O PanAsia lineage sequences collected from 1989 to 2001 in the Middle East, Asia, Europe and Africa were analysed, significant associations with host and region were found (Garabed et al., 2009). Contrasting findings were presented in a global study of FMDV serotypes O and A, sampled between 1939 and 2010, where no association with FMD evolution and time, country or host species was identified (Yoon et al., 2011a). The wide time range and geographical diversity of viruses included in the aforementioned epidemiological analysis may have resulted in low resolution (spatial and temporal) of the FMDV phylodynamics, preventing the detection of geographical or temporal associations.

The interpretation of all these epidemiological studies must take into account the fact that accumulated mutations observed in field samples over time are the result of the virus mutation rate and the selective forces exerted on the virus population in a given environment. Therefore, both the geographical diversity and temporal breadth of the samples studied can dramatically influence the epidemiological findings (Muellner et al., 2011).

In our study, we fitted a model that included genetic cluster as a hierarchy, to compute only the genetic distance to the earliest isolate in the cluster. In the final models, only geographical distance was significantly associated with aa and nt variation. However, when time between isolations replaced geographical distance, the association between time and aa or nt diversity was also significant (results not shown). Lack of time association with genetic variation was likely due to the strong correlation between geographical distance and time between isolation of the samples, which impairs our ability to understand whether sequences were different because they were collected at different locations or at different periods of time.

Interestingly, collection of samples from different species was not significantly associated with the pair-wise genetic distance assessed here. Moreover, we found three identical P1 nt sequences, two of them sampled 1 day apart, from buffalo and cattle, and in two different locations (Sargodha and Bahawalpur). These results support early findings, suggesting that FMDV-O Pan Asia lineage is adapted to multiple host species (Knowles et al., 2005) and is consistent with the frequent multi-species outbreaks observed in Pakistan and with the waves of disease outbreaks at specific locations and dates. These results have implications for disease prevention and control as it indicates transmission and circulation of similar viruses among different species.

The estimates given in this study add a geographical component to analysing genetic variability among field isolates, but they should be carefully interpreted as an average variation of the viruses studied. Some viruses may show lower or higher variation, having important implications to the disease epidemiology in endemic settings. For instance, in Fig. 2, most of the clusters are genetically diverse, except for the two smallest clusters of the Pan Asia III lineage, although they were collected within a time span of 761 and 694 days, respectively and within a spatial distance of 198 and 164 km. Only one nucleotide difference was observed in both clusters, resulting in one amino acid change in one of them. This sequence conservation for FMDV is unusual, given the fact that these viruses are interacting with hosts that have been either previously exposed or possibly vaccinated with FMDV. These conserved sequences of VP1 in space and time may suggest that these lineages use a mechanism for perpetuating in the animal population that does not require antigenic adaptation to host immune responses.

Arguably, the most important limitation of the study here was the impossibility to perform a probabilistic sampling design of outbreaks because of the nature and extent of the epidemiological information made available to us. Certainly, information on factors such as animal breed, age, gender, management practices, animals present in the herd, animal movement, details of the clinical disease and lesions would have helped to improve the model

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fitness and to reveal unexplored associations and influences. Unfortunately, implementation of structured study designs and availability of information has been impaired here by the particular prevailing conditions of the settings, as is typically the case for observational studies in FMD endemic regions.

In conclusion, this study contributes to the knowledge of FMDV dynamics in space and time, which, in addition to a comprehensive understanding of the molecular and structural changes related to virus antigenicity, will help to design appropriate FMD control strategies in endemic settings.

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Direct contact transmission of three different foot-and-mouth disease virus strains in swine demonstrates important strain-specific differences

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ABSTRACT

A novel direct contact transmission model for the study of foot-and-mouth disease virus (FMDV) infection of swine was utilized to investigate transmission characteristics of three FMDV strains belonging to serotypes A, O and Asia1. Each strain demonstrated distinct transmission characteristics and required different exposure times to achieve successful contact transmission. While a 4 h exposure was sufficient for strain A24 Cruzeiro (A24Cru), both O1 Manisa and Asia1 Shamir transmission required 18 h or more. Viral excretion levels from donors (for all three strains) and virus present in room air (for A24Cru and O1 Manisa) were evaluated and associated with clinical signs and observed transmission pattern. Although all directly inoculated donor animals showed acute FMD, A24Cru had the highest levels of viral shedding in saliva and nasal swabs followed by O1 Manisa and Asia1 Shamir. Virus levels in room air were higher and were detected longer for A24Cru than for O1 Manisa. These results provide direct evidence for important strain-specific variation in transmission characteristics and emphasize the need for thorough evaluation of different FMDV viral strains using a well defined contact transmission methodology. This information is critical for vaccine and biotherapeutic efficacy testing, pathogenesis and disease modeling of FMDV transmission.

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Introduction

Most foot-and-mouth disease virus (FMDV) pathogenesis and vaccine studies in pigs are performed by challenging via direct, intraepithelial injection in the heel bulb with virulent FMDV. Although this method is highly reproducible and results in clinical disease in most cases, it may not be appropriate to investigate certain virus–host interactions, particularly pre-viremic events and immune responses which occur during natural infection. Direct inoculation is inappropriate for measuring mucosal and innate responses as the inoculation bypasses many of the important host barriers and defense mechanisms. Swine are thought to play an important role as donors in the airborne transmission and spread of FMDV to other species and among different premises during outbreaks. However, previous reports indicate that higher viral doses and exposure times are necessary for aerosol infection in pigs (Kitching and Alexandersen, 2002). Direct contact transmission is more common (Salt et al., 1998), requires less time to cause severe disease (Alexandersen et al., 2001) and presents a higher rate of infection (Eble et al., 2006) when compared with indirect (aerosol) contact.

Direct contact is likely the most relevant manner of exposure for FMDV in pigs during the natural course of an outbreak. In studies which utilize direct contact exposure, strains of FMDV serotype O easily spread from pig to pig either in short (2–4 h) or continuous exposure time (Alexandersen et al., 2001; Barnett et al., 2002; Eble et al., 2004, 2007; Pacheco et al., 2010b; Pacheco and Mason, 2010; Parida et al., 2007). However, long direct exposure times are considered by some to result in overwhelming challenge doses for vaccine testing purposes (de Leeuw et al., 1979). Thus, determining the contact time required for transmission has similar value to determining the infectious dose in a direct inoculation scheme.

Vaccines against FMD are usually tested in cattle using well standardized protocols. Although in some regions of the world FMD vaccination campaigns focus on cattle, in other regions pigs are the main target of vaccination. Testing vaccine efficacy in swine is not commonly done; however, when it is performed, viral challenge is typically administered via direct inoculation of the heel-bulb, a route that bypasses most of the natural barriers and mucosal immune responses. A direct contact challenge method better reflects natural infection of FMDV and is necessary in order to adequately assess the mucosal immunity and other local responses.

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Additionally, for the investigation of pathogenesis and to develop data for modeling purposes, it is necessary to have reliable challenge models that are representative of natural infection (Arzt et al., 2010; Pacheco et al., 2010a; Pacheco and Mason, 2010).

In order to develop a successful swine challenge model, it is necessary to understand the transmission characteristics of different virus serotypes, subtypes and/or strains in controlled direct contact exposure experiments. Here we describe a methodology for the establishment of these conditions and use it to determine transmission characteristics for three commonly used FMDV challenge strains of the A, O and Asia1 serotypes. The contact exposure methodology described here has been recently successfully utilized in evaluating FMD vaccines and biotherapeutics (Dias et al., 2010).

Materials and methods

Virus strains and cell cultures

The FMDV strains A24 Cruzeiro (A24Cru), O1 Manisa and Asia1 Shamir used in this study were cattle-derived viruses that were amplified in pigs to adapt them to the host. The cell lines used for virus titration, isolation and characterization were BHK-21 (baby hamster kidney cell line, ATCC, catalogue number CCL-10), BHK alphaVbeta6 (Duque et al., 2004), LFBK (Swaney, 1988) and IBRS2 (de Castro, 1964).

Virus stock production

All animal procedures were performed following Protocol 151-R, approved by the Plum Island Animal Disease Center Institutional Animal Care and Use Committee (IACUC), which ensured ethical and humane treatment of experimental animals.

In order to produce a stock of inoculums for each viral strain, four 20–40 kg pigs were inoculated by the intradermal route in the heel bulb (Burrows, 1966). Two pigs received 10^5 and two received 10^4 infectious doses ($TCID_{50}$), distributed in four different sites. Pigs were observed for presence of vesicles once or twice a day without restraint (to avoid rupture of vesicles). When vesicles were visualized, usually at 48–72 h post inoculation (hpi), pigs were deeply sedated to harvest vesicular fluid from FMD lesions which was followed by humane euthanasia. Vesicular fluid was collected in tubes and an equal volume of Minimum Essential Medium (MEM) containing 25 mM Hepes (MEM/HEPES) was added to each tube before freezing at −70 °C. After harvesting was complete, vesicular fluids were thawed, pooled by strain, diluted 1:50 in MEM/HEPES, clarified, aliquoted and stored at −70 °C. One aliquot of these pig-adapted viruses was subsequently thawed for titration in cells and in swine by heel bulb inoculation.

The titer (plaque-forming units (PFU)/mL) in cells was obtained by standard methods, on previously described cell lines (BHK-21, BHK alphaVbeta6, LFBK and IBRS2). Determination of the viral infectious dose in pigs was performed as previously described (Pacheco et al., 2003; Pacheco and Mason, 2010) by minor modification of the method of Burrows (Burrows, 1966). Briefly, four outbred white pigs (20–40 kg) were sedated and inoculated intradermally in the heel bulb of each major digit of each foot with virus diluted in MEM/HEPES. Inoculation was achieved by inserting a 23G needle 1 cm along the superficial layer of the epidermis, and delivering virus dilutions adjusted to contain 10^2, 10^3, 10^4 or 10^5 PFU of virus/100 μL (estimated volume inoculated in each site). Formation of vesicles at the inoculation sites was scored as positive or negative 24 h after inoculation. The eight sites inoculated in the four animals for each dose were used to determine the 50% pig heel infectious dose per mL ($PHID_{50}$/mL) using the method of Reed and Muench (1938).

Direct inoculation and contact challenge

The contact experiments were designed in order to conduct 4 h and 18 h contact exposures in the same experiment while always maintaining the 1:2 ratio of donors: recipients (Fig. 1). For each strain, experimental groups containing four 20–40 kg donor pigs (D1–D4) housed in groups of two per room, and separated by a double fence, 1 m apart, were heel bulb inoculated at four sites in the right rear foot (100 μL/site) with 100 $PHID_{50}$ of FMDV per pig. In the case of A24Cru, we monitored the level of viral shedding by rRT-PCR in air samples, serum, oral and nasal swabs (see below) and determined that after 48 hpi shedding was optimal to begin exposure. At 52 hpi (0 h post contact, hpc) the four directly inoculated donor pigs (D1–D4) were moved into the same room in direct contact with eight naïve contact pigs (C1–C8). All twelve animals were kept inside a penned area of 2 m by 2 m and without food during the exposure time. At 4 hpc (56 hpi), two directly inoculated pigs (D1–D2) and four contact exposed pigs (C1–C4) were removed from the room, leaving six pigs (D3, D4, C5, C6, C7 and C8) in direct contact until 18 hpc (70 hpi), maintaining a 1:2 ratio of directly inoculated to contact exposed pigs. When contact inoculated animals were removed to a separate room, at both 4 and 18 hpc, they were segregated at a density of two pigs per room separated by double fencing as described above to avoid direct contact and minimize further exposure to virus shed by other pigs. All directly inoculated pigs D1, D2, D3 and D4 were held after the 18 hpc (70 hpi) in the exposure room and allowed to maintain direct contact for the duration of the experiment (Fig. 1).

Clinical evaluation and sampling

Clinical observations and sample collection were performed daily for 7 days post inoculation (dpi) or contact (dpc) for contact exposed animals. Swabs were collected from the mouth and nostrils with cotton swabs and immersed in 1 mL of MEM with 1% fetal calf serum, antibiotics and 25 mM HEPES. Serum was separated from blood collected from the jugular vein. All samples were frozen immediately

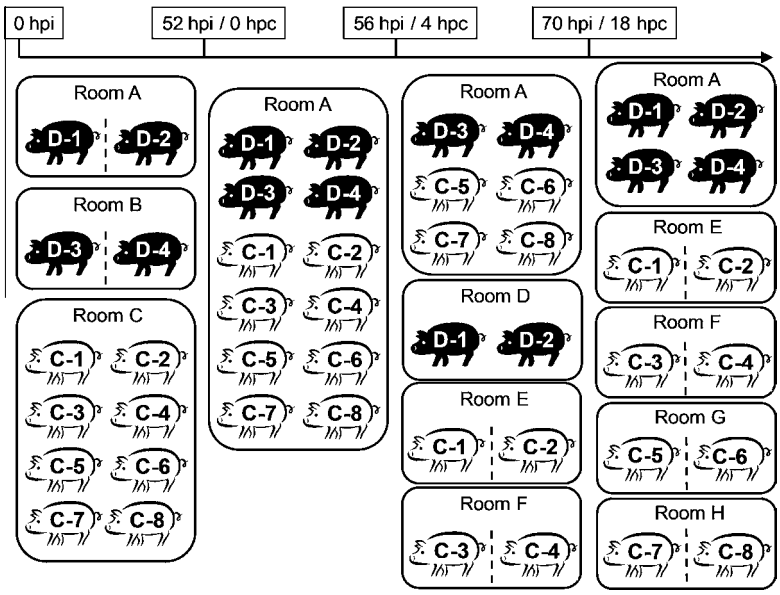

Fig. 1. Experimental design of the three identical experiments performed with FMDV A24Cru, O1 Manisa and Asia1 Shamir. Black pigs (D-1 to D-4) represent direct inoculated pigs and white pigs (C-1 to C-8) represent contact exposed pigs. Timeline, relative to inoculation of donors, on top is expressed in hours post inoculation (hpi) or hours post contact (hpc). The air sampler operated in Room A throughout the experiment. Dashed lines represent a double fence with a separation of 1 m to prevent contact between pen-mates.

Table 1
Detection of FMDV RNA (log_{10}RNA copies/1000 L) in room air samples.

Time period (hpi[a])	Number of pigs in room A (infected)	FMDV strain	
		A24Cru	O1 Manisa
−24 to 0	2 (0)	Negative	Negative
0–24	2 (2)	3.28	Negative
24–48	2 (2)	4.38	3.26
48–72[b]	2–12 (2–4)[c]	3.72	1.76
72–96	(4)	4.69	1.90
96–120	(4)	4.21	1.41
120–144	(4)	4.68	Negative
144–166	(4)	4.29	Negative
Average[d]		4.37	2.70

[a] Hours post inoculation.
[b] Contact exposure period.
[c] See Fig. 1 to determine the number of infected and contact animals in room A during this sampling period.
[d] Averages were calculated only from samples from which virus was detected.

after collection. Clinical signs were scored as described (Pacheco and Mason, 2010) with a maximum possible score of 20 points for contact exposed pigs and 16 for directly inoculated pigs since the inoculated foot was not used for scoring.

Air sampling

Air samples were collected from the exposure room (Room A, Fig. 1) containing A24Cru- or O1 Manisa-inoculated pigs, as described in Table 1. This room housed two directly inoculated pigs from 24 h before inoculation up to the moment when contact began (52 hpi), four direct inoculated and eight contact pigs for the next 4 h, two direct inoculated and eight contact pigs for the next 14 h and the four direct inoculated pigs for the remainder of the experiment.

Air sampling was performed as previously described (Mohamed et al., 2011). Briefly, a Dry Filter Unit (DFU) Model 1000 air pump developed by the Program Executive Office for Chemical Biological Defense (PEO-CBD) was used, fitted with either the original DFU filter assembly or with a Hi-Q filter holder (Hi-Q Environmental Products Company). In preliminary experiments no differences were found in FMDV detection between the two filter types (not shown). For the O1 Manisa experiment the DFU filter assembly was used. The air intake nozzle was located 1 m above the head of the pigs, close to the fenced area, and it held two separate Lockheed Martin polyester filter disks (1.0 μm filter, diameter 47 mm, Catalogue number DFU-P-24, Lockheed Martin). The air flow for this system was 8673 L/h. For A24Cru, the Hi-Q filter holder was used. This pump had an air flow of 2750 L/h. The air intake was 1 m above the pigs, and contained one Fluoropore membrane filter (1.0 μm filter pore size, diameter of 47 mm, Catalogue number FALP04700, Millipore).

For both systems, at the end of each sampling period, the filter holder was replaced with one containing a new filter and the old filter was removed from the holder inside a biosafety cabinet and stored at −70 °C. Holders were chemically decontaminated with Virkon (Dupont) before re-use. Proof-of-concept evaluation of this technique indicated that FMDV RNA could be detected from infectious virus suspension at a threshold of 1–100 infectious doses per filter (data not shown).

FMDV RNA detection

Sera and swabs samples were thawed and processed for RNA extraction and rRT-PCR as previously described (Arzt et al., 2010; Pacheco et al., 2010a). Air sampling filters were cut into four equal pieces (433 mm^2 each) using sterile scalpels and forceps. One quarter of each filter was placed in a sample tube (2 mL) containing 600 μL RLT/β-mercaptoethanol (RNeasy kit, Qiagen) and acid washed glass beads (Sigma, Catalog numbers: G8772-500G and G4649-500G). Filters were disrupted using a Retsch tissue Mixer Mill (model MM400) at 30 beats/s for 3 min. RNA extraction was performed using the RNeasy kit following manufacturer instruction and tested for the presence of viral RNA using rRT-PCR as previously described (Arzt et al., 2010; Pacheco et al., 2010a). Supplemental Fig. 1 shows the test sensitivity for these three FMDV strains (A24Cru, O1 Manisa and Asia1 Shamir).

Results

Animals inoculated for virus production with all three FMDV strains (A24Cru, O1 Manisa or Asia1 Shamir) showed vesicles by 48 or 72 hpi and a pool of 2–5 mL of vesicular fluid was obtained for each strain and used to prepare a virus stock (VS) as described in above. The titer of each VS showed little variation in four cell lines utilized for titration, ranging from $10^{7.44–7.95}$ PFU/mL for A24Cru, $10^{5.70–6.48}$ PFU/mL for O1 Manisa, and $10^{7.70–8.18}$ PFU/mL for Asia1 Shamir. However, for all viral strains the $PHID_{50}$/mL was 3–4 logs lower than the titers obtained in cells (Table 2).

Table 2
Titer of FMDV swine challenge stocks in various cell types (log_{10}PFU/mL) and in swine heel bulb ($log_{10}PHID_{50}$/mL).

Strain	Cell titration PFU[a] (mL)				Swine titer
	BHK-21	BHK-alphaVbeta6	LFBK	IBRS2	$PHID_{50}$[b] (mL)
A24Cru	7.95	7.44	7.87	7.68	3.00
O1 Manisa	5.70	6.06	5.81	6.48	3.25
Asia1 Shamir	7.70	8.10	8.18	8.00	3.42

[a] Plaque forming units.
[b] 50% pig heel infectious doses.

For A24Cru (Fig. 2) direct inoculation of 100 $PHID_{50}$ produced an acute and synchronous disease in the inoculated animals. Virus in blood and swabs was detected as early as 1 dpi peaking at 2–3 dpi when vesicles were observed at secondary sites of replication. By 72 hpi all direct-inoculated animals had acute FMD with generalized vesicles. Viral shedding in all animals was continuous throughout the observation period of 7 days and virus readily spread to direct-contact animals both in 4 h and 18 h exposure times. Contact animals showed acute and synchronous disease similar to directly inoculated animals except for a small delay in viremia, shedding and appearance of lesions in contact animals (Fig. 2).

In the case of O1 Manisa (Fig. 3), the direct inoculation of 100 $PHID_{50}$ produced acute and synchronous disease in the inoculated animals, similar to A24Cru. O1 Manisa was effectively transmitted to the pigs exposed by direct contact for 18 h causing clinical disease in all contact animals. However, in the 4 h direct contact period two of the four direct-contact animals never displayed clinical signs, viremia or virus shedding. One pig out of the four (217) had traces of FMDV RNA in sera and swabs on one single day; however, this pig never developed vesicles. Only one pig (218) had a single, clinically typical vesicle first detected at 6 dpc, and one more at day 7, but had no detectable viremia or viral shedding (Fig. 3). This animal's vesicles were presumed to be caused by FMDV; however laboratory confirmation was not performed. The O1 Manisa-exposed animals that showed no clinical signs of FMD, viremia or viral shedding at time of euthanasia (7 dpc), also had no FMDV-specific serum neutralizing antibodies (results not shown).

The Asia1 Shamir strain (Fig. 4) showed markedly different infection parameters. After direct inoculation of 100 $PHID_{50}$, pigs had delayed onset of disease with lower FMDV RNA loads detected in sera and swabs compared to both A24Cru and O1 Manisa. Detection and progression of vesicles was also delayed. Additionally, clinical scores reached maximum levels by 7 dpi. Additionally, after 4 h or 18 h direct contact, the Asia1 Shamir strain was inconsistently transmitted to direct contact animals. After 4 h exposure, only one of four pigs was detectably infected, and even in the 18 h exposure group, one of four animals remained uninfected. In all cases, all measured disease parameters were delayed by 2–3 days and levels of viremia and viral shedding were lower than those observed with A24Cru and O1 Manisa (Fig. 4). The Asia1 Shamir-exposed animals that showed no clinical signs of FMD, viremia or viral shedding at time or euthanasia (7 dpc), also had no FMDV-specific serum neutralizing antibodies (results not shown).

Although this study was not aimed at determining aerosol transmission, FMDV shedding from donor pigs was monitored in room air containing A24Cru- and O1 Manisa-inoculated pigs before, during, and after the direct contact exposure period. Marked strain-specific differences in the characteristics of viral shedding in room air were detected. In the case of A24Cru, over the course of the experiment an average of 4.37 log_{10}/m^3 (range 3.28–4.69) viral RNA copies were detected in the air starting at 0–24 hpi and

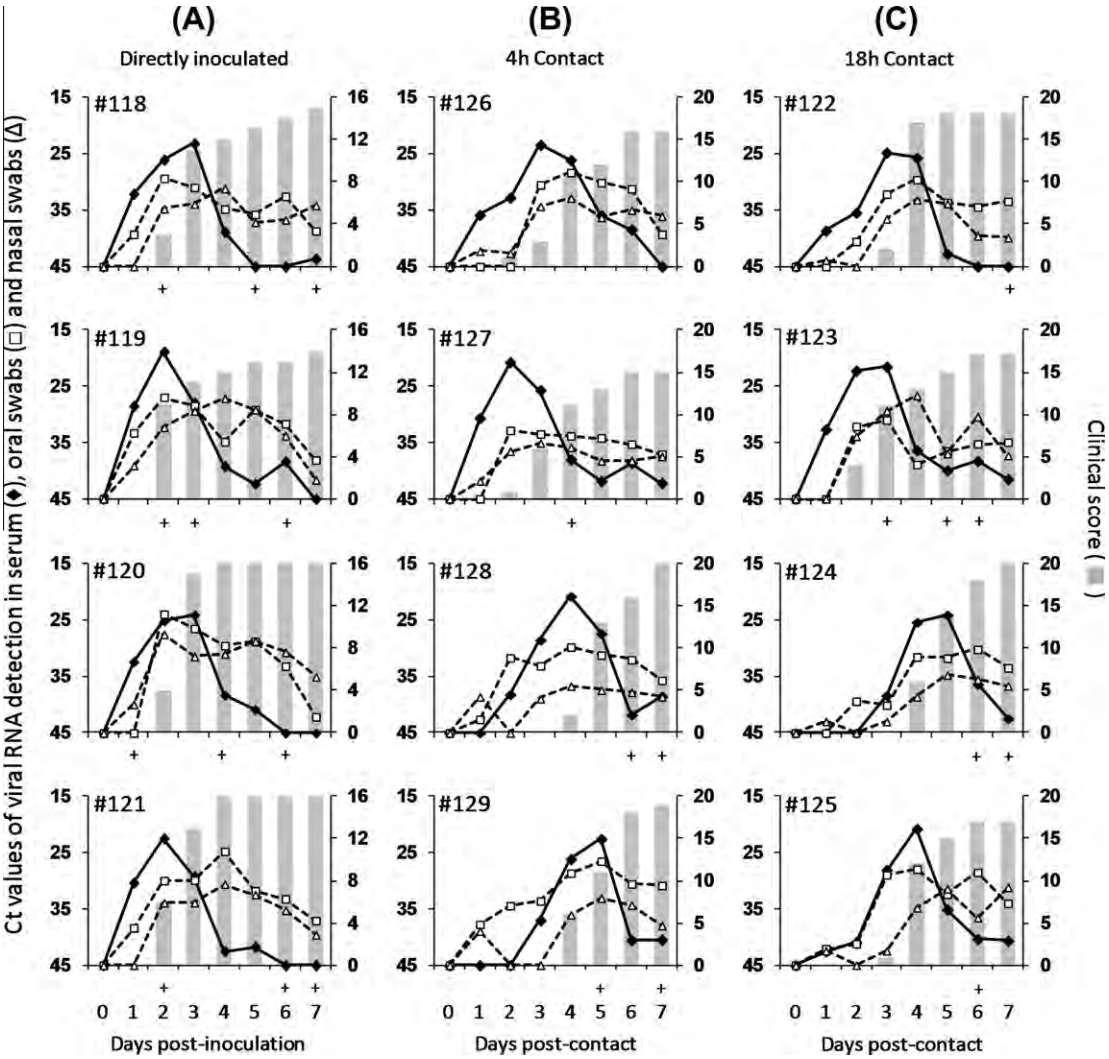

Fig. 2. Time course after intradermal heel bulb inoculation (panel A) with FMDV A24Cru, after 4 h-contact inoculation (panel B) and after 18 h-contact inoculation (panel C). Lines indicating Ct values of viral RNA detection in serum (♦), oral swabs (□) and nasal swabs (Δ) are expressed in the left *Y*-axes; the clinical score (bars) are expressed in the right *Y*-axes. +, fever (rectal temperature >40 °C).

continuing at high levels through the end of the study at 144–166 hpi. First detection of viral RNA in air coincided with onset of viral shedding detected in oral/nasal swabs and viremia (Table 1, Fig. 5a). In the case of O1 Manisa, virus in air was of lower quantity than that of A24Cru with an average of 2.70 $\log_{10}/m^3$ (range 1.41–3.26) viral RNA copies starting in the 24–48 hpi interval. Virus was not detected in air until 24 h after it was first detected in oral/nasal swabs and blood, peaked at 48 h and gradually decreased until it was no longer detected in air at 144 hpi, despite being still present in oral/nasal swabs and blood until 166 hpi (Table 1, Fig. 5b). For both strains of FMDV for which air sampling was performed, there was virus in the exposure room air during the exposure period (48–72 hpi) with 3.72 and 1.76 $\log_{10}/m^3$ viral RNA copies for A24Cru and, O1 Manisa strains respectively (Table 1).

Discussion

It has been previously documented that domestic pigs shed and aerosolize large quantities of FMDV and can serve as sources of aerosol infection to other susceptible animals particularly ruminants (Donaldson et al., 2001). It has also been previously shown that pigs are less susceptible than ruminants to aerosol infection and transmission requires direct contact (Kitching and Alexandersen, 2002). To our knowledge only one study has been done to characterize the ability of different viral strains to transmit and infect pigs under controlled direct contact exposure (Pacheco and Mason, 2010). This study was aimed at assessing the characteristics of transmission of different FMDV strains in swine using a controlled direct contact exposure method.

The first step in studying transmission in pigs is to have a reliable direct contact challenge methodology and this, in turn, relies on having consistent donor animals. The inoculation dose is important in determining the consistency of infection in the donor pigs. Low doses may be inconsistent, and transmission of infection is more efficient when donor pigs develop fulminant disease (Quan et al., 2009). To achieve consistency of infection, in this study we utilized a pig derived stock virus to inoculate the donor pigs. The use of 100 $PHID_{50}$ (regardless of tissue culture titer) meant that infection resulted in clinical disease and viral shedding for all three strains. Using challenge doses expressed as $TCID_{50}$ could be misleading as viral titers can vary substantially between cell culture and in vivo titration. For example, in the current study, heel bulb inoculation of 100 $PHID_{50}$ consistently resulted in clinical disease for O1 Manisa and A24Cru within 48 h despite the fact that this dose represented approximately 5 $\log_{10}$ PFU for O1 Manisa, and 7 $\log_{10}$ PFU for A24Cru when titrated in BHK-21 cells. In the case of Asia1 Shamir the 100 $PHID_{50}$ represented approximately

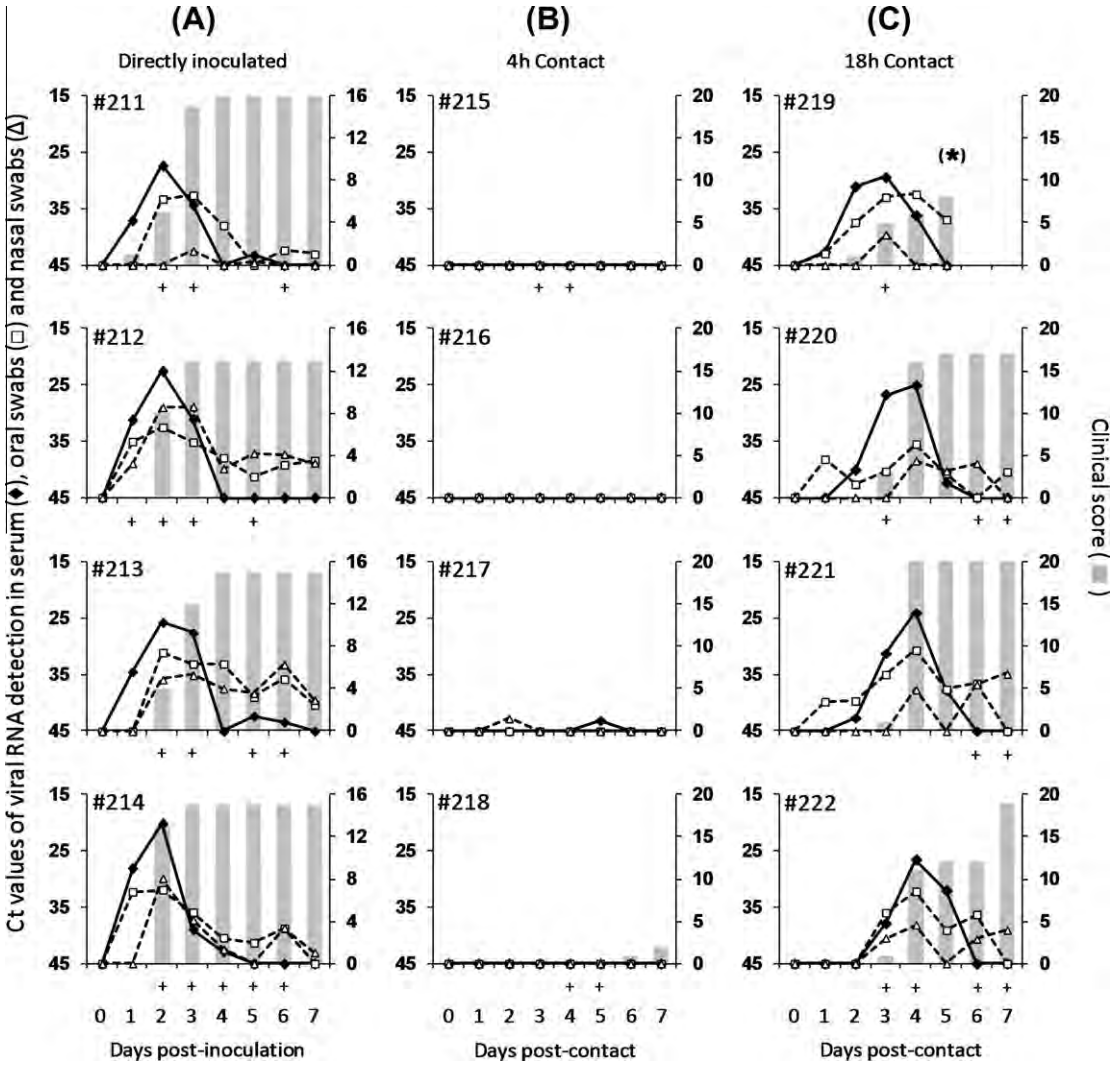

Fig. 3. Time course after intradermal heel bulb inoculation (panel A) with FMDV O1 Manisa, after 4 h-contact inoculation (panel B) and after 18 h-contact inoculation (panel C). Lines indicating Ct values of viral RNA detection in serum (♦), oral swabs (□) and nasal swabs (Δ) are expressed in the left *Y*-axes; the clinical score (bars) are expressed in the right *Y*-axes. +, fever (rectal temperature >40 °C); *, pig was found dead due to myocarditis.

6 $\log_{10}$ PFU titrated in BHK-21. Similar titers were obtained when these viruses infected BHK-21 (hamster) cells transfected with the FMDV integrin receptor (BHK-alphaVbeta6), a bovine cell line (LFBK), and a swine cell line (IBRS2) (Table 2).

It is likely that several different factors contribute to the efficacy of pig-to-pig transmission of FMDV. Group or individual exposure of pigs can influence transmission. A strong correlation exists between the dose shed by the donors (i.e. infectiousness of source and intensity of contact), length of incubation period, severity of clinical disease and efficiency of spread of FMDV. Lowering the density in a group of pigs during early stages of the infection may prolong the incubation period resulting in subclinical infection. Transmission and development of FMD is very rapid and efficient when pigs are exposed as a group, even when only exposed for a short time, provided the excretion levels of the donor pigs are high (Quan et al., 2009).

The current study compared shedding and transmission between three strains of FMDV, using controlled direct contact and keeping the other variables constant (density of pigs, time of exposure, etc.). In this work, FMD transmission was assessed using limited exposure times (4 and 18 h) in order to avoid continued re-exposure that might mask differences in transmissibility between viral strains. In this regard, establishing a minimum effective time for transmission has similar value to determining the minimum infectious dose in a direct inoculation system. This would be particularly important if the contact system were used as a pathogenesis model or challenge route in a vaccine study, since prolonged contact might result in a severe challenge, as previously described (Parida et al., 2007) and inappropriately suggest aberrant pathogenesis processes or vaccine failure (Pacheco et al., 2010b).

Interestingly, we found a striking difference between the three strains tested, in the time (relative to contact exposure) required for efficient transmission. While strain A24Cru was readily transmitted in 4 h, both O1 Manisa and Asia1 Shamir required 18 h for transmission to occur. Furthermore, in the case of Asia1 Shamir, even this longer direct contact exposure was insufficient to infect all contact animals in the room. There are several variables that could have influenced the observed differences including, the time at which direct contact exposure began, the clinical status of the donor animals, and their levels of viral shedding.

We fixed the time to begin the contact exposures at 52 hpi (donor) based on previous experiments performed in our laboratory with FMDV serotype O (Nfon et al., 2008; Pacheco et al., 2010b; Pacheco and Mason, 2010) or FMDV serotype A24Cru (Nfon

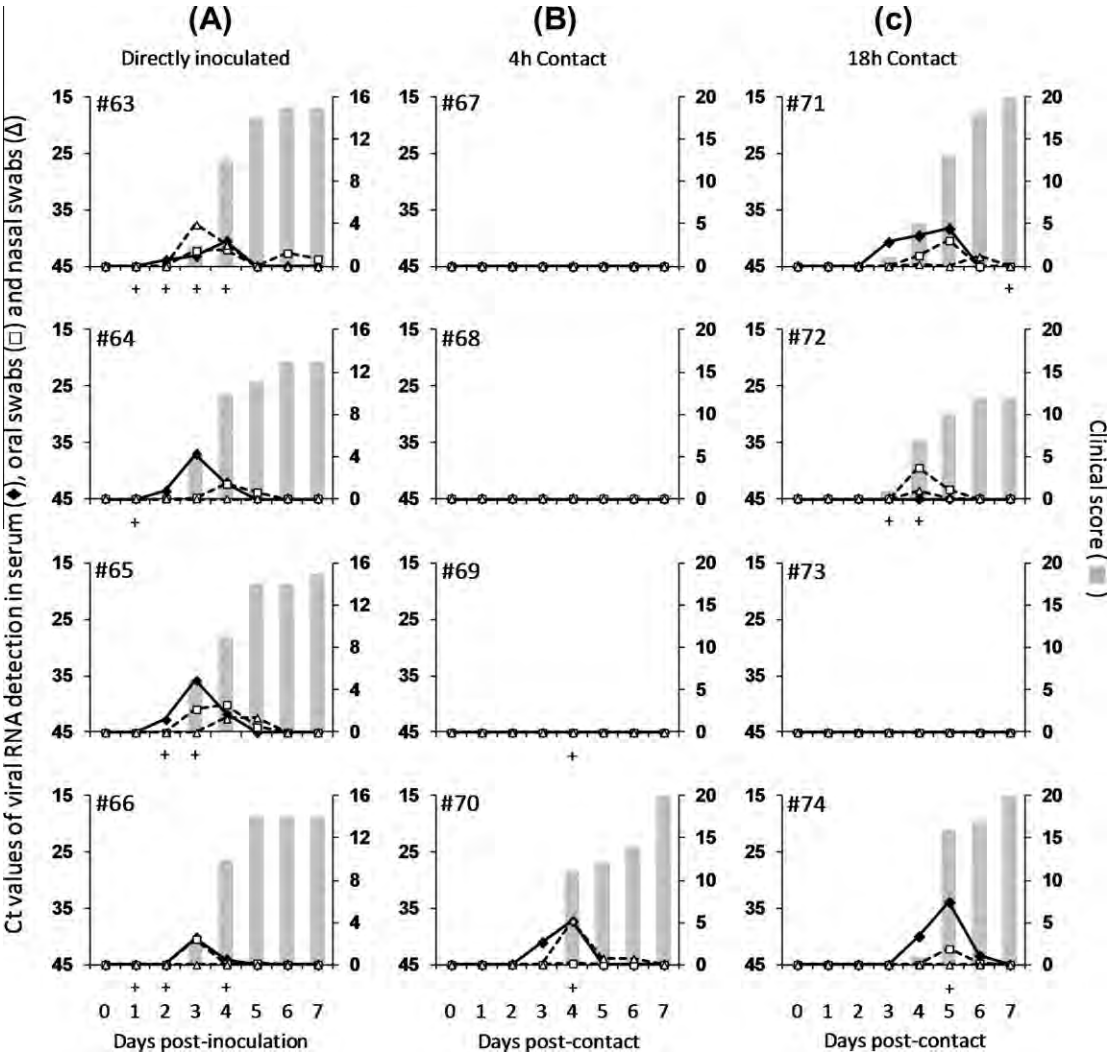

Fig. 4. Time course after intradermal heel bulb inoculation (panel A) with FMDV Asia1 Shamir, after 4 h-contact inoculation (panel B) and after 18 h-contact inoculations (panel C). Lines indicating Ct values of viral RNA detection in serum (♦), oral swabs (□) and nasal swabs (Δ) are expressed in the left *Y*-axes; the clinical score (bars) are expressed in the right *Y*-axes. +, fever (rectal temperature >40 °C).

et al., 2008). Additionally, results obtained with the A24Cru donors indicated that 48 hpi represented the peak of shedding based on viral detection in oral/nasal swabs and the onset of clinical signs. Peak viral shedding for O1 Manisa occurred at 48 h and at similar titers to those of A24Cru. By contrast, Asia1 Shamir showed onset of clinical signs and maximum detection of virus in blood and oral/nasal swabs occurring at 72 hpi. Viral RNA detection in serum and secretions was apparently lower for this strain, which is attributable to lower strain-specific sensitivity of our rRT-PCR system (supplementary Fig. 1). Retrospectively, it is clear that for the Asia1 Shamir strain, exposure should have started at a later time.

Comparing the donors' clinical disease and shedding of A24Cru and O1 Manisa, it is hard to explain the difference in transmission observed since in both cases donor animals were shedding high titers of virus in nasal and oral secretions and expired air, and had vesicular lesions at the time exposure began (52 hpi). This indicates that the O1 Manisa strain is less transmissible among pigs than A24Cru. In the case of Asia1 Shamir strain, donor animals showed milder clinical signs suggesting a possible explanation for the poor transmission observed.

Although intrinsic differences in virulence and/or transmissibility between the viruses is the most likely explanation of the findings described herein, it cannot be definitively ruled out that host-specific factors may have been variable across groups of animals and could have had confounding effects on the experimental results. Due to logistical constraints it was unavoidable that separate shipments of animals were used for each virus strain that was used. Thus, it is possible that there may have been underlying differences in genetic background or herd-level innate immune factors such as type I interferons (IFN) that have been reported to inhibit viral replication and affect the clinical course of disease (Chinsangaram et al., 2003; Moraes et al., 2003, 2007). We addressed this issue using a previously described Mx promoter/chloramphenicol acetyltransferase (CAT) reporter system (Fray et al., 2001) and confirmed no significant differences in IFN type I profiles across groups of pigs (results not shown). In order to minimize potential differences in genetic background, animals of the same breed, similar age and weight were obtained from the same vendor for all experiments performed.

The dose of virus received by each contact pig was not measurable, but judging by the clinical outcome we can extrapolate that all contact pigs were exposed to enough virus during the 18 h contact to cause infection and clinical disease. One detail of our exposure methodology that might be easily missed is the importance of avoiding feeding the pigs during the exposure time. Since it is well documented that transmission among pigs occurs mainly by the

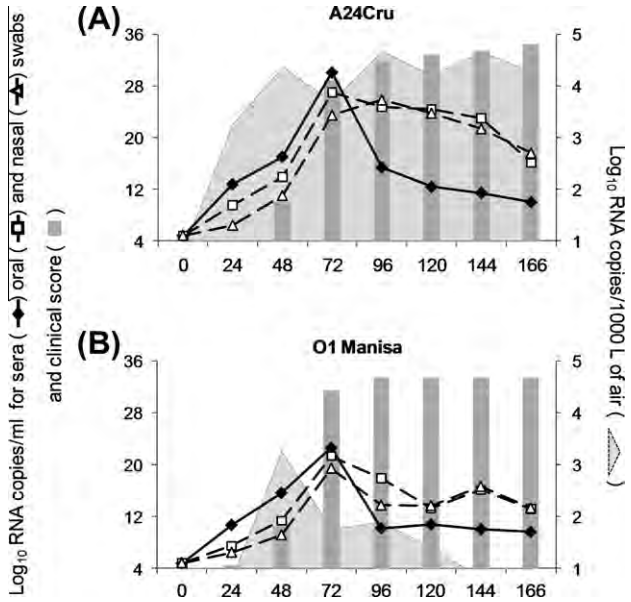

Fig. 5. Relationships between detection of FMDV in air samples and animal samples in the context of clinical scores of pigs inoculated intradermally with strains A24Cru (top panel) and O1 Manisa (bottom panel). The cumulative levels of FMDV RNA, as $\log_{10}$ genomes per mL, in serum samples (♦), oral swabs (□) and nasal swabs (Δ) are shown together with the average of clinical signs (bars, score of 0–16 scaled to fit on the 4–36 axis) on the left *Y*-axes. FMDV detected in air (shaded), as RNA/CN per 1000 L, is shown on the right *Y*-axes. Quantitation of FMDV RNA was performed by rRT-PCR where Ct values were converted to RNA copy numbers based on FMDV RNA-specific calibration curve developed with in vitro-synthesized RNA as previously described (Arzt et al., 2010).

oral route and through skin abrasions, we took advantage of typical porcine behavior when there is no food present. Pigs that have not been fed are more active and tend to interact with each other and with their environment more, creating opportunities for abrasions and chances of direct contact. In our experience, pigs that have been fed tend to lie down and have little interaction with each other.

By separating the exposed pigs in individual pens after contact challenge we were able to evaluate disease transmission based on the direct contact exposure time. The physical separation in individual pens was such that it precluded not only direct contact but also contact with feces or fluids and therefore minimized the potential for continued exposure due to viral shedding from penmates. Changing of gloves and washing boots, as well as surface decontamination with disinfectant was also performed between sampling of pigs to minimize fomite transmission during this post-exposure period. Previous work (Alexandersen et al., 2002; Alexandersen and Donaldson, 2002; Donaldson and Alexandersen, 2001; Pacheco and Mason, 2010) indicates that aerosol transmission is negligible among pigs in indirect contact even if they share the same room.

Air samples were collected before, during, and after the time of inoculation for strains A24Cru and O1 Manisa. Although this was not an indirect contact aerosol transmission study, determination of virus load in air samples was an indicator of viral shedding. We determined that air samples collected from rooms housing O1 Manisa infected pigs had 10–100 fold lower levels of viral RNA copies and were positive for a shorter period of time than those collected from rooms housing A24Cru infected pigs. This is an interesting finding since there were no noticeable differences in nasal or oral shedding between these two groups (as determined from swab samples). Thus, it is apparent that predicting the likelihood of transmission of a particular strain of FMDV is more complex than simply employing the conventional arsenal of quantitative techniques. Temporal and quantitative trends in detection of FMDV in room air, swabs, and serum surely contribute to transmissibility; but, in addition, less definable qualities such as virulence, tropism, and virus–host interactions are also relevant. These observations and considerations are similarly relevant when modeling FMDV transmission based on aerosol virus levels.

Conclusions

Important differences in transmission and pathogenesis among strains of FMDV belonging to serotypes A, O and Asia1 were demonstrated using direct-contact challenge methodology. Differences in transmission were variably associated with onset of clinical disease, viral shedding from oral/nasal swabs and with viral levels in room air. This work also demonstrates the importance of using a controlled exposure method to study pathogenesis and transmission dynamics of different FMDV strains. Finally, this research illustrates important differences in transmission dynamics between three FMDV strains of serotypes A, O and Asia1 that could have important implications for vaccine/biotherapeutic efficacy testing, pathogenesis and disease modeling.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tvjl.2012.01.012.

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Genus *Vesiculovirus*

4

Luis L. Rodriguez and Steven J. Pauszek

Abstract

The *Vesiculovirus* genus of the *Rhabdoviridae* includes viruses infecting mammals, insects and fish that have been taxonomically classified using a combination of serological relatedness, host range, genome organization, pathobiology and phylogenetic analysis of sequence data. There are 10 viruses assigned to the genus *Vesiculovirus* and a number of serologically related viruses that have been tentatively assigned to the genus. The best known members of the genus are vesicular stomatitis viruses which cause vesicular disease in farm animals including horses, cattle and pigs. Some vesiculoviruses cause disease in humans ranging from febrile syndromes (e.g. vesicular stomatitis virus) to fatal encephalitis (e.g. Chandipura virus). This chapter reviews the state-of-the-art knowledge about vesiculoviruses as well as some related viruses tentatively assigned to the genus.

Introduction

The first *Vesiculovirus* described was vesicular stomatitis virus (VSV), the prototype virus for the genus and one of the most studied RNA viruses. Although the first description of the virus was published in 1927 (Cotton, 1927) the vesicular stomatitis disease (initially known as sore mouth in cattle and horses) had been described affecting horses, pigs and cattle as early as 1801, along the eastern coast of the United States (Hanson, 1952). Outbreaks of 'sore tongue' were reported causing severe lameness in horses needed on the battlefield during the Civil War in 1862 (McClellan, 1864). Also VSV was carried from the US to Europe (France) with infected horses during World War I, and a vesicular disease was reported causing outbreaks in horses in South Africa in 1897 (Hanson, 1952). These two instances are the only reports of VSV causing disease outside the Americas. Reports of a vesicular disease in horses and cattle in Central and South America date back to the 1930s and continue to be reported every year in most endemic regions. There are well documented large epidemics of VSV occurring in the south-western US every 8–12 years from 1916 through 2006 (Rainwater-Lovett *et al.*, 2007).

Another vesiculovirus, Chandipura virus, was originally found as the cause of a febrile disease in humans in India in 1966. It was also found in sand flies, and it was shown that mosquitoes can be experimentally infected (Bhatt and Rodrigues, 1967; Rao *et al.*, 1967). More recently, Chandipura virus was associated with serious outbreaks of encephalitis, often fatal, in children from various parts of India (Hassler *et al.*, 2003; John, 2004; Rao *et al.*, 2004; Chadha *et al.*, 2005; Narasimha Rao *et al.*, 2008; Tandale *et al.*, 2008).

In 1977, Isfahan virus was found in sand flies in Iran and also in humans with febrile disease (Saidi *et al.*, 1977; Tesh *et al.*, 1977). This virus has also been isolated from sand flies collected in gerbil colonies in the former USSR (Gaidamovich *et al.*, 1980).

In the Americas, the majority of vesiculoviruses have been found in insects and mammals. The exception to this insect/mammal association is a virus that causes a disease in fish. This serious disease, referred to as 'infectious dropsy of carp,' appeared in 1930 and the aetiological agent was identified in the 1980s as spring viraemia of carp virus (SVCV) and has been currently classified as

a *Vesiculovirus* (Dietzgen *et al.*, 2011). This is the only member of the genus not infecting insects and/or mammals. It is very likely that many others, yet to be discovered vesiculoviruses exist in nature, circulating in insects, mammals or fish, perhaps without causing a visible disease.

Taxonomy and phylogeny

Vesiculoviruses have been taxonomically classified using a combination of morphological and serological relatedness, host range, genome organization, pathobiology and via phylogenetic analysis of gene sequences (when available). As defined in the ninth report of the International Committee on Taxonomy of Viruses, there are ten established *Vesiculovirus* species and nine related viruses tentatively assigned to the genus (Dietzgen, 2011) (Table 4.1). Based on a recent published report (Pauszek *et al.*, 2011), virus CoAr 171638 (Tesh *et al.*, 1987) has been identified as a vesiculovirus-related virus. Currently, only ten full length genomes of vesiculoviruses (MARAV, VSIV, COCV, VSAV, VSNJV, CHPV, ISFV, and SVCV) or related proposed vesiculoviruses (PFRV, EXEV) are present in GenBank (Table 4.2). Phylogenetic analyses based on the concatenated amino acid sequence of the five structural proteins show strongly supported grouping of the insect/mammal associated vesiculoviruses separate from those infecting fish (Fig. 4.1a). This monophyletic grouping was maintained when the amino acid sequence of the five structural proteins were analysed individually.

While full-length genomic sequences or even complete sequence of individual viral genes, are not available for the majority of viruses tentatively assigned to the *Vesiculovirus* genus, phylogenies derived from the amino acid sequence of block III of the L polymerase have been used recently to determine evolutionary relationships among a large number of unclassified rhabdoviruses (Bourhy *et al.*, 2005; Dacheux *et al.*, 2010). A phylogenetic tree of all available limited L gene sequences of classified or tentative vesiculoviruses shows three clearly demarcated groups: group I containing vesicular stomatitis viruses and viruses isolated from insects; group II containing vesiculoviruses infecting humans, and group III containing viruses of fish (Fig. 4.1b). This analysis provides support for the classification of the unassigned viruses CoAr 171638, PERV, JURV and MSPV as vesiculoviruses. Although viruses GLOV, PORV, KLAV and BTKV fall within the Dimarhabdovirus supergroup (rhabdoviruses infecting diptera and mammals) (Bourhy *et al.*, 2005), phylogenetic analysis based on the block III of the L polymerase does not support their classification as vesiculoviruses.

Antigenic properties

Vesiculoviruses have been identified and classified into serotypes based on their serological

Table 4.1 List of accepted and related/tentative vesiculoviruses discussed in this chapter. Assigned abbreviations are given in parenthesis

Vesiculovirus species	Related/tentative *Vesiculovirus* species
Carajas virus (CJSV)	Boteke virus (BTKV)
Chandipura virus (CHPV)	CoAr 171638 (CoArV 171638)
Cocal virus (COCV)	Eel virus European X (EXEV)
Isfahan virus (ISFV)	Grey Lodge virus (GLOV)
Maraba virus (MARAV)	Jurona virus (JURV)
Piry virus (PIRYV)	Klamath virus (KLAV)
Spring viraemia of carp virus (SVCV)	Malpais Spring virus (MSPV)
Vesicular stomatitis Alagoas virus (VSAV)	Perinet virus (PERV)
Vesicular stomatitis Indiana virus (VSIV)	Pike fry rhabdovirus (PFRV)
Vesicular stomatitis New Jersey virus (VSNJV)	Porton virus (PORV)

Table 4.2 Vesiculovirus genome organization and length

Species	Genome (nt)	N (aa)	P (aa)	M (aa)	G (aa)	L (aa)
VSIV	11,161–11,336	422	265	229	511	2109
COCV	11,003	422	264	229	512	2108
VSAV	11,070	422	260–261	229	511	2108
CoAr 171638	ND	ND	270	229[a]	513	ND
MARAV	11,135	422	265	229	512	2109
VSNJV	11,119–11,123	422	274	229	517	2109
CJSV	ND	ND	271	228	523	2109
CHPV	11,119–11,120	422	293	229	530	2092
ISFV	11088	423	289	226	523	2093
MSPV	ND	ND	273[a]	225[a]	521[a]	ND
PIRYV	ND	ND	327	229	529	ND
SVCV	11,019–11,047	418	309	223	509	2095–2101
PFRV	11097	419	309	222	508	2095
EXEV	11806	428	275	222	531	2075

ND, not determined.
[a]Previously unpublished.

relationships (Cartwright and Brown, 1972). Cross-neutralization, which is determined by antibodies recognizing epitopes located in the viral G protein, has been used to distinguish serotypes and subtypes of VSV. The G protein also determines protection in animals vaccinated with either inactivated whole virus or subunit vaccines (Mackett *et al.*, 1985). Furthermore, recombinant viruses containing the G protein of VSNJV, but all other proteins from VSIV, induced protective immune responses against challenge with VSNJV (Martinez *et al.*, 2004). The N protein is a more cross-reactive antigen than G and it is the base for complement fixation tests that helped define members of the genus (Brooksby, 1948; Jenney *et al.*, 1958). One of the criteria used for *Vesiculovirus* classification is cross-reactivity by complement fixation.

In the case of vesicular stomatitis viruses there are two major serotypes: New Jersey and Indiana (Federer *et al.*, 1967). The serotype Indiana has been subdivided into three distinct serological complexes. Indiana 1 comprises the classical Indiana viruses (VSIV) found from northern South America to southern US. The Indiana 2 subtype has Cocal virus (COCV) as the prototype virus which was originally isolated from mites collected from rice rats in Trinidad in 1961. Indiana 2 viruses cause disease in cattle and horses in Brazil and Argentina. The Indiana 3 subtype is represented by Alagoas virus (VSAV) which was first isolated from a mule in Alagoas, Brazil, in 1964. This subtype is the most common cause of vesicular stomatitis in livestock in Brazil (Pauszek *et al.*, 2011). In a recent study the phylogenetic relationships among South American vesicular stomatitis viruses were found to follow very closely the serological classification of these viruses. However, other vesiculoviruses found in South American insects but not causing clinical disease grouped into distinct genetic lineages from vesicular stomatitis viruses (Pauszek *et al.*, 2011).

In the case of the fish *Vesiculovirus* SVCV, there is a single serotype. Antibodies directed against SVCV cross-react to various degrees with other fish rhabdoviruses; pike fry rhabdovirus (PFRV), Grass carp rhabdovirus (GrCRV) and tench rhabdovirus (TenRV), indicating that these viruses are closely related which is reflected in the phylogenetic trees (Fig. 4.1). SVCV and PFRV have been shown to share common antigenic determinants on the G, N and M proteins, but can be differentiated by neutralization assays.

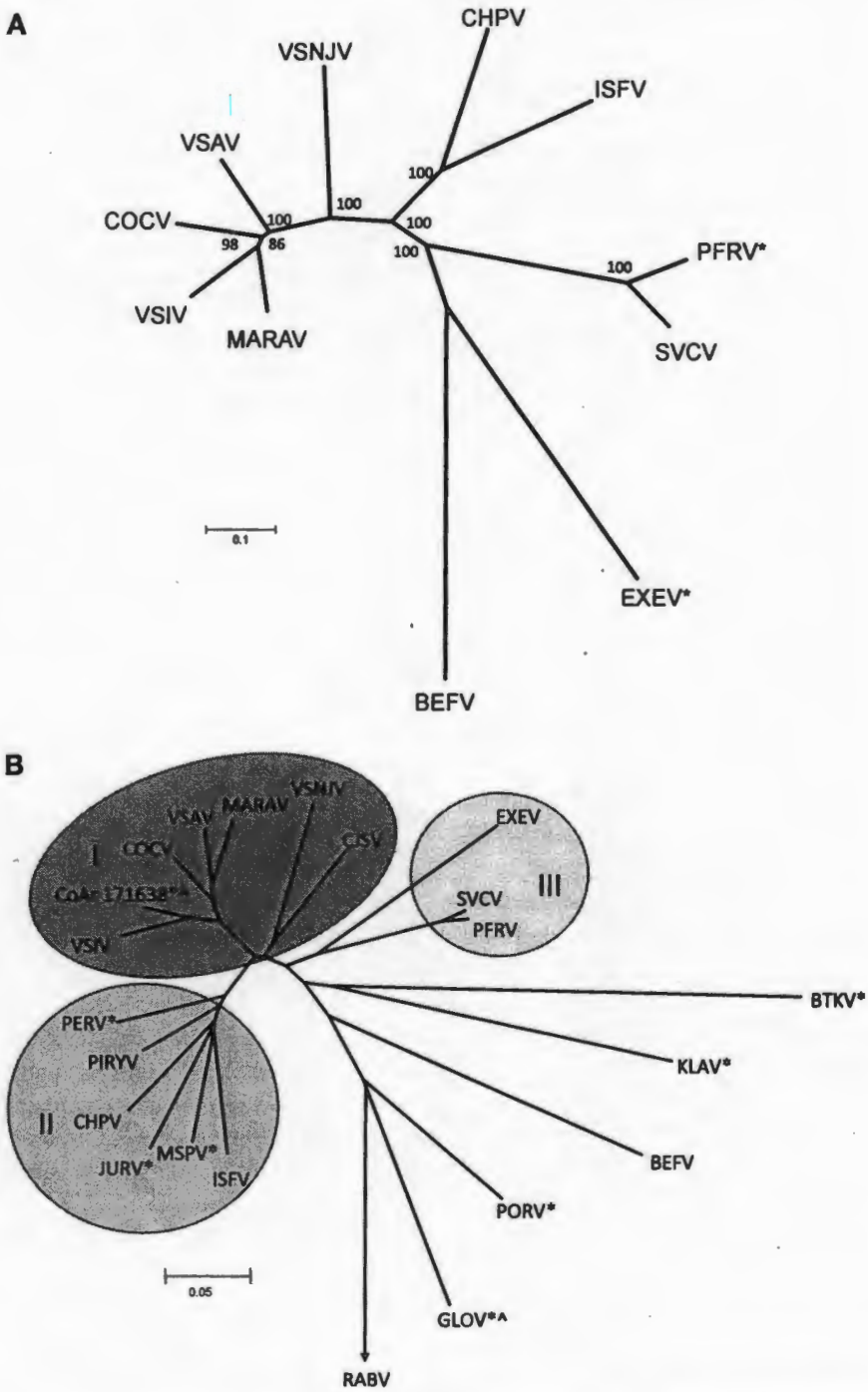

Figure 4.1 Phylogeny of the vesiculoviruses and related dimarhabdoviruses from concatenated full-length amino acid sequences of the five structural proteins (a) or block III of the L polymerase (b). Trees show three clearly demarcated groups: group I containing vesicular stomatitis viruses and viruses isolated from insects; group II containing vesiculoviruses infecting humans, and group III containing viruses of fish. The trees were inferred using the neighbour-joining method and numerical values indicate bootstrap support values (2000 replicates) for (a). The trees were calculated using the Poisson correction method and evolutionary distances represented as the number of amino acid substitutions per site. Gaps in the alignment were analysed with pairwise deletion. *Viruses related or tentatively assigned to the vesiculoviruses as defined in the ninth report of the International Committee on Taxonomy of Viruses; ^previously unpublished sequences.

Viral proteins, structure and morphology

Vesiculoviruses have five structural proteins (designated N, P, M, G and L).(Cartwright *et al.*, 1970; Kang and Prevec, 1970). Most vesiculoviruses also have two small, highly basic proteins coded in a second ORF within P (termed C′ and C) (Spiropoulou and Nichol, 1993). Three viral proteins (N, P and L) combine to form the transcription and replication complex which has RNA-dependent-RNA polymerase (RdRP) activity (Baltimore *et al.*, 1970; Davis and Wertz, 1982). The N protein encapsidates the viral RNA and functions in close association with the P protein. The P protein is a highly phosphorylated protein associated with viral polymerase activity. It mediates the binding of the L protein to the nucleocapsid core and facilitates access of the polymerase to the RNA template during transcription and replication (Wertz, 1983). Phosphorylation of P protein seems to be necessary for optimal transcriptase activity. The exact roles of the non-structural C and C′ proteins are unclear. Engineered viruses that do not express C proteins are indistinguishable from wild type virus in protein synthesis, virus production and host-protein synthesis shut off in tissue culture cells (Kretzschmar *et al.*, 1996). The L protein is multifunctional and performs most of the polymerase-associated functions including RNA synthesis, capping, methylation and poly (A) addition (Davis and Wertz, 1982). It also has protein kinase activity which preferentially phosphorylates serine residues on the P protein. The matrix protein (M) is the most abundant protein of virions. The M binds specifically to the G protein monomers and promotes their trimerization. The G protein is a typical class I membrane associated glycoprotein, with approximately 90% of the N-terminal region of the molecule projecting from the surface of the virion or infected cell, a hydrophobic transmembrane domain anchoring the protein in the membrane, and a C-terminal 28 amino acid cytoplasmic domain projecting to the interior of the infected cells (Rose *et al.*, 1980). The G protein forms trimers which constitute the approximately 400 spikes that are embedded on the virion bilayer phospholipid envelope. The G protein plays a major role in attachment and penetration of vesiculoviruses into susceptible cells and budding of virions from infected cells. It is the major target of serotype-specific neutralizing antibodies and is capable of inducing cell membrane fusion at low pH (Martinez and Wertz, 2005).

Virions are enveloped and bullet-shaped. The three-dimensional structure of VSV has been recently determined utilizing cryo-electron microscopy (Ge *et al.*, 2010). The virion structure is the result of the nucleocapsid forming a conical-shaped tip and a helical structure in the trunk. Matrix protein interacts with an inner helix of N protein and RNA as well as with neighbouring M and N subunits, providing rigidity to the ribonucleoprotein. Interactions among the N and M proteins provide scaffolding and stability to the complex and are critical in maintaining the bullet shape (Ge *et al.*, 2010). Two layers of lipids obtained from the host cell membrane with embedded trimers of the envelope glycoprotein form the outer layers of the virion and mediate the interaction with cellular receptor(s).

Genome organization and viral replication

Vesiculoviruses contain a single molecule of linear, negative-sense RNA of about 11 kb in length. The shortest genome is 11,003 nt (COCV) and the longest is 11,336 nt (VSIV) (Rodriguez *et al.*, 2002; Pauszek *et al.*, 2008). The genome organization for all vesiculoviruses consists of a short (~47 nt) leader sequence followed by the five structural protein genes in the order 3′ N–P–M–G–L, and a trailer sequence at the 5′ end. Most differences in length are found in non-translated regions, particularly in the M and G mRNAs. Despite the variability in lengths of the five mRNAs of the Indiana subtypes, four of the five predicted structural proteins (N, M, G and L) are within one amino acid residue in length from each other (Pauszek *et al.*, 2008).

Vesiculovirus replication is well studied and has served as a model for replication of rhabdoviruses. Transcription begins at a single entry site at the 3′ end of the genome with each gene expressed as a capped and polyadenylated monocistronic mRNA. The first transcript is the 3′ leader sequence, which is neither capped nor polyadenylated. It is transported to the nucleus

where it inhibits host cell transcription. The leader transcript is followed by the N mRNA, which is capped during synthesis by the virion polymerase complex (composed of N, P and L). The intergenic sequence (5'-AGUUUUUUUCAUA-3') serves as a signal for polyadenylation, termination and re-initiation of transcription in decreasing amounts as the polymerase complex moves away from the single promoter. Therefore, the gene order (i.e. 3' N>P>M>G>L 5') provides an efficient way of regulation of gene expression, by which proteins that are necessary in larger amounts (such as N) are located near the 3' end and are transcribed in larger amounts, and those needed in lesser amounts are located towards the 5' end and are transcribed less frequently (Ball and White, 1976).

After translation of the mRNAs to produce the viral proteins, genome replication starts. The RdRP initiates the replication process at the 3' end of the genome, ignores all signals for stop and polyadenylation of individual mRNAs, and instead synthesizes a full-length complementary antigenome. This antigenome serves as a template for synthesis of a negative-sense leader RNA, and also for synthesis of full-length progeny genomes. Full-length genomes can either serve as templates for secondary transcription, or can be assembled into infectious particles. Factors determining the RdRP functions in transcription or replication modes are not fully understood but the proportion of N protein and available RNP templates are thought to be critical for determining these functions. Following replication, further rounds of transcription (secondary transcription), translation and replication ensues (Barr *et al.*, 2002).

Host range and geographical distribution.

The host range of vesiculoviruses include a variety of mammals, birds, fish and insects, found to be infected either by direct virus isolation or by evidence of exposure to virus from serological tests. The viruses infecting mammals are all transmitted by insects and therefore are considered arthropod borne viruses (arboviruses). Vesicular stomatitis viruses have been found causing disease in horses, cattle, pigs and can also infect humans causing flu-like symptoms. Other vesiculoviruses cause febrile disease and encephalitis in humans (e.g. Piry virus and Chandipura virus).

The natural cycle of vesiculoviruses infecting mammals remains largely undefined. These viruses are commonly found in a number of insect species including sand flies (flebotomine flies), black flies (simulid flies), several species of *Culicoides* and mosquitoes. Serological evidence suggests they are capable of infecting not only a number of wild mammals, but also birds and even reptiles living in endemic areas (Tesh *et al.*, 1969, 1970). This wide range of hosts might explain the ability of vesiculoviruses to infect and replicate in a very diverse range of vertebrate and invertebrate cell cultures.

The incredibly broad host range of the vesiculoviruses most likely contributes to their expansive geographical distribution. Viruses of two species, VSNJV and VSIV, are found endemically from northern South America (Peru, Colombia, Ecuador, Venezuela), through Central America into southern Mexico with sporadic incursions into northern Mexico and south-western United States. Vesicular stomatitis virus subtypes Indiana-2 or Indiana-3 sporadically cause disease in Brazil while Indiana 2 has also occurred in Argentina (Pauszek *et al.*, 2011).

The geographic distribution of Chandipura virus seems to be mainly in India, with initial reports in northern India (Chandipur, Orissa) and later reports in Central and Southern India (Rao *et al.*, 2004). However, it has also been reported in Nigeria (Berge, 1975). Isfahan virus has been reported in Central Iran (Esfahan) (Saidi *et al.*, 1977) and has also been reported in neighbouring Turkmenistan (Gaidamovich *et al.*, 1980). Spring viraemia of carp virus has been reported in Europe, Asia and the Americas (Walker and Winton, 2010). Two vesiculoviruses (Carajas virus and Maraba virus) were isolated from phlebotomine sand flies in Brazil (Travassos da Rosa *et al.*, 1984) but have not been associated with disease in livestock or humans. Reports of naturally occurring Piry virus infection have been limited to Brazil but human disease associated to laboratory infections have been reported (Wilks and House, 1984).

Viral ecology

Vesicular stomatitis viruses are transmitted to cattle, horses and pigs by various blood-sucking insects (sandflies, blackflies, and culicoides) found to be infected during epidemics, and can also be transmitted between mammals by direct contact (Mead *et al.*, 2004). Additionally, experimental mechanical transmission has been achieved by feeding vesicular stomatitis virus laboratory infected grasshoppers to cattle (Nunamaker *et al.*, 2003; Drolet *et al.*, 2009). However, grasshoppers have never been shown to carry vesiculoviruses in natural infections. Vesiculoviruses have been shown to be transmitted transovarially and, interestingly, also horizontally between infected and non-infected black flies while co-feeding on mammalian hosts (Mead *et al.*, 2000). The latter mechanism of transmission might explain the noticeable absence of viremic mammalian hosts for vesiculoviruses, an unusual feature for an arbovirus (Smith *et al.*, 2011). Black flies have been shown to transmit VSNJV both biologically and mechanically to horses, pigs and cattle, and those species have also been shown to serve as sources of virus to uninfected flies feeding at or near lesion sites (Mead *et al.*, 2004; Howerth *et al.*, 2006; Mead *et al.*, 2009; Smith *et al.*, 2009). These multiple mechanisms of transmission offer a variety of possible life cycles by VSV in nature.

There is evidence of a correlation between ecological conditions and VSV RNA evolution (Rodriguez *et al.*, 1996). Studies in endemic areas have shown that specific genetic lineages of vesiculoviruses exist in specific ecological zones over long periods of time, causing local outbreaks on a regular basis. Some of these endemic areas exist in southern and central Mexico but specific viral lineages from these regions sporadically cause outbreaks in the western US (Rainwater-Lovett *et al.*, 2007). Although the mechanisms of introduction are not clear, these outbreaks usually occur in the spring and summer months and subside after the first frost, presumably when insect vector populations decrease (Rodriguez, 2002). These viral lineages are capable of overwintering and re-emerge in the US in the spring-summer but usually become extinct after one to two years. New outbreaks are associated with new genetic lineages closely related to those circulating in endemic areas (Perez *et al.*, 2010).

The fish *Vesiculovirus* SVCV affects cyprinid (carp, minnows) fish. Naturally occurring SVC infections have been recorded in the common carp (*Cyprinus carpio*), koi carp (*Cyprinus carpio koi*), crucian carp (*Carassius carassius*), sheatfish, (also known as European catfish or wels) (*Silurus glanis*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), grass carp (white amur) (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), orfe (*Leuciscus idus*), and tench (*Tinca tinca*).

The virus can be transmitted by ectoparasites such as carp louse (*Argulus foliaceus*) and the leech (*Pisicola geometra*), but waterborne transmission without any vector organism is also effective (Ahne *et al.*, 2002). Like other viruses from fish, SVCV is typically isolated in fish cell cultures at 15–25°C, a lower replication temperature than mammalian rhabdoviruses. The disease patterns are influenced by water temperature, age and condition of the fish, population density and stress factors. The immune status of the fish is also an important factor with both non-specific (interferon) and specific immunity (serum antibodies, cellular immunity). Clinical disease is usually observed at water temperature between 5–18°C and is most severe at temperatures below 10°C, when it is believed the host immune response is suppressed or delayed.

Pathobiology

Among vesiculoviruses the best characterized are those causing VSV in livestock, CHPV in humans, and SVCV in fish. There is also a great deal of information published on various vesiculoviruses and their effect in laboratory animals. We will only cover the pathobiology occurring after the natural infection. Vesicular stomatitis (VS) is a disease of cattle, horses and pigs that occurs in North, Central and South America. Serological evidence of the infection is also found in wild animals and in humans living in endemic regions (Tesh *et al.*, 1969). The clinical signs include the appearance of vesicular lesions in the mouth (tongue, gums) and hooves of animals, as well as on the udders of lactating cattle. In cattle and pigs the

clinical signs resemble those of foot-and-mouth disease (FMD), a devastating reportable disease of livestock that causes massive economic losses. When VS outbreaks occur, animal health authorities impose strict animal movement quarantines until FMD is ruled out with laboratory tests. In humans, VSV infection has been documented both naturally during outbreaks in livestock as well as a result of a laboratory exposure (Johnson *et al.*, 1966; Reif *et al.*, 1987). Vesicular lesions are not commonly observed, but rather a febrile, flu-like disease with signs of mild non-fatal encephalitis occurs (Quiroz *et al.*, 1988).

The basic mechanisms of infection leading to vesicular lesions remain unclear. Until recently, most pathogenesis studies have been done in laboratory rodents in which clinical signs are not vesicular in nature, but the infection manifests as a fatal encephalitis depending on host factors such as age, route of inoculation and virus strain. In cattle and pigs the development of lesions is dependent upon the site and the dose of inoculation. Vesicular lesions are observed only when cattle or swine are inoculated intradermally or by scarification at sites where lesions are found (snout, mouth, lip or coronary band of the hoof). Vesicular lesions are not observed after inoculation of the skin of the neck or abdomen, nor by the intranasal or intravenous routes (Scherer *et al.*, 2007). Likewise, clinical disease has been demonstrated in cattle and swine experimentally infected via black flies (*Simulim vittatum*) biting at lesion sites, but not when biting at non-lesion sites (Mead *et al.*, 2009). The viral and host determinants of virulence that define the clinical outcome are not clear. Few genetic determinants of virulence relevant to clinical disease in farm animals have been identified, except for the viral glycoprotein (Martinez *et al.*, 2003). The mechanisms of pathogenesis are not well elucidated, but recent data suggest that viral replication is limited at the non-lesion sites probably due to strong innate responses present at these sites (Reis *et al.*, 2010).

Early pathogenesis studies in cattle indicated that large amounts of virus inoculated in submucosa of the tongue resulted in local vesicular lesions (Cotton, 1926; Ribelin, 1958; Seibold and Sharp, 1960). However, these studies are unlikely representative of natural infection. Most natural infections in cattle living in endemic areas are subclinical and detected only by seroconversion without associated clinical signs (Rodriguez *et al.*, 1990; Vanleeuwen *et al.*, 1995). When clinical disease occurs in cattle in endemic regions, animals show vesicular lesions at specific sites: coronary bands, teats, mouth, or tongue, but rarely at more than one site (Heiny, 1945; Vanleeuwen *et al.*, 1995). The host and viral determinants for this tropism remain unclear. Little is known about the primary site of virus replication, cell types involved in supporting viral growth, or those involved in controlling the infection. In recent studies it was shown that bites by infected insects at lesion sites consistently resulted in lesion formation at much lower doses than the doses necessary to cause lesions via needle injection or scarification (Reis *et al.*, 2010). In pigs, generalized lesions are more common, and direct contact transmission between animals is readily observed in swine infected with VSNJV but not with VSIV (Stallknecht *et al.*, 2004). This is consistent with findings that the glycoprotein is a virulence determinant for VSV, indicating that the VSNJV G significantly increases VSIV virulence in swine (Martinez *et al.*, 2003).

Role of insects in VSV natural transmission cycle

The natural cycle of VSV remains obscure, although there is strong evidence that insects carry the virus in nature. In endemic regions VSV has been found infecting sand flies (*Lutzomyia* spp.), biting gnats (*Culicoides* spp.) and even mosquitoes (*Aedes* spp.) (Tesh *et al.*, 1970; Liu and Zee, 1976; Tesh *et al.*, 1987). During outbreaks in the southwestern United States, VSV has been isolated from *Culicoides* (*Culicoides* spp.), black flies (*Simulium* spp.) (Walton *et al.*, 1987; Francy *et al.*, 1988), mosquitoes and in non-biting insects such as eye gnats and house flies (Sudia *et al.*, 1967; Francy *et al.*, 1988). Members of three insect groups: sand flies (Diptera: Psychodidae), black flies (Diptera: Simuliidae) and *Culicoides* (Diptera: Ceratopogonidae) have experimentally been shown to be biological vectors of VSV (Tesh *et al.*, 1971; Cupp *et al.*, 1992). Biological vectors are those capable not only of supporting viral replication but

also of transmitting the virus to susceptible hosts. To date, only black flies (*S. vittatum*) have been shown to be capable of VSV transmission resulting in clinical disease in livestock species including pigs, cattle and horses (Mead *et al.*, 2004; Howerth *et al.*, 2006; Mead *et al.*, 2009). *Culicoides* sonorensis transmitted VSNJV to cattle but the infection was subclinical and resulted only in seroconversion (Perez de Leon and Tabachnick, 2006). However, in that work insect feeding was done at non-lesion sites which might explain the result. Other insects such as sand flies (*Lutzomyia shannoni*) have been shown to be capable of replicating VSV and transmitting the virus to laboratory mice (Comer *et al.*, 1991). A recent report showed that grasshoppers infected either by needle injection or by feeding on grass painted with VSNJV could transmit VSV to cattle fed with these infected insects. Although it is possible that they might play a role in VSV transmission, there are no reports of infected grasshoppers during VSV outbreaks (Nunamaker *et al.*, 2003; Drolet *et al.*, 2009). An interesting mechanism of VSNJV transmission between infected and non-infected black flies while co-feeding in naïve mice and cattle has been recently reported (Mead *et al.*, 2000; Smith *et al.*, 2009). This is the only example of horizontal transmission of an animal arbovirus by insects. Transmission did not require direct contact between infected and non infected flies and in some cases concurrent co-feeding was not necessary for transmission to occur. These authors also showed that vesicular lesions can serve as a source of insect infection (Smith *et al.*, 2009).

It has been previously documented that bites from arthropods can modulate vertebrate host functions by several mechanisms including modulation of the immune response, vasodilation and other physiological effects (Schoeler and Wikel, 2001). Arthropod salivary gland extracts can augment the growth of VSV in vitro in primary cell cultures and in laboratory mice, presumably by interfering with the host innate immune response (Limesand *et al.*, 2000, 2003). Although few studies exist in animals, tissues from cattle infected by scarification showed higher number of immune cells positive for MHC-II antigens than cattle infected by fly bite suggesting a down regulation of inflammatory response by insect factors. These data, combined with the fact that fly bite induced vesicular lesions with lower VSV titres than those required to see vesicular lesions after scarification, suggest that insect bites can potentiate VSV infection (Reis *et al.*, 2010).

Epidemiology and control

VS is endemic from southern Mexico to northern South America with outbreaks caused primarily by VSNJV, reported every year, mostly in cattle, but also in horses and pigs (Rodriguez, 2002). In Mexico disease outbreaks are more common in the central and southern states with less frequent incursions into northern states (Arroyo *et al.*, 2011). In the United States, VS outbreaks occur at approximately 8- to 10-year intervals in cycles lasting one to three years in the south-western states (Texas, Arizona, New Mexico, Utah, Colorado), with the last three outbreak cycles occurring in 1984/85, 1995/98 and 2004/06 (Rainwater-Lovett *et al.*, 2007). Phylogenetic and spatial analyses of the viruses causing the 2004–2006 outbreak cycles showed that the same viral lineage overwintered in the US and that the virus was not re-introduced each subsequent year (Perez *et al.*, 2010). Two additional small outbreaks of VSNJV confirmed only by serological tests were reported in Texas and New Mexico in 2009 and in Arizona in 2010 (http://www.aphis.usda.gov/vs/nahss/equine/vsv/vsv2010.htm). It is interesting to point out that large VSV outbreaks were reported in 2008 and 2009 in northern Mexico near the US border (Arroyo *et al.*, 2011).

Phylogenetic analyses show that each outbreak cycle in the US is caused by a single viral lineage that is genetically closer to viruses circulating concurrently in endemic areas of Mexico than to viruses causing previous outbreaks in the US (Rainwater-Lovett *et al.*, 2007). New viral outbreaks in the US typically start in the Mexico border states of Texas, Arizona or New Mexico in early spring and progress north following valleys and rivers along the Rocky Mountain range reaching as far north as Montana or Nebraska and usually end after the first frosts of the winter (Rodriguez, 2002). This pattern of VSV occurrence in the US is consistent with vector transmission rather than animal movement.

Control measures of vesiculoviruses depend on the knowledge of the viral ecology, transmission and epidemiology. In the case of VSV, data indicates that the virus persists in endemic regions in a stable manner, causing disease in a small proportion of the infected animals. The problem arises when virus incursions occur into non-endemic areas, spreading rapidly and affecting a large number of animals. Control measures include stop of animal movement (quarantine), separation of infected and non infected animals and recommendations to control exposure to biting insects (Hurd *et al.*, 1999). Vaccines to VSV have been produced and tested and show that immunization with a killed virus vaccine protects against homologous challenge with a virus of the same serotype (House *et al.*, 2003). However, implementing vaccination programmes for a sporadic disease has had limited acceptance and currently is not utilized even in endemic regions.

In the case of CHPV, several vaccines have been recently developed including subunit and inactivated vaccines (Venkateswarlu and Arankalle, 2009, 2010; Jadi *et al.*, 2011). However, routine vaccination is not commonly practiced. Likewise, SVCV experimental vaccines have been reported by routine vaccination is not practiced (Emmenegger and Kurath, 2008).

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Foot-and-mouth disease virus (FMDV) with a stable FLAG epitope in the VP1 G-H loop as a new tool for studying FMDV pathogenesis

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ABSTRACT

Foot-and-mouth disease virus (FMDV) VP1 G–H loop contains the major antigenic site. By replacing the sequence upstream of the RGD motif with a FLAG epitope, a marker virus for pathogenesis studies was generated. In cell culture, the recombinant virus containing FLAG (A24-FLAG) exhibited similar plaque phenotypes and growth kinetics to parental virus. A24-FLAG was distinguished, neutralized, and immunoprecipitated by FLAG anti-sera. A24-FLAG infected cattle exhibited FMD and an antibody response similar to parental virus. FLAG epitope stability was confirmed both *in vitro* and *in vivo*. Interestingly, no anti-FLAG antibodies were detectable in cattle up to 21 days post-inoculation. A24-FLAG G–H loop modeling suggested FLAG was rendered a cryptic site, inaccessible to the host immune system. These studies demonstrate the FMDV VP1 G–H loop tolerance to substitutions without detriment to pathogenesis and antigenicity. Finally, A24-FLAG manifested virulence in cattle as parental virus, and could be distinguished and tracked by tag-specific anti-sera.

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Introduction

Foot-and-mouth disease virus (FMDV) is responsible for the most economically important viral disease of cattle and other cloven-hoofed animals (Domingo et al., 2002; Grubman and Baxt, 2004; Mason et al., 2003; Saiz et al., 2002; Sobrino et al., 2001). It has been demonstrated that FMDV, the prototypic member of the *Aphthovirus* genus of the *Picornaviridae*, utilizes *in vitro* four integrin heterodimers ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$) for both attachment to host cells and subsequent entry via clathrin-coated pits (CCPs) (Baxt and Becker, 1990; Duque and Baxt, 2003; Jackson et al., 2000a,2000b, 2002; 2004; Neff et al., 1998; O'Donnell et al., 2005; Ruiz-Saenz et al., 2009). Specifically, a prominent surface-exposed loop connecting the βG-βH strands (known as the G–H loop) of the VP1 capsid protein (Acharya et al., 1989; Logan et al., 1993) contains a highly conserved Arg-Gly-Asp (RGD) motif, which has been shown to be a recognition sequence for the α_v-integrin family of cell surface receptors (Baxt and Becker, 1990; Brown et al., 1999; Burman et al., 2006; Fox et al., 1989). The VP1 G–H loop can be removed through limited trypsin proteolysis, resulting in FMDV particles that are considerably less infectious relative to untreated virions, thus highlighting the importance of this region for productive infection (Strohmaier et al., 1982; Wild and Brown, 1967). In addition to its importance to infectivity, the VP1 G–H loop has been described as one of the immuno-dominant sites (antigenic site 1 for serotype A) on the virus particle for several FMDV serotypes (Acharya et al., 1989; Aggarwal and Barnett, 2002; Barnett et al., 1989; Baxt et al., 1989; Broekhuijsen et al., 1987; Brown, 1988; Crowther et al., 1993a, 1993b; Kitson et al., 1990; Mateu et al., 1990; Thomas et al., 1988b). Additionally, FMDV field isolates continually passaged in cell culture adapt to utilize heparan sulfate (HS) as an alternative receptor, thus allowing for replication in cells expressing HS but not α_v-integrins (CHO K1 cell line), though such viruses exhibit attenuated pathogenicity (Baranowski et al., 2000; Fry et al., 1999; Jackson et al., 1996; Sa-Carvalho et al., 1997). Additionally, in isolated cases, FMDV can adapt to use an as yet unidentified third receptor (Baranowski et al., 2000; Zhao et al., 2003) permitting growth on cell lines devoid of both the integrin heterodimers and HS such as CHO 677 cells (Esko et al, 1988; Stephens et al., 2006).

We considered various known surface-exposed regions on the FMDV capsid as sites for the insertion or substitution of a foreign epitope to develop a recombinant FMDV vector for viral pathogenesis studies. Given the flexibility of the hypervariable G–H loop (Acharya et al., 1989), we anticipated that inserting a foreign tag sequence upstream of the RGD motif would be tolerated by the virus capsid. The FLAG epitope tag was selected for this proof-of-concept study based on its established utility in the differentiation of recombinant proteins from wild-type (WT) proteins,

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the array of commercially available anti-FLAG antibodies (Chubet and Brizzard, 1996; Einhauer and Jungbauer, 2001; Hopp et al., 1988) and importantly, the successful insertion of the FLAG epitope into a variety of viruses including FMDV (Baranowski et al., 2001; Laird and Desrosiers, 2007; Prentoe and Bukh, 2011; Seago et al., 2012; Tan et al., 2009; Upadhyay et al., 2011).

In this study, we have designed, produced, and characterized a recombinant FMDV with a FLAG tag embedded in the VP1 G–H loop just upstream of the RGD motif. The capacity of the FLAG-tagged virus (A24-FLAG) to replicate to similar titers as the WT progenitor virus (A24 Cruzeiro) on permissive cell lines was evaluated and confirmed. Moreover, the embedded FLAG tag was recognized by anti-FLAG antibodies, which allowed A24-FLAG to be distinguished by multiple immunological assays. Moreover, genetic and biochemical testing of clinical samples collected from cattle inoculated with A24-FLAG confirmed that the FLAG epitope was maintained post-infection of cattle. Although animals inoculated with the A24-FLAG virus did not develop an anti-FLAG epitope response, the recombinant virus could still be screened with commercially available anti-FLAG antibodies making this variant virus a useful tool in the study of FMDV pathogenesis, and to assess the significance of antigenic site 1 in serotype A FMDV.

Results

Derivation and preliminary characterization of FLAG-tagged FMDV.

Several sites within the P1 region of the FMDV genome were considered for insertion of the FLAG tag. Ultimately, the flexible and highly variable VP1 G–H loop of the VP1 capsid protein was selected. As depicted in Fig. 1A, within the A24 Cruzeiro (A24-Cru WT) backbone, the FLAG octapeptide (DYKDDDDK) replaced eight amino acid residues (SKYAVGGS) within the G–H loop upstream of the conserved RGD motif, which represents an antigenically significant site (Baxt et al., 1984; Brown et al., 1999). This site has also been successfully interchanged between different serotypes of FMDV (Wang et al., 2012). To minimize potential structural disruptions caused by the insertion, the YK of the FLAG epitope was reversed to KY as found in the original portion of the A24-Cru WT G–H loop sequence (Fig. 1A). Sequencing of the propagated recombinant virus confirmed the presence of the modified FLAG octapeptide (DKYDDDDK) upstream of the RGD motif (Table 1). Three additional amino acid substitutions were detected; one each in VP4, VP2, and VP1 (Table 1). All other nucleotides in the recombinant virus distinct from the consensus sequence for A24-Cru WT (Accession #AY593768) were silent mutations (Table 1).

The plaque morphologies of the WT and FLAG-tagged virus (hereafter referred to as A24-FLAG) grown on BHK-21 cells were compared, which showed similar plaque sizes (Fig. 1B). Additionally, one-step viral growth curves overlap for the WT and A24-FLAG viruses in BHK-21 cells (Fig. 1C). Sequencing of the recovered virus after multiple passages confirmed that the modification to the G–H loop was genetically stable (data not shown). Cumulatively, these preliminary results suggested that the insertion of the FLAG sequence upstream of the RGD motif did not alter the growth properties of A24-FLAG relative to its WT progenitor.

Replacement of FMDV residues within the G–H loop by FLAG tag renders a virus that maintains integrin recognition for entry into susceptible cells.

As described in Introduction, FMDV primarily utilizes specific αv-integrin heterodimers for attachment, though the virus can mutate to use HS and in some cases an unknown third receptor.

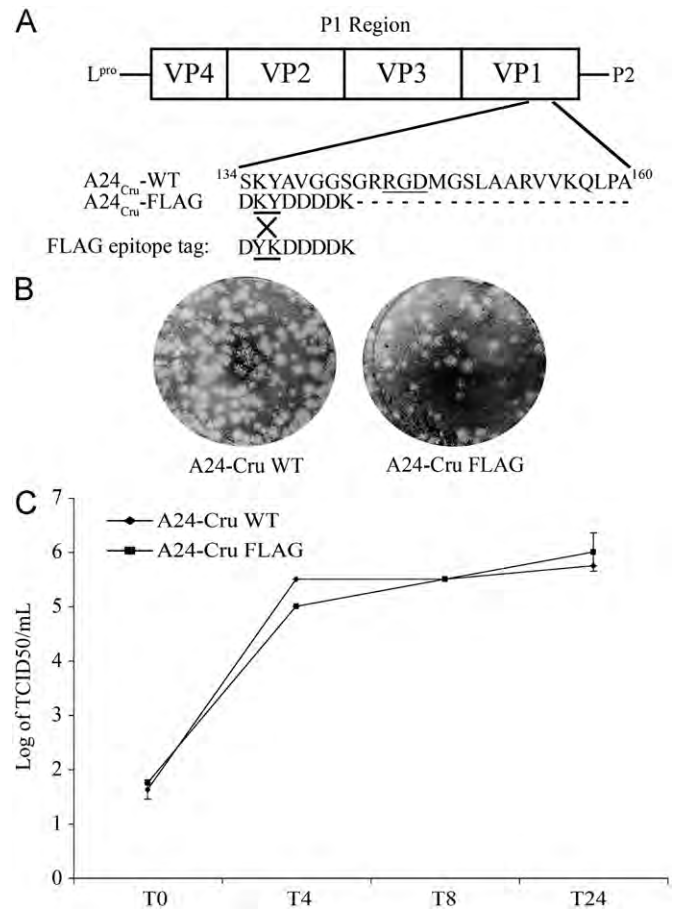


Fig. 1. Design of A24-FLAG. (A) Depiction of the insertion of the FLAG epitope within P1 region of the FMDV genome, specifically within the coding sequence for capsid protein VP1. As indicated, to minimize disruption to the normal structure of the G–H loop, the second and third amino acids of the FLAG epitope, YK, were reversed to KY. (B) Side-by-side comparison of the plaque morphologies of A24-Cru WT and A24-FLAG grown on a monolayer of BHK-21 cells at 24 hpi. (C) Growth curves of A24-Cru WT and A24-FLAG with measured time points at 0, 4, 8, and 24 hpi. Infections with each virus were performed in triplicate.

To evaluate if the substitution of the FLAG tag upstream of the RGD motif affects the ability of A24-FLAG to use α_v-integrin heterodimers for infection, transient expression of 4 different integrin heterodimers ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$) was achieved in a cell line (COS-1) devoid of α_v-integrin heterodimers (data not shown). The cells were then infected with either A24-Cru WT or A24-FLAG in the presence of ^{35}S-methionine, the infected cell lysates radioimmunoprecipitated (RIP) with anti-FMDV anti-sera, and the resulting eluates examined by autoradiography. FMDV-specific viral proteins (3D, VP0, and 2C) were detectable in the eluates from cells infected with both WT and A24-FLAG that transiently expressed $\alpha_v\beta_3$ or $\alpha_v\beta_6$ (Fig. 2A). To a much lesser extent, some viral protein can be seen in cells expressing $\alpha_v\beta_1$. No FMDV viral proteins were ever observed in RIP eluates from cells transiently expressing $\alpha_v\beta_5$, which is consistent with previous reports (Duque and Baxt, 2003; Duque et al., 2004). We inferred from these results that the embedded FLAG epitope upstream of the RGD motif in the G–H loop did not disrupt the interaction with the integrin receptor.

To corroborate the findings of the transient transfection infection assay (Fig. 2A), BHK-21, CHO K1, and CHO 677 cells were infected with A24-FLAG in parallel with cultures infected with the WT virus. In BHK-21 cells, A24-FLAG grew to within one half of a log of the titer achieved by the WT A24 Cruzeiro (Fig. 2B). However, only the HS-adapted FMDV VCRM4 variant of O1

Table 1
Sequence comparison of the P1 region of A24-Cru WT and A24-FLAG.

Region	A24 Cruzeiro WT			A24 Cruzeiro FLAG		
	Nucleotide[a]	Codon[b]	Amino acid[c]	Nucleotide[a]	Codon[b]	Amino acid[c]
VP4	G9	ggG	–	C9	ggC	Silent
VP4	A55	Agc	S19	G55	Ggc	G19
VP2	T275	aTg	M92	C275	aCg	T92
VP2	G417	cgG	–	A417	cgA	Silent
VP2	A600	caA	–	G600	caG	Silent
VP3	G939	ggG	–	T939	ggT	Silent
VP3	T1416	gcT	–	G1416	gcG	Silent
VP1	G1578	acG	–	C1578	acC	Silent
VP1	A1579	Act	T527	G1579	Gct	A527
VP1	A1972/ G1973	AGt	S658	G1972/ A1973	GAt	D658
VP1	C1982	gCt	A661	A1982	gAt	D661
VP1	T1985/ G1986	gTG	V662	A1985/ C1986	gAC	D662
VP1	G1988	gGt	G663	A1988	gAt	D663
VP1	G1991	gGt	G664	A1991	gAt	D664
VP1	T1993/ C1994	TCa	S665	A1993/ A1994	AAa	K665
VP1	C2143	Ctg	–	T2143	Ttg	Silent

[a] Nucleotide different between the viruses followed by number indicating its position within the coding sequence of the P1 region.
[b] Lowercase letters indicate the bases shared by the two viruses, and capital letters indicate the bases that differ between the two viruses.
[c] One-letter code of the encoded amino acid residues followed by number indicating residue position in the P1 polypeptide.

Campos (Neff et al., 1998) could appreciably replicate on the CHO K1 cell line, strongly suggesting that like A24-Cru WT, A24-FLAG utilizes integrins as the primary receptor for infection of host cells. Additionally, none of the viruses demonstrated significant replication in the CHO 677 cell line, which is consistent with what has been previously described for the receptor preferences of WT A24 and VCRM4. Based on the combined results of Fig. 2A and B, we concluded the FLAG epitope substitution did not negatively impact the ability of A24-FLAG to interact with $\alpha_v\beta_3$ or $\alpha_v\beta_6$, thus maintaining the established range of cell lines permissive to FMDV infection.

Antigenic detection of A24-FLAG.

To confirm that the introduction of the FLAG epitope within the antigenic site in the VP1 G–H loop (VP1-FLAG) could be utilized as a unique marker for detection, the A24-WT and A24-FLAG viruses were examined by immunocytochemistry (ICC) using commercially available antibodies against FLAG and a monoclonal antibody (anti-VP1) generated against an epitope located downstream of the RGD sequence within serotype A FMDV. As shown in Fig. 3A, both WT and A24-FLAG infected cells showed significant staining with the anti-VP1 antibody. However, only A24-FLAG infected cells reacted with the polyclonal rabbit anti-FLAG antibody.

To corroborate the ICC results, WT and A24-FLAG infected cell lysates were analyzed by western blot (Fig. 3B). Probing with anti-VP1 produced an immunoreactive band in both lysates that migrated at the approximate molecular weight predicted for VP1, but only the A24-FLAG lysate produced the corresponding band using the polyclonal anti-FLAG antibody. Considering that the second and third amino acids in the FLAG epitope were inverted from DKYDDDDK to DYKDDDDK (Fig. 1A), it seems likely that the altered amino acid sequence is not recognized by monoclonal anti-FLAG.

In a further elaboration of the utility of the embedded FLAG epitope within VP1 for molecular pathogenesis studies, A24-FLAG infected cells were examined by immunofluorescent microscopy (IFM) probing with both polyclonal anti-FLAG and monoclonal anti-VP1. As shown in Fig. 4, A24-FLAG infected cells stained positive for both FLAG and VP1. Consistent with the supposition that the FLAG and VP1 anti-sera reacted with FMDV particles displaying the FLAG epitope as well as the G–H loop epitope specific for the monoclonal anti-VP1, the fluorescence associated with both antibodies overlapped when the individual fluorescent channels were merged (Fig. 4A). When the assay was repeated using WT A24 lacking the FLAG epitope, only the VP1 antibody reacted with the FMDV infected cells (Fig. 4B). We concluded that the FLAG epitope substitution in A24-FLAG allowed for the antigenic distinction from WT virus without any discernable change to the replication cycle of FMDV.

A24-FLAG can be neutralized and isolated by polyclonal anti-FLAG.

Given the reactivity of the polyclonal anti-FLAG antibody to VP1-FLAG in A24-FLAG infected cell lysates, but not VP1 from A24-Cru WT, we investigated the capacity of polyclonal anti-FLAG to neutralize A24-FLAG. Preparations of A24-Cru WT and A24-FLAG were pre-incubated with various dilutions of the FLAG antibody and were subsequently applied to the LFBK cell line, which is permissive to all seven serotypes of FMDV and closely parallels bovine primary kidney cells (Swaney, 1988). Twenty-four hours post-infection, the virus-infected cells were harvested and viral protein synthesis was evaluated. Western blots of the virus-infected cell lysates were probed with an antibody directed against the FMDV 3D polymerase ($3D^{pol}$), which showed that A24-Cru WT was unaffected by the FLAG anti-sera at any of the dilutions tested (Fig. 5A, left panel). In strong contrast, A24-FLAG was effectively neutralized by the FLAG anti-sera at the highest antibody dilution tested (Fig. 5A, right panel).

The antibody neutralization samples were then tested for relative virus titer. Titrations revealed that pre-treatment with the anti-FLAG antibody significantly impacted the resulting titer of A24-FLAG (Fig. 5B). Pre-treatment of A24-FLAG at the highest dilution of the anti-FLAG antibody (1:100) produced a greater than 50% reduction in virus titer relative to the titer achieved by the A24-FLAG virus in the absence of anti-FLAG pre-treatment. The lowest dilution of the anti-FLAG antibody (1:10) neutralized A24-FLAG to such a degree that no plaque forming units were detected during the titration assay. In contrast, A24-Cru WT experienced no significant reduction in virus titer from the anti-FLAG pre-treatment (Fig. 5B).

Given that the polyclonal anti-FLAG could neutralize A24-FLAG virus, we wanted to explore if it could similarly be utilized to purify virus particles from infected cell lysates *in vitro*. As such, lysates from A24-FLAG infected cells were immunoprecipitated with anti-FLAG coupled to protein A/G beads in parallel with lysates from cells transiently expressing FLAG-tagged RNA Helicase A (FLAG-RHA). Western blots of the resulting eluates probed with anti-FLAG revealed a band corresponding to the molecular weight of VP1 in lanes containing A24-FLAG infected lysates (Fig. 4C). Similarly, in lanes containing FLAG-RHA expressing lysates, anti-FLAG detected a band consistent with the molecular weight of FLAG-RHA. When probed with anti-VP1, the VP1 band was only detected in the lanes of A24-FLAG infected cell lysates. When the anti-FLAG immunoprecipitation reaction was repeated with A24-Cru WT, VP1 was not detected on western blots of the eluates probed with anti-VP1 and anti-FLAG (data not shown). We concluded that the FLAG epitope was presented in the G–H loop of A24-FLAG such that it was accessible to polyclonal anti-FLAG, permitting the purification of A24-FLAG from infected cell culture.

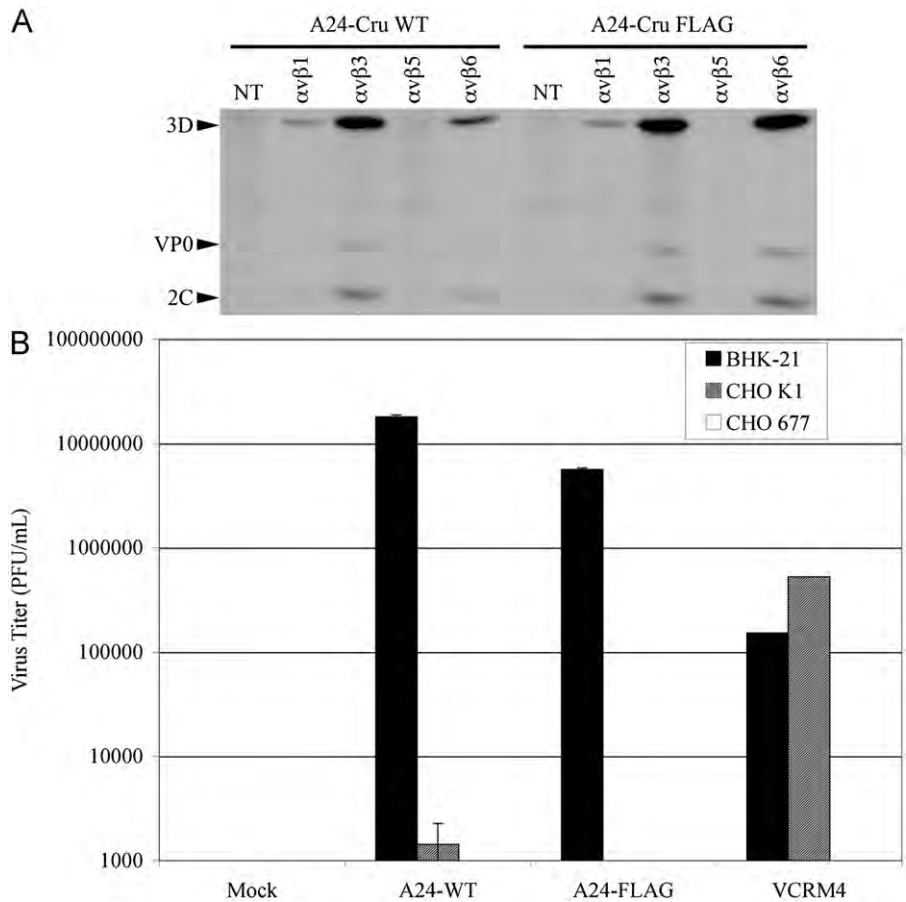


Fig. 2. Evaluation of growth on established cell lines. (A) Untransfected COS-1 cells and COS-1 cells transiently expressing $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, or $\alpha_v\beta_6$ were infected in parallel with A24-Cru WT and A24-FLAG in the presence of ^{35}S-methionine. The resulting virus-infected cell lysates were immunoprecipitated with serotype A specific anti-sera, separated by SDS-PAGE, and examined by autoradiography. Bands corresponding to viral proteins 3D, VP0, and 2C are indicated. (B) Virus titer assays were conducted on BHK-21, CHO K1, and CHO 677 cells comparing A24-Cru WT, A24-FLAG, and a genetic variant of O1 Campos designed to utilize heparan sulfate as an alternate receptor. At 24 hpi, plaque-forming units (PFUs) were counted, and virus titers calculated. Titers for virus-infected cells incubated 1 h at 37 °C were subtracted from the titers determined for samples evaluated at 24 hpi.

Virulence assessment of A24-FLAG in cattle.

Having established an immunoreactivity profile for A24-FLAG relative to the WT virus *in vitro*, we evaluated the kinetics of the antibody response generated after introduction into cattle. Virulence of A24-FLAG in cattle was assessed utilizing a well-established intradermolingual inoculation method (Henderson, 1949). Two cows (bovines #BR10-04 and #BR10-05, hereafter referred to as bovines #4 and #5) were inoculated with 10^7 $TCID_{50}$/animal and infection and clinical signs were monitored daily. Results demonstrated that the two animals developed clinical FMD and both animals showed vesicles in the feet by 2 or 3 days post-inoculation (dpi). Only bovine #4 showed fever (>40 °C) at 1 dpi. Fig. 6 shows that viremia was detected starting at 1 dpi and lasted 4–5 days, while virus shedding was detected in swabs from both animals beginning at 1 dpi. Moreover, biological fluids collected from the A24-FLAG infected animals showed viral RNA levels peak between days 1–2 post-inoculation (Fig. 6C), which was consistent with the pattern observed in cattle inoculated with A24-Cru WT (data not shown).

Plasma and serum collected from cattle inoculated separately with WT (bovines #393 and #394) and FLAG viruses (bovines #4 and #5) over 21 days exhibited similar antibody responses to FMDV for both viruses (Fig. 6A). For both A24-Cru WT and A24-FLAG, anti-FMDV antibodies (antibodies targeted to the viral RNA dependent RNA polymerase, $3D^{pol}$) were not detected over background until day 5, peaking at day 7 (Fig. 6A). Afterwards, the antibody response diminished until FMDV antibodies were undetectable at day 21.

The WT and A24-FLAG were also compared for cross-reactivity with heterologous serotype A FMDV isolates. While homologous challenge between A24-Cru WT and A24-FLAG showed cross-protection with relationship values (r_1) greater than 0.3, little cross-reactivity was observed with a small panel of heterologous related serotype A FMDVs (Fig. 6B). There was some serum cross-reactivity with A5 Westerwald for one cattle (bovine #393) inoculated with A24-Cru WT (r_1 of 0.840). However, there was no evidence that the antibody response against A24-FLAG was more cross-reactive than that of A24-Cru WT (no r_1 values exceeding 0.3).

FLAG epitope is preserved in A24-FLAG post-infection of cattle.

Next, we sought to determine if the FLAG epitope would be preserved in A24-FLAG upon replication in an animal host (cattle). RNA extracted from tissue samples and vesicular fluid collected from cattle infected with A24-FLAG was sequenced after RT-PCR for the presence of the FLAG tag within the G–H loop. As shown in Table 2, sequencing of the A24-FLAG VP1 revealed the FLAG epitope remained intact in virus found in biological samples collected at 2, 3, and 4 dpi in cattle. To corroborate the genetic analysis, vesicular fluid collected from the A24-FLAG infected cattle was examined by western blot probing with anti-FLAG (Fig. 7B). Consistent with the sequencing data, bands corresponding to the approximate molecular

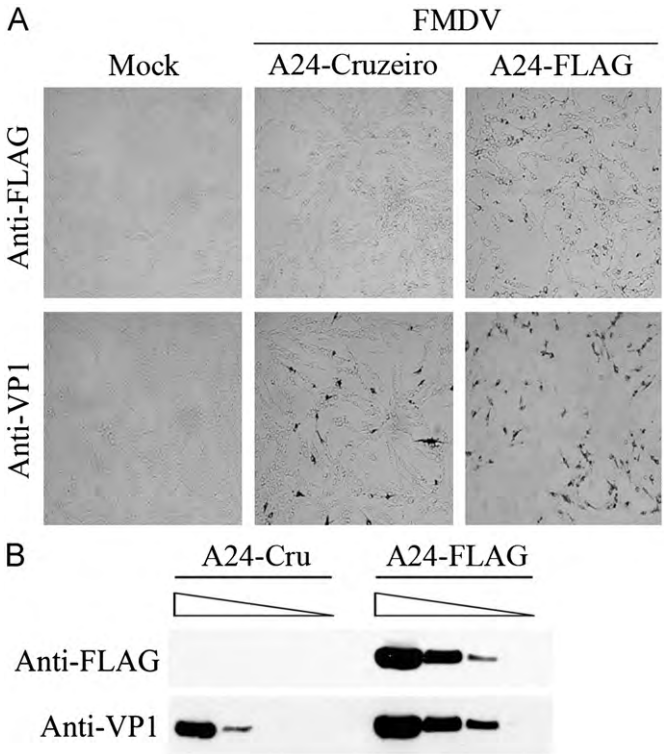


Fig. 3. Detection of A24-FLAG *in vitro*. (A) BHK-21 cells grown on coverslips were infected in parallel with either A24-Cru WT or A24-FLAG and examined by ICC at 5 hpi probing with either anti-VP1 (6HC4) or rabbit polyclonal anti-FLAG (Sigma). The cells were then incubated with goat-anti-mouse-HRP or goat-anti-rabbit-HRP, respectively, followed by DAB staining. (B) Serial dilutions of purified A24-Cru WT and A24-FLAG viruses were examined by western blot probing with the same antibodies in A.

weight of VP1 were reactive with the anti-FLAG polyclonal sera. Similarly, the same band was detected in serially diluted preparations of the same stock of A24-FLAG that was used to inoculate the cattle from which the biological samples were collected (Fig. 7A).

Absence of FLAG tag seroconversion.

Thus far, the antibody response of A24-FLAG paralleled A24-Cru WT from which it was derived (Fig. 6). Since previous studies with other viruses containing FLAG epitope insertion/substitutions elicited the production of FLAG-targeted antibodies *in vivo*, we wanted to investigate if a similar seroconversion occurred in the cattle infected with A24-FLAG. First, we evaluated for the presence of antibodies targeted to a mapped region of the VP1 G–H loop in sera collected from the A24-FLAG inoculated animals via a competitive ELISA (Fig. 8A). With the competitive ELISA, plates coated with A24-Cru WT were incubated with a mixture of sera collected from A24-FLAG infected cattle at either 0 and 28 dpi and a monoclonal antibody that recognizes VP1 (7SF3) (Robertson et al., 1984). The results showed that sera from A24-FLAG infected bovines at 28 dpi were able to compete effectively against the VP1 monoclonal antibody (Fig. 8A) similar to the sera from A24-Cru WT infected bovines (data not shown). This allowed us to conclude that the presence of the FLAG tag does not disrupt the reactivity of the G–H loop region with existing reference sera, which is consistent with results obtained *in vitro*.

Having established that the host immune system produced anti-sera to a mapped region of the G–H loop proximal to the site of the FLAG substitution, we wanted to determine if the A24-FLAG infected cattle also generated anti-sera to the FLAG epitope. As such, a western blot assay was performed using a commercially available FLAG-tagged protein: FLAG-bovine alkaline phosphatase (FLAG-BAP). A dilution series of FLAG-BAP was analyzed by western blot for reactivity with both the commercially available mouse monoclonal (M2) and rabbit polyclonal anti-FLAG antibodies. Both anti-sera successfully reacted with FLAG-BAP on the blots (Fig. 8B). Various dilutions of the sera collected from A24-FLAG inoculated bovines #4 and #5 failed to react with FLAG-BAP on the blots (data not shown). Finally, undiluted sera from bovines #4 and #5 were used to probe the blots, which similarly failed to react with FLAG-BAP (Fig. 8B). Based on the results of the ELISA and western blot panel (Fig. 8), we concluded that seroconversion to the FLAG epitope did not occur in A24-FLAG infected cattle.

Structural analysis of the FLAG epitope within the G–H loop.

With the successful detection, neutralization, and immunoprecipitation of A24-FLAG using FLAG polyclonal anti-sera, but the lack of seroconversion to the FLAG epitope in bovines inoculated with A24-FLAG, we sought to determine why the host immune system did not recognize the FLAG epitope. As such, we examined structural models of the G–H loop containing the FLAG tag. Using the coordinates from the solved structure for FMDV O1/BFS 1860/UK/67 (Protein Databank 1FOD) (Logan et al., 1993) as a template and the VP1 amino acid sequences of the WT and A24-FLAG, homology models were generated of the G–H loop (Fig. 8C). The structures of the G–H loops of the homologous viruses were similar, but not identical. Notably, the region with the FLAG epitope substitution was folded inward on itself, potentially rendering the site cryptic and inaccessible to detection by the host immune system. Molecular dynamics simulations (Amber 10, Case et al., 2008) were also performed using 1FOD (Logan et al., 1993) with the A24 sequence modeled into the G–H loop and the serotype O loop modified to contain the FLAG sequence as inserted into A24 (O1-FLAG). Fig. 8D shows these loops after 4 ns simulation. Notably, the O1-FLAG loop collapsed in on itself (to make salt bridge interactions between the FLAG sequence and other residues in the loop). While keeping in mind that this is *in silico* modeling performed in the absence of X-ray structures for the A24-Cru WT and A24-FLAG, it seems likely that animals infected with the A24-FLAG virus do not seroconvert to recognize the FLAG epitope due to the collapsed conformation assumed by a portion of the sequence in the G–H loop.

Discussion

Here, we sought to generate a stably tagged FMDV particle that could be broadly utilized in a variety of molecular assays with the aim of further delineating viral pathogenesis both *in vitro* and *in vivo*. To that end, we designed and characterized in tissue culture and in the natural host, a novel recombinant FMD virus with a FLAG epitope embedded within the VP1 G–H loop. While multiple sites on the capsid surface were considered for molecular manipulation, the hypervariable sequence upstream of the RGD motif was selected given the fact that these residues tolerate a high degree of variation, and are exposed on the virus capsid surface. Furthermore, this antigenic site, indeed the entire G–H loop, is not absolutely necessary for the development of protective immunity in cattle or pigs (Fowler et al., 2008, 2010; Rieder et al., 1994). Moreover, we were encouraged by three reports: the first describing the successful replacement of a portion of the G–H loop encompassing the conserved RGD motif and RGD+1 leucine with FLAG just downstream and adjacent to the site we selected for substitution (Baranowski et al., 2001), a second where FLAG

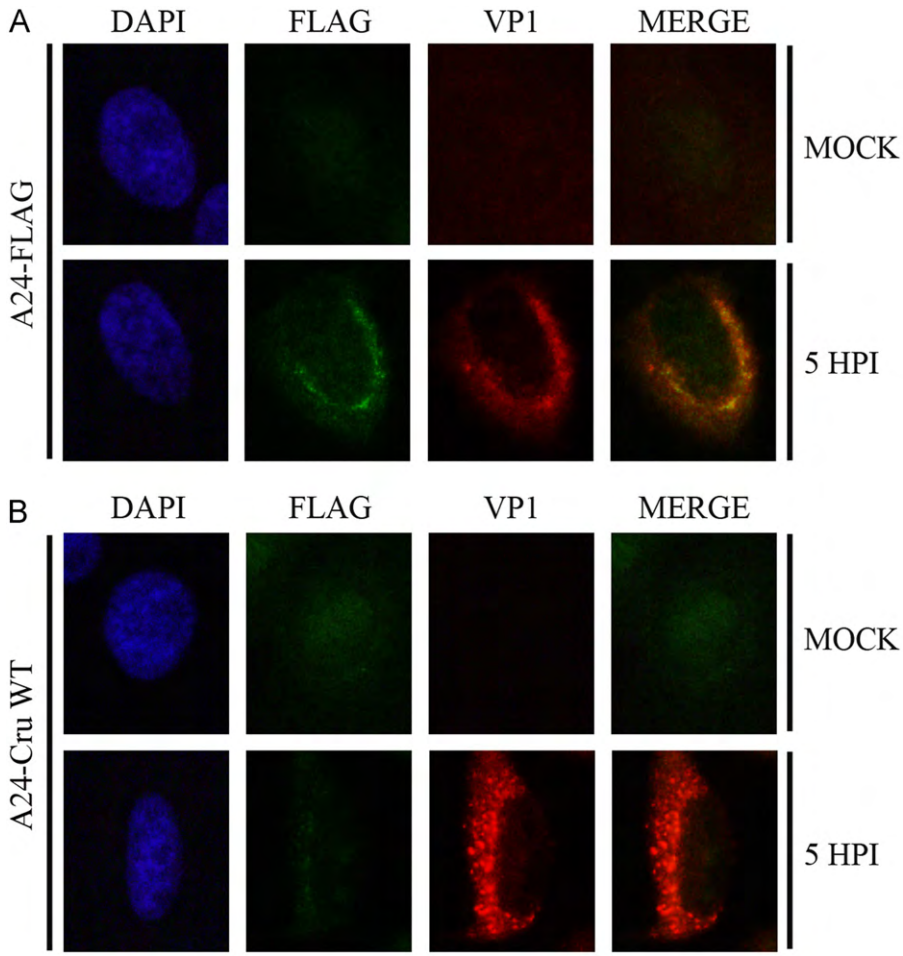


Fig. 4. Detection of A24-FLAG by immunofluorescent microscopy *in vitro*. (A) LFBK cells grown on glass coverslips were mock-infected (top row) or infected with A24-FLAG for 5 h (bottom row) and subsequently simultaneously probed with anti-FLAG (green) and anti-VP1 (red). (B) Same as in A except the cells were infected with A24-Cru WT. Mounting medium from all experiments was supplemented with DAPI nuclear stain (blue).

was inserted further downstream of the RGD and RGD+1 sites (which was published while this manuscript was under revision, Seago et al., 2012), and the third detailing that a specific neutralizing epitope within this region was successfully exchanged between FMDV serotypes O and Asia1, which was stably maintained, and expanded the immunoreactivity of the recombinant virus (Wang et al., 2012). Of note, the results of Baranowski et al. (2001) showed the G–H loop could tolerate the presence of the foreign epitope replacing amino acid residues, which participate in the recognition of the virus receptor (Baranowski et al., 2001; Dicara et al., 2008; Jackson et al., 2000b).

The stated goal of this endeavor was to produce a FMDV particle with a unique reactivity profile that could be tracked *in vitro* and *in vivo* without detriment to normal FMDV pathogenesis. The recombinant marker A24-FLAG with the unique substitution resulted in a virus that achieved high titers and formed plaques similar to that of parental A24-Cru on BHK-21 cell monolayers. Regardless of the proximity of the foreign sequence to the cell receptor binding site (RGD motif), and the presence of highly charged residues in the foreign peptide, the FLAG substitution neither affected the virus interaction with its cognate cell receptors $\alpha_v\beta_3$ and $\alpha_v\beta_6$ integrins (Burman et al., 2006; Jackson et al., 2000b; Neff et al., 2000), nor altered the established array of cell lines susceptible and refractory to FMDV infection. Of significance was the observation that the A24-FLAG virus maintained reactivity with established reference sera to VP1 but expanded reactivity to include polyclonal anti-FLAG as observed by IFM, ICC, and western blot assays. Importantly, given the proximity of the substitution to the RGD motif, the expanded reactivity was not at the expense of the recognition of integrins as the primary receptor. The positive reactivity with the polyclonal anti-FLAG successfully provided a means by which to differentiate the WT and FLAG-tagged virus particles *in vitro* as well as *in vivo*. Moreover, the anti-FLAG successfully neutralized A24-FLAG but not A24-Cru WT when treated prior to infection. By extension, A24-FLAG could be isolated from infected samples via FLAG antisera providing yet another advantage over WT particles. Thus, the tolerated substitution of the FLAG octapeptide within the G–H loop has allowed for the detection, tracking, neutralization, and purification of the recombinant marker virus distinct from its WT progenitor.

While the FLAG epitope has previously been incorporated into locations on the FMDV capsid (Baranowski et al., 2001; Seago et al., 2012), this study represents the first instance where a FLAG-tagged FMDV particle was examined in the natural host. Given the reported success for tag epitope insertions into the genomes of several other viruses (Laird and Desrosiers, 2007; Plemper et al., 2002; Prentoe and Bukh, 2011; Tan et al., 2009), it was hoped that the FLAG tag substitution would also redirect the host immune response to other antigenic site/s contained on the FMDV capsid (Baxt et al., 1984, 1989; Brown et al., 1999). While cattle infected with A24-FLAG induced levels of neutralizing antibodies comparable to the parental virus, we were unable to demonstrate the presence of host antibodies targeted to FLAG in infected

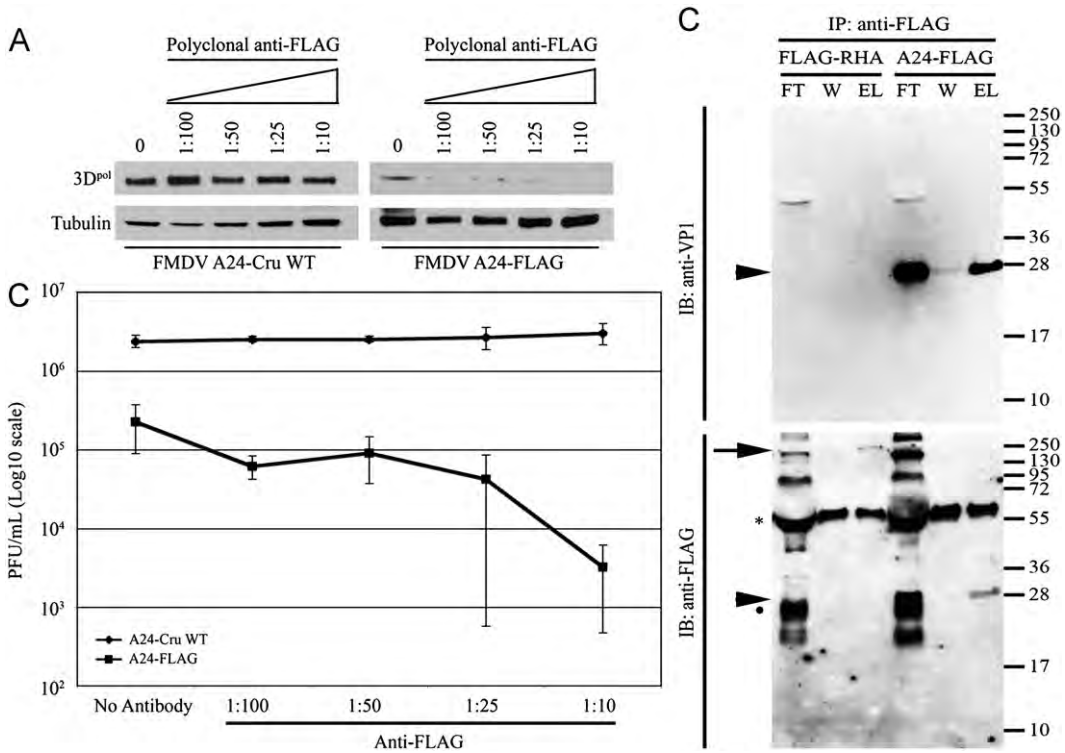


Fig. 5. Anti-FLAG antibodies can be used to neutralize and immunoprecipitate A24-FLAG but not A24-Cru WT. (A) The level of viral protein synthesis achieved for A24-Cru WT and A24-FLAG at 24 hpi after a virus neutralization assay that used anti-FLAG was evaluated by western blot probing with anti-FMDV $3D^{pol}$. Loading equivalency between lanes was confirmed by probing with anti-tubulin-α. (B) The relative virus titers achieved by A24-Cru WT and A24-FLAG after the virus neutralization assay was determined by virus titer assay (see Materials and methods). (C) LFBK cells were infected with A24-FLAG for 5 h and infected lysates were subject to immunoprecipitation with polyclonal anti-FLAG. The collected flow-through (FT), pooled washes (W), and eluate (EL) were examined by western blot probing with anti-VP1 (top) and anti-FLAG (bottom). FLAG-tagged VP1 is indicated by an arrowhead. To confirm the specificity of the anti-FLAG immunoprecipitation, LFBK cells expressing FLAG-tagged RHA were immunoprecipitated by the same method, and the resulting samples were analyzed on the same western blot as the A24-FLAG-containing samples. FLAG-tagged RHA is indicated by an arrow. Cross-reacting heavy chain and light chain from the immunoprecipitation reaction are indicated by an asterisk and a filled circle, respectively.

animals, despite successful detection with commercially available polyclonal anti-FLAG sera. Molecular modeling and simulation data suggested that a significant portion of the FLAG epitope was invaginated. Plausibly, the predicted invagination could impair the recognition of this epitope by the immune system of the cattle that were exposed to this virus. Seemingly though, this hypothesis is contradictory to the neutralization and immunoprecipitation of A24-FLAG by polyclonal anti-FLAG. However, the flexibility afforded antibodies in solution may allow for the recognition of an invaginated FLAG tag that a B-cell receptor fixed to a leukocyte surface could not.

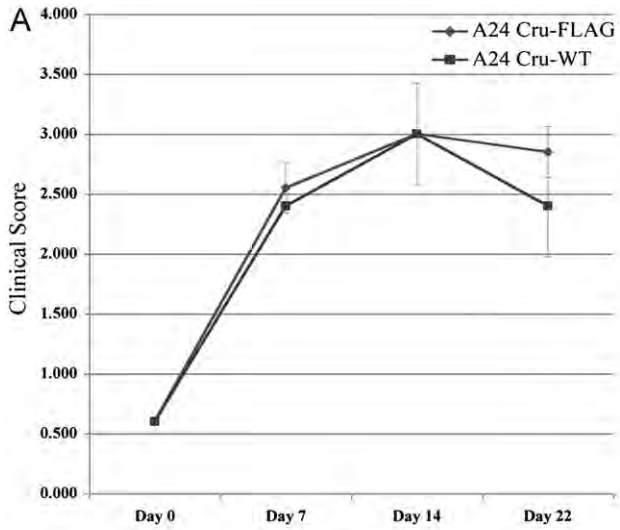
The toleration of the FLAG substitution within the hypervariable region upstream of the RGD motif also adds to the body of publications detailing the antigenicity of the FMDV capsid. Early work with FMDV identified several major antigenic sites on FMDV particles critical to antibody neutralization. The conserved RGD motif in the G–H loop of VP1 has been reported to be the major immuno-dominant site on the capsid of all seven serotypes of FMDV (Barnett et al., 1989; Baxt et al., 1984; Broekhuijsen et al., 1987; Brown, 1988; Crowther et al., 1993a, 1993b; Kitson et al, 1990; Mateu et al., 1990; Thomas et al., 1988a, 1988b). However, other reports have suggested just the opposite: that the G–H loop is dispensable in the context of the virus-host immune response. For example, vaccines prepared with chimeric FMDV capsids with the G–H loop exchanged with that of a different serotype were better protected against challenge with viruses homologous to the chimera backbone and not with the virus type providing the heterologous G–H loop (Fowler et al., 2008; Rieder et al., 1994). More recent work has revealed little to no effect to the elicitation of a protective immune response in animals vaccinated with a type A FMDV variant lacking a large portion of the VP1 G–H loop (Fowler et al., 2010). Our results showing that the recombinant A24-FLAG virus was capable of inducing an immune response in cattle nearly indistinguishable from that of the parental virus serves to reinforce the contention that this region of the capsid surface can be modified without detriment of the development of a robust neutralizing response. Indeed, given its apparent dispensability, additional epitope tags should be explored as possible molecular markers for tracking virus *in vitro* and *in vivo*, but also for possible seroconversion to the tag.

Taken together, the results reported here highlight the considerable utility of recombinant FMDVs with embedded sequence tags within capsid loop regions for investigations of FMDV virulence *in vitro* and *in vivo*. The FLAG epitope being unique without a naturally occurring counterpart allows FLAG-containing FMDV particles to be tracked and neutralized using no virus-specific anti-sera, and also provides an alternative single step method for virus purification from infected cell and tissue lysates unavailable for the parental virus. Importantly, the similar immune response elicited by the mutant and parental virus in cattle suggests that multiple epitope tags in the FMDV VP1 G–H loop could be utilized to trace the virus in susceptible animals for pathogenesis studies.

Materials and methods

Materials

FLAG-tagged BAP protein, 3X FLAG peptide, bovine serum albumin (BSA), glycine, normal goat serum, and paraformaldehyde were purchased from Sigma (St. Louis, MO). The Vectastain kit was



B

	Bov394 Serum A24-WT		BR10-04 Serum A24-FLAG		BR10-04 vs Bov394
Virus	Titer (log)	r_1 value	Titer (log)	r_1 value	
A24-WT	3.15	1.000	2.325	0.150	
A24-FLAG	3.75	3.980	3.15	1.000	R value = 77.27%
A Iran	2.325	0.150	1.35	0.016	
A Iraq	1.575	0.027	1.875	0.053	
Westerwald	2.4	0.178	1.65	0.031	
	Bov393 Serum A24-WT		BR10-05 Serum A24-FLAG		BR10-05 vs Bov393
Virus	Titer (log)	r_1 value	Titer (log)	r_1 value	
A24-WT	2.7	1.000	2.475	0.355	
A24-FLAG	2.78	1.202	2.925	1.000	R value = 65.27%
A Iran	2.025	0.211	1.8	0.075	
A Iraq	2.1	0.251	1.8	0.075	
Westerwald	2.625	0.840	2.325	0.251	

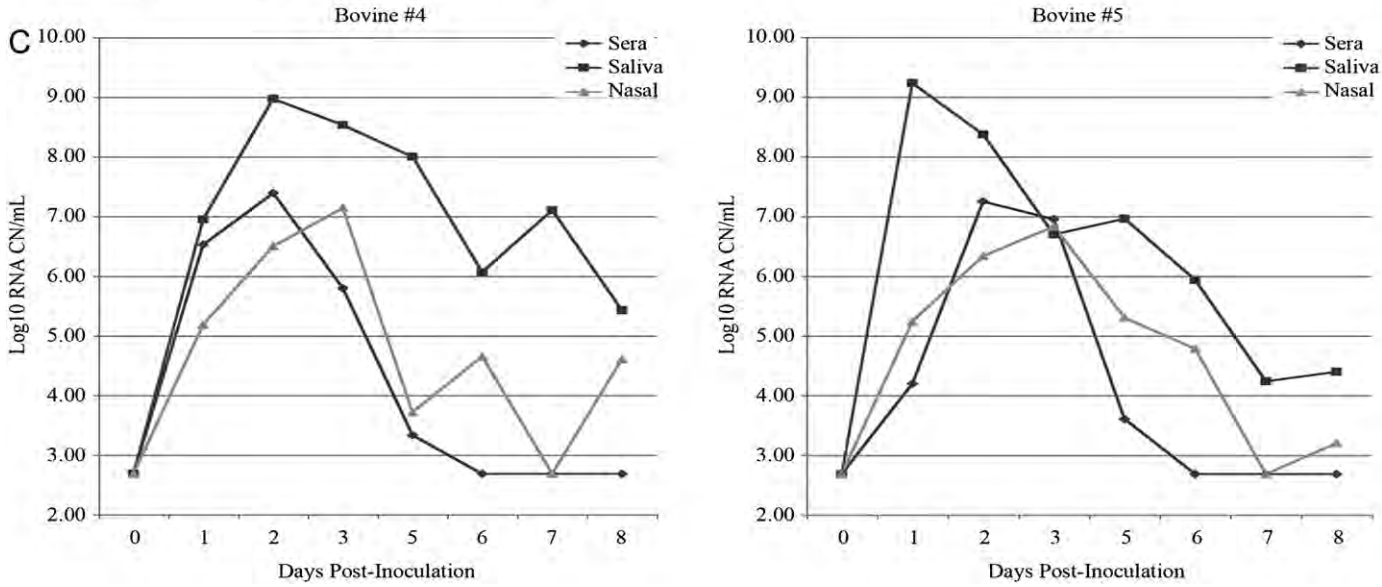


Fig. 6. A24-FLAG and A24-Cru WT elicit similar immune and physiological responses in infected cattle. (A) Comparison of the antibody response elicited by cattle homologously challenged with A24-Cru WT and A24-FLAG, and examined at 0, 7, 14, and 22 dpi. (B) Examination of the cross-reactivity of sera collected from A24-Cru WT and A24-FLAG inoculated cattle with heterologous serotype A FMDV isolates. Significant r_1 value: ≤ 0.3 = no relationship, > 0.3 = coverage. Significant R value: $> 64\%$. (C) The levels of viral RNA detected in sera, saliva, and nasal swabs collected over the course of 8 days post-inoculation with A24-FLAG in bovines #4 and #5.

Table 2
DNA sequencing of VP1 G–H loop of virus in fluids and tissue collected from A24-FLAG infected cattle.

Cow ID #	Extracted material	Day post-inoculation	Sequence obtained	Silent mutations
10-04	Tissue-left rear foot	3	GTDKYDDDDKGRRGD	3906, 3919
	Tissue-left rear foot	4	GTAKYDDDDKGRRGD	2775, 3906, 4685
10-05	Vesicular fluid-right rear foot	2	GTAKYDDDDKGRRGD	3906
	Vesicular fluid-left front foot	3	GTAKYDDDDKGRRGD	3228, 3906
	Tissue-left front foot	3	GTDKYDDDDKGRRGD	2925, 3906
	Tissue-left rear foot	3	GTAKYDDDDKGRRGD	3906
FMDV A24 Cruzeiro Reference Sequence		–	GTSKYAVGGSGRRGD	–
FMDV A24-FLAGReference Sequence		–	GTDKYDDDDKGRRGD	–

purchased from Vector Laboratories (Burlingame, CA). Immulon HB ELISA plates were purchased from Thermo Scientific (Rochester, NY). SureBlue Reserve substrate and BlueStop buffer were purchased from KPL (Gaithersburg, MD). Protein A/G Plus agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ProLong antifade mounting medium supplemented with DAPI stain was purchased from Invitrogen (Carlsbad, CA).

Antibodies

Rabbit polyclonal anti-FLAG, HRP-conjugated mouse monoclonal (M2) anti-FLAG, and anti-tubulin-α were purchased from Sigma (St. Louis, MO). Goat-anti-rabbit, goat-anti-mouse, and goat-anti-bovine secondary antibodies conjugated to HRP were purchased from Bethyl Laboratories (Montgomery, TX). AlexaFluor-488

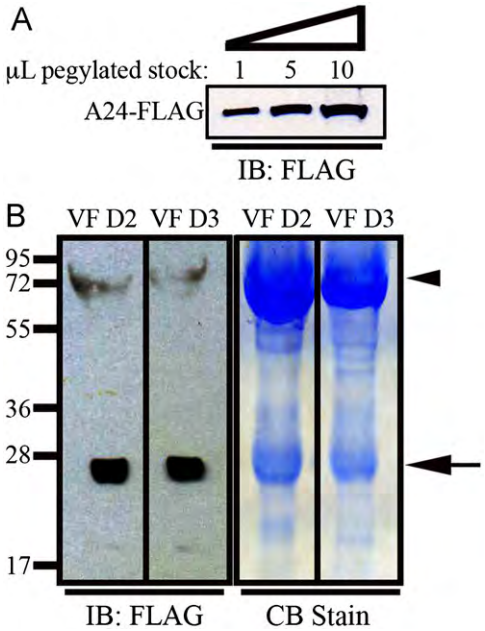


Fig. 7. FLAG epitope detection in virus present in biological samples collected from A24-FLAG infected cattle. (A) Dilution series of purified stock of A24-FLAG used to inoculate bovines #4 and #5 (Fig. 6) examined by western blot probing with polyclonal anti-FLAG. (B) Vesicular fluid (VF) collected from bovine #5 at day 2 (D2) and day 3 (D3) post-inoculation were examined by western blot probing with polyclonal anti-FLAG and Coomassie blue (CB) staining. FLAG-tagged VP1 is indicated by an arrow. An uncleaved P1 product is indicated by an arrowhead.

conjugated goat-anti-rabbit and AlexaFluor-594 conjugated goat-anti-mouse were purchased from Invitrogen (Carlsbad, *CA*). Mouse monoclonal anti-FMDV $3D^{pol}$ was generously provided Dr. Alfonso Clavijo (National Centre for Foreign Animal Diseases, Canada). Mouse monoclonal anti-FMDV VP1 (6HC4 and 7SF3) were generated as previously described (Robertson et al., 1984).

Cells, viruses, and plasmids

The BHK-21 cell line was purchased from the American Tissue Collection Company (ATCC; Manassas, VA) and cultured in Dulbecco's minimal eagle medium (DMEM) supplemented with 10% calf serum with 1% antibiotic/antimycotic (A/A) and incubated at 37 °C with 5% CO_2. The LFBK cell line was generated as previously described (Swaney, 1988), and cultured in DMEM supplemented with 10% fetal calf serum and incubated at 37 °C with 5% CO_2. The Chinese hamster ovary (CHO) K1 and CHO 677 cell lines were originally acquired from Esko et al. (1988) and cultured in Ham's minimal essential medium (Gibco) supplemented with 10% fetal calf serum and incubated at 37 °C with 5% CO_2. FMDV A24 Cruzeiro field strain was derived from pA24-Cru (Rieder et al., 2005) and the FMDV VCRM4 virus was derived from O1 Campos as previously described (Neff et al., 1998).

Virus titer assay

Following virus absorption for 1 h (h), the inoculum was removed, and the cells were washed in a mild acid solution followed by a wash in virus growth media (VGM). VGM was added to each well and the cells were returned to 37 °C. After 24 h at 37 °C, virus-infected cells were harvested and viral titers were determined by plaque assay as previously described (Rieder et al., 1993). Plates were fixed, stained with crystal violet (0.3% in Histochoice; Amresco, Solon, OH), and the plaques were counted. The values calculated for the number of plaque-forming units (PFUs) per milliliter (mL) were plotted using Microsoft Excel (Microsoft Corporation, Redmond, WA). All assays were performed in triplicate.

Transient transfection-infection assay

Assay was conducted as previously described (Neff et al., 2000). Briefly, COS-1 cells were transfected with two plasmids encoding either full-length α_v-integrin or 1 of 4 full-length β integrins (β_1, β_3, β_5, or β_6). The cells were then infected with A24-Cru WT, A24-FLAG, or VCRM4 viruses at a MOI of 1 in ^{35}S-methionine containing media. Subsequently, virus-infected cell lysates were examined by radioimmunoprecipitation for virus-specific bands: 3D, VP0, and VP1-3 as previously described.

Immunofluorescent microscopy

LFBK cells grown on glass coverslips were mock-infected or infected with A24-Cru WT and A24-FLAG for 1, 3, and 5 h at 37 °C. At each time point, the cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.1% Triton-X100 in PBS on ice, and blocked with buffer containing 3% BSA, 0.3 M glycine, and 10% normal goat serum. The cells were then probed simultaneously with polyclonal anti-FLAG (Sigma) and monoclonal anti-VP1 (6HC4) followed by goat-anti-rabbit-AF488 and goat-anti-mouse-AF594 (Invitrogen) with multiple PBS washes after each antibody treatment. Finally, the coverslips were air-dried and mounted onto glass slides with ProLong antifade mounting medium supplemented with DAPI stain (Invitrogen). Samples were examined using an Olympus fluorescent microscope with a 100X oil immersion objective.

Virus neutralization assay

A24-Cru WT and A24-FLAG were pre-incubated with antibodies directed against the FLAG epitope at dilutions of 1:100, 1:50, 1:25, or 1:10. Alternatively, the viruses were pre-incubated with an antibody targeted to a region within the VP1 G–H loop at dilutions of 1:100, 1:50, 1:10, or 1:5. After incubating for 1 h at RT, viruses were applied to LFBK cells and incubated 24 h at 37 °C. Virus-infected cell lysates were harvested and viral protein synthesis was evaluated by western blot probing with anti-FMDV $3D^{pol}$. Equivalent loading was confirmed by re-probing with anti-tubulin-α (Sigma).

Virus immunoprecipitation

A24-FLAG infected cell pellets were lysed using "FLAG lysis buffer" (Sigma), pre-cleared with normal rabbit serum (Pierce) and protein A/G beads (Santa Cruz Biotechnology), and incubated with polyclonal anti-FLAG (Sigma) and protein A/G beads at 4 °C for 24 h. After incubation with anti-FLAG bound beads, the samples were centrifuged at 2500 rpm for 5 min at RT, and the flow-through was collected for each sample. Afterwards, the samples were washed no less than five times with "FLAG 1X wash buffer" (Sigma), and the collected washes were pooled. Elution was performed by incubating samples with a 3X FLAG peptide (Sigma). The collected flow-through, pooled washes, and eluate were then examined by western blot.

Western blot

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out using a 12% Nu-PAGE ® pre-cast gel system (Invitrogen). Subsequently, the separated proteins were electro-blotted onto a nitrocellulose membrane (Sigma).

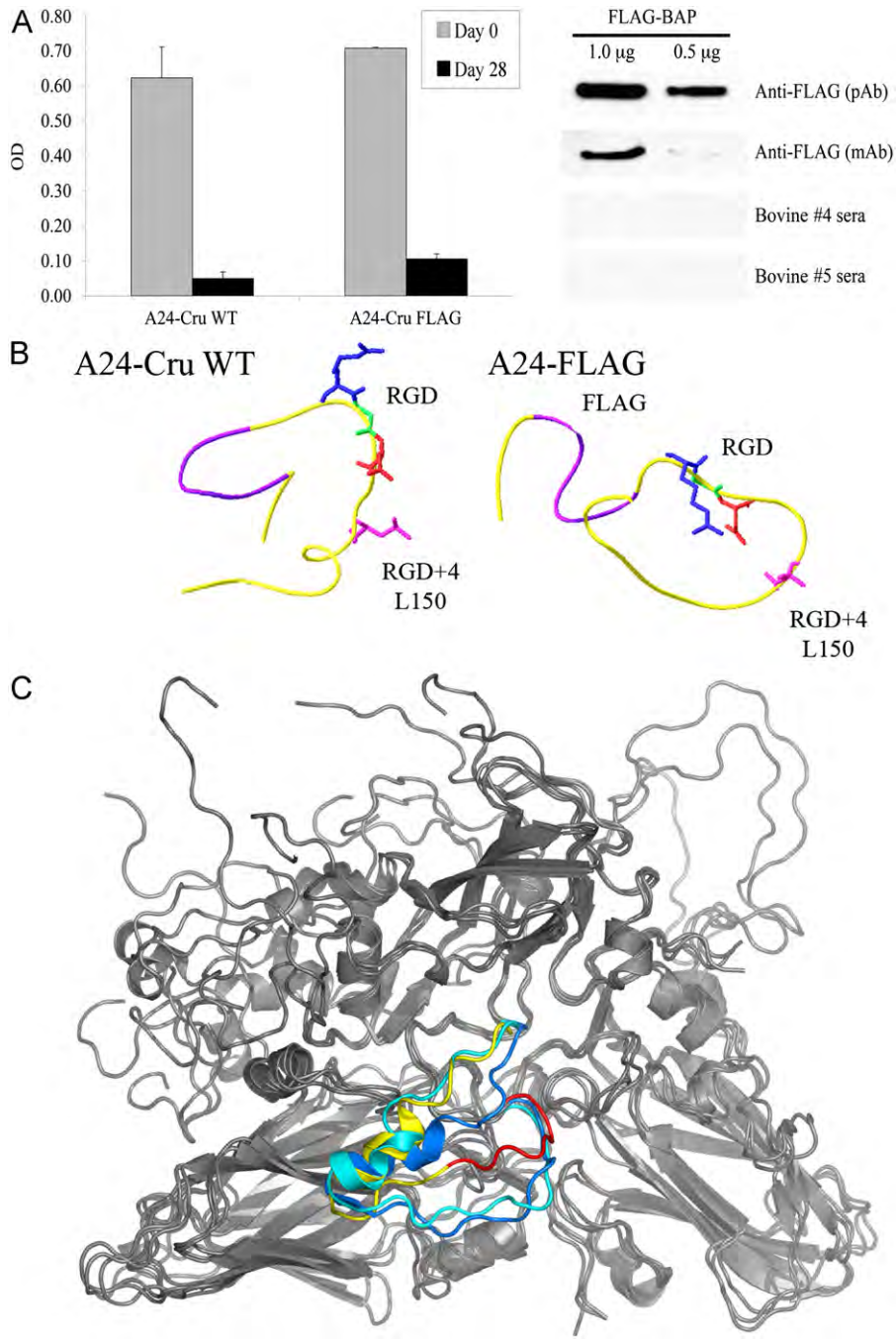


Fig. 8. Sera collected from A24-FLAG infected cattle do not react with FLAG epitope. (A) Competitive ELISA where A24-Cru WT and A24-FLAG viruses were incubated with equal quantities of day 0 (hatched) and 28 (black) sera collected from inoculated cattle and a reference mouse monoclonal antibody for VP1. Subsequently, the mixtures were tested for reactivity with HRP-conjugated goat-anti-mouse secondary antibody. (B) FLAG-tagged BAP protein was examined by western blot at two different concentrations (0.5 and 1 µg) and probed with rabbit polyclonal anti-FLAG, mouse monoclonal anti-FLAG, or the day 28 sera obtained from cattle (bovines #4 and #5) infected with A24-FLAG. (C) Homology models of the VP1 G–H loop of A24-Cru WT and A24-FLAG were generated using the coordinates from the crystal structure of FMDV O1/BFS 1860/UK/67 (Accession 1FOD). The RGD motif (blue, green, red, respectively), RGD+4 L150 position (pink), and FLAG sequence site (purple) are indicated. (D) Overlapping cartoon of the protomeric subunit with the G–H loop (after 4 ns simulation) highlighted by color: O1/BFS 1860/UK/67 (blue), A24-Cru WT (light cyan), and O1-FLAG (yellow, FLAG sequence in red).

After blocking with 5% milk in PBS-T, specific proteins were detected with primary antibodies at indicated dilutions followed by goat-anti-rabbit or goat-anti-mouse antibodies conjugated with HRP (Bethyl). Cellular tubulin, employed as an internal loading control protein, was detected with anti-tubulin-α (Sigma). The HRP-conjugated secondary antibodies were reacted with the WestDura SuperSignal chemiluminescent reagent (Thermo Scientific) according to the manufacturer's instructions and visualized on X-ray film (X-Omat; Kodak, N.Y., USA).

Competitive ELISA

Nunc Maxisorp ELISA plates were coated with 2×10^9 PFU/mL A24-Cru WT virus stock at 1:1000 and incubated overnight at 4 °C. Plates were washed with PBS-T and pre-mixed solutions containing reference and test sera (1:80) and anti-VP1 (7SF3) (1:10) were added and incubated 1 h at 37 °C. Goat-anti-mouse-HRP (1:2000) in 5% milk in PBS-T was added and the plates were incubated at 37 °C for an additional hour with subsequent

washing. Afterwards, 100 μl of SureBlue Reserve Substrate (KPL) was added to each well and incubated for 1 h at RT. The reactions were stopped with 50 μl of TMB BlueStop buffer (KPL) and the plates were read at 630 nm.

Virulence of A24-FLAG virus in cattle

Animal experiments were performed under biosafety level 3 conditions in the animal facilities at PIADC following a protocol approved by the Institutional Animal Care and Use Committee. Two steers (300–400 kg) were infected per the intradermolingual inoculation method (Henderson, 1949) with approximately 10^7 $TCID_{50}$ per cattle, diluted in MEM. For 8 days after inoculation, animals were clinically examined, including rectal temperature recordings and serum, oral and nasal swabs collection. After collection, clinical samples were aliquotted and frozen at −70 °C. One serum and one swab aliquot were used to perform viral titration by rRT-PCR as described previously (Pacheco et al., 2010). Clinical scoring was made based on lesions present in each foot, with a maximum of 4.

Structural analysis

The amino acid sequences of A24-Cru WT and A24-FLAG were used to construct homology models of the respective VP1 capsid proteins using the web algorithm Geno3D (Combet et al., 2002), which generates 10 most likely structures for the amino acid sequence supplied. Coordinates from the solved structure of the major immunogenic site of FMDV O1/BFS 1860/UK/67 (Accession 1FOD) served as a template for the homology models (Logan et al., 1993). The 10 most likely structures were examined using Deep View (Guex and Peitsch, 1997; Kaplan and Littlejohn, 2001), and a consensus structure for each amino acid sequence was selected. Molecular dynamics (MD) simulations were performed using 1FOD (Logan et al., 1993) allowing the G–H loop to move independently against the static capsid background with the A24 sequence modeled into the G–H loop and the serotype O loop modified to contain the FLAG sequence as inserted on A24 (O1-FLAG). The loops were subjected to 4 ns MD simulations using the Parm99 force-field implemented in AMBER 10 package (Case et al., 2008). The models were fully solvated in TIP3P waters in a rectangular box extending by 10 Å and Na^+ ions were used to neutralize the surface charge of the protein. Long-range electrostatic interactions were treated with periodic boundary conditions and Ewald sums as implemented in the AMBER package. The simulations were carried out at 310 K in the NVT ensemble with an integration time step of 2 fs and the non-bonded cut off distance was maintained at 12 Å. Long-range electrostatics were included using PME method. Prior to simulation the system was minimized in two steps, first the energy from water was minimized by keeping the protein restrained followed by the minimization of water and protein simultaneously. The system was heated to 310 K and equilibrated for 500 ps before performing an unrestrained 4 ns production run.

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Review
Future Virology

Use of replication-defective adenoviruses to develop vaccines and biotherapeutics against foot-and-mouth disease

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We have developed a replication-defective human adenovirus (Ad5) vectored foot-and-mouth disease (FMD) vaccine platform that protects both swine and cattle from subsequent challenge with homologous virus after a single immunization. This Ad5-FMD vaccine has undergone testing following the requirements of the Center for Veterinary Biologics of the Animal Plant and Health Inspection Service, US Department of Agriculture, and has recently been granted a conditional license for inclusion of the vaccine in the US National Veterinary Vaccine Stockpile. In this review, we will describe the approaches we have taken to improve the potency and efficacy of this vaccine platform. Furthermore, we will discuss the development of Ad5 vector-based biotherapeutics to generate rapid protection against FMD virus prior to vaccine-induced adaptive immunity and describe the use of a combination of these approaches to stimulate both fast and long-lasting immunity.

Foot-and-mouth disease (FMD) is a highly contagious disease of domestic cloven-hoofed animals including cattle, swine, sheep, goats and many wild animals [1]. The disease causes vesicular lesions on the tongue, nostrils, feet and teats of infected animals resulting in significant morbidity; however, mortality is low, except when young animals are affected. In addition, infection can have a significant adverse effect on animal productivity. FMD is a 'listed disease' by the World Organization for Animal Health (OIE) that requires member-states to notify the agency whenever they detect an outbreak. OIE recommendations suggest that FMD-free countries may impose a trade restriction of susceptible animals or their products on countries in which the disease is present. As a result, an FMD outbreak can have a major impact on the economy of affected countries, especially those that have substantial trade in susceptible livestock and their products.

The etiologic agent, FMD virus (FMDV), is a member of the *Picornaviridae* family, genus *Aphthovirus*. It contains a single-stranded positive-sense RNA genome of approximately 8300 bases encapsidated within an icosahedral particle consisting of 60 copies each of four structural proteins, VP1–4. The virus also codes for ten nonstructural (NS) proteins. FMDV is an antigenically variable virus that is composed of seven serotypes and numerous subtypes within each serotype [1,2].

FMDV replicates and spreads very rapidly. To control the disease, countries slaughter infected and FMD-susceptible in-contact animals, restrict susceptible animal movement, decontaminate infected premises and, in enzootic countries, vaccinate with an inactivated whole-virus vaccine. However, during an outbreak in previously FMD-free countries, the use of vaccination is problematic. Current OIE regulations favor slaughter or vaccination followed by slaughter rather than only vaccination since countries that slaughter all infected and susceptible in-contact animals can regain FMD-free status, and thus re-engage in trade, by documenting the absence of disease for 3 months after the last case, while countries that do not slaughter vaccinated animals must wait 6 months prior to regaining FMD-free status [1]. As a result of these policies, millions of animals, many of which were FMD-free, were slaughtered in outbreaks in the UK and The Netherlands in 2001 and in Japan in 2010 [3–5].

There are a number of concerns with the current inactivated whole-virus vaccine including: difficulty in differentiating infected from vaccinated animals; requirement of an expensive high-containment facility for vaccine

Keywords
■ foot-and-mouth disease ■ interferon ■ replication-defective human adenovirus ■ vaccine

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production; the possibility of escape of virulent virus from manufacturing facilities as occurred at the Pirbright facility (UK) in 2007; and the selection of antigenic variants during growth of virus for vaccine production. Furthermore, vaccinated animals, in particular cattle and African buffaloes, exposed to live virus can be asymptomatically infected and become carriers [6,7]. In addition, owing to current federal law, the USA cannot produce an inactivated FMD vaccine and is dependent on foreign manufacturers.

To address some of these concerns, we have constructed a novel FMD vaccine that is delivered by a replication-defective human adenovirus type 5 vector (Ad5-FMD) [8–10]. This Ad5 vector has previously been shown by a number of laboratories to be an efficient system for delivery of foreign antigens (see [8–10]). The Ad5-FMD vaccine contains the coding regions for the FMDV capsid polyprotein (P1–2A) and NS 3C protease, which is required for capsid polyprotein processing, as well as portions of NS proteins 2B and 3B [8]. However, the vaccine lacks the coding regions of the other FMDV NS proteins and therefore vaccinated animals can be easily distinguished from infected animals. Furthermore, since the vaccine does not contain infectious FMDV, it does not require a high-containment facility for production and, in fact, it has already been produced on the US mainland in a BSL2 facility [11]. Finally, since the vaccine is produced as a DNA molecule, it is not subject to the high error rate of replication of RNA molecules.

We have previously described the construction of this Ad5-FMD vector and a number of proof-of-concept potency and efficacy studies in both swine and cattle [9,10,12]. Furthermore, in a review article in *Future Virology* 2 years ago, we discussed the development, in collaboration with the Department of Homeland Security, Science and Technology Directorate (PIADC, NY, USA) and GenVec Inc. (MD, USA), of an Ad5 vector scalable manufacturing process, a master seed Ad5-FMDV serotype A24 Cruzeiro vector (Ad5-A24), a master seed cell line for vaccine vector production, a number of safety and efficacy studies in cattle and the production of Ad5-FMD vectors for other FMDV serotype A strains and other FMDV serotypes [11]. More recently, the Ad5-A24 vector has undergone a safety study at three sites on the US mainland. A report of this study was provided to the Center for Veterinary Biologics of the Animal Plant and Health Inspection Service, US Department of Agriculture, and on 7 June 2012, this agency granted a conditional license for use of the Ad5-A24 vaccine in cattle. With this license, the Ad5-A24 vaccine may be added to the US National Veterinary Vaccine Stockpile.

In this review, we will describe our recent studies on the development of improved second-generation Ad5-FMD vectors and the use of alternate delivery routes to enhance vaccine potency. In addition, we will describe our studies to develop Ad5-based biotherapeutics that can complement the Ad5-FMD vaccine and induce a rapid protective response prior to the stimulation of vaccine-induced adaptive immunity and also potentially act as a vaccine adjuvant [13].

Ad5-FMD vaccine development

As we have previously described, one dose of our first-generation Ad5-A24 vector administered intramuscularly (im.) conferred protection in both swine and cattle against a direct inoculation challenge as early as 7 days after vaccination and protection in swine lasted at least 42 days [10,12]. In a preliminary study in cattle, we also demonstrated that a boost given at 9 weeks after the initial inoculation significantly enhanced the FMDV-specific neutralizing antibody response, and the vaccinated animals, housed in the same room as a control animal, were protected when challenged 14 days later [14]. These later results suggested that the Ad5-FMD approach could also be useful in enzootic areas where vaccinations are given twice annually. However, in all of the above studies, the protective dose was 5×10^9 PFU per animal, a relatively high dose that could be an economical constraint in veterinary medicine.

FMDV serotype O has been circulating and causing outbreaks in many parts of the world for a number of years and is responsible for recent and recurring outbreaks in many parts of Asia including Taiwan, Japan, South Korea, China, Vietnam and Russia, among others [5,15]. It has been demonstrated that the inactivated serotype O vaccine induces a lower immune response as compared with serotype A antigen [16,17]. As a result of its poorer immunogenicity, commercial type O vaccines usually contain four- to fivefold more antigen that A vaccines. We constructed an Ad5-O1Campos (Ad5-O1C) vaccine and found that swine inoculated with 5×10^9 PFU developed lower levels of FMDV-specific neutralizing antibodies than Ad5-A24-inoculated swine. Furthermore, in efficacy studies, this dose of the Ad5-O1C vaccine did not protect pigs against homologous challenge, although disease signs were delayed and considerably less severe than in control animals, all of which died [18].

To enhance the potency and efficacy of the Ad5-FMD vector approach and produce a

commercially viable FMD vaccine candidate, we have examined a number of strategies that we discuss below.

Addition of complete 2B coding region

After infection of cells with FMDV, as well as other picornaviruses, there is a drastic rearrangement of intracellular membranes, which subsequently become the sites of viral replication [19–24]. A number of viral NS proteins have been implicated in these membrane rearrangements including the NS protein 2B. In an attempt to enhance the synthesis of FMDV capsid proteins and the efficiency and/or stability of capsid assembly, we constructed a second generation of Ad5-FMD vectors containing the full-length 2B coding region (Ad5-FMD-2B) [25,26]. Infection of cells with this vector induced significant rearrangement of cytoplasmic membranes similar to what is observed in FMDV-infected cells [25]. We then demonstrated that swine inoculated with the Ad5-A24-2B vector developed an enhanced FMDV-specific neutralizing antibody response as compared with our first-generation vector, which contained only a portion of the 2B coding region, and these animals showed no clinical signs of disease after challenge [25].

To address the limited immunogenicity of our first-generation Ad5-O1C vector, we also constructed a second-generation vector containing the complete 2B coding region, Ad5-O1C-2B [26]. Cattle were vaccinated with either of these vectors and challenged 21 days later with FMDV O1 Campos. At the day of challenge, both groups had similar levels of neutralizing antibodies, but in the group given Ad5-O1C, two of four animals were protected from clinical disease and the other two animals developed delayed and less severe disease than the control animals. In the group given Ad5-O1C-2B, three out of four animals were clinically protected and the remaining animal only developed one lesion that was detected 2–3 days later than all the clinically ill animals in the Ad5-O1C-inoculated group. In addition, all vaccinated animals had a significant reduction in the amount of virus shedding, for example 10–10,000-fold, as compared with the control animals, and furthermore, the Ad5-O1C-2B group had almost a tenfold reduction with respect to the Ad5-O1C group [26].

The role of cell-mediated immunity in protection against FMDV is unclear. A number of groups have shown that FMDV blocks various aspects of the host innate and adaptive immune response [27–30]. However, it has been demonstrated that FMDV infection or vaccination can induce a T-cell response in cattle and pigs [31,32] and that both viral structural and NS proteins contain T-cell epitopes [26,33–38]. To determine if induction of a cell-mediated adaptive immune response plays a role in the enhanced protection observed in the Ad5-O1C-2B-vaccinated group as compared with the Ad5-O1C group, we assayed for IFN-γ production by $CD4^+$ and $CD8^+$ cells [26]. We found that groups vaccinated with either vaccine had a statistically significant increase in IFN-γ production by $CD4^+$ and $CD8^+$ cells as early as 1 day postchallenge (dpc) and lasting for at least 7 days relative to the control animals. Furthermore, the Ad5-O1C-2B group had a statistically significantly higher IFN-γ $CD4^+$ T-cell response at 5 days and IFN-γ $CD8^+$ T-cell response at 3–5 dpc with respect to the Ad5-O1C vaccinated group.

These results demonstrate that the Ad5-FMD vaccine platform induces both a robust neutralizing antibody response and primes a cell-mediated immune response.

Effect of route & number of sites of inoculation on vaccine potency & efficacy

As already mentioned, swine vaccinated im. at one site with 5×10^9 PFU of Ad5-A24 were protected when challenged either at 7, 14 or 42 days postvaccination and enhanced efficacy was observed with the second-generation vaccine containing the full-length 2B coding region, Ad5-A24-2B. We then examined if we could further lower the protective dose with Ad5-A24-2B, by altering the route and number of sites of inoculation.

Groups of three swine were vaccinated im. at one site in the neck (our standard protocol) with 1×10^9 PFU Ad5-A24-2B, im. at two sites on either side of the neck with 1×10^9 or 2×10^8 PFU, or subcutaneously (sc.) at two sites on either side of the neck with 1×10^9 or 2×10^8 PFU (Table 1). A control group was inoculated sc. at two sites in the neck with 1×10^9 PFU Ad5-Blue, a vector containing the α-segment of β-galactosidase [39]. Animals were challenged in the heel bulb at 21 days postvaccination with 10^5 tissue culture infectious dose 50 FMDV serotype A24.

All control animals developed clinical disease by 2 dpc with scores of 14–15 (the maximum score is 17) (Table 1) [25]. These animals also had viremia 10^5–10^6 PFU/ml, were positive for viral RNA in the blood by real-time PCR and had 10^1–10^2 PFU/ml of virus in nasal swabs. All animals in the group vaccinated im. at one site with 1×10^9 PFU Ad5-A24-2B developed clinical disease at 4–5 dpc, a delay of 2–3 days as compared with the controls, and disease was less

Table 1. Comparison of route and number of sites on efficacy of adenovirus type 5-A24-2B inoculation in swine.

Vaccine	Dose[†] (PFU)	Animal number	Route/sites	Clinical score[‡]	Viremia[§]	RT-PCR[¶]	Shedding virus[#]	SN[††]
Ad5-Blue	1×10^9	28457	sc. 2	2/14	$1/2.3 \times 10^5/3$	SP	$2/4.8 \times 10^1/1$	0.1/2.4
		28458		2/15	$1/3.0 \times 10^6/3$	SP	$2/1.6 \times 10^2/2$	0.1/3.3
		28459		2/15	$2/1.9 \times 10^5/2$	SP	$2/4.5 \times 10^1/2$	0.1/3.0
Ad5-A24-2B	1×10^9	28448	im. 1	5/2	0	N	$5/4.0 \times 10^1/1$	1.2/3.6
		28449		4/6	0	N	0	1.2/3.6
		28450		4/12	0	N	0	0.9/3.6
Ad5-A24-2B	1×10^9	28445	im. 2	0	0	WP	0	1.2/2.7
		28446		0	0	N	0	1.8/1.8
		28447		0	0	SP	0	1.2/2.1
Ad5-A24-2B	2×10^8	28454	im. 2	5/2	0	N	0	1.2/3.9
		28455		6/1	0	N	0	1.5/3.3
		28456		5/1	0	N	0	1.2/4.2
Ad5-A24-2B	1×10^9	28442	sc. 2	0	0	N	0	1.8/2.7
		28443		0	0	N	0	1.2/3.3
		28444		0	0	WP	0	1.5/2.1
Ad5-A24-2B	2×10^8	28451	sc. 2	0	0	N	0	1.5/1.5
		28452		0	0	N	0	1.2/2.1
		28453		0	0	N	0	1.8/1.2

All animal experiments were performed under a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the Plum Island Animal Disease Center (NY, USA).
[†]Dose of Ad5 inoculum per animal expressed as number of PFU in a total volume of 2 ml.
[‡]dpc at first signs of lesions/highest lesion score. Clinical score is the number of toes with lesions plus the snout and tongue combined. The maximum score is 17.
[§]First dpc viremia was detected/maximum amount of viremia in PFU/ml detected in sera samples/duration (days) of viremia.
[¶]RT PCR for detection of foot-and-mouth disease virus RNA; 1–7 dpc sera tested.
[#]First dpc shedding virus was detected/maximum amount of shedding virus in PFU/ml detected in nasal swab samples/duration (days) of shedding.
[††]SN antibody response reported as tissue culture infectious dose 50 at 0/21 dpc.
Ad5: Adenovirus type 5; dpc: Day postchallenge; im.: Intramuscularly; N: Negative; RT: Real time; sc.: Subcutaneously; SN: Serum neutralizing; SP: Strong positive; WP: Weak positive.

severe. Only one animal in this group had detectable virus in nasal swabs and none of the animals had detectable viral RNA in the blood. None of the animals in the group vaccinated im. at two sites with 1×10^9 PFU vaccine developed clinical disease. The virus was not detectable in blood or nasal swabs, but two of the animals had detectable viral RNA in the blood. The group vaccinated im. at two sites with 2×10^8 PFU vaccine all developed clinical disease, but it was delayed 3–4 days as compared with the control group and was significantly less severe. Furthermore, this group, – im. two sites 2×10^8 PFU vaccine – did not have either detectable viral RNA in the blood or virus in nasal swabs. Both of the groups vaccinated sc. did not develop clinical disease, had no virus in nasal swabs and no detectable viral RNA in blood (one animal was weakly positive).

These results demonstrate that we can lower the protective dose at least 25-fold, for example, from 5×10^9 PFU to 2×10^8 PFU, by altering the route of inoculation, from im. to sc., and by changing the number of sites of inoculation to two sites instead of one.

Development of biotherapeutics to rapidly protect FMD-susceptible animals

Both the inactivated and the Ad5 vaccines require at least 7 days to fully protect cattle and swine against FMD [10,12,40]. However, in the event of an FMD outbreak in a disease-free country, the induction of rapid protection, prior to the development of vaccine-stimulated adaptive immunity, is necessary to block or limit disease spread and thus potentially reduce the number of animals that have to be slaughtered.

In all vertebrates, expression of interferons (IFNs) is the first line of the host innate immune defense against viral infection [41–46]. Three families of IFNs have been described – types I, II and III based on their receptor specificity [47]. Type I IFNs (IFN-α and IFN-β) signal through

a heterodimeric receptor complex formed by IFNAR1/IFNAR2; type II IFN (IFN-γ) signals through the complex IFN-γR1/IFN-γR2; and type III IFNs bind the receptor complex IL-28Rα/IL-10Rβ. Despite the receptor differences, the three IFN families transduce signals through the JAK–STAT pathways and type I and type III IFNs induce redundant responses [48]. Similar to many other viruses, FMDV counteracts the innate immune response by blocking the expression of IFN [49,50] and our group and others have demonstrated that FMDV is highly sensitive to type I IFN [47,51–53], suggesting that use of this type of IFN could be a successful strategy to control FMDV *in vivo*. However, since IFN-α/β protein is rapidly cleared, its clinical use requires multiple inoculations of relatively high doses for a prolonged time [54–56], which can lead to adverse effects [55]. To overcome this problem, we have chosen to deliver IFN proteins with the same replication-defective Ad5 system used for the FMD vaccine, thus allowing animals to produce the protein endogenously and spread it systemically. Using this system, we have expressed bovine and porcine IFN-α, IFN-β, IFN-γ and IFN-λ (Figure 1). All these proteins, except bovine IFN-α (boIFN-α), are glycosylated in mammalian cells [57,58].

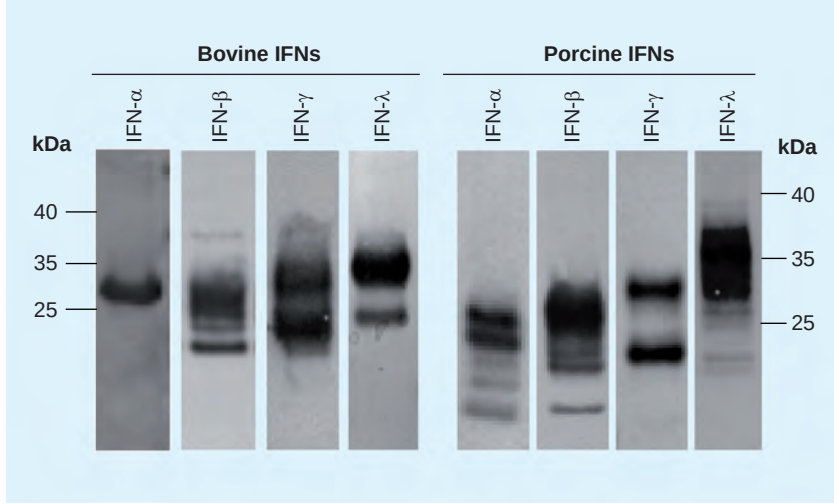

Figure 1. Expression of different types of interferons (bovine and porcine). IB-RS-2 cells were infected with adenovirus type 5 vectors expressing bovine and porcine IFN-α, IFN-β, IFN-γ and IFN-λ. A total of 24 h later, supernatants of infected cells were collected and tested by western blot using specific rabbit polyclonal antibodies.
IFN: Interferon.

Type I IFNs

In preliminary studies in cell culture, we demonstrated that in supernatants from Ad5-porcine IFN-α (Ad5-poIFN-α)-infected IB-RS-2 cells, poIFN-α was detected as early as 4 h postinfection (hpi), expression continued for at least 30 hpi and, more importantly, the protein obtained had antiviral activity [59]. Furthermore, all serotypes of FMDV were very sensitive to the supernatants of Ad5-poIFN-α-infected cells (Figure 2). Studies in swine demonstrated that after one im. inoculation with 1×10^9 PFU/animal of Ad5-poIFN-α, relatively high levels of antiviral activity (~800 U) were detected in plasma starting at 16 hpi, and animals were completely protected against FMDV A24 when challenged intradermally (id.) 24 h after the Ad5-poIFN-α treatment [59]. A reduction in the dose of Ad5-poIFN-α to 1×10^8 PFU/animal conferred partial protection with delayed onset of clinical signs and less severe disease. Furthermore, complete protection of swine inoculated with 1×10^9 PFU/animal of Ad5-poIFN-α lasted for 3–5 days, and a delay in disease development, reduced severity of clinical signs and a significant reduction in viremia was observed when the challenge was performed 7 days postinoculation (dpi) or 1 day prior to the treatment [60]. Similar results were obtained with an Ad5 vector expressing poIFN-β. In this case, although the systemic antiviral activity was lower than that induced by Ad5-poIFN-α with the same doses of adenovirus, animals were also completely protected against id. challenge with FMDV A24 [61].

Based on these results and considering that the previously described Ad5-FMD subunit vaccine could not completely protect swine prior to 7 dpi, our group tested the combination of Ad5-poIFN-α and Ad5-A24 subunit vaccine challenging swine id. at 5 dpi with FMDV A24. Treated animals were completely protected against disease and developed a significant adaptive immune response [60]. Further studies showed that the combination of the Ad5-FMD subunit vaccine with Ad5-poIFN-α enhanced the long-term level of immunity induced by the vaccine in swine [13], suggesting that the IFN was acting as an adjuvant, as has also been reported in other mammalian species [62–64].

Since FMDV is an antigenically variable virus [1,2], it is important to develop a control strategy that could rapidly block any virus serotype. As previously mentioned, cell culture studies showed that poIFN-α could inhibit replication of all seven serotypes of FMDV (Figure 2). Therefore, we performed swine experiments in which animals were inoculated with the Ad5-poIFN-α vector and challenged id. 24 h later with FMDV serotypes O1 Manisa or Asia. Using equivalent doses of the Ad5-poIFN-α, all animals were protected, independent of the FMDV serotype used for the challenge [61]. Although these proof-of-concept

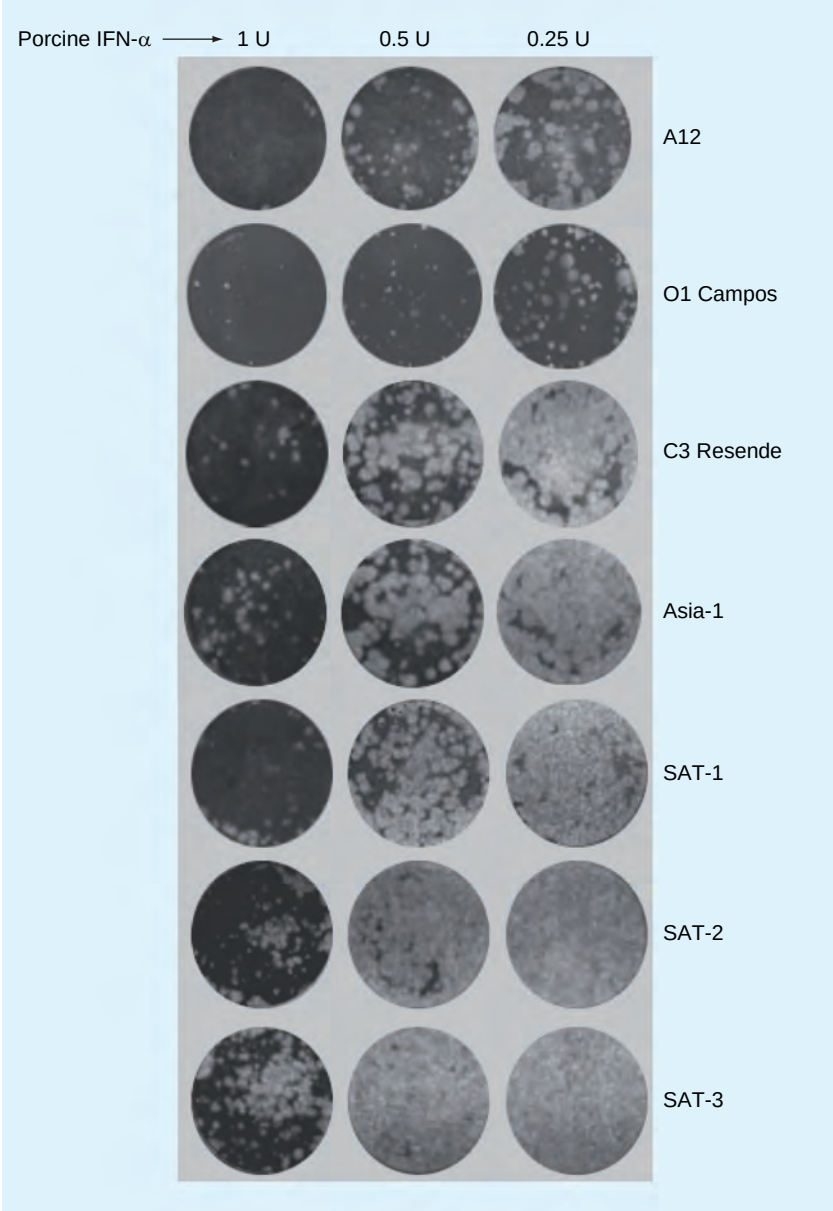

Figure 2. Effect of porcine IFN-α on replication of the seven foot-and-mouth disease virus serotypes. Different concentrations of porcine IFN-α (1, 0.5 and 0.25 U) were used to treat IB-RS-2 cells for 24 h. Cells were then challenged with the same dose of the seven foot-and-mouth disease virus serotypes (A [A12], O [O1 Campos], C [C3 Resende], Asia [Asia-1], SAT-1, SAT-2 and SAT-3). A total of 1 h after infection, cells were overlaid with gum tragacanth, and 24 h later, virus plaques were detected by staining with crystal violet.

studies demonstrated that Ad5-poIFN-α could rapidly protect swine against multiple serotypes of FMDV, all these experiments were performed by direct id. challenge. However, the natural route of FMDV infection is by aerosol [65–67]. We therefore examined if treatment with Ad5-poIFN-α could protect animals when exposed to aerosolized FMDV. A total of 24 h post-Ad5 treatment, swine were brought in direct contact with donor animals that had been directly infected 2 days earlier with FMDV and were displaying clinical signs of disease. After 18 h of co-mingling, the donor animals were removed and euthanized, while the exposed Ad5-poIFN-α-pretreated animals were monitored for clinical disease for 10 days. All Ad5-poIFN-α-inoculated animals were completely protected against disease, while the Ad5-null control group developed clinical disease, indicating that the same dose of Ad5-poIFN-α treatment is effective against multiple routes of FMDV exposure [61].

We also examined the effectiveness of this approach in bovines. Inoculation of bovines with 1×10^{10} PFU/animal of Ad5-poIFN-α or Ad5-boIFN-α induced a relatively low level of systemic antiviral activity (100–200 U/ml), and challenge of these animals with FMDV A24 by id. inoculation in the tongue only resulted in a short delay and reduced severity of disease as compared with control animals [57].

Type II IFNs

Another important biotherapeutic molecule is IFN-γ, the sole member of the type II IFN family. This protein is a multifunctional cytokine produced by Th1 and NK cells, and its biological functions include immunoregulatory, antineoplastic and antiviral properties [68]. The signal transduction pathways elicited by type II IFNs are different from those induced by type I IFN [69]. Interestingly, the combination of type I and type II IFNs can synergistically induce gene expression [69,70]. To examine the potential antiviral effect of IFN-γ on FMDV replication we constructed an Ad5 vector containing the porcine IFN-γ gene (Ad5-poIFN-γ). We demonstrated that our Ad5 construct produced IFN-γ protein (Figure 1) was able to block FMDV replication in cell culture [71]. Furthermore, we observed an enhanced protective effect against FMDV *in vitro* when type II IFN was combined with type I IFN [70]. Interestingly, the action of type II IFN in combination with type I IFN could synergistically block virus replication *in vivo*; swine inoculated with the combination of Ad5-poIFN-γ and Ad5-poIFN-α were completely protected against FMD challenge when used at doses that, individually, were not effective [71].

Treatment of bovines with a combination of type I and II IFNs (1×10^{10} PFU of each IFN/animal) and challenge id. 1 day later with FMDV A24 showed slightly improved levels of protection as compared with type I IFN alone. One animal never developed clinical disease while the other two animals developed clinical signs 4–6 days later than the control animals and disease was less severe [Moraes MP *et al.*, Unpublished Data]. However, all treated animals developed

viremia that was only somewhat lower than the control animals.

Type III IFNs

As mentioned above, use of type I and II IFNs was a very effective strategy to control FMD in swine. However, a similar approach only had limited efficacy in cattle [57].

Recently, a new family of IFNs – type III IFN or IFN-λ – has been identified in humans, mice, chicken and swine [72,73]. We identified and cloned bovine IFN-λ3 (boIFN-λ3), also known as IL-28B, and utilized the Ad5 expression system (Figure 1) for delivery in cattle. Inoculation of cattle with Ad5-boIFN-λ3 resulted in low levels of systemic antiviral activity but induced interferon stimulated genes (ISGs) in most tissues of the upper respiratory tract, which are targets of FMDV infection [58]. When compared side by side with Ad5-boIFN-α or Ad5-poIFN-α, inoculation of cattle with 1–1.5 × 10^{11} PFU/animal of Ad5-boIFN-λ3 followed by id. challenge in the tongue with FMDV 24 hpi resulted in a significant delay (4–6 days) and reduced severity of disease as compared with the controls [74]. Furthermore, the delay in the appearance of disease was significantly prolonged when treated cattle were challenged by aerosolization of FMDV, using a method that best resembles the natural route of infection [66]. In this later experiment, no clinical signs of FMD, viremia or viral shedding in nasal swabs were found in the Ad5-boIFN-λ3-treated animals for at least 9 dpc, and one animal remained free of disease during the entire experiment. These results indicated that boIFN-λ3 plays a critical role in the innate immune response of cattle against FMDV and suggested that a vaccine/boIFN-λ3 combination strategy may provide a viable approach to completely protect bovines from disease.

More recently, we have constructed an Ad5 vector expressing poIFN-λ3 (Figure 1). *In vitro*, this molecule induced lower levels of antiviral activity than poIFN-α; however, in a preliminary dose–response experiment in swine, a potency similar to type I IFN was detected. These results suggest that treatment with type III IFN is also a promising biotherapeutic strategy to control FMD in swine [Perez-Martin E *et al.*, Adenovirus expressing type III interferon protects swine against foot-and-mouth disease (2012), Manuscript in preparation].

Approaches to improve IFN treatment

A commercially viable biotherapeutic approach to block the spread of an animal pathogen must be economically affordable. Therefore, we have attempted to enhance the potency of our Ad5-IFN approach in several ways.

We were very interested in understanding the molecular mechanisms induced by the different types of IFN treatment that result in protection against FMDV challenge. Groups of animals were inoculated with either Ad5-poIFN-α or Ad5-poIFN-γ alone or in combination, and challenge id. with FMDV 1 day later. We examined ISGs in skin, peripheral blood mononuclear cells and lymphoid tissues and also evaluated possible immune cell recruitment to the skin and lymph nodes. We found that protection correlated with recruitment of dendritic cells (DCs) and NK cells to the skin and lymph nodes, respectively, and upregulation of a number of ISGs with antiviral activity including PKR and OAS, which block FMDV replication in cell culture [50,52], as well as cytokines and chemokines, including IP-10, which is involved in chemoattraction of DCs and NK cells [75]. Based on our results, we hypothesize that one or more of the above chemokines (or others not yet examined) may be involved in the recruitment/proliferation and activation of DCs and NK cells in IFN-treated animals and that the maturation and activation of these cells has a role in the control of FMDV replication.

Studies describing multiple-site immunization with various vaccines have shown an enhancement of the immune response as compared with single-site immunization [76,77]. As described in an earlier section, administration of Ad5-FMD vaccine sc. at two sites in the neck resulted in enhanced potency against FMDV challenge compared with inoculation at one site. Based on these results, we attempted to lower the protective dose of Ad5-poIFN-α by comparing single-site versus multiple-site im. inoculation of swine in the hind limb and neck. The data clearly showed that by inoculating at four sites in the neck, we were able to reduce the protective dose of Ad5-poIFN-α by tenfold [61]. In a subsequent experiment, we compared the efficacy of im. versus sc. inoculation at four sites in the neck. Swine were administered Ad5-poIFN-α im. or sc. at different doses and challenged 1 day later. The protective dose was reduced by twofold in sc. as compared with im. inoculated animals (Table 2). Furthermore, these animals did not develop viremia or virus in nasal swabs (Table 2).

Mimics of viral infection

The administration of replication-defective Ad5-IFN to cattle or swine upregulates a number of genes [58,71,75], which leads to protection against FMDV. However, during the course of

a productive viral infection, a broader response is initiated owing to the interaction of either unique viral molecules synthesized during replication or components of the pathogen ('pathogen-associated molecular patterns') with specific pattern recognition receptors present in host cells [78,79]. These interactions result in the induction and activation of a broad array of innate immune pathways, which ultimately leads to the expression of IFNs as well as to additional molecules. By directly administering IFN, the molecular interactions that normally occur during viral infection are bypassed. We hypothesized that treating animals with a molecular mimic of virus infection, such as dsRNA, alone or in combination with IFN, could induce a broader array of genes in comparison with IFN treatment alone and could also activate a number of signaling molecules that may potentially result in a positive feedback induction of additional IFN [79,80]. To test this hypothesis, we inoculated swine sc. at four sites in the neck using varying doses of Ad5-poIFN-α and poly-ICLC alone or in combination. Poly-ICLC is a synthetic, double-stranded polyriboinosinic-polyribocytidylic acid molecule stabilized with poly-L-lysine and carboxymethyl cellulose, which has enhanced biostability *in vivo* as compared with poly-IC [81]. Poly-IC is an agonist of Toll-like receptor 3 and the cytosolic receptor MDA-5 [82,83]. Our results indicated that a dose of 8 mg of poly-ICLC is able to protect swine against FMDV A24 id. challenge when used alone or combined with Ad5-poIFN-α (at a tenfold reduced dose as compared with the protective dose of Ad5-poIFN-α alone). These results demonstrated that the use of pattern recognition receptor agonists alone or in combination with IFNs may represent an effective and broad-spectrum antiviral strategy to combat FMDV infection and perhaps other viral diseases of livestock species [84].

Status & future perspective

In the last 12 years, significant progress has been made towards the development of a novel, effective Ad5-FMD vaccine. Our second-generation

Table 2. Comparison of route on efficacy of adenovirus type 5-porcine IFN-α administration in swine.

Group	Dose† (FFU)	Route	Animal number	Clinical score‡	Viremia§	Shedding virus¶	SN#
Ad5-poIFN-α	1×10^{10}	im.	1	5/14	$5/5.8 \times 10^{2}/1$	0	16,384
			2	0	0	0	128
			3	NA††	$4/2.0 \times 10^{3}/2$	$4/1 \times 10^{1}/1$	NA††
	5×10^{9}	im.	4	5/11	$5/1.7 \times 10^{3}/1$	$6/5 \times 10^{1}/1$	16,384
			5	0	0	0	<8
			6	4/14	$3/5 \times 10^{4}/3$	$4/2.3 \times 10^{2}/1$	2048
	1×10^{10}	sc.	7	0	0	0	32
			8	0	0	0	128
			9	0	0	0	128
	5×10^{9}	sc.	10	0	0	0	512
			11	0	0	0	8
			12	0	0	0	64
	2.5×10^{9}	sc.	13	3/14	$2/3.5 \times 10^{5}/3$	$4/5.8 \times 10^{2}/2$	4096
			14	3/14	$3/4.5 \times 10^{3}/2$	0	16,384
			15	0	0	0	64
Ad5-null	1×10^{10}	sc.	17	2/15	$3/1.0 \times 10^{4}/3$	$3/7.5 \times 10^{1}/3$	2048
			18	2/17	$3/5.9 \times 10^{4}/3$	$2/6 \times 10^{1}/4$	4096

All animal experiments were performed under a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the Plum Island Animal Disease Center (NY, USA).
†Dose of Ad5 inoculum per animal expressed as number of FFU in 2 ml of phosphate buffered saline.
‡dpc at first signs of lesions/highest lesion score. Clinical score is the number of toes with lesions plus the snout and tongue combined. The maximum score is 17.
§First dpc viremia was detected/maximum amount of viremia in PFU/ml detected in sera samples/duration (days) of viremia.
¶First dpc shedding virus was detected/maximum amount of shedding virus in PFU/ml detected in nasal swab samples/duration (days) of shedding.
#SN antibody response reported as serum dilution yielding a 70% reduction in the number of plaques at 21 dpc.
††Animal died 5 dpc from causes not related to foot-and-mouth disease.
Ad5: Adenovirus type 5; dpc: Day postchallenge; FFU: Focus forming units; im.: Intramuscularly; NA: Not applicable; poIFN-α: Porcine IFN-α; sc.: Subcutaneously; SN: Serum neutralizing.

vaccine with the full-length coding region of NS protein 2B has improved vaccine efficacy in both swine and bovines. The engineered Ad5-FMD vaccine has overcome most of the limitations of the current inactivated vaccine; however, additional improvements are still required to make this strategy affordable and practical worldwide. These improvements may include addition of coding regions of other FMDV NS proteins, use of alternative routes of inoculation, optimization of capsid protein expression and controlled expression of the toxic $3C^{pro}$, introduction of mutations to enhance capsid stability and a more efficient targeting of the Ad5 vector to APCs.

We have also made progress in the development of biotherapeutics that can rapidly protect swine against both direct inoculation and contact challenge exposure and significantly delay disease onset and severity in cattle. More recent information suggests that mimics of viral pathogen-associated molecular patterns alone or in combination with various IFNs may induce a broader and more robust innate immune response than IFNs alone. Furthermore, initial experiments combining the Ad5-FMD vaccine and IFNs indicate that the IFNs can also function as adjuvants to enhance the long-term adaptive immune response induced by the vaccine. Identification of adjuvants that induce a more potent innate response as well as a prolonged vaccine-induced protection are additional areas that we are pursuing.

We believe that this comprehensive approach will allow the development of an FMD vaccine platform that will be practical for use by FMD-free countries in the event of an outbreak, as well as an affordable strategy for use in countries in which FMD is currently enzootic.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Executive summary

Potency & efficacy of adenovirus type 5 foot-and-mouth disease vaccines

- We have improved the efficacy of the adenovirus type 5–24 (Ad5-A24) and Ad5-O1Campos (Ad5-O1C) vectored foot-and-mouth disease (FMD) vaccines by including the full-length coding region of FMD virus (FMDV) nonstructural protein 2B. The vaccine induces both an FMDV-specific neutralizing antibody response as well as a T-cell-mediated immune response.
- Delivery of the Ad5-vectored vaccine in swine subcutaneously at two sites on either side of the neck reduced the protective dose 25-fold as compared with intramuscular inoculation at one site in the hind limb.

Ad5 interferon biotherapeutic candidates: early development studies

- Ad5-porcine IFN-α/β can protect swine from multiple serotypes of FMDV. Protection is induced as early as 1 day post-treatment and can last for 3–5 days. Swine are protected when challenged either by direct inoculation in the heel bulb or by contact exposure to infected animals.
- The efficacy of Ad5-poIFN-α is enhanced by subcutaneous inoculation at multiple sites in the neck as compared with intramuscular inoculation at one site in the hind limb.
- Ad5-poIFN-γ vector also has antiviral activity against FMDV and in combination with type I IFN can protect swine.
- Although bovine type I and II IFNs were not as successful in cattle as the poIFNs in swine, cattle inoculated with Ad5-bovine IFN-λ and challenged with FMDV showed significantly delayed disease onset and reduced severity as compared with control animals. Furthermore, inoculation of cattle with this vector induced upregulation of interferon stimulated genes in multiple tissues susceptible to FMDV.
- Ad5-poIFN-λ vector has antiviral activity and can protect swine against FMD.

Viral mimics as biotherapeutic candidates

- Pathogen-associated molecular patterms induce a broad innate immune response. Poly-ICLC, a mimic of viral dsRNA, can rapidly protect swine against FMDV challenge.

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A Safe Foot-and-Mouth Disease Vaccine Platform with Two Negative Markers for Differentiating Infected from Vaccinated Animals

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Vaccination of domestic animals with chemically inactivated foot-and-mouth disease virus (FMDV) is widely practiced to control FMD. Currently, FMD vaccine manufacturing requires the growth of large volumes of virulent FMDV in biocontainment-level facilities. Here, two marker FMDV vaccine candidates ($A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$) featuring the deletion of the leader coding region (L^{pro}) and one of the 3B proteins were constructed and evaluated. These vaccine candidates also contain either one or two sets of mutations to create negative antigenic markers in the 3D polymerase ($3D^{pol}$) and 3B nonstructural proteins. Two mutations in $3D^{pol}$, $H_{27}Y$ and $N_{31}R$, as well as $RQKP_{9-12}$→PVKV substitutions, in $3B_2$ abolish reactivity with monoclonal antibodies targeting the respective sequences in $3D^{pol}$ and 3B. Infectious cDNA clones encoding the marker viruses also contain unique restriction endonuclease sites flanking the capsid-coding region that allow for easy derivation of custom designed vaccine candidates. In contrast to the parental A_{24}WT virus, single $A_{24}LL3D_{YR}$ and double $A_{24}LL3B_{PVKV}3D_{YR}$ mutant viruses were markedly attenuated upon inoculation of cattle using the natural aerosol or direct tongue inoculation. Likewise, pigs inoculated with live $A_{24}LL3D_{YR}$ virus in the heel bulbs showed no clinical signs of disease, no fever, and no FMD transmission to in-contact animals. Immunization of cattle with chemically inactivated $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$ vaccines provided 100% protection from challenge with parental wild-type virus. These attenuated, antigenically marked viruses provide a safe alternative to virulent strains for FMD vaccine manufacturing. In addition, a competitive enzyme-linked immunosorbent assay targeted to the negative markers provides a suitable companion test for differentiating infected from vaccinated animals.

Foot-and-mouth disease (FMD) is an extremely contagious viral disease of cloven-hoofed ungulates, including a variety of wild and domestic (cattle, pigs, and sheep, among others) animals. The disease is distributed worldwide and has great negative economic impact not only on livestock health and production but also on international trade. Disease outbreaks occur frequently on almost every continent; outbreaks in previously FMD-free countries often have devastating economic consequences. Regular prophylactic vaccination using vaccine antigens that antigenically match circulating viruses is practiced in many countries as a primary control measure. However, the emergence or introduction of new strains renders these vaccines ineffective and thus require the development of new viruses.

FMD virus (FMDV) is a member of the genus *Aphthovirus* in the family *Picornaviridae* and exists as an antigenically variable virus of 7 serotypes, including A, O, C, Asia-1, and South African Territories (SATs) 1 to 3, as well as multiple subtypes. The viral genome consists of 8,500 nucleotides of a single-stranded positive-sense RNA protected by an icosahedral capsid containing 60 copies of each of the four structural proteins (18). FMDV is translated as a single polyprotein that is posttranslationally cleaved to produce partial and full cleavage products resulting in four structural proteins (VP1, VP2, VP3, and VP4) and 10 nonstructural proteins (L^{pro}, 2A, 2B, 2C, 3A, $3B_{1-3}$, $3C^{pro}$, and $3D^{pol}$).

FMD control is largely based on the FMD status of a geographical region. In endemic countries, it is based on regular (twice a year) vaccinations to reduce disease and transmission. On the other hand, the control policy for FMD-free countries usually includes the slaughter of animals in affected regions, as well as in neighboring regions, regardless of the disease status. However, large outbreaks in the United Kingdom and the Netherlands in 2001, as well as more recent outbreaks in Japan and Korea, where millions of animals that were mostly noninfected were sacrificed and burned or buried, resulted in public outcry and questioning of these control measures. As a result, there is a need for emergency vaccination programs accompanied by "vaccinate to live" policies as an alternative to mass culling of infected animals (35).

Current FMD vaccines consist of FMD virus antigen that has been chemically inactivated and formulated with adjuvants. Although this vaccine has been very successful in reducing disease outbreaks and virus transmission in countries where FMD is endemic, there are numerous risks and limitations associated with the current product (18, 42). First, vaccine production requires large cultures of live virulent virus prior to inactivation, which poses the risk of virus escape from the manufacturing facilities. Due to the lack of cross protection between serotypes and subtypes, vaccine strains must be selected to provide adequate protection and control of the virus circulating in particular regions. Also, vaccine production requires additional antigen purification process to remove cellular contaminants, as well as nonstructural viral proteins (NSPs), in order to support DIVA (differentiating infected from vaccinated animals) diagnostic testing, which is critical in outbreak control and serosurveillance. Since vaccine antigens consisting of killed virus do not replicate or induce anti-

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bodies against NSPs, anti-NSP antibodies have often been used as markers of infection. The highly conserved FMDV 3D polymerase ($3D^{pol}$) has been long identified as the main determinant of infection and has been called the FMDV infection-associated antigen (3, 4, 12, 27, 46). Interestingly, studies by Newman and Brown (30, 31) suggested that FMDV purified 140S particle preparations contain small quantities of $3D^{pol}$ and therefore could account for seroconversion to $3D^{pol}$ in animals that have received multiple doses of inactivated FMD vaccines. At present, antibodies against NSP 3ABC are considered the most reliable indicator of infection in both bovine and porcine sera (23), and numerous 3AB/3ABC-based enzyme-linked immunosorbent assay (ELISA) diagnostic tests are available to differentiate between infected and vaccinated animals (4, 8, 13, 24, 41, 43, 44, 46).

Past strategies to generate attenuated vaccines for FMDV have failed due to unstable phenotypes, variable pathogenic profiles in different species, and failure to induce adequate protection (25, 28, 29, 50). However, stably attenuated FMDV could provide a safe platform to produce inactivated antigen vaccines. Recent advances in infectious cDNA technology have led to opportunities to study and produce genetically engineered FMDV with altered virulence. FMDV virulence factor, leader protease (L^{pro}), is a papain-like proteinase (22, 40, 45) and is produced in two forms, Lab and Lb, through translation initiation codon at the first or the second AUG site, respectively. The presence of this inter-AUG region (the 84 nucleotides [nt] between the two AUG codons), as well as second AUG initiation codon of the L^{pro}, has been shown to be essential for viral virulence and replication of the FMDV type A_{24} virus (6, 36, 37). Both forms of L^{pro} can autocatalytically remove itself from the N terminus of the nascent polyprotein as well as cleave the eukaryotic initiation factor 4G, resulting in the shutoff cap-dependent host cell translation machinery. However, studies have demonstrated that polyprotein translation from the first AUG codon results in low translational efficiency with no viable virus production *in vitro*, and polyprotein translation is favored from the second AUG site through an unknown mechanism (6, 37). L^{pro} has also been shown to induce degradation of nuclear factor κB (NF-κB) in FMDV-infected cells, leading to inhibition of the host innate immune response (14, 15). Numerous studies with genetically engineered serotype A FMDVs lacking L^{pro} have been shown to be infectious but grow more slowly in cell culture (37) and is attenuated in both cattle and swine (5, 7, 26).

In the present study, we developed experimental marker FMD vaccine candidates that can aid in FMD control while providing DIVA capabilities. The negative antigenic marker virus candidates, $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$ derived by infectious cDNA technology, lack the functional L^{pro} but contain the 84-nt inter-AUG sequence, as well as the second initiation codon of L^{pro}. These vaccine candidates also lack one of three 3B copies ($3B_1$) and also contain replacements of immunodominant epitopes in 3B and $3D^{pol}$ with sequences corresponding to the closely related bovine rhinitis virus 2 (BRV-2). Aerosol inoculation of cattle with the live leaderless marker viruses results in very limited virus replication and the absence of FMD signs. Moreover, none of the infected animals shed significant amounts of virus into the environment. Likewise, swine inoculated in the heel bulbs with live $A_{24}LL3D_{YR}$ virus showed no clinical signs of FMD, nor did the inoculated animals transmit the disease to contact animals. Both $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$ vaccines produced by chemical inactivation with binary ethylenimine (BEI) proved to be as effective as a commercially available FMD vaccine in protecting cattle from challenge with the parental virus. Finally, serum from animals inoculated with these marker viruses can be readily distinguished from parental FMDV-infected animals utilizing companion DIVA serological ELISAs based on either one or two built-in antigenic markers.

MATERIALS AND METHODS

Viruses and cells. FMDV type A_{24} Cruzeiro was derived from the infectious cDNA clone, $pA_{24}Cru$ (referred to here as $A_{24}WT$ for simplicity [39]). A plasmid containing the BRV-2 (pBRV2, accession number EU236594) sequence from poly(C) to poly(A) described previously (20) was used as a source of bovine rhinitis virus 2 genetic material. The baby hamster kidney strain 21, clone 13, cell line (BHK-21) was maintained in Eagle basal medium (BME; Life Technologies, Gaithersburg, MD) supplemented with 10% calf serum (HyClone, South Logan, UT), 10% tryptose phosphate broth, and antibiotic/antimycotic. Cells were grown at 37°C with 5% CO_2 in a humidified atmosphere.

Derivation of $A_{24}LL$ negative marker FMDVs. The $3D^{pol}$ region of $pA_{24}Cru$ ($A_{24}WT$) was modified by PCR utilizing the mutagenic oligonucleotides P1266 (5′-ACCGTTGCGTACGGTGTGTTCCGTCCTGAGTTCGGG) and P1267 (5′-CCCGAACTCAGGACGGAACACACCGTACGCAACGGT) engineered to introduce mutations at codons 27 and 31 of $3D^{pol}$ protein (see Fig. 1B). The deletion of L^{pro} and the introduction of FseI at the beginning of the coding region for the capsid viral protein VP4 and NheI site in 2A were generated by overlap PCR fusion, created by mixing PCR amplified fragments, and reamplifying through the product of the fusion of these two fragments. This was accomplished by using the oligonucleotides P819 (5′-CGAGCCACAGGAAGGATGGGGGCCGGCCAATCCAG) and P820 (5′-CTGGATTGGCCGGCCCCCATCCTTCCTGTGGCTCG) containing an FseI site added by silent mutation in VP4 and sense (5′-GACCTGCTTAAGCTAGCCGGAGACGTTGA) and antisense (5′-TCAACGTCTCCGGCTAGCTTAAGCAGGTC) oligonucleotides containing a silent mutation that introduces NheI in 2A. To introduce these unique restriction sites and the deletion of the functional L^{pro} into $pA_{24}Cru$, two mutant PCR products (PCR-LLfseI and PCR-NheI) were generated. PCR-LLfseI (a 1,315-bp product) lacking the L^{pro} and containing the unique restriction sites XbaI, FseI, and SpeI was digested with XbaI and SpeI and cloned into pA24Cru digested with the same enzymes to generate pA24-LLNheI. The PCR-SacII/MfeI fragment (2,333 bp) containing unique restriction sites SacII, NheI and MfeI was digested with SacII and MfeI, and this mutant product was cloned into pA24-LLNheI plasmid digested with the same restriction enzymes. Although all leaderless plasmids generated in the present study lack the functional leader protein, they do contain the 84-nt region between the first and the second AUG codons of the leader-coding region. The generated plasmids $pA_{24}Cru$, $pA_{24}WT3D_{YR}$, $pA_{24}LL$, and $pA_{24}LL3D_{YR}$ all contain a T7 promoter sequence in front of a hammerhead ribozyme at the 5′ terminus of the S fragment of the FMDV genome, terminate with a poly(A) tract of 15 residues, and possess a unique restriction site (SwaI) that can be used for linearization. The double-negative epitope mutants, $A_{24}WT3B_{PVKV}3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$, were derived from plasmids $pA_{24}WT3D_{YR}$ and $pA_{24}LL3D_{YR}$, respectively, and lack one of the 3B ($3B_1$; also known as VPg_1) proteins but contain a substitution in $3B_2$ that abolishes reactivity with monoclonal antibody (MAb) F83B (a gift from Alfonso Clavijo, National Centre for Foreign Animal Disease, Winnipeg, Manitoba, Canada). The cDNA template corresponding to the $3B_1$ deletion mutant virus, $A_{24}WT$-5853, arose from transfection of a RNA that contained a transposon insertion in $3B_1$ at position 5853 (33). This virus has been shown to grow *in vitro* and produce signs of FMD in cattle similar to $A_{24}WT$. Therefore, to derive these double mutant plasmids, a PCR product spanning sequences between the unique restriction sites SalI and AgeI was produced that lacked $3B_1$ and harbored a substitution in $3B_2$ $RQKP_{9-12}$ with PVKV found at a similar position in BRV-2. The sequence encoding $3B_2$ was modified by utilizing mutagenic sense (5′-GCCCGATGGAGAGACCAG

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TTAAAGTTAAAGTGAAAGCAAAAGCC) and antisense (5′-GGCTTTT GCTTTCACTTTAACTTTAACTGGTCTCTCCATCGGGC) oligonucleotides to introduce mutations in $3B_2$ (also known as VPg_2; see Fig. 1B). Full-length genomic clones were linearized with SwaI and were *in vitro* transcribed using the T7 Megascript system (Ambion, Austin, TX). Transcript RNAs were transfected into BHK-21 cells by electroporation as previously described (38). The transfected cells were seeded in six-well plates and incubated for 24 to 48 h at 37°C. All six viruses were passaged up to four times in BHK-21 cells, and complete viral genomes were verified by sequence analysis. Virus stocks at passage 4 were stored at −70°C. Passage 4 virus stocks were used in animal experiments and for the production of inactivated vaccines. Virus titers were determined by plaque assays as described below.

Rescue of parental and mutant viruses, viral growth, and plaque assays. For virus growth curves, BHK-21 monolayers were infected with A_{24}WT, A_{24}WT3D_{YR}, A_{24}LL, A_{24}LL3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, and A_{24}LL3B_{PVKV}3D_{YR} at a multiplicity of infection (MOI) of 5 PFU/cell. After 1 h of adsorption at 37°C, the monolayers were rinsed once with MES buffer (morpholine ethanesulfonic acid [25 mM], 145 mM NaCl [pH 5.5]) and then twice with phosphate-buffered saline (PBS), followed by the addition of fresh BME containing no serum. At various times postinfection, virus titers were determined by plaque assays (38) using 0.6% gum tragacanth overlay and incubated for 48 to 72 h at 37°C. Plates were fixed and then stained with crystal violet (0.3% in Histochoice; Amresco, Solon, OH), and the plaques were counted. Titers were expressed as PFU/ml and determined in duplicate.

Western blotting. BHK-21 cells were either mock infected or infected with A_{24}WT, A_{24}WT3D_{YR}, A_{24}LL, A_{24}LL3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, or A_{24}LL3B_{PVKV}3D_{YR} at an MOI of 5. The following day, cells were lysed with radioimmunoprecipitation assay buffer (PBS supplemented with 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and protease inhibitors). Next, 10 μl of each cell lysate was run under denaturing conditions in a SDS–12% PAGE gel (Invitrogen) and transferred onto nitrocellulose membranes using an XCell II transfer system (Invitrogen). The blots were blocked with 5% skim milk in PBS-Tween (PBS-T) for 1 h at room temperature, followed by an additional incubation of 1 h with 1:200 FMDV-specific MAbs (MAb F32-44, MAb F19-2, and MAb F83B) in PBS-T and 1% skim milk. Blots were then washed twice with PBS-T for 5 min each and incubated for an additional hour with goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP; Bethyl Laboratories) at 1:20,000 diluted in PBS-T and 1% skim milk. The blots were subsequently washed three times with PBS-T and developed with SuperSignal West Dura extended duration substrate (Thermo Scientific). Mouse anti-tubulin IgG conjugated to HRP (Abcam) was used at a dilution of 1:2,000 for a loading control.

Immunohistochemistry assay. The MAbs used in the present study were the MAbs F19-2 and F32-44 directed against the FMDV $3D^{pol}$ (49) and MAb F83B directed against the 3B nonstructural protein (19). Briefly, cell monolayers grown in 24-well plates were infected with A_{24}WT, A_{24}WT3D_{YR}, A_{24}LL, A_{24}LL3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, or A_{24}LL3B_{PVKV}3D_{YR} at an MOI of 5. The following day, the infected cells were fixed with cold acetone-methanol (50/50) mix for 20 min, followed by two washes with PBS. Fixed cells were stained using FMDV-specific MAbs according to the manufacturer's instructions in a Vectastain ABC alkaline phosphatase kit (Vector Labs).

Antigen production and vaccine formulation. The A_{24}LL3D_{YR} and A_{24}LL3B_{PVKV}3D_{YR} vaccine antigens were harvested from infected BHK-21 monolayers (a total of 3×10^8 cells) and inactivated with 5 mM BEI for 24 h at 25°C. The inactivated antigens were then concentrated and partially purified with 8% polyethylene glycol 8000. The yield of the inactivated antigens ranged between 18 and 23 μg. The vaccines were prepared as water-in-oil-in-water (WOW) emulsion with Montadine ISA 206 (Seppic, Paris, France) according to the manufacturer's instructions. Briefly, the oil adjuvant was mixed into the aqueous antigen phase (50:50) at 30°C for 15 min and stored at 4°C for 24 h, followed by another brief mixing cycle for 10 min. The integrity of 146S particles and antigen concentration present in the experimental vaccines (15 μg/dose of chemically inactivated A_{24}LL3D_{YR} or A_{24}LL3B_{PVKV}3D_{YR} antigen) were determined by 10 to 30% sucrose density gradient and 260 nm densitometry. The commercial vaccine used for comparison was a polyvalent vaccine (Biogenesis-Bagó, Bioaftogen series 565 composed of O_1 Campos, A_{24} Cruzeiro, A Arg 2001, and C_3 Indaial) with an antigen load of 4 to 6 μg/dose.

Virulence study in cattle. Groups of Holstein steers—two animals each for A_{24}WT, A_{24}WT3B_{PVKV}3D_{YR}, and A_{24}LL3B_{PVKV}3D_{YR} viruses and three animals each for A_{24}WT3D_{YR} and A_{24}LL3D_{YR} viruses—were marked and housed in a single room for a week of acclimation. Prior to infection, the animals were moved to separate rooms, and each of them was inoculated by aerosol with either 1×10^7 50% tissue culture infective dose(s) ($TCID_{50}$) (for live A_{24}WT) or 1×10^6 to 3×10^6 $TCID_{50}$ (for live A_{24}WT3D_{YR}, A_{24}LL3D_{YR}, and A_{24}WT3B_{PVKV}3D_{YR} mutants) using a method previously described (32). A dose of 7×10^6 $TCID_{50}$ of live A_{24}LL3B_{PVKV}3D_{YR} virus was inoculated intradermolingually into cattle. Sera and oral secretions were collected daily for up to 9 days for A_{24}WT and for up to 21 days for A_{24}WT3D_{YR}, A_{24}LL3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, and A_{24}LL3B_{PVKV}3D_{YR}, and temperature and clinical evaluation were recorded for the same period of time. Shedding of virus in the air was also monitored using a dry filter unit model 1000 air pump developed by the Program Executive Office for Chemical Biological Defense (PEO-CBD), as previously published (34). Clinical signs were scored as one credit for each affected foot and one credit for the affected head (vesicles in mouth, nostrils, tongue, or lips). FMDV RNA was measured in sera, swabs, and air samples by real-time reverse transcription-PCR (rRT-PCR) as described below.

Swine study. Mutant (A_{24}LL3D_{YR}) virus was tested for its virulence in swine. Briefly, two 20-kg pigs were inoculated intradermally in the heel bulb of the foot with 10^5 $TCID_{50}$ of live virus, and 24 h later two naive pigs were added in direct contact. Sera and nasal and oral secretions were collected daily for up to 9 days postinoculation (dpi) and twice a week thereafter. Infected animals underwent clinical evaluation, and rectal temperature was determined daily throughout the experiment. Shedding of virus in the air was also monitored as described above. FMDV RNA was measured in sera, swabs, and air samples by rRT-PCR as described below.

Vaccination and challenge of cattle. Sixteen Holstein steers, between 250 and 300 kg, were allowed to acclimatize from shipping for 1 week before testing was initiated. Groups of four steers were vaccinated intramuscularly in the neck with the commercial vaccine (cattle 863, 864, 865, and 866), with chemically inactivated A_{24}LL3D_{YR}/water-in-oil-in-water (WOW, cattle 867, 868, 869, and 870) vaccine or A_{24}LL3B_{PVKV}3D_{YR}/water-in-oil-in-water (WOW, cattle 1018, 1019, 1020, and 1021) vaccine. Cattle 871, 872, 1022, and 1023 were vaccinated with sterile PBS to be used as unvaccinated controls. On day 21 postvaccination (dpv), all 16 cattle were challenged intradermolingually with 10^4 $BTID_{50}$ (50% bovine tongue infectious doses [10]) of parental A_{24}WT. The animals were then monitored at 0, 4, 7, and 10 days postchallenge (dpc) for the appearance of localized and generalized lesions. Sera, nasal swabs (cotton tip, immersed in 2 ml of minimum essential medium with 25 mM HEPES and 1% fetal bovine sera), and temperature data were collected daily. Clinical signs were scored as one credit for each affected foot, and the presence of vesicles in the head was not considered due to lingual inoculation of challenge. FMDV RNA was measured in sera, swabs, and air samples by rRT-PCR as described below.

Foot-and-mouth disease virus RNA detection and DNA sequence analysis. Sera and swabs were processed for RNA extraction and rRT-PCR as previously described (2, 32). Briefly, 50 μl of each sample (sera, nasal, or oral swab suspension) for each cow was transferred to 96-well plates (King Fisher no. 97002540) containing 150 μl of lysis/binding solution. RNA was then extracted using MagMax-96 viral RNA isolation kit (Ambion, catalog no. 1836) on a King Fisher-96 magnetic particle processor (Thermo Electron Corp.). After an initial 5-min lysis/binding step, the RNA samples underwent a series of four washing steps, a drying step, and

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a final elution step. RNA was eluted in a final volume of 25 μl. At each of the above steps, RNA was magnetically bound to the beads contained in the lysis/binding solution and was transferred to the different extraction solutions. For filters containing the air samples, 1/4 of the filters were processed with 600 μl of RLT/β-mercaptoethanol and 106-μm acid-washed glass beads (Sigma). The sample was then disrupted using a Retsch tissue Mixer Mill (model MM400) at 30 beats/s for 3 min, and the liquid suspension was used for RNA extraction with the standard RNeasy RNA extraction. RNA extracted from all of the previous described samples was analyzed by rRT-PCR using 2.5 μl of RNA on the ABI 7000 as previously described (36). The cutoff to consider a positive value for preclinical samples (sera and swabs) was $10^{2.69}$ RNA copies/ml, while the cutoff for air samples was $10^{0.8}$ RNA copies number/1,000 liters of air. When necessary, PCR amplicons were sequenced using gene-specific primers, BigDye termination cycle sequencing kits (Applied Biosystems, Foster City, CA) and a Prism 3700 automated sequencer (Applied Biosystems). Primers and probes were designed using Primer Express software (Applied Biosystems).

Expression of recombinant FMDV $3D^{pol}$ protein. Expression clone for $3D^{pol}$ was prepared by using standard recombinant DNA methods. Briefly, PCR was used to amplify the $3D^{pol}$-coding sequence of a type A FMDV. Forward primer P727 (5′-GCGGAATTCCCGCGGTGGAGGGT TAATCGTTGATAC) containing a SacII restriction site was designed to fuse the three amino acids of C terminus of ubiquitin (48) to the coding sequence for $3D^{pol}$. The antisense primer, P728 (5′- GCGGAATTCGGA TCCTGCGTCACCGCACACGGCGTTCACCC), encoding the C-terminal residues of $3D^{pol}$ also contains a BamHI restriction site for cloning purposes. The PCR product was cloned into pET26cHis, and the protein was expressed and purified from *E. coli*.

Serology and antigen differentiation assays. Serum samples from all animals were tested for the presence of neutralizing antibodies against FMDV in a serum neutralization assay. Neutralizing titers were reported as the reciprocal of the last serum dilution to neutralize 100 $TCID_{50}$ of homologous FMDV in 50% of the wells (17). To study the anti-$3D^{pol}$ response in the animals, we utilized sera collected from cattle following aerosol inoculation with live A_{24}WT (bovines 7109 and 7110), $A_{24}WT3D_{YR}$ (bovines 7199, 1, and 2), and $A_{24}WT3B_{PVKV}3D_{YR}$ (bovines 9143 and 9144) viruses and four animals introdermolingually inoculated with live A_{24}WT virus (bovines 871, 872, 1022, and 1023). A competitive ELISA (cELISA) was performed according to the protocol of Yang et al. (49) with minor modifications. Briefly, recombinant $3D^{pol}$ was diluted in buffer carbonate-bicarbonate (pH 9.6) to obtain 0.25 μg/ml, and 100 μl/well was used to coat Nunc Maxisorp plates (Fisher Scientific). After 2 h of incubation at 37°C on a rotary shaker, the plates were washed four times with 0.01 M PBS and 0.05% Tween 20 (PBS-T). Triplicates of 50 μl of test sera/well (1/15 in PBS-T) and 50 μl of F32-44 hybridoma culture supernatant/well (1/5 in PBS-T) were applied to the coated plates, followed by incubation at 37°C for 1 h on a rotary shaker. After four washes, 100 μl of peroxidase labeled goat antibody to mouse-IgG (H+L) (KPL)/well diluted 1:2,000 in 5% skim milk in PBS-T was added, followed by incubation for 1 h at 37°C. After four additional washes, the antigen-antibody complexes were detected by the addition of 100 μl/well of SureBlue Reserve (KPL) and stopped in 25 min with 50 μl of TMB BlueSTOP solution (KPL)/well. The optical density (OD) was determined at 630 nm on an automated ELISA plate reader. For cELISA based on MAb F83B, a similar protocol was utilized with minor modifications. In particular, the antigen consisted of a peptide encoding the 3B sequence GPYAGPLETQ KPLK and was applied at a concentration of 0.5 μg/well. Test sera were assayed at a 1/15 dilution in PBS-T, and MAb F83B was used at a 1/125 dilution. The results were expressed as the percentage of inhibition using mean OD values of test sera as well as known FMDV-positive and -negative bovine sera. The percent inhibition (PI) of samples was derived according to the following formula: PI = [(negative reference serum OD − test sample OD)/(negative reference serum OD − positive reference serum OD)] × 100.

RESULTS

Generation of negative marker FMDVs. Two mutant plasmids designated $pA_{24}WT3D_{YR}$ and $pA_{24}LL3D_{YR}$ were derived by site-directed mutagenesis using either FMDV pA_{24}Cru (39) or the backbone of functional leader-deleted pA_{24}LL infectious cDNA clones (Fig. 1A). Plasmids $pA_{24}WT3D_{YR}$, and $pA_{24}LL3D_{YR}$ were engineered with a substitution in $3D^{pol}$ for residues found in BRV-2 at the respective locations that would eliminate an important antigenic epitope in FMDV $3D^{pol}$; His_{27} was replaced by Tyr ($H_{27}Y$), and Asn_{31} was changed to the basic amino acid, Arg ($N_{31}R$) (Fig. 1B). Likewise, the two double-negative marker plasmids, $pA_{24}WT3B_{PVKV}3D_{YR}$ and $pA_{24}LL3B_{PVKV}3D_{YR}$, were derived by deleting one of the 3B copies ($3B_1$) and introducing a substitution in $3B_2$. The substitution in $3B_2$ was at amino acid positions 9 to 12 (RQKP), which were replaced by PVKV based on the sequence found at similar position in BRV-2 (Fig. 1B). The integrity of each construct for the genetic alterations was verified by DNA sequencing. Infectious virus was derived from each of the six infectious cDNA plasmids by electroporation of transcript RNA into BHK-21 cells. All mutant viruses were sequenced to confirm the presence of marker mutations in 3B and/or $3D^{pol}$. The genetic stability of 3B and $3D^{pol}$ epitope mutations were confirmed by nucleotide sequence analysis of virus recovered after up to 15 serial passages in BHK-21 cells (data not shown).

***In vitro* characterization of negative marker FMDVs.** *In vitro* characterization studies of negative marker viruses were performed to compare these viruses to their respective parental A_{24}WT and A_{24}LL viruses. The plaque phenotypes of $A_{24}WT3D_{YR}$ and $A_{24}WT3B_{PVKV}3D_{YR}$ viruses were a mix of various sizes after 48 h, similar to the parental A_{24}WT virus (Fig. 1A). However, the plaques of A_{24}LL, $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$ viruses were significantly smaller after 48 h of incubation and therefore required a 72-h overlay incubation.

In vitro growth kinetics of A_{24}WT and A_{24}LL viruses with their respective negative marker mutants were determined by using a high MOI of 5 on BHK-21 monolayers (Fig. 2A). Although the growth of the A_{24}WT, $A_{24}WT3D_{YR}$, and $A_{24}WT3B_{PVKV}3D_{YR}$ viruses reached similar titers by 24 h postinfection (hpi), A_{24}LL and the two marker viruses, $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$, were slightly delayed in their initial proliferation. By 24 hpi, virus production in A_{24}LL-, $A_{24}LL3D_{YR}$-, and $A_{24}LL3B_{PVKV}3D_{YR}$-infected cells yielded titers that were lower by 0.5 to 1 log compared to their respective parenteral WT strains.

In order to assay the antigenic profile of mutant versus parental viruses, virus-infected cells were examined by Western blot analysis and immunohistochemistry (Fig. 2B and C, respectively). As shown in Fig. 2, BHK cells infected with each of the six viruses were immunoreactive with MAb F19-2, which detects an epitope in $3D^{pol}$ protein that was not altered. In contrast, only A_{24}WT and A_{24}LL viruses were immunoreactive with MAb F32-44. Failure of MAb F32-44 to react with $A_{24}WT3D_{YR}$, $A_{24}WT3B_{PVKV}3D_{YR}$, $A_{24}LL3D_{YR}$, and $A_{24}LL3B_{PVKV}3D_{YR}$ mutant viruses indicated that the mutation of the $3D^{pol}$ epitope (a two-amino-acid replacement) affected the ability of mutant FMDVs to be recognized by MAb F32-44 but not by MAb F19-2. Likewise, both $A_{24}WT3B_{PVKV}3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$ viruses failed to react against MAb F83B antibody, which recognizes an epitope that was altered in the $3B_2$ region of the double-negative marker viruses.

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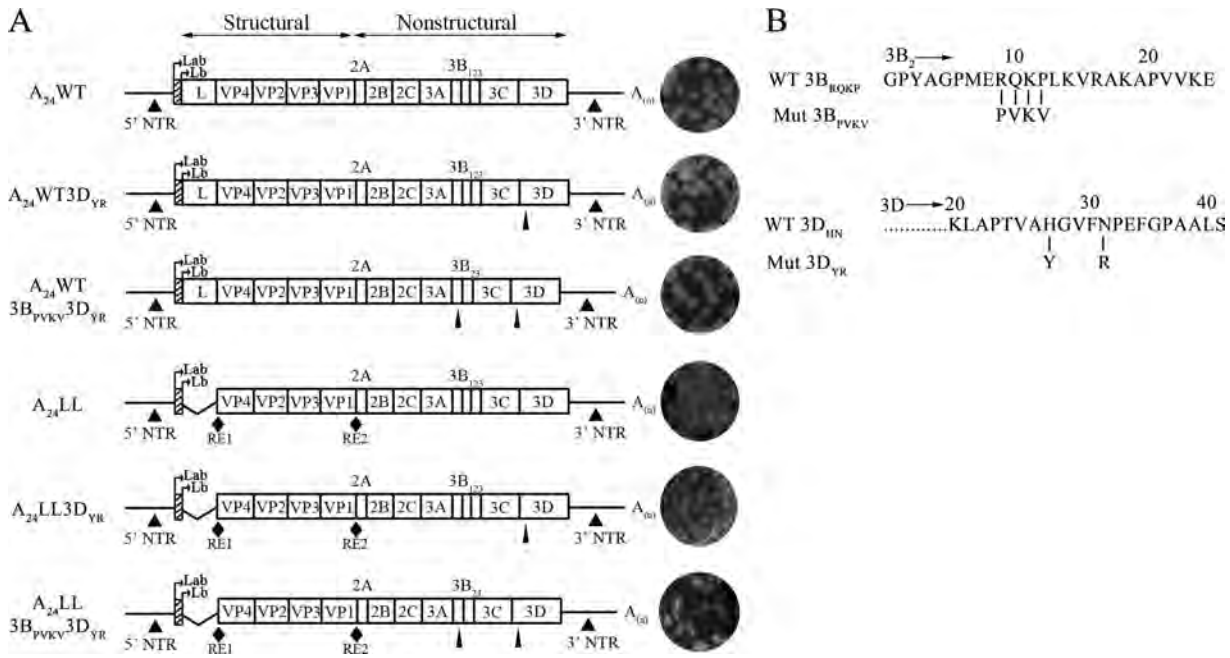

FIG 1 Schematic representation of the WT and mutant FMDV genomes and the modifications introduced in the present study. (A) A_{24}WT3D_{YR} and A_{24}LL3D_{YR} viruses were generated by site-directed mutagenesis of a full-length clone pA_{24}Cru of the FMDV outbreak strain A_{24} Cruzeiro. In addition, A_{24}WT3B_{PVKV}3D_{YR} and A_{24}LL3B_{PVKV}3D_{YR} viruses lack one of the 3B ($3B_1$) proteins and contain a substitution in $3B_2$. Additional modifications present in the mutant plasmids are indicated: Lab and Lb, deletion of the functional leader-coding region but including the two AUG codons as well as the 84-nt inter-AUG region between Lab and Lb; $3B_{23}$, contains only two copies of 3B; and two unique restriction endonuclease enzyme cloning sites 1 and 2 (◆; RE1 and RE2). The plaque phenotypes of A_{24}WT and mutant FMDVs on BHK-21 monolayers are also shown. (B) $3B_2$ contains a substitution at $RQKP_{9-12}$ with PVKV, while $3D^{pol}$ contains two amino acid substitutions at positions H_{27}Y and N_{31}R.

Assessment of attenuation of live recombinant A_{24}LL3D_{YR} virus in pigs. An experiment protocol for swine challenge model was developed by Pacheco et al. (34). In that and subsequent experiments, naive pigs were inoculated intradermally in the heel bulbs with a range of 10^5 to 10^7 $TCID_{50}$ of live A_{24}WT. These animals developed disease with fever and lesions starting at 2 dpi (16, 34; unpublished data). In addition, these directly inoculated pigs were able to transmit the virus to naive pigs by direct contact, all of which succumbed to disease. Therefore, we wanted to determine the virulence of the recombinant A_{24}LL3D_{YR} in pigs. For this, 10^5 $TCID_{50}$ (similar to the experiment used above) of live A_{24}LL3D_{YR} virus was used to inoculate the heel bulbs of two animals (Table 1, animals 40 and 41), and two naive animals (animals 43 and 44) were moved to the room at 24 hpi and housed together for 20 days. Among the directly inoculated pigs, only one (animal 41) showed detectable viral RNA in serum ($10^{5.44}$ viral RNA copies/ml) at 1 dpi and $10^{5.45}$ viral RNA copies/ml in oral swabs at 2 dpi. This animal also developed low serum neutralizing antibodies titers starting at 5 dpi, but in the absence of any clinical manifestation of FMD. Interestingly, no virus transmission occurred from this pig to the second directly inoculated pig or to the two in-contact animals (animals 43 and 44). None of the pigs were ever pirexic (temperatures remained below 40°C) during the course of this experiment. No virus was detected in the air samples collected in this room during 21 days (results not shown).

Assessment of attenuation of live single- and double-negative marker viruses in cattle. To examine the influence of mutations on pathogenicity and the serological response to live recombinant A_{24}WT3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, A_{24}LL3D_{YR}, and A_{24}LL3B_{PVKV}3D_{YR} compared to the parental A_{24}WT virus in cattle, we performed aerosol or intradermolingual inoculation of these viruses, followed by measurement of clinical disease and virus shedding to the environment. After inoculation, several parameters, including fever, clinical score, viremia, neutralizing antibodies, and the presence of virus in air and oral swabs samples were recorded and analyzed (see Materials and Methods and Table 2).

Two animals (bovines 7109 and 7110) that received aerosol inoculation with 10^7 $TCID_{50}$ of live A_{24}WT showed viremia, virus in saliva, and fever by 2 dpi. Clinical signs appeared by 2 to 4 dpi and reached a high clinical score by 5 to 7 dpi when neutralizing antibodies were first detectable. Virus was also detected in air samples when collected, but only from animal 7109. For the negative single marker viruses, six steers housed in separate rooms were aerosol inoculated with approximately 1×10^6 to 3×10^6 $TCID_{50}$ of either live A_{24}LL3D_{YR} or A_{24}WT3D_{YR} virus. Bovines 7199, 1, and 2 inoculated with live A_{24}WT3D_{YR} virus showed viremia by 2 dpi and reached a peak at 3 dpi. Virus was detectable in saliva starting at 2 to 3 dpi and peaking at 4 to 6 dpi. Fever appeared at 2 or 6 dpi and lasted up to 3 or 8 dpi. Clinical signs appeared by 4 to 6 dpi, and virus was detected in air samples when collected (only from bovines 1 and 2). Virus was shed in the air starting by 3 dpi and peaked at 4 to 7 dpi. Serum neutralizing antibodies were first detectable by 5 to 6 dpi in all three cows (Table 2). Vesicular fluid was also collected from lesions on 4 and 6 dpi (bovines 1, 2, and 7199), and each sample was separately processed for rRT-PCR and sequencing. These fluids contained viruses that were indistinguishable from the inoculated virus in their genome sequences,

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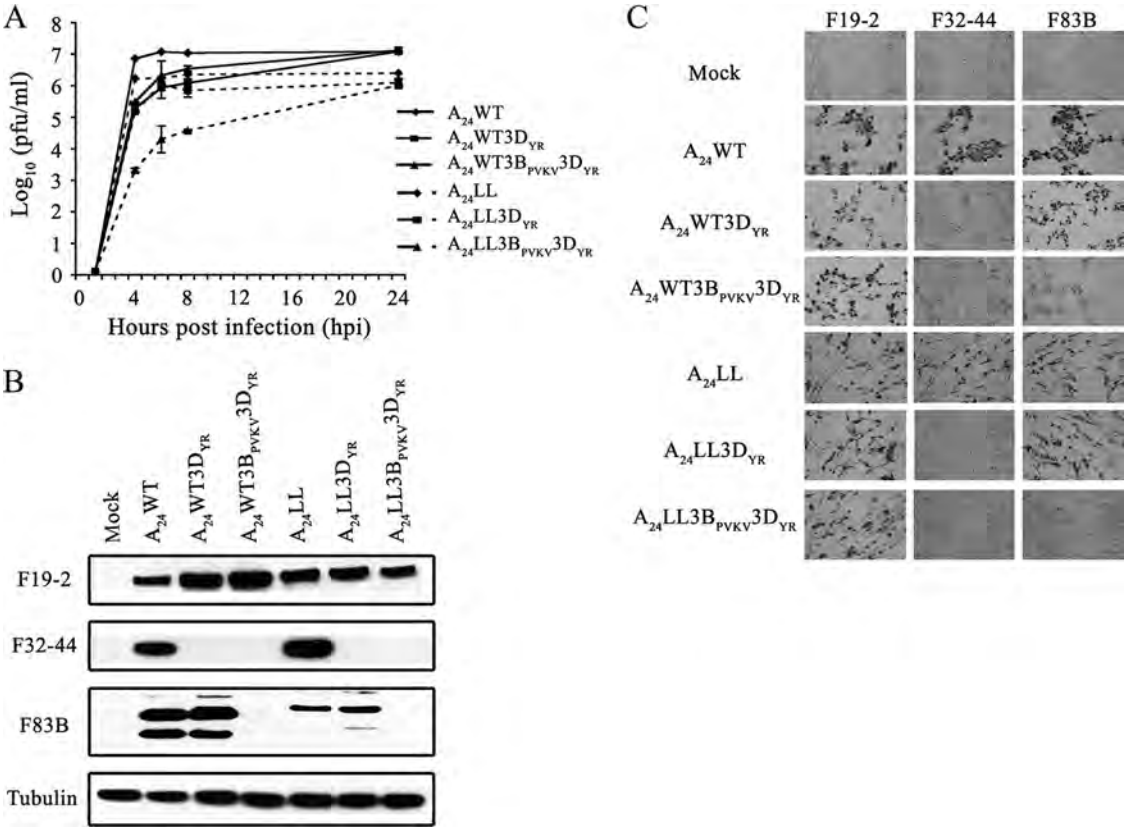

FIG 2 *In vitro* characterization of marker vaccine candidates. (A) FMDVs. Monolayers were mock infected or infected with A_{24}WT, A_{24}WT3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, A_{24}LL, A_{24}LL3D_{YR}, or A_{24}LL3B_{PVKV}3D_{YR} at an MOI of 5 PFU/cell. (B) Analysis of the marker epitope expression of mutant FMD viruses by Western blotting. Cells were mock infected or infected with A_{24}WT, A_{24}WT3D_{YR}, A_{24}LL, A_{24}LL3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, or A_{24}LL3B_{PVKV}3D_{YR} at an MOI of 5. The following day, the cell lysates were collected and run under denaturing conditions using a SDS–12% PAGE. The nitrocellulose blots were probed with MAbs F83B for FMDV 3B protein and F19-6 and F32-44 for FMDV $3D^{pol}$ protein. (C) Analysis of the marker epitope expression of mutant FMD viruses by immunohistochemistry. Cells were mock infected or infected with A_{24}WT, A_{24}WT3D_{YR}, A_{24}LL, A_{24}LL3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, or A_{24}LL3B_{PVKV}3D_{YR} at an MOI of 5. The following day, the cells were fixed and processed for immunohistochemistry using MAbs specific for 3B (F83B) and $3D^{pol}$ proteins (F19-2 and F32-44).

further indicating that the A_{24}WT3D_{YR} virus had not changed during growth in bovines. In clear contrast to the pathogenic profiles of A_{24}WT and A_{24}WT3D_{YR} viruses, the three animals that received aerosol inoculation with live A_{24}LL3D_{YR} (bovines 7201, 3, and 4; Table 2) showed absence of fever, viremia, clinical manifestation, or shedding of virus in saliva or air samples. The level of attenuation was such that these animals did not develop significant levels of neutralizing antibodies during the course of the experiment (Table 2), even though antibodies against viral structural proteins were demonstrated by 21 dpi by radioimmunoprecipitation assays (data not shown).

In order to determine the virulence profiles of double-negative marker viruses, groups of two steers were inoculated with either live A_{24}WT3B_{PVKV}3D_{YR} or the A_{24}LL3B_{PVKV}3D_{YR} virus. Bovines

TABLE 1 Response of swine inoculation with live FMDV A_{24}LL3D_{YR}

Pig	Route of exposure[a]	Maximum titer (dpi)[b]			Clinical score[c]	Neutralization titer maximum[d] (starting dpi)
		Viremia	Virus in saliva	Virus in nasal passages		
40	Direct	Negative[e]	Negative	Negative	0/5	<0.9
41	Direct	5.44 (1)	5.45 (2)	Negative	0/5	1.2 (5)
42	Contact	Negative	Negative	Negative	0/5	<0.9
43	Contact	Negative	Negative	Negative	0/5	<0.9

[a] Direct, 10^5 $TCID_{50}$ of FMDV A_{24}LL3D_{YR}/pig.
[b] Values are expressed as the $\log_{10}$ RNA copies/ml. dpi indicates the day(s) postinoculation that the virus peak was detected.
[c] Animals were monitored daily for 9 days and once a week thereafter.
[d] That is, the FMDV-specific neutralizing antibody titer ($\log_{10}$ of the reciprocal of the last serum dilution to neutralize 100 $TCID_{50}$ of virus in 50% of the wells). Sensitivity, >0.9. The starting dpi refers to the first day after inoculation that neutralizing antibodies were detected.
[e] That is, an RNA copy number minimum detection level of $10^{2.69}$/ml.

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TABLE 2 Responses of cattle directly inoculated with live A_{24}WT, A_{24}WT3D_{YR}, A_{24}LL3D_{YR}, A_{24}WT3B_{PVKV}3D_{YR}, or A_{24}LL3B_{PVKV}3D_{YR} virus

Bovine[a]	Virus	Maximum titer (dpi)[b]		Fever[c] (dpi)	Maximum clinical score/maximum achievable[d] (dpi)	Neutralization titer maximum[e] (starting dpi)	Shedding in air, maximum titer[f] (dpi)
		Viremia	Virus in saliva				
7109	A_{24}WT	7.60 (3)	8.90 (3)	Yes (2 and 3)	5/5 (7)	2.4 (5)	5.57 (5)
7110	A_{24}WT	7.31 (4)	10.18 (2)	Yes (2 to 5)	5/5 (5)	2.4 (6)	ND
7199	A_{24}WT3D_{YR}	6.73 (3)	9.58 (6)	Yes (6 and 8)	1/5 (6)	2.7 (6)	ND
1	A_{24}WT3D_{YR}	7.55 (3)	8.00 (4)	Yes (2 and 3)	5/5 (6)	3.0 (5)	3.66 (7)
2	A_{24}WT3D_{YR}	7.95 (3)	9.47 (4)	Yes (2 and 3)	2/5 (7)	3.0 (5)	2.55 (4)
7201	A_{24}LL3D_{YR}	Negative[g]	Negative	No	0/5	<0.9	Negative[h]
3	A_{24}LL3D_{YR}	Negative	Negative	No	0/5	<0.9	Negative
4	A_{24}LL3D_{YR}	Negative	Negative	No	0/5	<0.9	Negative
9143	A_{24}WT3B_{PVKV}3D_{YR}	7.40 (4)	9.03 (5)	Yes (3 to 5)	1/5 (5)	3.6 (6)	6.29 (6)
9144	A_{24}WT3B_{PVKV}3D_{YR}	7.92 (4)	8.85 (5)	Yes (3 to 6)	4/5 (7)	3.0 (6)	5.45 (7)
9145	A_{24}LL3B_{PVKV}3D_{YR}	Negative	Negative	No	0/5	1.5 (9)	ND
9146	A_{24}LL3B_{PVKV}3D_{YR}	Negative	Negative	No	0/5	2.4 (9)	Negative

[a] Cattle were inoculated with 1×10^7 $TCID_{50}$ of A_{24}WT, 1×10^6 to 3×10^6 $TCID_{50}$ of A_{24}WT3D_{YR} and A_{24}LL3D_{YR}, or 7×10^6 $TCID_{50}$ of A_{24}WT3B_{PVKV}3D_{YR} virus by aerosolization or with 10^6 $TCID_{50}$ of A_{24}LL3B_{PVKV}3D_{YR}virus by the intradermolingual route.
[b] Values are expressed as the $\log_{10}$ RNA copies/ml. dpi here indicates the day(s) postinoculation that the virus peak was detected.
[c] Fever defined as rectal temperature ≥ 40.0°C. dpi here indicates the days when fever was detected.
[d] Clinical scores were based on the number of feet with vesicular lesions and lesions in the head (mouth, nostrils, lips, or tongue), with a maximum of 5. dpi here indicates the day after inoculation that the maximum score was reached.
[e] That is, the FMDV-specific neutralizing antibody titer ($\log_{10}$ of the reciprocal of the last serum dilution to neutralize 100 $TCID_{50}$ of virus in 50% of the wells). Sensitivity, >0.9. dpi here indicates the first day after inoculation that neutralizing antibodies were detected.
[f] Values are expressed as the $\log_{10}$ RNA copy/1,000 liters. ND, not determined. dpi here indicates the day(s) postinoculation that the virus peak was detected.
[g] The RNA copy number minimum detection level was $10^{2.69}$/ml.
[h] The RNA copy number minimum detection level was $10^{0.8}$/1,000 liters.

9143 and 9144 that received aerosol inoculation with 7×10^6 $TCID_{50}$ of live A_{24}WT3B_{PVKV}3D_{YR} showed a pathogenic profile similar to A_{24}WT and A_{24}WT3D_{YR} viruses. These animals showed viremia by 2 dpi and reached a peak at 4 dpi. Likewise, virus was detected in saliva starting at 2 to 3 dpi and peaking at 5 dpi. Fever was detected by 3 dpi and lasted up to 6 dpi, while clinical signs appeared by 4 to 5 dpi. Virus shedding in the air was detected by 6 to 7 dpi, and serum neutralizing antibodies appeared by 6 dpi in both cows. On the other hand, the lack of virulence of A_{24}LL3B_{PVKV}3D_{YR} was similar to that of A_{24}LL3D_{YR}. Two cows, animals 9145 and 9146, were inoculated intradermolingually with 10^6 $TCID_{50}$ of the live virus, and the results shown in Table 2 demonstrate that this virus is attenuated with lack of fever, disease, viremia, and viral shedding in air and saliva. However, unlike bovines infected with A_{24}LL3D_{YR}, these animals were able to develop neutralizing antibodies starting at 9 dpi.

Efficacy of chemically inactivated antigen vaccines prepared with single- or double-negative marker viruses in cattle. To determine the efficacy of inactivated antigen vaccines prepared using attenuated single or double marker viruses in providing immunological protection against challenge with parental A_{24}WT virus, BEI-inactivated A_{24}LL3D_{YR} or A_{24}LL3B_{PVKV}3D_{YR} vaccines were tested in cattle and compared to a commercial FMDV vaccine. As shown in Table 3, four cattle (animals 863 to 866) were each inoculated intramuscularly with one dose of a commercial polyvalent vaccine, while groups of four steers, animals 867 to 870 and animals 1018 to 1021, received the A_{24}LL3D_{YR} and A_{24}LL3B_{PVKV}3D_{YR} BEI-inactivated vaccine, respectively. Steers 871, 872, 1022, and 1023 were mock vaccinated with PBS. All A_{24}LL3D_{YR}- and A_{24}LL3B_{PVKV}3D_{YR}-vaccinated animals except one (animal 1020) developed a detectable FMDV-specific neutralizing antibody response by 7 days postvaccination (dpv). Bovine 1020 had no detectable neutralizing antibodies at 7 dpi, but they appeared by 14 dpi. Vaccination with A_{24}LL3D_{YR} or A_{24}LL3B_{PVKV}3D_{YR} yielded slightly higher neutralizing titers compared to vaccination with the commercial vaccine, which is more likely due to a high content of antigen mass (approximately 15 μg/dose of A_{24}LL3D_{YR} or A_{24}LL3B_{PVKV}3D_{YR}). By day 21, all animals but one (bovine 864) had increased titers of serum neutralizing antibodies. In contrast, the unvaccinated animals (bovines 871, 872, 1022, and 1023) that received PBS had no detectable FMDV-specific antibody response (Table 3). Furthermore, the 12 immunized cattle, regardless of the vaccine, did not show any FMD clinical signs (data not shown).

At 21 dpv, all animals were challenged by intradermal inoculation at four sites in the tongue with 10^4 bovine infectious doses ($BTID_{50}$) of parental FMDV A_{24}WT. All control animals developed fever within 1 to 3 dpc, followed by typical FMD lesions on all four feet. In contrast, none of the vaccinated animals showed clinical signs of FMD during the course of the experiment and were fully protected (up to 10 dpc, Table 3). Only one animal vaccinated with the commercial vaccine showed fever at 3 dpc, but it did not succumb to FMDV. Unfortunately, cattle 866 and 1020 were euthanized due to illnesses unrelated to FMDV. All unvaccinated animals developed viremia from 1 to 5 dpc. In contrast, no virus was detected in the sera of any of the vaccinated animals (data not shown).

Antibody responses against 3B and $3D^{pol}$ in animals inoculated with negative marker viruses determined using competitive ELISA. Preimmune (0 dpv) or convalescent-phase (9 to 21 dpv) sera collected from animals inoculated with live A_{24}WT by the intradermolingual route or infected by aerosol exposure to live A_{24}WT or A_{24}WT3D_{YR} were assayed for reactivity to FMDV $3D^{pol}$ protein in cELISA (see Materials and Methods). The assay (Fig. 3A) allows the distinction of the serological responses of cattle inoculated with either parental (A_{24}WT) from those inoculated with mutant (A_{24}WT3D_{YR}) virus. Although seroconversion after

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TABLE 3 Specific neutralizing antibody response and protection against challenge with the FMDV A_{24}WT strain after vaccination with commercial polyvalent, $A_{24}LL3D_{YR}$, or $A_{24}LL3B_{PVKV}3D_{YR}$ chemically inactivated antigen vaccine

Treatment	Bovine	Neutralization titer (dpv)[a]							Protection[b]
		0	7	14	21	28	35	42	
Commercial vaccine	863	<0.9	0.9	1.2	1.5	3.9	4.2	3.9	Yes
	864	<0.9	1.2	0.9	<0.9	3.6	3.6	3.6	Yes
	865	<0.9	0.9	0.9	1.8	3.9	3.6	3.9	Yes
	866	<0.9	1.2	1.5	2.1	ND[c]	ND	ND	ND
$A_{24}LL3D_{YR}$ vaccine	867	<0.9	2.1	2.4	2.7	3.3	3.3	3.0	Yes
	868	<0.9	2.4	2.1	2.7	3.3	3.6	3.9	Yes
	869	<0.9	2.1	2.4	2.7	3.0	2.7	3.0	Yes
	870	<0.9	2.1	1.8	2.4	3.0	3.3	3.0	Yes
$A_{24}LL\ 3B_{PVKV}3D_{YR}$ vaccine	1018	<0.9	1.8	2.1	2.1	3.0	3.3	3.0	Yes
	1019	<0.9	2.1	2.1	2.1	3.3	3.6	3.0	Yes
	1020	<0.9	<0.9	2.1	1.8	2.7	ND	ND	ND
	1021	<0.9	1.5	1.8	2.1	2.1	2.7	3.6	Yes
PBS (controls)	871	<0.9	<0.9	<0.9	<0.9	2.1	3.0	3.6	NO
	872	<0.9	<0.9	<0.9	<0.9	2.7	3.3	3.6	NO
	1022	<0.9	<0.9	<0.9	<0.9	2.4	3.3	2.7	NO
	1023	<0.9	<0.9	<0.9	<0.9	2.4	2.7	2.1	NO

[a] dpv, day postvaccination. Day 0 = day of vaccination; day 21 = day of challenge. Virus neutralizing titers of serum antibodies responses ($\log_{10}$ of the reciprocal of the last serum dilution to neutralize 100 $TCID_{50}$ of virus in 50% of the wells) are shown. Sensitivity, >0.9.
[b] Cattle were challenged by intradermolingual inoculation of 4 $\log_{10}$ bovine infectious doses of FMDV A_{24}WT.
[c] ND, not determined (the animal was euthanized due to an illness unrelated to FMDV).

inoculation with A_{24}WT resulted in significant inhibition of the anti-$3D^{pol}$ response in cELISA, sera from animals inoculated with $A_{24}WT3D_{YR}$ showed little inhibition, demonstrating that the YR mutation effectively removes reactivity with MAb F32-44. Likewise, sera from animals inoculated with $A_{24}WT3B_{PVKV}3D_{YR}$ via an aerosol route also showed a decreased antibody inhibition compared to sera collected from A_{24}WT-infected animals (Fig. 3B). Therefore, cELISA using MAb F32-44 allows for differentiation of animals infected with A_{24}WT from those inoculated with the negative marker viruses. In addition, antibody responses to FMDV 3B epitopes were measured by cELISA developed using MAb F83B and a 3B peptide (see Materials and Methods). The results demonstrate that convalescent-phase sera from animals infected with $A_{24}WT3B_{PVKV}3D_{YR}$ (bovines 9143 and 9144) were unable to compete with MAb F83B, unlike sera obtained from A_{24}WT-infected animals (Fig. 3C). Finally, sera collected from convalescent animals inoculated with the marker $A_{24}LL3B_{PVKV}3D_{YR}$ virus by the intradermolingual route were analyzed against both FMDV $3D^{pol}$ protein and 3B peptide in cELISAs, but no antibodies against nonstructural $3D^{pol}$ and 3B were detected (data not shown).

DISCUSSION

Currently, FMD vaccines are produced in expensive biological containment facilities where cell culture-adapted, live virulent FMDVs are grown in large volumes. This process has resulted in

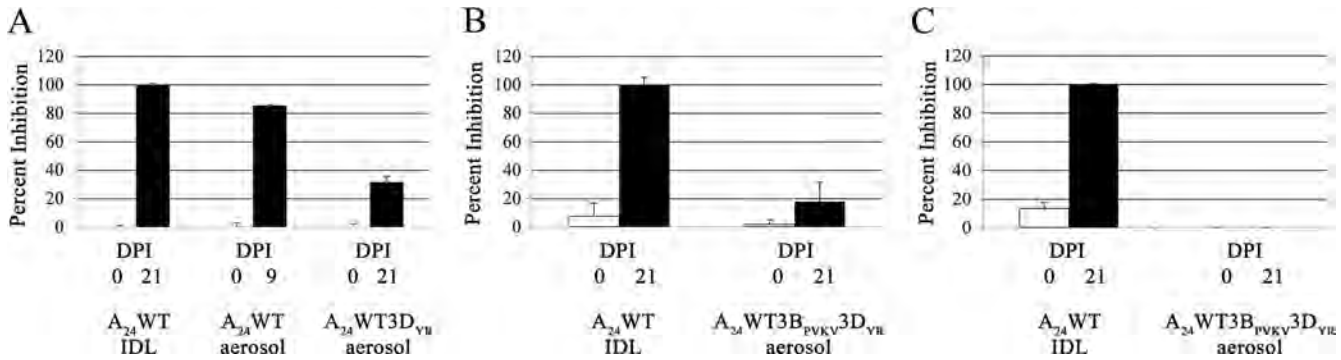

FIG 3 cELISA. (A) Differential antibody response in animals infected with live A_{24}WT and $A_{24}WT3D_{YR}$ using MAb F32-44 raised against $3D^{pol}$. (B) Differential antibody response in animals infected with live A_{24}WT and $A_{24}WT3B_{PVKV}3D_{YR}$ using MAb F32-44 raised against $3D^{pol}$. (C) Differential antibody response in animals infected with live A_{24}WT and $A_{24}WT3B_{PVKV}3D_{YR}$ using MAb F83B raised against 3B. For each group, the average ± the standard deviation of two to three cows infected with either virus is shown. Samples were collected before inoculation and at day 21. DPI, days postinoculation; IDL, intradermolingual. Neutralization titers against A_{24}Cru for all samples used in the cELISA were determined to be about ≥2.1 (see Tables 2 and 3). No significant difference in the day 9 and day 21 neutralizing titers against A_{24}Cru were observed.

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the escape of virulent virus from manufacturing facilities, causing costly outbreaks in livestock (11, 21). After growth, the live virus is then inactivated using chemicals, and antigen concentrates are prepared by purification steps required to remove contaminant proteins that make it difficult to differentiate infected from vaccinated animals (DIVA) through serological diagnostic tests. Since there is little to no cross-protection across serotypes and subtypes, appropriate matching between vaccine and circulating field strains is required to achieve protection. Despite these limitations, billions of vaccine doses are manufactured every year around the world. Their use has been the basis for eradicating FMDV from Europe and controlling the associated disease in many parts of the world through mass vaccination campaigns. Thus, there is an urgent need for the development of effective marker FMD vaccine candidates with improved DIVA capabilities.

We present here a rational approach to design novel negative marker FMD vaccine viruses for use in conjunction with a companion serological test. The vaccine candidate viruses, $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$, harbor negative antigenic markers for potential DIVA capabilities which are encoded in either the $3D^{pol}$ alone or both in 3B and $3D^{pol}$, respectively. An additional modification of the vaccine candidates included the deletion of the nonessential L^{pro} and one of three 3B coding sequence that rendered these leaderless viruses markedly attenuated in both cattle and swine. Our data also showed that the leaderless marker viruses were not transmissible between cattle or swine. These results are consistent with previous studies on $A_{12}LLV2$ FMD virus showing that deletion of the FMDV L^{pro} coding sequence produces viruses that maintained the ability to infect BHK-21 cells but displayed low virulence for cattle or pigs (1, 5, 7, 26). The vaccine platform also includes strategically located restriction enzyme sites that allow easy exchange of the capsid region of any virus serotype and subtype into the vaccine backbone. This feature allows rapid development of new vaccines in response to emerging FMDV antigenic variants.

Marker mutations introduced on the vaccine viruses were based on similar sequences found in BRV-2, the closest relative of FMDV. Comparison of the $3D^{pol}$ sequences of FMDV and BRV-2 viruses revealed 64% identity (20) at the amino acid level. Amino acids 16 to 32 comprise an important antigenic site in the FMDV $3D^{pol}$ protein (49), and this peptide is 76% identical between these two closely related viruses. MAbs F32-44 and F83B targeted against native FMDV $3D^{pol}$ and 3B protein, respectively, showed high reactivity against $A_{24}WT$ protein but did not react with BRV-2. These MAbs also failed to react with the mutants, $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$, suggesting that these antigenic markers and MAbs are of significant value for the development of companion DIVA diagnostic tests for the vaccine platform.

The two mutant viruses, $A_{24}WT3D_{YR}$ and $A_{24}WT3B_{PVKV}3D_{YR}$, carrying the leader coding sequence but lacking the $3D^{pol}$ epitope did not react with MAb F32-44. Moreover, the additional deletion of 3B epitope in the double-mutant $A_{24}WT3B_{PVKV}3D_{YR}$ failed to react with MAb F83B. Regardless of these mutations in the NSP, these marker viruses containing the leader proteinase showed similar tissue culture phenotypes as the parental $A_{24}WT$ virus. Furthermore, mutant viruses containing leader were virulent in the natural hosts, demonstrating that the mutations introduced in 3B and $3D^{pol}$ do not significantly attenuate the virus and that deletion of leader is critical to abrogate viral virulence. It is notable that the 3B and $3D^{pol}$ mutations were stably maintained in tissue culture (unchanged even after 15 serial passages in BHK-21) and also during replication in animals.

Attenuation of FMDV virulence is a critical aspect to be addressed in developing a safe vaccine production platform particularly for regions that are FMD free. Given that cattle infected with live $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$ by the aerosol or the intradermolingual route, and pigs inoculated intradermally in the heel bulb of the foot with live $A_{24}LL3D_{YR}$ showed no signs of FMD and no transmission to naive animals in direct contact, an outbreak in animals due to escape or through incomplete inactivation of these marker vaccine viruses from the manufacturing is highly unlikely. Also notable is that infection with the live leaderless marker viruses resulted in low or no antibody responses to FMDVs, indicating that very little or no replication of these viruses occurred. Therefore, in order to test the serological profile induced by the markers in 3B and $3D^{pol}$, we used live single- and double-marker FMDVs with the leader containing backbone ($A_{24}WT3D_{YR}$ and $A_{24}WT3B_{PVKV}3D_{YR}$, respectively). Convalescent-phase sera from animals infected with these WT marker viruses had a serological profile that was easily distinguished by cELISA from that observed in WT-infected animals. The presence of a $3D^{pol}$ marker in the vaccine candidate is particularly important since the FMDV $3D^{pol}$ protein is known to stimulate a strong humoral response that is detectable early after infection and therefore is valuable in assessing infectious status during control of an outbreak (9, 12). In the present study, we utilized a modified cELISA (49) to capture the $3D^{pol}$ antigen to the solid phase, and the ability of test sera to inhibit the binding of the MAb F32-44 to the antigen was evaluated. Likewise, a similar in-house cELISA based on MAb F83B (targeted against 3B protein) was used to detect serological responses to the epitope contained in the 3B viral protein. Therefore, the presence of these 3B and $3D^{pol}$ epitopes in the parental virus but the lack of them in the mutant $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$ viruses provides a potential DIVA companion test to be used in conjunction with these marker FMD vaccine candidates.

Killed FMD vaccines are currently commercially available and have been shown to be safe and effective for the control of FMD. The killed-virus vaccine is prepared from virus grown in BHK-21 cells, chemically inactivated using BEI and formulated with adjuvants. In this report, we have demonstrated that both BEI-inactivated vaccine candidates, $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$, as well as a commercial polyvalent FMD vaccine (with a standard antigen payload), protected cattle from direct challenge after only one vaccine dose. The analysis of the humoral responses against FMDV revealed that the three vaccine formulations were able to induce levels of neutralizing antibodies predictive of protection before challenge. However, the higher performance by the experimental vaccines in the induction of neutralizing antibodies was most likely due to higher amounts of antigens present in the experimental vaccines. We did not detect a significant antibody response against NSPs after a single-dose vaccination with either $A_{24}LL3D_{YR}$ or $A_{24}LL3B_{PVKV}3D_{YR}$ (data not shown). On the other hand, animals infected with the live $A_{24}WT3D_{YR}$ or $A_{24}WT3B_{PVKV}3D_{YR}$ virus developed antibody responses to NSPs that were unable to compete with the 3B and $3D^{pol}$ MAbs targeted against the marker epitopes. This demonstrates the robustness of the DIVA markers built into the vaccine platform (Fig. 3).

In control programs, particularly in countries where FMD is endemic, multiple doses of inactivated FMDV vaccines are ap-

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plied for the control and prevention of FMD, and antibodies against $3D^{pol}$ are commonly detected in animals vaccinated multiple times (44). Our results suggest that vaccines prepared using our platform would consistently allow for easy differentiation of vaccinated from infected animals, even after multiple vaccinations. Furthermore, the absence of the two epitopes in 3B and $3D^{pol}$ would simplify the downstream processing during vaccine production, making it unnecessary to remove NSPs from the vaccine antigens. This will not only decrease production cost but could also potentially increase the potency of the vaccine since it is often hypothesized that presence of NSPs is desirable for the induction of a stronger and potentially cross-reactive immune response to FMD. Nonstructural proteins of FMDV are much more conserved across all clades (see the introduction). Some additional desirable characteristics of $A_{24}LL3D_{YR}$ and $A_{24}LL3B_{PVKV}3D_{YR}$ marker vaccine candidates include the fact that they can be differentiated from field FMDV strains by genetic (e.g., PCR targeting the leader region) and immunological methods targeting the two antigenic markers.

Considering the high economic damage that FMD can elicit on livestock (47), current vaccination programs are now supporting the "vaccinate-to-live" policy for FMD outbreaks. In this scenario, the $A_{24}LL3D_{YR}$, and $A_{24}LL3B_{PVKV}3D_{YR}$ vaccine candidates have built-in capabilities (negative markers) to differentiate between vaccinated and WT-infected animals. As a result, these vaccines can be used in conjunction with a companion DIVA test (cELISA) that could assist in FMD control measurements and support the differentiation of infected versus vaccinated animals.

Taken together, the marker viruses developed here provide a viable alternative to using antigen produced from virulent virus for vaccine, allowing for safe production of FMD vaccines using existing production facilities with little or no modification. This innovative vaccine platform allows for the easy exchange sequences encoding the capsid proteins that can be utilized to rapidly derive contemporary outbreak strains of other subtypes and serotypes. The safety features of these marker vaccine viruses open the door for the interesting possibility of vaccine production in regions that do not currently allow the use of live FMDV for vaccine production.

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FULL MANUSCRIPT

Novel Antiviral Therapeutics to Control Foot-and-Mouth Disease

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Foot-and-mouth disease virus (FMDV) causes a highly contagious disease of cloven-hoofed animals. Vaccines require ~7 days to induce protection; thus, before this time, vaccinated animals are still susceptible to the disease. Our group has previously shown that swine inoculated with 1×10^{11} focus forming units (FFU) of a replication-defective human adenovirus containing the gene for porcine interferon alpha (Adt-pIFN-α) are sterilely protected from FMDV serotypes A24, O1 Manisa, or Asia 1 when the animals are challenged 1 day postadministration, and protection can last for 3–5 days. Polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine and carboxymethyl cellulose (poly ICLC) is a synthetic double-stranded RNA that is a viral mimic and activates multiple innate immune pathways through interaction with toll-like receptor 3 and MDA-5. It is a potent inducer of IFNs. In this study, we initially examined the effect of poly IC and IFN-α on FMDV replication and gene induction in cell culture. Poly ICLC alone or combined with Adt-pIFN-α was then evaluated for its therapeutic efficacy in swine against intradermal challenge with FMDV A24, 1 day post-treatment. Groups of swine were subcutaneously inoculated either with poly ICLC alone (4 or 8 mg) or in combination with different doses of Adt-pIFN-α (2.5×10^9, 1×10^9, or 2.5×10^8 FFU). While different degrees of protection were achieved in all the treated animals, a dose of 8 mg of poly ICLC alone or combined with 1×10^9 FFU of Adt-pIFN-α was sufficient to sterilely protect swine when challenged 24 h later with FMDV A24. IFN-stimulated gene (ISG) expression in peripheral blood mononuclear cells at 1 day post-treatment was broader and higher in protected animals than in nonprotected animals. These data indicate that poly ICLC is a potent stimulator of IFN and ISGs in swine and at an adequate dose is sufficient to induce complete protection against FMD.

Introduction

Foot-and-mouth disease (FMD) is a severe, highly contagious disease of cloven-hoofed animals that is caused by a member of the *Aphthovirus* genus of the Picornaviridae family. The disease has a significant economic impact on affected countries. Currently, FMD is controlled by restriction of animal movement, slaughter of infected and in-contact susceptible animals, and in enzootic countries vaccination with an inactivated whole virus vaccine (Grubman and Baxt 2004). However, administration of this vaccine or an experimental vaccine based on a replication-defective human adenovirus (Ad5) vector that delivers the FMD virus (FMDV) capsid and 3C proteins requires ~7 days to induce protective immunity in animals (Moraes and others 2003; Golde and others 2005; Pacheco and others 2005). Since FMDV replicates and spreads rapidly, we have focused on developing strategies that induce the innate immune response as a mechanism to initiate protection before the development of vaccine-induced adaptive immunity. This approach can also aid in the generation of a more robust and long-lasting adaptive immune response (Le Bon and others 2001; Cull and others 2002; Proietti and others 2002; Moraes and others 2003; de Avila Botton and others 2006).

Type I interferon (IFN-α/β) is the first line of host defense against a viral infection, and it plays an important role in antiviral innate immunity (Stark and others 1998; Goodbourn and others 2000). Induction and secretion of this cytokine by virally infected cells up-regulates hundreds of IFN-stimulated genes (ISGs) and their products, resulting in potent antiviral and immunomodulatory activity (Der and others 1998; Takaoka and Yanai 2006). Previous studies by

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our group have shown that swine inoculated intramuscularly (IM) at 1 site in the hind limb with an Ad5 vector containing porcine IFN-α (Ad5-pIFN-α) are completely protected when challenged 1 day later either by intradermal (ID) inoculation or by direct contact with FMDV serotype A24 Cruzeiro (Chinsangaram and others 2003; Moraes and others 2003; Dias and others 2011). Furthermore, protection lasts for 3–5 days, and even treatment 1 day postchallenge (dpc) reduces viremia and clinical disease as compared with control animals (Moraes and others 2003). More recently, we demonstrated that Ad5-pIFN-α is also able to protect swine against challenge with a number of other FMDV serotypes, including O1 Manisa and Asia-1, and Ad5-pIFN-β protects against FMDV A24 challenge (Dias and others 2011). Moreover, we have also demonstrated that inoculation of cattle with an Ad5 vector containing a member of the bovine type III IFN family (Ad5-pIFN-λ3) can protect 1 of 3 animals and significantly delay disease onset in the other 2 animals in the group challenged by aerosol 1 day later (Perez-Martin and others 2012). Thus, treatment with IFN has proved, so far, to be the best biotherapeutic approach tested against FMDV.

A commercially viable biotherapeutic approach or vaccine to block the spread of an animal pathogen should be economically affordable. Therefore, we have attempted to enhance the potency of our Ad5-IFN approach in several ways including (1) administration IM at 4 sites in the neck reduced the protective dose 10-fold as compared with our initial protocol that involved IM inoculation in the right hind limb (Dias and others 2011), (2) subcutaneous (SC) rather than IM inoculation at 4 sites resulted in a 20-fold lower protective dose (Grubman and others 2012), and (3) initiated studies to understand the molecular mechanisms induced by IFN treatment that result in protection against FMDV challenge and found a correlation between protection and both specific ISG up-regulation and tissue-specific infiltration of dendritic cells and natural killer (NK) cells (Moraes and others 2007; Diaz-San Segundo and others 2010).

Poly ICLC is a synthetic, double-stranded polyriboinosinic-polyribocytidylic acid molecule stabilized with poly-L-lysine and carboxymethyl cellulose (poly ICLC) that has enhanced biostability in animals as compared with poly IC (Nordlund and others 1970) and is a known toll-like receptor 3 (TLR3) and MDA-5 agonist (Meylan and Tschopp 2006; Stahl-Hennig and others 2009). In rodents and primates, poly ICLC is a strong IFN-α inducer that provides antiviral and adjuvant activity (Levy and others 1976; Harrington and others 1979; Caskey and others 2011). It has been effective in providing protection in various animal models against a number of viral infections, including those caused by yellow fever, Venezuelan equine encephalomyelitis, Rift Valley fever, influenza, papilloma, and rabies viruses (Houston and others 1976; Stephen and others 1977, 1979; Baer and others 1979; Kende and others 1985, 1987; Stahl-Hennig and others 2009; Li and others 2011). Moreover, 2 intranasal (IN) doses of poly ICLC (8 and 48 h before infection) protected mice against IN challenge with a lethal dose of influenza and was found to be more efficacious than 2 IN doses of IFN-α or IFN-γ (Wong and others 1995). Likewise, the duration of protection against influenza virus provided by poly ICLC or liposome encapsulated poly ICLC in mice can persist for approximately 2 or 3 weeks post-treatment, respectively, although poly ICLC was less effective in the postexposure treatment of influenza virus infection than it was for prophylaxis (Wong and others 1995, 2007, 2009). The antiviral activity of poly ICLC is related to its ability to modulate the immune response by inducing the production of IFNs, α, β, and λ *in vitro* and *in vivo* (Levy and others 1975; Kende 1985; Lauterbach and others 2010) and by stimulating specific components of the cellular and humoral immune systems, including the activation of NK cells (Wiltrout and others 1985). By inducing pro-inflammatory cytokines, poly ICLC up- and down-regulates a broad variety of cellular genes (Geiss and others 2001; Stahl-Hennig and others 2009; Caskey and others 2011).

In the current study we examined the ability of poly ICLC alone or in combination with Ad5-pIFN-α (designated Adt-pIFN-α) to inhibit FMDV replication in cell culture and to protect swine against FMDV challenge. Initially, we found that treatment of porcine and bovine cells lines with poly IC significantly reduced FMDV replication. Interestingly, in bovine cells, the combination of poly IC and bovine IFN (bIFN)-α had a synergistic effect as compared with each treatment alone, and there was up-regulation in the expression of a number of cytokines, chemokines, transcription factors, and genes with direct antiviral activity. Based on these results, we performed 2 animal studies in which different doses of Adt-pIFN-α and poly ICLC were combined and evaluated for efficacy against ID challenge with FMDV serotype A24 Cruzeiro. Our results indicate that 8 mg of poly ICLC is able to protect swine against FMDV A24 challenge when used alone or combined with 1×10^9 focus forming units (FFU) of Adt-pIFN-α. At lower individual or combination doses, some animals were protected, while others had a delay in disease onset and reduced disease severity and viremia as compared with controls. A broader array of up-regulated ISGs was detected in the peripheral blood mononuclear cells (PBMCs) of protected animals in comparison with animals partially protected. These results suggest that the use of TLR and/or cytosolic agonists alone or in combination with IFNs may represent an effective and broad-spectrum antiviral strategy that combats FMDV infection and perhaps other viral diseases of livestock species.

Materials and Methods

Cells and viruses

Baby hamster kidney cells (BHK-21, clone 13) were used to measure FMDV titers in plaque assays, and a swine kidney cell line (IB-RS-2) was used to measure antiviral activity in plasma from inoculated animals by a plaque reduction assay (Chinsangaran and others 2001). Embryonic bovine kidney (EBK) cells, LF-BK cells, a bovine kidney cell line (Swaney 1988), and SK6 cells, a swine kidney cell line, were used to test the antiviral activity of poly IC, bIFN-α, and pIFN-α against FMDV serotype A12. EBK cells treated with different concentrations of poly IC and bIFN-α were also analyzed for up-regulation of ISGs. FMDV serotype A24 Cruzeiro was obtained from the vesicular fluid of infected swine, titered in both swine and in tissue culture, stored in aliquots at −70°C, and used for the swine challenge studies.

The replication-defective human Ad5 vector containing the pIFN-α gene, constructed as previously described (Gall and others 2007) and the control vector AdNull were obtained from GenVec, Inc. through an agreement with the Department of Homeland Security, Office of Science and Technology. The pIFN-α gene was supplied by our lab to

GenVec, Inc. for use in the construction of the Ad5 vector containing type I IFN (designated Adt-pIFN-α). All experiments were performed with the same vector lot. Poly ICLC was provided by Oncovir, Inc.

Production of IFN and antiviral activity from in vitro *samples*

IB-RS-2 cells in a T-75 flask were infected with either Ad5-pIFN-α or Ad5-bIFN-α at a multiplicity of infection (moi) of 20 for 24 h. The supernatant, containing p/bIFN-α, was removed, centrifuged at 2,500 rpm to remove cell debris, and filtered through an Amicon Ultra 100K centrifugal filter (Millipore Corp.) at 2,500 rpm for 30 min in a Sorvall centrifuge. The filtered supernatant was aliquoted and stored at −70°C. IB-RS-2 cells were treated with dilutions of p/bIFN-α for 24 h and subsequently infected with ~100 plaque forming units (PFU) of FMDV A12. Antiviral activity, U/mL, was reported as the reciprocal of the highest supernatant dilution that resulted in a 50% reduction in the number of plaques relative to the number of plaques in the mock-treated infected cells.

EBK, LF-BK, or SK6 cells were treated with various concentrations of poly IC, bIFN-α, or pIFN-α, (the IFNs derived from the supernatants of IB-RS-2 cells infected with Ad5-bIFN-α or Ad5-pIFN-α, respectively, and titrated for antiviral activity) for ~24 h, the media were removed, and the cells were washed 1× with minimal essential medium (MEM; Gibco BRL, Invitrogen) and infected at different times post-treatment with FMDV A12 at an moi of 1. At 1 hour post infection (hpi), the media were removed, cells were washed once with 2-(N-morpholino) ethanesulfonic acid buffer (MES; pH 6) to inactivate nonadsorbed virus, once with MEM, followed by incubation in MEM for ~20–24 h. Cells were frozen and thawed 1×, centrifuged, and supernatants were collected to determine virus yield in BHK-21 cells by a standard plaque assay (Chinsangaram and others 1999). Results were expressed as $\log_{10}$/mL of sample.

Animal studies

Two independent swine experiments were performed under a protocol reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Plum Island Animal Disease Center. Yorkshire pigs weighing about 35–40 lbs each were used in all experiments and acclimated for 5 days before the start of the experiments. Based on previous studies (Dias and others 2011), we selected an FMDV A24 Cruzeiro challenge dose of 10^5 $TCID_{50}$ for both experiments that is 10-fold higher than the challenge dose recommended by the World Organization of Animal Health (OIE) (OIE 2004) to be certain that the control animals develop clinical disease.

Three swine per group were inoculated SC with 2 mL Adt vector and/or poly ICLC (0.5 mL per site) in 4 sites in the neck. In the first experiment, animals were treated with either 2.5×10^9 FFU of Adt-pIFN-α or 4 mg of poly ICLC alone or in combination. For the second experiment, different dose combinations were used: 2.5×10^9 FFU or 1×10^9 FFU of Adt-pIFN-α combined with 4 mg of poly ICLC; 1×10^9 FFU or 2.5×10^8 FFU of Adt-pIFN-α combined with 8 mg of poly ICLC; and 8 mg of poly ICLC alone. Swine inoculated SC with 2.5×10^9 FFU of AdNull were used as a control for both experiments. Adt-pIFN-α and/or poly ICLC inoculated animals were housed in double-gated rooms (2 animals per room) so that they had no direct contact, while the control group (AdNull) was housed together.

All animals were challenged ID at 1 day postinoculation (dpi) with FMDV A24 Cruzeiro, using 4 sites of inoculation in the hind heel bulb, 100 μL per site. The animals were monitored daily for 10 days for clinical signs, including fever, alertness, lameness, and development of vesicles on the coronary band of the hooves, snout, and mouth. Lesion scores of the animals were determined by the number of digits (16) plus snout and mouth with vesicles (maximum score is 17).

Blood and nasal swab sampling

Blood samples were drawn from the anterior vena cava at the times indicated in each experiment. Serum was obtained from blood drawn into nonheparinized tubes and tested for viremia and neutralizing antibodies using a standard plaque reduction assay as described below. Plasma was obtained from the blood drawn into heparinized tubes at 0, 1, and 2 dpi for an analysis of antiviral activity or at 0, 1, 2, and 4 dpi for extraction of RNA from PBMCs for an analysis of the expression of several ISGs (see next). Nasal swabs were collected starting the day of challenge and for the next 7 days and tested for the presence of FMDV by titration in BHK-21 cells.

IFN biological assays

Antiviral activity was evaluated in plasma samples as previously described (Moraes and others 2003; de Avila Botton and others 2006). Briefly, samples obtained at 0–4 dpi were diluted and incubated on IB-RS-2 cells and after 24 h, supernatants were removed, and the cells were infected for 1 h with ~100 PFU of FMDV serotype A12 and then overlaid with gum tragacanth. Plaques were visualized 24 h later by staining with crystal violet. Antiviral activity (U/mL) was reported as just described.

IFN-α ELISA

ELISA was performed as previously described (Moraes and others 2003). Porcine IFN-α concentrations were expressed in picograms per milliliter and calculated by linear regression analysis of a standard curve generated with serial 2-fold dilutions of recombinant pIFN-α (PBL Biomedical Laboratories). All samples were assayed in duplicate. Serum levels of pIFN-α protein of <200 pg/mL were considered background.

Detection of FMDV RNA by real-time reverse transcription–polymerase chain reaction

One to 7 dpc frozen sera samples from animals that had no detectable clinical disease were thawed and processed for RNA extraction and real-time reverse transcription–polymerase chain reaction (rRT-PCR) as previously described (Arzt and others 2010). Samples were considered positive when Ct values were <40.

Plaque reduction neutralization (PRN_{70}) assay

Sera samples were collected at 0, 7, 14 and 21 dpc for each experiment, heated at 56°C for 30 min, and stored at −70°C. The presence of neutralizing antibodies against FMDV was determined in a plaque reduction neutralization (PRN) assay (Mason and others 1997). Neutralizing titers were reported

as the serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}).

3ABC ELISA assay

Swine sera from 0 and 21 dpc were examined for the presence of antibodies against FMDV nonstructural (NS) protein 3ABC using a PrioCHECK™ FMDV-NS ELISA kit (Prionics AG) following the manufacturer's instructions (Sorensen and others 1998).

Radioimmunoprecipitation of [^{35}S]methionine/[^{35}S]cysteine-labeled FMDV A24 infected cell lysates

In order to investigate the presence of antibodies specific to FMDV structural and NS proteins, lysates of FMDV A24 infected BHK-21 cells were incubated with convalescent serum from an FMDV infected bovine or individual swine sera samples from 0 and 21 dpc (Grubman and others 1984). After 60 min of incubation at room temperature, antibodies were precipitated with *Streptococcus aureus* protein A. Proteins were resolved by SDS-PAGE on a 15% gel and visualized by autoradiography.

Analysis of ISGs

Total RNA was isolated from EBK cells using an RNeasy isolation kit (Qiagen) following the manufacturer's directions. RNA yield and quality was determined in a NanoDrop 1000 spectrophotometer (Thermo Fisher) and in a Bioanalyzer (Agilent Technologies). A quantitative rRT-PCR (qrRT-PCR) assay was used to evaluate the mRNA levels of a number of bovine genes as previously described (de los Santos and others 2006). Glyceraldehyde-3-phosphate dehyrogenase (GAPDH) was used as the internal control to normalize the values of each sample. Primer and probe sequences used are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/jir). Reactions were performed in an ABI Prism 7000 sequence detection system (Applied Biosystems). Relative mRNA levels were determined by comparative cycle threshold analysis (user bulletin 2; Applied Biosystems) using mock-treated cells as a reference. We only considered genes up-regulated if there was a 2-fold or greater induction.

PBMCs were purified from heparinized blood and RNA extracted as previously described (Diaz-San Segundo and others 2010). A qRT-PCR was used to evaluate the mRNA levels of a number of ISGs utilizing as a reference the 0 dpi samples for each animal. The primer and probe sequences used to analyze swine gene up-regulation are listed in Supplementary Table S2.

Results

Effect of poly IC or bIFN-α on FMDV replication in cell culture

EBK cells were treated for ~24 h with various amounts of poly IC or bIFN-α alone or in combination and then infected with FMDV in the absence of the inhibitors. Virus yield was determined at 24 hpi. As shown in Table 1, increasing

Table 1. Effect of IFN-α/Poly IC Alone or in Combination on FMDV Replication in EBK or SK6 Cells

Cells	*Treatment*[a]	*FMDV titer (PFU/mL) ± SD*	*Fold-reduction*
EBK			
	MEM	$9.8\times10^6\pm2.3\times10^6$	—
	1 U bIFN-α	$2.5\times10^6\pm7.7\times10^5$	3.9
	5 U bIFN-α	$1.4\times10^6\pm4.4\times10^5$	7.0
	10 U bIFN-α	$4.4\times10^5\pm1.6\times10^5$	22.2
	100 U bIFN-α	$2.5\times10^4\pm1.3\times10^4$	392
	1.25 ng poly IC	$8.9\times10^4\pm4.1\times10^4$	110
	2.5 ng poly IC	$1.6\times10^2\pm6.4\times10^1$	61,250
	12.5 ng poly IC	$0\pm3.9\times10^1$	$>10^6$
	50 ng poly IC	$0\pm3.3\times10^1$	$>10^6$
	1 U bIFN-α + 1.25 ng poly IC	$3.2\times10^2\pm1.1\times10^2$	$>10^5$
	1 U bIFN-α + 2.5 ng poly IC	$7.8\times10^1\pm7.1\times10^1$	$>10^5$
	1 U bIFN-α + 12.5 ng poly IC	$0\pm6.9\times10^1$	$>10^6$
SK6			
	MEM	$1.8\times10^6\pm2.2\times10^5$	—
	1 U pIFN-α	$8.1\times10^4\pm2.3\times10^4$	22.2
	5 U pIFN-α	$6.0\times10^3\pm2.1\times10^3$	300
	10 U pIFN-α	$4.6\times10^3\pm3.4\times10^2$	391
	100 U pIFN-α	$7.4\times10^3\pm2.1\times10^3$	243
	1.25 ng poly IC	$8.3\times10^3\pm2.2\times10^2$	217
	2.5 ng poly IC	$3.6\times10^3\pm5.4\times10^2$	500
	12.5 ng poly IC	$5.0\times10^3\pm1.5\times10^2$	360
	25 ng poly IC	$3.8\times10^3\pm2.3\times10^2$	474
	50 ng poly IC	$2.5\times10^3\pm2.3\times10^2$	720
	1 U pIFN-α + 1.25 ng poly IC	$7.8\times10^3\pm4.9\times10^2$	231
	1 U pIFN-α + 2.5 ng poly IC	$6.4\times10^3\pm8.4\times10^2$	281
	1 U pIFN-α + 12.5 ng poly IC	$6.2\times10^3\pm4.0\times10^2$	290

[a]Each reagent or combination was diluted in 1 mL MEM and added to 1×10^6 cells.

SD, standard deviation; FMDV, foot-and-mouth disease virus; EBK, embryonic bovine kidney; MEM, minimal essential medium; PFU, plaque forming units; bIFN, bovine interferon.

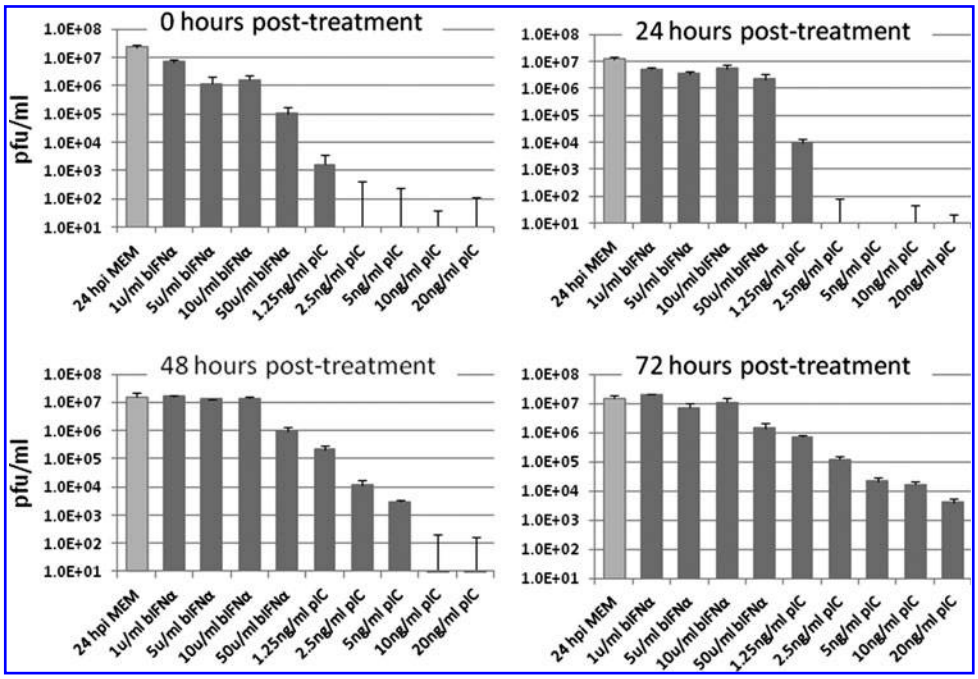

FIG. 1. Duration of protection of cells treated with poly IC (pIC) or bovine interferon (bIFN)-α. Embryonic bovine kidney (EBK) cells were treated overnight with various concentrations of bIFN-α, poly IC, or untreated [24 hours post infection (hpi) minimal essential medium (MEM), *light gray bars*]. The media were removed, and cells were either immediately infected at a multiplicity of infection (moi) of 1 with foot-and-mouth disease virus (FMDV) A12 for 1 h (0 h post-treatment) or incubated with MEM for 24, 48, or 72 h before infection with FMDV. After the 1 h infection, the media were removed, cells were washed with (N-morpholino) ethanesulfonic acid buffer (MES), pH6 buffer, and incubated overnight with MEM. The FMDV yield was determined as described in the Materials and Methods section.

amounts of either poly IC or bIFN-α resulted in an increased inhibition of FMDV yield. We repeated the experiment a number of times with both EBK cells and a bovine cell line, LF-BK cells, and obtained similar results, although the degree of inhibition varied (data not shown). Treatment of a swine cell line, SK6 cells, with various amounts of pIFN-α and poly IC also significantly reduced the yield of FMDV (Table 1). We obtained similar results with pIFN-α in a second swine cell line, IB-RS-2 cells (data not shown).

To examine whether the combination of poly IC and bIFN-α could synergistically inhibit FMDV replication, we chose concentrations of each reagent that alone only had a limited effect on virus yield. The combination had a significant synergistic inhibitory effect in reducing FMDV yield in EBK cells at least 100-fold in comparison with poly IC or bIFN-α alone (Table 1). Surprisingly, although there was a dose response with either poly IC or pIFN-α, we did not see a synergistic effect with the combination in the SK6 swine cell line.

We next examined the duration of protection afforded by either poly IC or bIFN-α. EBK cells were treated with various amounts of either molecule for 24 h, the media were removed, and cells were infected with FMDV immediately (1 day post-treatment) or at 1, 2, or 3 days after drug removal (2, 3 or 4 dpi) (Fig. 1). The level of inhibition was dose dependent and lasted for at least 4 days after treatment with poly IC at all doses tested. Likewise, after treatment with bIFN-α, FMDV replication was reduced for at least 2 days, although at high amounts, that is, 50 U/mL, inhibition was maintained for at least 4 days.

Identification of ISGs induced in vitro

EBK cells were treated with different concentrations of bIFN-α (1–100 U/mL), poly IC (1.25–5.0 ng/mL), or the combination. RNA was analyzed for up-regulation of ISGs by qRT-PCR (Table 2). In EBK cells treated with different concentrations of poly IC a broader array of ISGs was induced as compared with cells treated with different concentrations of bIFN-α. In addition, at all concentrations of poly IC used, there was an up-regulation of type I and III IFNs, but not at any concentration of IFN (Table 2). Furthermore, the combination of poly IC and bIFN-α did not result in a synergistic up-regulation of these ISGs, but rather reflected the level of induction after poly IC treatment. We obtained very similar results in poly IC treated SK6 cells (manuscript in preparation).

Effect of pretreatment of swine with poly ICLC, Adt-pIFN-α, or the combination on resistance to FMDV challenge in swine

Earlier, we demonstrated that IM inoculation at 4 sites in the neck with 1×10^{10} FFU of Adt-pIFN-α protected swine against challenge with FMDV A24 Cruzeiro at 1 dpi (Dias and others 2011). However, this is still a relatively high dose. In an attempt to lower the protective dose further, we hypothesized that the use of a TLR and/or cytosolic receptor ligand alone or in combination with IFN may help. To address this hypothesis, we performed 2 experiments in swine using poly ICLC, a more stable derivative of poly IC (Levy and others 1975). None of the animals inoculated with poly

Table 2. Effect of bIFN-α/Poly IC Alone or in Combination on Induction of IFNs, IRFs, and ISGs in EBK Cells

	Treatment											
		bIFN-α[a]				poly IC[a]			bIFN-α + poly IC[a]			
Gene symbol	Lipofectamine	1 U	5 U	10 U	100 U	1.25 ng	2.5 ng	5 ng	1 U + 2.5 ng	5 U + 2.5 ng	1 U + 5 ng	5 U + 5 ng
CCL2	**2.1**[b]	1.4	**2.1**	1.6	**2.8**	**6.7**	**11.1**	**18.7**	**6.7**	**5.9**	**10.1**	**19.8**
CCL3	0.8	0.9	1.2	0.8	1.1	1.1	1.1	1.3	1.1	1.0	1.1	1.4
CCL5	**2.0**	**7.0**	**19.0**	**21.1**	**51.4**	**399.4**	**3327.2**	**9946.2**	**3076.9**	**3083.7**	**9493.7**	**18661.0**
CCL20	0.8	0.9	1.2	0.8	1.1	**55.4**	**71.2**	**153.2**	**41.9**	**34.0**	**53.1**	**215.7**
IP-10	0.8	0.9	1.2	0.8	**5.2**	**159.8**	**5824.2**	**12367.8**	**5286.0**	**3297.4**	**10467.0**	**14771.3**
IL-28B[c]	1.2	0.5	0.4	1.7	1.2	**92.5**	**1584.1**	**4097.8**	**1027.5**	**708.8**	**1873.5**	**5550.3**
IFN-α	0.8	0.6	0.7	0.8	1.2	1.4	**49.5**	**181.2**	**31.1**	**22.2**	**65.7**	**131.2**
IFN-β	1.1	0.9	1.0	0.6	0.4	**9.8**	**301.3**	**361.0**	**173.7**	**182.3**	**144.0**	**249.4**
IFNγ	0.8	0.9	1.2	1.1	1.1	1.1	1.1	1.3	1.1	1.0	1.1	1.4
INDO	1.4	1.2	**5.3**	**8.0**	**39.6**	**226.4**	**5401.9**	**13172.8**	**3923.7**	**2268.3**	**10507.8**	**17350.0**
iNOS	1.0	0.9	1.2	1.3	1.1	**2.6**	**5.1**	**3.5**	**2.2**	**2.2**	**2.1**	**3.9**
IRF1	1.0	1.3	**2.1**	1.9	**3.4**	**7.7**	**18.2**	**19.2**	**10.9**	**8.5**	**11.2**	**16.5**
IRF7	1.2	**3.4**	**6.1**	**7.2**	**12.3**	**10.1**	**26.0**	**29.5**	**20.3**	**18.1**	**22.0**	**22.1**
ISG15	1.3	**314.2**	**1031.6**	**1166.5**	**3268.9**	**2102.1**	**6279.7**	**8605.2**	**5562.8**	**5180.8**	**6680.9**	**9180.4**
MDA-5	0.9	**5.7**	**18.5**	**20.5**	**57.5**	**46.5**	**127.1**	**172.2**	**111.3**	**99.0**	**114.3**	**177.9**
Mx1	1.0	**89.6**	**211.3**	**217.4**	**405.0**	**305.4**	**495.6**	**676.7**	**467.9**	**379.8**	**457.6**	**641.4**
OAS	1.2	**569.5**	**1139.9**	**1298.7**	**2001.9**	**1423.7**	**3287.6**	**3611.6**	**2919.5**	**2854.5**	**2852.2**	**3663.8**
PKR	0.9	**5.4**	**11.5**	**10.9**	**18.5**	**14.9**	**29.7**	**32.5**	**21.6**	**22.6**	**23.2**	**29.3**
PERF1	1.1	1.5	1.4	0.8	1.5	0.4	**2.9**	**3.0**	1.5	1.2	1.8	**4.0**
RIG-I	0.8	0.9	1.2	0.8	1.1	1.1	1.1	1.3	1.1	1.0	1.1	1.4
STAT1	0.8	1.6	**3.6**	**3.3**	**6.8**	**5.3**	**9.1**	**11.8**	**8.5**	**8.0**	**7.6**	**11.0**
TNF-α	0.8	0.9	1.2	0.8	1.1	**3.4**	**9.0**	**59.9**	**14.5**	**6.1**	**21.5**	**69.4**

[a]Each reagent or combination was diluted in 1 mL of media and added to 1×10^6 cells.
[b]Genes up-regulated 2-fold or greater are expressed with bold font.
[c]IFN-λ3.
ISG, IFN-stimulated gene; IRF, interferon regulatory factor.

ICLC showed any site reaction nor was their appetite or behavior affected in contrast to previously reported data showing a local reaction and influenza-like symptoms in human volunteers inoculated with a lower dose of poly ICLC (Caskey and others 2011).

In the first experiment groups of 3 animals were administered either 2.5×10^9 FFU Adt-pIFN-α, 4 mg of poly ICLC, the combination of 2.5×10^9 FFU Adt-pIFN-α and 4 mg of poly ICLC, or 2.5×10^9 FFU AdNull (control group). All groups were challenged at 1 dpi with 10^5 $TCID_{50}$ FMDV A24. The control animals developed viremia, virus in nasal swabs, and clinical disease by 2 or 4 dpc (Table 3). All the animals in the group treated with 2.5×10^9 FFU Adt-pIFN-α developed disease at 4 dpc, but it was less severe than in the control group. Both the level of viremia and virus in nasal swabs were 10–1,000-fold lower than in the control group. In the 4 mg poly ICLC-treated group, one of the animals, No. 43, did not develop clinical signs, nor had detectable viremia or virus in nasal swabs and was negative for FMDV NS protein 3ABC and FMDV by RT-PCR. However, despite the absence of antibodies against the NS protein 3D, which are indicative of challenge virus replication, this animal developed a low FMDV-specific neutralizing antibody response and low levels of antibodies against the viral structural proteins (Table 3 and Supplementary Fig. S1). Another animal in this group, No. 41, developed clinical signs by 4 dpc, but viremia was not detectable and virus in nasal swabs was 10–100-fold lower than in the controls. The third animal in this group, No. 42, died 3 days postchallenge from causes related to FMD, although it had no detectable viremia or virus in nasal swabs at 1 and 2 dpc (Table 3). In the group given the combination treatment, 2 of the 3 animals, No. 36 and 37, did not develop clinical disease, viremia, or virus in nasal swabs, while the third animal, No. 35, only developed clinical disease at 7 dpc. These animals developed the highest levels of antiviral activity and pIFN-α protein in the blood. The two clinically protected animals in this group were negative by 3ABC ELISA, RT-PCR and had very low levels of FMDV-specific neutralizing antibodies, but had no detectable antibodies against either the viral structural proteins or 3D by radioimmunoprecipitation (RIP), indicating that they were either sterilely protected or had a very low level of challenge virus replication (Table 3 and Supplementary Fig. S1).

Based on the results of this experiment, we performed a second experiment lowering the Adt-pIFN-α dose and increasing the poly ICLC dose. Groups of 3 swine were inoculated with (1) 2.5×10^9 FFU and 4 mg of poly ICLC, (2) 1×10^9 FFU Adt-pIFN-α and 4 mg of poly ICLC, (3) 1×10^9 FFU and 8 mg of poly ICLC, (4) 2.5×10^8 FFU Adt-pIFN-α and 8 mg of poly ICLC, (5) 8 mg of poly ICLC alone, or (6) 2.5×10^9 AdNull. Animals were challenged 24 h later with 10^5 $TCID_{50}$ of FMDV A24 (Table 3).

All control animals developed clinical disease by 2 dpc and high levels of viremia by 1–2 dpc (Table 3). As in the previous experiment 2 of 3 animals in the group given 2.5×10^9 FFU Adt-pIFN-α and 4 mg of poly ICLC, No. 76 and 77, did not develop clinical disease, viremia, or virus in nasal swabs and were either sterilely protected or had very low levels of challenge virus replication, that is, negative 3ABC ELISA, RT-PCR and RIP, and undetectable or very low levels of FMDV-specific neutralizing antibody. The third animal in this group,

Table 3. Clinical Performance of Swine Treated with Different Concentrations of Poly ICLC and/or Adt-pIFN-α and Challenged 24 h Later with FMDV A24

Trial	Group	Dose[a] (FFU)	Animal No.	Antiviral Act./ pIFN-α[b]	Viremia[c]	Shedding Virus[d]	Clinical Score[e]	PRN_{70}[f]	3ABC ELISA[g]	RT-PCR[h]	RIP[i] 3D/SP
1	AdNull	2.5×10^9	44	<25/0	$2/2.4\times10^5/3$	$3/2.2\times10^3/2$	2/17	2,048	P	P	ND
			45	50/248	$4/1.7\times10^3/1$	$3/2.7\times10^2/1$	4/13	1,024	P	ND	+/+++
	Adt-pIFN-α	2.5×10^9	38	100/417	$3/1.0\times10^2/1$	$5/5.0\times10^0/1$	4/8	512	P	ND	+++/+++
			39	50/375	$3/6.0\times10^2/2$	$5/1.0\times10^1/1$	4/8	256	P	ND	++/++
			40	25/189	0	$5/1.0\times10^2/2$	4/9	4,096	P	ND	ND
	poly ICLC	4 mg	41	<25/200	0	$5/3.0\times10^1/1$	4/10	2,048	P	ND	ND
			42	50/330	0/D[j]	0/D[j]	D[j]	D[j]	D[j]	D[j]	D[j]
			43	<25/87	0	0	0	64	N	N	−/+
	Adt-pIFN-α+poly ICLC	$2.5\times10^9+4$ mg	35	200/1220	0	0	7/7	2,048	P	P	++/+++
			36	200/897	0	0	0	8	N	N	N/N
			37	100/318	0	0	0	64	N	N	N/N
2	AdNull	2.5×10^9	90	<25/226	$2/3.2\times10^5/3$	$3/5.2\times10^2/2$	2/15	1,024	P	ND	ND
			91	<25/74	$2/2.8\times10^5/2$	$3/4.9\times10^2/2$	2/16	2,048	P	ND	ND
			92	<25/71	$1/2.9\times10^5/3$	$2/7.1\times10^2/3$	2/17	1,024	P	P	ND
	Adt-pIFN-α+poly ICLC	$2.5\times10^9+4$ mg	75	50/830	$4/6.4\times10^4/3$	$5/2\times10^2/2$	4/7	512	P	ND	+++/+++
			76	50/806	0	0	0	<8	N	N	−/−
			77	50/954	0	0	0	8	N	N	−/+
	Adt-pIFN-α+poly ICLC	$1\times10^9+4$ mg	78	25/378	0	0	4/7	D[k]	D[k]	D[k]	D[k]
			79	50/845	$5/1.9\times10^3/2$	$5/4\times10^2/1$	6/12	512	P	ND	ND
			80	25/762	$3/2.9\times10^3/2$	$4/4.1\times10^2/2$	5/12	512	P	ND	ND
	Adt-pIFN-α+poly ICLC	$1\times10^9+8$ mg	81	25/699	0	0	0	<8	N	N	−/−
			82	100/1310	0	0	0	<8	N	N	−/−
			83	<25/252	0	0	0	<8	N	N	−/−
	Adt-pIFN-α+poly ICLC	$2.5\times10^8+8$ mg	84	25/359	0	0	0	8	N	N	−/−
			85	<25/235	$6/3\times10^1/1$	$5/3.2\times10^2/2$	7/12	2,048	P	ND	ND
			86	25/585	$3/2.8\times10^3/2$	$4/5.1\times10^2/3$	5/14	1,024	P	P	ND
	poly ICLC	8 mg	87	<25/290	D[l]	D[l]	D[l]	D[l]	D[l]	D[l]	D[l]
			88	25/617	0	0	0	8	N	N	−/−
			89	<25/386	0	0	0	8	N	N	−/−

[a]Dose of inoculum per animal expressed as number of FFU in 2 mL of phosphate-buffered saline.
[b]Antiviral activity (U/mL) of swine plasma in IB-RS-2 cells and pIFN-α (pg/mL) detected in swine plasma by ELISA at 1 dpi.
[c]First dpc that viremia was detected; maximum amount of viremia in PFU/mL detected in sera samples; and the duration (days) of viremia.
[d]First dpc that shedding virus was detected; maximum amount of shedding virus in PFU/mL detected in nasal swab samples; and the duration (days) of shedding.
[e]dpc first signs of lesions/highest lesion score.
[f]Neutralizing antibody response of 21 dpc serum reported as serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}).
[g]Twenty-one dpc sera tested; N, negative; P, positive.
[h]One to 7 dpc sera tested; N, negative; P, positive; ND, not done.
[i]RIP assay; NS protein 3D/SP; −, negative; +++, strong positive; ++, positive; +, weak positive; ND, not done.
[j]Animal No. 42 probably died from FMD related causes at 3 dpc.
[k]Animal No. 78 died from non-FMD causes at 7 dpc.
[l]Animal No. 87 died from non-FMD causes at 1 dpc.

FFU, focus forming unit; Adt-pIFN-α, adenovirus containing the gene for porcine interferon alpha; dpi, day postinoculation; RT-PCR, reverse transcription–polymerase chain reaction; PRN, plaque reduction neutralization; NS, nonstructural; RIP, radioimmunoprecipitation; dpc, day postchallenge; SP, structural proteins; poly ICLC, polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine and carboxymethyl cellulose.

No. 75, developed clinical signs 2 days later than the control animals, and the disease was less severe. When the dose of Adt-pIFN-α was decreased to 1×10^9 FFU and 4 mg of poly ICLC was added, all the animals developed clinical disease, but there was a delay of 2–4 days compared with the controls. One animal in this group, No. 78, never developed viremia or virus in nasal swabs, while the other 2 animals, No. 79 and 80, had 100-fold lower levels of viremia as compared with the controls. All the animals in the group administered 1×10^9 FFU Adt-pIFN-α and 8 mg of poly ICLC were sterilely protected. There was no evidence of virus replication by 3ABC ELISA, RT-PCR, and no detectable FMDV-specific neutralizing antibody response. Furthermore, the RIP gel analysis showed no evidence of antibodies against the viral structural proteins or the NS protein 3D supporting the absence of any detectable challenge virus replication (Table 3 and Supplementary Fig. S1). When the Adt-pIFN-α dose was reduced to 2.5×10^8 FFU, 1 animal, No. 84, did not develop clinical disease, while in the other 2 animals, No. 85 and 86, disease was delayed 3 or 5 days and viremia was reduced 100 or 10,000-fold as compared with the control group. In the group given only 8 mg of poly ICLC 1 animal, No. 87, died of causes unrelated to FMD, while the other 2 animals, No. 88 and 89, had no clinical disease, viremia, or virus in nasal swabs, and were 3ABC ELISA and RT-PCR negative. However, very low levels of FMDV-specific neutralizing antibodies were detected, although no antibodies against the viral structural proteins and NS protein 3D were evident in the RIP assay, suggesting that they were either sterilely protected or only had very low levels of challenge virus replication.

IFN-α levels and antiviral activity in plasma

All animals were assayed for the presence of pIFN-α protein and antiviral activity in plasma. Animals treated with 2.5×10^9 FFU Adt-pIFN-α combined with 4 mg of poly ICLC showed higher antiviral activity and higher levels of pIFN-α protein in plasma at 1 day post-treatment than the other treatment groups, with the exception of animals, No. 79 and 82, in the groups treated with 1×10^9 FFU Adt-pIFN-α combined with 4 or 8 mg of poly ICLC, respectively (Table 3). These levels are considerably lower than those previously found in swine inoculated with 1×10^{10} FFU Adt-pIFN-α alone (Dias and others 2011).

Identification of ISGs

In the second experiment, we also evaluated the effect of Adt-pIFN-α and poly ICLC treatment on the expression of 21 ISGs in PBMCs at 1 day post-treatment (Table 4 and Fig. 2). We selected ISGs that have various functional roles. All treatments were able to up-regulate some ISGs. However, among all treatment groups, 1×10^9 FFU Adt-pIFN-α combined with 8 mg of poly ICLC induced a broader array and higher levels of ISGs (Fig. 2 and Table 4). In 2 of the protected animals in this group, 21 and 17 genes were up-regulated from 5- to 84 fold. RIG-I, ISG-15, OAS, IRF7, IP-10, MDA-5, CCL5, Mx1, and IFN-αR1 were the ISGs up-regulated in most protected animals.

Discussion

In previous swine studies, we demonstrated that direct delivery of IFN genes can induce up-regulation of several ISGs and rapidly protect animals against challenge with multiple serotypes of FMDV (Chinsangaram and others 2003; Moraes and others 2003, 2007; Dias and others 2011; Diaz-San Segundo and others 2011; Perez-Martin and others 2012). Nevertheless, this strategy is currently quite costly, because it requires relatively high levels of IFN delivered by a recombinant Ad5 vector.

During the course of a viral infection, several "pathogen associated molecular patterns" are recognized by specific pattern recognition receptors present in host cells (Medzhitov and Janeway 1998; Honda and Taniguchi 2006). These interactions result in the induction of a broad array of innate immune pathways that ultimately lead to the expression of IFNs. However, by directly administering IFN, the host-pathogen interactions that usually occur during viral infection are bypassed. Recent studies have shown that the highly efficacious live-attenuated yellow fever vaccine induces a large number of innate immune pathways before the initiation of a broad and persistent adaptive immune response (Gaucher and others 2008; Querec and others 2009; Caskey and others 2011). The administration of poly ICLC to human volunteers induces a very similar and extensive array of genes, representing 10 canonical innate immune pathways (Caskey and others 2011).

We hypothesized that treatment of animals with a molecular mimic of virus infection, such as dsRNA, could induce a broader array of genes in comparison to IFN treatment alone, and could also activate a number of signaling molecules that may potentially result in a positive feedback induction of additional type I IFN (Marie and others 1998; Honda and Taniguichi 2006; Yoneyama and others 2005). Furthermore, we postulated that a combination of type I IFN and dsRNA may induce a more robust and sustained innate response than either treatment alone.

In cell culture, we found that both IFN-α and poly IC inhibited FMDV replication, while the combination had a synergistic effect in bovine cells but not in swine cells. Interestingly, poly IC induced the up-regulation of all genes examined, including type I and III IFNs, in both bovine and swine cells (Table 2, manuscript in preparation), while IFN-α mainly induced the up-regulation of genes that have direct antiviral activity, including INDO, ISG15, Mx1, OAS, and PKR as well as IRF1 and 7. Presumably, this effect is the result of the ability of poly IC, but not IFNs, to activate IRF7, which has a major role in the induction of type I IFN genes (Honda and others 2005; Honda and Taniguchi 2006). Using mouse embryonic fibroblasts from IRF7 knockout mice, Honda and others (2005) demonstrated that IRF7 is the master regulator of type I IFN immune responses and after its involvement in activating the initial phase of type I IFN gene induction, it is subsequently up-regulated by type I IFN and plays a crucial role in the induction of additional IFN-β and various subtypes of IFN-α (Honda and Taniguchi 2006).

Based on the *in vitro* experiments, we performed animal studies to examine the efficacy of various individual or combination treatments on rapid protection against FMDV challenge. We found that a combination of 1×10^9 FFU Adt-pIFN-α plus 8 mg poly ICLC resulted in sterile protection as measured by the absence of viremia and virus in nasal swabs, negative 3ABC ELISA, RT-PCR, and RIP for both structural and NS proteins, and the absence of detectable FMDV-specific neutralizing antibodies. We obtained essentially similar results in the group inoculated with only 8 mg

Table 4. Induction of Genes in PBMCs of Swine Treated with Different Concentrations of Adt-pIFN-α and/or Poly ICLC at 1 dpi

		Fold induction[a]							
		Protected animals				Unprotected animals			
Function class	Symbol	1×10^9 FFU Adt-pIFN-α + 8 mg poly ICLC[b]	8 mg poly ICLC[c]	2.5×10^9 FFU Adt-pIFN-α + 4 mg poly ICLC[d]	2.5×10^8 FFU Adt-pIFN-α + 8 mg poly ICLC[e]	2.5×10^9 FFU Adt-pIFN-α + 4 mg poly ICLC[f]	2.5×10^8 FFU Adt-pIFN-α + 8 mg poly ICLC[g]	1×10^9 FFU Adt-pIFN-α + 4 mg poly ICLC[h]	AdNull[i]
ISG	IRF1	5.14 ± 6.13	0.92 ± 0.27	0.38 ± 0.21	0.16	1.28	0.35 ± 0.26	0.28 ± 0.12	0.88 ± 0.82
	IRF3	7.42 ± 7.26	0.66 ± 0.43	1.64 ± 1.36	0.43	1.12	0.51 ± 0.46	0.80 ± 0.17	0.65 ± 0.20
	IRF7[j]	24.43 ± 24.02	2.89 ± 0.34	5.07 ± 3.42	1.72	1.12	3.55 ± 3.60	2.57 ± 1.42	1.26 ± 0.32
	IFN-αR1[j]	17.34 ± 27.85	3.07 ± 1.49	1.32 ± 0.85	2.63	3.43	1.53 ± 0.41	0.57 ± 0.22	1.08 ± 0.45
	IFN-β	63.25 ± 106.83	4.38 ± 5.18	2.89 ± 3.75	11.93	1.34	9.54 ± 10.94	2.39 ± 1.84	1.23 ± 1.37
	IFNγ	2.43 ± 2.12	0.52 ± 0.16	2.12 ± 2.65	0.68	1.15	0.39 ± 0.06	0.71 ± 0.44	0.70 ± 0.56
	ISG15[j]	45.37 ± 57.15	5.01 ± 0.95	9.56 ± 7.87	3.83	1.02	5.38 ± 5.25	3.37 ± 1.79	0.42 ± 0.11
	OAS[j]	39.28 ± 42.18	9.75 ± 5.92	5.50 ± 2.75	3.47	0.81	7.03 ± 5.86	3.20 ± 0.71	1.42 ± 0.69
	INDO	84.40 ± 143.82	1.26 ± 0.40	1.96 ± 1.01	0.55	1.23	9.74 ± 13.44	1.51 ± 1.34	1.19 ± 1.04
	Mx1[j]	15.44 ± 20.71	10.36 ± 5.06	1.72 ± 0.88	0.56	0.47	6.74 ± 5.51	3.44 ± 2.66	4.23 ± 3.90
Protein degradation	PRF1	13.06 ± 19.76	1.08 ± 0.54	2.16 ± 2.51	1.89	1.10	0.80 ± 0.26	1.14 ± 0.91	0.62 ± 0.57
Chemokines	CCL5[j]	17.28 ± 24.61	1.67 ± 1.01	3.56 ± 4.35	1.69	1.01	0.59 ± 0.05	1.26 ± 0.66	0.71 ± 0.71
	IP-10[j]	56.55 ± 58.06	3.07 ± 0.49	3.13 ± 1.63	1.47	1.07	3.43 ± 2.47	1.68 ± 0.38	1.00 ± 0.50
Cytokines and growth factors	IL-1β	19.44 ± 21.07	1.94 ± 0.27	0.80 ± 0.77	3.61	161.21	1.18 ± 0.85	2.44 ± 2.98	1.54 ± 1.42
	IL-6	10.21 ± 9.16	1.96 ± 1.58	0.68 ± 0.16	1.32	178.32	2.18 ± 1.38	2.08 ± 2.80	0.60 ± 0.57
	IL-12	16.45 ± 21.02	2.27 ± 0.92	1.10 ± 0.32	0.68	0.69	0.76 ± 0.79	0.74 ± 0.42	10.31 ± 16.07
	TNF-α	5.12 ± 5.95	0.79 ± 0.17	1.12 ± 0.40	0.21	1.34	0.55 ± 0.55	0.52 ± 0.17	1.27 ± 1.30
dsRNA recognition and cell signaling	RIG-I[j]	72.98 ± 75.53	9.85 ± 0.68	12.12 ± 11.28	2.74	0.90	9.99 ± 10.77	5.49 ± 3.11	1.80 ± 0.81
	MDA-5[j]	26.28 ± 25.72	4.96 ± 3.41	5.92 ± 5.70	1.83	1.03	2.91 ± 2.73	2.63 ± 0.90	1.13 ± 0.37
TNF-induced signaling	TRAF-6	22.51 ± 19.38	1.76 ± 0.52	2.75 ± 2.70	3.48	381.98	0.78 ± 0.22	1.33 ± 0.55	1.54 ± 1.03
	TBK-1	11.37 ± 11.87	1.28 ± 0.48	1.20 ± 0.44	0.45	0.72	1.58 ± 0.69	2.92 ± 3.10	2.55 ± 1.97

[a]The fold induction is the mean of animals in each protected and unprotected group ± standard deviation.
[b]Values correspond to 3/3 animals.
[c]Values correspond to 3/3 animals. We included the data for animal No. 87 that died at 1 dpc.
[d]Values correspond to 2/3 animals.
[e]Value corresponds to 1/3 animals.
[f]Value corresponds to 1/3 animals.
[g]Values correspond to 2/3 animals.
[h]Values correspond to 3/3 animals.
[i]Values correspond to 3/3 animals.
[j]Genes up-regulated in the majority of protected animals from different groups.
Light gray boxes indicate genes that qualified as up-regulated (≥2-fold induction).
PBMCs, peripheral blood mononuclear cells.

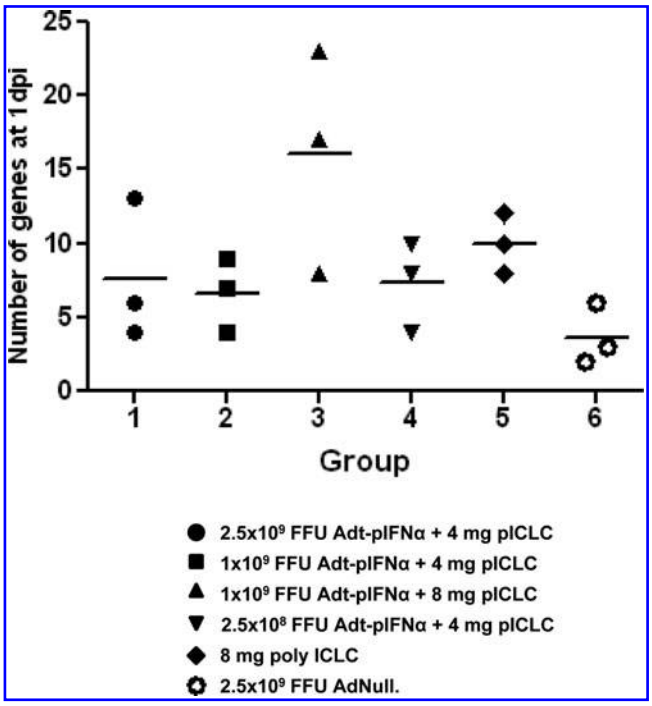

FIG. 2. Number of IFN-stimulated genes (ISGs) up-regulated in peripheral blood mononuclear cells of swine 1 day post-treatment with poly ICLC alone or combined with different doses of Adt-pIFN-α. Group 1: 2.5×10^9 FFU of Adt-pIFN-α + 4 mg of poly ICLC; group 2: 1×10^9 FFU of Adt-pIFN-α + 4 mg of poly ICLC; group 3: 1×10^9 FFU of Adt-pIFN-α + 8 mg of poly ICLC; group 4: 2.5×10^8 FFU of Adt-pIFN-α + 4 mg of poly ICLC; group 5: 8 mg of poly ICLC; group 6: 2.5×10^9 FFU of AdNull.

poly ICLC, although these animals developed a very low level of FMDV-specific neutralizing antibodies (1:8). Surprisingly, in the group given a 4-fold lower dose of Adt-pIFN-α, 2.5×10^8 FFU, plus 8 mg poly ICLC, only 1 animal was clinically protected and developed a low neutralizing antibody response (1:8), while the other 2 animals developed disease 3 or 5 days after the control animals. Although we do not have a clear explanation about these later results, we believe that the individual animal response is an uncontrolled factor that has to be taken into account. Different degrees of protection including delay in disease onset and less severe clinical signs were obtained with all the other combinations tested.

We examined gene induction at 1 dpi in PBMCs of treated swine. The pattern of gene induction followed the degree of protection. The group with the highest number of up-regulated genes was the group inoculated with 1×10^9 FFU Adt-pIFN-α plus 8 mg poly ICLC in which all animals were protected against challenge. This group was followed by the 8 mg poly ICLC group in which all animals were also protected (Fig. 2 and Table 4). The groups in which 2 of 3 or 1 of 3 animals were protected had the next highest number of genes induced, while the group in which all the animals developed clinical disease, but lesions were detected 2–4 days later than the control animals had more genes induced than the control group. Some genes were up-regulated in all, RIG-I and ISG15, or most of the protected animals, IRF7, IP-10, OAS, MDA-5, Mx1, CCL5, and IFN-αRI, but were also induced in some unprotected animals. We have previously shown that OAS and PKR have a role in the inhibition of FMDV replication (Chinsangaram and others 2001; de los Santos and others 2006), and we found that in tissues of swine inoculated with Ad5-pIFN-α and Ad5-pIFN-γ or bovines inoculated with Ad5-bIFN-λ, there was an induction of IP-10 and these animals had increased numbers of DCs and NK cells in the skin and lymph nodes (Diaz-San Segundo and others 2010, 2011).

It is difficult to directly correlate protection with gene induction, as we only analyzed a very limited number of genes and examined gene induction only in PBMCs. Nevertheless, the cell culture and animal data indicate that poly ICLC induces a broader array of genes than IFN. While both IFN and poly ICLC pretreatment can protect swine as early as 1 day postadministration, the utility of an animal biotherapeutic approach requires the protective dose to be affordable. Combining Adt-pIFN-α and poly ICLC as well as altering the route and number of sites of inoculation have allowed us to use a 100-fold lower dose of the Adt vector as compared with our initial studies (Dias and others 2011). In addition, even in the group given a 4-fold lower dose of Adt vector, 2.5×10^8 FFU, and poly ICLC, 1 animal was protected while the other 2 animals only developed lesions 3 or 5 days later than the control inoculated animals, and the level of viremia was reduced by 100–10,000-fold. Since our goal is to combine biotherapeutics with an FMD vaccine, even this lower dose of Adt-pIFN-α appears to be sufficient to delay disease onset until the vaccine-induced adaptive response is effective (Grubman 2003).

Our results indicate a viral mimic, such as poly ICLC, administered with IFN, and the FMD vaccine may represent a successful and more economical approach in providing rapid and long-lasting protection in animals against this feared disease.

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Author Disclosure Statement

No competing financial interests exist.

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Foot-and-Mouth Disease Virus Nonstructural Protein 2C Interacts with Beclin1, Modulating Virus Replication

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Foot-and-mouth disease virus (FMDV), the causative agent of foot-and-mouth disease, is an *Apthovirus* within the *Picornaviridae* family. Replication of the virus occurs in association with replication complexes that are formed by host cell membrane rearrangements. The largest viral protein in the replication complex, 2C, is thought to have multiple roles during virus replication. However, studies examining the function of FMDV 2C have been rather limited. To better understand the role of 2C in the process of virus replication, we used a yeast two-hybrid approach to identify host proteins that interact with 2C. We report here that cellular Beclin1 is a specific host binding partner for 2C. Beclin1 is a regulator of the autophagy pathway, a metabolic pathway required for efficient FMDV replication. The 2C-Beclin1 interaction was further confirmed by coimmunoprecipitation and confocal microscopy to actually occur in FMDV-infected cells. Overexpression of either Beclin1 or Bcl-2, another important autophagy factor, strongly affects virus yield in cell culture. The fusion of lysosomes to autophagosomes containing viral proteins is not seen during FMDV infection, a process that is stimulated by Beclin1; however, in FMDV-infected cells overexpressing Beclin1 this fusion occurs, suggesting that 2C would bind to Beclin1 to prevent the fusion of lysosomes to autophagosomes, allowing for virus survival. Using reverse genetics, we demonstrate here that modifications to the amino acids in 2C that are critical for interaction with Beclin1 are also critical for virus growth. These results suggest that interaction between FMDV 2C and host protein Beclin1 could be essential for virus replication.

Foot-and-mouth disease virus (FMDV), a single-stranded positive-sense RNA virus, is the causative agent of foot-and-mouth disease (FMD), a highly contagious viral disease of domestic and wild cloven-hoofed animals. Seven serotypes of FMDV exist (A, O, C, Asia, SAT1, SAT2, and SAT3), and recovery from one serotype does not provide immunity against the others (7, 22). The infectious virion is a nonenveloped icosahedron composed of four structural proteins: VP1, VP2, VP3, and VP4. The genome of approximately 8,400 nucleotides has a single open reading frame (ORF) that is translated into a polyprotein, which is processed by the three viral proteases Lpro, 2A, and 3C into the polypeptide products P1 (VP1 to VP4), P2 (2A, 2B, and 2C), and P3 (3A, 3B, 3Cpro, and 3Dpol). Further cleavage of these regions yields 14 mature virus proteins, along with several protein intermediates, that are needed for viral replication (18, 19).

During replication, FMDV forms a replication complex produced by the rearrangement of intracellular membranes into vesicular structures containing viral nonstructural proteins (2, 31). Many other positive-strand RNA viruses also initiate production of replication complexes upon infection of a cell (3, 4, 11, 38, 39). FMDV 2C, a 318-amino-acid protein, is the largest membrane-binding component of the virus RNA replication complex (30). FMDV 2C binds ssRNA nonspecifically, has ATPase activity (44), and is involved in the RNA replication complex (25). 2C prediction studies suggest that an amphipathic helix in its N terminus would be responsible for its ability to bind the intracellular membranes (46). The structure and size of 2C suggests that it plays multiple roles in the process of virus replication, including interactions with several host cellular factors during infection.

In order to better understand the role of FMDV 2C in virus replication, we attempted to identify host cell proteins that interact with 2C utilizing a yeast two-hybrid approach. Our screen identified a host protein, Beclin1, as a binding partner for 2C of FMDV serotypes O1 Campos and A24 Cruzeiro. Beclin1 is a central regulator of the autophagy process that regulates multiple steps of the autophagy pathway (48). Beclin1 is involved in the initiation of the autophagy pathway by marking membranes to form the first double membrane structure in the autophagy pathway, the phagophore (21, 37). Later in the autophagy pathway, Beclin1 functions to mediate autophagosome to lysosome fusion (28, 37). We have previously reported that FMDV 2C colocalized with autophagosome marker LC3 and that downregulation of the autophagy pathway resulted in decreased viral yields, while induction of the autophagy pathway resulted in an increase in virus titer (35). Thus, the cellular autophagy pathway appears to be critical for FMDV replication.

Here we show that interaction between FMDV 2C and cellular Beclin1, initially identified using yeast two-hybrid screening, actually occurs in FMDV-infected cells, as confirmed using coimmunoprecipitation and confocal microscopy. Importantly, modulations of the expression of Beclin1, as well as Bcl-2 (another host protein playing a critical role in the autophagy pathway), can have a negative effect on FMDV replication in cell culture. We also provide evidence that binding of 2C to Beclin1 may block the fusion of FMDV-containing autophagosomes to lysosomes, preventing virus degradation. In addition, identification of areas

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within 2C interacting with Beclin1 was performed by alanine scanning mutagenesis. These mutations were introduced into an infectious clone of FMDV and were determined to be critical for virus replication, suggesting that the 2C-Beclin1 interaction may play a significant role in virus replication.

MATERIALS AND METHODS

Cell lines, viruses, and plasmids. Human mammary gland epithelial cells (MCF-10A) were obtained from the American Type Culture Collection (catalogue no. CRL-10317) and maintained in a mixture of Dulbecco minimal essential medium (Life Technologies, Grand Island, NY) and Ham F-12 medium (1:1; Life Technologies) containing 5% heat-inactivated fetal bovine serum (Thermo Scientific, Waltham, MA), 20 ng of epidermal growth factor (Sigma-Aldrich, St. Louis, MO)/ml, 100 ng of cholera toxin (Sigma-Aldrich)/ml, 10 μg of insulin (Sigma-Aldrich)/ml, and 500 ng of hydrocortisone (Sigma-Aldrich)/ml.

FMDV type O1 strain Campos (O1C) was derived from the vesicular fluid of an experimentally infected steer. The virus was grown in baby hamster kidney-21 (BHK-21) cells, and the titer was determined by plaque assay on BHK-21 cells according to standard techniques (35).

Plasmids were purchased from Addgene, i.e., green fluorescent protein (GFP)-Bcl-2 (47) (Addgene plasmid, catalog no. 17999) and PCDNA4-Beclin1-FL (43) (Addgene plasmid, catalog no. 24388), or bought commercially, i.e., phrGFP II-N mammalian expression vector (Agilent Technologies, catalog no. 240145).

For viral replication studies, MCF-10A cells were plated at a density of 1×10^6 per well in a six-well plate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). The indicated plasmids were transfected into cells using Fugene (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. After 24 h, the cells were infected with FMDV type O1C at the specified multiplicity of infection (MOI) or mock infected. Virus was allowed to absorb for 1 h and then acid washed, and then fresh medium was added containing 0.5% serum. Samples were taken at the indicated time points.

Antibodies and reagents. Monoclonal antibody (MAb) 12FB, directed against the FMDV type O1 structural protein VP1, has been previously described (42). MAb 3D10, directed against the FMDV type O1 nonstructural protein 2C, was developed at the Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna, Brescia, Italy. Rabbit antibodies to LC3 (AnaSpec, Fremont, CA), Lamp1 (BD Biosciences, San Jose, CA), and UVRAG1 (Sigma-Aldrich) were used as autophagy markers. Protein disulfide isomerase (PDI; Affinity Bioreagents, Golden, CO) and bafilomycin A1 (Sigma-Aldrich) from *Streptomyces griseus*, an inhibitor of autophagosome fusion to lysosomes, were prepared as a 0.5 mM stock solution in dimethyl sulfoxide (DMSO) and then diluted in minimal essential medium (MEM) to 0.5 μM.

Infection and transfection of cells for confocal and deconvolution microscopy. Subconfluent monolayers of MCF-10A cells grown on 12-mm glass coverslips in 24-well tissue culture dishes were transfected with the indicated plasmids. After 24 h, the samples were infected with FMDV O1C at an MOI of 10 50% tissue culture infective dose(s) ($TCID_{50}$)/cell in MEM (Life Technologies) containing 0.5% heat-inactivated fetal bovine serum, 25 mM HEPES (pH 7.4), and 1% antibiotics. After the 1-h adsorption period, the supernatant was removed, and the cells were rinsed with ice-cold 2-morpholinoethanesulfonic acid (MES)-buffered saline (25 mM MES [pH 5.5], 145 mM NaCl) to remove unabsorbed virus. The cells were washed once with medium before fresh medium was added, followed by incubation at 37°C. At the indicated time points after infection, the cells were fixed with 4% paraformaldehyde (EMS, Hatfield, PA) and analyzed by confocal or deconvolution microscopy. To express GFP-BCL2, Beclin1-FLAG, or GFP protein, monolayers of MCF-10A cells were transfected with 0.5 μg of plasmid DNA using FuGene (Roche, Mannheim, Germany) according to the manufacturer's recommendations. At 19 to 24 h posttransfection, the cells were infected as described above and then fixed with 4% paraformaldehyde (EMS) at the appropriate times.

After fixation, the paraformaldehyde was removed, and the cells were permeabilized with 0.5% Triton X-100 for 5 min at room temperature, followed by incubation in blocking buffer (phosphate-buffered saline [PBS], 5% normal goat serum, 2% bovine serum albumin, 10 mM glycine, 0.01% thimerosal) for 1 h at room temperature. The fixed cells were then incubated with the primary antibodies overnight at 4°C. When double labeling was performed, the cells were incubated with both antibodies together. After three washes with PBS, the cells were incubated with the appropriate secondary antibody, goat anti-rabbit immunoglobulin G (IgG; 1/400; Alexa Fluor 594 or Alex Flour 647; Molecular Probes) or goat anti-mouse isotype-specific IgG (1/400; Alexa Fluor 488 or Alexa Fluor 594; Molecular Probes), for 1 h at room temperature. After this incubation, the coverslips were washed three times with PBS, counterstained with the nuclear stain TOPRO-iodide 642/661 (Molecular Probes) or DAPI (Life Technologies) for 5 min at room temperature, washed as described above, mounted, and examined using either a Leica scanning confocal microscope or a Nikon Eclipse 90i deconvolution microscope. The data were collected utilizing appropriate prepared controls lacking the primary antibodies, as well as anti-FMDV antibodies in uninfected cells, to give the negative background levels and to determine channel crossover settings. The captured images were adjusted for contrast and brightness using Adobe Photoshop software.

Knockdown of Beclin1 using siRNA. Small interfering RNA (siRNA) SMARTpools consisting of four RNA duplexes targeting Beclin1 (20) and a control siRNA (siRNA Glo) were purchased from Dharmacon (Lafayette, CO). MCF-10A cells were grown to densities of 10^5 cells per well in 24-well tissue culture dishes in 1 ml of medium without antibiotics, followed by transfection using DharmaFECT (Dharmacon) as recommended by the manufacturer's protocol. At 48 h posttransfection, the cells were infected with FMDV O1C at an MOI of 1 for 1 h at 37°C. After adsorption, the inoculum was removed, and the cells were rinsed with ice-cold MES to remove residual virus particles. The cells were then rinsed with MEM containing 1% fetal bovine serum and 25 mM HEPES (pH 7.4), followed by incubation at 37°C. At the indicated times postinfection, samples were drawn for titer assays using BHK-21 cell monolayers.

Viral replication in the presence of bafilomycin A1. MCF-10A cells were incubated with bafilomycin A1 for 30 min at 37°C prior to infection. The cells were then infected with FMDV O1C at an MOI of 10 in the presence or absence of bafilomycin A1. At the end of the adsorption period, the supernatant was removed, followed by a single rinse with ice-cold MES-buffered saline to inactivate the unabsorbed virus. The cells were washed once with medium before fresh medium with or without bafilomycin A1 was added. One set of cultures was immediately frozen at −70°C, whereas the other set of plates was incubated for an additional 4 h at 37°C and then moved to −70°C. The plates were then thawed, the cell debris was removed by centrifugation, and virus titers were determined based on the $TCID_{50}$ on BHK-21 cell monolayers.

Coimmunoprecipitation of FMDV 2C and Beclin1. MCF-10A cells were grown to 90% confluence and then infected at an MOI of 10 or mock infected. The cells were lysed at 2.5 h postinfection using protease inhibitors (Protea Biosciences, Morgantown, WV) and radioimmunoprecipitation assay (RIPA) buffer (Teknova, Hollister, CA). The protein lysate was used for immunoprecipitation using protein G-beads (Sigma-Aldrich) coupled to an MAb directed against Beclin1 (H-300). The cell lysate was incubated with anti-Beclin1 antibody for 2.5 h and then incubated with antibody and beads overnight at 4°C. The beads were washed five times using RIPA buffer, and then protein elutes were collected for each sample and examined by Western blot probing for anti-2C (3D10).

Development of the cDNA library. A cDNA expression library was constructed (Clontech, Mountain View, CA) using tissues susceptible to FMDV infection (dorsal soft palate, interdigital skin, middle tongue epithelium, and middle anterior lung) from a healthy, uninfected bovine. Total RNA was extracted using an RNeasy extraction kit (Qiagen, Valen-

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cia, CA). Contaminant genomic DNA was removed by DNase treatment using Turbo DNA-free (Ambion, Austin, TX). After DNase treatment, genomic DNA contamination of RNA stocks was assessed by real-time PCR amplification targeting the bovine β-actin gene. RNA quality was assessed using RNA nanochips on an Agilent Bioanalyzer 2100. Cellular proteins were expressed as GAL4-AD fusion proteins, while FMDV 2C was expressed as GAL 4-BD fusion proteins.

Library screening. The GAL4-based yeast two-hybrid system provides a transcriptional assay for detection of protein-protein interactions (10, 14). The bait protein, FMDV strain O1C 2C protein, was expressed with an N terminus fusion to the GAL4-binding domain (BD). Full-length 2C protein (amino acids 1082 to 1399 of the FMDV polyprotein) was used for screening and for full-length mutant protein construction. The previously described bovine cDNA library of proteins fused to GAL4-AD were used as prey. The reporter genes used here are histidine and adenine for growth selection. The bovine library used contains more than 3×10^6 independent cDNA clones. For screening, the yeast strain AH109 (Clontech) carrying 2C protein was transformed with library plasmid DNA and selected on plates lacking tryptophan, leucine, histidine, and adenine. Tryptophan and leucine are used for plasmid selection, and histidine and adenine are used for the identification of positive interacting library fusions. Once identified, the positive library plasmids were recovered in *Escherichia coli* and sequenced to identify the cellular interacting protein. Sequence analysis also determined whether the library proteins (cellular) were in frame with the activation domain. To eliminate false-positive interactions, all library-activation domain fusion proteins were retransformed into strains carrying the 2C-binding domain fusion protein, as well as into strains carrying Lam-binding domain fusion. Lam is human lamin C, commonly used as a negative control in the yeast two-hybrid system since lamin C does not form complexes or interact with most other proteins. The Beclin1 recovered from the library contained amino acids 1 to 211 of the bovine Beclin1 (NCBI reference sequence NP_001028799.1) amino terminus fused to GAL4 activation domain.

Site-directed mutagenesis. Full-length pO1Ca (5) or 2C-BD was used as a template in which amino acids were substituted with alanine, introduced by site-directed mutagenesis using a QuikChange XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) performed according to the manufacturer's instructions, where the full-length plasmid was amplified by PCR, digested with DpnI to leave only the newly amplified plasmid, transformed into XL10-Gold ultracompetent cells, and grown on Terrific broth plates containing ampicillin. Positive colonies were grown for plasmid purification using a Qiagen maxiprep kit. The full-length pO1Ca was sequenced to verify that only the desired mutation was present in the plasmid. The primers were designed using the Stratagene primer mutagenesis program, which limited us to a maximum of seven amino acid changes and was the basis for deciding on the regions to be mutated. Primers were designed using the manufacturer's primer design program (https://www.genomics.agilent.com/collectionsubpage.aspx?pagetype=tool&subpagetype=toolqcpd&pageid=15).

Construction of mutant FMDV viruses. Plasmid pO1Ca or its mutant version was linearized at the EcoRV site following the poly(A) tract and used as a template for RNA synthesis using the MegaScript T7 kit (Ambion) according to the manufacturer's protocols. BHK-21 cells were transfected with these synthetic RNAs by electroporation (Electrocell Manipulator 600; BTX, San Diego, CA) as previously described (5, 29). Briefly, 0.5 ml of BHK-21 cells at a concentration of 1.5×10^7 cells/ml in PBS was mixed with 10 μg of RNA in a 4-mm-gap BTX cuvette. The cells were then pulsed once at 330 V, infinite resistance, and a capacitance of 1,000 μF; the cells were then diluted in cell growth medium and allowed to attach to a T-25 flask. After 4 h, the medium was removed, fresh medium was added, and the cultures were incubated at 37°C for up to 24 h.

The supernatants from transfected cells were passaged in LF-BK αVβ6 cells until a cytopathic effect appeared. After successive passages in these cells, virus stocks were prepared, and the viral genome was completely sequenced using a Prism 3730xl automated DNA sequencer (Applied Biosystems), as previously described (5).

RESULTS

FMDV nonstructural protein 2C is highly conserved among different serotypes. Nonstructural FMDV 2C is a 318-amino-acid protein that is essential for virus replication (7). Comparison of the amino acid sequence of 2C from multiple serotypes of FMDV revealed a high degree of similarity, with >85% of the amino acids being identical in all reported isolates from all seven serotypes (Fig. 1). In addition, 2C is made up of 72% invariant residues, or residues that are 100% conserved among all reported isolates. These invariant residues include the proposed ATP/GTP binding domain of 2C (amino acid residues at positions 110 to 116, 160 to 163, and 243 to 246) (8). This high degree of similarity among isolates suggests that 2C has evolved to have essential functions during infection with FMDV 2C.

FMDV nonstructural 2C protein interacts with the bovine host protein Beclin1. A yeast two-hybrid system (15) was used to identify host cellular proteins that interact with FMDV 2C protein. An N-terminal fusion of the Gal4 protein DNA binding domain (BD) with FMDV 2C protein from FMDV O1 Campos (FMDV O1C) was used as "bait." For "prey," we used a custom cDNA library that was derived from RNA extracted from FMDV-susceptible bovine tissues (dorsal soft palate, interdigital skin, middle tongue epithelium, and middle anterior lung), expressed as N-terminal fusion of the Gal4 activation domain (AD). More than 1×10^7 independent yeast colonies were screened from the library containing more than 3×10^6 independent clones, representing a >3-fold screening of the library. Putative protein interactions were selected after cotransforming yeast strain AH109 with BD-2C fusion and cDNA library-AD fusion. Colonies were selected for growth in defined media lacking amino acids leucine, tryptophan, histidine, and nucleobase adenine. Plasmids were recovered from positive colonies and sequenced. In-frame ORFs were retested for specificity of the observed protein interaction with the 2C protein. To address the issue of false-positive interaction with FMDV 2C protein, human lamin C protein expressed as a fusion with BD (BD-LAM) was used as a negative control in AH109 yeast cotransfected with the positive colonies from the 2C library screen. One specific protein binding partner for 2C, Beclin1, was selected for further study due to the involvement of Beclin1 in the autophagy pathway (Fig. 2A). The plasmid that was recovered from the yeast two-hybrid screen containing Beclin1 was found to be truncated, containing amino acids 1 to 211 of bovine Beclin1 (NCBI reference sequence NP_001028799.1). However, this truncated portion of Beclin1 still contained the BH3 domain involved in binding Bcl-2 (amino acids 114 to 123) (6, 13) and part of the CCD domain known to bind to UVRAG (amino acids 144 to 269), suggesting the possibility that 2C could bind Beclin1 in a similar manner as Bcl2 or UVRAG.

To confirm that the interaction identified using the two-hybrid system in yeast occurs during FMDV infection of host cells, coimmunoprecipitation experiments were performed using MAbs specifically recognizing both proteins. Human epithelial cell line MCF-10A was infected (MOI = 10) with FMDV O1C, and samples were harvested at 2 h postinfection (hpi), the time point when 2C is beginning to accumulate in MCF-10A cells as determined by Western blotting (data not shown). MCF-10A cell lysates were collected from infected or mock-infected cells and immunopre-

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                10        20        30        40        50        60        70        80
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O       LKARDINDIFAILKNGEWLVKLILAIRDWIKAWIASEEKFVTMTDLVPGILEKQRDLNDPSKYKEAKEWLDNARQACLKS
Asia    ................................E............N.................................
C       ............................................................D.......T..V...N
SAT-1   ................................S....YIS......R...C.HN.......Q.S....E...E....N
Sat-2   ................................S............................H......E........N
Sat-3   ................................S....YIS......R...C..N.......Q.S....E...E....N
A       ..........................................T.....S.....Q.........................
                90       100       110       120       130       140       150       160
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O       GNVHIANLCKVVAPAPSKSRPEPVVVCLRGKSGQGKSFLANVLAQAISAHFTGRTDSVWYCPPDPDHFDGYNQQTVVVMD
Asia    ...................G.D..........................TL...........................L....
C       ..........................................T.L.............................
SAT-1   ..H........N....................................T...............................
Sat-2   ...........T....................................T..........................A.....
Sat-3   ...........N....................................T.....V....................A.....
A       ..................R.............................T...............................
               170       180       190       200       210       220       230       240
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O       DLGQNPDGKDFKYFAQMVSTTGFIPPMASLEDKGKPFNSKVIIATTNLYSGFTPRTMVCPDALNRRFHFDIDVSAKDGYK
Asia    ..................................................................................
C       ....................................S................K.........................
SAT-1   ..........................................S..............................G...
Sat-2   ..........................................S................................R
Sat-3   ..........................................S..............................R...R
A       ..................................................................................
               250       260       270       280       290       300       310
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
O       INNKLDIIKALEDTHTNPVAMFQYDCALLNGMAVEMKRMQQDMFKPQPPLQNVYQLVQEVIDRVELHEKVSSHPIFKQ
Asia    T.......................................V............................N......
C       ...............A......H...............L...V...................E................
SAT-1   V..R.............AP...N........S......L...V...L...NSL....D...E..K.............
Sat-2   V......T..............................L..........IL......D...E..N.....A.Q.....
Sat-3   V..R.............AP...N........S......L...V...L...NSL....D...E..K.............
A       ..........................................................E................
```

FIG 1 Multiple sequence alignment using Bioedit software was performed using a representative sequence for each of serotypes of FMDV. The sequences used were from serotypes O, Asia, C, SAT1, SAT2, SAT3, and A with the respective GenBank accession numbers AJ320488.1, NP_937964, NC_002554, NC_011451, NC_003992, NC_011452, and AY593768.1.

cipitated with an anti-Beclin1 MAb, H-300 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a Western blot with an FMDV 2C-specific MAb, 3D10 (Izler, Brescia, Italy). A single band at the expected molecular mass of FMDV 2C (40 kDa) was clearly obtained, indicating that during FMDV infection, 2C coimmunoprecipitates with Beclin1, confirming the previous yeast two-hybrid results, suggesting a 2C-Beclin1 interaction (Fig. 2B). Attempts to perform reverse coimmunoprecipitation, immunoprecipitating 2C and detecting Beclin1 on the blot, were inconclusive since the presence Beclin1 was masked by the immunoglobulin heavy chain.

The localization of 2C and Beclin1 during infection was assessed using double-label immunofluorescence and confocal microscopy in cells infected with FMDV. MCF-10A cells were infected (MOI = 10) or mock infected with FMDV O1C. The cells were fixed on glass coverslips at 30-min intervals for up to 4 h after infection and stained with MAbs that exhibit specific fluorescence for FMDV 2C (3D10) or Beclin1 (H-300). The results indicated that a clear colocalization of FMDV 2C and Beclin1 proteins occurred at between 2 and 2.5 hpi, with both of the proteins displaying a small punctuated distribution pattern (Fig. 2C), supporting the hypothesis that the interaction between these two proteins occurs during viral infection. However, at other time points examined, no clear colocalization occurred, suggesting that the FMDV 2C and Beclin1 protein interaction occurs only during the early phase of infection.

Overexpression of the autophagy protein Beclin1 results in decreased FMDV replication. To understand how Beclin1 may affect FMDV replication, we measured fluctuations in the intracellular levels of Beclin1 and changes in virus yield from cells infected with FMDV. MCF-10A cells were infected (MOI = 10) with FMDV O1C, and cell lysates were collected every 30 min during the course of infection from 1 to 4 hpi. Samples were tested by Western blotting to assess the levels of Beclin1 present in the cell lysate. The results demonstrated that levels of Beclin1 remain unaltered during the course of infection with FMDV (Fig. 2D), ruling out the possibility that 2C may promote the degradation of Beclin1.

In order to assess the role of Beclin1 in FMDV replication, we attempted to manipulate the levels of Beclin1 in infected cells. Specific siRNA targeting Beclin1 was transfected into MCF-10A cells infected (MOI = 0.01) with FMDV O1C. Beclin1 expression decreased >80% in the treated cells, as determined by Western blotting (Fig. 3A) and as quantified using ImageJ software (Fig. 3B) (obtained from the National Center for Biotechnology Information) using the recommended procedure to calculate relative density compared to a control treated sample (siGlow). The decrease of Beclin1 expression in the Beclin1-specific siRNA-treated cells did not affect viral yields compared to siGlow (as a control)-treated cells (Fig. 3C). This result may indicate that decreased translation of Beclin1 does not affect virus replication or, alternatively, that remaining low levels of Beclin1 are still enough to allow FMDV replication.

The effect of overexpression of Beclin1 on FMDV replication was also assessed. MCF-10A cells were transfected with a plasmid utilizing a cytomegalovirus (CMV) promoter that overexpresses

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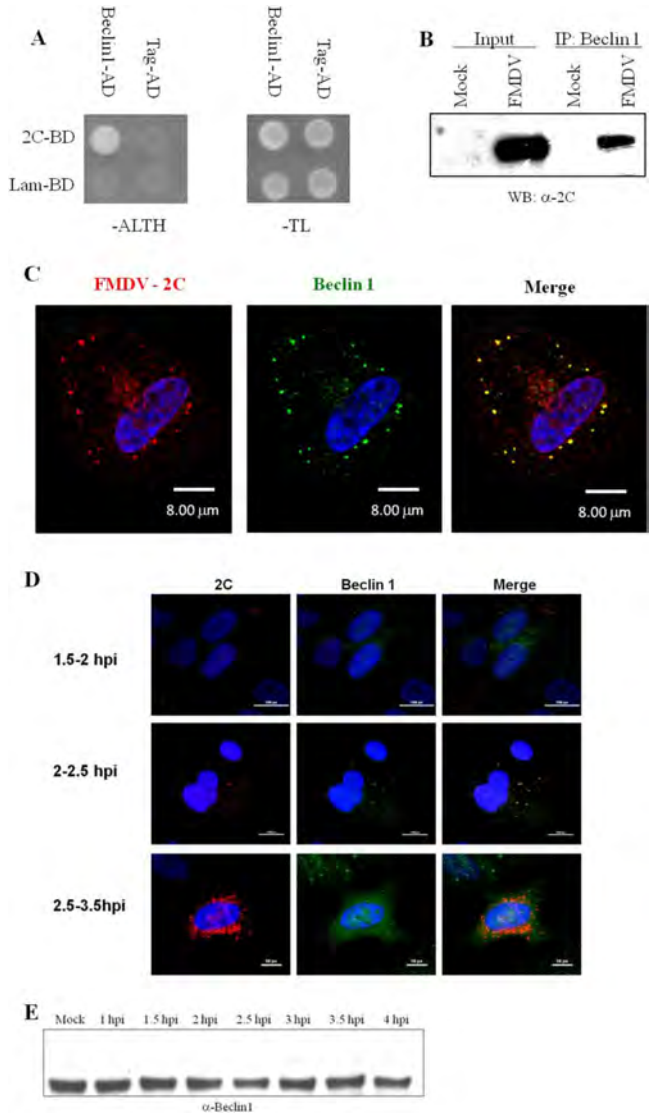

FIG 2 Protein-protein interaction of FMDV 2C with bovine Beclin1 in the yeast two-hybrid system (A), coimmunoprecipitation (B), confocal microscopy (C), and deconvolution microscopy (D). (A) Yeast strain AH109 was transformed with either GAL4-binding domain (BD) fused to FMDV 2C (2C-BD) or as a negative control human lamin C (Lam-BD). These strains were then transformed with GAL4 activation domain (AD) fused to Beclin1 (Beclin1-AD) or T antigen (Tag-AD) as indicated above each lane. Spots of strains expressing the indicated constructs containing 2×10^6 yeast cells were spotted on selective media for protein-protein interaction in the yeast two-hybrid system, either SD−Ade/His/Leu/Trp plates (−ALTH) or and nonselective SD−Leu/Trp (−TL) for plasmid maintenance only. (B) Western blot probing for FMDV 2C (band is ~40 kDa). Input cell lysate was from mock-infected (lane 1) or FMDV-infected (lane 2) cell lysates. Coimmunoprecipitation of FMDV 2C from mock-infected (lane 3) or FMDV O1C-infected (lane 4) cell lysates was performed using an antibody specific to Beclin1. (C and D) Analysis of the distribution of Beclin1 and FMDV 2C protein in MCF 10A cells. Cells were infected with FMDV O1C and processed by immunofluorescence staining as described in Materials and Methods. FMDV 2C was detected with mouse MAb (3D10) and visualized with Alexa Fluor 594 (red). Beclin1 was detected with monoclonal antibody (H-300) and visualized with Alexa Fluor 488 (green). Yellow indicates colocalization of the Alexa Flour 594 and 288 in the merged image. (E) Analysis of Beclin1 expression in MCF-10A cells. Cells were infected with FMDV O1C and processed for Western blotting at the indicated time points. Beclin1 was detected using MAb (H-300).

Beclin1-FLAG (pBeclin1-Flag; Addgene, plasmid no. 24388) or GFP (control plasmid) (vitality hrGFP; Agilent, catalog no. 240145). Overexpression of Beclin1 in transfected cells was assessed at 19 h posttransfection by Western blotting (Fig. 4A). Furthermore, MCF-10A cells overexpressing Beclin1-FLAG or GFP were infected (MOI = 0.1) with FMDV O1C, and virus present in the supernatant was measured hourly between 0 and 5 hpi and at 24 hpi. A considerable reduction (~2 logs) in virus titer was observed in cells overexpressing Beclin1-FLAG compared to cells overexpressing GFP and containing endogenous levels of Beclin1 (Fig. 4B). In addition, no differences were found when virus titers at the intracellular and extracellular compartment were compared (Fig. 4C). These results indicate that overexpression of Beclin1 causes a decrease in virus yield.

Effect of Beclin1 overexpression on viral VP1 and cellular UVRAG. UVRAG (UV radiation resistance-associated gene) binds the coiled-coil region of Beclin1 getting incorporated into autophagosomes and is later involved in autophagosome maturation to autophagolysosomes (12, 28), a process known to initiate the degradation of the material inside the autophagosome. It is possible that FMDV 2C prevents the incorporation of UVRAG into autophagosomes, which would later prevent UVRAG-mediated fusion of autophagosomes to lysosomes, thus preventing viral protein degradation. To examine this hypothesis, the colocalization of FMDV structural protein VP1 with cellular UVRAG was tested using FMDV-infected (MOI = 10) MCF-10A cells. No colocalization of VP1 and UVRAG was observed (Fig. 5A) at 4 hpi. However, when the same experiment was performed in cells overexpressing Beclin1, autophagosomes containing FMDV VP1 were seen to localize with UVRAG. MCF-10A cells were transfected with pBeclin1-FLAG and 19 h later infected (MOI = 10) with FMDV O1C. Four hours later, the colocalization of UVRAG and VP1 was clearly observed (Fig. 5B). Thus, the overexpression of Beclin1 would promote the incorporation of UVRAG into autophagosomes containing viral proteins, facilitating the process of autophagosome-lysosome fusion and provoking the degradation of virus particles.

To further examine that overexpression of Beclin1 associates with the fusion of autophagosomes that contain viral proteins with lysosomes, we examined the localization of FMDV structural protein VP1 with cellular lysosome-associated membrane protein 1 (LAMP1), a late lysosome marker. VP1-LAMP1 colocalization was first tested in MCF-10A cells infected with FMDV (MOI = 10). No colocalization of VP1 and LAMP1 was observed in these cells at 4 hpi (Fig. 6B). However, when the same experiment was performed in cells overexpressing Beclin1, FMDV VP1 was seen to localize with LAMP1 (Fig. 6A). MCF-10A cells were transfected with pBeclin1-FLAG and 19 h later infected (MOI = 10) with FMDV O1C. Four hours later, the colocalization of LAMP1 and VP1 was clearly observed (Fig. 6A). Thus, overexpression of Beclin1 associates with the fusion of autophagosomes containing viral proteins, with lysosomes perhaps causing virus degradation and the concomitant reduction in viral yield.

To provide additional confirmation that autophagosome fusion to lysosomes is not required during FMDV infection, we used bafilomycin A1, a specific inhibitor of vacuolar H^+-ATPase, which blocks UVRAG-induced autophagosome fusion to lysosomes (28). To determine the effect of bafilomycin A1 on FMDV replication, MCF-10A cells were pretreated for 30 min at 37°C with 0.5 μM bafilomycin A1. The medium was removed, and the

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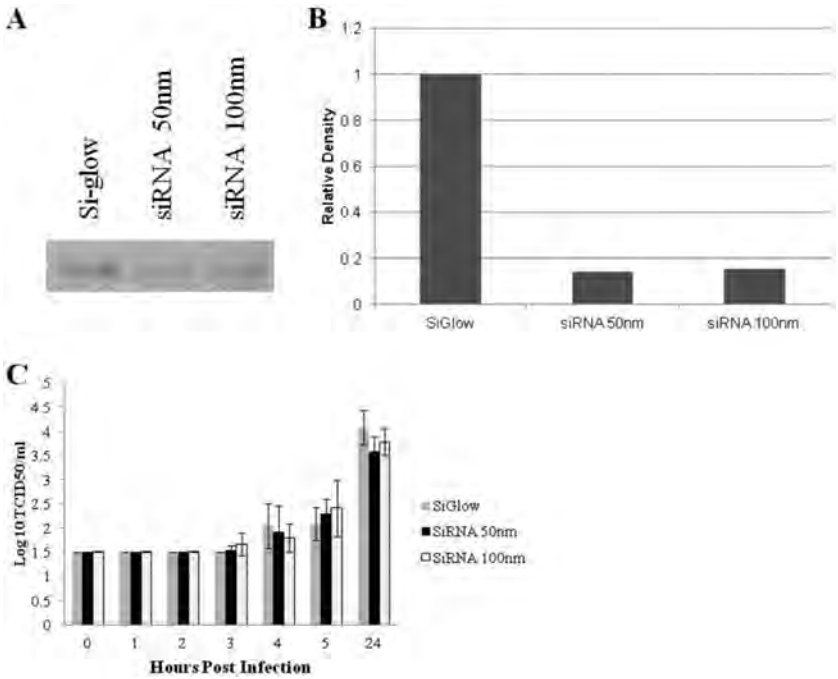

FIG 3 Effect of Beclin1-specific siRNA treatment on FMDV replication. MCF-10A cells were transfected with four RNA duplexes targeted Beclin1 or with a control siRNA, siGlo, for 48 h at 37°C as described in Materials and Methods. (A) Whole-cell lysates were collected 48 h after siRNA treatment and analyzed by Western blotting with a Beclin1-specific antibody (band is ~65 kDa). (B) Quantification of Beclin1 bands in Western blot showed in panel A using ImageJ analysis software. (C) Viral yields from FMDV infections in MCF-10A cells treated with siRNA to reduce the intracellular concentration of Beclin1. After transfection, triplicate plates were infected with FMDV O1C (MOI = 1). Titers were determined in BHK-21 cells and expressed as the $\log_{10}$ $TCID_{50}$/ml.

cells were infected with FMDV O1C (MOI = 1) for 1 h, acid washed, and then incubated with or without bafilomycin A1. Samples were taken at 1 and 5 hpi. The results (Fig. 7) showed that treatment with bafilomycin A1 had no effect on virus replication, indicating that autophagosome fusion to lysosomes is not required for FMDV replication.

Overexpression of the autophagy protein Bcl-2 blocks FMDV replication. Beclin1-dependent autophagy is known to be partially regulated by endogenous Bcl-2. Bcl-2 binds Beclin1, tethering it to the endoplasmic reticulum (9, 37). Overexpression of Bcl-2 inhibits the dissociation of Beclin1 from the endoplasmic reticulum, preventing the progression of Beclin1-induced autophagy (37). To determine the affect of overexpression of Bcl-2 on FMDV replication, Bcl-2 was overexpressed using a plasmid encoding GFP-Bcl-2 (pGFP-Bcl-2) under the CMV promoter (47); a plasmid encoding GFP alone, phrGFP II-N (Stratagene, catalog no. 240145), was used as a negative control. MCF-10A cells were transfected with either plasmid and, 24 h later, a high rate of GFP-Bcl-2 or GFP expression was observed by immunofluorescence (Fig. 8A). Transfected cells were then infected (MOI = 0.1) with FMDV O1C, and the virus yield assessed at 0, 5, and 24 hpi. A decrease (~1.5 to 2 $\log_{10}$) in virus titers in cell cultures overexpressing Bcl-2 (Fig. 8B) was measured.

Evidence supporting that Beclin1 is tethered to the endoplasmic reticulum in MCA-10A cells expressing Bcl-2GFP was obtained by demonstrating the colocalization of Bcl-2, Beclin1, and PDI, a marker for the endoplasmic reticulum (Fig. 8C). Therefore, tethering of Beclin1 to the endoplasmic reticulum, which could prevent the progression of Beclin1-induced autophagy, may be the reason that the overexpression of Bcl-2 is detrimental virus replication.

To examine whether the overexpression of Bcl-2 may impede viral entry into the autophagy pathway, we assessed the colocalization of FMDV VP1 and cellular LC3 (a microtubule-associated protein and well-known autophagosome marker) in infected cells overexpressing Bcl-2. MCF-10A cells transfected with GFP-Bcl-2 and 19 h later infected (MOI = 10) with FMDV O1C exhibited colocalization of FMDV VP1 and LC3 at 4 hpi (Fig. 9). This result suggests that a decreased FMDV yield in cells overexpressing Bcl-2 must be mediated by an unknown mechanism not affecting the entry of FMDV into the autophagy pathway.

To determine whether overexpression of Bcl-2 had any effect on autophagosome-lysosome fusion (as the overexpression of Beclin1 has), the colocalization of FMDV VP1 and UVRAG in infected cells overexpressing Bcl-2 was assessed. The same cell preparations used to determine FMDV VP1 and LC3 colocalization were used to analyze colocalization between FMDV VP1 and UVRAG. No colocalization was observed between these two proteins (data not shown) in FMDV-infected cells overexpressing Bcl-2, suggesting that the mechanism of viral inhibition in cells overexpressing Bcl-2 should be different than that triggered in cells overexpressing Beclin1.

Identification of the Beclin1 binding site on FMDV 2C. To determine the binding site(s) for Beclin1 present in 2C, an alanine scanning mutagenesis approach was used. We used site-directed mutagenesis to construct a set of 46 mutant 2C proteins containing sequential stretches of seven amino acids where the native amino acid residues were substituted by alanine residues (Fig. 10A). These mutated 2C proteins were assessed for their ability to bind Beclin1 utilizing the yeast two-hybrid system. 2C proteins containing mutations in areas 15, 16, 17, 19, 20, 29, 33 to 40, and 42 were unable to bind Beclin1 (Fig. 10A). To ensure that all 2C

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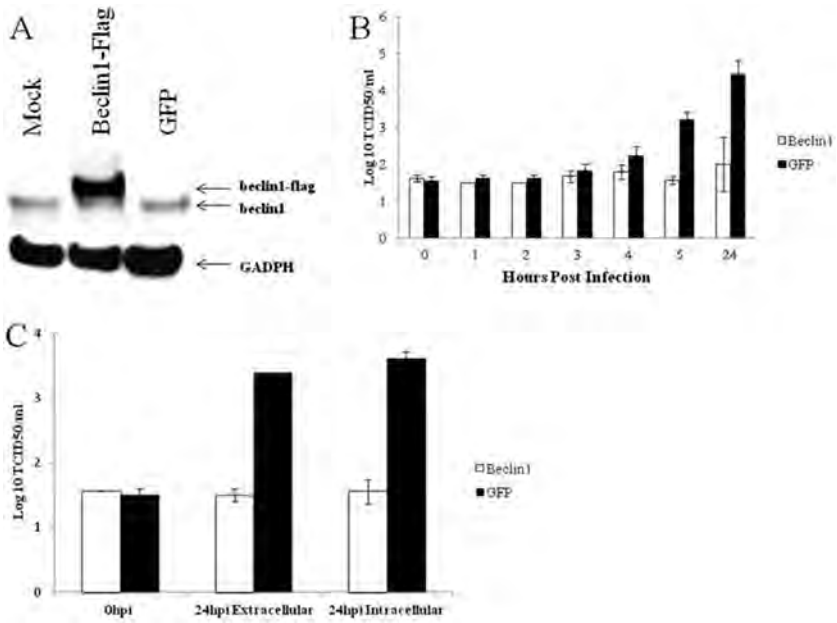

FIG 4 Viral yields from FMDV infections in MCF-10A cells overexpressing Beclin1-Flag. MCF-10A cells were transfected either with a plasmid encoding Beclin1-Flag (pBeclin-Flag) or GFP (pGFP), as a control as described in Materials and Methods. (A) Western blot showing endogenous intracellular levels of Beclin1, as well as overexpressed levels of Beclin1-Flag in MCF-10A cells transfected either with pBeclin-Flag or pGFP. As a loading control, the detection of the levels of intracellular GADPH (40 kDa) was performed. (B) After transfection, triplicate plates were infected with FMDV O1C (MOI = 0.1). Titers were determined in BHK-21 cells and are expressed as the $\log_{10}$ $TCID_{50}$/ml. (C) Triplicate plates were infected with FMDV O1C (MOI = 0.1). Extracellular and intracellular samples were taken at 24 hpi. Titers were determined in BHK-21 cells and are expressed as the $\log_{10}$ $TCID_{50}$/ml.

alanine mutants were still able to be expressed in the yeast two-hybrid system, protein MCM7-AD (another bovine host protein that was detected as a binding partner for 2C) was used as an internal control. MCM7-AD was able to interact with all 2C mutants lacking Beclin1 binding (Fig. 10C), thereby demonstrating

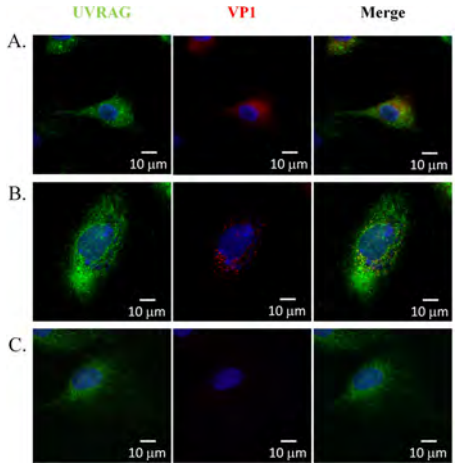

FIG 5 Analysis of distribution of FMDV VP1 and UVRAG in FMDV-infected MCF-10A cells. Mock-transfected cells (A) or cells transfected with Beclin1-Flag (B) were infected (MOI = 10) 24 h posttransfection with FMDV O1C. These preparations were processed for immunofluorescence staining as described in Materials and Methods, along with uninfected control cells (C). UVRAG was detected with rabbit polyclonal antiserum (Sigma-Aldrich, catalog no. U7508) and visualized with Alexa Fluor 488 (green). FMDV VP1 was detected with a MAb (10GA) and visualized with Alexa Flour 594 (red). Yellow indicates colocalization of the Alexa Flour 594 and 288 in the merged image.

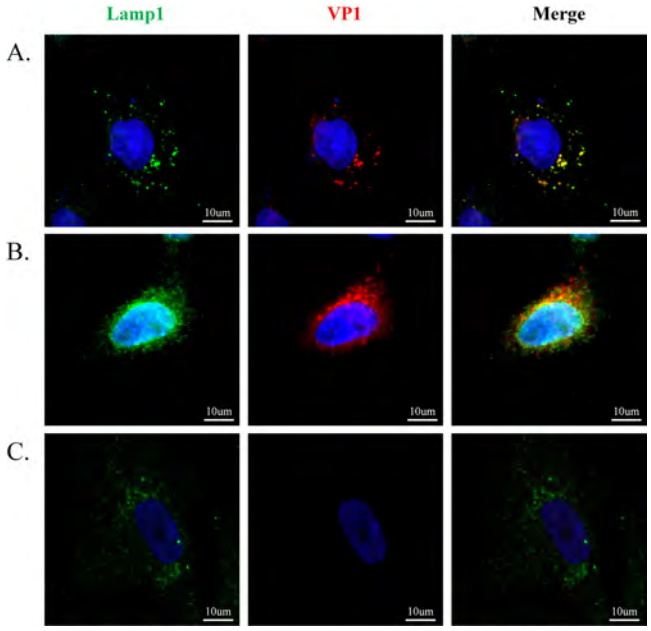

FIG 6 Analysis of distribution of FMDV VP1 and Lamp1 in FMDV-infected MCF-10A cells. (A) Cells transfected with Beclin1-Flag or (B) Mock transfected cells were infected (MOI = 10) 24 h posttransfection with FMDV O1C. These preparations were processed for immunofluorescence staining as described in Materials and Methods, along with (C) uninfected control cells. Lamp1 was detected with mouse MAb (BD Biosciences 555798) and visualized with Alexa Fluor 488 (green). FMDV VP1 was detected with a MAb (10GA) and visualized with Alexa Flour 594 (red). Yellow indicates colocalization of the Alexa Flour 594 and 288 in the merged image.

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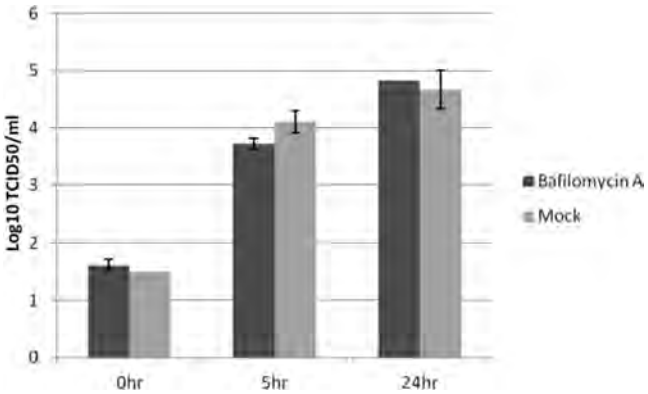

FIG 7 Effect of bafilomycin A1 on FMDV replication in MCF-10A cells. Cells were pretreated with bafilomycin A1 at a concentration of 0.5 μM for 30 min and then infected with FMDV O1Ca (MOI = 10) for 1 h at 37°C. The cells were then treated or mock treated with bafilomycin A1. Samples were taken at 1 h and at 5 and 24 h after infection. Titers were determined in BHK-21 cells and are expressed as the $\log_{10}$ $TCID_{50}$/ml.

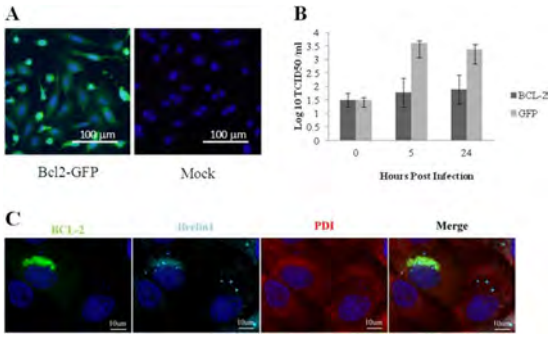

FIG 8 Viral yields in FMDV-infected MCF-10A cells overexpressing Bcl-2. MCF-10A cells were mock transfected or transfected with a plasmid encoding Bcl-2-GFP (pBcl-2-GFP) or GFP (pGFP) as a control. (A) Detection of Bcl-2 overexpression in Bcl-2-GFP transfected (left panel) or mock transfected (right panel) MCF-10 cells by fluorescence microscopy. (B) After transfection, triplicate plates were infected (MOI = 0.1 $TCID_{50}$/cell) with FMDV O1C. Titers were determined in BHK-21 cells and expressed as the $\log_{10}$ $TCID_{50}$/ml for the extracellular virus. (C) Analysis of distribution of Beclin1 and PDI in GFP-Bcl-2-expressing MCF10A cells. Cells were processed for immunofluorescence staining as described in Materials and Methods. Bcl-2 was visualized by GFP (Green). Beclin1 was detected with a MAb (H-300) and visualized with Alexa Flour 647 (Light blue). PDI was detected with an MAb (Affinity Bioreagents MA3-018) and visualized with Alexa Flour 488 (red).

that mutating these areas specifically interrupted the binding between Beclin1 and 2C.

Reverse genetics were used to assess the effect of 2C mutations identified as critical in mediating the interaction between 2C and Beclin1. Infectious clones of FMDV O1C (5) contained areas of 2C harboring the same alanine substitutions for 2C-15, 2C-16, 2C-17, 2C-19, 2C-20, and 2C-36 selected from areas shown to alter 2C-Beclin1 reactivity in the yeast two-hybrid model that were sequentially introduced into the infectious clone construct. These infectious clone constructs were then used to produce the corresponding RNAs by *in vitro* transcription, which were then used in cell transfections to produce their respective FMDV progeny. Although transfection with parental FMDV O1C RNA produced viable virus progeny, mutations in 2C-15, 2C-16, 2C-17, 2C-19, 2C-20, and 2C-36 consistently resulted in nonviable virus. A more detailed mapping of the area of interaction between Beclin1 and 2C was performed to exclude the possibility of detrimental effects from unidentified causes by reducing the number of amino acids mutated while still disrupting the Beclin1-2C interaction. Regions in mutants 2C-16, 2C-17, and 2C-19 were further subdivided into three separate subareas that were individually assessed for their reactivities with Beclin1 in the yeast two-hybrid system. This procedure resulted in several smaller areas (comprising two to three residues each) responsible for the Beclin1-2C interaction: 16A, 16B, 17A, 17C, and 19A, while maintaining the MCM7-2C interaction (Fig. 10B). Interestingly, none of the infectious clones harboring these mutated areas was able to produce viable progeny, suggesting that amino acid residues critical to FMDV 2C-Beclin1 interaction are also critical for virus replication. These results suggest that the interaction between 2C and Beclin1 is essential to the process of virus replication. However, we cannot rule out the possibility that these mutations in 2C have other effects by changing the structure of 2C or by inhibiting 2C to interact with other proteins.

DISCUSSION

Viruses have developed complex mechanisms to manipulate normal cellular pathways to facilitate replication and to evade host defense mechanisms. To do so, viruses often interact with cellular proteins to modify their function, thus modifying natural cellular pathways. We report here that FMDV nonstructural protein 2C binds to a central regulator of autophagy pathway, Beclin1. Beclin1 plays dual roles in the autophagy pathway: involvement at the initiation step of autophagosome formation, and later, at autophagosome fusion to the lysosome. Several studies have suggested that the autophagy pathway can function as an antiviral pathway by degrading viruses or as a proviral pathway, helping viruses replicate or exit the cell (17, 32, 40). In this report, a yeast two-hybrid model was used to show that Beclin1 is a specific protein binding partner for viral 2C. In concordance with this result, we also demonstrated the occurrence of coimmunoprecipitation of 2C with Beclin1 and colocalization of 2C with Beclin1 in cells infected with FMDV.

Manipulation of the autophagy pathway has been shown to occur by a wide range of viruses (17) and, interestingly, all of them produce their effect by binding to Beclin1. Three proteins in different herpesviruses have been shown to inhibit the formation of autophagosomes: herpes simplex virus 1 (HSV-1) protein ICP35.5 (26), Kaposi's sarcoma herpesvirus protein orf16 (Bcl-2 homologue) (45), and murine gamma herpesvirus 68 protein M11 (Bcl-2 homologue) (23, 41). All three proteins antagonize Beclin1 to prevent its incorporation into autophagy complexes, possibly by anchoring Beclin1 to the endoplasmic reticulum, resulting in the suppression of the initial steps of autophagy. As an example, in HSV-1, deletion of the binding domain in ICP35.5 that binds Beclin1 causes reduced neurovirulence in mouse models. The neurovirulence in mouse models can be regained by using knockout mice with a defect in the autophagy pathway (26). RNA viruses utilize the cellular autophagy pathway differently than DNA viruses, using it to benefit their replication strategy by stabilizing autophagosomes and preventing autophagosome-lysosome fusion. Infection by poliovirus, HCV, or FMDV results in an accumulation of autophagosomes, benefiting viral replication through mechanisms not well understood (1, 20, 35). In the case

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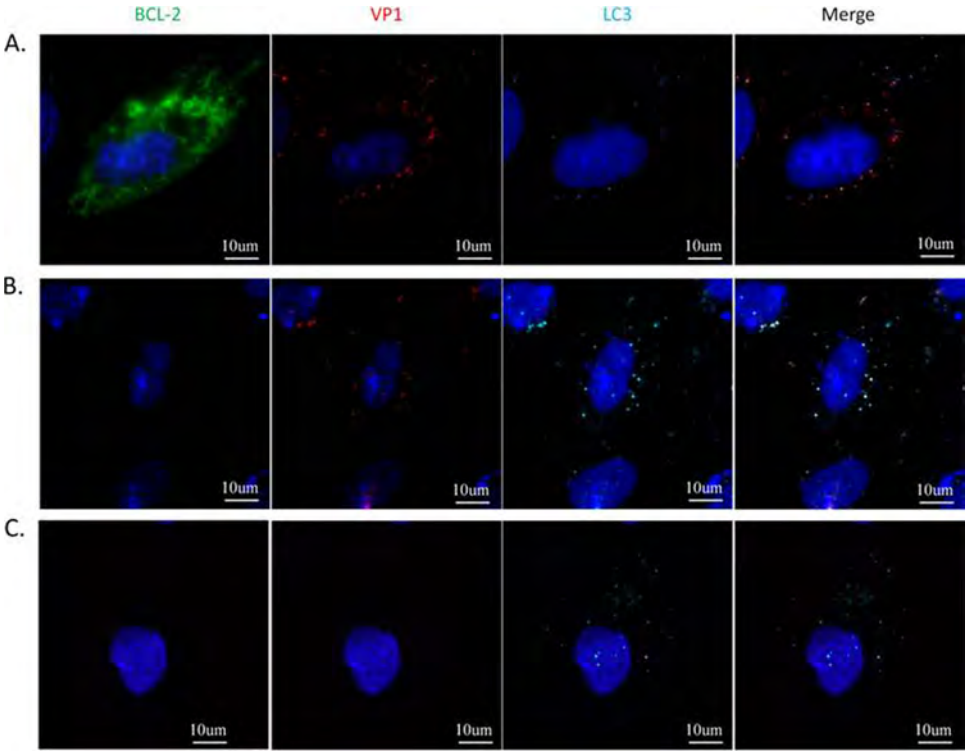

FIG 9 Analysis of distribution of LC3 and FMDV VP1 in GFP-Bcl-2-expressing (A) and mock-transfected (B) MCF10A cells infected with FMDC O1C (MOI = 10) 24 h posttransfection with FMDV O1C. These preparations were processed for immunofluorescence staining as described in Materials and Methods, along with uninfected control cells (C). Bcl-2 was visualized by using GFP (green). FMDV VP1 was detected with a MAb (10GA) and visualized with Alexa Flour 488 (red). LC3 was detected with rabbit polyclonal antiserum (catalog no. 53341; Anaspec, Fremont, CA) and visualized with Alexa Fluor 647 (light blue). Pink indicates colocalization of the Alexa Flour 488 and Alexa Flour 647 in the merged image.

of influenza virus A, autophagosomes are stabilized by protein M2, which binds Beclin1 to prevent the fusion of autophagosomes to lysosomes (16). In a similar manner, human immunodeficiency virus (HIV) stabilizes autophagosomes by binding of viral protein nef to Beclin1 (24). Blocking autophagosome fusion to lysosomes prevents RNA virus degradation by the lysosomes (17).

In a previous study we demonstrated that FMDV triggers the autophagy machinery, enhancing viral replication (35). Furthermore, chemical stimulation or inhibition of the autophagy process directly correlated with an increase or decrease in virus production. To understand how Beclin1 plays a role during FMDV replication, we analyzed the effect of overexpression of Beclin1 (Fig. 4B). The overexpression of Beclin1 resulted in a severe decrease in viral yields. It is possible that Beclin1overexpression favors UVRAG-mediated incorporation of viral proteins into autophagosomes and later the fusion of these phagosomes to lysosomes, as evidenced by the sequential colocalization of viral VP1 with UVRAG (Fig. 5B) and LAMP1 (Fig. 6A). Both events are absent during the normal process of FMDV replication (Fig. 5 and 6B). It is possible that during viral infection 2C binding to Beclin1 would prevent autophagosome incorporation of UVRAG by blocking the UVRAG binding site in Beclin1, thereby inhibiting UVRAG-mediated autophagosome maturation. These results also suggest that FMDV 2C may function in a similar form as HIV nef (24) or influenza virus A M2 (16), by binding to Beclin1 to help the virus escape the autophagy-induced protein degradation pathway.

To determine whether autophagosome maturation is necessary for FMDV replication, we tested the effect of bafilomycin A1, an inhibitor of autophagosome-lysosome fusion, on FMDV replication. Our results clearly support the fact that autophagosome-lysosome fusion is not required for FMDV replication (Fig. 7) and determined that there was no difference in viral yield, suggesting that fusion with lysosomes is not a required process for FMDV replication.

It is well known that Beclin1 is partially regulated by Bcl-2 (9, 37). Therefore, we evaluated the effect of overexpression of Bcl-2 in MCF-10A cells infected with FMDV. As shown in Fig. 8B, the overexpression of Bcl-2 is detrimental for virus replication. Interestingly, these cells still showed colocalization of 2C viral protein with LC3 (Fig. 9), an autophagy hallmark, but not with UVRAG. This result suggests the virus was still able to enter the autophagy pathway (perhaps in a Beclin1-independent way) even though the autophagosome-lysosome fusion did not occur. These observations suggest that overexpressing Bcl-2 should trigger a mechanism for disrupting FMDV replication mediated by a different pathway than that induced by overexpression of Beclin1. The difference in the inhibitory mechanisms mediated by the overexpression of Beclin1 and Bcl-2 suggests the possible existence of multiple mechanisms allowing FMDV to interact with the autophagy process in favor of viral replication.

Interestingly, Beclin1 as it was identified in the yeast two-hybrid screen contained only amino acids 1 to 211 of bovine Beclin1 (NCBI reference sequence NP_001028799.1), indicating that the Beclin1 protein recovered in the yeast two-hybrid experiment was a truncated version containing areas responsible for binding Bcl-2

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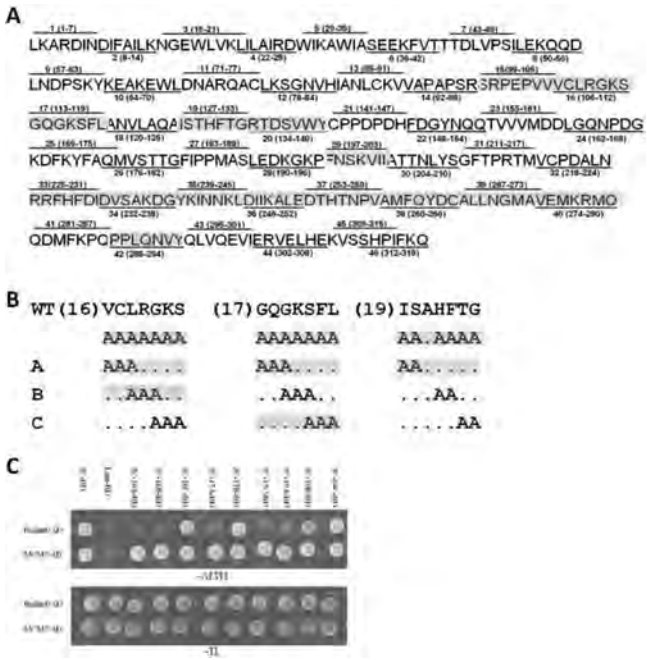

FIG 10 Scheme showing FMDV 2C alanine mutants used in the present study. (A) Each alanine 2C name mutant is followed, in parentheses, by the amino acid residues mutated for that mutant. All indicated residues were mutated to an alanine. The highlighted 2C mutants represent mutations that resulted in lack of binding of 2C to Beclin1 in the yeast two-hybrid system. (B) Subdivisions (labeled A, B, and C) of the original alanine scanning mutants 16, 17, and 19. Highlighted mutants were found not to bind Beclin1 in the yeast two-hybrid system. (C) Yeast strain AH109 was transformed with either GAL4-binding domain (BD) fused to FMDV 2C (2C-BD), the indicated FMDV 2C mutation, or as a negative control human lamin C (Lam-BD). These strains were then transformed with GAL4 activation domain (AD) fused to Beclin1 (Beclin1-AD) or MCM7 (MCM7-AD) as indicated above. Strains expressing the indicated constructs containing 2×10^6 yeast cells were spotted onto selective media to evaluate protein-protein interaction in the yeast two-hybrid system, using either SD−Ade/His/Leu/Trp plates (−ALTH) or else nonselective SD−Leu/Trp (−TL) for plasmid maintenance only.

and UVRAG: the BH3 domain (amino acids 114 to 123) (6, 13) and part of the CCD domain (amino acids 144 to 269) (27). Therefore, it is possible that FMDV 2C competes with UVRAG or Bcl-2 for binding to Beclin1 and that, to prevent autophagosome-lysosome fusion and subsequent virus destruction, FMDV 2C blocks the UVRAG binding site in Beclin1. However, additional studies mapping the binding domain in Beclin1 would be needed to determine whether this is true.

Although it appears that the 2C-Beclin1 interaction is necessary for virus replication and that the autophagy pathway is used for replication by FMDV, the precise mechanism still needs to be elucidated for how FMDV enters the autophagy pathway and manipulates the autophagosome to facilitate its replication and why in previous studies the chemical induction of autophagosomes increased viral yield (35). Previous studies have shown that FMDV enters the cell via endosomes (33); however, there was no colocalization between MPR (mannose 6-phosphate receptor), an endosomal marker, and FMDV proteins (34), suggesting that the mechanism of FMDV entering the autophagy pathway is not due to endosome fusion to the autophagosome, as was shown with dengue virus (36). Therefore, FMDV could enter the autophagy pathway by different means that are independent of both endosome fusion to the autophagosome and of Beclin1-induced autophagy. Further studies are required to explore this possibility.

The results reported here identify, for the first time, a cellular host protein, Beclin1 that interacts with a viral protein of FMDV, 2C. This interaction appears to be critical for virus growth since mutated FMDV genomes harboring the 2C mutations that disrupted the interaction between 2C and Beclin1 resulted in viruses completely unable to replicate in cell cultures (Fig. 10). Importantly, 2C-Beclin1 interaction appears to modulate the autophagy pathway by preventing virus destruction in the final steps of autophagy. This presents new possibilities of exploration for FMDV pathogenesis, and further work still needs to be done to explore other cellular proteins that may play a role during FMDV infection. Further understanding host protein-viral protein relationships will encourage the design of novel therapeutic strategies that disrupt viral-host interactions.

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Identification of peptides from foot-and-mouth disease virus structural proteins bound by class I swine leukocyte antigen (SLA) alleles, SLA-1*0401 and SLA-2*0401

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Summary

Characterization of the peptide-binding specificity of swine leukocyte antigen (SLA) class I and II molecules is critical to the understanding of adaptive immune responses of swine toward infectious pathogens. Here, we describe the complete binding motif of the SLA-2*0401 molecule based on a positional scanning combinatorial peptide library approach. By combining this binding motif with data achieved by applying the NetMHCpan peptide prediction algorithm to both SLA-1*0401 and SLA-2*0401, we identified high-affinity binding peptides. A total of 727 different 9mer and 726 different 10mer peptides within the structural proteins of foot-and-mouth disease virus (FMDV), strain A24 were analyzed as candidate T-cell epitopes. Peptides predicted by the NetMHCpan were tested in ELISA for binding to the SLA-1*0401 and SLA-2*0401 major histocompatibility complex class I proteins. Four of the 10 predicted FMDV peptides bound to SLA-2*0401, whereas five of the nine predicted FMDV peptides bound to SLA-1*0401. These methods provide the characterization of T-cell epitopes in response to pathogens in more detail. The development of such approaches to analyze vaccine performance will contribute to a more accelerated improvement of livestock vaccines by virtue of identifying and focusing analysis on bona fide T-cell epitopes.

Keywords binding motif, prediction, SLA, T-cell epitope

Introduction

The major histocompatibility complex class I (MHC I) molecules are highly polymorphic proteins that bind 8 to 11 amino acid (AA) peptides derived from the intracellular protein metabolism. In humans, such MHC class I molecules are referred to as human leukocyte antigens (HLA) and in swine are termed swine leukocyte antigens (SLA). These integral membrane proteins are found on all nucleated cells in vertebrates and play a crucial role in the cell-mediated immune response against viruses, other intracellular pathogens and transformed cells (cancer). When an endogenous peptide engages in a productive interaction with a MHC class I heavy chain and the non-polymorphic MHC I-stabilizing subunit molecule, beta-2-microglobulin (β_2m), they generate a heterotrimeric peptide/MHC/β_2m (pMHC) complex that is displayed at the cell surface. When surface-exposed MHC complexes incorporate foreign peptides, derived from a virus for instance, these complexes serve as targets for the T-cell receptor (TCR) on CD8$^+$, MHC I-restricted, peptide-specific, cytotoxic T lymphocytes (CTLs). In this way, endogenous peptides are constantly presented on the cell surface, signaling the cell's current state of integrity to the immune system of the host. If the peptide presented is of foreign origin, T cells may become activated and kill the infected or transformed cell (Doherty & Zinkernagel 1975; Zinkernagel & Doherty 1975; Bevan 1995; Harty *et al.* 2000).

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The effect of polymorphism of the MHC I molecule is reflected in the different peptide-binding motifs that different class I molecules have, with peptide-binding anchor positions (APs) typically in positions 1, 2, 3 and 9 (Sette & Sidney 1999; Lamberth *et al.* 2008). More than 5400 different HLA-I alleles have been registered according to the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/stats.html), and this number is growing rapidly as more efficient HLA typing techniques are employed worldwide. The same has been seen for SLA typing in recent years, with highly specific primer-based typing systems being used with success for both class I and class II genes in inbred as well as outbred pigs (Ho *et al.* 2006, 2010a,b; Yeom *et al.* 2010). As such, there is an equivalent need for biochemical data. However, characterizing the peptide-binding motif for each of these alleles is not feasible. Rather, application of the NetMHCpan peptide-binding predictor algorithm (Nielsen *et al.* 2007; Hoof *et al.* 2009) developed from the extensive human database available provides a method for quantitative predictions of peptide binding to any MHC protein. Thus, potential peptides that will bind can be reduced from hundreds (or even thousands) to a more dexterous number that can be tested.

Previously, we reported that the bioinformatics tools originally developed to cover all HLA-I molecules, despite the evolutionary distance, can be applied to SLA-I molecules (Hoof *et al.* 2009; Pedersen *et al.* 2011). Here, we have used the NetMHCpan predictor to predict peptides likely to be bound by the SLA-1*0401 and SLA-2*0401 molecules using a NetMHCpan rank score threshold of 2.00%. In this way, we were able to limit the number of possible peptides derived from the structural proteins of foot-and-mouth disease virus (FMDV), strain A24, from more than 700 to <16. Furthermore, by comparing the sequences of these peptides with the AA binding preferences exhibited by the SLA molecule in question, we were able to further reduce the number of peptides to be tested. The final peptide candidates were then analyzed *in vitro* for the ability to induce the formation of pMHC complexes with a high enough affinity to allow proper folding using the recombinant SLA-1*0401 and SLA-2*0401 class I heavy chains.

Through the production of recombinant SLA molecules, mapping of their peptide-binding motifs using universal peptide libraries, that is, the positional scanning combinatorial peptide libraries (PSCPL) (Stryhn *et al.* 1996; Lamberth *et al.* 2008), and the screening of AA sequences from FMDV structural proteins for possible peptide/MHC matches using NetMHCpan (Hoof *et al.* 2009), we were able to identify several *in vitro* binding peptides and characterize the SLA-2*0401 binding motif. Such data add to the knowledge of the binding characteristics for SLA molecules in general and could eventually lead to further insight into specific viral-derived peptides that induce porcine CTLs during infection. The result of taking these approaches is a rapid and accurate capacity to identify T-cell epitopes and the subsequent ability to measure specific T-cell immunity following vaccination or infection (Patch *et al.* 2011).

Materials and methods

Recombinant construct encoding the SLA-2*0401 protein

SLA-2*0401 recombinant protein was produced using a previously described standard expression system (Pedersen *et al.* 2011). Briefly, a transmembrane, truncated fragment encompassing residues 1–276 of the SLA-2*0401 alpha chain (Genscript, Appendix S1) followed by an FXa-BSP-HAT tag (FXa = factor Xa cleavage site comprising the AA sequence IEGR = I (isoleucine), E (glutamine acid), G (glycine), R (arginine), BSP = biotinylation signal peptide, HAT = histidine affinity tag for purification purposes) was inserted into a pET28a expression vector (Novagen). The construct was transformed into DH5α cells and sequenced (ABI Prism 3100Avant; Applied Biosystems) (Hansen *et al.* 2001). The validated construct of interest was transformed into an *Escherichia coli* (E. coli) production cell line, BL21 (DE3), containing the pACYC184 expression plasmid (Avidity) with an IPTG (Isopropyl-beta-D-thiogalactoside)-inducible BirA gene to express biotin-ligase. This leads to almost complete *in vivo* biotinylation of the desired product (Schmidt *et al.* 2009).

Expression and purification of recombinant SLA-2*0401 and human beta-2 microglobulin

SLA-2*0401 heavy chain proteins were produced as previously described (Pedersen *et al.* 2011). *E. coli* BL21(DE3) cells containing the SLA proteins were lyzed in a cell disrupter (Constant Cell Disruptor Systems set at 2300 bar), and the released inclusion bodies were isolated by centrifugation (Sorval RC6, 20 min, 17 000 ***g***). The inclusion bodies were washed twice in PBS, 0.5% NP-40 (Sigma), 0.1% deoxycholic acid (DOC; Sigma) and extracted into urea–Tris buffer (8 M urea, 25 mM Tris, pH 8.0). SLA-2*0401 heavy chain proteins extracted in 8 M urea were purified as previously described for the SLA-1*0401 molecule (Pedersen *et al.* 2011). In brief, this was carried out by successive immobilized metal adsorption, hydrophobic interaction, and Superdex-200 size exclusion chromatography. Throughout purification and storage, the MHC I heavy chain proteins were dissolved in 8 M urea to keep them denatured. The pre-oxidized, denatured proteins were stored at −20 °C in Tris-buffered 8 M urea. Recombinant human β_2m was expressed and purified as described elsewhere (Ostergaard *et al.* 2001; Ferre *et al.* 2003). Complexing the porcine alpha chains with human β_2m and peptide allowed the application of the ELISA for class I MHC folding using mouse anti-human β_2m, as described below and previously (Pedersen *et al.* 2011). Reagents for porcine β_2m are not available.

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Peptides and peptide libraries

All peptides were purchased from Schafer-N. Briefly, they were synthesized by standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, purified by reverse-phase high-performance liquid chromatography (to at least >80% purity, frequently 95–99% purity), validated by mass spectrometry and quantitated by weight.

PSCPL peptides were used to determine the SLA-2*0401-binding motif. The experimental strategy of PSCPL has previously been described for both HLA (Stryhn *et al.* 1996) and SLA (Pedersen *et al.* 2011) proteins. Briefly, pools of random peptides with a single fixed AA in a fixed peptide position for every AA in every peptide position were used to determine the MHC-binding pocket motif of the MHC I protein. PSCPL peptides were used in a quantitative ELISA as described for the SLA-1*0401 molecule (Pedersen *et al.* 2011). The relative binding (RB) affinity of each PSCPL sublibrary was determined as RB (PSCPL) = $ED_{50}(X_9)/ED_{50}$(PSCPL) and normalized, so that the sum of the RB values of the 20 naturally occurring AA equaled 20 (ED_{50} being the concentration needed to half-saturate a low concentration of MHC class I molecules). Anchor position values were calculated by the equation $\sum(RB - 1)^2$.

PSCPL peptides were synthesized using standard solid-phase Fmoc chemistry on 2-chlorotrityl chloride resins. Briefly, an equimolar mixture of 19 of the common Fmoc AAs (excluding cysteine) was prepared for each synthesis and used for coupling in eight positions, whereas a single type of Fmoc AA (including cysteine) was used in one position. This position was changed in each synthesis starting with the N-terminus and ending with the C-terminus. In one synthesis, the AA pool was used in all nine positions. The library therefore consisted of $20 \times 9 + 1 = 181$ individual peptide libraries:

- 20 PSCPL sublibraries describing position 1: AX_8, CX_8, DX_8, … YX_8
- 20 PSCPL sublibraries describing position 2: XAX_7, XCX_7, XDX_7, … XYX_7
- etc.
- 20 PSCPL sublibraries describing position 9: X_8A, X_8C, X_8D, … X_8Y
- a completely random peptide library: X_9

X denotes the random incorporation of AA from the mixture, whereas the single-letter AA abbreviation is used to denote the identity of the fixed AA. Following synthesis, the peptides were cleaved from the resin in trifluoroacetic acid/1,2-ethanedithiol/triisopropylsilane/water 95:2:1:3 v/v/v/v, precipitated in cold diethyl ether and extracted with water before desalting on C18-columns, freeze drying and weighing.

ELISA for affinity of peptide binding

Peptides were tested for their ability to form complexes with the SLA-1*0401 and SLA-2*0401 proteins using a previously described immunosorbent assay (Sylvester-Hvid *et al.* 2002). Complexes of heavy chain with human β_2m and peptide were formed in 96-well plates (Thermo Scientific). All peptides were dissolved in 150 μl standard folding buffer [PBS, 0.1% pluriol (Lutrol F-68, BASF), pH 7.0] using sonication for 5 min. A fivefold dilution series of peptide in 12 consecutive points was performed in standard folding buffer. Following a 48-h incubation (18 °C), samples were transferred to streptavidine-coated plates (Nunc, DK-4000) and incubated for 3 h at 4 °C. Plates were washed in wash buffer (PBS, 0.05% Tween-20) and probed with a mouse anti-hβ_2m mAb (Brodsky *et al.* 1979), BBM1 (70 nM), for 1 h at room temperature. All wells were washed in wash buffer and detection antibody [HRP-conjugated goat-anti-mouse IgG (1.13 nM); Sigma] was added followed by a 1-h incubation at room temperature. Plates were washed and substrate (TMB; Kirkegaard & Perry Laboratories) was added to all wells. Reactions were stopped using H_2SO_4 (0.3 M) and plates read at 450 nm using a ELx808 Microplate reader (Biotek). Data were analyzed using EXCEL and PRISM software.

A pre-folded, biotinylated FLPSDYFPSV/HLA-A*02:01 (Kast *et al.* 1994) complex was used as a standard to convert OD_{450} values to the amount of complex formed using the second-order polynomial ($Y = a + bX + cX^2$), thereby enabling a direct conversion of the actual peptide concentration offered to the actual concentration of correctly formed pMHC complex. Because the effective concentration of MHC (2–5 nM) used in these assays is below the equilibrium dissociation constant (K_D) of most high-affinity peptide–MHC interactions, the peptide concentration, ED_{50}, leading to half-saturation of the MHC is a reasonable approximation of the affinity of the interaction.

Results

Analysis of the peptide-binding specificity of SLA-2*0401 with the PSCPL

The porcine MHC class I heavy chain, SLA-2*0401, was tested for binding PSCPL using human β_2m to support folding, as previously described (Stryhn *et al.* 1996; Pedersen *et al.* 2011). Similar to the SLA-1*0401 molecule (Pedersen *et al.* 2011), no differences were seen for the SLA-2*0401 allele when comparing human β_2m and porcine β_2m in supporting MHC peptide binding and complex folding (data not shown). Each peptide sublibrary was titrated to determine the EC50 (K_D) for SLA-2*0401. The peptide-binding preferences for the SLA-2*0401 heavy chain protein are shown in Table 1. Amino acids in each peptide position were assigned a RB value as described in Materials and Methods. Amino acid residues with RB values above 2 (shown in boldface, Table 1) indicate support of peptide binding, whereas AAs having RB values below 0.5 (shown in italics, Table 1) are considered disfavored. A

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Table 1 SLA-2*0401-binding motif

	Amino acid position in peptide								
SLA-2*0401	1	2	3	4	5	6	7	8	9
A	*0.3*	*0.4*	1.3	1.9	0.8	0.6	**2.6**	0.8	*0.0*
C	*0.4*	*0.1*	0.6	0.9	*0.2*	*0.4*	*0.5*	1.0	*0.0*
D	*0.5*	*0.3*	0.7	*0.4*	1.5	**2.2**	1.3	1.5	*0.1*
E	0.7	*0.1*	1.1	0.8	0.8	*0.1*	**3.7**	**2.7**	*0.0*
F	**2.0**	*0.2*	*0.3*	0.9	1.3	1.5	*0.4*	0.8	**3.0**
G	*0.1*	0.9	*0.4*	*0.2*	1.0	*0.2*	*0.4*	1.6	*0.1*
H	1.0	*0.1*	*0.1*	*0.5*	0.9	**2.3**	*0.4*	0.7	*0.3*
I	1.2	0.7	0.6	0.5	1.2	0.8	1.7	0.6	*0.3*
K	*0.5*	*0.2*	*0.1*	1.1	*0.4*	*0.3*	*0.2*	0.6	*0.1*
L	*0.5*	*0.3*	0.9	0.8	0.8	1.0	0.7	0.7	*0.1*
M	**2.2**	*0.3*	1.4	0.8	1.1	1.5	1.2	1.0	*0.3*
N	0.8	**8.9**	1.6	0.7	1.8	*0.3*	0.8	0.7	*0.1*
P	*0.1*	*0.5*	0.7	**3.3**	1.4	1.6	0.6	0.8	*0.0*
Q	1.4	*0.2*	0.8	1.5	**3.3**	**2.5**	**2.8**	1.0	*0.0*
R	**2.2**	*0.2*	1.4	1.4	0.5	1.2	*0.4*	1.1	*0.1*
S	1.6	**3.3**	1.0	0.8	0.9	0.6	0.8	0.9	*0.1*
T	0.9	1.8	1.1	0.8	0.5	1.2	*0.4*	0.7	*0.1*
V	1.1	0.8	0.8	*0.4*	0.7	*0.2*	*0.3*	0.8	*0.0*
W	0.9	*0.2*	**2.1**	1.0	0.5	0.9	*0.3*	1.0	**11.2**
Y	1.9	*0.5*	**3.1**	1.3	0.6	0.6	*0.4*	1.1	**4.1**
Sum	20	20	20	20	20	20	20	20	20
AP value	9	77	10	9	8	10	19	5	132

Normalized relative binding (RB) values indicate whether an amino acid is favored (RB $\geq$ 2.0, bold numbers) or disfavored (RB $\leq$ 0.5, *italic* numbers) for binding of each position in the peptide. Anchor position (AP) values are given by the equation $\sum(\mathrm{RB} - 1)^2$. Important AP values are underlined.
SLA, swine leukocyte antigen.

primary AP is characterized by AAs being strongly preferred, and many AAs were unaccepted by the class I molecule in question. Anchor position values of 15 or more indicate primary anchors (underlined values, Table 1) (Lamberth *et al.* 2008). Such anchors are of critical importance for peptide binding.

The PSCPL analysis of SLA-2*0401 identifies positions 2 and 9 as APs, as is commonly seen for many class I molecules (Sette & Sidney 1999; Lamberth *et al.* 2008). The motif in Table 1 shows that the AAs asparagine (N) and tryptophan (W) are favored for peptide binding in positions 2 and 9 respectively. The preference of AAs with larger side chains such as tryptophan (W), tyrosine (Y) and phenylalanine (F) in position 9 was revealed by RB values of 11.2, 4.1 and 3.0 respectively (Table 1). Such RB values indicate that this anchor must align with a large and deep F pocket. The B pocket responsible for binding the AA in position 2 of the peptide is most stable binding hydrophilic side chains, resulting in a preference for the AAs serine (S) and asparagine (N) with RB values of 3.3 and 8.9 respectively.

These preferences are illustrated in Fig. 1, where logos displaying single-letter AA codes were generated to compare the *in vitro* SLA-binding motifs for the PSCPL with those of *in silico* NetMHCpan-based peptide preference predictions. NetMHCpan is a mathematical algorithm developed for MHC peptide-binding predictions in several different species of interest (Nielsen *et al.* 2007; Hoof *et al.* 2009). For SLA-1*0401, the data show that the PSCPL-based matrix detected preferences only for aspartic acid (D) and glutamic acid (E) in position 3 of the peptide. Furthermore, the NetMHCpan-based logo had tyrosine (Y) preferred in position 9 with phenylalanine (F) second most preferred, whereas the PSCPL data indicate a preference of phenylalanine (F) over tyrosine (Y) (Fig. 1). The PSCPL showed the same preference for the SLA-2*0401 molecule regarding tyrosine (Y) and phenylalanine (F) in position 9 (Fig. 1). Additionally, a shared preference for serine (S) was seen between the two SLA-2*0401 logos in position 2, as well as a unique preference for asparagine (N) within the PSCPL matrix only. These differences between the NetMHCpan-based logo and the PSCPL-based logo likely reflect the limited amount of peptide-binding data available for the NetMHCpan algorithm-based predictions with regard to MHC molecules that share specificity with these two SLA molecules (Hoof *et al.* 2009). Increasing the data available

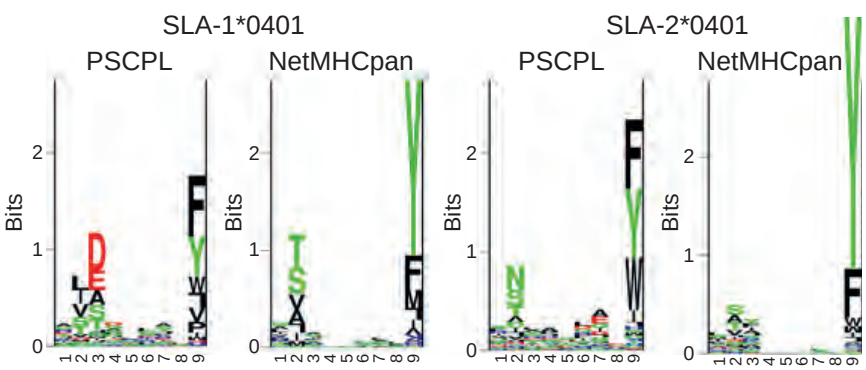

Figure 1 Sequence logo representations of the SLA-1*0401- and SLA-2*0401-binding motifs. The logos were calculated from the top 1% highest scoring peptides selected from a pool of 200 000 natural 9mer peptides using either the positional scanning combinatorial peptide library matrix or NetMHCpan as predictors. In the sequence logo, each peptide position is represented by a stack of letters indicating its significance for binding (information content) and the height of each letter (amino acid) is proportional to its relative frequency (Schneider & Stephens 1990). Acidic residues are displayed in red, basic in blue, neutral in green, and hydrophobic in black. Underrepresented amino acids are displayed upside down. SLA, swine leukocyte antigen.

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for swine MHC class I proteins will improve the NetMHCpan algorithm prediction profile.

Identification of peptides derived from FMDV bound by SLA-1*0401 and SLA-2*0401 alleles

The P1 region of FMDV, which codes for the viral capsid or structural proteins, was screened for 9mer and 10mer peptides that would potentially bind SLA-1-*0401 and SLA-2*0401 using the NetMHCpan version 2.3 (Nielsen *et al.* 2007). Nine such peptides were identified for the SLA-1*0401 allele (four 9mers and five 10mers), and 10 peptides (five 9mers and five 10mers) were identified for SLA-2*0401 (Table 2). Selection of peptides was based on the NetMHCpan algorithm predictions and further analyzed for the AA preference in positions 2, 3 and 9 of the individual peptides based on the PSCPL-binding motifs (Table 1 and Pedersen *et al.* 2011). Some peptides were selected because of their NetMHCpan rank despite not satisfying one of the primary anchors according to the PSCPL motif. Others were left out despite expressing an anchor–residue match and a proper NetMHCpan rank because of in-house peptide limitations. Within a pool of 726 possible peptide combinations from the P1 region, eight of the nine peptides selected for either SLA-1*0401 or SLA-2*0401 were within the top 10 highest ranking peptides for predicted binding (Appendix S2). All chosen peptides had a NetMHCpan rank score of <2% (Erup *et al.* 2011), meaning that <2% of random natural peptides had a predicted binding affinity stronger than the selected peptides.

These peptides, predicted to be bound, were tested for actual binding by SLA-1 and SLA-2 in an *in vitro* ELISA binding assay. Five of the nine FMDV-derived peptides predicted to be bound by the SLA-1*0401 molecule tested positive in the assay. These peptides include four 9mers (TVYNGTSKY, MTAHITVPY, SSVGVTHGY and YLSGIAQYY) and one 10mer (NMTAHITVPY). Additionally, we observed that four of the 10 peptides predicted to be bound by SLA-2*0401 produced correctly folded peptide–MHC complexes. These included three 9mers (MTAHITVPY, SSVGVTHGY and YLSGIAQYY) and one 10mer (NTYLSGIAQY). The respective binding affinity (K_D) values for all bound peptides ranged between 0.8 and 433 nM. Peptides with K_D values below 500 nM are considered strong (50–500 nM) or very strong (0.1–50 nM) binders (Table 2).

Two additional peptides, from the VP35 region of the Sudan *Ebola* virus and from the conserved hypothetical protein of the *Vibrio cholera* bacteria respectively, also were tested for binding by the SLA-2*0401 protein for comparison (Table 2). These peptides previously have been shown to be bound by SLA-1*0401 with high affinity (Pedersen *et al.* 2011). As for SLA-1*0401, both peptides were found to be strongly bound by the SLA-2*0401 molecule with K_D values of 1 and 2 nM respectively (Table 2).

Discussion

Previously, we defined the peptide motifs that are bound by the class I MHC protein of swine, SLA-1*0401 (Pedersen *et al.* 2011). We now expand this analysis to an additional SLA protein, SLA-2*0401, and describe a method for the analysis of a virus proteome to identify the peptides that will be bound by porcine MHC I proteins. Peptides from the P1 region of the FMDV genome, which encodes the structural proteins of the virus, were identified using this method for the gene products of two class I alleles, SLA-1*0401 and SLA-2*0401. These two class I proteins are commonly expressed alleles in commercial pig breeds as well as in different porcine cell lines

Table 2 Peptide candidates for SLA-1*0401 and SLA-2*0401 affinity analysis

	FMDV peptides tested for SLA-1*0401			FMDV peptides tested for SLA-2*0401		
Peptide sequence	NetMHCpan predicted% rank score	K_D (nM)	Actual binding status	NetMHCpan predicted% rank score	K_D (nM)	Actual binding status
TVYNGTSKY	0.17	87	Intermediate	0.05	NB	Non-binder
MTAHITVPY	0.01	0.8	Strong	0.05	7	Strong
SSVGVTHGY	0.10	433	Intermediate	0.40	14	Strong
AAKHMSNTY	1.00	ND	ND	0.40	NB	Non-binder
YLSGIAQYY	1.50	17	Strong	0.25	42	Strong
LAAKHMSNTY	0.20	NB	Non-binder	0.12	NB	Non-binder
NMTAHITVPY	2.0	67	Strong	0.80	NB	Non-binder
NTYLSGIAQY	0.20	NB	Non-binder	0.12	22	Strong
ATVYNGTSKY	0.15	NB	Non-binder	0.80	NB	Non-binder
QSSVGVTHGY	0.80	NB	Non-binder	1.50	NB	Non-binder

Peptide sequence	SLA-2*0401 *Ebola*/Cholera peptides NetMHCpan predicted% rank score	K_D (nM)	Actual binding status
ASYQFQLPY	0.01	2	Strong
ATAAATEAY	1.00	1	Strong

ND, not determined; NB, non-binding peptide having a $K_D > 5000$ nM; FMDV, foot-and-mouth disease virus; SLA, swine leukocyte antigen.

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used for experimental research (Smith *et al.* 2005; Ho *et al.* 2006, 2009). Hence, the determination of binding motifs for such proteins is of great value as a tool in the analysis of porcine immune responses and vaccine development.

A comparison of *in silico* predictions with *in vitro* analysis of peptide binding, expressed as the formation of correctly folded porcine MHC class I, supports previously published data describing the capacity of the NetMHCpan predictor to identify successful peptide/MHC binding beyond humans (Hoof *et al.* 2009; Pedersen *et al.* 2011). All 12 different peptides tested for the SLA-2*0401 molecule were predicted by the NetMHCpan to be bound, and six formed complexes with the SLA class I alpha chain and β_2m *in vitro* (Table 2). These data show the NetMHCpan predicted actual peptide binding at a rate of 50%, based on the limited data available to date. Of the nine peptides selected for SLA-1*0401-binding tests, all were predicted to be bound and five formed complexes with the heavy chain during the *in vitro* analysis. For the SLA-1*0401 molecule, 55.6% of the predictions were confirmed. These results were obtained using a prediction algorithm based on human MHC proteins. As such, they illuminate the value of using the extensive human database to apply to swine MHC peptide predictions to identify potential immune epitopes.

Of the 10 peptides from the sequence of the FMDV structural protein region identified by the algorithm, four of the six that failed to be bound by SLA-2*0401 have random binding scores. Contrarily, none of the four peptides that were bound by SLA-2*0401 have random binding scores calculated from the PSCPL matrix values and the approximation for longer peptide-binding prediction described by Lundegaard *et al.* (2008) (data not shown). This indicates that improvement could be achieved by adding quantitative SLA peptide-binding data derived using the PSCPL matrix for the NetMHCpan neural network to refine the algorithm and generate more accurate predictions (Nielsen *et al.* 2007; Hoof *et al.* 2009). In addition, one peptide (YLSGIAQYY) was predicted not to be bound by SLA-2*0401 but in fact was strongly bound. Considering several peptides predicted to be bound failed to do so *in vitro* illustrates the need for more data to enhance the NetMHCpan for species other than humans.

Differences in peptide motifs expressed by the two SLA alleles, SLA-1*0401 and SLA-2*0401, are illustrated by peptides bound by one allele but not by the other. The 10mer peptide, NTYLSGIAQY, was bound by SLA-2*0401 with an affinity (K_D) value of 22 nM but was not bound by the SLA-1*0401 alpha chain (Table 2). This supports the higher preference for the AA tyrosine (Y) in position 3 (RB = 3.1) by the SLA-2*0401 molecule and that the residue is disfavored in the same position for SLA-1*0401 (RB = 0.5) peptide binding. SLA-1*0401 is unique from most other alleles in that this alpha chain has a peptide-binding anchor in position 3 as well as in the more commonly observed APs 2 and 9 (Pedersen *et al.* 2011). Thus, the side chain of the AA in position 3 factors into peptide binding, and so the NTYLSGIAQY peptide does not bind despite the positions 2 and 9 anchors being satisfied (T: RB = 2.5 and Y: RB = 4.3 respectively). The opposite effect is seen for the 10mer peptide NMTAHITVPY. This peptide is readily bound by SLA-1*0401 with a K_D value of 67 nM but is not bound by SLA-2*0401. Methionine (M) is disfavored in position 2 in the SLA-2*0401 protein, whereas it is favored by the SLA-1*0401 peptide-binding motif (RB = 0.3 and 1.9 respectively). Furthermore, threonine (T) in position 3 is less favored for SLA-2*0401 than for SLA-1*0401.

As with the differences in binding preferences, similarities also are seen for the two SLA alpha chains analyzed when analyzing these viral peptides. Some of the identified binding peptides are shared, and this sharing can be explained by the identical preferences for tyrosine (Y) in the important position 9 anchor and for methionine (M) in position 1. There is also the shared preference for serine (S) and threonine (T) in the position 2 anchor (Table 1; Pedersen *et al.* 2011). In addition, our data show that both SLA proteins can bind 9mer peptides as well as 10mer peptides with similar affinities. This observation may indicate that peptide epitopes longer than nine AAs may be common for these MHC proteins. Such peptides would lead to different recognition patterns expressed by TCRs than the more often reported 9mers. The reason for this lies in the conformation of the peptide–MHC complex, which would differ in the way the peptide bulges away from the groove. This is caused by the bound peptide now having one or even two additional AAs in the center available for TCR recognition, depending on which peptide AA interacts with the respective anchors within the MHC groove (Fig. 2).

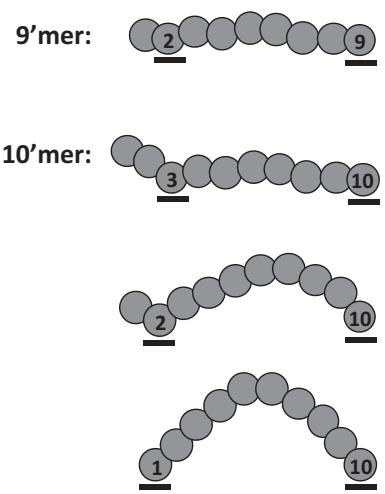

Figure 2 Illustration of commonly known 9mer peptide-binding vs. three different proposed 10mer peptide-binding scenarios and the effect on the exposure of the central amino acids. Black bars illustrate important interactions between the peptide and the anchor position residues in the B- and F-pockets of the major histocompatibility complex (MHC) class I-binding groove. Numbers indicate the peptide position of the primary MHC-interacting amino acids within the peptide.

To date, most analysis has focused on 9mer peptides as candidates for strong class I complex folding. Longer peptides, specifically the 10mers, were included in this study to expand the pool of possible peptides for analysis and, hence, possible T-cell epitope candidates. For human class I (Chen *et al.* 1994; Urban *et al.* 1994; Tynan *et al.* 2005), binding of peptides other than 9mers has been readily shown with resulting diverse peptide conformations within the HLA class I-binding groove. Longer peptides not only provide a larger pool of combinations of potential peptide sequences and binding configurations (Fig. 2), but also contribute to greater diversity in the center region of the peptide available for TCR recognition as a result of the peptide bulging away from the binding cleft. Hence, depending on the individual binding pattern of the peptide, binding of a 10mer peptide could include additional AAs between the anchors of the bound peptide available for TCR interactions. Such a change in conformation could have the potential to expand pMHC-mediated activation of virus-specific CTLs.

The ability of MHC molecules to bind the same peptide in different combinations, depending on the identity of the peptide anchor residues, would appear to have distinct advantages to the immune system. Based on the PSCPL RB values of the respective pockets expressed by the SLA-1*0401 and SLA-2*0401 molecules, and in combination with the respective 10 AA peptides bound, the model predicts that the two 10mer peptides bind by a position 2/position 10 (P2/P10) or P1/P10 anchor manner (NMTAHITVPY) for SLA-1*0401 and a P3/P10 anchor manner (NTYLSGIAQY) for SLA-2*0401 (Table 1; Pedersen *et al.* 2011). These binding patterns are only speculations and rely on a comparison of the AA in peptide positions 1–3 with the MHC preferences expressed for the individual AA in P1–3, combined with the comparison of the AA in peptide positions 9 and 10 with the preferences expressed for the individual AA in P9. Future mapping of MHC class I-binding motifs using an X10-based peptide library as compared to exclusively X9 will further illuminate which AA positions of the 10mer peptides account for binding interactions between the peptide residues and the anchors of the MHC class I protein groove.

In conclusion, we determined the binding motif of an additional swine MHC class I molecule, SLA-2*0401. Further, the NetMHCpan prediction algorithm based on human MHC proteins identified peptides from a viral proteome of interest. Based on *in silico* predictions and *in vitro* verifications, we identified four FMDV peptides bound by SLA-2*0401: MTAHITVPY, SSVGVTHGY, YLSGIAQYY and NTYLSGIAQY; and five FMDV peptides bound by SLA-1*0401: TVYNGTSKY, MTAHITVPY, SSVGVTHGY, YLSGIAQYY and NMTAHITVPY. Three of these peptides were observed to be commonly shared between the two heavy chain molecules (MTAHITVPY, SSVGVTHGY and YLSGIAQYY), whereas one peptide (NTYLSGIAQY) was bound only by the SLA-2*0401 protein and two peptides (TVYNGTSKY and NMTAHITVPY) were bound only by the SLA-1*0401 protein. All these peptides are candidate T-cell epitopes by virtue of being strongly bound by two of the most highly expressed porcine class I MHC proteins.

Analysis of antigen-specific, MHC I-restricted T cells using MHC tetramers made with the peptides identified here showed that at least one of these, MTAHITVPY bound by SLA-1 *0401, reacts with FMDV-specific T cells (Patch *et al.* 2011). The reason for including *Ebola* and *V. cholera* peptides in this study was an ambition of adding the SLA-2*0401 molecule to the experimental setup of such tetramer staining analyses. FMDV-specific porcine CTLs should not recognize either *Ebola*- or *V. cholera*-derived peptides bound and presented by the SLA-2*0401 molecule, and hence, these peptides should make for good negative controls. Subsequent studies indicate that other peptides represented in the data presented here are T-cell epitopes, and confirmation of these preliminary results is underway, now also including the SLA-2*0401 molecule. Minimally, these data, together with our previous reports (Patch *et al.* 2011; Pedersen *et al.* 2011), show that the approach of using the NetMHCpan prediction algorithm, developed for human MHC molecules, can be effective in predicting MHC peptide interactions in other species.

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Conflict of interest

The authors claim no conflict of interest in the publication of this information.

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Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 The nucleic acid and amino acid sequence of SLA-2*0401 expressed in *E. coli*.

Appendix S2 NetMHCpan version 2.3 predictions

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Research paper

Time-dependent biodistribution and transgene expression of a recombinant human adenovirus serotype 5-luciferase vector as a surrogate for rAd5-FMDV vaccines in cattle

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ABSTRACT

Replication-defective recombinant adenovirus 5 (rAd5) vectors carrying foot-and-mouth disease virus (FMDV) transgenes elicit a robust immune response to FMDV challenge in cattle; however mechanistic functions of vaccine function are incompletely understood. Recent efforts addressing critical interactions of rAd5 vectors with components of the bovine immune system have elucidated important aspects of induction of protective immunity against FMDV. In the current study, a rAd5-Luciferase (rAd5-Luc) surrogate vector was utilized for indirect assessment of rAd5-FMDV distribution during the first 48 hours post inoculation (hpi). To compare vector distribution dynamics and time-dependent transgene expression, bovine cells were inoculated in vitro with rAd5-FMDV and rAd5-Luc vectors. Superior transgene expression was detected in cells infected with rAd5-Luc compared to rAd5-FMDV. However, both vectors behaved remarkably similar in demonstrating elevated mRNA transcription at 24 and 48 hpi with peak occurrence of transgene expression at 48 hpi. Injection sites of cattle inoculated with rAd5-Luc contained mononuclear inflammatory infiltrates with hexon and transgene proteins associated with antigen-presenting cells. Luciferase activity, as well as microscopic detection of luciferase antigens, peaked at 24 hpi. Presence of viral mRNA also peaked at 24 hpi but unlike luciferase, remained strongly detected at 48 hpi. Cell-associated luciferase antigens were detected as early as 6 hpi at the cortical interfolicullar areas of local LN, indicating rapid trafficking of antigen-presenting cells to lymphoid tissues. This work provides mechanistic insights on rAd5-mediated immunity in cattle and will contribute to ongoing efforts to enhance rAd5-FMDV vaccine efficacy.

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1. Introduction

Foot-and-mouth disease virus (FMDV), an Aphtovirus of the Picornaviridae family (Fauquet et al., 2005), can cause severe disease in susceptible livestock (Grubman and Baxt, 2004). In enzootic regions, FMD-associated morbidity causes food insecurity and limits trade. In FMDV-free regions, considerable resources are directed towards preventing introduction of the virus. Control strategies are of upmost importance and have historically relied in the use of an inactivated whole virus vaccine that can effectively control clinical disease (Doel, 2003; Rodriguez and Gay, 2011). However, because of a number of limitations of the current vaccine preparations, new vaccination approaches have been envisioned and explored in recent

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years (Grubman and Baxt, 2004; Rodriguez and Gay, 2011; Rodriguez and Grubman, 2009). One novel approach utilizes a replication-defective recombinant human adenovirus type 5 (rAd5) vaccine containing FMDV coding sequences (rAd5-FMDV) (Grubman et al., 2010; Mayr et al., 1999; Moraes et al., 2002). This platform has been demonstrated to be a highly efficient system for delivering antigens to the host and eliciting protective immune responses against FMDV challenge (Grubman et al., 2010; Grubman and Baxt, 2004; Montiel et al., 2012; Pacheco et al., 2005). Vaccination trials using rAd5-FMDV have demonstrated complete protection against virulent challenge at 21 days post vaccination (dpv) and partial protection before 7 dpv in presence of low levels of anti-FMDV antibodies (Grubman et al., 2010; Sanz-Parra et al., 1999a,b). Furthermore, recent studies on rAd5 vector biodistribution in cattle indicated that during the first 48 hours after inoculation with rAd5-FMDV, adenoviral and transgene proteins were found highly associated with cells phenotypically consistent with antigen presenting cells (APC) primarily at the injection site and secondarily at various local lymph nodes (Montiel et al., 2012). These results implicate the innate immune system in early events associated with generation of protective immunity, which are of critical importance in driving a protective response against FMDV.

Several groups have explored novel approaches using sensitive reporter systems based on expression of the luciferase gene, both in vitro and in vivo. Porcine cell lines infected with a rAd5-Luciferase (rAd5-Luc) vector (Torres et al., 1996), and plasmid DNA containing the firefly luciferase gene directly inoculated into the muscle of pigs (Everett et al., 2000) and mice (Manthorpe et al., 1993; Wolff et al., 1990), have shown detectable and stable levels of biologically active recombinant proteins, demonstrating the feasibility of using such systems in gene transfer-related studies. To establish validity as a surrogate vector for rAd5-FMDV vaccine biodistribution studies, our lab recently demonstrated transgene expression in human and bovine cells lines infected with a rAd5-Luc construct, and correlated these data with PCR and microscopy (Montiel et al., 2012). We also demonstrated that upon intramuscular inoculation of cattle with rAd5-Luc, luciferase was strongly detected at the injection site and, to a lesser extent, at the local lymph nodes. The luciferase transgene was also detected by PCR and luciferase antigens by immunomicroscopy in bovine tissues (Montiel et al., 2012).

The current study provides a comprehensive investigation of rAd5 biodistribution and transgene expression using recently validated detection techniques in tissues of cattle (Montiel et al., 2012), and should contribute to enhancing our current understanding of host–vector interactions for the improvement of vaccine effectiveness against FMD.

2. Materials and methods

2.1. Cells and viruses

The replication-defective recombinant (r) Ad5 vectors used in this study have deletions in the E1, E3, and E4 regions that render them incapable of generating progeny virus in non-complementary cell lines. The first vector, rAd5-FMDV strain A24 Cruzeiro (Ad5-A24; Adt.A24.11D), contains the FMDV P1-2A and $3C^{pro}$-coding regions (Grubman et al., 2010). The second (i.e. surrogate) vector, rAd5-Luciferase (rAd5-Luc; AdL.11D), contains the firefly (*Photinus pyralis*) luciferase gene. Both vectors' transgene coding regions are under the control of a cytomegalovirus promoter. In vitro infections were carried out as previously described with minor variation (Pacheco et al., 2010). Briefly, bovine LFBK (Swaney, 1988) and human 293 (Graham et al., 1977) cells were seeded onto 6-well plates and 1-well tissue culture slides (cat# 154453, Lab-Tek®II Chamber Slide™ System; Nalgene Nunc, International; Naperville, IL), inoculated with rAd5 vectors at m.o.i. 100 and 300, and incubated at 37 °C for varying times. Following incubation of chamber slides, the monolayers were rinsed twice with media and the upper structure removed. The slides were then fixed in acetone at room temperature for 10 min and stored at −70° C until assayed by microscopy.

2.2. Experimental animals and inoculation system

Six 300 kg Holstein steers were obtained from an experimental-livestock provider accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (Thomas-Morris Inc., Reistertown, MD). All studies were conducted under an approved Institutional Animal Care and Use Committee (IACUC) protocol and animals were individually housed in a biosafety level-3 (BSL-3) animal facility from time of inoculation until time of euthanasia. Animals were examined prior to inoculation to ensure absence of pre-existing medical conditions. On Day 0 each animal received three simultaneous 3-ml intramuscular injections with a total dose of 1.5×10^{11} PU of the rAd5-Luc vector resuspended in Final Formulation Buffer (cat# B5003, FFB; Lonza, Walkersville, MD, USA) containing adjuvant D. Adjuvant D is a proprietary lipid containing emulsion designed for producing defined particle size formulas for delivery of live virus or virus-vector vaccines and immunotherapeutics and was provided by Benchmark Biolabs (Lincoln, NE) through an agreement with DHS S&T PIADC.

2.3. Animal sample collection

In vivo experiments were terminated by euthanasia via intravenous barbiturate overdose at predetermined end points at 6, 24, and 48 hours post-inoculation (hpi). Necropsies were performed immediately thereafter. Sample collection and processing was performed as previously described (Montiel et al., 2012). In order to maximize retrieval of the injection depot from the injection site (IS) area, 8 specimens of the regional muscle were collected based on anatomic location and visualization of focal hemorrhage and/or edema associated with deposit of the inoculum. Non-IS specimens collected from each animal were spleen, thymus, liver, lung, pancreas, adrenal, kidney, and local and distant lymph nodes.

Table 1
Antibodies used in the study.

Antibody	Clone	Specificity	Isotype	Manufacturer
Anti-Adenovirus	2Q1850; 8C4	Hexon protein; hexon protein	IgG_1; IgG_{2a}	cat# A0977; USBiological, Swampscott, MA; cat# ab8249; Abcam, Cambridge, MA
Anti-FMDV	6HC4	VP1 capsid protein	IgG_{2b}	Hybridoma-A12 VP1
Anti-Luciferase	LUC-1	Firefly luciferase	IgG_1	cat# L2164; Sigma–Aldrich, St. Louis, MO
CD11c	BAQ153A	Mono[a], MΦ[b], sub T, NK, sub B, DC[c]	IgM	cat# BAQ153A; VMRD, Inc., Pullman, WA
MHCII (HLA-DR α)	TH14B	MHC class II	IgG_{2a}	cat# TH14B; VMRD
CD14 (M-M9)	CAM36A	Mono, MΦ, DC subset	IgG_1	cat# CAM36A; VMRD
CD172a	CC149	Mono, granulo	IgG_{2b}	cat# MCA2041G; AbD Serotec
MAC387	MAC387	Granulo[d], mono, histiocytes	IgG_1	cat# M0747; Dako, Carpinteria, CA
Von Willebrand Factor (vWf)	Polyclonal	Endothelial cells	Rabbit IgG	cat# A0082; Dako
Anti-Collagen I	COL-1	Extracellular matrix (ECM)	IgG_1	cat# ab90395; Abcam
ACTA-1	Polyclonal	Actin (N-terminus)	Rabbit IgG	cat# LS-B3339; LSBio, Seattle, WA
CAR	3C100	Human CAR receptor	IgG_1	cat# sc-70493; Santa Cruz Biotechnology, Inc., Santa Cruz, CA

[a] Mono = monocytes.
[b] MΦ = macrophages.
[c] DC = dendritic cells.
[d] Granulo = granulocytes.

2.4. Extraction of viral nucleic acids and transgenic product from cell lines and tissues

Nucleic acids (DNA and RNA) and total protein were extracted from cell cultures and tissues as previously described (Montiel et al., 2012), following the manufacturer's recommendations. The nucleic acid and luciferase-containing supernatants were frozen and stored at −70 °C until assayed.

2.5. Real-time PCR (rPCR and rRT-PCR) and luciferase assays

The strategy to characterize rAd5 vector biodistribution and infection distinctly utilizing two separate nucleic acid detection systems has been recently described in vitro and in vivo (Montiel et al., 2012). Systems used for detection of rAd5 DNA (PIX) and rAd5 mature mRNA transcripts (splice, representing transgene expression in infected cells) including probe and primer sequences were utilized as described previously (Montiel et al., 2012). For the in vitro assessment, values correspond to the mean Ct values of 3 separate experiments performed independently in triplicate. For the ex vivo assessment, for each time-point, PCR results were expressed as the Ct values of 39 distinct tissues, each of which was collected and analyzed independently in duplicate. Results are expressed as cycle threshold (Ct) mean values with specific detection defined for positive samples at Ct < 40. Luciferase activity was also determined based on a recently published methodology (Montiel et al., 2012). Briefly, luciferase content of each sample was recorded as relative light units (RLU) and normalized by the respective total protein concentration of the samples (RLU/mg total protein) obtained by the bicinchoninic acid assay (BCA) (Smith et al., 1985).

2.6. Immunomicroscopy

Localization of rAd5 and transgene products (FMDV-A24 capsid proteins and luciferase) in cell lines and bovine tissues was similarly determined by immunohistochemistry (IHC) and multichannel immunofluorescence (mIF) techniques as previously described (Arzt et al., 2009; Montiel et al., 2012) with minor variation. Primary antibodies (Table 1) were used individually for IHC or in combination for mIF assays. For mIF, detection was performed using goat-anti-rabbit and isotype-specific, anti-mouse secondary antibodies labeled with Alexa Fluor dyes (Invitrogen) diluted in Fluorescence Antibody Diluent (Biocare Medical, Concord, CA). For IHC, immunoreactivity was detected with a micropolymer alkaline phosphatase kit (Biocare Medical). Isotype controls were included with each experiment. Slides were evaluated with a wide-field epifluorescence microscope and images captured with a Nikon DS-Qi1 digital camera. Images of individual detection channels were adjusted for contrast and brightness and merged in commercially available software (Adobe Photoshop CS2). Differential interference contrast (DIC) microscopy was utilized on selected images to enhance cellular and extracellular matrix structural detail.

2.7. Statistical analysis

Data analyses and graphic representations were performed by using Microsoft Office Excel 2007. Statistical differences were determined by using a Student's t test when applicable. A cutoff value of $p < 0.05$ was considered significant.

3. Results

3.1. Time-dependent detection and quantitation of rAd5 nucleic acids and luciferase in LFBK cells

Luminometry, rPCR, and rRT-PCR were utilized to assess vector dynamics that occurred in LFBK cells during the first 72 hpi after inoculation with rAd5-FMDV or rAd5-Luc vectors at fixed m.o.i. (Fig. 1). Vector DNA of both constructs was detected at similar levels throughout the time-course 3–72 hpi (Ct: 27.64–26.16 for rAd5-FMDV and 26.93–25.08 for rAd5-Luc). In rAd5-FMDV infected cells,

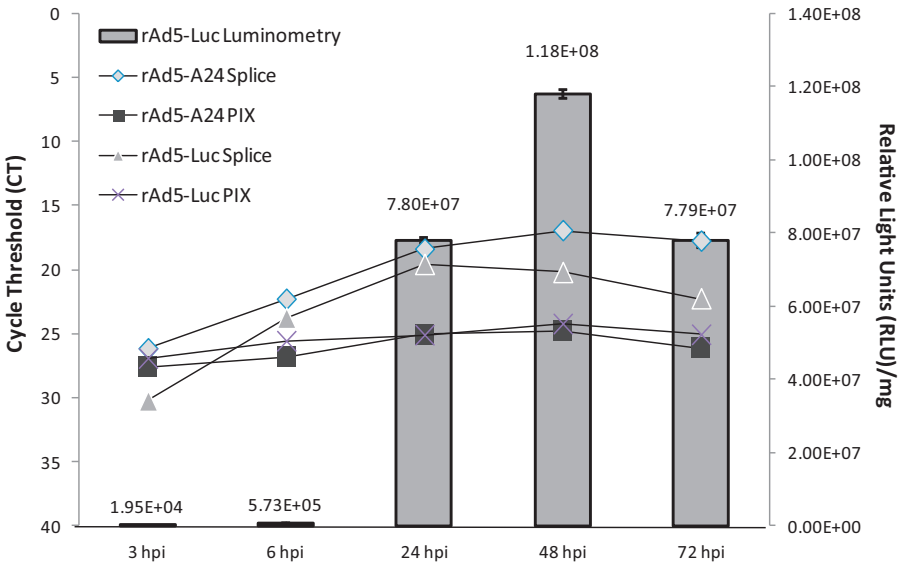

Fig. 1. Detection of nucleic acids (mRNA and DNA) and luciferase activity in cells infected with rAd5-Luc or rAd5-A24 vectors at different time-points. Each column represents mean luciferase activity (relative light units (RLU) per mg of total protein) ± SD of 3 replicates assayed simultaneously. Lines correspond to levels of rAd5-A24 mRNA (diamonds), rAd5-A24 DNA (squares), rAd5-Luc mRNA (triangles), and rAd5-Luc DNA (crossing lines) as determined by rRT-PCR (splice) and rPCR (PIX) in LFBK cells infected at fixed m.o.i. = 300 (rAd5-A24) or 100 (rAd5-Luc). Ct values are reported as mean Ct values of 3 different experiments assayed in triplicate.

mRNA signal increased with time post-inoculation with a peak observed at 48 hpi (Ct 16.94). Relative to rAd5-Luc-infected cultures, mRNA content in rAd5-FMDV-infected cultures was consistently higher (lower Ct values) at all time-points. Nonetheless, detection of mRNA in rAd5-Luc cultures followed a remarkable similar pattern than the observed in rAd5-FMDV positive cells during the first 24 hpi (Ct 30.27 at 3 hpi, Ct 23.80 at 6 hpi, and Ct 19.58 at 24 hpi). However, mRNA signal peaked at 24 hpi (Fig. 1).

In rAd5-Luc-infected cultures, luciferase activity increased with time post-exposure between 3 and 48 hpi followed by a decrease between 48 and 72 hpi (Fig. 1). The most significant difference regarding luciferase activity levels across consecutive time-points was observed between 6 and 24 hpi (136 ± 24-fold; $p < 0.05$). Moreover, the peak of luciferase activity was detected at 48 hpi ($1.18 \times 10^8 \pm 1.25 \times 10^6$ RLU/mg protein). It is noteworthy that luciferase activity levels at 24 ($7.80 \times 10^7 \pm 6.87 \times 10^5$) and 72 ($7.79 \times 10^7 \pm 1.88 \times 10^6$) hpi were approximately 50% lower than at the peak (1.18×10^8 RLU). These results demonstrate that bovine LFBK cells provide a suitable model for the study of time-dependent kinetics of rAd5 infection in vitro, which can be further applied for the study of rAd5 vaccine pathogenesis in vivo.

3.2. Immunomicroscopy in rAd5-infected LFBK cells

In order to further characterize suitability of rAd5-Luc infection as a surrogate model for rAd5-FMDV, LFBK cells were inoculated with either rAd5-FMDV or rAd5-Luc and analyzed at similar times post inoculation (Fig. 2). Adenoviral protein (hexon) and transgene product of both constructs (FMDV capsids and luciferase) were detected using specific antibodies (Table 1). Luciferase-expressing cells were found as early as 3 hpi with an average of one luc+ cell per field. A clear peak of detection occurred at 48 hpi with more than 70% of cells identified as luc+. At 24 and 72 hpi, LFBK cell cultures had a similar protein expression pattern and were visualized containing substantially less luc+ cells (50% or less) than at 48 hpi. Localization of the Coxsackievirus and Adenovirus Receptor (CAR) in rAd5-Luc+ LFBK cultures was consistently membrane-associated and unaltered by the presence of luciferase or hexons throughout the time-course.

By contrast, FMDV antigens (FMDV-A24 capsids) were not detected in rAd5-FMDV-infected LFBK cell cultures until 24 hpi, suggesting expression of transgene antigens was not substantial until after 6 hpi. At 24–48 hpi, around 30% of cells were immunoreactive to the anti-FMDV antibody, and at 72 hpi only few A24+ cells were found in the cultures. From 24 to 72 hpi in rAd5-FMDV cultures, there was progressive accumulation of clusters of degenerate, rounded cells and autofluorescent cell bodies (Fig. 2, arrows). Detection of CAR in rAd5-FMDV-inoculated cultures was similarly strong and uniform at 3, 6, and 24 hpi; however, altered patterns of CAR detection were observed at 48 and 72 hpi with significantly reduced expression intensity (Fig. 2).

3.3. Time-dependent detection and quantitation of rAd5-Luc nucleic acids and luciferase in bovine tissues

In order to characterize vector dynamics in vivo, tissue specimens from rAd5-Luc inoculated cattle were collected from animals euthanized at 6, 24, and 48 hpi and subsequently processed by rPCR, rRT-PCR, and luciferase bioassay (Fig. 3).

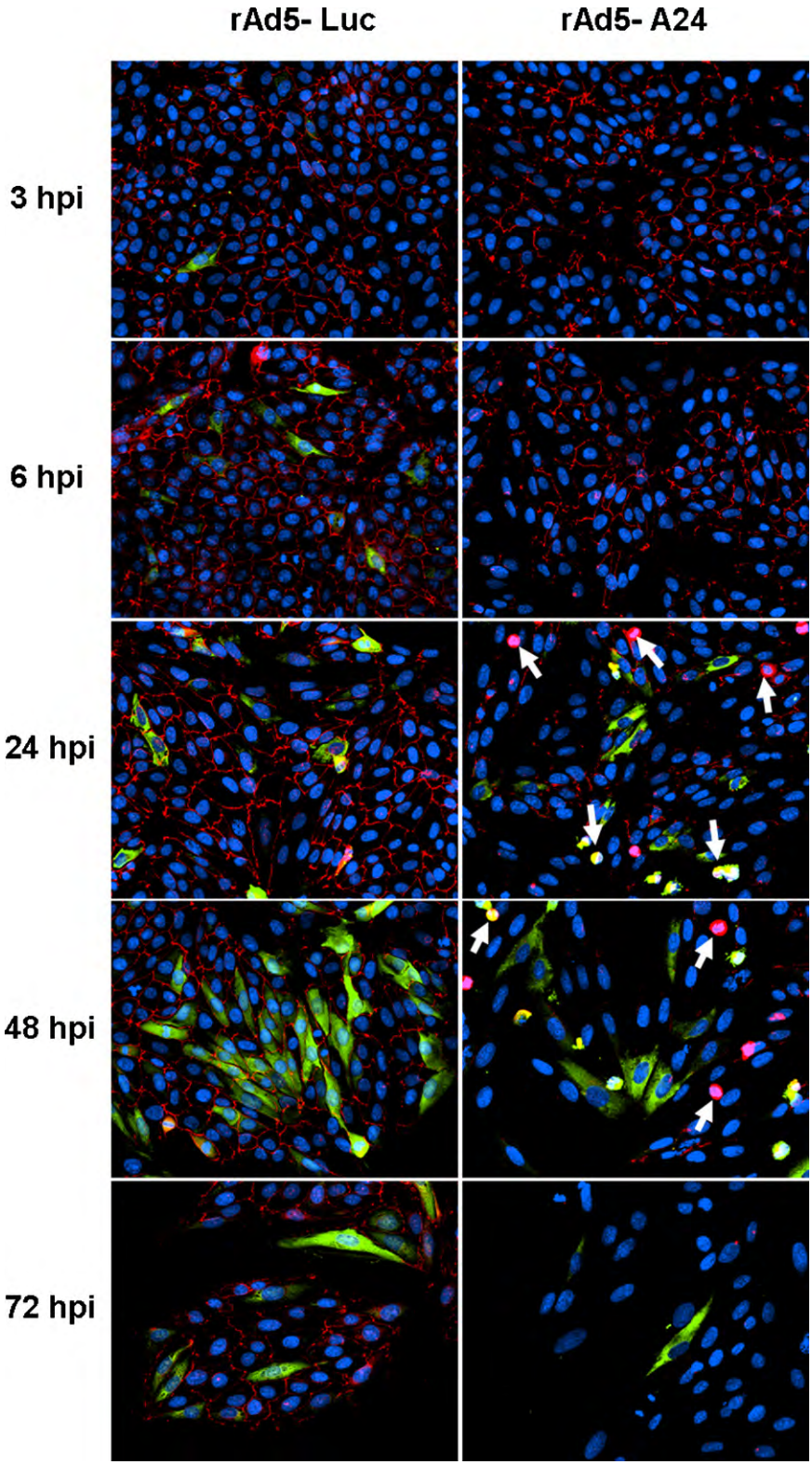

Fig. 2. Detection of transgene products in LFBK cells infected with rAd5 vectors during time-course experiments. Diffuse cytoplasmic distribution of FMDV capsids and luciferase (green) in the cytoplasm of rAd5-A24 or rAd5-Luc infected cells at m.o.i. 300 or 100, respectively. Membrane-associated expression of the coxsackie and adenovirus receptor (CAR) is shown in red. Multichannel immunofluorescence (mIF) microscopy. Degenerate autofluorescent cells (arrows) only seen in rAd5-A24 infected cells. Nuclei DAPI counterstain. 20× magnification.

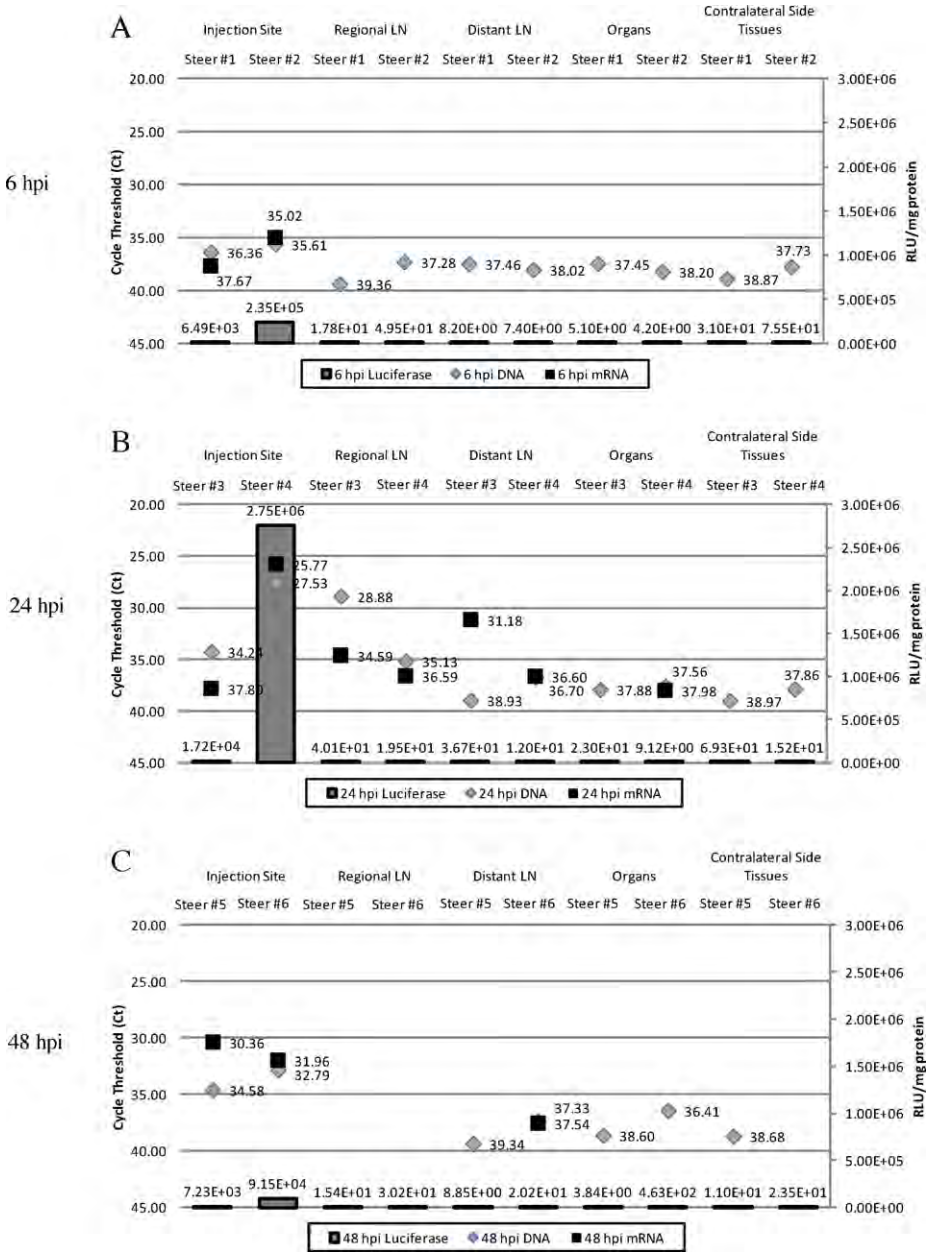

Fig. 3. Detection of rAd5-Luc nucleic acids (mRNA and DNA) and luciferase activity in tissues of inoculated animals euthanized at 6, 24, and 48 hpi. Luciferase detection (columns) was only substantial at injection sites (relative light units (RLU) per mg of total protein). Diamonds correspond to levels of rAd5-Luc DNA (PIX); squares represent detection of rAd5-Luc mRNA (splice). PCR results are expressed as mean Ct values of individual samples assayed in duplicate with minimum detection threshold of Ct < 40.

As early as 6 hpi (steers #1 and #2), rAd5 DNA was detected at the injection site (IS), local LN, and systemically disseminated to visceral organs and distant lymph nodes (Fig. 3A). Ct values ranged between 35 and 40 which were interpreted as low level detection. Mature mRNA transcripts were also detected at low levels at the IS, but not in LN or organs of 6 hpi steers (Ct > 40). Similarly, low level luciferase activity was present in the IS but not in other tissues of the 6 hpi steers (mean = $1.2 \times 10^5 \pm 1.6 \times 10^5$ RLU/mg protein) (Fig. 3A).

At 24 hpi (steers #3 and #4), rAd5 DNA was detected locally at IS and systemically distributed (Fig. 3B). Overall, greater quantities of DNA and RNA (Ct values between 25 and 35) were detected relative to the corresponding tissues of 6 hpi animals. Unlike at 6 hpi, mature (splice) mRNA transcripts were detected in several tissue specimens beyond the IS (local and distant LN and viscera). The lowest Ct value (strongest PCR signal) (Ct = 25.77) was detected at the IS. Moderate quantities of mRNA were also detected in regional (Ct 34.59) and distant (Ct 31.18) LN. Luciferase activity (mean = $1.38 \times 10^6 \pm 1.9 \times 10^6$ RLU/mg

protein) correlated with the strongest detection by PIX (DNA; Ct = 27.53) and splice (mRNA; Ct = 25.77) PCR (Fig. 3B). Interestingly, luciferase activity was detected at the IS, but not in LN or any other tissues.

Adenovirus biodistribution (DNA) and gene expression (mature mRNA transcripts and luciferase) at 48 hpi (steers # 5 and 6) was largely similar to that of the 6 hpi group (Fig. 3C). As with earlier time points DNA distributed to distant LN and organs. Beyond the IS, rAd5 mRNA transcripts were also found within distant LN but not in other organs. Interestingly, nucleic acids (DNA and mRNA) were undetectable at regional LN. Luciferase activity at 48 hpi was the lowest (mean = $4.8 \times 10^4 \pm 6 \times 10^4$ RLU/mg protein) of the time-course; similar to 6 and 24 hpi, luciferase was generally not detected in regional LN or systemically distributed (Fig. 3C). The only exception was in steer #6 (48 hpi) which had elevated luciferase activity (99-fold above background controls) in the adrenal glands (3×10^3 RLU/mg protein) (data not shown). Similar to 6 and 24 hpi, at 48 hpi luciferase activity correlated well with detection of mature mRNA transcripts at the IS of the two replicates (steer #5 and #6; 7.2×10^3 and 9.1×10^4 RLU/mg protein, and Ct 30.36 and 31.96, respectively) (Fig. 3C).

3.4. Gross pathology and microscopy of injection sites of rAd5-Luc inoculated cattle

Injection sites of all animals were identified post-mortem as multifocal, 0.5–1.5 cm intramuscular hemorrhages with variable edema (Fig. 4A–C). One of the 48 hpi steers (#5) had a moderately demarcated, firm, edematous granuloma at the IS in the splenius muscle (Supplemental Figure). The mass was composed of multiple individual firm nodules of 3 cm × 3 cm surrounded by gelatinous edema.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2012.10.003.

Histologically, all IS had variable extents of regions in which normal muscle architecture was multifocally disrupted by extensive edema and cellular infiltrates with limited regional myocyte degeneration and necrosis. Inflammatory infiltrates composed predominantly of large mononuclear cells with fewer neutrophils and small mononuclear cells dissected myofibers following the interstitial connective tissue of the endomysium and perimysium (Fig. 4A–C). At 6 hpi, infiltrates were mild and predominantly perivascular (Fig. 4A). Small foci of granular basophilic material (suggestive of adjuvant) were identified at the IS of one 6 hpi steer (Fig. 4A, inset). At 24–48 hpi, inflammation was more extensive (Fig. 4B and C) with effacement and replacement of myocytes with regionally extensive aggregates of infiltrates and occasional intralymphatic myocyte emboli (Fig. 4B, inset). The intramuscular mass of steer #5 was comprised of numerous microgranulomas often centered upon pools of amphophilic liquid.

Hexon proteins were immunohistochemically localized within or in close proximity to inflammatory infiltrates in all the subjects included in the study. Throughout the time-course, rAd5 hexon signal intensity was low with an observable peak at 24 hpi. Hexon antigens were found inconsistently associated with cells expressing CD14, MHCII (Fig. 4D) and/or CD11c (Fig. 4E) antigens, presumably antigen-presenting cells (APC; dendritic cells and macrophages). There was a rapid influx and increase in signal intensity of cells consistent with the APC phenotype from 6 to 24 hpi (Fig. 4F and G). However, in contrast to hexon signal decrease after 24 hpi, expression of APC antigens reached a plateau by 48 hpi.

The transgene product luciferase was observed within IS tissues immunomicroscopically at all time-points (6, 24, and 48 hpi) studied. Distribution of luciferase immunoreactivity was consistently similar to hexon, associated with mononuclear cells within perimysial connective tissue. At 6 hpi, IHC, but not IF, demonstrated presence of scarce amounts of luciferase antigens at injection sites (Fig. 5A) which increased substantially by 24 hpi (Fig. 5B). As with hexon, signal intensity associated with luciferase antigen detection declined by 48 hpi (data not shown). Within infiltrates, luciferase antigen colocalized to hexon+ cells and in close association with cells of APC phenotype (Fig. 5C and D).

3.5. Microscopy of non-injection site tissues of rAd5-Luc inoculated cattle

Hexon antigens were found in several LN (lateral and medial retropharyngeal LN and infrequently at the axillary and prescapular LN) as early as 6 hpi in the lymphofollicular dark (marginal) zone (Fig. 6A). Luciferase antigens were also detected in lymph nodes at 6 hpi, although at lesser quantities and with distinct distribution relative to hexon. Small clusters of luc+ cells were present in subcapsular and trabecular sinuses and in interfollicular paracortex regions of the prescapular and lateral retropharyngeal LN. Hexon detection in LN was minimal at 24 hpi and completely absent at 48 hpi. However, at 24 hpi, abundant luc+ cells were found at the axillary LN paracortex and sinus regions (Fig. 6B). No luc+ cells were found within lymphoid follicles. Luciferase signal (IHC) was also present at 48 hpi and followed a similar pattern that at 24 hpi; however, at 48 hpi, detection was only limited to the axillary and the prescapular LN. Luciferase (and hexon) immunoreactivity was absent from all other non-injection site tissues analyzed.

4. Discussion

We have previously demonstrated that a replication-defective recombinant human adenovirus 5-Luciferase (rAd5-Luc) vector can be used as a surrogate agent for rAd5-FMDV, providing a convenient and effective screening method in host–vector interaction and vaccination studies in cattle (Montiel et al., 2012). Results of those studies indicated that molecular signatures and cellular events occurring after intramuscular inoculation with a rAd5-Luc vector were remarkably similar to those induced by vaccination with a rAd5-FMDV vector. Expression of the luciferase transgene in tissues of vaccinated animals facilitated localization of the adenovector that further allowed examination of key interactions with various cellular phenotypes of the immune system. In the current work, we hypothesized that rAd5-Luc could be implemented as

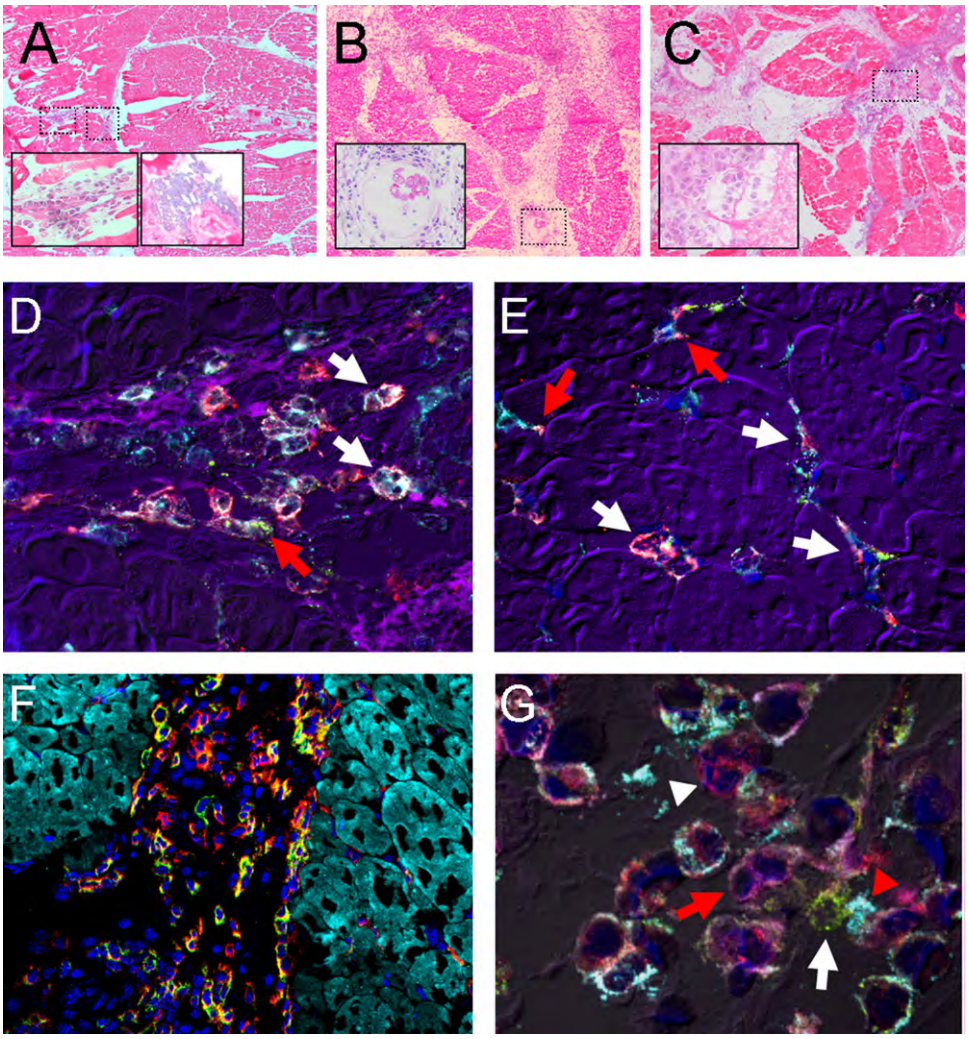

Fig. 4. Microscopy of injection sites (IS) of steers inoculated with rAd5-Luc at 24 hpi. (A–C) Progression of skeletal muscle lesions at IS of steers (#3 and 4) inoculated with adjuvanted rAd5-Luc. (A) 6 hpi, mild perivascular lymphoplasmacytic inflammation (box 1, inset 1) and rare aggregates of extracellular basophilic material (presumptive adjuvant; box 2, inset 2). (B) 24 hpi, severe endomysial–perimysial lymphohistiocytic myositis with intralymphatic myoemboli (inset). (C) 48 hpi, modestly less cellular myositis with edema, fibrin, and predominantly large mononuclear infiltrates (inset). H&E stain. (D) Steer #4, immunofluorescent localization of hexon (green, red arrow), MHCI (light blue), and MHCII (red) antigens to interstitial tissue of endomysium of the IS. Cells expressing MHCI or MHCII complexes, and colocalization of both markers (white arrows) are shown. Collagen (pink). (E) Steer #4, immunofluorescent visualization of hexon (green), MHCII (red), and CD11c (light blue) in between myocytes. Areas with varying degrees of double MHCII-CD11c (red arrows) or triple MHCII-CD11c-hexon (white arrows) colocalization can be seen at the endomysium. Myocyte architecture in purple using differential interference contrast (DIC) technique (in D and E). (F) Steer #4, infiltrated region of intermuscular connective tissue (perymisium) showing separation of myocytes (actin, light blue). Cells positive for CD14 (green) or CD11c (red) are shown. Double positive CD14-CD11c cells are seen as yellow (F). (G) Steer #4, expanded region of infiltrates of IS showing colocalization of cells expressing either CD14 (pink, red arrow), MHCII (green, white arrow), CD172a (red, white arrow head), and CD11c (light blue, red arrow head) or multiple combinations of these markers. Nuclei are shown in blue.

a surrogate agent for time-dependent studies on biodistribution and transgene expression of rAd5-based FMDV vaccines, in vivo. For this purpose, critical events related to rAd5-Luc vector dissemination and expression of transgene products were investigated during a time-course of 72 hours post-inoculation in vitro and during the first 48 h subsequent to intramuscular administration to cattle.

The need for an alternative vector for FMDV adenovirus-based vaccination studies (such as rAd5-Luc) is based upon observations of cytotoxicity (presumably induced by the 3C protease ($3C^{pro}$) in rAd5-FMDV-vectored vaccines) (Moraes et al., 2011). In the current study when rAd5-FMDV and rAd5-Luc were compared, cytopathogenic effects were notably absent in rAd5-Luc inoculated cell cultures but present in rAd5-FMDV-infected cultures. Moreover, both transgene expression levels and cell stability were superior in rAd5-Luc inoculated cells. Detection of CAR, was altered in rAd5-FMDV (all time-points post inoculation), but not in rAd5-Luc cultures, further demonstrating deleterious effects of rAd5-FMDV vectors on cell integrity.

With the use of rAd5-Luc, we demonstrated increased transgene expression (luciferase activity) in inoculated cultures at 24 hpi in relation to earlier time points, with a further peak of luciferase activity at 48 hpi. Microscopy

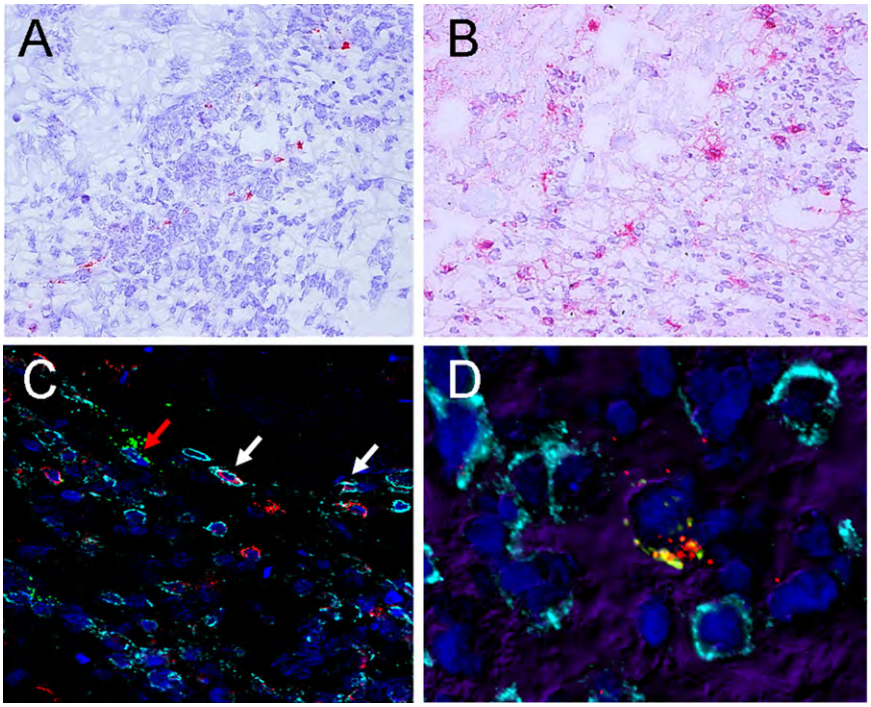

Fig. 5. (A and B) Presence of luciferase antigens in large mononuclear cells is shown by immunohistochemistry at 6 hpi (A) and at 24 hpi (B). (C) Immunofluorescent localization of luciferase (green), MHCII (red), and CD11c (light blue) antigens within infiltrates of the IS of a 24 hpi animal. Luc+CD11c+ colocalization (red arrows) in the proximity to single CD11c+ or MHC II+, or double CD11c+MHCII+ cells (white arrows), presumably corresponding to dendritic cells or macrophages. (D) Serial section of panel C, a double hexon+ (red) luc+ (green) positive cell can be observed surrounded by CD11c+ (light blue) cells. Nuclei are shown in blue.

data also followed the same detection pattern observed by luminometry throughout the time-course. By immunomicroscopy, detection of luciferase proteins was superior to detection of FMDV capsid products. Additionally, detection of increasing levels of luciferase (microscopy) lagged behind detection of rAd5 mRNA transcripts (representing transgene expression) by PCR; rAd5 DNA levels remained steady throughout the time-course for both vectors. As it was previously suggested (Montiel et al., 2012), it is possible that differences in efficiency of post-transcriptional expression of transgenes or reduced sensitivity of the anti-FMDV antibody compared to the anti-Luc antibody may account for differences in microscopic visualization of adenovirus transgene products in cells. However, in the current study, direct comparisons between luminometry results (integrated with PCR data) and microscopy, confirmed high sensitivity and a high degree of correlation of both protein detection-based techniques. This phase of the study demonstrated the utility of the rAd5-Luc vector as a suitable surrogate for study of biodistribution and gene expression in vitro and in vivo.

Consistent with our previously published results (Montiel et al., 2012), subsequent to inoculation with rAd5-Luc, luciferase activity was detected only at the injection site of all the experimental subjects with a statistically significantly higher peak of activity at 24 hpi followed by a rapid decrease by 48 hpi. Unlike luciferase activity, severity of the inflammatory infiltrates in response to inoculation also reached a plateau at 24 hpi and remained high at 48 hpi. Together, these observations confirmed (1) active production of transgene protein products (luciferase) and (2) rapid and efficient recruitment of leukocytes to the sites of rAd5 inoculum-induced inflammation. Steady levels of inflammatory infiltrates, including presence of macrophages, and a rapid rise and fall of luciferase activity during the first 48 hpi is consistent with previous studies that indicate that during the first 24 h after muscle injury phagocytic macrophages are attracted to inflamed areas to degrade damaged muscle (Schiaffino and Partridge, 2008). These phagocytic functions of macrophages could partially explain the short-lived presence of luciferase and the substantial differences in luciferase detection between 24 and 48 hpi in muscle. Furthermore, immunomicroscopy of injection sites demonstrated that luciferase followed a multifocal and heterogeneous distribution in muscle with varying degrees of association with cells of the infiltrates. Consistent with luciferase activity, visualization of luciferase proteins in muscle increased from 6 to 24 hpi followed by a rapid decrease in detection at 48 hpi. Therefore, it is likely that following inoculation with rAd5-Luc, specific target cells are efficiently infected, luciferase is effectively produced and detected at the highest levels at 24 hpi, and rapidly commences to be degraded within phagocytic macrophages.

Alternatively, it has been proposed that IFN-γ induced by innate anti-adenoviral immune responses inhibit adenovirus-mediated transgene expression (Sung et al., 2001), and that the actions of anti-IFN-γ antibodies can

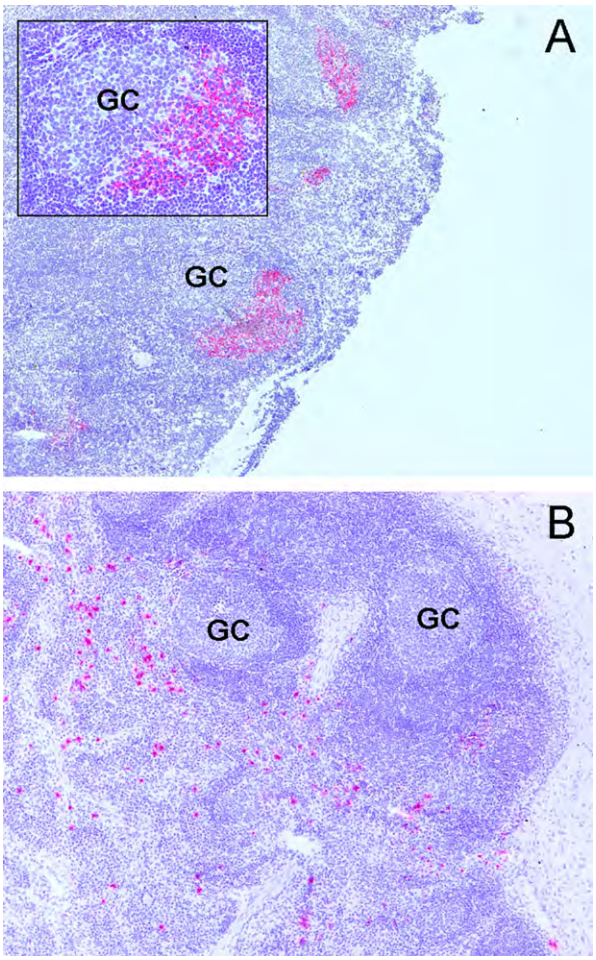

Fig. 6. Immunohistochemical detection of hexon and luciferase antigens in local LN of rAd5-Luc inoculated steers. (A) Steer #1, hexon signal is observed at the cortex of one of the medial retropharyngeal LN within primary lymphoid follicles, at 6 hpi. Note hexon-laden mantle zones of primary lymphoid follicle (A, inset). (B) Steer #3, luciferase positive cells are seen scattered throughout the perifollicular cortex, subcortex, and corticomedullary junction of the axillary LN of rAd5-Luc inoculated steer #3, at 24 hpi. GC, germinal center. Micropolymer alkaline phosphatase technique.

reverse these effects, enhancing immunogenicity of recombinant adenovirus vectors (Jackson et al., 2011). During the acute phase of inflammation and immune responses, IFN-γ is produced by NK cells and antigen-presenting cells (APCs), especially interleukin-12 -stimulated macrophages and dendritic cells (Frucht et al., 2001). Thus, it is plausible that our observations could also be attributed to a combination of both phagocytic effects of macrophages and actions of IFN-γ during and after the first 24 hpi. While it is possible that discrepancies observed on levels of luciferase detected by luminometry and/or microscopy may be due to intrinsic variability of the techniques and or to inter-animal variation factors, our data strongly suggest that the majority of the effects observed are attributable to true biological processes in response to rAd5 infection.

We previously reported that detection of rAd5 DNA and rAd5 mRNA transcripts in tissues of rAd5-FMDV-vaccinated cattle elucidated important aspects of adenovirus distribution and transgene expression (Montiel et al., 2012). In the current study, patterns of biodistribution and expression of rAd5-Luc-derived nucleic acids were remarkably similar to those of a rAd5-FMDV (reported in our previous study). A cross-analysis of luminometry, PCR, and immunomicroscopy results clearly demonstrates correlation across detection techniques. In muscle, the highest levels of mRNA transgene expression corresponded to strong detection of luciferase (by luminometry) at 24 hpi. However, at 48 hpi, despite abundant rAd5 mRNA (Ct values between 30 and 32) at the IS, luciferase activity was found at surprisingly lower levels. This may be explained by selective phagocytic elimination of the subset of infected cells with active translation of luciferase; thus PCR signal may persist from infected (translationally inactive) cells that remain in tissues. Alternatively, luciferase may be degraded within the phagolysosomal compartment, whereas nucleic acids (particularly viral DNA) persist in a distinct cellular compartment.

While detection of luciferase in muscle was consistently demonstrated by microscopy and luminometry, luciferase activity was only marginally present in all other tissue specimens examined, including local and regional LN. This finding is consistent with observations for our previous report where luciferase was only present at the injection site of a single rAd5-Luc inoculated steer. It is likely that lack of detection is due to the dilution effect created by inoculation of a replication-incompetent vector into a 300 kg steer. Alternatively this may represent suboptimal sensitivity of the luciferase bioassay in non-injection sites as compared to immunomicroscopy. It has been shown that both liver and adrenal are major targets for both wild-type adenoviruses and recombinant vectors (Bernt et al., 2003; Duncan et al., 1978; Wang et al., 2003; Ying et al., 2009). While hepatic tissue has been demonstrated to be the principal organ for adenovirus sequestration (Shayakhmetov et al., 2004), persistent transgene expression and functional gene activity in adrenal tissue of animals undergoing gene transfer experiments using rAd has been reported by several groups (Alesci et al., 2007; Senoo et al., 2000; Ying et al., 2009). Our observations of low level luciferase activity detected in adrenal tissue are supported by the published studies. Complete lack of detection in liver remains confounding. However, non-canonical tropism may be the result of atypical tropism of a human rAd5 vector interacting with bovine tissues and cells.

In agreement with previous reports (Banchereau and Steinman, 1998; Guidotti and Chisari, 2001; Montiel et al., 2012; Steinman et al., 1999), the rapid recruitment of dendritic cells and macrophages to injection sites in areas where hexon proteins and transgene product (luciferase) localize, is highly suggestive of critical early immunological events triggered by inoculation with the rAd5 vector. Importantly, subsequent to inoculation with rAd5-Luc, a peak of hexon and transgene protein expression and detection occurs at 24 hpi followed by a rapid decrease by 48 hpi, while expression of various combinations of CD14+, CD11c+, MHCI+, MHCII+ phenotypes within cellular infiltrates reach steady levels after 24 hpi. In addition, during

the first 48 hpi, luciferase proteins were detected in cortical and interfollicular regions of some of the local LN. Overall, these findings suggest that at the site of inoculation, adenovirus and transgene products are first captured and processed for antigen presentation by APCs through MHC I and/or II -dependent mechanisms, followed by rapid trafficking of transgene product (free or cell-associated) to lymphoid tissues (lymph nodes) where induction of protective immune responses is presumably initiated. The extent to which this trafficking was modulated by the adjuvant used in this study remains undetermined; however microscopic localization of adjuvant at 6 hpi and adjuvant-associated inflammation at 48 hpi suggest a contributory role in the immunopathogenesis.

5. Conclusions

Recent work (Montiel et al., 2012) has documented the biodistribution in cattle of a recombinant Ad5 construct encoding FMDV capsid antigens (rAd5-FMDV) which is the first recombinant vaccine granted conditional license for use in cattle by the U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service's Center for Veterinary Biologics (CVB) (Press release[1,2]). In the present study, we assessed biodistribution and transgene expression of a rAd5 containing the luciferase gene and demonstrated that it behaves remarkably similar to rAd5-FMDV. Thus, these baseline descriptions obtained by the former and the current work characterizing rAd5 inoculation in cattle will contribute to a better understanding of rAd5-FMD vaccine immunopathogenesis and toward efforts to enhance adenoviral-based FMDV vaccine potency and efficacy.

Acknowledgments

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[1] http://dairybusiness.com/dairyline_headline.php?item=New+Foot-And-Mouth+Disease+Vaccine+Gets+Licensed.

[2] http://benchmarkbiolabs.com/2012/06/a-world-free-one-most-virulent-animal-diseases-planet/.

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Early Adaptive Immune Responses in the Respiratory Tract of Foot-and-Mouth Disease Virus-Infected Cattle

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Foot-and-mouth disease (FMD) is a highly contagious viral disease which affects both domestic and wild biungulate species. This acute disease, caused by the FMD virus (FMDV), usually includes an active replication phase in the respiratory tract for up to 72 h postinfection, followed by hematogenous dissemination and vesicular lesions at oral and foot epithelia. The role of the early local adaptive immunity of the host in the outcome of the infection is not well understood. Here we report the kinetics of appearance of FMDV-specific antibody-secreting cells (ASC) in lymphoid organs along the respiratory tract and the spleen in cattle infected by aerosol exposure. While no responses were observed for up to 3 days postinfection (dpi), all animals developed FMDV-ASC in all the lymphoid organs studied at 4 dpi. Tracheobronchial lymph nodes were the most reactive organs at this time, and IgM was the predominant isotype, followed by IgG1. Numbers of FMDV-ASC were further augmented at 5 and 6 dpi, with an increasing prevalence in upper respiratory organs. Systemic antibody responses were slightly delayed compared with the local reaction. Also, IgM was the dominant isotype in serum at 5 dpi, coinciding with a sharp decrease of viral RNA detection in peripheral blood. These results indicate that following aerogenous administration, cattle develop a rapid and vigorous genuine local antibody response throughout the respiratory tract. Time course and isotype profiles indicate the presence of an efficient T cell-independent antibody response which drives the IgM-mediated virus clearance in cattle infected by FMDV aerosol exposure.

Foot-and-mouth disease (FMD) is a highly contagious viral disease which affects a wide range of domestic and wild biungulate species. Although fatal cases usually occur in young animals, the high transmissibility and morbidity observed in adult animals infected by the FMD virus (FMDV) result in major economic losses to the livestock industry during disease outbreaks (1). The potentially devastating economic, social, and environmental consequences of the disease have been demonstrated dramatically during the last 2 decades by a number of different outbreaks of the disease reported around the world (2–4).

FMDV pathogenesis presents particular features depending on the host. Routes of entry, primary replication sites, and, consequently, the associated symptoms and immune responses elicited differ among species (reviewed in reference5). Cattle are highly susceptible to FMDV, and the virus usually gains entry through the respiratory tract of these animals (6). Moreover, the ability of FMDV to replicate in tissues of the upper respiratory system, demonstrated in early reports (7) and widely confirmed later using more sensitive approaches, identified the soft palate and pharynx as sites of FMDV replication and persistence in ruminants (8, 9). Sutmoller and McVicar further expanded their initial findings to include the lung as an additional portal of virus entry (10), although many researchers considered the evidence for both sites of entry to be controversial. Recently, a detailed description of the previremic stages after experimental aerosol infection of cattle confirmed these early results, identifying both the pharynx and lung as primary sites for viral entry (11). Cattle infected by controlled aerosol exposure of infectious FMDV (12) exhibit a primary replication event in epithelial cells of the pharynx followed by extensive replication in pneumocytes in the lungs, which in turn allows the establishment of a sustained viremia (11).

The detection of adaptive immune responses at the local level after natural or experimental infection of cattle was performed in the past through the detection of secretory immunoglobulins in the oronasal cavities and esophageal-pharyngeal fluids (OPF), and most of the time these efforts were focused on the discovery of markers of infection or a carrier state (13–19). In an early report, Figueroa et al. (13) reported neutralizing activity in samples of saliva and nasal fluids taken from cattle infected by FMDV administered by intranasal spray. Later, McVicar and Sutmoller (18), also using an intranasal route for infection, detected neutralizing antibodies in saliva and OPF, suggesting a putative independence between the local response and the antibody response detected in serum. Other reports also showed the detection of FMDV-specific IgM and IgA in pharyngeal fluids 1 week after infection, but they proposed a systemic origin for these antibodies (14, 20). In these reports, only IgA antibodies detected at late infection times were attributed to genuine mucosal responses. None of these studies were able to identify a local immune response earlier than the 7th day postinfection.

Despite these studies, the role of the early host local immune response in the outcome of the infection is not well characterized.

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In this report, we approached the study of the local immunity against FMDV in cattle experimentally infected through the oronasal route by identifying organs and tissues involved in the local production of antibodies. Lymphoid tissues from the respiratory system were analyzed at early times postinfection by use of an FMDV-specific antibody-secreting cell enzyme-linked immunosorbent spot (FMDV-ASC ELISPOT) assay developed for this purpose. We provide evidence of the existence of antibody mucosal responses soon after infection, and we determined their origin, time course, and immunoglobulin isotype profiles. The results show a strong stimulation of FMDV-specific B lymphocytes to locally produce antibodies all along the respiratory tract, including in the tracheobronchial lymph nodes (TBL). This local response precedes the appearance of the systemic humoral immune response and demonstrates a close correlation with the disappearance of viremia, mediated mainly by FMDV-specific IgM antibodies.

MATERIALS AND METHODS

Experimental animals. Fifteen 15- to 18-month-old Hereford steers weighing 300 to 350 kg each were used for the experiments. Animals were obtained from an FMDV-free region and checked for the absence of FMDV-specific antibodies by a liquid-phase blocking enzyme-linked immunosorbent assay (lpELISA) before infection. During the experimental period, steers were kept in biosafety level 3A (BSL-3A) animal facilities according to internal and federal regulations on biosecurity and animal welfare (Institute of Virology, CICVyA-INTA, Argentina).

Virus, experimental infections, and clinical assessment of cattle. Virulent FMDV strain O1 Campos was provided by the OIE FMD Reference Laboratory at SENASA, Argentina. Experimental infection through the oronasal route was performed with a jet nebulizer attached to an aerosol delivery system (10^7 50% tissue culture infective doses [$TCID_{50}$] in a 2-ml volume per animal) according to the protocols described by Pacheco et al. (12). Cattle were monitored daily for clinical signs of FMD after challenge. These included vesiculation, lameness, increased salivation, loss of appetite, and fever (rectal temperature of >39.5°C). Clinical scores were determined by assigning a score of 1 for fever or behavioral modifications, 1 for lesions in the oral and nasal cavities, and 1 for each foot that developed vesicles, with a maximum clinical score of 6.

Inactivated FMDV antigens. Inactivated suspensions of FMDV O1 Campos were kindly provided by Biogénesis-Bagó S.A. (Argentina), and whole viral particles were purified following a standard sucrose density gradient (SDG) centrifugation method (21) further optimized in our laboratory. Briefly, inactivated FMDV suspensions were mixed 4:1 (vol/vol) with 6% *N*-lauroylsarcosine (Sigma-Aldrich) in NET buffer (0.1 M NaCl-0.004 M EDTA-0.05 M Tris, pH 8.0). This preparation was placed on a discontinuous 15 to 45% SDG and ultracentrifuged at 45,000 × *g* for 16 h at 4°C in an SW-28 rotor in a Beckman Coulter ultracentrifuge. Fractions were collected by selecting the peak absorbance at 260 nm and were pooled. This preparation was further centrifuged at 100,000 × *g* for 16 h at 4°C, and pelleted virus was resuspended in NET buffer, quantified by spectrophotometry at 260 nm, and stored at −80°C. The integrity of 140S particles was assessed by SDG and a monoclonal antibody (MAb)-based indirect ELISA applied to each gradient fraction, as described previously (22).

Serum and tissue sample collection. Serum samples were collected on a daily basis from the arrival of the animals until the time of necropsy. One steer (C135) was euthanized and necropsied for tissue collection prior to the experimental infections to provide negative-control samples for immunological assays. The rest of the animals were aerosol inoculated and then euthanized and necropsied at different time points from 2 to 6 days postinfection (dpi), except for two steers that were kept until 14 dpi and were sampled daily only for serum. Tissue collection was based on previous reports showing the main sites of FMDV replication along the respiratory tract (11, 23, 24). Samples obtained postmortem included 6 anatomically distinct organs and tissues: mandibular lymph nodes (ML), medial and lateral retropharyngeal lymph nodes (MRL and LRL, respectively), pharyngeal tonsils (PhT), tracheobronchial lymph nodes (TBL), and the spleen. Tissues were collected aseptically and placed in ice-cold wash buffer (RPMI 1640, 10 mM HEPES, 10^6 units/ml penicillin G sodium, 700 mg/ml streptomycin, and 500 mg/ml gentamicin) until processing.

Isolation of mononuclear cells from lymphoid tissues. All tissues were processed to obtain mononuclear cell suspensions according to previously published protocols (25), with minor modifications. Briefly, tissues were first ground in cell collectors with 80-mesh screens (Sigma-Aldrich, St. Louis, MO) to obtain single-cell suspensions. Cells were then pelleted, resuspended in 90% Percoll solution (GE Healthcare, Uppsala, Sweden), and centrifuged at 5,000 × *g* (30 min at 4°C). Cell pellets were resuspended in 43% Percoll solution, transferred to a 70% Percoll solution, and centrifuged at 4,000 × *g* (30 min at 4°C). Mononuclear cells were collected at the 43 to 70% interface and resuspended in complete medium (wash buffer with 10% fetal bovine serum), and their viability was determined by trypan blue exclusion.

ELISPOT assay for FMDV-specific ASC. An FMDV-ASC ELISPOT assay was developed for this study. Ninety-six-well nitrocellulose plates (Millipore, MA) were coated overnight with inactivated purified FMDV O1 Campos and blocked with 4% skim milk for 1 h at room temperature (RT). Mononuclear cells were seeded in FMDV-coated plates in 2-fold dilutions (5×10^6 and 2.5×10^6 cells per ml) in triplicate wells and incubated overnight at 37°C with 5% CO_2. After 5 washes with phosphate-buffered saline (PBS), mouse anti-bovine IgG1 or IgG2 monoclonal antibodies (BD-Serotec, Oxford, United Kingdom) were added (1:500 dilution) and incubated for 1 h at RT. Reactions were revealed with anti-mouse IgG horseradish peroxidase (HRP)-labeled conjugate (KPL, United Kingdom) for 1 h at RT, followed by addition of True Blue peroxidase substrate (KPL, United Kingdom). IgM and IgA ASC were detected with HRP-labeled sheep anti-bovine IgM and IgA sera (Bethyl, TX), diluted 1:5,000 and revealed as described above. Spots corresponding to ASC were visualized and counted manually under a stereomicroscope. Spots from control wells were subtracted from experimental wells, and results were expressed, unless otherwise indicated, as the mean number of ASC per 5×10^5 cells for triplicate wells.

FMDV RNA detection in serum samples. Specific sense and antisense primers were utilized to amplify a 259-nucleotide fragment of the viral polymerase gene (positions 7079 to 7338). Viral RNA was extracted from serum samples (140 μl) by use of a QIAamp viral RNA minikit (Qiagen), and reverse transcription was carried out using the antisense primer and reverse transcriptase (from Moloney murine leukemia virus [M-MLV]; Promega) under standard conditions. The resulting template cDNAs or 10-fold dilutions of a standard plasmid containing the 3D gene were used for PCRs, which were performed with a real-time PCR master mix (Mezcla Real; Biodynamics) and ROX reference dye (Invitrogen). The reaction was started with a 10-min incubation at 95°C, followed by 45 amplification cycles (95°C for 15 s followed by 1 min at 60°C), and after cycling, a dissociation stage was carried out to detect specific amplification. A standard curve built using plasmid dilutions with the specific primers was utilized to correlate threshold cycle (C_T) values obtained from serum samples with the number of FMDV genome copies per ml. The standard curve presented a slope of −3.332 (primer efficiency, >99%) and an R^2 value of 0.993. Samples and standards were run in triplicate in an ABI 7500 thermocycler (Applied Biosystems) and analyzed using model 7500 SDS software v 1.3.1. Both primers and the standard plasmid were kindly provided by Guido König.

IgM purification from serum samples. Total IgM was purified from pooled serum samples by affinity chromatography in an Äkta Explorer system (GE Healthcare). Serum samples were diluted 1:3 in binding buffer [20 mM sodium phosphate, 0.8 M $(NH_4)_2SO_4$, pH 7.5] and applied to HiTrap IgM HP purification columns (GE Healthcare). Unbound input

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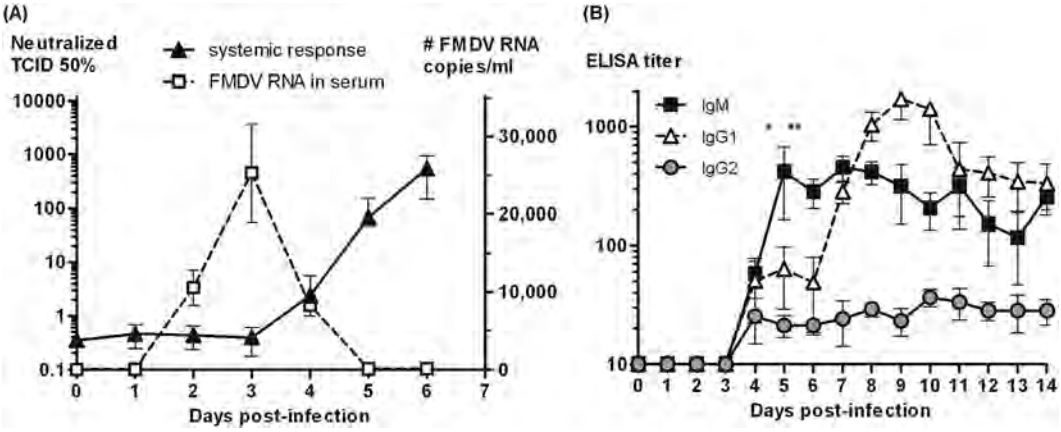

FIG 1 Time course of viral RNA detection and systemic adaptive immune responses in cattle after FMDV aerosol exposure. (A) Viral RNA detection in sera from infected cattle at each time point is indicated as the mean number of FMDV genome copies/ml ± standard deviation (SD) (right axis). Systemic antibody responses are represented by the number of serum neutralizing doses detected for each animal, and results are expressed as the mean $TCID_{50}$ neutralized by the diluted sera from all surviving animals at each time ± SD (left axis). (B) Isotypes of FMDV-specific serum antibodies were analyzed by an FMDV-specific isotype indirect ELISA. Each line corresponds to a particular isotype, and each point represents the average ELISA titer obtained for all animals assayed. Titers are expressed as the highest dilution of serum reaching an optical density of 0.2 (mean value for 25 negative sera plus 2 SD). *, significant differences between IgM titers at 4 and 5 dpi ($P < 0.05$); **, significant differences between IgM and IgG1 titers at 5 dpi ($P < 0.05$).

sample was collected and preserved as the IgM-depleted fraction, and IgM-purified fractions were eluted in 20 mM sodium phosphate buffer, pH 7.5. A purified commercial bovine IgM standard (Sigma) was also included as a control for the whole purification procedure. The IgM content in all fractions was confirmed by 10% SDS-PAGE and Western blot assay, using an HRP-labeled sheep anti-bovine IgM (Bethyl) as a probe (data not shown). All fractions assayed for virus-neutralizing activity were further adjusted to match a 1:8 dilution of the original whole-serum pools.

Serology determinations. FMDV-specific total antibody titers were determined by an lpELISA originally developed by Hamblin et al. (26) and further modified by Periolo et al. (27).

Neutralizing antibodies were detected by a microplate virus neutralization assay modified in our laboratory (28). Briefly, serum samples (50 μl; 1:16 dilution in RPMI) were added to 96-well cell culture plates and incubated at 37°C for 1 h with four 10-fold dilutions of infective FMDV O1 Campos (50 μl; 1 to 1,000 $TCID_{50}$). BHK-21 cell suspensions (100 μl; 3×10^5 cells/ml) were then added and incubated for 72 h at 37°C with 5% CO_2. Virus dilutions were prepared from a 10^8-$TCID_{50}$ FMDV O1 Campos stock suspension, and the concentration was assessed for each test, allowing a variation of $\pm 0.5 \log_{10}$ from the expected value. Neutralization assays performed for purified IgM and pooled sera were carried out essentially in the same way, except that samples were diluted 1:8 (50 μl), mixed with the different virus dilutions (50 μl), and applied to BHK-21 cell monolayers already seeded in 96-well cell culture plates. For both assays, neutralizing antibody titers were expressed as the $TCID_{50}$ neutralized by the diluted samples, according to the method of Reed and Muench (29).

FMDV-specific isotype ELISA was performed as reported by Capozzo et al. (30), except that sheep anti-bovine IgG1 and IgG2 HRP-conjugated antibodies were used at a dilution of 1:750, and sheep anti-bovine IgM HRP-conjugated antibody was used at 1:5,000 (BD-Serotec, Oxford, United Kingdom).

Statistical analysis. Differences in the number of ASC produced per isotype per animal and between FMDV-specific serum IgM titers at different times were analyzed by one-way analysis of variance (ANOVA), and pairwise comparisons were carried out using the Tukey test. Differences in mean FMDV-specific serum isotype titers at different times were analyzed by two-way ANOVA following the Bonferroni method. Analyses were performed using Graph Pad Prism 5.0 software (Graph Pad Software Inc.).

RESULTS

Progression of viremia and clinical symptoms following experimental oronasal FMDV infection in cattle. A total of 12 animals were experimentally infected by the oronasal route following the procedure described by Pacheco et al. (12). Clinical symptoms were similar across the infected cattle. Animals started exhibiting fever and general lameness between 1 and 2 days after exposure, and the first vesicles were observed from day 2 postinfection. As expected, clinical scores progressed in a time-dependent manner, up to the maximum score of 6, corresponding to a generalized infection with lesions in all feet and in oral and nasal cavities at 6 dpi (data not shown). FMDV RNA was detected in serum after 24 h postinfection; the number of viral genome copies sharply increased to a peak at 3 dpi and disappeared from the bloodstream of infected animals by 5 dpi, coinciding with the onset of systemic antibody responses (Fig. 1A).

Systemic adaptive antibody responses in FMDV-infected cattle. Circulating FMDV-neutralizing antibodies were initially detected at 4 dpi in 2 of 8 assayed animals, and they were observed in all animals ($n = 5$) at 5 dpi (Fig. 1A). Similar results were observed when total FMDV-reacting antibodies were studied by lpELISA (data not shown). Isotype profiles at 4 dpi showed that mean FMDV-specific IgM titers were at similarly low levels as IgG1 antibodies, while IgG2 was barely evident. Mean FMDV-specific IgM titers, however, increased significantly at 5 dpi ($P < 0.05$) and were significantly higher than IgG1 or IgG2 titers at 5 dpi ($P < 0.05$). FMDV-specific IgG1 and IgG2 levels remained unchanged between 4 and 6 dpi. Following this initial phase, IgG1 levels dramatically increased, peaking at 9 dpi and remaining stable between 11 and 14 dpi, the last sampling time point. IgM titers remained mostly unchanged from 5 dpi, showing little fluctuation between 12 and 14 dpi, and IgG2 titers remained low throughout the study period (Fig. 1B).

Mucosal adaptive antibody responses in FMDV-infected cattle. Animals were sacrificed at 2 ($n = 1$), 3 ($n = 3$), 4 ($n = 3$), 5 ($n = 2$), or 6 ($n = 3$) dpi to study FMDV-specific mucosal im-

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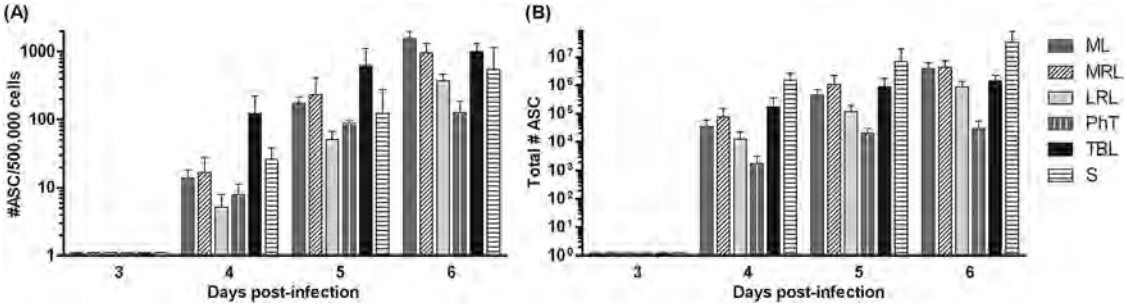

FIG 2 Detection of FMDV-specific antibody-secreting cells in lymphoid organs of the respiratory tract of infected cattle. After oronasal infections, FMDV-specific ASC were detected by an FMDV-ASC ELISPOT assay. Mandibular lymph nodes (ML), pharyngeal tonsils (PhT), lateral (LRL) and medial (MRL) retropharyngeal lymph nodes, tracheobronchial lymph nodes (TBL), and the spleen (S) were processed and assayed by the FMDV-ASC ELISPOT assay. Results are expressed as either the number of FMDV-specific ASC per 5×10^5 extracted mononuclear cells (A) or the total number of FMDV-specific ASC per organ (B). Each bar corresponds to the average value for 3 individuals (except for 5 dpi, where $n = 2$) plus SD for each particular organ.

mune responses along the respiratory tract by FMDV-ASC ELISPOT assay. An additional naïve animal (C135) was also sacrificed and processed for use as a negative control. Onset of local responses was established between 3 and 4 days postinfection. While FMDV-specific ASC were not detected in any of the individuals studied at 3 ($n = 3$) or 2 ($n = 1$) dpi, all animals analyzed at 4 dpi ($n = 3$) developed adaptive responses in most of the lymphoid tissues sampled. The mean number of FMDV-specific ASC (per 5×10^5 total mononuclear cells) detected in the organs of the respiratory tract, considering all animals and organs analyzed at each time, rose ~7-fold between 4 (mean ± standard deviation [SD], 166.4 ± 104.0) and 5 (mean ± SD, 1,172.5 ± 726.2) dpi and ~3.4-fold between 5 and 6 dpi (mean ± SD, 4,011.0 ± 1,003.9) (see Table S1 in the supplemental material). A strong FMDV-specific antibody response was detected in all infected cattle at 6 dpi: FMDV-ASC represented up to 0.39% of the total mononuclear cells extracted from mandibular lymph nodes and 0.23% of those from the tracheobronchial lymph nodes (Fig. 2; see Table S1 in the supplemental material). When ELISPOT assay results were considered in terms of the total number of FMDV-specific ASC per organ, no major changes in the general profile were observed, with the exception of values corresponding to the largest organ, the spleen, and the smallest one, the pharyngeal tonsil.

Lymphoid organs involved in local production of FMDV antibodies in infected cattle. Five different lymphoid tissues from the lower and upper respiratory systems were sampled: mandibular and retropharyngeal (lateral and medial) lymph nodes, pharyngeal tonsil, and TBL. The spleen was also analyzed as a representative measurement of systemic responses elicited in each animal. TBL were the most reactive organs in all the individuals studied 4 days after FMDV infection (Fig. 2). The spleen (S), medial retropharyngeal (MRL), and mandibular (ML) lymph nodes followed the TBL in the mean number of anti-FMDV ASC, although no differences were registered among these organs compared with the lateral retropharyngeal lymph nodes (LRL) or the pharyngeal tonsils (PhT), which presented only a marginal reactivity.

A similar profile was found at 5 dpi, with TBL as the predominant organ for FMDV-specific ASC, followed by MRL (steer C118) or ML (steer C146). Differences in FMDV-specific ASC numbers in MRL and ML were small with respect to those in TBL and LRL; PhT had increased anti-FMDV ASC numbers relative to those in TBL. ASC numbers in the spleen were augmented in one of the animals (steer C118), without exhibiting significant differences within the other organs. The remaining steer did not display a further increase in spleen stimulation relative to the value from 4 dpi (Fig. 2; see Table S1 in the supplemental material).

The increasing relevance of the upper respiratory organs in the mucosal response initiated at 5 dpi was confirmed at 6 dpi. ML were significantly the most reactive organs in all animals studied at this time ($n = 3$), followed by the TBL and MRL, with similar values for all animals analyzed. The number of FMDV-ASC in ML from the 3 steers increased ~9-fold, on average, relative to the number from the previous day, while the LRL, which displayed relatively low levels at 4 and 5 dpi, also increased their average number >7-fold. Rises registered in the PhT were moderate, reaching an average of 128 FMDV-specific ASC/5×10^5 mononuclear cells. Responses in the spleen were modestly higher than those of the previous day for two of the animals (steers C115 and C126), although the third animal analyzed (steer B995) presented a marked increase in the anti-FMDV reactivity in this organ as measured by ELISPOT assay, reaching a total of 1,220 ASC/5×10^5 mononuclear cells (see Table S1 in the supplemental material).

Immunoglobulin isotype profiles induced by local adaptive immunity in FMDV-infected cattle. As mentioned above, TBL were the most stimulated organs in all three animals 4 days after infection, with IgM as the dominant isotype among the FMDV-ASC developed in these organs. IgG1 was consistently second with regard to detection levels, with levels 3- to 4-fold lower than those for IgM. IgA and IgG2 ASC were detected at very low levels or were undetectable. This pattern was repeated overall, with lower magnitudes, in all the local organs assayed. FMDV-specific ASC in the spleen also exhibited increased levels of IgM-producing cells compared to cells expressing the rest of the isotypes. Splenic IgG2 ASC were not detectable at this time, and IgA and IgG1 values, which were 8 to 10 times lower than the IgM value, showed no significant differences between them (Fig. 3; see Table S1 in the supplemental material).

Five days after infection, TBL were still the most stimulated organs in both animals studied. The isotype pattern observed was similar to that of the previous day, although the IgM/IgG1 and IgM/IgA ratios increased 3- to 4-fold. The total number of FMDV-specific ASC in TBL was also augmented 5-fold relative to that on the previous day, and thus an important fraction of this increase was due to the IgM ASC. The same figure was registered in the

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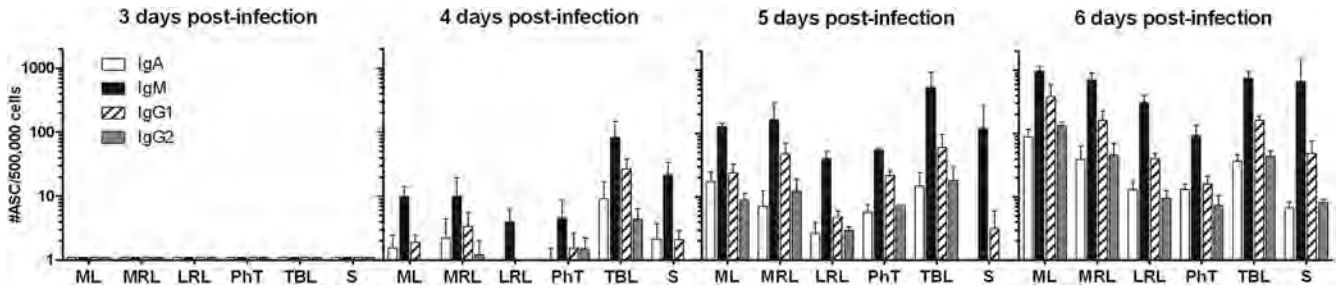

FIG 3 Isotype profiles of the FMDV-ASC developed in cattle after FMDV aerosol exposure. Mononuclear cells were purified from mandibular lymph nodes (ML), pharyngeal tonsils (PhT), lateral (LRL) and medial (MRL) retropharyngeal lymph nodes, tracheobronchial lymph nodes (TBL), and the spleen (S) and assayed by the FMDV-ASC ELISPOT assay, using monoclonal (IgG1 and IgG2) or polyclonal (IgM and IgA) antibodies against bovine immunoglobulin isotypes as probes. Results are expressed as the number of FMDV-specific ASC per 5×10^5 extracted cells, and each bar corresponds to the average value for 3 individuals (except for 5 dpi, where $n = 2$) plus SD for each particular isotype and organ.

spleen, since IgG1 and IgA isotypes showed little differences from the previous day, while mean IgM ASC numbers went from 21 to 118 ASC per 5×10^5 extracted cells (Fig. 3). IgM was also the dominant isotype in the FMDV-ASC detected in the rest of the organs tested, and differences with the other isotypes remained mostly constant.

The general isotype pattern at 6 dpi was still dominated by IgM ASC for all animals and organs assayed, although a relative rise in the numbers of FMDV-specific IgG1 and IgA ASC was observed, mainly in the ML, TBL, and spleen (Fig. 3). The number of FMDV-specific IgG2 ASC was also augmented, particularly in ML, reaching IgA levels. One of the animals (steer B995) developed a large number of FMDV-specific IgG1 ASC (789 ASC/5×10^5 cells), close to the IgM level in the same animal and organ (1,003 ASC/5×10^5 cells). Interestingly, this was the same individual that also presented a significantly larger number of anti-FMDV splenic ASC than the other 2 animals studied at that time (see Table S1 in the supplemental material).

Neutralizing activity of FMDV-specific serum IgM antibodies isolated from infected cattle. In order to evaluate the contributions of the different isotypes to the phenomenon of virus neutralization during infection in cattle, the neutralizing ability of purified serum IgM was tested using a pool of sera obtained daily from 4 to 6 days after infection. For each time point, serum IgM was affinity purified and its neutralizing activity compared to those of whole pooled sera, IgM-depleted serum fractions, and pooled sera from uninfected animals. As shown in Fig. 4, neutralizing activity of the tested samples was clearly detected after 4 dpi, and the serum IgM fractions from 5 and 6 dpi neutralized infective FMDV to levels similar to those with whole serum. In contrast, the neutralizing capacity of the IgM-depleted serum fractions resembled that of the normal serum for all time points. This result is in line with a central role of this immunoglobulin subclass in the early clearance of viremia in infected animals.

DISCUSSION

The detection of specific neutralizing antibodies in oronasal and esophageal-pharyngeal fluids has previously been described for cattle infected by oronasal routes with different FMDV O1 strains (13, 15, 18). All of these reports were based on results obtained starting at least 1 week after the experimental infections. Consequently, they did not clearly determine the onset of the local anti-FMDV antibody response in naturally infected cattle or indubitably identify the exact organ/tissue originating that response.

Neutralizing activity in cattle infected by nasal spray exposure was reported for nasal fluids and saliva 10 and 21 days after nasal spray infection, respectively (13), and around 2 weeks after instillation for OPF samples (18). An even earlier report by Kaaden and Matthaeus (16) revealed a poor correlation between serum and saliva neutralization titers, with IgA as the predominant isotype in salivary secretions. McVicar and Sutmoller (18) also compared the kinetics of the serum and mucosal antibody responses up to 30 weeks after primary infection and concluded that the lack of temporal correlation between them might reflect the independence of the FMDV-neutralizing activity at systemic and local levels. This perspective, however, was later set aside by other authors. Francis et al. (14) detected FMDV-specific IgM and IgA antibodies in pharyngeal fluid in naïve cattle 7 days after virus exposure but proposed that these were actually due to serum and tissue fluid escaping from the damaged mucosa during the acute inflammatory phase of infection. According to these authors, active local antibody production was evident only at later stages (20 to 60 days after virus exposure) and corresponded to the rise in specific IgA antibodies. Archetti et al. also followed the kinetics of serum and OPF antibodies in naïve cattle by use of ELISA (20). Even when the earliest positive reaction was detected at day 14 postinfection, they proposed that only the IgA responses peaking after healing of the vesicular lesions (between 4 and 8 weeks postinfection) were produced by mucosa-resident antibody-secreting B lymphocytes.

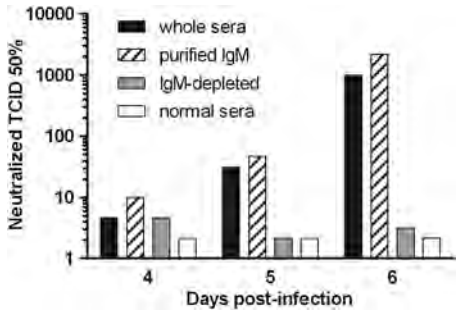

FIG 4 Virus-neutralizing activity in whole sera and antibody fractions. IgM antibodies were purified from pooled serum samples taken between 4 and 6 dpi. Neutralizing antibodies were determined for sera from nonimmune cattle, as well as pooled sera, IgM, and IgM-depleted fractions from infected animals, as described in Materials and Methods. Results are expressed as $TCID_{50}$ neutralized by the different diluted samples.

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Therefore, the role of the early host local immune response in the outcome of the infection is still not well understood.

We approached the study of the mucosal humoral responses induced by FMDV infection by the aerosol route in cattle by analyzing the induction of ASC specific for the virus in lymphoid tissues located along the respiratory tract to obtain information about the source of the antibodies detected at local lymphoid organs in relation to tissues involved in the initial viral replication stages (11, 23, 24).

Interestingly, patterns of immune reactivity found in lymphoid organs after infection were associated with the early stages of replication of aerosolized FMDV. Arzt et al. (11) used the same aerosol exposure method to show that after primary replication (during 6 h postexposure) in the follicle-associated epithelium overlying the pharyngeal mucosa-associated lymphoid tissue, the virus followed an extensive replication in pneumocytes between 24 and 48 h postexposure. They proposed that this replication in the lungs allows the virus to establish and sustain viremia. Accordingly, our results demonstrated that the onset of local adaptive responses at 4 dpi was dominated by the TBL, with poor stimulation of the lymph nodes from the upper respiratory system, and that only at 6 dpi were lymph nodes from the upper tract able to exceed TBL ASC counts.

It is possible to hypothesize that this first extensive replication of FMDV in large organs such as the bovine lungs is directly related to the predominant stimulation of the tracheobronchial lymph nodes draining these tissues observed on days 4 and 5 after infection and thus independent from the humoral systemic response. It is probable that primary replication in nasopharynx and larynx tissues produced only limited amounts of FMDV, and therefore only a modest number of FMDV-specific B cells were activated in lymph nodes from the upper tract at the onset of the response. Only when the virus was extended in a generalized infection through the peripheral circulation could a larger number of FMDV-specific B lymphocytes be activated in these lymph nodes. Progression of the response from 6 dpi led to a more extended immunoglobulin class switching and to an increasing importance of the systemic immunity, as reflected by the spleen ASC responses and IgG1 serum levels detected between 7 and 14 dpi. Interestingly, IgM mediated most of the local ASC responses coexisting with the end of the viremia phase at 5 dpi.

The FMDV capsid possesses key structural features compatible with type 2 T cell-independent antigens, which are able to stimulate antibody production in the absence of major histocompatibility complex (MHC) class II-restricted T cell help (31). In addition, as demonstrated for poliovirus (32) and vesicular stomatitis virus (33), the high local antigen concentrations reached during infection, which allow replicating virus to be present at high levels for a prolonged time, represent a key factor in the efficient induction of T cell-independent responses (34). An early work from our laboratory demonstrated that FMDV-specific primary and short-term memory protective antibody responses were not dependent on the $CD4^+$ T-helper cells in a mouse model (35). Only recently, Juleff et al. extended these results to cattle, showing that a rapid and class-switched antibody response could be elicited by FMDV infection in bovines transiently depleted of $CD4^+$ T cells (36). Our results at local and systemic levels are consistent with these previous findings, even when differences can be pointed out regarding this later work. Juleff et al. first detected FMDV-specific serum IgG1 antibodies at 5 dpi, with FMDV-specific IgM antibodies identified 1 day later. In our work, FMDV-specific IgM responses were rapidly induced at the local level 4 days after infection, and IgG1 ASC numbers were comparable to those of the IgM-secreting cells only at 6 dpi. Serum responses followed a similar kinetics; while specific IgM and IgG1 antibodies were detected between 4 and 5 dpi, IgM titers peaked at 5 dpi, exhibiting significantly higher titers than the other isotypes at that time.

A previous report by Abu Elzein and Crowther indicated that FMDV-neutralizing activity in infected cattle started between 4 and 5 dpi, showing a biphasic development with a slow initial rise until 10 dpi, when a sharp increase in titer was observed (37). These authors found that both FMDV-specific IgM and IgA were present from 4 dpi, but since IgG1 and IgG2 were also detected in smaller amounts, they could not attribute the neutralizing activity to a determined isotype. We showed here that anti-FMDV IgM predominated in the adaptive response at both the local and systemic levels, and interestingly, the peak of anti-FMDV IgM in serum closely correlated with a sharp decrease in viral RNA detection in blood at 5 dpi. Supporting the role of IgM as a critical element in controlling/clearing FMDV infection in cattle, we demonstrated here that a considerably large proportion of the neutralizing activity of sera sampled 5 and 6 days after infection was due to the FMDV-specific IgM antibodies.

Altogether, our observations suggest that the development of local adaptive responses coincides with tissues where the virus undergoes significant productive replication and that IgM antibodies are the main effectors mediating FMDV clearance from peripheral circulation and thus represent a key factor in the development of early protective responses.

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(12) **United States Patent**
Borca et al.

(10) **Patent No.: US 8,114,852 B2**
(45) **Date of Patent: Feb. 14, 2012**

(54) **N-LINKED GLYCOSYLATION ALTERATION IN E1 GLYCOPROTEIN OF CLASSICAL SWINE FEVER VIRUS AND NOVEL CLASSICAL SWINE FEVER VIRUS VACCINE**

(75) Inventors: **Manuel Borca**, Westbrook, CT (US); **Guillermo Risatti**, Westbrook, CT (US)

(73) Assignee: **The United States of America as represented by the Secretary of Agriculture**, Washington, DC (US)

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(22) Filed: **Jan. 15, 2008**

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A61K 39/12 (2006.01)
A61K 48/00 (2006.01)

(52) **U.S. Cl.** **514/44**; 424/93.1; 435/91.2; 435/236

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

E1, along with Erns and E2 is one of the three envelope glycoproteins of Classical Swine Fever Virus (CSFV). Our previous studies indicated that glycosylation status of either E2 or Erns strongly influence viral virulence in swine. Here, we have investigated the role of E1 glycosylation of highly virulent CSFV strain Brescia during infection in the natural host. The three putative glycosylation sites in E1 were modified by site directed mutagenesis of a CSFV Brescia infectious clone (BICv). A panel of virus mutants was obtained and used to investigate whether the removal of putative glycosylation sites in the E1 glycoprotein would affect viral virulence/pathogenesis in swine. We observed that rescue of viable virus was completely impaired by removal of all three putative glycosylation sites in E1. Single mutations of each of the E1 glycosylation sites showed that CSFV amino acid N594 (E1.N3 virus), as well the combined mutation of N500 and N513 (E1.N1N2 virus) resulted in BICv attenuation. Infection of either E1.N1N2 or E1.N3 viruses were able to efficiently protected swine from challenge with virulent BICv at 3 and 28 days post-infection. These results, along with those demonstrating the role of glycosylation of E^{rns} and E2, suggest that manipulation of the pattern of glycosylation could be a useful tool for development of CSF live-attenuated vaccines.

11 Claims, 6 Drawing Sheets
(1 of 6 Drawing Sheet(s) Filed in Color)

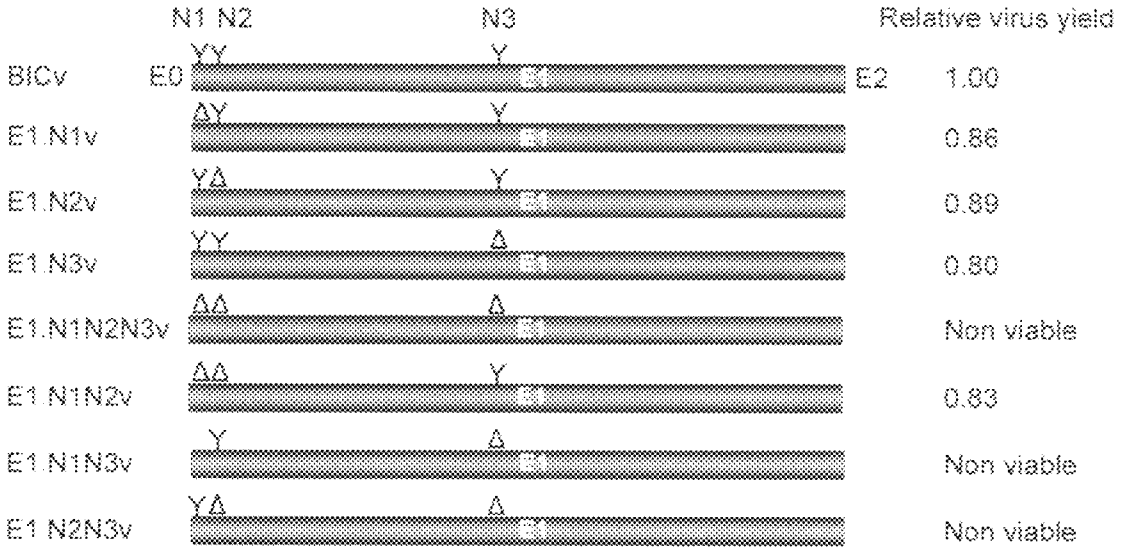
N1 N2
N3
Relative virus yield
BICv
E0
E1
E2
1.00
E1.N1v
0.86
E1.N2v
0.89
E1.N3v
0.80
E1.N1N2N3v
Non viable
E1.N1N2v
0.83
E1.N1N3v
Non viable
E1.N2N3v
Non viable

Figure 1A

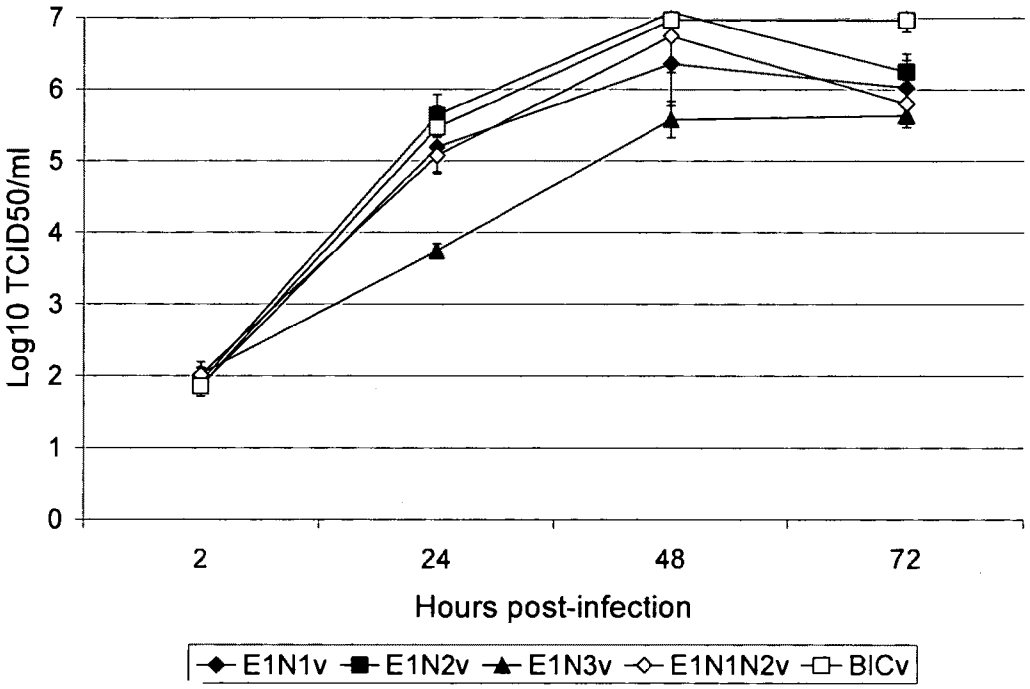
7
6
5
4
3
2
1
0
Log10 TCID50/ml
2
24
48
72
Hours post-infection
E1N1v
E1N2v
E1N3v
E1N1N2v
BICv

Figure 1B

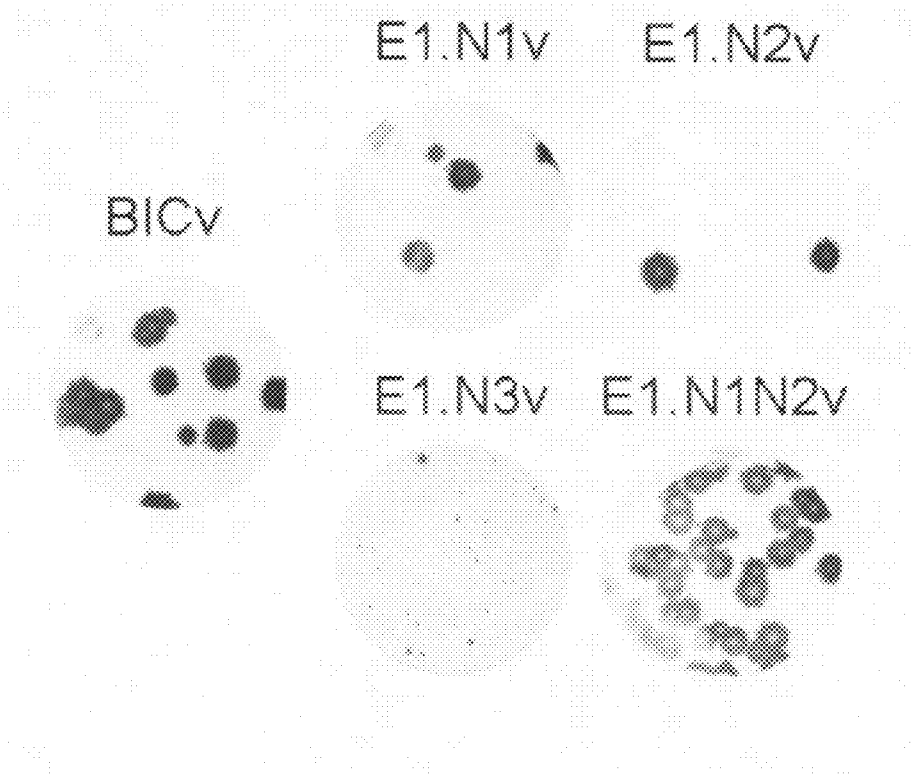

Figure 1C

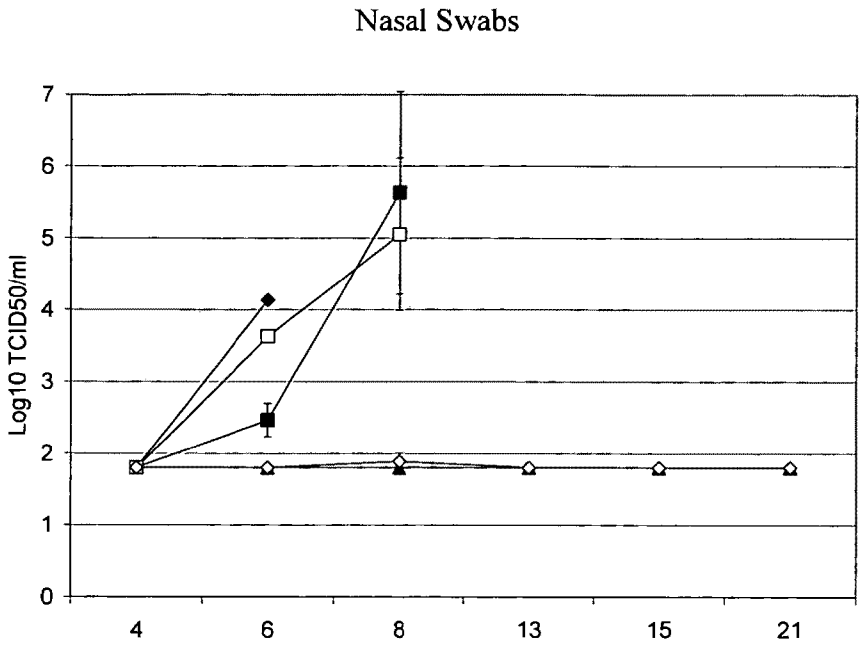

Figure 2A

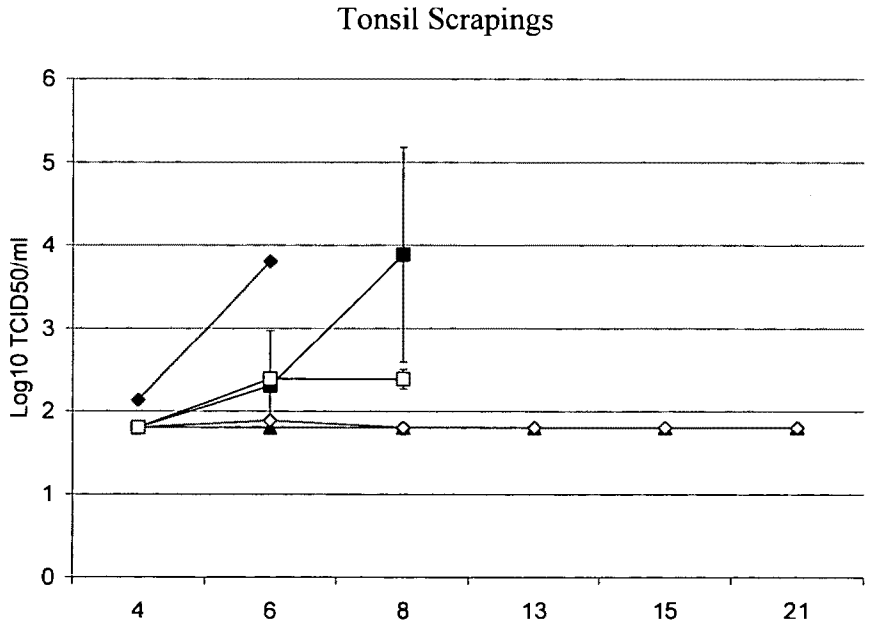

Figure 2B

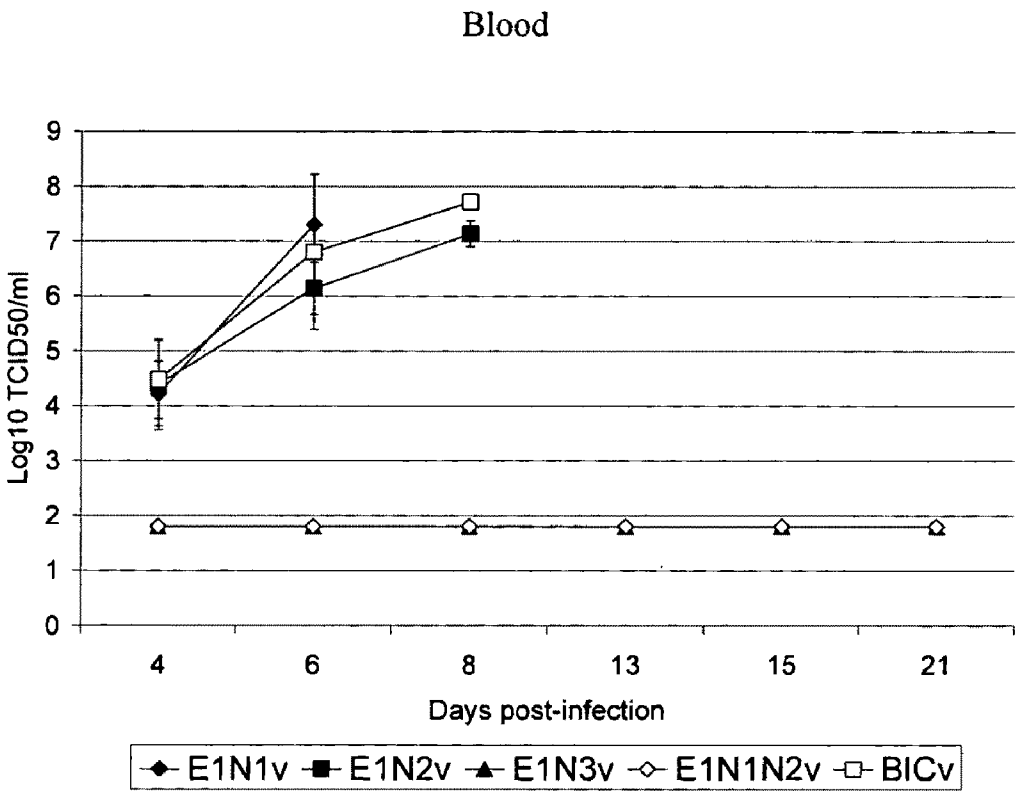
Blood
Log10 TCID50/ml
9
8
7
6
5
4
3
2
1
0
4
6
8
13
15
21
Days post-infection
E1N1v
E1N2v
E1N3v
E1N1N2v
BICv

Figure 2C

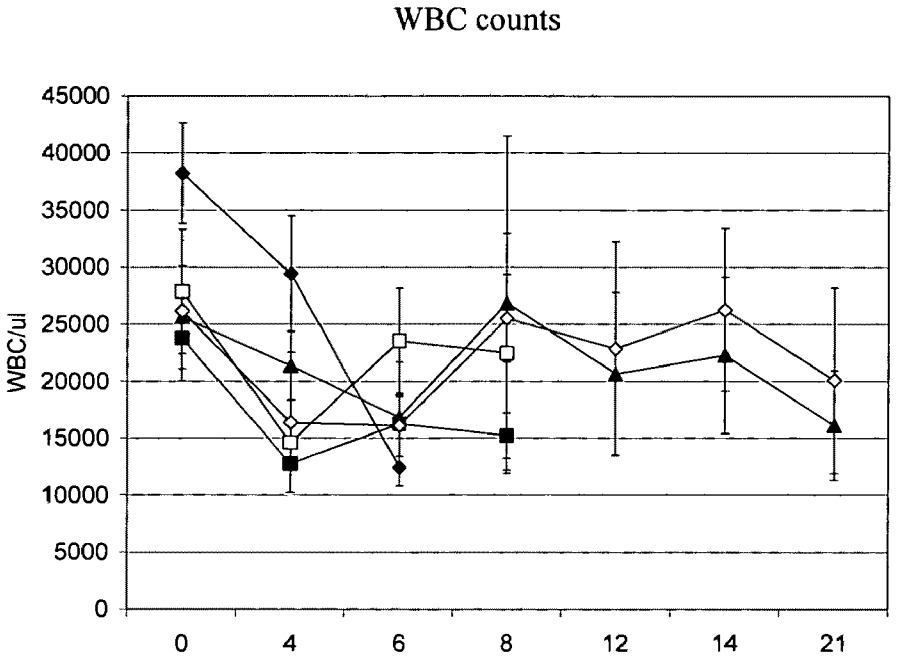

Figure 2D

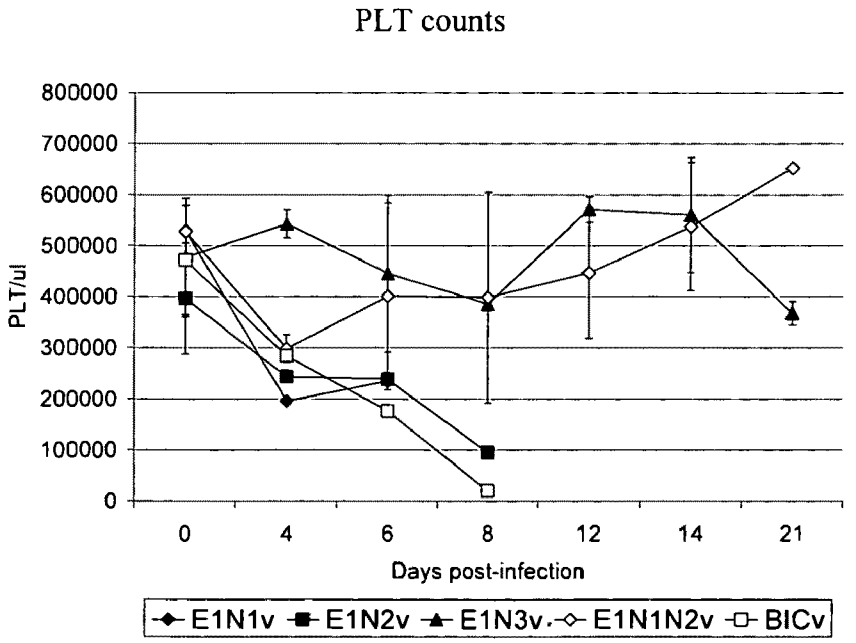

Figure 2E

N-LINKED GLYCOSYLATION ALTERATION IN E1 GLYCOPROTEIN OF CLASSICAL SWINE FEVER VIRUS AND NOVEL CLASSICAL SWINE FEVER VIRUS VACCINE

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to the characterization of the role that glycosylation of the transmembrane glycoprotein E1 of highly virulent Classical Swine Fever Virus (CSFV) strain Brescia plays during infection in the natural host and to the utilization of a strategy for manipulating the pattern of glycosylation for particular E1 glycosylation sites in order to alter CSFV virulence, providing a useful tool in the design and development of CSF live-attenuated vaccines.

2. Description of the Relevant Art

Classical swine fever (CSF) is a highly contagious disease of swine. The etiological agent, CSF virus (CSFV), is a small, enveloped virus with a positive, single-stranded RNA genome and, along with Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV), is classified as a member of the genus Pestivirus within the family Flaviridae (Becher et al. 2003. *Virology* 311: 96-104). The 12.5 kb CSFV genome contains a single open reading frame that encodes a 3898-amino-acid polyprotein and ultimately yields 11 to 12 final cleavage products (NH_2—Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B—NS5A-NS5B—COOH) through co- and post-translational processing of the polyprotein by cellular and viral proteases (Rice, C. M. 1996. In: *Fundamental Virology,* 3rd edition, Knipe et al., eds., Lippincott Raven, Philadelphia, Pa., pages 931-959). Structural components of the CSFV virion include the capsid (C) protein and glycoproteins E^{rns}, E1, and E2. E1 and E2 are anchored to the envelope at their carboxyl termini and Ems loosely associates with the viral envelope (Slater-Handshy et al. 2004. Virology 319: 36-48; Weiland et al. 1990. *J. Virol.* 64: 3563-3569; Weiland et al. 1999. *J. Gen. Virol.* 80: 1157-1165). E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor (Thiel et al. 1991. *J. Virol.* 65: 4705-4712). E1 has been implicated (Wang et al. 2004. *Virology* 330: 332-341), along with E^{rns} and E2 (Hulst et al. 1997. *J. Gen Virol.* 78: 2779-2787), in viral adsorption to host cells. Importantly, modifications introduced into these glycoproteins appear to have an important effect on CSFV virulence (Meyers et al. 1999. *J. Virol.* 73: 10224-10235; Risatti et al. 2005a. *J. Virol.* 79: 3787-3796; Risatti et al. 2005. *Virology* 355: 94-101; Risatti et al. 2005b. *Virology* 343: 116-127; Van Gennip et al. 2004. *J. Virol.* 78: 8812-8823).

Glycosylation is one of the most common types of protein modifications. N-linked oligosaccharides are added to specific asparagine residues in the context of the consensus sequence Asn-X-Ser/Thr (Kornfeld and Kornfeld. 1985. *Annu. Rev. Biochem.* 54: 631-664). According to a glycosylation analysis algorithm (Retrieved from the Internet: cbs.dtu.dk/services/), E1 of the CSFV strain Brescia has three putative N-linked glycosylation sites although this is not confirmed by experimental evidence. Predicted E1 glycosylation sites (at CSFV amino acid residue position N500, N513 and N594) are highly conserved among CSFV isolates and two of them (N513 and N594) are also conserved in other Pestiviruses. However, the significance of viral envelope protein glycosylation in virus replication, pathogenesis, and virulence in the natural host is not completely defined. It has just been recently described that specific removal of certain putative glycosylation sites in E^{rns} and E2 significantly alters the virulence of highly virulent Brescia strain in swine (Fernandez Sainz et al. 2008. *Virology* (370:122-129); Risatti et al. 2007. *J. Vinci.* 81: 924-933).

Strategies for controlling disease in the event of a CSFV outbreak include the production of rationally designed live attenuated vaccine CSFV strains. Thus, the effect of modification of glycosylation sites of other of the CSFV virion glycoproteins need to be evaluated. Here, we report the effects of modification of particular predicted E1 glycosylation sites. We used oligonucleotide site-directed mutagenesis of the E1 gene of the highly virulent CSFV strain Brescia to construct a panel of glycosylation mutants. These mutants were evaluated to determine whether the removal of each of these glycosylation sites in the E1 glycoprotein could affect viral infectivity and virulence in swine.

SUMMARY OF THE INVENTION

We have discovered glycosylation sites within the classical swine fever virus (CSFV) E1 glycoprotein where modification of the sites results in CSFV having novel virulence determinants.

In accordance with this discovery, it is an object of the invention to provide a recombinant CSFV comprising DNA encoding a modified CSFV E1 glycoprotein wherein specific glycosylation sites within E1 have been mutated resulting in an alteration in the site, i.e., the formerly glycosylated amino acid being altered and replaced by a non-glycosylated amino acid.

It is also an object of the invention to provide an isolated polynucleotide molecule comprising a genetically modified DNA sequence encoding a genetically modified infectious RNA molecule encoding a genetically modified CSFV. The CSFV is genetically modified such that when it infects a porcine animal it is unable to produce CSFV in the animal and it is able to elicit an effective immunoprotective response against infection by a CSFV in the animal. Mutated sequences or sequences homologous thereto contain a mutation that renders the encoded CSFV attenuated and able to elicit an effective immunoprotective response against infection by a CSFV in the animal.

It is additionally an object of the invention to provide an isolated infectious RNA molecule encoded by the isolated polynucleotide molecule recited above, and isolated infectious RNA molecules homologous thereto, which isolated infectious RNA molecules each encode a genetically modified CSFV, disabled in its ability to produce CSF.

An added object of the invention is to provide immunogenic compositions comprising a viable recombinant CSFV comprising a modified CSFV E1 glycoprotein displaying a glycosylation pattern different from that of the non-mutated E1 glycoprotein.

An additional object of the invention is to provide a rationally designed live attenuated CSFV vaccine which lessens severity of CSF disease when challenged with virulent Brescia CSFV wherein said vaccine comprises an altered glycosylation pattern as compared to that of the infectious, non-mutated virus.

Another object of the invention is to provide a rationally designed live attenuated CSFV vaccine effective to protect an animal from clinical CSF disease when challenged with virulent Brescia CSFV wherein said vaccine comprises an altered glycosylation pattern as compared to that of the infectious, non-mutated virus.

A further object of the invention is to provide a marker vaccine which allows a serological distinction between vaccinated animals and animals infected with CSFV.

A still further object of the invention is to provide a method for making a genetically modified CSFV, which method comprises mutating an infectious cDNA sequence, transforming the modified DNA into a modified infectious RNA molecule encoding a modified CSFV, and rescuing the genetically modified CSFV there from subsequent to said mutation.

Yet another object of the invention is to provide a method for protecting an animal against CSF by administering an effective amount of rationally designed live attenuated CSFV vaccine.

An additional object of the invention is to provide a method for delaying onset or severity of CSF in an animal by administering an effective amount of rationally designed live attenuated CSFV vaccine.

Other objects and advantages of this invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the U.S. Patent and Trademark Office upon request and payment of the necessary fee.

FIG. **1**A is a schematic representation of glycosylation mutants of Classical Swine Fever Virus E1 protein, generated by site-directed mutagenesis of a cDNA full-length clone PBIC (Terpstra et al. 1990. *Dtsch Tierarztl Wochenschr* 97: 77-79). Wild type E1 glycoprotein shown at the top. Y: putative glycosylation sites. Mutants were named with an N (N-linked glycosylation) followed by a number that represents the relative position of putative glycosylation sites within E1 amino acid sequence (500, 513, 594). Relative virus yield is final point virus yield as proportion of final end point (72 hours post-infection) virus yield of parental BICv. FIG. **1**B) shows the in vitro growth characteristics of E1 glycosylation mutants and parental BICv. Primary swine macrophage cell cultures were infected (MOI=0.01) with each of the mutants or BICv and virus yield was titrated at times post infection in SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: >1.8 $TCID_{50}$/ml. FIG. **1**C shows plaque formation of E1 glycosylation mutants and BICv. SK6 monolayers were infected, overlaid with 0.5% agarose and incubated at 37° C. for 3 days. Plates were fixed with 50% (vol/vol) ethanol-acetone and stained by immunohistochemistry with mAb WH303.

FIGS. **2**A, **2**B, and **2**C depict the virus titers and FIGS. **2**D and **2**E, the hematological data, in nasal swabs, tonsil scrapings, and blood from animals infected with CSFV E1 glycosylation mutants or parental BICv. Peripheral white blood cell and platelet counts are expressed as numbers/ul of blood. Data represent means and standard deviations from at least two animals. Sensitivity of virus detection: >1.8 $TCID_{50}$/ml.

DETAILED DESCRIPTION OF THE INVENTION

Virus glycoproteins are crucial in key steps of the virus cycle such as attachment to host cell receptors, entry, assembly of newly produced viral progeny, and exit. In vivo viral glycoproteins have been shown to influence infectivity (Ansari et al. 2006. *J. Virol.* 80: 3994-4004), virulence (Hulse et al. 2004. *J. Virol.* 78: 9954-9964; Panda of al. 2004. *J. Virol.* 78: 4965-4975), and host immune response (Abe of al. 2004. *J. Virol.* 78: 9605-9611). Added oligosaccharides confer proper function to viral glycoproteins since alteration of those glycosylation sites have shown dramatic consequences for viruses affecting protein folding (Herbert et al. 1997. *J. Cell Biol.* 139: 613-623; Kornfeld, supra; Shi et al. 2005. *J. Virol.* 79: 13725-13734; Shi and Elliott. 2004. *J. Virol.* 78: 5414-5422; Slater-Handshy, supra) and protein active conformation (Meunier et al. 1999. *J. Gen. Virol.* 80: 887-896). In this study, we analyzed glycosylation of the CSFV E1 glycoprotein and evaluated its effect on the virulence of CSFV in swine. DNA encoding CSFV strain Brescia E1 glycoprotein contains 3 N-linked putative glycosylation sites (Retrieved from the Internet: cbs.dtu.dk/services/) (Moorman et al. 1990. *Vet. Microbiol.* 23: 185-191). Sequence analysis of CSFV E1 glycoprotein showed that 3 of the N-linked glycosylation sites are highly conserved in CSV strains CSFV and two of them (N513 and N594) also among BVDV type I and II, and BDV strains (data not shown), implying an important role for these sites in all Pestiviruses. However, very little is known about the role of glycosylation on the function of Pestivirus glycoproteins. All putative glycosylation sites in E1 were modified by site-directed mutagenesis using a full-length cDNA infectious clone of virulent strain Brescia as the target sequence. Here, we showed that some of these sites have a major role in virulence and protection; some of the sites seem to be critical for the production of viable virus.

Cleavage and glycosylation patterns of the hemagglutinin gene of H5 avian influenza viruses have been shown to affect pathogenicity in chickens (Deshpande et al. 1987. *Proc. Natl. Acad. Sci. USA* 84: 36-40; Horimoto and Kawaoka. 1994. *J. Virol.* 68: 3120-3128). More recently it has been shown that glycosylation patterns of the neuraminidase gene of highly pathogenic H5N1 avian flu viruses are important for increased virulence in chickens (Hulse, supra). The mechanisms by which these patterns affect avian flu virulence are unknown. Similarly, a single mutation (E1.N3v) or multiple mutations (E1.N1N2v) within E1 resulted in attenuated viruses with restricted in vivo replication ability (see Table 2). Unlike the acute fatal disease induced by BICv, infections caused by these mutants were sub-clinical in swine and characterized by decreased viral replication in target organs and reduced virus shedding. Interestingly, mutants E1.N1v, and E1.N2v retained the same capability of causing severe disease in swine as parental BICv, showing that in vivo E1 functions are retained and not influenced by the lack of glycans at positions N500 and N513. As with avian flu, the genetic basis and the molecular mechanisms underlying CSFV virulence remain unknown.

As shown in this study, single mutations of E1 putative glycosylation sites have no effect on in vitro or in vivo infectivity of CSFV, with the exception of residue N594 in the E1.N3v mutant. However, when multiple site mutations were introduced in E1, we observed that any multiple mutations involving residue N594 (E1.N1N2, E1.N1N3 or E1.N1N2N3) render non-viable viruses (data not shown).

Production and manipulation of the isolated polynucleotide molecules described herein are within the skill in the art and can be carried out according to recombinant techniques described, among other places, in Sambrook et al. 1989. *Molecular Cloning: A Laboratory Manual,* 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Innis et al. (eds). 1995. *PCR Strategies*, Academic Press, Inc., San Diego, which are incorporated herein by reference.

The subject invention provides isolated polynucleotide molecules comprising genetically modified DNA sequences that encode genetically modified infectious RNA molecules that encode genetically modified Classical Swine Fever Viruses (CSFVs).

In particular, the subject invention provides an isolated polynucleotide molecule comprising a genetically modified

DNA sequence encoding a genetically modified infectious RNA molecule that encodes a genetically modified CSFV, wherein said DNA sequences are SEQ ID NO:1. (E1.N1N2) and SEQ ID NO: 2 (E1.N3) or sequences homologous thereto encoding the mutated viruses. Said DNA sequences encode infectious RNA molecules that are the RNA genomes of the mutated CSF viruses E1.N1N2 and E1.N3, respectively.

It is understood that terms herein referring to nucleic acid molecules such as "isolated polynucleotide molecule" and "nucleotide sequence include both DNA and RNA molecules and include both single-stranded and double-stranded molecules whether it is natural or synthetic origin.

For example, SEQ ID NO:1 is a DNA sequence corresponding to the genetically modified RNA genome of a genetically modified CSFV. Thus, a DNA sequence complementary to the DNA sequence set forth in SEQ ID NO:1 is a template for, i.e. is complementary to or "encodes", the RNA genome of the SF virus (i.e., RNA that encodes the CSFV).

Furthermore, when reference is made herein to sequences homologous to a sequence in the Sequence Listing, it is to be understood that sequences are homologous to a sequence corresponding to the sequence in the Sequence Listing and to a sequence complementary to the sequence in the Sequence Listing.

An "infectious RNA molecule", for purposes of the present invention, is an RNA molecule that encodes the necessary elements for viral replication, transcription, and translation into a functional virion in a suitable host cell, provided, if necessary, with a peptide or peptides that compensate for any genetic modifications, e.g. sequence deletions, in the RNA molecule.

An "isolated infectious RNA molecule" refers to a composition of matter comprising the aforementioned infectious RNA molecule purified to any detectable degree from its naturally occurring state, if such RNA molecule does indeed occur in nature. Likewise, an "isolated polynucleotide molecule" refers to a composition of matter comprising a polynucleotide molecule of the present invention purified to any detectable degree from its naturally occurring state, if any.

For purposes of the present invention, two DNA sequences are substantially homologous when at least 80% (preferably at least 85% and most preferably 90%) of the nucleotides match over the defined length of the sequence using algorithms such as CLUSTRAL or PILEUP. Sequences that are substantially homologous can be identified in a Southern hybridization experiment under stringent conditions as is known in the art. See, for example, Sambrook et al., supra. Sambrook et al. describe highly stringent conditions as a hybridization temperature 5-10° C. below the T_m of a perfectly matched target and probe; thus, sequences that are "substantially homologous" would hybridize under such conditions.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the polypeptide encoded by the nucleotide sequence. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of nucleotides that do not substantially affect the functional properties of the resulting transcript. It is therefore understood that the invention encompasses more than the specific exemplary nucleotide or amino acid sequences and includes functional equivalents thereof. Alterations in a nucleic acid fragment that result in the production of a chemically equivalent amino acid at a given site, but do not affect the functional properties of the encoded polypeptide, are well known in the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule would also not be expected to alter the activity of the polypeptide. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. A method of selecting an isolated polynucleotide that affects the level of expression of a polypeptide in a virus or in a host cell (eukaryotic, such as plant, yeast, fungi, or algae; prokaryotic, such as bacteria) may comprise the steps of: constructing an isolated polynucleotide of the present invention or an isolated chimeric gene of the present invention; introducing the isolated polynucleotide or the isolated chimeric gene into a host cell; measuring the level of a polypeptide in the host cell containing the isolated polynucleotide; and comparing the level of a polypeptide in the host cell containing the isolated polynucleotide with the level of a polypeptide in a host cell that does not contain the isolated polynucleotide.

Moreover, substantially similar nucleic acid fragments may also be characterized by their ability to hybridize. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art (1985. *Nucleic Acid Hybridization*, Hames and Higgins, Eds., IRL Press, Oxford, U.K.). Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms.

Thus, isolated sequences that encode a modified CSFV E1 glycopeptide (E1.N1N2 or E1.N3) and which hybridize under stringent conditions, as described herein, to the modified CSFV E1 sequences disclosed herein, i.e., SEQ ID NO:1 (E1.N1N2) or SEQ ID NO: 2 (E1.N3) or to fragments thereof, are encompassed by the present invention. Fragments of a nucleotide sequence that are useful as hybridization probes may not encode fragment proteins retaining biological activity.

Substantially similar nucleic acid fragments of the instant invention may also be characterized by the percent identity of the amino acid sequences that they encode to the amino acid sequences disclosed herein, as determined by algorithms commonly employed by those skilled in this art.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988. *CABIOS* 4:11-17), the local homology algorithm of Smith et al. (1981. *Adv. Appl. Math.* 2:482); the homology alignment algorithm of Needleman and Wunsch (1970. *J. Mol. Biol.* 48:443-453); the search-for-similarity-method of Pearson and Lipman (1988. *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990. *Proc. Natl. Acad. Sci. USA* 87:2264), modified as in Karlin and Altschul (1993. *Proc. Natl. Acad. Sci. USA* 90:5873-5877).

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignments using these programs can be performed using the default parameters.

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins, it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule.

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 80% sequence identity, preferably at least 85%, more preferably at least 90%, most preferably at least 95% sequence identity compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 80%, preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman et al. (1970. *J. Mol. Biol.* 48:443).

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1° C. to about 20° C., depending upon the desired degree of stringency as otherwise qualified herein.

A "substantial portion" of an amino acid or nucleotide sequence comprises an amino acid or a nucleotide sequence that is sufficient to afford putative identification of the protein or gene that the amino acid or nucleotide sequence comprises. Amino acid and nucleotide sequences can be evaluated either manually by one skilled in the art, or by using computer-based sequence comparison and identification tools that employ algorithms such as BLAST. In general, a sequence of ten or more contiguous amino acids or thirty or more contiguous nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising 30 or more contiguous nucleotides may be used in sequence-dependent methods of gene identification and isolation. In addition, short oligonucleotides of 12 or more nucleotides may be use as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises a nucleotide sequence that will afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions at those sequences as defined above.

By "variants" substantially similar sequences are intended. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the CSFV E1 glycoproteins of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR), a technique used for the amplification of specific DNA segments. Generally, variants of a particular nucleotide sequence of the invention will have generally at least about 90%, preferably at least about 95% and more preferably at least about 98% sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein.

By "variant protein" a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein is intended. Variant proteins encompassed by the present invention are biologically active, that is they possess the desired biological activity, that is, a modified CSFV E1 glycoprotein activity, i.e., E1.N1N2 or E1.N3 glycoprotein activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a modified CSFV E1 glycoprotein of the invention, i.e., E1.N1N2 or E1.N3, will have at least about 90%, preferably at least about 95%, and more preferably at least about 98% sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, or even 1 amino acid residue.

The polypeptides of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Novel proteins having properties of interest may be created by combining elements and fragments of proteins of the present invention, as well as with other proteins. Methods for such manipulations are generally known in the art. Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired modified CSFV E1 glycoprotein activity, i.e., E1.N1N2 or E1.N3 glycoprotein activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays where the effects of modified CSFV E1 glycoprotein, i.e., E1.N1N2 or E1.N3 glycoprotein activity, can be observed.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment comprising a nucleotide sequence that encodes all or a substantial portion of the amino acid sequences set forth herein.

It is furthermore to be understood that the isolated polynucleotide molecules and the isolated RNA molecules of the present invention include both synthetic molecules and molecules obtained through recombinant techniques, such as by in vitro cloning and transcription.

As used herein, the term "CSF" encompasses disease symptoms in swine caused by a CSFV infection. Examples of such symptoms include, but are not limited to, anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough. As used herein, a CSFV that is "unable to produce CSF" refers to a virus that can infect a pig, but which does not produce any disease symptoms normally associated with a CSF infection in the pig, or produces such symptoms, but to a lesser degree, or produces a fewer number of such symptoms, or both.

The terms "porcine" and "swine" are used interchangeably herein and refer to any animal that is a member of the family Suidae such as, for example, a pig. "Mammals" include any warm-blooded vertebrates of the Mammalia class, including humans.

The terms "classical swine fever virus" and "CSFV", as used herein, unless otherwise indicated, mean any strain of CSF viruses.

The term "open reading frame", or "ORF", as used herein, means the minimal nucleotide sequence required to encode a particular CSFV protein without an intervening stop codon.

Terms such as "suitable host cell" and "appropriate host cell", unless otherwise indicated, refer to cells into which RNA molecules (or isolated polynucleotide molecules or viral vectors comprising DNA sequences encoding such RNA molecules) of the present invention can be transformed or transfected. "Suitable host cells" for transfection with such RNA molecules, isolated polynucleotide molecules, or viral vectors, include mammalian, particularly porcine cells, and are described in further detail below.

A "functional virion" is a virus particle that is able to enter a cell capable of hosting a CSFV, and express genes of its particular RNA genome (either an unmodified genome or a genetically modified genome as described herein) within the cell. Cells capable of hosting a CSFV include swine kidney cells (SK6) and primary porcine macrophage cell cultures. Other mammalian cells, especially other porcine cells, may also serve as suitable host cells for CSF virions.

The isolated polynucleotide molecules of the present invention encode CSF viruses that can be used to prepare live attenuated vaccines using art-recognized methods for protecting swine from infection by a CSFV, as described in further detail below. Furthermore, these isolated polynucleotide molecules are useful because they can be mutated using molecular biology techniques to encode genetically-modified CSF viruses useful, inter alia, as vaccines for protecting swine from CSF infection. Such genetically-modified CSF viruses, as well as vaccines comprising them, are described in further detail below.

Accordingly, the subject invention further provides a method for making a genetically modified CSFV, which method comprises mutating the DNA sequence encoding an infectious RNA molecule which encodes the CSFV as described above, and expressing the genetically modified CSFV using a suitable expression system. A CSFV, either wild-type or genetically modified, can be expressed from an isolated polynucleotide molecule using suitable expression systems generally known in the art, examples of which are described in this application. For example, the isolated polynucleotide molecule can be in the form of a plasmid capable of expressing the encoded virus in a suitable host cell in vitro.

The term "genetically modified", as used herein and unless otherwise indicated, means genetically mutated, i.e. having one or more nucleotides replaced, deleted and/or added. Polynucleotide molecules can be genetically mutated using recombinant techniques known to those of ordinary skill in the art, including by site-directed mutagenesis, or by random mutagenesis such as by exposure to chemical mutagens or to radiation, as known in the art.

The subject invention further provides an isolated polynucleotide molecule comprising a DNA sequence encoding an infectious RNA molecule which encodes a genetically modified CSFV that is unable to produce CSF in a porcine animal, wherein the DNA sequence encoding the infectious RNA molecule encoding said modified CSFV is SEQ ID NO:1 or SEQ ID NO: 2 or a sequences homologous thereto, contains one or more mutations that genetically disable the encoded CSFV in its ability to produce CSF. "Genetically disabled" means that the CSFV is unable to produce CSF in a swine animal infected therewith.

In one embodiment, the genetically modified CSFV disabled in its ability to cause CSF is able to elicit an effective immunoprotective response against infection by a CSFV in a swine animal. Accordingly, the subject invention also provides an isolated polynucleotide molecule comprising a DNA sequence encoding an infectious RNA molecule which encodes a CSFV that is genetically modified such that when it infects a porcine animal it: a) is unable to produce CSF in the animal, and b) is able to elicit an effective immunoprotective response against infection by a CSFV in the animal, wherein the DNA sequence encoding said modified CSFV is SEQ ID NO:1 (E1.N1N2) or SEQ ID NO: 2 (E1.N3) or sequences homologous thereto, contains one or more mutations that genetically disable the encoded CSFV in its ability to produce CSF.

The term "immune response" for purposes of this invention means the production of antibodies and/or cells (such as T lymphocytes) that are directed against, or assist in the decomposition or inhibition of, a particular antigenic epitope or particular antigenic epitopes. The phrases "an effective immunoprotective response", "immunoprotection", and like terms, for purposes of the present invention, mean an immune response that is directed against one or more antigenic epitopes of a pathogen so as to protect against infection by the pathogen in a vaccinated animal. For purposes of the present invention, protection against infection by a pathogen includes not only the absolute prevention of infection, but also any detectable reduction in the degree or rate of infection by a pathogen, or any detectable reduction in the severity of the disease or any symptom or condition resulting from infection by the pathogen in the vaccinated animal as compared to an unvaccinated infected animal. An effective immunoprotective response can be induced in animals that have not previously been infected with the pathogen and/or are not infected with the pathogen at the time of vaccination. An effective immunoprotective response can also be induced in an animal already infected with the pathogen at the time of vaccination.

An "antigenic epitope" is, unless otherwise indicated, a molecule that is able to elicit an immune response in a particular animal or species. Antigenic epitopes are proteinaceous molecules, i.e. polypeptide sequences, optionally comprising non-protein groups such as carbohydrate moieties and/or lipid moieties.

The genetically modified CSF viruses encoded by the above-described isolated polynucleotide molecules are, in one embodiment, able to elicit an effective immunoprotective response against infection by a CSFV. Such genetically modified CSF viruses are preferably able to elicit an effective immunoprotective response against any strain of CSF viruses.

In one embodiment, the mutation or mutations in the isolated polynucleotide molecule encoding the genetically disabled CSFV are non-silent and occur in one or more open reading frames of the nucleotide sequence encoding the CSFV.

As used herein, unless otherwise indicated, "coding regions" refer to those sequences of RNA from which CSFV proteins are expressed, and also refer to cDNA that encodes such RNA sequences. Likewise, "ORFs" refer both to RNA sequences that encode CSFV proteins and to cDNA sequence encoding such RNA sequences.

Determining suitable locations for a mutation or mutations that will encode a CSFV that is genetically disabled so that it is unable to produce CSF yet remains able to elicit an effective immunoprotective response against infection by a CSFV can be made based on SEQ ID NO:1 and SEQ ID NO: 2 provided herein. One of ordinary skill can refer to the sequence of the infectious cDNA clone of CSFV provided by this invention, make sequence changes which will result in a mutation altering the glycosylation pattern of the glycoprotein, and test the viruses encoded thereby both for their ability to produce CSF in swine, and to elicit an effective immunoprotective response against infection by a CSFV. In so doing, one of ordinary skill can refer to techniques known in the art and also those described and/or exemplified herein.

For example, an ORF of the sequence encoding the infectious RNA molecule encoding the CSFV can be mutated and the resulting genetically modified CSFV tested for its ability to cause CSF.

In a further preferred embodiment, an antigenic epitope of the genetically modified CSFV of the present invention is a detectable antigenic epitope. Such isolated polynucleotide molecules and the CSF viruses they encode are useful, inter alia, for studying CSF infections in swine, determining successfully vaccinated swine, and/or for distinguishing vaccinated swine from swine infected by a wild-type CSFV. Preferably, such isolated polynucleotide molecules further contain one or more mutations that genetically disable the encoded CSFV in its ability to produce CSF, and more preferably are able to elicit an effective immunoprotective response in a porcine animal against infection by a CSFV.

Antigenic epitopes that are detectable, and the sequences that encode them, are known in the art. Techniques for detecting such antigenic epitopes are also known in the art and include serological detection of antibody specific to the heterologous antigenic epitope by means of, for example, Western blot, ELISA, or fluorescently labeled antibodies capable of binding to the antibodies specific to the heterologous antigenic epitope. Techniques for serological detection useful in practicing the present invention can be found in texts recognized in the art, such as Coligan, J. E., et al. (eds), 1998, *Current Protocols in Immunology*, John Willey & Sons, Inc., which is hereby incorporated by reference in its entirety. Alternatively, the antigenic epitope itself can be detected by, for example, contacting samples that potentially comprise the antigenic epitope with fluorescently-labeled antibodies or radioactively-labeled antibodies that specifically bind to the antigenic epitopes.

The present invention further provides an isolated polynucleotide molecule comprising a DNA sequence encoding an infectious RNA molecule which encodes a genetically modified CSFV that detectably lacks a CSFV antigenic epitope, wherein the DNA sequence encoding the RNA molecule encoding the modified CSFV is SEQ ID NO:1 (E1.N1.N2) or SEQ ID NO: 2 (E1.N3) or sequences homologous thereto, except that it lacks one or more nucleotide sequences encoding a detectable CSFV antigenic epitope. Such isolated polynucleotide molecules are useful for distinguishing between swine infected with a recombinant CSFV of the present invention and swine infected with a wild-type CSFV. For example, animals vaccinated with killed, live or attenuated CSFV encoded by such an isolated polynucleotide molecule can be distinguished from animals infected with wild-type CSF based on the absence of antibodies specific to the missing antigenic epitope, or based on the absence of the antigenic epitope itself; If antibodies specific to the missing antigenic epitope, or if the antigenic epitope itself, are detected in the animal, then the animal was exposed to and infected by a wild-type CSFV. Means for detecting antigenic epitopes and antibodies specific thereto are known in the art, as discussed above. Preferably, such an isolated polynucleotide molecule further contains one or more mutations that genetically disable the encoded CSFV in its ability to produce CSF. More preferably, the encoded virus remains able to elicit an effective immunoprotective response against infection by a CSFV.

Vaccines of the present invention can be formulated following accepted convention to include acceptable carriers for animals, including humans (if applicable), such as standard buffers, stabilizers, diluents, preservatives, and/or solubilizers, and can also be formulated to facilitate sustained release. Diluents include water, saline, dextrose, ethanol, glycerol, and the like. Additives for isotonicity include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others. Stabilizers include albumin, among others. Other suitable vaccine vehicles and additives, including those that are particularly useful in formulating modified live vaccines, are known or will be apparent to those skilled in the art. See, e.g.,

Remington's Pharmaceutical Science, 18th ed., 1990, Mack Publishing, which is incorporated herein by reference.

Vaccines of the present invention can further comprise one or more additional immunomodulatory components such as, e.g., an adjuvant or cytokine, among others. Non-limiting examples of adjuvants that can be used in the vaccine of the present invention include the RIBI adjuvant system (Ribi Inc., Hamilton, Mont.), alum, mineral gels such as aluminum hydroxide gel, oil-in-water emulsions, water-in-oil emulsions such as, e.g., Freund's complete and incomplete adjuvants, Block copolymer (CytRx, Atlanta Ga.), QS-21 (Cambridge Biotech Inc., Cambridge Mass.), SAF-M (Chiron, Emeryville Calif.), AMPHIGEN™ adjuvant, saponin, Quil A or other saponin fraction, monophosphoryl lipid A, and Avridine lipid-amine adjuvant. Non-limiting examples of oil-in-water emulsions useful in the vaccine of the invention include modified SEAM62 and SEAM ½ formulations. Modified SEAM62 is an oil-in-water emulsion containing 5% (v/v) squalene (Sigma), 1% (v/v) SPAN™ 85 detergent (ICI Surfactants), 0.7% (v/v) TWEEN™ 80 detergent (ICI Surfactants), 2.5% (v/v) ethanol, 200 μg/ml Quil A, 100 μg/ml cholesterol, and 0.5% (v/v) lecithin. Modified SEAM ½ is an oil-in-water emulsion comprising 5% (v/v) squalene, 1% (v/v) SPAN™ 85 detergent, 0.7% (v/v) Tween 80 detergent, 2.5% (v/v) ethanol, 100 μg/ml Quil A, and 50 μg/ml cholesterol. Other immunomodulatory agents that can be included in the vaccine include, e.g., one or more interleukins, interferons, or other known cytokines.

Vaccines of the present invention can optionally be formulated for sustained release of the virus, infectious RNA molecule, plasmid, or viral vector of the present invention. Examples of such sustained release formulations include virus, infectious RNA molecule, plasmid, or viral vector in combination with composites of biocompatible polymers, such as, e.g., poly(lactic acid), poly(lactic-co-glycolic acid), methylcellulose, hyaluronic acid, collagen and the like. The structure, selection and use of degradable polymers in drug delivery vehicles have been reviewed in several publications, including Domb et al. 1992. *Polymers for Advanced Technologies* 3: 279-292, which is incorporated herein by reference. Additional guidance in selecting and using polymers in pharmaceutical formulations can be found in texts known in the art, for example M. Chasin and R. Langer (eds), 1990, "Biodegradable Polymers as Drug Delivery Systems" in: *Drugs and the Pharmaceutical Sciences*, Vol. 45, M. Dekker, NY, which is also incorporated herein by reference. Alternatively, or additionally, the virus, plasmid, or viral vector can be microencapsulated to improve administration and efficacy. Methods for microencapsulating antigens are well-known in the art, and include techniques described, e.g., in U.S. Pat. No. 3,137,631; U.S. Pat. No. 3,959,457; U.S. Pat. No. 4,205,060; U.S. Pat. No. 4,606,940; U.S. Pat. No. 4,744,933; U.S. Pat. No. 5,132,117; and International Patent Publication WO 95/28227, all of which are incorporated herein by reference.

Liposomes can also be used to provide for the sustained release of virus, plasmid, or viral vector. Details concerning how to make and use liposomal formulations can be found in, among other places, U.S. Pat. No. 4,016,100; U.S. Pat. No. 4,452,747; U.S. Pat. No. 4,921,706; U.S. Pat. No. 4,927,637; U.S. Pat. No. 4,944,948; U.S. Pat. No. 5,008,050; and U.S. Pat. No. 5,009,956, all of which are incorporated herein by reference.

An effective amount of any of the above-described vaccines can be determined by conventional means, starting with a low dose of virus, plasmid or viral vector, and then increasing the dosage while monitoring the effects. An effective amount may be obtained after a single administration of a vaccine or after multiple administrations of a vaccine. Known factors can be taken into consideration when determining an optimal dose per animal. These include the species, size, age and general condition of the animal, the presence of other drugs in the animal, and the like. The actual dosage is preferably chosen after consideration of the results from other animal studies.

One method of detecting whether an adequate immune response has been achieved is to determine seroconversion and antibody titer in the animal after vaccination. The timing of vaccination and the number of boosters, if any, will preferably be determined by a doctor or veterinarian based on analysis of all relevant factors, some of which are described above.

The effective dose amount of virus, infectious RNA molecule, plasmid, or viral vector, of the present invention can be determined using known techniques, taking into account factors that can be determined by one of ordinary skill in the art such as the weight of the animal to be vaccinated. The dose amount of virus of the present invention in a vaccine of the present invention preferably ranges from about 10^1 to about 10^9 pfu (plaque forming units), more preferably from about 10^2 to about 10^8 pfu, and most preferably from about 10^3 to about 10^7 pfu. The dose amount of a plasmid of the present invention in a vaccine of the present invention preferably ranges from about 0.1 g to about 100 mg, more preferably from about 1 μg to about 10 mg, even more preferably from about 10 μg to about 1 mg. The dose amount of an infectious RNA molecule of the present invention in a vaccine of the present invention preferably ranges from about 0.1 μg to about 100 mg, more preferably from about 1 μg to about 10 mg, even more preferably from about 10 μg to about 1 mg. The dose amount of a viral vector of the present invention in a vaccine of the present invention preferably ranges from about 10^1 pfu to about 10^9 pfu, more preferably from about 10^2 pfu to about 10^8 pfu, and even more preferably from about 10^3 to about 10^7 pfu. A suitable dosage size ranges from about 0.5 ml to about 10 ml, and more preferably from about 1 ml to about 5 ml.

In summary, our studies determined that individual N-linked glycosylation in glycoprotein E1 sites are not essential for viral particle formation or virus infectivity in cultured swine macrophages or the natural host, with one individual site, N594, involved in attenuation of the virulent parental virus. This study also showed that in the context of two or more putative glycosylation site modifications, residue N594 is critical for virus viability. The effective protective immunity elicited by E1.N3v and E1.N1N2v suggests that glycosylation of E1 could be modified for the development of live-attenuated vaccines. An improved understanding of the genetic basis of virus virulence and host range will permit future rational design of efficacious biological tools for controlling CSF.

EXAMPLES

Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein only to further illustrate the invention and are not intended to limit the scope of the invention as defined by the claims.

Example 1

Viruses and Cell Cultures

Swine kidney cells (SK6) (Terpstra et al., supra) free of Bovine Viral Diarrhea Virus (BVDV) were cultured in Dul-

becco's Minimal Essential Medium (DMEM, GIBCO, Grand Island, N.Y.) with 10% fetal calf serum (FCS, Atlas Biologicals, Fort Collins, Colo.). CSFV Brescia strain (obtained from the Animal and Plant Health Inspection Service, Plum Island Animal Disease Center) was propagated in SK6 cells and used for an infectious cDNA clone (Risatti et al. 2005a, supra). Growth kinetics were assessed on primary swine macrophage cell cultures prepared as described by Zsak et al. (1996. *J. Virol.* 70: 8865-8871). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, Mass.). Viral infectivity was detected, after 4 days in culture, by an immunoperoxidase assay using the CSFV monoclonal antibodies WH303 (Edwards et al. 1991. *Vet Microbiol.* 29:101-108) and the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.). Titers were calculated using the method of Reed and Muench (1938. *American J. Hygiene* 27: 493-497) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was ≧1.8 TCID50/ml. Plaque assays were performed using SK6 cells in 6-well plates (Costar). SK6 monolayers were infected, overlaid with 0.5% agarose and incubated at 37° C. for 3 days. Plates were fixed with 50% (vol/vol) ethanol-acetone and stained by immunohistochemistry with mAb WH303.

Example 2

Construction of CSFV Glycosylation Mutants

A full-length infectious clone of the virulent Brescia isolate (pBIC) (Risatti of et al. 2005a, supra) was used as a template in which N-linked glycosylation sites in the E1 glycoprotein were mutated. Glycosylation sites were predicted using analysis tools from the Center for Biological Sequence Analysis (Retrieved from the Internet: cbs.dtu.dk/services/). Mutations were introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, Tex.) performed per manufacturer's instructions and using the following primers (only forward primer sequences are shown); E1.N1v: TATGCCCTATCACCT TATTGT GCTGTGACAAGCAAAATAGGGTAC (SEQ ID NO:5); E1.N2v: GGGTACATA TGGTACACTAAC GCCTGTACCCCGGCTTGCCTCCCC (SEQ ID NO:6); E1.N3v: GAAGGCTGTGACACA AACCAGCTGGCTT-TAACAGTGGAACTCAGGACT (SEQ ID NO:7).

Example 3

In Vitro Rescue of CSFV Brescia and Glycosylation Mutants

Full-length genomic clones were linearized with SrfI and in vitro transcribed using the T7 Megascript system (Ambion, Austin, Tex.). RNA was precipitated with LiCl and transfected into SK6 cells by electroporation at 500 volts, 720 ohms, 100 watts with a BTX 630 electroporator (BTX, San Diego, Calif.). Cells were seeded in 12-well plates and incubated for 4 days at 37EC and 5% CO_2. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at −70EC.

Infectious RNA was in vitro transcribed from full-length infectious clones of the CSFV Brescia strain or a set of glycosylation mutants (Table 1, FIG. **1**) and used to transfect SK6 cells. Mutants referred to as E1.N1, E1.N2, E1.N3 represent each of three putative glycosylation sites starting from the N terminus of E1 (Table 1), whereas multiple mutants are represented by combinations of indicated sites (FIG. **1**A). Viruses were rescued from transfected cells by day 4 post-transfection. Nucleotide sequences of the rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at predicted glycosylation sites were reflected in rescued viruses.

TABLE 1

Set of CSFV E1 glycosylation mutant viruses constructed.

E1 Position	Wild-Type Sequence	Mutant Sequence	Codon Change	Mutant
500	NVTS	AVTS	AAT → GCT	E1.N1
513	NCTP	ACTP	AAC → GCC	E1.N2
594	NLTV	ALTV	AAT → GCT	E1.N3
500/513	NVTS/NCTP	AVTS/ACTP	AAT → GCT/ AAC → GCC	E1.N1N2
513/594	NCTP/NLTV	ACTP/ALTV	AAC → GCC/ AAT → GCT	E1.N2N3
500/594	NVTS/NLTV	AVTS/ALTV	AAT → GCT/ AAT → GCT	E1.N1N3
500/513/594	NVTS/NCTP/NLTV	AVTS/ACTP/ALTV	AAT → GCT/ AAC → GCC/ AAT → GCT	E1.N1N2N3

Example 4

DNA Sequencing and Analysis

Full-length infectious clones and in vitro rescued viruses were completely sequenced with CSFV specific primers by the dideoxynucleotide chain-termination method (Sanger et al. 1977. *Proc. Natl. Acad. Sci. USA* 74: 5463-5467). Viruses recovered from infected animals were sequenced in the mutated area. Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit. (Applied Biosystems, Foster City, Calif.). Reaction products were sequenced on a PRISM 3730xl automated DNA Sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3 (Huang et al. 1999. *Genome Res.* 9: 868-877). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (Retrieved from the Internet: mbio.ncsu.edu/BioEdit/bioedit.html).

The DNA sequence encoding a modified CSFV E1 glycoprotein, i.e., E1.N1N2 is identified by SEC) ID NO: 1. The DNA sequence encoding a modified CSFV E1 glycoprotein,

i.e., E1.N3 is identified by SEQ ID NO: 2. The glycoproteins encoded by these DNA molecules are identified by SEQ ID NOs: 3 and 4, respectively.

Example 5

In Vitro and In Vivo Analysis of Glycosylation Mutants

In vitro growth characteristics of mutant viruses E1.N1v, E1.N2v, E.1N3v and E1.N1N2v were evaluated relative to parental BICv in a multistep growth curve (FIG. **1**B). Primary porcine macrophage cell cultures were infected at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time zero), and samples were collected at times post-infection through 72 h.

All single glycosylation site mutants exhibited titers approximately an order lower than those corresponding to BICv. Additionally, when viruses were tested for their plaque size in SK6 cells, E1.N3v exhibited a noticeable reduction in plaque size relative to BICv (FIG. **1**C). Interestingly, some viruses were not rescued from SK-6 cells transfected with RNA transcribed from full-length cDNA clones carrying multiple glycosylation site mutations (E1.N1N2N3, E1.N1N3 and E1.N2N3) that included substitutions at the E1.N3 position (N594).

To examine the effect of E1 glycosylation on CSFV virulence, and establish the impact of mutations at individual glycosylation sites in swine virulence, individual mutants were intranasally inoculated with 10^5 $TCID_{50}$ and monitored for clinical disease relative to the parental virus. Swine used in all animal studies were 10 to 12 weeks old, forty-pound commercial bred pigs. For screening, 10 pigs were randomly allocated into 5 groups of 2 animals each, and pigs in each group were inoculated with one of the single glycosylation mutants, E1.N1N2v or BICv. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment and scored as previously described (Mittelholzer et al. 2000. *Vet. Microbiol.* 74: 293-308).

BICv exhibited a characteristic virulent phenotype (Table 2). Animals infected with E1.N3v survived the infection and remained normal throughout the observation period (21 days). All animals infected with E1.N1v and E1.N2v presented clinical signs of CSF starting 5 to 8 DPI, with clinical presentation and severity similar to those observed in animals inoculated with BICv. White blood cell and platelet counts dropped by 4 to 6 DPI in animals inoculated with E1.N1 and E1.N2v, and BICv and kept declining until death, while a transient decrease was observed in animals inoculated with E1.N1v (FIG. **2**). Since E1.N1v and E1.N2v were as virulent as the wild type BICv it was interesting to assess the influence on viral virulence of the simultaneous removal of both glycosylation sites. Two animals were infected with E1.N1N2v under the same conditions above described. Infected animals remained normal throughout the observation period (21 days) along with a transient decrease in the hematological (Table 2 and FIG. **2**).

TABLE 2

Swine survival and fever response following infection with CSFV E1 glycosylation mutants and parental BICv.

Virus	Survivor/ total	Mean time to death: Days ∀SD	Mean time of fever onset: Days ∀SD	Mean time of fever duration: Days ∀SD
BICv	6/6*	8.2 (0.9)	3.4 (1.1)	6.5 (0.9)
E1.N1v	0/2	5.5 (0.7)	2 (0.0)	3.5 (0.7)
E1.N2v	0/2	9.5 (2.1)	3 (0.0)	6.5 (2.1)
E1.N3v	6/6*	—	—	—
E1.N1N2v	6/6*	—	—	—

The capability of E1.N3v and E1.N1N2v to establish a systemic infection in intranasally inoculated animals was compared with that of virulent parental virus BICv. To assess the effect of the E1.N3v and E1.N1N2v mutation on virus shedding and distribution in different organs during infection, pigs were randomly allocated into 3 groups of 9 animals each and intranasally inoculated (see above) with E1.N3v, E1.N1N2v or BICv. One pig per group was sacrificed at 2, 4, 6, 8 and 12 DPI. Blood, nasal swabs and tonsil scraping samples were obtained from pigs at necropsy. Tissue samples (tonsil, mandibular lymph node, spleen and kidney) were snap-frozen in liquid nitrogen for subsequent virus titration. The remaining 4 pigs in each room were monitored to check for appearance of clinical signs during a 21-day period.

Virus shedding and viremia in E1.N3v and E1.N1N2v inoculated animals was undetectable while values in E1.N1v, E1.N2v were 1.5-2.5 logs below of those of BICv infected swine depending on the time post-infection (FIG. **2**). In all cases partial nucleotide sequences of E2 protein from viruses recovered from infected animals were identical to those of stock viruses used for inoculation (data not shown).

Titers measured in tissue samples are shown in Table 3. In vivo replication of E1.N3v and E1.N1N2v were transient in tonsils with titers reduced up to 10^2 to 10^5, depending on the time post-infection, relative to those of BICv. Differences between E1.N3v and E1.N1N2v and BICv virus titers were also observed in mandibular lymph nodes, spleen, and kidney, indicating a limited capability of E1.N3v and E1.N1N2v to spread within the host.

TABLE 3

Titers of virus in tissues after intranasal inoculation with mutant E1.N1N2v, E1.N3v or parental BICv.

		$Log_{10}TCID_{50}$/g in:			
Virus	DPI	Tonsil	Mandibular Lymph Node	Spleen	Kidney
E1.N1N2v	2	n.d.*	2.63	1.97	n.d.
	4	4.47	n.d.	n.d.	1.97
	6	3.63	n.d.	2.13	2.47
	8	2.8	n.d.	n.d.	n.d.
	12	n.d.	n.d.	n.d.	n.d.
E1.N3v	2	2.3	n.d.	2.13	2.8
	4	1.97	n.d.	1.97	n.d.
	6	2.3	n.d.	2.47	2.47
	8	7.8	n.d.	1.97	n.d.
	12	n.d.	n.d.	1.97	2.13
BICv	2	3.12	1.97	n.d.	n.d.
	4	7.13	3.8	2.97	2.8
	6	6.8	6.13	6.13	5.13
	8	7.13	4.97	7.13	5.47
	12	D#	D	D	D

*n.d. (not detectable): virus titers equal or less than 1.8 $TCID_{50}$ (log_{10}).
D, animals in this group were all dead by this time point.

Example 6

Immunization, Challenge, and Clinical Analysis

For protection studies, 18 pigs were randomly allocated into 5 groups of 4 animals each. Pigs in groups 1 and 2 were inoculated with E1.N1N2v, pigs in groups 3 and 4 were inoculated with E1.N3v and pigs in group 5 were mock infected. At 3 DPI (groups 1 and 3) or 28 DPI (groups 2 and 4), animals were challenged with BICv along with animals in group 5. Clinical signs and body temperature were recorded daily throughout the experiment as described above. Blood, serum, nasal swabs and tonsil scrapings were collected at times post-challenge, with blood obtained from the anterior vena cava in EDTA-containing tubes (Vacutainer) for total and differential white blood cell counts. Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman, Coulter, Calif.).

The limited in vivo replication kinetics of E1.N3v and E1.N1N2v is similar to that observed with CSICv (Risatti et al. 2005a, supra), a CSFV vaccine strain. However, restricted viral in vivo replication could also impair protection against wild-type virus infection. Thus, the ability of E1.N3v and E1.N1N2v to induce protection against virulent BICv was assessed in early and late vaccination-exposure experiments.

Mock-vaccinated control pig groups receiving BICv only (n=2) developed anorexia, depression, and fever by 4 days post-challenge (DPC), and a marked reduction of circulating leukocytes and platelets by 4 DPC (data not shown), and died or were euthanized in extremis by 9 DPC (Table 4). Notably, E1.N3v and E1.N1N2v induced complete protection by 3 DPI. All pigs survived infection and remained clinically normal, without significant changes in their hematological values (data not shown). Pigs challenged at 28 days post N1v infection were also protected, remaining clinically normal, without alterations of hematological profiles (data not shown).

Viremia and virus shedding of vaccinated-exposed animals was examined at 4, 6, 8, 14 and 21 DPC (Table 4). As expected, in mock-vaccinated control animals, viremia was observed by 4 DPC, with virus titers remaining high by 8 DPC (approximately 10^7 $TCID_{50}$/ml). Furthermore, virus was detected in nasal swabs and tonsil scrapings of these animals after 4 DPC. Conversely, viremia was detected by 4 DPC in all clinical samples of one of the four E1.N1N2v-infected animals challenged at 3 DPI, while no virus was detected in any sample from E1.N3v-infected animals at any time post challenge (Table 4). Virus was not detected in clinical samples obtained from any E1.N3v- or E1.N1N2v-infected pigs challenged at 28 DPI. Therefore, even though E1.N3v and E1.N1N2v showed a limited in vivo growth, a solid protection was induced shortly after vaccination.

All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

The foregoing description and certain representative embodiments and details of the invention have been presented for purposes of illustration and description of the invention. It is not intended to be exhaustive or to limit the invention to the precise forms disclosed. It will be apparent to practitioners skilled in this art that modifications and variations may be made therein without departing from the scope of the invention.

TABLE 4

Detection of virus in nasal swabs, tonsil scrapings, and blood samples obtained after challenge of E1.N1N2v- or E1.N3v-vaccinated animals with virulent BICv.

		Days Post-Challenge						
Challenge Group	Sample	0	4	6	8	12	14	21
E1.N1N2v	Nasal	0/4*	1/4 (1.9)	1/4 (2.5)	1/4 (3.1)	0/4	0/4	0/4
3DPI	Tonsil	0/4	1/2 (2.1)	1/4 (2.8)	2/4 (2.7)	0/4	0/4	0/4
	Blood	0/4	1/2 (2.9)#	3/4 (4)	1/4 (4.8)	0/4	0/4	0/4
E1.N1N2v	Nasal	0/4	0/4	0/4	0/4	0/4	0/4	0/4
28DPI	Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
E1.N3v	Nasal	0/4	0/4	0/4	0/4	0/4	0/4	0/4
3DPI	Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
E1.N3v	Nasal	0/4*	0/4	0/4	0/4	0/4	0/4	0/4
28DPI	Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Control	Nasal	0/2	0/2	2/2 (2.1)	2/2 (5.1)	D		
3DPI	Tonsil	0/2	1/2 (2.1)	2/2 (2.7)	2/2 (4.1)			
	Blood	0/2	1/2 (2.2)	2/2 (6.4)	2/2 (7.2)			
Control	Nasal	0/2	0/2	1/2 (2.1)	2/2 (3.6)	D		
28DPI	Tonsil	0/2	1/2 (1.9)	2/2 (4.2)	2/2 (3.6)			
	Blood	0/2	1/2 (2.1)	2/2 (5.1)	2/2 (7.1)			

*Number of animals positive for isolated virus over total number of challenged animals.

#Number in parentheses indicates average virus titers expressed as $\log_{10}$ $TCID_{50}$/ml for four animals.

D Animals in this group were all dead by this time point.

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agagaatcta cagaccccgg gtttacataa gaaactaatg gaagttttca atgcattgaa  10500
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tgactttgtg gacgagaaga aacccagagt catacaatac cctgaagcaa aaacaaggct  10800
ggccatcacc aaggtgatgt ataagtgggt gaagcagaag ccagtagtca tacccgggta  10860
tgaagggaag acacctctgt tccaaatttt tgacaaagta aagaaggaat gggatcaatt  10920
ccaaaatcca gtggcagtga gcttcgacac taaggcgtgg gacacccagg tgaccacaaa  10980
tgatctggag ctgataaagg acatacaaaa gtactacttc aagaagaaat ggcataaatt  11040
tattgacacc ctgactatgc atatgtcaga agtacccgta atcactgctg atggggaggt  11100
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gtatataagg aaagggcaaa gaggtagtgg acagcccgac acaagcgcag gcaacagcat  11160

gctaaatgtg ttaacaatgg tttatgcctt ctgcgaggcc acaggggtac cctacaagag  11220

ttttgacagg gtggcaaaaa ttcatgtgtg cggggacgat ggtttcctga tcacagagag  11280

agctctcggc gagaaattcg caagcaaggg agtccaaatc ctgtatgaag ctgggaagcc  11340

ccagaagatc actgaagggg acaaaatgaa agtggcctac caatttgatg atattgagtt  11400

ttgctcccat acaccaatac aagtaaggtg gtcagataac acttctagct acatgccagg  11460

gagaaataca accacaatcc tggctaaaat ggccacaagg ttagattcca gtggtgagag  11520

gggtaccata gcgtacgaga aagcagtagc attcagcttc ctgctaatgt attcctggaa  11580

cccactaatc agaaggattt gcttattggt actatcaact gaactgcaag tgaaaccagg  11640

gaagtcaacc acttactatt atgaagggga cccgatatct gcctacaagg aagtcatcgg  11700

ccacaatctt ttcgatctca agagaacaag cttcgagaag ctggccaagt taaatctcag  11760

catgtccgta ctcggggcct ggactagaca caccagcaaa agactactac aagactgtgt  11820

caatatgggt gttaaagagg gcaactggtt agtcaatgca gacagactgg tgagtagtaa  11880

gactggaaat aggtatgtac ctggagaagg ccacaccctg caagggagac attatgaaga  11940

actggtgttg gcaagaaaac agatcaacag cttccaaggg acagacaggt acaatctagg  12000

cccaatagtc aacatggtgt taaggaggct gagagtcatg atgatgaccc tgatagggag  12060

aggggtatga gtgcgggtga cccgcgatct ggacccgtca gtaggaccct attgtagata  12120

acactaattt tttatttatt tagatattac tatttattta tttatttatt tattgaatga  12180

gtaagaactg gtacaaacta cctcatgtta ccacactaca ctcattttaa cagcacttta  12240

gctggaagga aaattcctga cgtccacagt tggactaagg taatttccta acggccc     12297
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<210> SEQ ID NO 3
<211> LENGTH: 3898
<212> TYPE: PRT
<213> ORGANISM: Classical Swine Fever Virus

<400> SEQUENCE: 3

Met Glu Leu Asn His Phe Glu Leu Leu Tyr Lys Thr Asn Lys Gln Lys
1               5                   10                  15

Pro Met Gly Val Glu Glu Pro Val Tyr Asp Val Thr Gly Arg Pro Leu
            20                  25                  30

Phe Gly Asp Pro Ser Glu Val His Pro Gln Ser Thr Leu Lys Leu Pro
        35                  40                  45

His Asp Arg Gly Arg Gly Asn Ile Lys Thr Thr Leu Lys Asn Leu Pro
    50                  55                  60

Arg Arg Gly Asp Cys Arg Ser Gly Asn His Leu Gly Pro Val Ser Gly
65                  70                  75                  80

Ile Tyr Val Lys Pro Gly Pro Val Phe Tyr Gln Asp Tyr Met Gly Pro
                85                  90                  95

Val Tyr His Arg Ala Pro Leu Glu Phe Phe Asp Glu Ala Gln Phe Cys
            100                 105                 110

Glu Val Thr Lys Arg Ile Gly Arg Val Thr Gly Ser Asp Gly Lys Leu
        115                 120                 125

Tyr His Ile Tyr Val Cys Ile Asp Gly Cys Ile Leu Leu Lys Leu Ala
    130                 135                 140

Lys Arg Gly Glu Pro Arg Thr Leu Lys Trp Ile Arg Asn Leu Thr Asp
145                 150                 155                 160

Cys Pro Leu Trp Val Thr Ser Cys Ser Asp Asp Gly Ala Ser Ala Ser
                165                 170                 175
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Lys Glu Lys Lys Pro Asp Arg Ile Asn Lys Gly Lys Leu Lys Ile Ala
            180                 185                 190

Pro Lys Glu His Glu Lys Asp Ser Arg Thr Lys Pro Pro Asp Ala Thr
        195                 200                 205

Ile Val Val Glu Gly Val Lys Tyr Gln Val Lys Lys Lys Gly Lys Val
    210                 215                 220

Lys Gly Lys Asn Thr Gln Asp Gly Leu Tyr His Asn Lys Asn Lys Pro
225                 230                 235                 240

Pro Glu Ser Arg Lys Lys Leu Glu Lys Ala Leu Leu Ala Trp Ala Val
                245                 250                 255

Ile Ala Ile Met Leu Tyr Gln Pro Val Ala Ala Glu Asn Ile Thr Gln
            260                 265                 270

Trp Asn Leu Ser Asp Asn Gly Thr Asn Gly Ile Gln His Ala Met Tyr
        275                 280                 285

Leu Arg Gly Val Ser Arg Ser Leu His Gly Ile Trp Pro Glu Lys Ile
    290                 295                 300

Cys Lys Gly Val Pro Thr Tyr Leu Ala Thr Asp Thr Glu Leu Arg Glu
305                 310                 315                 320

Ile Gln Gly Met Met Asp Ala Ser Glu Gly Thr Asn Tyr Thr Cys Cys
                325                 330                 335

Lys Leu Gln Arg His Glu Trp Asn Lys His Gly Trp Cys Asn Trp Tyr
            340                 345                 350

Asn Ile Asp Pro Trp Ile Gln Leu Met Asn Arg Thr Gln Ala Asn Leu
        355                 360                 365

Ala Glu Gly Pro Pro Ser Lys Glu Cys Ala Val Thr Cys Arg Tyr Asp
    370                 375                 380

Lys Asn Ala Asp Ile Asn Val Val Thr Gln Ala Arg Asn Arg Pro Thr
385                 390                 395                 400

Thr Leu Thr Gly Cys Lys Lys Gly Lys Asn Phe Ser Phe Ala Gly Thr
                405                 410                 415

Val Ile Glu Gly Pro Cys Asn Phe Asn Val Ser Val Glu Asp Ile Leu
            420                 425                 430

Tyr Gly Asp His Glu Cys Gly Ser Leu Leu Gln Asp Thr Ala Leu Tyr
        435                 440                 445

Leu Val Asp Gly Met Thr Asn Thr Ile Glu Arg Ala Arg Gln Gly Ala
    450                 455                 460

Ala Arg Val Thr Ser Trp Leu Gly Arg Gln Leu Arg Thr Ala Gly Lys
465                 470                 475                 480

Arg Leu Glu Gly Arg Ser Lys Thr Trp Phe Gly Ala Tyr Ala Leu Ser
                485                 490                 495

Pro Tyr Cys Ala Val Thr Ser Lys Ile Gly Tyr Ile Trp Tyr Thr Asn
            500                 505                 510

Ala Cys Thr Pro Ala Cys Leu Pro Lys Asn Thr Lys Ile Ile Gly Pro
        515                 520                 525

Gly Lys Phe Asp Thr Asn Ala Glu Asp Gly Lys Ile Leu His Glu Met
    530                 535                 540

Gly Gly His Leu Ser Glu Phe Leu Leu Leu Ser Leu Val Val Leu Ser
545                 550                 555                 560

Asp Phe Ala Pro Glu Thr Ala Ser Ala Leu Tyr Leu Ile Leu His Tyr
                565                 570                 575

Val Ile Pro Gln Ser His Glu Glu Pro Glu Gly Cys Asp Thr Asn Gln
            580                 585                 590

Leu Asn Leu Thr Val Glu Leu Arg Thr Glu Asp Val Ile Pro Ser Ser
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        595                 600                 605

Val Trp Asn Val Gly Lys Tyr Val Cys Val Arg Pro Asp Trp Trp Pro
    610                 615                 620

Tyr Glu Thr Lys Val Ala Leu Leu Phe Glu Glu Ala Gly Gln Val Val
625                 630                 635                 640

Lys Leu Ala Leu Arg Ala Leu Arg Asp Leu Thr Arg Val Trp Asn Ser
                645                 650                 655

Ala Ser Thr Thr Ala Phe Leu Ile Cys Leu Ile Lys Val Leu Arg Gly
            660                 665                 670

Gln Val Val Gln Gly Val Ile Trp Leu Leu Leu Val Thr Gly Ala Gln
        675                 680                 685

Gly Arg Leu Ala Cys Lys Glu Asp His Arg Tyr Ala Ile Ser Thr Thr
    690                 695                 700

Asn Glu Ile Gly Leu Leu Gly Ala Glu Gly Leu Thr Thr Thr Trp Lys
705                 710                 715                 720

Glu Tyr Asn His Asn Leu Gln Leu Asp Asp Gly Thr Val Lys Ala Ile
                725                 730                 735

Cys Met Ala Gly Ser Phe Lys Val Thr Ala Leu Asn Val Val Ser Arg
            740                 745                 750

Arg Tyr Leu Ala Ser Leu His Lys Asp Ala Leu Pro Thr Ser Val Thr
        755                 760                 765

Phe Glu Leu Leu Phe Asp Gly Thr Ser Pro Leu Thr Glu Glu Met Gly
    770                 775                 780

Asp Asp Phe Gly Phe Gly Leu Cys Pro Tyr Asp Thr Ser Pro Val Val
785                 790                 795                 800

Lys Gly Lys Tyr Asn Thr Thr Leu Leu Asn Gly Ser Ala Phe Tyr Leu
                805                 810                 815

Val Cys Pro Ile Gly Trp Thr Gly Val Ile Glu Cys Thr Ala Val Ser
            820                 825                 830

Pro Thr Thr Leu Arg Thr Glu Val Val Lys Thr Phe Arg Arg Glu Lys
        835                 840                 845

Pro Phe Pro Tyr Arg Arg Asp Cys Val Thr Thr Thr Val Glu Asn Glu
    850                 855                 860

Asp Leu Phe Tyr Cys Lys Trp Gly Gly Asn Trp Thr Cys Val Lys Gly
865                 870                 875                 880

Glu Pro Val Thr Tyr Thr Gly Gly Pro Val Lys Gln Cys Arg Trp Cys
                885                 890                 895

Gly Phe Asp Phe Asn Glu Pro Asp Gly Leu Pro His Tyr Pro Ile Gly
            900                 905                 910

Lys Cys Ile Leu Ala Asn Glu Thr Gly Tyr Arg Ile Val Asp Ser Thr
        915                 920                 925

Asp Cys Asn Arg Asp Gly Val Val Ile Ser Thr Glu Gly Ser His Glu
    930                 935                 940

Cys Leu Ile Gly Asn Thr Thr Val Lys Val His Ala Leu Asp Glu Arg
945                 950                 955                 960

Leu Gly Pro Met Pro Cys Arg Pro Lys Glu Ile Val Ser Ser Ala Gly
                965                 970                 975

Pro Val Arg Lys Thr Ser Cys Thr Phe Asn Tyr Ala Lys Thr Leu Arg
            980                 985                 990

Asn Arg Tyr Tyr Glu Pro Arg Asp  Ser Tyr Phe Gln Gln  Tyr Met Leu
        995                 1000                 1005

Lys Gly  Glu Tyr Gln Tyr Trp  Phe Asp Leu Asp Val  Thr Asp Arg
    1010                 1015                 1020
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His Ser  Asp Tyr Phe Ala Glu  Phe Ile Val Leu Val  Val Val Ala
    1025                 1030                 1035

Leu Leu  Gly Gly Arg Tyr Val  Leu Trp Leu Ile Val  Thr Tyr Ile
    1040                 1045                 1050

Val Leu  Thr Glu Gln Leu Ala  Ala Gly Leu Gln Leu  Gly Gln Gly
    1055                 1060                 1065

Glu Val  Val Leu Ile Gly Asn  Leu Ile Thr His Thr  Asp Ile Glu
    1070                 1075                 1080

Val Val  Val Tyr Phe Leu Leu  Leu Tyr Leu Val Met  Arg Asp Glu
    1085                 1090                 1095

Pro Ile  Lys Lys Trp Ile Leu  Leu Leu Phe His Ala  Met Thr Asn
    1100                 1105                 1110

Asn Pro  Val Lys Thr Ile Thr  Val Ala Leu Leu Met  Val Ser Gly
    1115                 1120                 1125

Val Ala  Lys Gly Gly Lys Ile  Asp Gly Gly Trp Gln  Arg Leu Pro
    1130                 1135                 1140

Glu Thr  Asn Phe Asp Ile Gln  Leu Ala Leu Thr Val  Ile Val Val
    1145                 1150                 1155

Ala Val  Met Leu Leu Ala Lys  Lys Asp Pro Thr Thr  Val Pro Leu
    1160                 1165                 1170

Val Ile  Thr Val Ala Thr Leu  Arg Thr Ala Lys Ile  Thr Asn Gly
    1175                 1180                 1185

Leu Ser  Thr Asp Leu Ala Ile  Ala Thr Val Ser Thr  Ala Leu Leu
    1190                 1195                 1200

Thr Trp  Thr Tyr Ile Ser Asp  Tyr Tyr Lys Tyr Lys  Thr Leu Leu
    1205                 1210                 1215

Gln Tyr  Leu Ile Ser Thr Val  Thr Gly Ile Phe Leu  Ile Arg Val
    1220                 1225                 1230

Leu Lys  Gly Val Gly Glu Leu  Asp Leu His Thr Pro  Thr Leu Pro
    1235                 1240                 1245

Ser Tyr  Arg Pro Leu Phe Phe  Ile Leu Val Tyr Leu  Ile Ser Thr
    1250                 1255                 1260

Ala Val  Val Thr Arg Trp Asn  Leu Asp Ile Ala Gly  Leu Leu Leu
    1265                 1270                 1275

Gln Cys  Val Pro Thr Leu Leu  Met Val Phe Thr Met  Trp Ala Asp
    1280                 1285                 1290

Ile Leu  Thr Leu Ile Leu Ile  Leu Pro Thr Tyr Glu  Leu Thr Lys
    1295                 1300                 1305

Leu Tyr  Tyr Leu Lys Glu Val  Lys Ile Gly Ala Glu  Arg Gly Trp
    1310                 1315                 1320

Leu Trp  Lys Thr Asn Phe Lys  Arg Val Asn Asp Ile  Tyr Glu Val
    1325                 1330                 1335

Asp Gln  Ala Gly Glu Gly Val  Tyr Leu Phe Pro Ser  Lys Gln Lys
    1340                 1345                 1350

Thr Gly  Thr Ile Thr Gly Thr  Met Leu Pro Leu Ile  Lys Ala Ile
    1355                 1360                 1365

Leu Ile  Ser Cys Ile Ser Asn  Lys Trp Gln Phe Ile  Tyr Leu Leu
    1370                 1375                 1380

Tyr Leu  Ile Phe Glu Val Ser  Tyr Tyr Leu His Lys  Lys Ile Ile
    1385                 1390                 1395

Asp Glu  Ile Ala Gly Gly Thr  Asn Phe Ile Ser Arg  Leu Val Ala
    1400                 1405                 1410

Ala Leu  Ile Glu Ala Asn Trp  Ala Phe Asp Asn Glu  Glu Val Arg
    1415                 1420                 1425
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Gly Leu  Lys Lys Phe Phe Leu  Leu Ser Ser Arg Val  Lys Glu Leu
    1430                 1435                 1440

Ile Ile  Lys His Lys Val Arg  Asn Glu Val Met Val  His Trp Phe
    1445                 1450                 1455

Gly Asp  Glu Glu Val Tyr Gly  Met Pro Lys Leu Val  Gly Leu Val
    1460                 1465                 1470

Lys Ala  Ala Thr Leu Ser Lys  Asn Lys His Cys Ile  Leu Cys Thr
    1475                 1480                 1485

Val Cys  Glu Asn Arg Glu Trp  Arg Gly Glu Thr Cys  Pro Lys Cys
    1490                 1495                 1500

Gly Arg  Phe Gly Pro Pro Val  Thr Cys Gly Met Thr  Leu Ala Asp
    1505                 1510                 1515

Phe Glu  Glu Lys His Tyr Lys  Arg Ile Phe Phe Arg  Glu Asp Gln
    1520                 1525                 1530

Ser Glu  Gly Pro Val Arg Glu  Glu Tyr Ala Gly Tyr  Leu Gln Tyr
    1535                 1540                 1545

Arg Ala  Arg Gly Gln Leu Phe  Leu Arg Asn Leu Pro  Val Leu Ala
    1550                 1555                 1560

Thr Lys  Val Lys Met Leu Leu  Val Gly Asn Leu Gly  Thr Glu Val
    1565                 1570                 1575

Gly Asp  Leu Glu His Leu Gly  Trp Val Leu Arg Gly  Pro Ala Val
    1580                 1585                 1590

Cys Lys  Lys Val Thr Glu His  Glu Lys Cys Thr Thr  Ser Ile Met
    1595                 1600                 1605

Asp Lys  Leu Thr Ala Phe Phe  Gly Val Met Pro Arg  Gly Thr Thr
    1610                 1615                 1620

Pro Arg  Ala Pro Val Arg Phe  Pro Thr Ser Leu Leu  Lys Ile Arg
    1625                 1630                 1635

Arg Gly  Leu Glu Thr Gly Trp  Ala Tyr Thr His Gln  Gly Gly Ile
    1640                 1645                 1650

Ser Ser  Val Asp His Val Thr  Cys Gly Lys Asp Leu  Leu Val Cys
    1655                 1660                 1665

Asp Thr  Met Gly Arg Thr Arg  Val Val Cys Gln Ser  Asn Asn Lys
    1670                 1675                 1680

Met Thr  Asp Glu Ser Glu Tyr  Gly Val Lys Thr Asp  Ser Gly Cys
    1685                 1690                 1695

Pro Glu  Gly Ala Arg Cys Tyr  Val Phe Asn Pro Glu  Ala Val Asn
    1700                 1705                 1710

Ile Ser  Gly Thr Lys Gly Ala  Met Val His Leu Gln  Lys Thr Gly
    1715                 1720                 1725

Gly Glu  Phe Thr Cys Val Thr  Ala Ser Gly Thr Pro  Ala Phe Phe
    1730                 1735                 1740

Asp Leu  Lys Asn Leu Lys Gly  Trp Ser Gly Leu Pro  Ile Phe Glu
    1745                 1750                 1755

Ala Ser  Ser Gly Arg Val Val  Gly Arg Val Lys Val  Gly Lys Asn
    1760                 1765                 1770

Glu Asp  Ser Lys Pro Thr Lys  Leu Met Ser Gly Ile  Gln Thr Val
    1775                 1780                 1785

Ser Lys  Ser Thr Thr Asp Leu  Thr Glu Met Val Lys  Lys Ile Thr
    1790                 1795                 1800

Thr Met  Asn Arg Gly Glu Phe  Arg Gln Ile Thr Leu  Ala Thr Gly
    1805                 1810                 1815

Ala Gly  Lys Thr Thr Glu Leu  Pro Arg Ser Val Ile  Glu Glu Ile
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    1820                1825                1830

Gly Arg  His Lys Arg Val Leu  Val Leu Ile Pro Leu  Arg Ala Ala
    1835                1840                1845

Ala Glu  Ser Val Tyr Gln Tyr  Met Arg Gln Lys His  Pro Ser Ile
    1850                1855                1860

Ala Phe  Asn Leu Arg Ile Gly  Glu Met Lys Glu Gly  Asp Met Ala
    1865                1870                1875

Thr Gly  Ile Thr Tyr Ala Ser  Tyr Gly Tyr Phe Cys  Gln Met Pro
    1880                1885                1890

Gln Pro  Lys Leu Arg Ala Ala  Met Val Glu Tyr Ser  Phe Ile Phe
    1895                1900                1905

Leu Asp  Glu Tyr His Cys Ala  Thr Pro Glu Gln Leu  Ala Ile Met
    1910                1915                1920

Gly Lys  Ile His Arg Phe Ser  Glu Asn Leu Arg Val  Val Ala Met
    1925                1930                1935

Thr Ala  Thr Pro Ala Gly Thr  Val Thr Thr Thr Gly  Gln Lys His
    1940                1945                1950

Pro Ile  Glu Glu Tyr Ile Ala  Pro Glu Val Met Lys  Gly Glu Asp
    1955                1960                1965

Leu Gly  Ser Glu Tyr Leu Asp  Ile Ala Gly Leu Lys  Ile Pro Val
    1970                1975                1980

Glu Glu  Met Lys Ser Asn Met  Leu Val Phe Val Pro  Thr Arg Asn
    1985                1990                1995

Met Ala  Val Glu Thr Ala Lys  Lys Leu Lys Ala Lys  Gly Tyr Asn
    2000                2005                2010

Ser Gly  Tyr Tyr Tyr Ser Gly  Glu Asp Pro Ser Asn  Leu Arg Val
    2015                2020                2025

Val Thr  Ser Gln Ser Pro Tyr  Val Val Val Ala Thr  Asn Ala Ile
    2030                2035                2040

Glu Ser  Gly Val Thr Leu Pro  Asp Leu Asp Val Val  Val Asp Thr
    2045                2050                2055

Gly Leu  Lys Cys Glu Lys Arg  Ile Arg Leu Ser Pro  Lys Met Pro
    2060                2065                2070

Phe Ile  Val Thr Gly Leu Lys  Arg Met Ala Val Thr  Ile Gly Glu
    2075                2080                2085

Gln Ala  Gln Arg Arg Gly Arg  Val Gly Arg Val Lys  Pro Gly Arg
    2090                2095                2100

Tyr Tyr  Arg Ser Gln Glu Thr  Pro Val Gly Ser Lys  Asp Tyr His
    2105                2110                2115

Tyr Asp  Leu Leu Gln Ala Gln  Arg Tyr Gly Ile Glu  Asp Gly Ile
    2120                2125                2130

Asn Ile  Thr Lys Ser Phe Arg  Glu Met Asn Tyr Asp  Trp Ser Leu
    2135                2140                2145

Tyr Glu  Glu Asp Ser Leu Met  Ile Thr Gln Leu Glu  Ile Leu Asn
    2150                2155                2160

Asn Leu  Leu Ile Ser Glu Glu  Leu Pro Met Ala Val  Lys Asn Ile
    2165                2170                2175

Met Ala  Arg Thr Asp His Pro  Glu Pro Ile Gln Leu  Ala Tyr Asn
    2180                2185                2190

Ser Tyr  Glu Thr Gln Val Pro  Val Leu Phe Pro Lys  Ile Lys Asn
    2195                2200                2205

Gly Glu  Val Thr Asp Ser Tyr  Asp Asn Tyr Thr Phe  Leu Asn Ala
    2210                2215                2220
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Arg Lys  Leu Gly Asp Asp Val  Pro Pro Tyr Val Tyr  Ala Thr Glu
    2225                 2230                 2235

Asp Glu  Asp Leu Ala Val Glu  Leu Leu Gly Leu Asp  Trp Pro Asp
    2240                 2245                 2250

Pro Gly  Asn Gln Gly Thr Val  Glu Ala Gly Arg Ala  Leu Lys Gln
    2255                 2260                 2265

Val Val  Gly Leu Ser Thr Ala  Glu Asn Ala Leu Leu  Val Ala Leu
    2270                 2275                 2280

Phe Gly  Tyr Val Gly Tyr Gln  Ala Leu Ser Lys Arg  His Ile Pro
    2285                 2290                 2295

Val Val  Thr Asp Ile Tyr Ser  Ile Glu Asp His Arg  Leu Glu Asp
    2300                 2305                 2310

Thr Thr  His Leu Gln Tyr Ala  Pro Asn Ala Ile Lys  Thr Glu Gly
    2315                 2320                 2325

Lys Glu  Thr Glu Leu Lys Glu  Leu Ala Gln Gly Asp  Val Gln Arg
    2330                 2335                 2340

Cys Met  Glu Ala Met Thr Asn  Tyr Ala Arg Asp Gly  Ile Gln Phe
    2345                 2350                 2355

Met Lys  Ser Gln Ala Leu Lys  Val Lys Glu Thr Pro  Thr Tyr Lys
    2360                 2365                 2370

Glu Thr  Met Asp Thr Val Ala  Asp Tyr Val Lys Lys  Phe Met Glu
    2375                 2380                 2385

Ala Leu  Ala Asp Ser Lys Glu  Asp Ile Ile Lys Tyr  Gly Leu Trp
    2390                 2395                 2400

Gly Thr  His Thr Thr Leu Tyr  Lys Ser Ile Gly Ala  Arg Leu Gly
    2405                 2410                 2415

Asn Glu  Thr Ala Phe Ala Thr  Leu Val Val Lys Trp  Leu Ala Phe
    2420                 2425                 2430

Gly Gly  Glu Ser Ile Ala Asp  His Val Lys Gln Ala  Ala Thr Asp
    2435                 2440                 2445

Leu Val  Val Tyr Tyr Ile Ile  Asn Arg Pro Gln Phe  Pro Gly Asp
    2450                 2455                 2460

Thr Glu  Thr Gln Gln Glu Gly  Arg Lys Phe Val Ala  Ser Leu Leu
    2465                 2470                 2475

Val Ser  Ala Leu Ala Thr Tyr  Thr Tyr Lys Ser Trp  Asn Tyr Asn
    2480                 2485                 2490

Asn Leu  Ser Lys Ile Val Glu  Pro Ala Leu Ala Thr  Leu Pro Tyr
    2495                 2500                 2505

Ala Ala  Thr Ala Leu Lys Leu  Phe Ala Pro Thr Arg  Leu Glu Ser
    2510                 2515                 2520

Val Val  Ile Leu Ser Thr Ala  Ile Tyr Lys Thr Tyr  Leu Ser Ile
    2525                 2530                 2535

Arg Arg  Gly Lys Ser Asp Gly  Leu Leu Gly Thr Gly  Val Ser Ala
    2540                 2545                 2550

Ala Met  Glu Ile Met Ser Gln  Asn Pro Val Ser Val  Gly Ile Ala
    2555                 2560                 2565

Val Met  Leu Gly Val Gly Ala  Val Ala Ala His Asn  Ala Ile Glu
    2570                 2575                 2580

Ala Ser  Glu Gln Lys Arg Thr  Leu Leu Met Lys Val  Phe Val Lys
    2585                 2590                 2595

Asn Phe  Leu Asp Gln Ala Ala  Thr Asp Glu Leu Val  Lys Glu Ser
    2600                 2605                 2610

Pro Glu  Lys Ile Ile Met Ala  Leu Phe Glu Ala Val  Gln Thr Val
    2615                 2620                 2625
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Gly Asn  Pro Leu Arg Leu Val  Tyr His Leu Tyr Gly  Val Phe Tyr
    2630                 2635                 2640

Lys Gly  Trp Glu Ala Lys Glu  Leu Ala Gln Arg Thr  Ala Gly Arg
    2645                 2650                 2655

Asn Leu  Phe Thr Leu Ile Met  Phe Glu Ala Val Glu  Leu Leu Gly
    2660                 2665                 2670

Val Asp  Ser Glu Gly Lys Ile  Arg Gln Leu Ser Ser  Asn Tyr Ile
    2675                 2680                 2685

Leu Glu  Leu Leu Tyr Lys Phe  Arg Asp Ser Ile Lys  Ser Ser Val
    2690                 2695                 2700

Arg Gln  Met Ala Ile Ser Trp  Ala Pro Ala Pro Phe  Ser Cys Asp
    2705                 2710                 2715

Trp Thr  Pro Thr Asp Asp Arg  Ile Gly Leu Pro Gln  Asp Asn Phe
    2720                 2725                 2730

Leu Arg  Val Glu Thr Lys Cys  Pro Cys Gly Tyr Lys  Met Lys Ala
    2735                 2740                 2745

Val Lys  Asn Cys Ala Gly Glu  Leu Arg Leu Leu Glu  Glu Glu Gly
    2750                 2755                 2760

Ser Phe  Leu Cys Arg Asn Lys  Phe Gly Arg Gly Ser  Arg Asn Tyr
    2765                 2770                 2775

Arg Val  Thr Lys Tyr Tyr Asp  Asp Asn Leu Ser Glu  Ile Lys Pro
    2780                 2785                 2790

Val Ile  Arg Met Glu Gly His  Val Glu Leu Tyr Tyr  Lys Gly Ala
    2795                 2800                 2805

Thr Ile  Lys Leu Asp Phe Asn  Asn Ser Lys Thr Ile  Leu Ala Thr
    2810                 2815                 2820

Asp Lys  Trp Glu Val Asp His  Ser Thr Leu Val Arg  Val Leu Lys
    2825                 2830                 2835

Arg His  Thr Gly Ala Gly Tyr  Arg Gly Ala Tyr Leu  Gly Glu Lys
    2840                 2845                 2850

Pro Asn  His Lys His Leu Ile  Glu Arg Asp Cys Ala  Thr Ile Thr
    2855                 2860                 2865

Lys Asp  Lys Val Cys Phe Leu  Lys Met Lys Arg Gly  Cys Ala Phe
    2870                 2875                 2880

Thr Tyr  Asp Leu Ser Leu His  Asn Leu Thr Arg Leu  Ile Glu Leu
    2885                 2890                 2895

Val His  Lys Asn Asn Leu Glu  Asp Lys Glu Ile Pro  Ala Val Thr
    2900                 2905                 2910

Val Thr  Thr Trp Leu Ala Tyr  Thr Phe Val Asn Glu  Asp Ile Gly
    2915                 2920                 2925

Thr Ile  Lys Pro Ala Phe Gly  Glu Lys Ile Thr Pro  Glu Met Gln
    2930                 2935                 2940

Glu Glu  Ile Thr Leu Gln Pro  Ala Val Val Val Asp  Ala Thr Asp
    2945                 2950                 2955

Val Thr  Val Thr Val Val Gly  Glu Thr Pro Thr Met  Thr Thr Gly
    2960                 2965                 2970

Glu Thr  Pro Thr Thr Phe Thr  Ser Ser Gly Pro Asp  Pro Lys Gly
    2975                 2980                 2985

Gln Gln  Val Leu Lys Leu Gly  Val Gly Glu Gly Gln  Tyr Pro Gly
    2990                 2995                 3000

Thr Asn  Pro Gln Arg Ala Ser  Leu His Glu Ala Ile  Gln Ser Ala
    3005                 3010                 3015

Asp Glu  Arg Pro Ser Val Leu  Ile Leu Gly Ser Asp  Lys Ala Thr
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    3020                3025                3030

Ser Asn  Arg Val Lys Thr Val  Lys Asn Val Lys Val  Tyr Arg Gly
    3035                3040                3045

Arg Asp  Pro Leu Glu Val Arg  Asp Met Met Arg Arg  Gly Lys Ile
    3050                3055                3060

Leu Val  Ile Ala Leu Ser Arg  Val Asp Asn Ala Leu  Leu Lys Phe
    3065                3070                3075

Val Asp  Tyr Lys Gly Thr Phe  Leu Thr Arg Glu Thr  Leu Glu Ala
    3080                3085                3090

Leu Ser  Leu Gly Arg Pro Lys  Lys Lys Asn Ile Thr  Lys Ala Glu
    3095                3100                3105

Ala Gln  Trp Leu Leu Arg Leu  Glu Asp Gln Met Glu  Glu Leu Pro
    3110                3115                3120

Asp Trp  Phe Ala Ala Gly Glu  Pro Ile Phe Leu Glu  Ala Asn Ile
    3125                3130                3135

Lys His  Asp Arg Tyr His Leu  Val Gly Asp Ile Ala  Thr Ile Lys
    3140                3145                3150

Glu Lys  Ala Lys Gln Leu Gly  Ala Thr Asp Ser Thr  Lys Ile Ser
    3155                3160                3165

Lys Glu  Val Gly Ala Lys Val  Tyr Ser Met Lys Leu  Ser Asn Trp
    3170                3175                3180

Val Met  Gln Glu Glu Asn Lys  Gln Ser Asn Leu Thr  Pro Leu Phe
    3185                3190                3195

Glu Glu  Leu Leu Gln Gln Cys  Pro Pro Gly Gly Gln  Asn Lys Thr
    3200                3205                3210

Ala His  Met Val Ser Ala Tyr  Gln Leu Ala Gln Gly  Asn Trp Met
    3215                3220                3225

Pro Thr  Ser Cys His Val Phe  Met Gly Thr Ile Ser  Ala Arg Arg
    3230                3235                3240

Thr Lys  Thr His Pro Tyr Glu  Ala Tyr Val Lys Leu  Arg Glu Leu
    3245                3250                3255

Val Glu  Glu His Lys Met Lys  Thr Leu Cys Pro Gly  Ser Ser Leu
    3260                3265                3270

Arg Lys  His Asn Glu Trp Val  Ile Gly Lys Ile Lys  Tyr Gln Gly
    3275                3280                3285

Asn Leu  Arg Thr Lys His Met  Leu Asn Pro Gly Lys  Val Ala Glu
    3290                3295                3300

Gln Leu  His Arg Glu Gly His  Arg His Asn Val Tyr  Asn Lys Thr
    3305                3310                3315

Ile Gly  Ser Val Met Thr Ala  Thr Gly Ile Arg Leu  Glu Lys Leu
    3320                3325                3330

Pro Val  Val Arg Ala Gln Thr  Asp Thr Thr Asn Phe  His Gln Ala
    3335                3340                3345

Ile Arg  Asp Lys Ile Asp Lys  Glu Glu Asn Leu Gln  Thr Pro Gly
    3350                3355                3360

Leu His  Lys Lys Leu Met Glu  Val Phe Asn Ala Leu  Lys Arg Pro
    3365                3370                3375

Glu Leu  Glu Ser Ser Tyr Asp  Ala Val Glu Trp Glu  Glu Leu Glu
    3380                3385                3390

Arg Gly  Ile Asn Arg Lys Gly  Ala Ala Gly Phe Phe  Glu Arg Lys
    3395                3400                3405

Asn Ile  Gly Glu Ile Leu Asp  Ser Glu Lys Asn Lys  Val Glu Glu
    3410                3415                3420
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Ile Ile  Asp Asn Leu Lys Lys  Gly Arg Asn Ile Lys  Tyr Tyr Glu
    3425                 3430                 3435

Thr Ala  Ile Pro Lys Asn Glu  Lys Arg Asp Val Asn  Asp Asp Trp
    3440                 3445                 3450

Thr Ala  Gly Asp Phe Val Asp  Glu Lys Lys Pro Arg  Val Ile Gln
    3455                 3460                 3465

Tyr Pro  Glu Ala Lys Thr Arg  Leu Ala Ile Thr Lys  Val Met Tyr
    3470                 3475                 3480

Lys Trp  Val Lys Gln Lys Pro  Val Val Ile Pro Gly  Tyr Glu Gly
    3485                 3490                 3495

Lys Thr  Pro Leu Phe Gln Ile  Phe Asp Lys Val Lys  Lys Glu Trp
    3500                 3505                 3510

Asp Gln  Phe Gln Asn Pro Val  Ala Val Ser Phe Asp  Thr Lys Ala
    3515                 3520                 3525

Trp Asp  Thr Gln Val Thr Thr  Asn Asp Leu Glu Leu  Ile Lys Asp
    3530                 3535                 3540

Ile Gln  Lys Tyr Tyr Phe Lys  Lys Lys Trp His Lys  Phe Ile Asp
    3545                 3550                 3555

Thr Leu  Thr Met His Met Ser  Glu Val Pro Val Ile  Thr Ala Asp
    3560                 3565                 3570

Gly Glu  Val Tyr Ile Arg Lys  Gly Gln Arg Gly Ser  Gly Gln Pro
    3575                 3580                 3585

Asp Thr  Ser Ala Gly Asn Ser  Met Leu Asn Val Leu  Thr Met Val
    3590                 3595                 3600

Tyr Ala  Phe Cys Glu Ala Thr  Gly Val Pro Tyr Lys  Ser Phe Asp
    3605                 3610                 3615

Arg Val  Ala Lys Ile His Val  Cys Gly Asp Asp Gly  Phe Leu Ile
    3620                 3625                 3630

Thr Glu  Arg Ala Leu Gly Glu  Lys Phe Ala Ser Lys  Gly Val Gln
    3635                 3640                 3645

Ile Leu  Tyr Glu Ala Gly Lys  Pro Gln Lys Ile Thr  Glu Gly Asp
    3650                 3655                 3660

Lys Met  Lys Val Ala Tyr Gln  Phe Asp Asp Ile Glu  Phe Cys Ser
    3665                 3670                 3675

His Thr  Pro Ile Gln Val Arg  Trp Ser Asp Asn Thr  Ser Ser Tyr
    3680                 3685                 3690

Met Pro  Gly Arg Asn Thr Thr  Thr Ile Leu Ala Lys  Met Ala Thr
    3695                 3700                 3705

Arg Leu  Asp Ser Ser Gly Glu  Arg Gly Thr Ile Ala  Tyr Glu Lys
    3710                 3715                 3720

Ala Val  Ala Phe Ser Phe Leu  Leu Met Tyr Ser Trp  Asn Pro Leu
    3725                 3730                 3735

Ile Arg  Arg Ile Cys Leu Leu  Val Leu Ser Thr Glu  Leu Gln Val
    3740                 3745                 3750

Lys Pro  Gly Lys Ser Thr Thr  Tyr Tyr Tyr Glu Gly  Asp Pro Ile
    3755                 3760                 3765

Ser Ala  Tyr Lys Glu Val Ile  Gly His Asn Leu Phe  Asp Leu Lys
    3770                 3775                 3780

Arg Thr  Ser Phe Glu Lys Leu  Ala Lys Leu Asn Leu  Ser Met Ser
    3785                 3790                 3795

Val Leu  Gly Ala Trp Thr Arg  His Thr Ser Lys Arg  Leu Leu Gln
    3800                 3805                 3810

Asp Cys  Val Asn Met Gly Val  Lys Glu Gly Asn Trp  Leu Val Asn
    3815                 3820                 3825
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Ala Asp  Arg Leu Val Ser Ser  Lys Thr Gly Asn Arg  Tyr Val Pro
    3830                 3835                 3840

Gly Glu  Gly His Thr Leu Gln  Gly Arg His Tyr Glu  Glu Leu Val
    3845                 3850                 3855

Leu Ala  Arg Lys Gln Ile Asn  Ser Phe Gln Gly Thr  Asp Arg Tyr
    3860                 3865                 3870

Asn Leu  Gly Pro Ile Val Asn  Met Val Leu Arg Arg  Leu Arg Val
    3875                 3880                 3885

Met Met  Met Thr Leu Ile Gly  Arg Gly Val
    3890                 3895


<210> SEQ ID NO 4
<211> LENGTH: 3898
<212> TYPE: PRT
<213> ORGANISM: Classical Swine Fever Virus

<400> SEQUENCE: 4

Met Glu Leu Asn His Phe Glu Leu Leu Tyr Lys Thr Asn Lys Gln Lys
1               5                   10                  15

Pro Met Gly Val Glu Glu Pro Val Tyr Asp Val Thr Gly Arg Pro Leu
            20                  25                  30

Phe Gly Asp Pro Ser Glu Val His Pro Gln Ser Thr Leu Lys Leu Pro
        35                  40                  45

His Asp Arg Gly Arg Gly Asn Ile Lys Thr Thr Leu Lys Asn Leu Pro
    50                  55                  60

Arg Arg Gly Asp Cys Arg Ser Gly Asn His Leu Gly Pro Val Ser Gly
65                  70                  75                  80

Ile Tyr Val Lys Pro Gly Pro Val Phe Tyr Gln Asp Tyr Met Gly Pro
                85                  90                  95

Val Tyr His Arg Ala Pro Leu Glu Phe Phe Asp Glu Ala Gln Phe Cys
            100                 105                 110

Glu Val Thr Lys Arg Ile Gly Arg Val Thr Gly Ser Asp Gly Lys Leu
        115                 120                 125

Tyr His Ile Tyr Val Cys Ile Asp Gly Cys Ile Leu Leu Lys Leu Ala
    130                 135                 140

Lys Arg Gly Glu Pro Arg Thr Leu Lys Trp Ile Arg Asn Leu Thr Asp
145                 150                 155                 160

Cys Pro Leu Trp Val Thr Ser Cys Ser Asp Asp Gly Ala Ser Ala Ser
                165                 170                 175

Lys Glu Lys Lys Pro Asp Arg Ile Asn Lys Gly Lys Leu Lys Ile Ala
            180                 185                 190

Pro Lys Glu His Glu Lys Asp Ser Arg Thr Lys Pro Pro Asp Ala Thr
        195                 200                 205

Ile Val Val Glu Gly Val Lys Tyr Gln Val Lys Lys Lys Gly Lys Val
    210                 215                 220

Lys Gly Lys Asn Thr Gln Asp Gly Leu Tyr His Asn Lys Asn Lys Pro
225                 230                 235                 240

Pro Glu Ser Arg Lys Lys Leu Glu Lys Ala Leu Leu Ala Trp Ala Val
                245                 250                 255

Ile Ala Ile Met Leu Tyr Gln Pro Val Ala Ala Glu Asn Ile Thr Gln
            260                 265                 270

Trp Asn Leu Ser Asp Asn Gly Thr Asn Gly Ile Gln His Ala Met Tyr
        275                 280                 285

Leu Arg Gly Val Ser Arg Ser Leu His Gly Ile Trp Pro Glu Lys Ile
    290                 295                 300
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Cys Lys Gly Val Pro Thr Tyr Leu Ala Thr Asp Thr Glu Leu Arg Glu
305                 310                 315                 320

Ile Gln Gly Met Met Asp Ala Ser Glu Gly Thr Asn Tyr Thr Cys Cys
                325                 330                 335

Lys Leu Gln Arg His Glu Trp Asn Lys His Gly Trp Cys Asn Trp Tyr
            340                 345                 350

Asn Ile Asp Pro Trp Ile Gln Leu Met Asn Arg Thr Gln Ala Asn Leu
        355                 360                 365

Ala Glu Gly Pro Pro Ser Lys Glu Cys Ala Val Thr Cys Arg Tyr Asp
    370                 375                 380

Lys Asn Ala Asp Ile Asn Val Val Thr Gln Ala Arg Asn Arg Pro Thr
385                 390                 395                 400

Thr Leu Thr Gly Cys Lys Lys Gly Lys Asn Phe Ser Phe Ala Gly Thr
                405                 410                 415

Val Ile Glu Gly Pro Cys Asn Phe Asn Val Ser Val Glu Asp Ile Leu
            420                 425                 430

Tyr Gly Asp His Glu Cys Gly Ser Leu Leu Gln Asp Thr Ala Leu Tyr
        435                 440                 445

Leu Val Asp Gly Met Thr Asn Thr Ile Glu Arg Ala Arg Gln Gly Ala
    450                 455                 460

Ala Arg Val Thr Ser Trp Leu Gly Arg Gln Leu Arg Thr Ala Gly Lys
465                 470                 475                 480

Arg Leu Glu Gly Arg Ser Lys Thr Trp Phe Gly Ala Tyr Ala Leu Ser
                485                 490                 495

Pro Tyr Cys Asn Val Thr Ser Lys Ile Gly Tyr Ile Trp Tyr Thr Asn
            500                 505                 510

Asn Cys Thr Pro Ala Cys Leu Pro Lys Asn Thr Lys Ile Ile Gly Pro
        515                 520                 525

Gly Lys Phe Asp Thr Asn Ala Glu Asp Gly Lys Ile Leu His Glu Met
    530                 535                 540

Gly Gly His Leu Ser Glu Phe Leu Leu Leu Ser Leu Val Val Leu Ser
545                 550                 555                 560

Asp Phe Ala Pro Glu Thr Ala Ser Ala Leu Tyr Leu Ile Leu His Tyr
                565                 570                 575

Val Ile Pro Gln Ser His Glu Glu Pro Glu Gly Cys Asp Thr Asn Gln
            580                 585                 590

Leu Ala Leu Thr Val Glu Leu Arg Thr Glu Asp Val Ile Pro Ser Ser
        595                 600                 605

Val Trp Asn Val Gly Lys Tyr Val Cys Val Arg Pro Asp Trp Trp Pro
    610                 615                 620

Tyr Glu Thr Lys Val Ala Leu Leu Phe Glu Glu Ala Gly Gln Val Val
625                 630                 635                 640

Lys Leu Ala Leu Arg Ala Leu Arg Asp Leu Thr Arg Val Trp Asn Ser
                645                 650                 655

Ala Ser Thr Thr Ala Phe Leu Ile Cys Leu Ile Lys Val Leu Arg Gly
            660                 665                 670

Gln Val Val Gln Gly Val Ile Trp Leu Leu Leu Val Thr Gly Ala Gln
        675                 680                 685

Gly Arg Leu Ala Cys Lys Glu Asp His Arg Tyr Ala Ile Ser Thr Thr
    690                 695                 700

Asn Glu Ile Gly Leu Leu Gly Ala Glu Gly Leu Thr Thr Thr Trp Lys
705                 710                 715                 720

Glu Tyr Asn His Asn Leu Gln Leu Asp Asp Gly Thr Val Lys Ala Ile
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                725                 730                 735

Cys Met Ala Gly Ser Phe Lys Val Thr Ala Leu Asn Val Val Ser Arg
            740                 745                 750

Arg Tyr Leu Ala Ser Leu His Lys Asp Ala Leu Pro Thr Ser Val Thr
        755                 760                 765

Phe Glu Leu Leu Phe Asp Gly Thr Ser Pro Leu Thr Glu Glu Met Gly
    770                 775                 780

Asp Asp Phe Gly Phe Gly Leu Cys Pro Tyr Asp Thr Ser Pro Val Val
785                 790                 795                 800

Lys Gly Lys Tyr Asn Thr Thr Leu Leu Asn Gly Ser Ala Phe Tyr Leu
                805                 810                 815

Val Cys Pro Ile Gly Trp Thr Gly Val Ile Glu Cys Thr Ala Val Ser
            820                 825                 830

Pro Thr Thr Leu Arg Thr Glu Val Val Lys Thr Phe Arg Arg Glu Lys
        835                 840                 845

Pro Phe Pro Tyr Arg Arg Asp Cys Val Thr Thr Thr Val Glu Asn Glu
    850                 855                 860

Asp Leu Phe Tyr Cys Lys Trp Gly Gly Asn Trp Thr Cys Val Lys Gly
865                 870                 875                 880

Glu Pro Val Thr Tyr Thr Gly Gly Pro Val Lys Gln Cys Arg Trp Cys
                885                 890                 895

Gly Phe Asp Phe Asn Glu Pro Asp Gly Leu Pro His Tyr Pro Ile Gly
            900                 905                 910

Lys Cys Ile Leu Ala Asn Glu Thr Gly Tyr Arg Ile Val Asp Ser Thr
        915                 920                 925

Asp Cys Asn Arg Asp Gly Val Val Ile Ser Thr Glu Gly Ser His Glu
    930                 935                 940

Cys Leu Ile Gly Asn Thr Thr Val Lys Val His Ala Leu Asp Glu Arg
945                 950                 955                 960

Leu Gly Pro Met Pro Cys Arg Pro Lys Glu Ile Val Ser Ser Ala Gly
                965                 970                 975

Pro Val Arg Lys Thr Ser Cys Thr Phe Asn Tyr Ala Lys Thr Leu Arg
            980                 985                 990

Asn Arg Tyr Tyr Glu Pro Arg Asp  Ser Tyr Phe Gln Gln  Tyr Met Leu
        995                 1000                 1005

Lys Gly  Glu Tyr Gln Tyr Trp  Phe Asp Leu Asp Val  Thr Asp Arg
    1010                 1015                 1020

His Ser  Asp Tyr Phe Ala Glu  Phe Ile Val Leu Val  Val Val Ala
    1025                 1030                 1035

Leu Leu  Gly Gly Arg Tyr Val  Leu Trp Leu Ile Val  Thr Tyr Ile
    1040                 1045                 1050

Val Leu  Thr Glu Gln Leu Ala  Ala Gly Leu Gln Leu  Gly Gln Gly
    1055                 1060                 1065

Glu Val  Val Leu Ile Gly Asn  Leu Ile Thr His Thr  Asp Ile Glu
    1070                 1075                 1080

Val Val  Val Tyr Phe Leu Leu  Leu Tyr Leu Val Met  Arg Asp Glu
    1085                 1090                 1095

Pro Ile  Lys Lys Trp Ile Leu  Leu Leu Phe His Ala  Met Thr Asn
    1100                 1105                 1110

Asn Pro  Val Lys Thr Ile Thr  Val Ala Leu Leu Met  Val Ser Gly
    1115                 1120                 1125

Val Ala  Lys Gly Gly Lys Ile  Asp Gly Gly Trp Gln  Arg Leu Pro
    1130                 1135                 1140
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Glu Thr  Asn Phe Asp Ile Gln  Leu Ala Leu Thr Val  Ile Val Val
    1145                 1150                 1155

Ala Val  Met Leu Leu Ala Lys  Lys Asp Pro Thr Thr  Val Pro Leu
    1160                 1165                 1170

Val Ile  Thr Val Ala Thr Leu  Arg Thr Ala Lys Ile  Thr Asn Gly
    1175                 1180                 1185

Leu Ser  Thr Asp Leu Ala Ile  Ala Thr Val Ser Thr  Ala Leu Leu
    1190                 1195                 1200

Thr Trp  Thr Tyr Ile Ser Asp  Tyr Tyr Lys Tyr Lys  Thr Leu Leu
    1205                 1210                 1215

Gln Tyr  Leu Ile Ser Thr Val  Thr Gly Ile Phe Leu  Ile Arg Val
    1220                 1225                 1230

Leu Lys  Gly Val Gly Glu Leu  Asp Leu His Thr Pro  Thr Leu Pro
    1235                 1240                 1245

Ser Tyr  Arg Pro Leu Phe Phe  Ile Leu Val Tyr Leu  Ile Ser Thr
    1250                 1255                 1260

Ala Val  Val Thr Arg Trp Asn  Leu Asp Ile Ala Gly  Leu Leu Leu
    1265                 1270                 1275

Gln Cys  Val Pro Thr Leu Leu  Met Val Phe Thr Met  Trp Ala Asp
    1280                 1285                 1290

Ile Leu  Thr Leu Ile Leu Ile  Leu Pro Thr Tyr Glu  Leu Thr Lys
    1295                 1300                 1305

Leu Tyr  Tyr Leu Lys Glu Val  Lys Ile Gly Ala Glu  Arg Gly Trp
    1310                 1315                 1320

Leu Trp  Lys Thr Asn Phe Lys  Arg Val Asn Asp Ile  Tyr Glu Val
    1325                 1330                 1335

Asp Gln  Ala Gly Glu Gly Val  Tyr Leu Phe Pro Ser  Lys Gln Lys
    1340                 1345                 1350

Thr Gly  Thr Ile Thr Gly Thr  Met Leu Pro Leu Ile  Lys Ala Ile
    1355                 1360                 1365

Leu Ile  Ser Cys Ile Ser Asn  Lys Trp Gln Phe Ile  Tyr Leu Leu
    1370                 1375                 1380

Tyr Leu  Ile Phe Glu Val Ser  Tyr Tyr Leu His Lys  Lys Ile Ile
    1385                 1390                 1395

Asp Glu  Ile Ala Gly Gly Thr  Asn Phe Ile Ser Arg  Leu Val Ala
    1400                 1405                 1410

Ala Leu  Ile Glu Ala Asn Trp  Ala Phe Asp Asn Glu  Glu Val Arg
    1415                 1420                 1425

Gly Leu  Lys Lys Phe Phe Leu  Leu Ser Ser Arg Val  Lys Glu Leu
    1430                 1435                 1440

Ile Ile  Lys His Lys Val Arg  Asn Glu Val Met Val  His Trp Phe
    1445                 1450                 1455

Gly Asp  Glu Glu Val Tyr Gly  Met Pro Lys Leu Val  Gly Leu Val
    1460                 1465                 1470

Lys Ala  Ala Thr Leu Ser Lys  Asn Lys His Cys Ile  Leu Cys Thr
    1475                 1480                 1485

Val Cys  Glu Asn Arg Glu Trp  Arg Gly Glu Thr Cys  Pro Lys Cys
    1490                 1495                 1500

Gly Arg  Phe Gly Pro Pro Val  Thr Cys Gly Met Thr  Leu Ala Asp
    1505                 1510                 1515

Phe Glu  Glu Lys His Tyr Lys  Arg Ile Phe Phe Arg  Glu Asp Gln
    1520                 1525                 1530

Ser Glu  Gly Pro Val Arg Glu  Glu Tyr Ala Gly Tyr  Leu Gln Tyr
    1535                 1540                 1545
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Arg Ala  Arg Gly Gln Leu Phe  Leu Arg Asn Leu Pro  Val Leu Ala
    1550                 1555                 1560

Thr Lys  Val Lys Met Leu Leu  Val Gly Asn Leu Gly  Thr Glu Val
    1565                 1570                 1575

Gly Asp  Leu Glu His Leu Gly  Trp Val Leu Arg Gly  Pro Ala Val
    1580                 1585                 1590

Cys Lys  Lys Val Thr Glu His  Glu Lys Cys Thr Thr  Ser Ile Met
    1595                 1600                 1605

Asp Lys  Leu Thr Ala Phe Phe  Gly Val Met Pro Arg  Gly Thr Thr
    1610                 1615                 1620

Pro Arg  Ala Pro Val Arg Phe  Pro Thr Ser Leu Leu  Lys Ile Arg
    1625                 1630                 1635

Arg Gly  Leu Glu Thr Gly Trp  Ala Tyr Thr His Gln  Gly Gly Ile
    1640                 1645                 1650

Ser Ser  Val Asp His Val Thr  Cys Gly Lys Asp Leu  Leu Val Cys
    1655                 1660                 1665

Asp Thr  Met Gly Arg Thr Arg  Val Val Cys Gln Ser  Asn Asn Lys
    1670                 1675                 1680

Met Thr  Asp Glu Ser Glu Tyr  Gly Val Lys Thr Asp  Ser Gly Cys
    1685                 1690                 1695

Pro Glu  Gly Ala Arg Cys Tyr  Val Phe Asn Pro Glu  Ala Val Asn
    1700                 1705                 1710

Ile Ser  Gly Thr Lys Gly Ala  Met Val His Leu Gln  Lys Thr Gly
    1715                 1720                 1725

Gly Glu  Phe Thr Cys Val Thr  Ala Ser Gly Thr Pro  Ala Phe Phe
    1730                 1735                 1740

Asp Leu  Lys Asn Leu Lys Gly  Trp Ser Gly Leu Pro  Ile Phe Glu
    1745                 1750                 1755

Ala Ser  Ser Gly Arg Val Val  Gly Arg Val Lys Val  Gly Lys Asn
    1760                 1765                 1770

Glu Asp  Ser Lys Pro Thr Lys  Leu Met Ser Gly Ile  Gln Thr Val
    1775                 1780                 1785

Ser Lys  Ser Thr Thr Asp Leu  Thr Glu Met Val Lys  Lys Ile Thr
    1790                 1795                 1800

Thr Met  Asn Arg Gly Glu Phe  Arg Gln Ile Thr Leu  Ala Thr Gly
    1805                 1810                 1815

Ala Gly  Lys Thr Thr Glu Leu  Pro Arg Ser Val Ile  Glu Glu Ile
    1820                 1825                 1830

Gly Arg  His Lys Arg Val Leu  Val Leu Ile Pro Leu  Arg Ala Ala
    1835                 1840                 1845

Ala Glu  Ser Val Tyr Gln Tyr  Met Arg Gln Lys His  Pro Ser Ile
    1850                 1855                 1860

Ala Phe  Asn Leu Arg Ile Gly  Glu Met Lys Glu Gly  Asp Met Ala
    1865                 1870                 1875

Thr Gly  Ile Thr Tyr Ala Ser  Tyr Gly Tyr Phe Cys  Gln Met Pro
    1880                 1885                 1890

Gln Pro  Lys Leu Arg Ala Ala  Met Val Glu Tyr Ser  Phe Ile Phe
    1895                 1900                 1905

Leu Asp  Glu Tyr His Cys Ala  Thr Pro Glu Gln Leu  Ala Ile Met
    1910                 1915                 1920

Gly Lys  Ile His Arg Phe Ser  Glu Asn Leu Arg Val  Val Ala Met
    1925                 1930                 1935

Thr Ala  Thr Pro Ala Gly Thr  Val Thr Thr Thr Gly  Gln Lys His
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    1940                1945                1950

Pro Ile  Glu Glu Tyr Ile Ala  Pro Glu Val Met Lys  Gly Glu Asp
    1955                1960                1965

Leu Gly  Ser Glu Tyr Leu Asp  Ile Ala Gly Leu Lys  Ile Pro Val
    1970                1975                1980

Glu Glu  Met Lys Ser Asn Met  Leu Val Phe Val Pro  Thr Arg Asn
    1985                1990                1995

Met Ala  Val Glu Thr Ala Lys  Lys Leu Lys Ala Lys  Gly Tyr Asn
    2000                2005                2010

Ser Gly  Tyr Tyr Tyr Ser Gly  Glu Asp Pro Ser Asn  Leu Arg Val
    2015                2020                2025

Val Thr  Ser Gln Ser Pro Tyr  Val Val Val Ala Thr  Asn Ala Ile
    2030                2035                2040

Glu Ser  Gly Val Thr Leu Pro  Asp Leu Asp Val Val  Val Asp Thr
    2045                2050                2055

Gly Leu  Lys Cys Glu Lys Arg  Ile Arg Leu Ser Pro  Lys Met Pro
    2060                2065                2070

Phe Ile  Val Thr Gly Leu Lys  Arg Met Ala Val Thr  Ile Gly Glu
    2075                2080                2085

Gln Ala  Gln Arg Arg Gly Arg  Val Gly Arg Val Lys  Pro Gly Arg
    2090                2095                2100

Tyr Tyr  Arg Ser Gln Glu Thr  Pro Val Gly Ser Lys  Asp Tyr His
    2105                2110                2115

Tyr Asp  Leu Leu Gln Ala Gln  Arg Tyr Gly Ile Glu  Asp Gly Ile
    2120                2125                2130

Asn Ile  Thr Lys Ser Phe Arg  Glu Met Asn Tyr Asp  Trp Ser Leu
    2135                2140                2145

Tyr Glu  Glu Asp Ser Leu Met  Ile Thr Gln Leu Glu  Ile Leu Asn
    2150                2155                2160

Asn Leu  Leu Ile Ser Glu Glu  Leu Pro Met Ala Val  Lys Asn Ile
    2165                2170                2175

Met Ala  Arg Thr Asp His Pro  Glu Pro Ile Gln Leu  Ala Tyr Asn
    2180                2185                2190

Ser Tyr  Glu Thr Gln Val Pro  Val Leu Phe Pro Lys  Ile Lys Asn
    2195                2200                2205

Gly Glu  Val Thr Asp Ser Tyr  Asp Asn Tyr Thr Phe  Leu Asn Ala
    2210                2215                2220

Arg Lys  Leu Gly Asp Asp Val  Pro Pro Tyr Val Tyr  Ala Thr Glu
    2225                2230                2235

Asp Glu  Asp Leu Ala Val Glu  Leu Leu Gly Leu Asp  Trp Pro Asp
    2240                2245                2250

Pro Gly  Asn Gln Gly Thr Val  Glu Ala Gly Arg Ala  Leu Lys Gln
    2255                2260                2265

Val Val  Gly Leu Ser Thr Ala  Glu Asn Ala Leu Leu  Val Ala Leu
    2270                2275                2280

Phe Gly  Tyr Val Gly Tyr Gln  Ala Leu Ser Lys Arg  His Ile Pro
    2285                2290                2295

Val Val  Thr Asp Ile Tyr Ser  Ile Glu Asp His Arg  Leu Glu Asp
    2300                2305                2310

Thr Thr  His Leu Gln Tyr Ala  Pro Asn Ala Ile Lys  Thr Glu Gly
    2315                2320                2325

Lys Glu  Thr Glu Leu Lys Glu  Leu Ala Gln Gly Asp  Val Gln Arg
    2330                2335                2340
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Cys Met  Glu Ala Met Thr Asn  Tyr Ala Arg Asp Gly  Ile Gln Phe
    2345                 2350                 2355

Met Lys  Ser Gln Ala Leu Lys  Val Lys Glu Thr Pro  Thr Tyr Lys
    2360                 2365                 2370

Glu Thr  Met Asp Thr Val Ala  Asp Tyr Val Lys Lys  Phe Met Glu
    2375                 2380                 2385

Ala Leu  Ala Asp Ser Lys Glu  Asp Ile Ile Lys Tyr  Gly Leu Trp
    2390                 2395                 2400

Gly Thr  His Thr Thr Leu Tyr  Lys Ser Ile Gly Ala  Arg Leu Gly
    2405                 2410                 2415

Asn Glu  Thr Ala Phe Ala Thr  Leu Val Val Lys Trp  Leu Ala Phe
    2420                 2425                 2430

Gly Gly  Glu Ser Ile Ala Asp  His Val Lys Gln Ala  Ala Thr Asp
    2435                 2440                 2445

Leu Val  Val Tyr Tyr Ile Ile  Asn Arg Pro Gln Phe  Pro Gly Asp
    2450                 2455                 2460

Thr Glu  Thr Gln Gln Glu Gly  Arg Lys Phe Val Ala  Ser Leu Leu
    2465                 2470                 2475

Val Ser  Ala Leu Ala Thr Tyr  Thr Tyr Lys Ser Trp  Asn Tyr Asn
    2480                 2485                 2490

Asn Leu  Ser Lys Ile Val Glu  Pro Ala Leu Ala Thr  Leu Pro Tyr
    2495                 2500                 2505

Ala Ala  Thr Ala Leu Lys Leu  Phe Ala Pro Thr Arg  Leu Glu Ser
    2510                 2515                 2520

Val Val  Ile Leu Ser Thr Ala  Ile Tyr Lys Thr Tyr  Leu Ser Ile
    2525                 2530                 2535

Arg Arg  Gly Lys Ser Asp Gly  Leu Leu Gly Thr Gly  Val Ser Ala
    2540                 2545                 2550

Ala Met  Glu Ile Met Ser Gln  Asn Pro Val Ser Val  Gly Ile Ala
    2555                 2560                 2565

Val Met  Leu Gly Val Gly Ala  Val Ala Ala His Asn  Ala Ile Glu
    2570                 2575                 2580

Ala Ser  Glu Gln Lys Arg Thr  Leu Leu Met Lys Val  Phe Val Lys
    2585                 2590                 2595

Asn Phe  Leu Asp Gln Ala Ala  Thr Asp Glu Leu Val  Lys Glu Ser
    2600                 2605                 2610

Pro Glu  Lys Ile Ile Met Ala  Leu Phe Glu Ala Val  Gln Thr Val
    2615                 2620                 2625

Gly Asn  Pro Leu Arg Leu Val  Tyr His Leu Tyr Gly  Val Phe Tyr
    2630                 2635                 2640

Lys Gly  Trp Glu Ala Lys Glu  Leu Ala Gln Arg Thr  Ala Gly Arg
    2645                 2650                 2655

Asn Leu  Phe Thr Leu Ile Met  Phe Glu Ala Val Glu  Leu Leu Gly
    2660                 2665                 2670

Val Asp  Ser Glu Gly Lys Ile  Arg Gln Leu Ser Ser  Asn Tyr Ile
    2675                 2680                 2685

Leu Glu  Leu Leu Tyr Lys Phe  Arg Asp Ser Ile Lys  Ser Ser Val
    2690                 2695                 2700

Arg Gln  Met Ala Ile Ser Trp  Ala Pro Ala Pro Phe  Ser Cys Asp
    2705                 2710                 2715

Trp Thr  Pro Thr Asp Asp Arg  Ile Gly Leu Pro Gln  Asp Asn Phe
    2720                 2725                 2730

Leu Arg  Val Glu Thr Lys Cys  Pro Cys Gly Tyr Lys  Met Lys Ala
    2735                 2740                 2745
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```
Val Lys  Asn Cys Ala Gly Glu  Leu Arg Leu Leu Glu  Glu Glu Gly
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Lys Trp  Val Lys Gln Lys Pro  Val Val Ile Pro Gly  Tyr Glu Gly
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Asp Gln  Phe Gln Asn Pro Val  Ala Val Ser Phe Asp  Thr Lys Ala
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    3620                 3625                 3630

Thr Glu  Arg Ala Leu Gly Glu  Lys Phe Ala Ser Lys  Gly Val Gln
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Ile Leu  Tyr Glu Ala Gly Lys  Pro Gln Lys Ile Thr  Glu Gly Asp
    3650                 3655                 3660

Lys Met  Lys Val Ala Tyr Gln  Phe Asp Asp Ile Glu  Phe Cys Ser
    3665                 3670                 3675

His Thr  Pro Ile Gln Val Arg  Trp Ser Asp Asn Thr  Ser Ser Tyr
    3680                 3685                 3690

Met Pro  Gly Arg Asn Thr Thr  Thr Ile Leu Ala Lys  Met Ala Thr
    3695                 3700                 3705

Arg Leu  Asp Ser Ser Gly Glu  Arg Gly Thr Ile Ala  Tyr Glu Lys
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Ala Val  Ala Phe Ser Phe Leu  Leu Met Tyr Ser Trp  Asn Pro Leu
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Ser Ala  Tyr Lys Glu Val Ile  Gly His Asn Leu Phe  Asp Leu Lys
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We claim:

1. An isolated polynucleotide molecule comprising a DNA sequence encoding an infectious RNA molecule that encodes a genetically modified classical swine fever virus (CSFV) mutant of a highly pathogenic native Brescia strain, wherein said CSFV mutant encodes a genetically modified E1 glycoprotein set forth in SEQ ID NO:4 that said genetic modified E1 gene has a mutated amino acid asparagine in position 594 to alanine, said altered amino acid having a glycosylation pattern different from the amino acid present in position 594 of said highly pathogenic native Brescia strain, wherein said genetically modified CSFV mutant is attenuated and unable to produce a pathogenic CSFV infection in a porcine animal.

2. An isolated recombinant classical swine fever virus comprising the RNA molecule according to claim **1**, said RNA molecule encodes a genetically modified mutated CSFV E1 glycoprotein having a sequence identified by SEQ ID NO: 4.

3. The isolated polynucleotide molecule of claim **1**, wherein said DNA sequence is SEQ ID NO: 2 or its complement thereof.

4. A CSF vaccine comprising a genetically modified CSFV mutant that does not produce CSF disease in swine, wherein said virus is encoded by the polynucleotide of claim **1**.

5. A genetically modified CSFV mutant, wherein the CSF virus is encoded by the isolated polynucleotide molecule of claim **1**.

6. A vaccine for protecting a porcine animal against infection by a CSFV, which vaccine comprises (a) a genetically modified CSFV encoded by an infectious RNA molecule encoded by the polynucleotide molecule according to claim **1**, (b) said infectious RNA molecule of claim **1**, wherein the vaccine is in an effective amount to produce immunoprotection against a CSFV infection; and a carrier acceptable for veterinary use.

7. A method of producing an attenuated recombinant classical swine fever virus comprising DNA encoding a modified CSFV E1glycoprotein, comprising:

(a) mutating a region of the E1 gene of the highly pathogenic strain Brescia, wherein said region encodes the modified CSFV E1 glycoprotein set forth in SEQ ID NO: 4, and whereby mutations in said DNA result in a change in the glycosylation pattern characteristic of CSFV E1 glycoprotein; and

(b) achieving attenuation of CSFV as a result of such modification.

8. A method for generating a genetically modified CSFV, which method comprises transfecting a host cell with an infectious RNA molecule that encodes the genetically modified CSFV mutant according to claim **1** and obtaining the genetically modified CSFV mutant generated by the transfected host cell.

9. A rationally designed live attenuated CSF vaccine comprising a recombinant classical swine fever virus according to claim **2**.

10. A method of immunizing an animal against CSF, comprising administering to said animal a vaccine comprising a recombinant classical swine fever virus according to claim **2**.

11. A method of protecting an animal against CSF, comprising administering to said animal an effective amount of the vaccine of claim **9** effective to protect said animal from clinical CSF.

* * * * *

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(12) **United States Patent**
Borca et al.

(10) **Patent No.: US 8,133,495 B2**
(45) **Date of Patent: Mar. 13, 2012**

(54) **LIVE ATTENUATED ANTIGENICALLY MARKED CLASSICAL SWINE FEVER VIRUS**

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(73) Assignees: **The United States of America as represented by the Secretary of Agriculture**, Washington, DC (US); **The University of Connecticut**, Farmington, CT (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 180 days.

(21) Appl. No.: **11/805,278**

(22) Filed: **May 22, 2007**

(65) **Prior Publication Data**

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(51) **Int. Cl.**
A61K 39/155 (2006.01)
C12P 19/34 (2006.01)

(52) **U.S. Cl.** **424/211.1**; 435/91.32

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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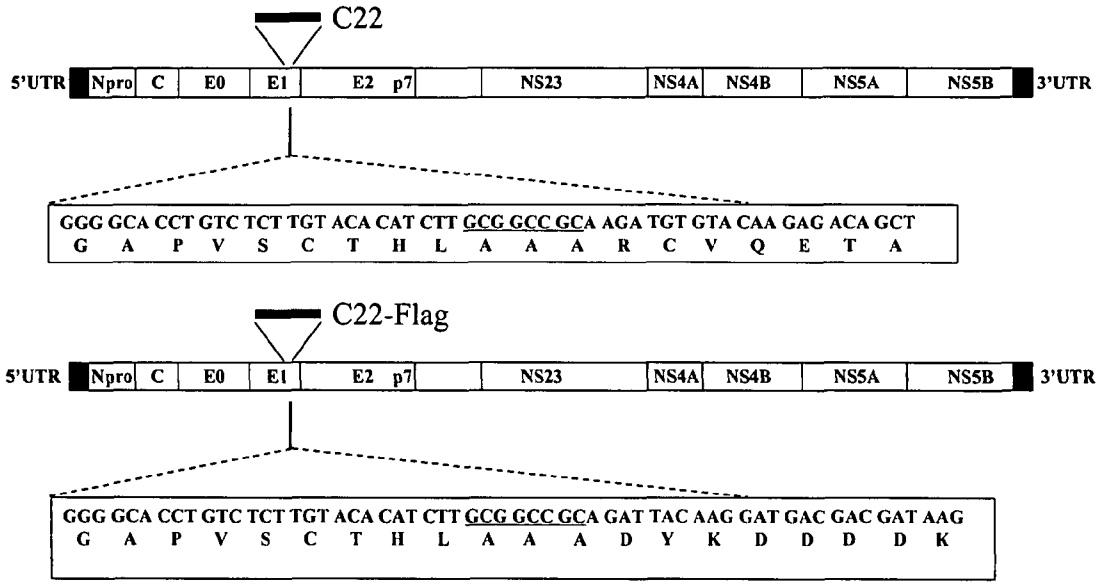
(74) *Attorney, Agent, or Firm* — John D. Pado; Evelyn M. Rabin

(57) **ABSTRACT**

Classical swine fever virus is a world-wide distributed highly-contagious disease affecting swine. The two main strategies for diseases control are prophylactic vaccination and non-vaccination stamping out policies. Marker vaccines are a promising strategy. Here we report the rational development of a doubly antigenic marker CSFV experimental live attenuated candidate strain vaccine (Flag/T4 virus). Flag/T virus (Flag/T4v) is based in the combination of two Brescia derived recombinant attenuated viruses: RB-C22 and T4. RB-C22v contains a 19mer insertion in the structural glycoprotein E1, while T4v posses mutated CSFV amino acid residues 830 to 834 in the structural glycoprotein E2, deleting the highly conserved epitope recognized by monoclonal antibody (mAb) WH303. Flag/T4 virus contains a positive foreign antigenic marker, due to the insertion of the highly antigenic epitope Flag in the 19mer insertion of E1, as well as a negative antigenic marker, the lack of reactivity with mAb WH303. Immunized with Flag/T4v induced a complete protection against the challenge with virulent strain Brescia both at 3 and 28 days post infection when nasally administered and since the second day post infection when intramuscularly administered. These results constitute an example of rational design of a CSFV antigenically marked LAV.

10 Claims, 2 Drawing Sheets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C
E0
E1
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5'UTR
Npro
C
E0
E1
E2
p7
NS23
NS4A
NS4B
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NS5B
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GGG GCA CCT GTC TCT TGT ACA CAT CTT GCG GCC GCA GAT TAC AAG GAT GAC GAC GAT AAG
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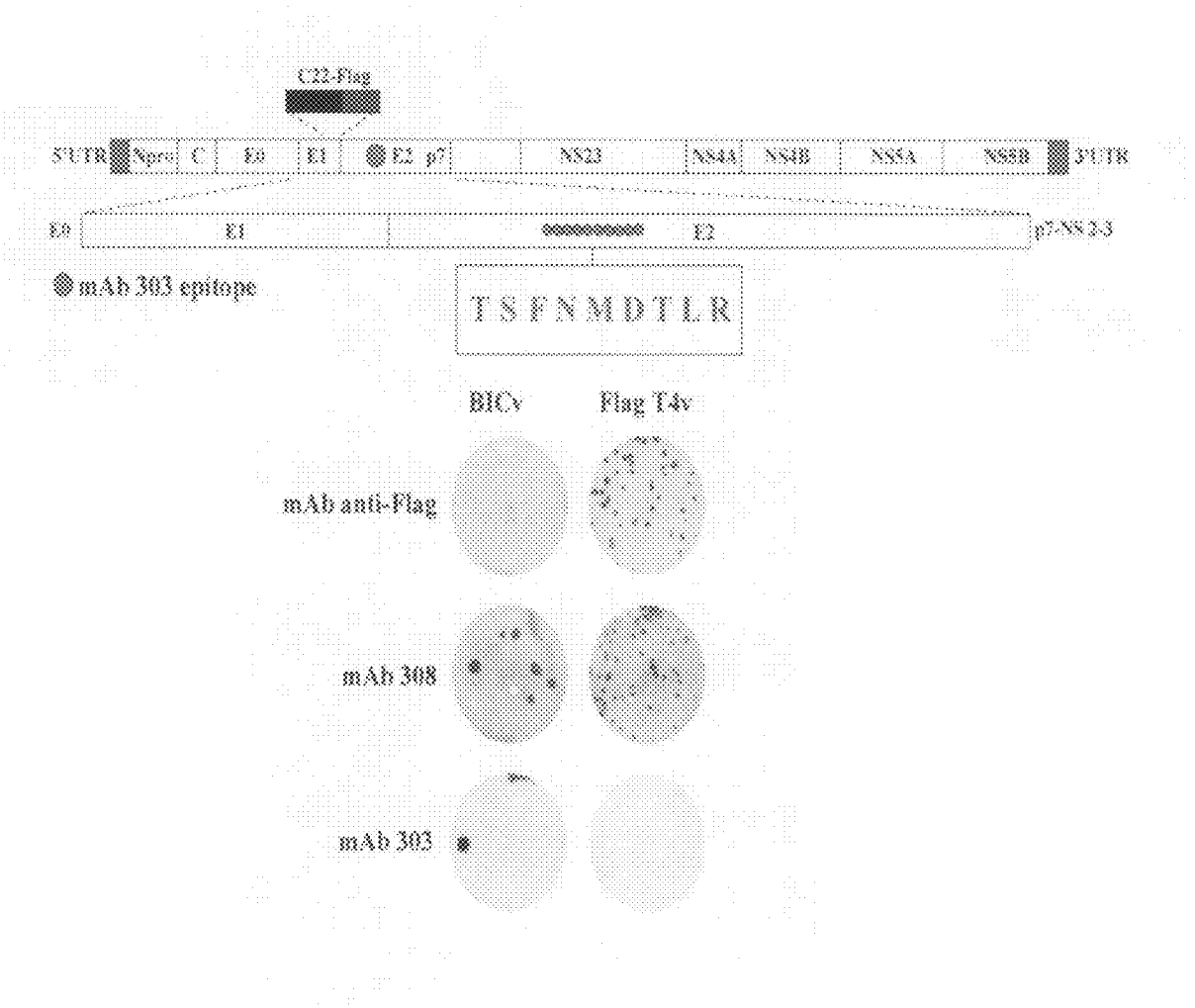
Fig. 1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E0
E1
E2
p7-NS2-3
mAb 303 epitope
T S F N M D T L R
BICv
Flag T4v
mAb anti-Flag
mAb 308
mAb 303

Fig. 2

LIVE ATTENUATED ANTIGENICALLY MARKED CLASSICAL SWINE FEVER VIRUS

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to the design of a double antigenically marked classical swine fever virus (CSFV) live attenuated candidate strain vaccine. The Flag/T4 virus is a recombinant CSFV which contains a positive foreign antigenic marker due to the insertion of the highly antigenic epitope, Flag, into the 19mer insertion of the E1 structural protein of recombinant virus RB-C22, as well as a negative antigenic marker, i.e., changes in the WH303 epitope of the E2 protein which result in the lack of reactivity with monoclonal antibody WH303.

2. Description of the Relevant Art

Classical swine fever (CSF) is a highly contagious disease of swine that can be either acute or chronic in nature (van Oirschot, J. T. 1986. In: *Diseases of Swine*, 6th edition, Leman et al., eds., Iowa State University Press, Ames, Iowa, page 289). The etiological agent, CSF virus (CSFV), is a small, enveloped virus with a positive, single-stranded RNA genome. Along with bovine viral diarrhea virus (BVDV) and border disease virus (BDV), CSFV is classified as a member of the genus *Pestivirus* within the family Flaviridae (Becher et al. 2003. *Virology* 311: 96-104). The 12.5 kb CSFV genome contains a single open reading frame that encodes a 3898 amino acid polyprotein and ultimately yields 11 to 12 final cleavage products (NH2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B—COOH) through co- and post-translational processing of the polyprotein by cellular and viral proteases (Rice, C. M. 1996. In: *Fundamental Virology*, 3rd edition, Fields and Howley, eds., Lippincott Raven, Philadelphia, pp. 931-959).

CSFV is enzootic in all continents, and actively circulating in South and Central America, southern Mexico, and the Caribbean. Disease outbreaks occur intermittently in Europe where control programs of the disease (Westergaard et al. 1998. In: *Vaccines in Agriculture: Immunological Applications To Animal Health and Production*, Wood et al., eds., CSIRO, East Melbourne, Australia, pages 13-20) include quarantine and eradication of infected herds, resulting in the elimination of a large number of animals, including noninfected animals, thus engendering significant economic losses. Vaccination, quarantine and testing protocols offer an alternative to these measures and may become the only means to control and eradicate an outbreak of CSF, reducing the economic impact that results from the heretofore elimination of such a vast number of pigs.

Safe and effective CSFV vaccines that prevent clinical symptoms of the disease and virus spreading, including during the early post-vaccination period, have been used around the world (Aynaud, J. M. 1988. In: *Classical Swine Fever and Related Viral Infections*, B. Liess, ed., Nijhoff, Boston, Mass., pages 165-176; Biront and Leunen. 1988. ibid., pages 181-197). Among available CSFV vaccines, live attenuated vaccines, such as C strain, GPE-strain, Thiversal strain, and PAV-250, confer effective and long lasting immunity against CSF (Biront and Leunen, ibid.). In general, these vaccines have been obtained after serial passages of CSFV isolates in tissue culture or rabbits; however, the genetic bases of the attenuation in the above cases are unknown. Additionally, it is not currently possible to distinguish, serologically, between animals vaccinated with live attenuated vaccines and animals infected with wild-type virus.

Development of infectious CSFV cDNA clones has enabled genetic approaches for defining mechanisms of viral replication and pathogenesis. Infectious clones of the attenuated CSFV C-strain and the pathogenic Alfort/Tübingen strain have been constructed, enabling identification of E^{ms} and N^{pro} as virulence factors in swine and the role of different E^{ms} mutations in virus attenuation (Meyers et al. 1996, supra, Moormann et al. 1996. *J. Virol.* 70: 763-770, Ruggli et al. 1996. *J. Virol.* 70:3478-3487, Mayer et al. 2004. *Vaccine* 22: 317-328). CSFV infectious clones have been used to identify viral proteins or protein domains functioning in viral replication and virulence, and to engineer attenuated marker CSF live attenuated vaccines (Meyers et al. 1999, supra, Moser et al. 2001. *Virus Genes* 23: 63-68, Tratschin et al. 1998. *J. Virol.* 72: 7681-7684, van Gennip et al. supra).

The capsid protein, and glycoproteins E^{rns}, E1, and E2 are the structural components of the CSFV virion with E1 and E2 anchored to the envelope by their carboxyl termini and E^{rns} loosely associated with the viral envelope (Thiel et al. 1991. *J. Virol.* 65: 4705-4712; Weiland et al. 1990. *J. Virol.* 64: 3563-3569; Weiland et al. 1999. *J. Gen. Virol.* 80: 1157-1165).

E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor (Thiel et al., supra). E2, the major structural protein, is considered essential for CSFV replication, as virus mutants containing partial or complete deletions of the E2 gene have proven non-viable (van Gennip et al. 2000. *Vaccine* 19:447-459). E2 is the most immunogenic of the CSFV glycoproteins (Konig et al. 1995. *J. Virol.* 69: 6479-6486; van Gennip et al. and Weiland et al. 1990, supra), inducing neutralizing antibodies and protection against lethal CSFV challenge (Wensvoort et al. 1989. *Vet. Microbiol.* 21: 9-20; Van Zijl et al. 1991. *Vaccine* 17: 433-440; Hulst et al. 1993. *J. Virol.* 67: 5435-5442; Rumenapf et al. 1991. *J. Virol.* 65: 589-597; Van Rijn et al. 1996. *J. Gen. Virol.* 77: 2737-2745; Van Rijn et al. 1998. *Vaccine* 17: 433-440). CSFV E2 also contains, between residues 829 and 837, an epitope recognized by monoclonal antibody (mAb) WH303 (Lin et al. 2000. *J. Virol.* 74:11619-11625), a reagent which is routinely used for CSF diagnostics. WH303 mAb does not react with E2 of BVDV or BDV.

Candidate CSFV subunit marker vaccines have been developed using recombinant E2 envelope protein (Van Zijl et al., supra; Hooft van Iddkinge et al. 1996. *Vaccine* 14: 6-12; Hulst et al, 1993, supra; Van Rijn et al., supra). Different E2 protein domains have been described as targets for neutralizing monoclonal antibodies (Wensvoort et al., supra), but E2 subunit vaccines have not been found to be as efficacious as traditional live attenuated vaccines, particularly when animals are challenged shortly after vaccination (Hulst et al., 1993, supra; Van Rijn et al., supra; Risatti et al. 2003. *J. Clin. Microbiol.* 41: 500-505). The failure to induce rapid and efficient protective immunity precludes the use of subunit vaccines as an emergency control measure during a CSFV outbreak. DNA vaccines encoding E2, when expressed, also induced protection in pigs; however, again, rapid elicitation of protection was not proven (Andrew et a. 2000. *Vaccine* 17: 1932-1938; Yu et al. 2001. *Vaccine* 19: 1520-1525).

Recently, infectious clone technology has enabled antigenic modification of attenuated CSFV strains for use as experimental marker live attenuated CSF vaccines. Infectious clones of the attenuated C-strain were used to replace the antigenic region of E2 and/or the complete E^{ms} gene with analogous sequences from Bovine Viral Diarrhea Virus (BVDV). Preliminary data indicated that both chimeric viruses were able to induce protection in pigs at one week after vaccination. Significantly, chimera-induced anti-CSFV

antibody responses could be discriminated from those produced by parental virus (van Gennip et al., supra).

The development of disease control strategies in the event of a CSFV outbreak requires rapid onset of protection, which becomes a more important parameter of vaccine performance than, for example, duration of protection. The development of such vaccines would imply the production of rationally designed live attenuated vaccine CSFV strains.

SUMMARY OF THE INVENTION

We have developed a novel classical swine fever mutant virus, the Flag/T4 virus.

In accordance with this discovery, it is an object of the invention to provide a recombinant classical swine fever virus (CSFV) mutant virus, the Flag/T4v virus, comprising DNA encoding a CSFV E1 glycoprotein modified by insertion of the Flag epitope and DNA encoding a CSFV E2 glycoprotein lacking an immunodominant WH303 epitope.

It is also an object of the invention to provide a recombinant classical swine fever virus comprising DNA encoding CSFV E2 glycoprotein which has been modified by progressively mutating a portion of the E2 gene of the highly pathogenic strain Brescia, resulting in the mutated E2 virus more closely resembling the WH303 epitope sequence of the homologous E2 gene from BVDV, a modification resulting in attenuation of CSFV.

An added object of the invention is to provide immunogenic compositions comprising a viable recombinant classical swine fever virus mutant, Flag/T4v.

An additional object of the invention is to provide a rationally designed live attenuated CSFV effective to protect an animal from clinical CSF disease when challenged with virulent Brescia CSFV.

A further object of the invention is to provide a marker vaccine which can potentially distinguish between vaccinated animals and animals infected with CSFV.

A still further object of the invention is to provide a vaccine comprising at least one additional vaccine strain capable of inducing protection against CSF or against another porcine pathogen.

Another object of the invention is to provide a method for protecting an animal against CSF by administering an effective amount of rationally designed live attenuated CSFV vaccine.

Other objects and advantages of this invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts a schematic representation of the Flag virus. Flag virus is a derivative of CSFV RB-C22 strain (Risatti et. al., 2006. *Virology* 355: 94-101) where a portion of the 19mer insert (GAPVSCTHLAAARCVQETA; SEQ ID NO:8, encoded by SEQ ID NO:10) has been replaced by the FLAG™ epitope (DYKDDDDK; SEQ ID NO:5) resulting in a 20 amino acid insertion (GAPVSCTHLAAADYKDDDDK; SEQ ID NO:9, encoded by SEQ ID NO:11) in the wild-type E2 glycoprotein sequence. The unique NotI site is indicated in italics.

FIG. **2** shows the antigenic phenotype of Flag-T4 and BIC viruses. SK6 monolayers were infected, overlaid with 0.5% agarose and incubated at 37° C. for 3 days. Plates were fixed with 50% (vol/vol) ethanol-acetone and stained by immunohistochemistry with mAbs WH303, anti-Flag or WH308.

DETAILED DESCRIPTION OF THE INVENTION

We have developed an experimental live attenuated CSFV vaccine strain, Flag/T4 (SEQ ID NO:1), which contains a positive foreign antigenic marker in CSFV structural protein E1 as well as a negative antigenic marker in structural protein E2. Immunization with Flag/T4v induced a complete protection against the challenge with the virulent strain Brescia, both at 3 and 28 days post infection when nasally administered and at the second day post infection when intramuscularly administered. A serological response against the Flag epitope in Flag/T4v-immunized animals allows the discrimination between a Flag/T4v-immunized and a Brescia-infected animal.

The genetic basis and the molecular mechanisms underlying Pestivirus virulence are still unclear. In the case of CSFV, different reports have described associations between viral proteins or specific genomic region with virulence. An in frame insertion of 19 amino acids into E1 gene of CSFV strain Brescia led to attenuation in vivo (Risatti et al. 2005b, supra). A viral mutant, RB-C22v, with a 19 amino acid insertion at nucleotide position 2429, near the carboxyl terminus of E1 glycoprotein, was obtained. Insertion of the 19 amino acid peptide results in complete in vivo attenuation of the virulent CSFV strain Brescia, rendering a virus that resembles CSFV live attenuated vaccines in infected pigs. Unlike the acute fatal disease induced by Brescia and its infectious clone, BICv, RB-C22v infection was subclinical, characterized by decreased viral replication in tonsils, limited generalization of infection, a significant reduction of virus shedding, and significantly lower virus titers in blood, lymph nodes, bone marrow, kidney, spleen, and brain.

Mutations introduced into the WH 303 epitope (epitope which specifically binds to mAb WH303) in E2 glycoprotein of highly pathogenic CSFV strain Brescia also resulted in attenuation of the virus. Progressive changes were introduced into the CSFV Brescia WH 303 epitope (TAVSPTTLR; SEQ ID NO:2) to resemble residues found at the same position (TSFNMDTLA; SEQ ID NO:3) in BVDV strain NADL E2 glycoprotein. The mutant virus T4v (TSFNMDTLR; SEQ ID NO:4) lacks reactivity to mAb WH 303, shows small plaque morphology, i.e., an approximately 70% plaque size reduction compared with parental BICv in SK6 cells, and is significantly attenuated in vivo. Unlike the acute fatal disease induced by BICv, T4v infection is sub-clinical and is characterized by decreased viral replication in target organs and reduced virus shedding. Thus, a novel CSFV genetic virulence determinant associated with E2 glycoprotein has been identified. Although the mechanism underlying the attenuation remains unknown, interestingly, the gradual loss of reactivity with the mAb WH 303 correlated with a loss of virulence in vivo, leading to the final attenuation of CSFV.

The relevance of WH 303 as a major immunodominant epitope has been recently observed during characterization of neutralizing monoclonal antibodies specific for CSFV E2 and Erns using a phage-displayed random peptide library (Zhang et al. 2006. *Archives of Virology* 151 (1): 37-54). Those monoclonal antibodies were found to bind a common motif SPTxL (SEQ ID NO:12) that also map to WH 303 epitope (SPTTL; SEQ ID NO:13). Furthermore, multi peptide-vaccines, containing the WH 303 epitope, consisting of six overlapping peptides ranging between 20 to 25 amino acids length induced immunity against CSFV (Dong et at. 2005. *Vaccine* 23:3630-3633).

Construction of the live attenuated vaccine of the invention, a Flag/T4v mutant, is based on two previously described viral mutants: the RB-C22v mutant, which has a 19 amino acid insertion at nucleotide position 2429, near the carboxyl terminus of the E1 glycoprotein (Risatti et al. 2006, supra) and the mutant T4v, a mutant virus having a mutation in the CSFV E2 glycoprotein, i.e., in the highly specific and con-

served CSFV WH303 epitope of E2 (Risatti et al. 2005. *Virology* 343: 116-127). The Flag/T4v mutant virus vaccine was constructed by inserting the oligonucleotide encoding the highly antigenic epitope Flag, DYKDDDDK(SEQ ID NO:5), into the 19 mer insertion of the CSFV E1 polypeptide and introducing a mutation into the region covered by amino acid positions 829-834 of the WH303 epitope of E2, changing TAVSPTTLR to TSFNMDTLR. Thus, the resulting Flag/T4v mutant virus vaccine has dual advantages. Those animals vaccinated with Flag/T4v exhibit a positive specific response to the Flag epitope and at the same time, are expected to lack reactivity with viral epitopes recognized by a monoclonal antibody which specifically recognizes WH303. Thus, this viral mutant has the potential of being utilized as an attenuated marker vaccine, useful for the discrimination between Flag/T4v-immunized and Brescia-infected animals.

A vaccine is defined herein as a biological agent which is capable of providing a protective response in an animal to which the vaccine has been delivered and is incapable of causing severe disease. Administration of the vaccine results in immunity from a disease; the vaccine stimulates antibody production or cellular immunity against the pathogen causing the disease. Immunity is defined herein as the induction of a significant higher level of protection in a population of swine against mortality and clinical symptoms after vaccination compared to an unvaccinated group. In particular, the vaccine according to the invention protects a large proportion of vaccinated animals against the occurrence of clinical symptoms of the disease and mortality. The vaccine of the invention herein is a genetically engineered mutant virus vaccine. A marker vaccine is defined as a vaccine that, in conjunction with a diagnostic test, enables serological differentiation of vaccinated animals from infected animals. A mutation is understood to be a change in the genetic information of a "wild-type" or unmodified E1 or E2 gene of a parent CSFV strain which is able to express native E1 and E2 proteins. Thus, the E1 and E2 polypeptides expressed by the Flag/T4v mutant virus are changed: the E1 protein displays a Flag epitope within the altered (by insertion) E1 protein and the E2 protein lacks a wild-type immunodominant WH303 epitope.

A Flag/T4v mutant according to the present invention was generated by introducing a mutation in the gene encoding the WH303 epitope such that the ability of the immunodominant CSFV epitope to react with mAb WH303, the mAb specific for the WH303 epitope was lost. Example 5 demonstrates that the Flag/T4v (FTv) mutant according to the invention is able to protect FTv-infected animals challenged with BICv. No challenge virus was detected in nasal, tonsil, or blood samples. Detection was performed using mAb WH303, the mAb specific for the challenge virus BICv.

A further requirement of the mutation introduced in the E1 and E2 genes is that the altered E1 and E2 genes allow the recovery of infectious Flag/T4v virus from cell culture after transfection of these cells with the appropriate plasmids. Example 3 describes an experiment that is suitable for determining the ability of the infectious Flag/T4v to respond to WH303-specific and FLAG-specific antibodies and the permissibility of the mutation to generate infectious virus recoverable from cell culture transfected with the appropriate plasmids.

A vaccine against CSFV is provided that comprises a Flag/T4v mutant as defined above in a live form, and a pharmaceutically acceptable carrier or diluent. The vaccine according to the invention containing the live virus can be prepared and marketed in the form of a suspension or in a lyophilized form and additionally contains a pharmaceutically acceptable carrier or diluent customary used for such compositions. Carriers include stabilizers, preservatives and buffers. Suitable stabilizers are, for example SPGA (sucrose, phosphate, glutamate, and human albumin), carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers are for example alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

If desired, the live vaccines according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity are well known in the art. Furthermore, nucleic acid sequences encoding polypeptides for pharmaceutical or diagnostic applications, in particular immunomodulators such as lymphokines, interferons or cytokines, may be incorporated into the vaccine.

A vaccine according to the invention can be prepared by conventional methods such as those commonly used for the commercially available live attenuated CSFV vaccines. Briefly, a susceptible substrate is inoculated with the Flag/T4v mutant and propagated until the virus has replicated to a desired titer after which Flag/T4v-containing material is harvested. Subsequently, the harvested material is formulated into a pharmaceutical preparation with immunizing properties.

Every substrate which is able to support the replication of Flag/T4v viruses can be used in the present invention, including Swine kidney cells (SK6) and primary cultures of swine peripheral blood macrophages.

The vaccine may be administered by intramuscular, intradermal, subcutaneous or intranasal inoculation or injection in an amount which is effective to protect the animal against challenge by a virulent strain of CSFV. This amount may vary according to the animal being inoculated, taking into consideration the size and weight of the animal. The vaccine according to the invention comprises an effective dosage of the Flag/T4v mutant as the active component, i.e. an amount of immunizing Flag/T4v material that will induce immunity in the vaccinated animals, swine, against challenge by a virulent CSFV. Immunity is defined herein as the induction of a significant higher level of protection in a population of swine against mortality and clinical symptoms after vaccination compared to an unvaccinated group. In particular, the vaccine according to the invention prevents a large proportion of vaccinated animals against the occurrence of clinical symptoms of the disease and mortality. Typically, the live vaccine can be administered in a dose of 10^4-10^5 $TCID_{50}$. Effective amounts may be experimentally determined as necessary by those of skill in the art by following the guidance provided by specific Example 5.

In addition to the Flag/T4v mutant, the invention can also include combination vaccines comprising a vaccine strain capable of inducing protection against another porcine pathogen.

The Flag/T4v marker vaccine described above, in conjunction with a diagnostic method, has the potential of distinguishing between animals that are vaccinated with it and animals that are infected with naturally occurring CSFV strains or vaccinated with conventional CSFV vaccines.

The present invention also provides an invaluable tool to monitor CSFV control measures that may lead to eradication of CSFV if applied in large scale stamping out programs. This tool concerns a method for determining CSFV infection in swine comprising the step of examining a sample of the animal for the presence or absence of antibodies reactive with the immunodominant epitopes FLAG and WH303. The

sample of the animal used in this method may be any sample in which CSFV or Flag/T4v antibodies can be detected, e.g. a blood, serum or tissue sample.

The design of the immunoassay may vary. For example, the immunoassay may be based upon competition or direct reaction. Furthermore, protocols may use solid supports or may use cellular material. The detection of the antibody-antigen complex may involve the use of labeled antibodies; the labels may be, for example, enzymes, fluorescent, chemiluminescent, radioactive or dye molecules.

Suitable methods for the detection of WH303 and FLAG in the sample include, for example, the enzyme-linked immunosorbent assay (ELISA), immunofluorescent tests and Western blot analysis.

Porcine anti-CSFV sera raised against the Flag/T4v mutant according to the present invention has the potential of being distinguishable from porcine sera raised against naturally occurring CSFV strains and conventional CSFV vaccine strains. Thus, the Flag/T4v mutant has the potential of being a marker vaccine.

Thus, the particular CSFV mutant, Flag/T4v, according to the present invention has the potential of producing antiserum in pigs wherein the antisera are lacking in antibodies that react with an immunodominant epitope, WH303. Such antiserum would score negative in a direct- or blocking WH303 enzyme-linked immunosorbant assay (ELISA).

In an ELISA to detect porcine anti-FLAG and anti-WH303 epitope porcine antibodies, microtitration plates are coated with FLAG-bearing E1 protein (or a FLAG antigen) or wild type E2 protein bearing the WH303 epitope (or an E2 fragment bearing the WH303 epitope). Next, the wells of the coated plates are filled with porcine serum and serial dilutions are made. After incubation, porcine anti-FLAG or anti-WH303 epitope protein serum antibodies are determined by detecting antibody (monoclonal or polyclonal) with the same specificity as the coated one, but which is labeled (e.g. with biotin). The labeled antibody will occupy the free antigens that have not been occupied by anti-WH303 epitope or anti-FLAG antibodies in the porcine serum. For example, horse radish peroxidase coupled to avidin may be added and the amount of peroxidase is measured by an enzymatic reaction. If no antibodies against FLAG-marked E1 or the E2 WH303 epitope are present in the porcine serum sample then a maximum absorption is obtained. If the serum contains many antibodies against the WH303 epitope then a low absorption is expected. Alternatively, after the incubation with porcine serum, the amount of antibodies present in the serum that bound to the WH303 epitope antigen may be determined directly by using an anti-porcine conjugate followed by the enzymatic reaction.

In a sandwich ELISA the wells of a polystyrene microtitration plate can be coated with a monoclonal antibody directed against the WH303 epitope protein, i.e., mAb WH303. Next, the wells of these coated plates are incubated with antigen. After the antigen is captured, the wells are filled with the porcine serum and serial dilutions are made. Subsequently, the protocol as described above may be followed.

In another diagnostic test (Western blot analysis), the FLAG-marked E1 polypeptide or FLAG fragment or the WH303 epitope-containing material is subjected to SDS-PAGE. Next, the separated proteins are electroblotted onto nitro-cellulose membrane. Thereafter, the membranes can be cut into lanes and the lanes are incubated with the porcine serum. The presence in the sample of antibodies specific for the WH303 epitope or antibodies specific for FLAG can be determined by examining whether antibodies bound to the WH303 epitope or the FLAG antigen, for example by using an anti-porcine conjugate followed by an enzymatic reaction. If antibodies against the WH303 epitope or FLAG are present then a band of the appropriate size is identifiable.

The WH303 epitope-containing polypeptide may be any WH303 epitope-containing protein or fragment comprising material which allows the formation of the WH303 epitope antigen-WH303 mAb complex. Similarly, the FLAG-containing polypeptide may be any FLAG-containing protein or fragment comprising material which allows the formation of the FLAG (antigen)-anti-FLAG (antibody) complex. Preferably, the WH303 epitope antigen and the FLAG antigen comprise the expression product of a conventional recombinant host cell or virus, e.g. such as *E. coli* expressed or baculovirus expressed protein. In a further embodiment of the present invention, a diagnostic test kit is provided which is suitable for performing the diagnostic test according to the invention as described above.

In particular, a diagnostic test kit is provided which comprises in addition to the components usually present, the WH303 epitope antigen or FLAG antigen (if desired, coated onto a solid phase) as the immunological reagent. Other components usually present in such a test kit include, biotin or horseradish peroxidase conjugated antibodies, enzyme substrate, washing buffer etc.

To determine CSFV (BICv) WH303 antigen or FLAG antigen in a test sample from an animal in the field, WH303 mAb and anti-FLAG mAb are used as the immunological reagent, preferably fixed to a solid phase. The test sample is added, and after an incubation time allowing formation of the antibody-antigen complex, a second labeled antibody may be added to detect the complex.

Typically, the absorbance (OD) cut-off value for the ELISA to differentiate positive from negative samples is set at three standard deviations above the average P/N ratios of negative control samples from pigs (where P=the OD of samples from wells coated with a relevant peptide coupled to a carrier molecule and; N=the OD of samples from wells coated with the carrier molecule). A carrier molecule can be a carrier protein, such as BSA, ovalbumin, KLH, a carbohydrate chain or a synthetic amino acid chain.

In an alternative embodiment of the diagnostic method the presence of specific antibodies in porcine serum is examined by incubating the serum and an appropriate antigen in the presence of a monoclonal antibody that specifically reacts with an epitope located within the E2 region.

The recombinant FLAG/T4v mutant according to the invention is obtained after transfection of suitable cells (e.g., SK6 cells) with the synthetic RNA transcript of the FLAG/T4v mutant genome by electroporation. Finally the recombinant FLAG/T4v mutant is harvested from the supernatant of the transformed cells.

It has also been found that FLAG/T4v mutant according to the present invention is able to induce a protective immune response, i.e. animals immunized with a vaccine comprising the FLAG/T4v mutant are protected against virulent challenge. Moreover, it has been found that antisera of animals infected with naturally occurring CSFV comprise antibodies directed to the WH303 epitope of BICv. Antisera from animals infected with the FLAG/T4v mutant virus according to the present invention can be tested according to the methods described above to determine their reactivity with the Flag and WH303 epitope. In addition, it has been found that the FLAG/T4v mutant virus as described above is attenuated if compared with the parent BICv virus which is able to produce the native E1 and E2 proteins.

EXAMPLES

Having now generally described this invention, the same will be better understood by reference to certain specific

examples, which are included herein only to further illustrate the invention and are not intended to limit the scope of the invention as defined by the claims.

Example 1

Viruses and Cell Cultures

Swine kidney cells (SK6) (Terpstra et al. 1990. *Dtsch. Tierarztl. Wochenschr*. 97: 77-79), free of BVDV, were cultured in Dulbecco' minimal essential medium (DMEM) (Gibco, Grand Island, N.Y.) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, Colo.). CSFV Brescia strain was propagated in SK6 cells and used for the construction of an infectious cDNA clone (Risatti et al. 2005. *J. Virol*. 79: 3787-3796). Titration of CSFV from clinical samples was performed using SK6 cells in 96 well plates (Costar, Cambridge, Mass.). Viral infectivity was detected, after 4 days in culture, by immunoperoxidase assay using the CSFV monoclonal antibodies 174 (kindly provided by Georgina Ibata, Veterinary Laboratory Agency, UK) and the Vectastain ABC kit (Vector Laboratories, Buringames, Calif.). Titers were calculated using the method of Reed and Muench (1938. *Amer. J. Hygiene* 27: 493-497) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was >1.8 $TCID_{50}$/ml. Plaque assays were performed using SK6 cells in 6 well plates (Costar). SK6 monolayers were infected, overlaid with 0.5% agarose and incubated at 37° C. for 3 days. Plates were fixed with 50% (vol/vol) ethanol-acetone and stained by immunohistochemistry with mAbs 174, WH303, WH308 (Edwards et al. 1991. *Vet Microbiol*. 29:101-108) or anti-Flag (Sigma).

Example 2

Construction of CSFV Flag-T4 Virus

A full-length CDNA copy of RB-C22v (Risatti et al. 2006., supra) was NotI-NheI digested and the excised 46 nucleotide fragment replaced with an oligonucleotide, forward sequence 5'GGCCGCAGATTACAAGGATGACGAC-GATAAGGGGGCACAAGGCCGG 3' (SEQ ID NO:6) and reverse complementary sequence 5'CTAGCCGGCCTTGT-GCCCCCTTATCGTCGTCATCCTTGTAATCTGC 3' (SEQ ID NO:7), encoding for the flag epitope DYKDDDDK (bold case). One hundred pmoles of the forward and reverse oligonucleotides were annealed in buffer containing 10 mM Tris, pH 7.5-8.0, 50 mM NaCl, 1 mM EDTA. Oligos were heated at 95° C. for 5 min and cooled at room temperature for 30 min. Annealed oligonucleotides were directionally cloned into NotI-NheI-digested pRB-C22 using T4 DNA ligase (New England Biolabs, Beverley, Mass.). The introduction of mutations in amino acids at positions 829-834 of the CSFV polyprotein resulting in a shift of the Brescia amino acid sequence (TAVSPT) to the corresponding residues in bovine viral diarrhea virus (BVDV) strain NADL (TSFNMD) were performed on the pFlag-T4 exactly as described by Risatti et al. (2005. *Virology* 343: 116-127) for the development of the T4 virus.

Infectious RNA was in vitro transcribed from full-length infectious clones of the Flag-T4 virus (FTv) (FIGS. **1** and **2**) and used to transfect SK6 cells. Viruses were rescued from transfected cells by day 4 post-transfection. Nucleotide sequences of the rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at predicted glycosylation sites were reflected in rescued viruses.

Example 3

In Vitro Rescue of CSFV Brescia and Glycosylation Mutants

Full-length genomic infectious clones were linearized with SrfI and in vitro transcribed using the T7 Megascript system (Ambion, Austin, Tex.). RNA products were precipitated with LiCl and transfected into SK6 cells by electroporation at 500 volts, 720 ohms, 100 watts with a BTX 630 electroporator (BTX, San Diego, Calif.). Cells were plated in 12 well plates and incubated for 4 days at 37° C. and 5% CO_2 atmosphere. Virus was detected by immunoperoxidase staining as described above and stocks of rescued viruses were stored at −70° C.

In vitro growth characteristics of FTv were evaluated relative to parental BICv in a single step growth curve. SK6 cell cultures were infected at a MOI of one $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time zero), and samples were collected at 48 h post-infection (PI). Results demonstrated that FTv exhibited growth characteristics practically indistinguishable from BICv reaching titers of 6.10 (SD: 0.45) $TCID_{50/ml}$ versus 6.04 (SD:07) of BICv.

Additionally, FTv was tested for their plaque size in SK6 cells, exhibiting a noticeable reduction in plaque size relative to BICv (FIG. **2**). This small plaque phenotype is expected since both parental viruses, RB-C22 and T4v, possess small plaque compared with their parental virus BIC (Risatti et al. 2005, 2006, supra).

Reactivity of Flag-T4 virus against mAbs anti-Flag, WH303 and WH308 was performed in order to analyze its antigenic phenotype. Flag-T4 virus strongly reacts with mAbs anti-Flag and WH308 and completely fails to react with mAb WH303. The WH308 epitope is located between residues 882 and 1064 of the CSFV E2 polypeptide, a region of E2 different from the epitope identified by WH303 (Risafti et al. *Virology* (in press). Conversely, BICv reacts with mAbs WH303 and WH308 and fails to react with mAb anti-Flag (FIG. **2**).

Example 4

DNA Sequencing and Analysis

Full-length infectious clones and in vitro rescued viruses were completely sequenced with CSFV specific primers by the dideoxynucleotide chain-termination method (Sanger et al. 1977. *Proc. Natl. Acad. Sci*. USA 74: 5463-5467). Viruses recovered from infected animals were sequenced in the mutated area. Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA Sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (www.phrap.org), with confirmatory assemblies performed using CAP3 (Huang and Madan. 1999. *Genome Res*. 9: 868-877). The final DNA consensus sequence represented, on average, five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (Retrieved from the Internet: <URL: mbio.ncsu.edu/BioEdit/bioedit.html)

Example 5

Animal Infections

The ability of Flag-T4 virus to induce protection against virulent BICv was assessed in early and late vaccination-

exposure experiments. For protection studies, 12 pigs were randomly allocated into 3 groups of 4 animals each. Pigs in Groups 1 and 2 were inoculated intranasally with 10^5 $TCID_{50}$ Flag-T4, and pigs in Group 3 were mock infected. At 3 DPI (Group 1) or 28 DPI (Group 2), animals were challenged with 10^5 $TCID_{50}$ BICv along with animals in Group 3. Clinical signs and body temperature were recorded daily-throughout the experiment as described above. Blood, serum, nasal swabs and tonsil scrapings were collected at times post-challenge, with blood obtained from the anterior vena cava in EDTA containing tubes (Vacutainer) for total and differential white blood cell counts. Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman, Coulter, Calif.).

Mock-vaccinated control pigs receiving BICv only (Group 3) developed anorexia, depression, and fever by 4 days post-challenge (Table 1), and a marked reduction of circulating leukocytes and platelets by 4 DPC (data not shown), and died or were euthanized in extremis by 9-10 DPC. Notably, FTv induced complete protection by 3 DPI. All pigs survived infection and remained clinically normal, without significant. changes in their hematological profiles after challenge (data not shown).

TABLE 1

Survival and fever response following challenge with BICv post FT4v infection.

Challenge Group	No. of Survivors/ Total No.	Mean time to Death (Days ± SD)	Fever Days to Onset (Days ± SD)	Fever Duration (Days ± SD)
3 DPI	4/4	—	—	—
28 DPI	4/4	—	—	—
Mock Vaccinated	0/4	12 (0)	4.5 (0.7)	9.5 (0.7)

Viremia and virus shedding of vaccinated-exposed animals were examined at 3, 4, 5, 8, 12, 14, and 21 DPC (Table 2). Detection was performed using mAb WH303, the mAb specific for the challenge virus BICv. As expected, in mock-vaccinated control animals, viremia was observed by 4DPC, with virus titers remaining high by 8 DPC ($10^{7.05}$ $TCID_{50}$/ml, SD: 0.11). Furthermore, virus was detected in nasal swabs and tonsil scrapings of mock-vaccinated animals after 4 DPC. Conversely, in FTv-infected animals, challenged either at 3 or 28 DPI, no challenge virus was detected in nasal, tonsil, or blood samples during the 21 day observation period (Table 2). These results indicated that protection induced by FTv was solid, preventing both the presentation of CSF related clinical signs and the replication of the challenge virus.

TABLE 2

Detection of virus[a] in nasal swabs, tonsil scrapings, and blood samples after challenge of N1v-vaccinated animals with virulent BICv.

Challenge Group[e]	Sample	Days Post-Challenge 3	4	6	8	12	14	21
3 DPI	Nasal	nd[b]	nd	nd	nd	nd	nd	nd
	Tonsil	nd	nd	nd	nd	nd	nd	nd
	Blood	nd	nd	nd	nd	nd	nd	nd
28 DPI	Nasal	nd	nd	nd	nd	nd	nd	nd
	Tonsil	nd	nd	nd	nd	nd	nd	nd
	Blood	nd	nd	nd	nd	nd	nd	nd

TABLE 2-continued

Detection of virus[a] in nasal swabs, tonsil scrapings, and blood samples after challenge of N1v-vaccinated animals with virulent BICv.

Challenge Group[e]	Sample	Days Post-Challenge 3	4	6	8	12	14	21
Mock Vaccinated	Nasal	nd	1.9 (0)[c]	3.5 (0)	4.3 (1)	D[d]	D	D
	Tonsil	nd	2.8 (0.4)	2.9 (0.7)	4.5 (0.1)	D	D	D
	Blood	nd	3.9 (0.4)	6.7 (0.6)	7.0 (0.1)	D	D	D

[a]Virus detection was performed using mAb WH303.
[b]n.d. (not detectable): virus titers equal or less than 1.8 $TCID_{50}$ (log 10).
[c]number in parentheses indicate Standard Deviation for 4 animals.
[d]D: Dead animals.
[e]n = 4 animals/group

CSFV, when intranasally inoculated, replicate primarily in tonsils, and from there the infection expands to secondary target lymph organs. Therefore, virus replication in tonsils is a critical step in the animal infection after the intranasal inoculation of CSFV. In order to gain insight into the kinetics of viral replication at early times after infection, the presence of FTv and BICv in the main target organs was analyzed at different times after FTv infection or after the challenge with BICv. A group of animals (n=6) were intranasally infected with 10^5 $TCID_{50}$ of either Flag-T4 or BIC viruses, or with FTv and 3 days later challenged with BICv. Two animals per group were euthanized at 3, 5, and 7 DPI in groups infected with FTv or BICv and at 1, 3, 5, and 7 days post-challenge in animals infected with FTv and challenged with BICv. Tonsils, mandibular, MLN, RPLN, and spleen were immediately removed after necropsy. The presence of each virus in all collected organs was examined by virus titration on SK6 cells. As expected, the highest titers are shown in tonsils at 3 OPI with increasing levels at 5 and 7 DPI (Table 3). In contrast, animals infected with FTv presented virus titers (detected by mAb anti-Flag) in tonsils at 3 DPI with a slight increase by day 5 and 7 PI, although titers were 100 to 10000 times lower when compared with tonsils in BICv-infected animals (Table 3). RPLN and MNL in FTv-infected animals showed virus titers only at 3 and 5 DPI with levels 10-100 fold lower than in BICv-infected animals (Table 3). These results indicate that replication of FTV in intranasally infected animals remains restricted to the tonsils and its draining lymph nodes. Remarkably, in FTv-infected/BICv-challenged animals, all organs presented negative BICv titers at all time points tested (1, 3, 5, and 7 DPC) (Table 3). These results support the observation that after challenge, BICv does not replicate in FTv-infected animals.

TABLE 3

Titers of virus in organs of animals infected with Flag-T4 virus, BICv or infected with Flag-T4 virus and challenge with BICv.

Virus	DPI or DPC	Log 10 $TCID_{50}$/g in: Tonsil	RFLN	MLN	Spleen
Flag-T4[e]	3[a]	2.1 (0.2)[g]	nd[d]	1.9 (0)	nd
	5	4.8 (0)	2.6 (0.6)	2.1 (0.2)	nd
	7	4.1 (1.6)	nd	nd	nd
BIC[f]	3[a]	4.4 (1)	2.6 (0.4)	2.88 (0.3)	2.1 (0.1)
	5	6.3 (0)	4.3 (0)	4.6 (0.7)	4.5 (0)
	7	6.9 (0.8)	6.3 (0.2)	6.9 (1.1)	6.2 (1)
Flag-T4/ BICv[g,f]	1[b]	nd	nd	nd	nd
	3	nd	nd	nd	nd

TABLE 3-continued

Titers of virus in organs of animals infected with Flag-T4 virus, BICv or infected with Flag-T4 virus and challenge with BICv.

Virus	DPI or DPC	Log 10 $TCID_{50}$/g in: Tonsil	RFLN	MLN	Spleen
	5	nd	nd	nd	nd
	7	nd	nd	nd	nd

[a]DPI: days post-infection.
[b]DPC: days post-challenge with BICv.
[c]Animals were infected with Flag-T4 and 3 days later challenged with BICv.
[d]not detectable: virus titers equal or less than 1.8 $TCID_{50}$ (log 10).
[e]Virus titers detected with mAb anti-Flag.
[f]Virus titers detected with mAb WH303.
[g]Number in Parentheses indicate Standard Deviation for 2 animals.

Intramuscular (IM) administration of Flag-T4 virus induces protection at second day post infection. The early induction of protection after vaccination is of paramount importance during a disease outbreak. Therefore, the ability of FTv to induce protection earlier then 3 DPI was assessed. Four groups of animals (n=2) were IM infected with 10^5 $TCID_{50}$ of FTv and challenged intranasally with 10^5 $TCID_{50}$ of BICv at 24, 48, or 72 h later. Interestingly, animals challenged at 24 and 48 h post FTv infection remained clinically normal during the 21 day experimental period. One of the animals challenged at 24 h after FTv virus infection presented CSFV clinical signs being euthanized at 15 DPC while the other remained clinically normal.

Thus, an experimental live attenuated vaccine strain has been rationally developed. The strain contains positive and negative antigenic markers which confer solid protection at 3 DPI, when delivered intranasally and at 2 DPI when administered by the intramuscular route.

Example 6

Immunofluorescence and Confocal Microscopy

To confirm the results cited above in Example 5, the presence of FTv and BICv in tonsil tissues were evaluated by immunofluorescence using mAbs anti-Flag or WH303. Triplicate samples were collected postmortem of selected tissues (palatine tonsil, mandibular lymph node (MLN), retropharyngeal LN (RPLN), and spleen). The tissues were mounted n blocks using Optimal cutting temperature ( ) OCT) compound (TissueTek), promptly frozen in liquid nitrogen and stored at −70° C. Four μm thick sections of cryopreserved tissues from all three samples of all animals were sectioned with a cryomicrotome and fixed with acetone for 10 min at −20° C. After fixation, the sections were incubated in blocking buffer (PBS, 2% bovine serum albumin, 20% normal bovine serum) for 1.5 hours at room temperature (RT). Primary mAbs (mAb anti-Flag M2 and WH303) were diluted in blocking buffer and incubated with the sections overnight at 4° C. in a humid chamber. After washing five times with PBS, the sections were incubated with the appropriate secondary antibody, goat anti-mouse isotype-specific IgG (AlexaFluor 488 or AlexaFluor 594, Molecular Probes) diluted in blocking buffer, for 1.5 hours at 37° C. following this incubation, the sections were washed 5 times with PBS, counterstained with TOPRO-iodide 642/661 (Molecular Probes) for 5 min at RT, washed as before, mounted, and examined in a Leica scanning confocal microscope. Data were collected utilizing an appropriate control lacking the primary antibodies in order to determine channel crossover settings, as well as using these antibodies in uninfected tissues to give the negative background level and to determine channel cross-over settings. The captured images were adjusted-for contrast and brightness using Adobe Photoshop software.

Results completely corroborate the results obtained by virus titration (Table 4). Tonsils from animals infected with FTv showed a weak but clear fluorescence signal with mAb anti-Flag on days 3, 5, and 7 PI. Moreover, tonsils of BICv-infected animals showed strong reactivity with mAb WH303 at all days tested. Although mAb anti-Flag reactivities, comparable to that of FTv-infected animals, were detected in tonsils of FTv-infected/BICv challenged animals, no signal with mAb WH303 was detected in tonsils at any of the times tested (1, 3, 5, and 7 DPC).

TABLE 4

Detection by immunofluorescence of Flag-T4 and BIC viruses in swine tonsils from animals[f] infected with Flag-T4 virus, BICv, or Flag-T4 virus/BICv challenge.

Virus	DPI or DPC	Monoclonal Antibody: Anti-Flag	WH303
Flag-T4	3[a]	+\+[e]	neg[g]\neg
	5	++++\+++	neg\neg
	7	++\ND[d]	neg\neg
BIC	3[a]	neg\neg	++++\++++
	5	neg\neg	++++\++++
	7	neg\neg	+++++\+++++
Flag-T4/BICv	1[b]	+++\+	neg\neg
	3	+++\++++	neg\neg
	5	+++\ND	neg\ND
	7	+++\++	neg\neg

[a]DPI: days post-infection.
[b]DPc: days post-challenge with BICv.
[c]Animals were infected with Flag-T4 and 3 days later challenged with BICv.
[d]ND: not determined.
[e]Reactivity in fluorescence was quantified from (+) to (+++++)
[f]Two animals were processed per group per time point..
[g]neg: staining indistinguishable from background.

All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

The forgoing description and certain representative embodiments and details of the invention have been presented for purposes of illustration and description of the invention. It is not intended to be exhaustive or to limit the invention to the precise forms disclosed. It will be apparent to practitioners skilled in this art that modifications and variations may be made therein without departing from the scope of the invention.

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acactaattt tttatttatt tagatattac tatttattta tttatttatt tattgaatga  12240

gtaagaactg gtacaaacta cctcatgtta ccacactaca ctcattttaa cagcacttta  12300

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We claim:

1. A recombinant classical swine virus (CSFV) mutant comprising a DNA sequence encoding a mutation in the E1 glycoprotein of said CSFV mutant, wherein the mutation comprises a 20 mer insertion (SEQ ID NO: 9) at nucleotide position 2429, wherein said 20 mer insertion comprises the FLAG™ epitope in the E1 glycoprotein of the CSFV mutant, said epitope consisting of the amino acid sequence, DYDDDDK (SEQ ID NO: 5) as the last eight amino acids of the insertion, wherein said recombinant CSFV mutant is a live attenuated CSFV and said FLAG™ epitope carried by said CSVF mutant is capable of being recognized by monoclonal antibodies specifically binding to said FLAG™ epitope, which serve as a positive mutation marker for said live attenuated CSFV mutant.

2. The recombinant live attenuated CSFV mutant of claim **1** further comprising a mutation in CSFV E2 glycoprotein in addition to said positive mutation marker in CSFV E1 glycoprotein, wherein the mutation of CSFV E2 glycoprotein comprises a substitution mutation in the wild-type WH303 immunodominant epitope, TAVSPTTLR set forth in SEQ ID NO: 2, at amino acids positions 829-837 with the epitope, TSFNMDTLR set forth in SEQ ID NO:4 as a negative marker for the mutation, thus resulting in an elimination of the wild-type WH303 immunodominant epitope and wherein said recombinant CSFV mutant is a live attenuated CSFV having both the positive mutation marker as a result of the mutation in CSFV E1 glycoprotein of claim **1** and the negative marker of the mutation as a result of the mutation in the wild-type WH303 immunodominant epitope in CSFV E2 glycoprotein.

3. The recombinant CSFV mutant of claim **2** comprising DNA having the sequence identified by SEQ ID NO: 1.

4. The recombinant CSFV mutant according to claim **1** wherein the CSFV mutant comprises additional attenuating mutations.

5. A vaccine composition comprising the recombinant CSFV mutant according to any one of claims **1**, **2**, **3** and **4**.

6. An isolated cell infected with the CSFV mutant of any one of claims **1**, **2**, **3** and **4**.

7. A method for the protection of swine against CSF, comprising administering to swine a live attenuated CSF vaccine comprising a recombinant CSFV mutant according to claim **1** in an amount effective to protect said swine from clinical CSF.

8. A method of distinguishing animals infected with CSFV from animals vaccinated with a live attenuated CSF vaccine comprising a recombinant CSFV mutant according to claim **1**, comprising: analyzing serum from an animal under evaluation to determine if said serum binds specifically to the FLAG™ epitope.

9. A method for the protection of swine against CSF, comprising administering to swine a live attenuated CSF vaccine comprising a recombinant CSFV mutant according to any one of claims **2** and **3** in an amount effective to protect said swine from clinical CSF.

10. A method of distinguishing animals infected with CSFV from animals vaccinated with a live attenuated CSF vaccine comprising a recombinant CSFV mutant according to any one of claims **1**, **2** and **3**, comprising analyzing serum from an animal under evaluation to determine if the positive marker, the FLAG™ epitope, is present and if the negative marker, the WH303 epitope immunodominant, is absent.

* * * * *

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