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Plum Island Animal Disease Center

Foreign Animal Disease Research Unit

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Foreword

It is my pleasure and honor to introduce the 2011 collection of published scientific works by the scientists and collaborators of the Foreign Animal Disease Research Unit (FADRU) at the Plum Island Animal Disease Center (PIADC). Foreign or, more appropriately called, Transboundary Animal Diseases remain one of the largest burdens to animal health, production and trade worldwide. The FADRU scientists had one of their most productive years, with continuing international research collaborations in multiple countries such as Denmark, South Africa, Pakistan, Korea, Vietnam, Spain, India, Argentina, the Republic of Georgia, Russia and expanding new research collaborations in Uganda and Cameroon this year. Also this past year the FADRU continued working on foot-and-mouth disease (FMD), classical swine fever (CSF) and vesicular stomatitis (VS) but expanded its research mission to include again African Swine Fever (ASF). This disease has been expanding its range since its introduction to the Republic of Georgia in 2007. It has traveled into neighboring countries such as Armenia, Azerbajan and is expanding its range in Russia putting countries like Finland on high alert.

During 2011 FADRU scientists made great progress toward understanding FMDV and CSFV functional genomics, pathogenesis and immunology, identifying a number of critical interactions between host-cell and viral proteins and understanding the role of these interactions on the mechanisms of viral pathogenesis. They also made great progress in understanding the immune responses against FMD, including the T-cell responses that had been previously poorly characterized. With new tools developed by PIADC scientists and collaborators, we can now measure specific cellular responses to infection.

There were also great advances made in understanding the innate responses including the discovery of novel interferons in cattle and swine that proved to be effective against FMD virus infection. Also, progress was made in understanding the role of insect transmission on the pathogenesis of VS virus in cattle. Through collaborations with scientists in the US and overseas specializing in epidemiology and disease modeling, FADRU scientists also made contributions to the understanding of the effect of vaccination in prevention and control of FMD under field conditions.

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The work highlighted below and detailed in the enclosed scientific papers and patents would not have been possible without the contributions made by the FADRU collaborators from the US and overseas and also from the visiting scientists and research fellows that made their contributions to the science and put the hard work and long hours to get the job done, many times in less than ideal conditions due to weather conditions and despite the heavy regulatory constraints imposed on our scientists working in the BSL3 laboratory with select agents. I also must acknowledge the support and contributions to the scientific program by the other government agencies. The Department of Homeland Security provided not only all of the local logistics and operational support at PIADC, but also provided the largest extramural support to the FADRU research program. Other agencies such as the National Science Foundation, the Environmental Protection Agency, and the Department of Energy (ORISE program) also made important contributions to our research program. Finally, the contributions made by the ARS administrative team in maintaining the high level of productivity and efficacy of our program must be recognized.

Please enjoy the following collection of some of the best scientific work in animal health published in 2011.

2011 Research Highlights

- **Induction of Foot-and-Mouth disease Virus Specific Cytotoxic T Cell Killing by Vaccination.** One of the primary goals of vaccine development for foot-and-mouth disease (FMD) is cross protection for the many serotypes and subtypes of the virus. Many investigators have assumed that targeting the cellular immune response mediated by T cells would be the best chance to achieve cross protection. Anti-viral immunity mediated by T cells involves the induction of cytotoxic T lymphocytes (CTLs) which can lyse virus infected cells in vivo thereby stopping the infection. No one has ever demonstrated antigen specific, CTL killing of virus infected cells in FMD. Here we describe a vaccine strategy that targets the CTL response to FMD virus infection. We clearly demonstrate the killing of infected cells by CTLs from vaccinated animals and show antigen specificity of that response. Further, we confirm the CTL induction by this vaccine using the cutting edge technology of staining with major histocompatibility complex tetramers for the first time in swine. The information is valuable for advancing the technology to control FMD.

- **Characterization of the Temporal and Spatial Distribution and Reproductive Ration of Vesicular Stomatitis Outbreaks in Mexico in 2008.** Vesicular Stomatitis (VS) is a disease of livestock caused by vesicular stomatitis virus (VSV). The disease is characterized by the appearance of vesicles and ulceration of tongue, mouth, feet, and teats. The disease affects horses, cattle, and pigs, lesions in the later two species resemble those caused by foot-and-mouth disease a devastating disease of livestock. Occasionally humans and some wildlife species may become infected. The United States is regularly affected by VSV incursions and genetic evidence suggests that viruses associated with such incursions likely originate from enzootic areas of Mexico. The study here was aimed at characterizing the temporal and spatial distribution and reproductive ratio of VS outbreaks reported in Mexico in 2008. Such knowledge will help to prevent and control the occurrence of VS epidemics in both Mexico and the United States and will help to improve the efficiency of the national surveillance systems for the disease.

- **Foot-and-Mouth Disease Virus Utilizes an Autophagic Pathway during Viral Replication.** Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed livestock caused by foot-and-mouth disease virus (FMDV), which results in large economic losses to countries where it occurs. It has been shown that after initiation of picornavirus infection, a number of changes take place in the cell, which includes the rearrangement of intracellular membranes into vesicular structures where viral genome replication takes place. Characterization of these double membrane vesicular structures suggests an autophagic origin. Autophagy is a cell regulated pathway designed to degrade and recycle long-lived proteins and cellular components, which are important in organelle turnover and management of starvation. Recent studies have shown that a number of positive-strand RNA viruses, including picornaviruses, induce the formation of autophagic membranes. In this study, we determine the role of autophagy in FMDV replication by using confocal microsopy and monitoring the co-localization of specific autophagosome and viral proteins in infected cells. To extend our studies, we explore the effect of biochemical compounds that inhibit or induce autophagy, and RNA interference, on FMDV replication. Our results suggest that, although autophagy has both antiviral and antibacterial functions, it appears to play an important role in the replication of FMDV. Understanding better the mechanisms that are involved during viral replication, let us examine the role of drugs which inhibit these processes as possible anti-viral agents.

- **Identification of an NTPase Motif in Classical Swine Fever Virus NS4B Protein** Classical swine fever (CSF) is a highly contagious and often fatal disease of swine caused by CSF virus (CSFV). The virus in composed, besides its nucleic acids, by structural proteins (SP), which are part of the virus particles itself. Besides the SP, the genome of the virus encodes for the non structural proteins (NSP) which are mainly involved in the process of virus replication. There are ten NSP and the functions of most of them are unclear. In this paper we identify, by genetic analysis first, that one of the NSP, the NS4B, can work facilitating the synthesis of virus nucleic acid during the replication of the virus in the cell. We present data demonstrating experimentally that the isolated NS4B protein plays its role in vitro. We also determined the amino acid residues in the protein that mediate the function. These results have important implications for developing novel antiviral strategies against CSFV that may affect the efficacy of virus replication.

- **Development of Vaccines toward the Global Control and Eradication of Foot-and-Mouth Disease.** Foot and mouth disease (FMD) is one of the most economically and socially devastating diseases affecting animal agriculture throughout the world. Although mortality is low, millions of animals have been killed in efforts to rapidly control and eradicate FMD. The causing virus (FMDV) is a highly variable RNA virus occurring in seven serotypes (A, O, C, Asia 1, Sat 1, Sat 2 and Sat 3) and a large number of subtypes. FMDV is one of the most infectious agents known, affecting cloven-hoofed animals with significant variations in infectivity and virus transmission. Although inactivated FMD vaccines have been available for decades, there is little or no cross-protection across serotypes and subtypes, requiring vaccines that are matched to circulating field strains. Current inactivated vaccines require growth of virulent virus posing a threat of escape from manufacturing plants, have limited shelf life and induce short lived immunity. These vaccines have aided in eradication of FMD from Europe and control of clinical disease in many parts of the world, albeit at a very high cost. However, FMDV persists in endemic regions impacting millions of people dependent on livestock for food and their livelihood. Usually associated with developing countries that lack the resources to control it, FMD is a global problem and the World Animal Health Organization (OIE) and the United Nations' Food Agriculture Organization (FAO) have called for its global control and eradication. One of the main limitations to FMDV eradication is the lack of vaccines designed for this purpose, vaccines that not only protect against clinical signs but that can actually prevent infection and effectively interrupt the natural transmission cycle. These vaccines should be safely and inexpensively produced, easy to deliver, and capable of inducing lifelong immunity against multiple serotypes and subtypes. Also there is need for better integrated strategies that fit the specific needs of endemic regions. Only when these critical components are available will the global eradication of FMDV be possible.

- **Interaction between Core Protein of Classical Swine Fever Virus with Cellular IQGAP1 Protein Appears Essential for Virulence in Swine.** Core is one of the four structural proteins of Classical Swine Fever Virus (CSFV) a virus causing a severe disease in swine. Knowledge of Core function is very limited. Recently, we report the identification of two swine proteins that interact with CSFV Core during the virus infection. We showed that that interaction was critical in the process of the disease produced by the virus in pigs. In this new report we extended those studies identifying a new swine protein, named IQGAP, that specifically interact with the virus Core protein. The sites of the Core protein which actually interact with this host protein were identified. CSFV harboring alteration of regions in Core that interact with IQGAP resulted completely attenuated in swine. Therefore CSFV Core protein specifically interacts with at least three swine host protein during the cycle of virus replication and this interaction appears to be critical in the mechanism of virus virulence. These results will be useful in vaccine development against this important disease.

- **Custom-engineered Chimeric Foot-and-Mouth Disease Vaccine Elicits Protective Immune Responses in Pigs.** Foot-and-mouth disease (FMD) is a devastating disease of cloven hooved animals caused by any of seven serotypes (A, O, C, Asia, SAT1, SAT2 and SAT3) and multiple subtypes of FMD virus. Current vaccines do not induce protective immune responses across serotypes or, in many cases, subtypes. In this report, the feasibility of an FMDV vaccine containing parts of the SAT1/SAT2 serotypes (chimeric vaccine) was assessed by determining the immunogenicity and protective ability following immunization of pigs. We demonstrated that the chimeric vaccine exhibited similar tissue culture growth characteristics, virion stability and antigenic profiles to the SAT1 parental virus. Pigs vaccinated with the chimeric vaccine showed good immune responses and the majority of the animals were protected against homologous (SAT1) FMDV challenge. Furthermore, these results suggest that custom-engineered chimeric FMD vaccines can be produced and applied in a fashion similar to the current inactivated vaccines.

- **Rapid and Transient Activation of Gamma/Delta T Cells to Interferon Gamma Production, NK Cell-Like Killing and Antigen Processing during Acute Virus Infection.** Foot-and-mouth disease virus (FMDV) infection of cattle and swine is a highly acute infection. Disease is expressed within a few days of exposure, infected animals shed large amounts of virus spreading the infection through a herd or premises, and virus is cleared from the individual quickly, followed by resolution of disease symptoms. Different early immune responses, specific and nonspecific, combine to mediate rapid elimination of the virus. These responses are potential targets of rapid acting vaccines. Here we show results of analysis of a prominent population of lymphocytes in the circulation of ruminants, the gamma/delta T cells, during infection of cattle with FMDV. The data presented show that this cell population is rapidly activated to multiple functions that are involved in combating the viral infection. Remarkably, these activated cells detected within 72 hours of infection with the virus but was no longer found in the blood 5 days after infection. These cells appear to mediate a rapid and highly transient antiviral response and may play a role in early immunity to FMDV. Therefore, vaccines that target gamma/delta T cell activation have the potential to provide rapid protection against FMDV infection.

- **Lesion Development and Replication Kinetics during Early Infection in Cattle Inoculated with Vesicular Stomatitis New Jersey Virus via Scarification and Black Fly (Simulium Vittatum) Bite.** Vesicular Stomatitis Virus (VSV) affects cattle, horses and pigs. The virus is spread by biting flies, causes a disease of significant economic impact in horses and in cattle and pigs, clinical signs can be a confounded for those of foot-and-mouth disease. The mechanisms of this disease have not been well elucidated. For example despite the fact that is causes lesions in mouth and feet, there is no evidence of blood invasion and little is known regarding virus distribution during early infection in livestock. Here we inoculated Holstein steers on the feet with VS New Jersey virus, either by directly scratching the surface of the skin (scarification) or by allowing infected black flies (Simulium vittatum) to feed in the same areas. Our findings confirmed that in both cases the virus remained localized to the inoculated skin and lymphatic nodes draining the inoculated legs (i.e. no virus was found in the blood) and lesions appeared only in the infected leg. Control animals inoculated on the skin of the neck did not develop any lesions. Animals infected by insects developed more severe lesions suggesting that factors contained in the insect saliva facilitate virus growth and lesion development. Taken together, the results indicate that VSV infection is dependent on the site of inoculation and that insects play an important role in transmission and severity of disease in cattle. This information is useful in guiding prevention and control methods during VSV outbreaks.

- **Antiviral Activity of Bovine Type III Interferon against Foot-and-Mouth Disease Virus.** Interferons are the first line of defense against viral infections and administration of interferons as biotherapeutics has been demonstrated to be effective in controlling several viral infections. Here we report for the first time the identification and characterization of a member of the bovine type III IFN family, boIFN-lambda3, also named interleukin (IL) 28B. We have cloned and expressed boIFN-lambda3 using a recombinant replication defective human adenovirus type 5 (Ad5). We demonstrated that the identified boIFN-lambda3 has antiviral activity against foot-and-mouth disease virus (FMDV) and vesicular stomatitis virus (VSV) in bovine cells. Furthermore we showed that inoculation of cattle with Ad5-boIFN-lambda3 induces a systemic antiviral state and up-regulates the expression of IFN stimulated genes with antiviral properties in mucosal tissues of the upper airways and in the skin, tissues that are the primary and preferred sites of FMDV replication. These results indicate that the identified boIFN-lambda3 deserves future attention in efficacy studies against FMDV and potentially could be evaluated in controlling other bovine viral diseases that affect mucosal tissues or skin.

- **Substitution of Specific Cysteine Residues in E1 Glycoprotein of Classical Swine Fever Virus Strain Brescia affects Formation of E1-E2 Heterodimers and Alters Virulence in Swine.** Classical swine fever virus (CSFV) has four proteins forming the structure of the virus particle: Core, E0, E1 and E2. Core protein forms the capside of the virus, surrounding and protecting virus genome while the other three are part of the external layer (envelope) of the virus particle. E1 can be presented as monomer or linked to a molecule of E2. This interaction is mediated by a specific type of amino acid residue named Cystein (CysR). A molecule of E1 contains six CysR but it is unknown which ones are involved in the interaction with E2, and the possible function of these CysR has been largely ignored in virus biology. In this report we identify the two CysR critical for the interaction with E2, and also show that that these two CysR are critical for the production of disease in swine. Additionally, we show that a genetically engineered virus, having deleted these two critical CysR, can be used as live attenuated vaccine as it induces complete protection against the infection with a very virulent virus.

- **Chemical Disinfection of High-Consequence Transboundary Animal Disease Viruses on Nonporous Surfaces.** Border disease viruses such as foot-and-mouth disease (FMDV), classical swine fever, and African swine fever (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 animals, 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. While there are many commercial disinfectants registered by the US Environmental Protection Agency (EPA) for virus disinfection, there is little published data describing the efficacy of ordinary chemicals against dried forms of the viruses. We were tasked by the EPA to develop a model for nonporous surface disinfection to test various chemicals to inactivate virus. We found that FMDV and ASFV were susceptible to sodium hypochlorite and citric acid resulting in complete disinfection, but dried CSFV (Classical Swine Fever Virus) was not completely disinfected by 2% citric acid. 4% Sodium carbonate is commonly used for surface disinfection of vehicles and aircraft leaving FMDV-endemic areas, however, we were unable to completely disinfect FMDV with sodium carbonate in our assay. We conclude that disinfectants formulated with a minimum of 1000 ppm sodium hypochlorite be used for ASFV and CSFV (Classical Swine Fever Virus) disinfection, and a minimum of 1% citric acid be used for FMDV disinfection. These findings will help animal welfare agencies determine what disinfectants to use in response to border disease outbreaks.

- **Evidence of Activation and Suppression during the Early Immune Response to Foot-and-Mouth Disease Virus.** Foot-and-mouth disease virus (FMDV) causes a serious disease of livestock species, threatening free global trade and food security. The disease spreads rapidly between animals, and in order to ensure a window of opportunity for such spread the virus has evolved multiple mechanisms to subvert the early immune response. The cycle of infection in the individual animal is very short, infection is initiated, disseminated throughout the body and infectious virus produced in less than seven days. FMDV has been shown to disrupt the innate response of its very first target cells in the epithelium of the soft palate and pharynx, but also interacts directly with antigen-presenting cells and their precursors. This interaction results in sub-optimal immune function, favoring viral replication and the delayed onset of effective immunity. Detailed understanding of this cycle is crucial to effectively control disease in livestock populations. Improved understanding will inform the design of vaccines and antiviral therapies and add more precision to disease transmission models, which will ultimately refine disease control protocols.

- **The Pathogenesis of Foot-and-Mouth Disease I: Viral Pathways in Cattle.** In 1898 foot-and-mouth disease (FMD) earned a place in history as the first disease of animals shown to be caused by a virus. Yet, despite over a century of active investigation and elucidation of many aspects of FMD pathogenesis, critical knowledge about the virus-host interactions is still lacking. The aim of this review is to provide a comprehensive overview of FMD pathogenesis in cattle spanning from the earliest studies to recently acquired insights emphasizing works which describe animals infected by methodologies most closely resembling natural infection (predominantly aerosol or direct/indirect contact). The 3 basic phases of FMD pathogenesis in vivo will be dissected and characterized as: (1) previremia characterized by infection and replication at the primary replication site(s), (2) sustained viremia with generalization and vesiculation at secondary infection sites, and (3) postviremia/convalescence including resolution of clinical disease that may result in long-term persistent infection. Critical evaluation of the current status of understanding will be used to identify knowledge gaps to guide future research efforts.

- **The Pathogenesis of Foot-and-Mouth Disease II: Viral Pathways in Swine, Small Ruminants, and Wildlife, Myotropism, Chronic Syndromes, and Molecular Virus-Host Interactions.** Investigation of the pathogenesis of foot-and-mouth disease (FMD) has focused on study of the disease in cattle with less emphasis on pigs, small ruminants, and wildlife. “Atypical” FMD-associated syndromes such as myocarditis, reproductive losses, and chronic heat-intolerance have also received little attention. Yet, all of these manifestations of FMD are reflections of distinct pathogenesis events. For example, naturally occurring porcinophilic strains and unique virus-host combinations that result in high mortality outbreaks surely have their basis in molecular, cellular, and tissue-level interactions between host and virus (i.e. pathogenesis). The goal of the current review is to emphasize how the less commonly studied FMD syndromes and host species contribute to the overall understanding of pathogenesis and how extensive in vitro studies have contributed to our understanding of disease processes in live animals.

- **Factors Associated with Within-Herd Transmission of Serotype A Foot-and-Mouth Disease Virus during the 2001 Outbreak in Argentina: A Protective Effect of Vaccination.** Foot-and-Mouth Disease (FMD) is one of the most important animal diseases in terms of the economic impact that it inflicts to regions affected by the disease. Argentina, recognized by the World Organisation for Animal Health (OIE) as a FMD-free-without vaccination country, was affected by a major FMD epidemic in 2001. The epidemic was caused by serotype A virus strains and was controlled by application of systematic vaccination. Here, we quantified the protective effect that emergency vaccination had in reducing the within-herd transmission rate of the disease through the epidemic. This information will contribute to estimate the impact that vaccination may have in the event of a FMD epidemic in the United States.

- **Predicting Antigenic Sites on the Foot-and-Mouth Disease Virus Capsid of the South African Territories (SAT) Types using Virus Neutralization Data.** We have investigated the genetic variation of the capsid-coding region of FMDV SAT1 and SAT2 strains in the sub-Saharan Africa during a 28 year period (1974 to 2002). In this study we have combined information of variability on virus capsid sequences (those containing the antigens responsible for the protective immune response), structural data and serological relatedness measurements to predict areas on the surface of the FMD virion that are antigenically significant. We were able to predict areas on the surface of the FMD virion that are relevant for antigenicity. These sites were mostly consistent with antigenic sites previously determined for types A, O and C using monoclonal antibodies and escape mutant studies. Our methodology offers a quick alternative to determine antigenic relevant sites for FMDV field strains. This work is relevant to the development of more effective FMD vaccines.

- **Mapping of Amino Acid Residues Responsible for Adhesion of Cell Culture-Adapted Foot-and-Mouth Disease SAT Type Viruses.** In this report novel amino acid residues within the capsid proteins of Foot-and-Mouth Disease Virus (FMDV) South African Territories (SAT) serotype 1 and SAT serotype 2 viruses were identified that affect virus host range in cell culture and plaque phenotype. We demonstrated that cell culture adaptation phenotype is acquired following repeated passages of the field strains in cell cultures. Furthermore, we illustrated that this phenotype can be transferred to an infectious copy DNA (cDNA) clone of FMDV field strains from which viable cell culture adapted viruses were recovered. This information is valuable for understanding the mechanism of FMDV adaptation to the host cell in vitro and the identification of amino acid in the 9 outer capsid region responsible for binding to heparan sulfate psoteoglycan (HSPG).

- **Effects of Glycosylation on Antigenecity and Immunogenicity of Classical Swine Fever Virus Envelop Proteins.** Classical swine fever virus (CSFV) has three glycoproteins (proteins with a high content of sugars in their molecules) in its external membrane E (rns), E1 and E2. Here we analyzed the effects of lack of sugar in the immunogenicity of E (rns), E1, and E2 proteins. All three proteins lacking glycosylation, failed to induce a detectable virus neutralizing antibody response and protection against CSFV. In addition, it was observed that single administration of purified E (rns) glycoprotein induced an effective protection against CSFV infection.

- **Genetic and Antigenic Relationships of Vesicular Stomatitis Viruses from South America.** Vesicular stomatitis (VS) is a viral disease of cattle, horses and pigs that, in pigs and cattle, is clinically similar to foot and mouth disease, a devastating disease of livestock. The disease is caused by a group of viruses that have been serologically classified into two serotypes: New Jersey (VSNJV) and Indiana (VSIV). These viruses are endemic in northern South America (Peru, Colombia, Ecuador, and Venezuela), Central America and southern Mexico and cause sporadic outbreaks in Bolivia, northern Mexico and southwestern United States. In the rest of South America, VS has only been reported in Brazil and occasionally in Argentina, and the disease is caused by viruses related to VS Indiana that have been serologically classified as either Indiana-2 or Indiana-3, however little is known about the epidemiology and genetic relationships among these viruses. Here we characterized 10 viral strains recovered from outbreaks in Brazil and Argentina between 1963 and 1998 and showed that the viruses causing clinical VS in livestock in Brazil and Argentina are classified into two distinct groups: Indiana-2 (VSIV-2) and Indiana-3 (VSIV-3). Our results will help in understanding the virus distribution and classification of VSV in the Americas.

- **Use of Inactivated E.Coli Enterotoxins to Enhance Respiratory Mucosal Adjuvanticity during Vaccination in Swine.** Foot-and-mouth disease (FMD) is a devastating disease of livestock, decreasing animal productivity and disrupting trade of millions of animals, particularly in developing countries. Currently available vaccines are effective in preventing clinical disease but do not always protect against infection. This is thought to be the result of poor induction of immunity by these vaccines on the mucosal membranes overlaying the respiratory and digestive tracts. This work reports the application of detoxified bacterial enterotoxins as mucosal adjuvants in pigs, in order to increase mucosal immunity. Administration of these adjuvants enhanced mucosal immunity in nasal wash samples when compared to controls. This work will be useful not only for improving FMD, but is also relevant to the general field of vaccines and to the enhancement of mucosal immunity.

- **Increased Efficacy of an Adenovirus-Vectored Foot-and-Mouth Disease Capsid Subunit Vaccine Expressing Nonstructural Protein 2B is Associated with a Specific T Cell.** Foot-and-mouth disease virus (FMDV) causes an economically devastating disease of cloven-hoofed animals. Vaccines produced by chemical inactivation of the virus are available, but there are concerns about vaccine safety and the ability to distinguish vaccinated animals from infected animals.

  A possible alternative approach to develop safe, effective FMD vaccines is to produce vaccines from portions of the virus which do not contain infectious FMDV and lack the genetic information for a number of viral nonstructural proteins. We have developed a method to produce the vaccine (Ad5-FMDV) that does not require expensive high-containment manufacturing facilities, and thus can be made in the U.S., which currently prohibits work with infectious FMDV on the mainland. The animals inoculated with this marker vaccine can readily be differentiated from infected animals using available diagnostic tests. Previous work in our laboratory has demonstrated that this Ad5-FMD vaccine can protect both swine and bovines after one inoculation. However, relatively large amounts of the vaccine are required.

  In this work, we have improved the protective efficacy of the Ad5-FMD vaccine by examining the potential booster effect of certain additions when included as part of the vaccine. Cattle were inoculated with both the original Ad5-FMD vaccine and the modified Ad5-FMD vaccine were infected with FMDV for 21 days. The group inoculated with the modified vaccine had enhanced protection as compared to the groups inoculated with the other Ad5-FMD vaccines. These results suggest that the 2B protein can enhance the potency and efficacy of the Ad5-FMD vaccine platform.

- **Porcine Major Histocompatibility Complex (MHC) Class I Molecules and Analysis of Their Peptide-Binding Specificities.** The analysis of immune responses, particularly to viral infections, historically focuses on the induction of "cellular" immunity. This refers to the response of cytotoxic T lymphocytes (CTLs) that can kill virus infected cells. A critical aspect of mammalian immune responses is that the antigen specific receptors that mediate the response have evolved to exquisitely differentiate a normal cell from a cell infected with virus, termed "self verses non-self". The highly variable major histocompatibility complex (MHC) proteins are the determining molecules for self. These are the "transplantation" antigens that have to be matched between donors and recipients of organ transplants. These molecules scan proteins for peptides. When a peptide is derived from normal proteins of the cell and bound by MHC, the cell looks like self to the immune system. However, when the protein is derived from a virus, the cell looks foreign and triggers CTL responses.

  The work presented in this paper takes years of study of the human MHC molecules and applies the knowledge of peptide scanning by human MHC to swine MHC molecules. Here we show that molecules with domains derived from both human and swine behave essentially the same. Further, we can use peptide binding predictions from the large database of human MHC molecules and predict what peptides the swine proteins will bind. This now allows us to predict immune responses in pigs and use this information in the design of vaccines to prevent important viral infections like foot-and-mouth disease.

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Induction of Foot-and-Mouth Disease Virus-Specific Cytotoxic T Cell Killing by Vaccination[▽]§

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Foot-and-mouth disease (FMD) continues to be a significant threat to the health and economic value of livestock species. This acute infection is caused by the highly contagious FMD virus (FMDV), which infects cloven-hoofed animals, including large and small ruminants and swine. Current vaccine strategies are all directed toward the induction of neutralizing antibody responses. However, the role of cytotoxic T lymphocytes (CTLs) has not received a great deal of attention, in part because of the technical difficulties associated with establishing a reliable assay of cell killing for this highly cytopathic virus. Here, we have used recombinant human adenovirus vectors as a means of delivering FMDV antigens in a T cell-directed vaccine in pigs. We tested the hypothesis that impaired processing of the FMDV capsid would enhance cytolytic activity, presumably by targeting all proteins for degradation and effectively increasing the class I major histocompatibility complex (MHC)/FMDV peptide concentration for stimulation of a CTL response. We compared such a T cell-targeting vaccine with the parental vaccine, previously shown to effectively induce a neutralizing antibody response. Our results show induction of FMDV-specific CD8$^+$ CTL killing of MHC-matched target cells in an antigen-specific manner. Further, we confirm these results by MHC tetramer staining. This work presents the first demonstration of FMDV-specific CTL killing and confirmation by MHC tetramer staining in response to vaccination against FMDV.

Foot-and-mouth disease (FMD) is a highly infectious viral disease that affects cloven-hoofed animals and remains an important threat to livestock throughout much of the world (reviewed in reference 18). Many species of wild animals are susceptible to infection, but agricultural concerns are particularly focused on swine, cattle, sheep, and goats. This acute disease is characterized by fever and viremia that last 1 to 2 days, the formation of vesicular lesions in the mouth and on the feet and teats, lethargy, lameness, and loss of meat and milk production. Clinical signs of disease resolve within 7 to 10 days; however, an asymptomatic persistent infection (carrier state) can develop, particularly in previously vaccinated cattle (14, 17). The etiological cause of disease is FMD virus (FMDV), which has a single-stranded, positive-sense RNA genome of 8 kb. FMDV is a member of the *Picornaviridae* family, genus *Aphthovirus*. Following entry of the virus into the cell and uncoating, the released viral genome serves as an mRNA for the translation of viral proteins. Initially synthesized as a single polypeptide, the protein is subsequently cleaved by viral proteases, including leader (L^{pro}) and $3C^{pro}$, into mature structural and nonstructural proteins (53).

FMDV is highly cytopathic, and several viral proteins are known to play a role in disrupting the host immune response (reviewed in reference 19). L^{pro} cleaves the host translation initiation factor eIF4G, which plays a critical role in cap-dependent mRNA translation (12). An internal ribosomal entry site (IRES) in the 5′ untranslated region of the FMDV genome allows synthesis of viral proteins to continue while effectively shutting down host protein synthesis (2, 27). L^{pro} also promotes the degradation of NF-κB, a regulator of beta interferon (IFN-β) transcription (10). The combined actions of L^{pro} allow FMDV to block synthesis of the antiviral proteins IFN-α and -β, to which FMDV is sensitive (7, 9). In addition to the role of $3C^{pro}$ in cleaving the viral polyprotein into mature protein products, it has been shown to induce the cleavage of eIF4A and eIF4G (3). This protease has also been implicated in the cleavage of histone H3 (15, 54), thus further inhibiting host transcription and translation. In addition, both the precursor protein 2BC and the coexpressed mature products, 2B and 2C, are involved in disruption of the cellular secretory pathway (33, 34). Combined with the loss of host protein synthesis, this disruption of the secretory pathway appears to be responsible for inhibition of new MHC class I translation and surface expression in FMDV-infected cells (51).

The immunopathology of this acute infection includes a transient lymphopenia (1, 13). Viral infection was shown to lead to inhibition of the type I IFN response of multiple populations of dendritic cells (36, 37). *In vitro*, natural killer (NK) cells have been shown to lyse FMDV-infected cells (56). However, analysis of cells from FMDV-infected animals shows a loss of NK cell function during the acute phase of infection (55) (reviewed in reference 16).

The development of neutralizing antibodies plays a central

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role in FMDV immunity and often correlates with protection of vaccinated animals against live virus challenge (32). Currently available vaccines consist of killed virus in adjuvant and are effective at providing protection but do not allow for the "differentiation of infected from vaccinated animals" (DIVA) (48). A leading candidate for a next-generation FMDV vaccine is a replication-defective human adenovirus 5 (Ad5) viral vector delivering FMDV capsid precursor (P1) and $3C^{pro}$, an "empty capsid" vaccine. This platform has DIVA capability and is very compatible with diagnostic tests in use at present. The parent polypeptide is processed by the $3C^{pro}$ into the protein components of the capsid (reviewed in references 20 and 48). This vaccine strategy is effective in swine (30, 31, 35) and cattle (39). However, like the killed-virus vaccine and natural infection, neutralizing antibodies raised against the vaccine strain do not provide cross-serotype protection. This limits the value of vaccination during an FMDV outbreak or in disease eradication (48), as virus strain matching is required for vaccine efficacy.

$CD8^+$ cytotoxic T lymphocytes (CTLs) play an important role in adaptive immunity and are capable of directly killing virus-infected cells in an antigen-specific manner through the release of perforin and granzymes as well as through Fas-Fas ligand interactions (reviewed in references 5 and 58). Unlike antibodies which recognize complex antigens, CTLs recognize antigens that have been processed and cleaved into 8- to 12-amino-acid peptides by the proteasome and then loaded into MHC class I molecules in the lumen of the endoplasmic reticulum (reviewed in reference 46). These peptide/MHC complexes are then transported to the surface of the infected cell, where they are displayed to T cell surveillance. CTL recognition occurs when an appropriate T cell receptor (TCR), in association with the CD8 molecule, stably binds to the peptide/MHC complex, resulting in stimulatory signaling cascades within the CTL (58). The ability of CTLs to recognize epitopes that remain hidden to neutralizing antibodies makes them attractive candidates for enhancing FMDV immunity.

In contrast to the focus on neutralizing antibodies, the role of CTLs in FMDV immunity has gone largely unexplored. The ability of the virus to inhibit MHC synthesis and transport and the speed with which the virus is cleared presumably result in limited antigen exposure for MHC class I-restricted T cells. In addition to the virus-induced lymphopenia, data from *in vitro* studies would predict that FMDV-specific CTLs are not elicited by natural FMDV infection if MHC class I expression is blocked *in vivo* as has been described *in vitro* (51). This would make FMDV-infected cells invisible to class I-restricted CTLs. Given the cytopathic nature of the virus, this hypothesis has gone largely untested, as FMDV-infected cells are rapidly lysed *in vitro* and cannot be used as target cells in assays for antigen-specific cell lysis.

We have overcome these limitations with two alternative approaches. First, we employed recombinant Ad5 FMDV vaccine technology as a method for delivering FMDV antigens to both stimulating and target cells for the *in vitro* CTL killing assay. Here, we describe our development of an assay using swine leukocyte antigen (SLA) homozygous pigs and the MHC-matched PK (15) cells as target cells. Second, in order to maximize the CTL response, we used a vaccine construct containing a mutation in $3C^{pro}$ that inhibits its ability to process P1 into mature capsids. This approach tests the hypothesis that one result of reduced processing of the primary polypeptide precursor would be an increase in the pool of FMDV proteins available for loading of FMDV-derived peptides into MHC class I molecules. This is then predicted to enhance stimulation of CTLs specific for FMDV peptides.

Further, to confirm that CTL killing was antigen specific and MHC restricted, we developed class I MHC tetramers using the NetMHCpan prediction algorithm developed for human class I MHC (38) and extended to other species, including swine (reference 25 and this report). Here we report that the T cell-targeting vaccine elicited a greater overall CTL killing response than the parental vaccine and correlated with the induction of FMDV-specific CTLs by MHC tetramer staining.

MATERIALS AND METHODS

Cell lines. Porcine kidney [PK(15)] cells (ATCC CCL-33) and PK(15)-EGFP cells (described below) were maintained in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA) and 3.4 mM L-glutamine, 3 mM dextrose, 1 mM sodium pyruvate, 5% sodium bicarbonate, nonessential and essential amino acids, 2-mercaptoethanol, and antibiotics (MEM-10). Baby hamster kidney (BHK-21) cells (ATCC CCL-10) were maintained in basal medium Eagle (BME) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 10% tryptose phosphate broth, 2 mM L-glutamine, and antibiotics (BME-10). Human 293 cells (ATCC CRL-1573) were maintained in MEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. Peripheral blood mononuclear cells (PBMCs) were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 3.4 mM L-glutamine, 3 mM dextrose, 1 mM sodium pyruvate, 5% sodium bicarbonate, nonessential and essential amino acids, 2-mercaptoethanol, and antibiotics. IBRS2 (swine kidney) cells were maintained in MEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics.

Animals. Yucatan 4b.0/4b.0 (24) pigs were obtained from Sinclair Research, Inc., Columbia, MO. Animals were held for at least 1 week before being entered into a study. All animal procedures were reviewed and approved by the Plum Island Institutional Animal Care and Use Committee.

Antibodies. A mouse monoclonal antibody (MAb) against porcine CD6, clone MIL8, was purchased from AbD Serotec (Raleigh, NC). Mouse MAbs against porcine CD8 (R-phycoerythrin [RPE], clone 76-2-11) and CD4 (fluorescein isothiocyanate [FITC], clone 74-12-4) were purchased from BD Biosciences (San Jose, CA). Monoclonal anti-FMDV-VP1 (clone 6HC4.1.3 [45]) and anti-VP2 (VP0) (clone F1412SA [57]) were used in combination to probe for Western blotting. In addition, a mixture of rabbit polyclonal antibodies raised against the FMDV capsid proteins (VP1, VP2, and VP3) were used for Western blotting (43). Anti-tubulin-α MAb was used for control staining of the Western blot (clone DM1A from Thermo Scientific, Fremont, CA).

Viruses. The production of viruses Ad5A24 (Ad5-FMDV-B), Ad5VSV-G (Ad5-VSV-G) (35), and Ad5-Blue (Ad5-Empty) (35) have been previously described. A mutation in the active site of the $3C^{pro}$ gene (21) was subcloned into pAd5-A24, replacing the parental, wild-type $3C^{pro}$, and was termed pAd5A24-3C-mut. Production of this new adenovirus, Ad5A24-3C-mut (Ad5-FMDV-T), was otherwise identical to that of the parental virus. All adenoviruses were grown in 293 cells. FMDV, strain A24 Cruzeiro, was propagated in BHK-21 cells.

Western blotting. IBRS2 cells were seeded in a 96-well microplate at 20,000 cells/well in 50 μl supplemented, phenol red-free MEM and incubated for 24 h at 37°C. Following attachment, cells were infected with adenovirus constructs at a multiplicity of infection (MOI) of 20, or with FMDV A24 at an MOI of 10, and then incubated for 24 h or 5 h, respectively. Whole-cell lysates were prepared, and protein expression was evaluated by separation by SDS-PAGE followed by Western blotting.

Vaccination. Six Yucatan 4b.0/4b.0 pigs were divided into two groups of three pigs each and vaccinated with either Ad5-FMDV-B or Ad5-FMDV-T. PBS was administered to one additional pig as a negative control. For both vaccination and boosting, animals were given a total of 5×10^9 PFU of the appropriate adenovirus. Virus was delivered intradermally in a total of 2 ml to four sites on the neck (0.5 ml/site) using the DERMA-VAC NF needle-free system (Merial, Duluth, GA). Pigs were boosted 12 weeks postvaccination and again 11 or 13 months postvaccination.

Isolation of PBMCs. Porcine blood was collected in Vacutainer tubes containing heparin. Typically, 20 ml of blood was diluted with 14 ml of phosphate-buffered saline (PBS), followed by the addition of a 12-ml underlay of Lymphoprep (Axis-Shield, Oslo, Norway), and then centrifuged at 1,000 × *g* for 20 min at room temperature. The band of peripheral blood mononuclear cells (PBMCs) was harvested and washed three times with PBS, followed by suspension in RPMI.

Stimulator/target cell preparation. PK(15) cells were chosen because they are MHC haplotype 4b.0/4.b0 (23), matching the MHC locus of the Yucatan swine under study. To prepare stimulator cells, PK(15) cells were seeded into 24-well plates and allowed to attach overnight. Cell medium was removed and cells were infected with adenovirus at an MOI of 10 in 0.2 ml of RPMI 1640 (nonsupplemented) for 1 h at 37°C with occasional rocking. For target cells, we developed PK(15)-EGFP cells. pEGFP-C1 (Clontec, Mountain View, CA) was linearized by digestion with PciI (New England BioLabs, Ipswich, MA) and transfected into PK(15) cells using the ProFection mammalian transfection system (Promega, Madison, WI). Stably transfected cells were selected by growth in the presence of Geneticin (500 μg/ml) (Invitrogen, Carlsbad, CA) and cloned by two rounds of limiting dilution. For the assay, 2×10^6 cells were infected with the appropriate virus at an MOI of 10 for 1 h in a low volume of MEM. Target cells were then given MEM-10 and incubated overnight at 37°C.

CD6$^+$ purification. PBMCs isolated from vaccinated pigs were suspended at a concentration of 10^7 cells/ml, and 1 ml was added to each well of infected PK(15) cells (typically 10 to 16 replicate wells), followed by the addition of 1 ml/well of RPMI 1640 (supplemented). Plates were incubated at 37°C for 3 to 5 days. Stimulated PBMCs were recovered from wells and reisolated on Lymphoprep in order to remove dead cells. To positively select CD6$^+$ cells, purified PBMCs were labeled with mouse anti-porcine CD6 MAb (1 μl/10^6 cells) followed by MACS goat anti-mouse IgG microbeads and separated on LS columns using a MidiMACS magnetic separator (Miltenyi Biotec, Gladbach, Germany), according to the manufacturer's instructions, resulting in approximately 95% CD6$^+$ cells (data not shown).

Cytotoxic T lymphocyte killing assay. *In vitro* expanded, CD6$^+$-enriched cells were suspended in supplemented RPMI 1640 at a concentration of 10^7 cells/ml, plated (100 μl/well) in a low-binding 96-well plate (Thermo Fisher Scientific, Waltham, MA), and diluted 2-fold to obtain final effector-to-target-cell (E:T) ratios of 50:1, 25:1, 12:1, and 6:1. Trypsinized PK(15)-EGFP target cells were suspended at 10^5 cells/ml and added to the effector cells (100 μl/well). In order to discriminate dead target cells from live cells, 5 μl of 7-amino-actinomycin D (7-AAD) (BD Biosciences, San Jose, CA) was added to each well. The plate was centrifuged at 233 × *g* for 1 min and then incubated at 37°C for 4 h. Following incubation, cells were resuspended and data were acquired by a FACSCalibur flow cytometer with a high-throughput sampler (HTS) (BD Biosciences, San Jose, CA). Data were analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA) by gating on PK(15)-EGFP cells and determining the percentage of 7-AAD-positive (lysed) cells. Background lysis was determined by 7-AAD staining of target cells in the absence of effectors, and this percentage was subtracted from all groups.

Serum neutralization assay. Endpoint titers of serum neutralization of FMDV A24 were determined according to the procedure described by Pacheco and coworkers (40). Briefly, 4-fold serum dilutions were incubated at 37°C with 100 50% tissue culture infective doses ($TCID_{50}$) of FMDV serotype A24 for 1 h in a microtiter plate, after which BHK-21 cells were added (10^4 cells/well). Following incubation for 3 days, wells were scored for cytopathic effect and endpoint titers were calculated, which represent the reciprocal of the log dilution of serum able to neutralize 100 $TCID_{50}$ of virus.

Tetramer construction and staining. Peptides and recombinant SLA-1*0401 and human β_2-microglobulin (β_2m) were produced and complexed into tetramers as described previously (28) with minor modifications. Briefly, recombinant β_2m and peptides were added to 10-fold and 40-fold excesses, respectively, of SLA-1*0401 in a reaction buffer containing a cocktail of protease inhibitors [0.68 mM EDTA, 4.8 μM pepstatin A, 94.4 μM 1-10 phenanthroline, 0.1 mM *N*-ethylmaleimide, 8.9 μM tosyl-L-lysine chloromethyl ketone (TLCK), 9.3 μM tosyl-L-phenylalanine chloromethyl ketone (TPCK), 66.8 μM phenylmethylsulfonyl fluoride (PMSF)] followed by the addition of streptavidin-allophycocyanin (SA-APC). For tetramer staining, 2×10^5 to 5×10^5 cells were washed with cold FACS buffer (PBS, 0.3% bovine serum albumin, 0.1% sodium azide) and resuspended in solution containing tetramer previously mixed 1:1 with 2× FACS buffer. Cells were incubated at room temperature with rocking for 20 min and then washed three times in cold FACS buffer. Cells were resuspended in FACS buffer containing fluorescent MAbs against porcine CD8 and CD4 and then incubated at room temperature for 20 min. After being washed three times in FACS buffer, cells were fixed in PBS containing 1% formaldehyde and stored at 4°C until being analyzed by flow cytometry.

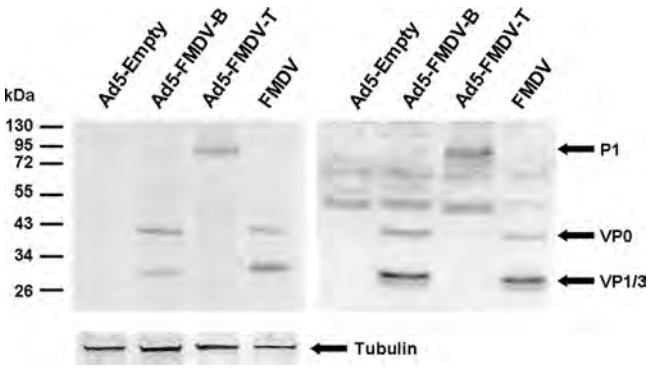

FIG. 1. Reduced capsid processing of Ad5-FMDV-T. IBRS2 cells were infected with the indicated virus as described in Materials and Methods. Following infection, cell lysates were made and proteins were separated by SDS-PAGE and visualized by Western blotting using either MAbs against VP2 (visualized as the VP0 precursor) and VP1 (left) or a mix of rabbit polyclonal antibodies against FMDV capsid proteins (right).

RESULTS

Mutation of FMDV 3C protease results in reduced capsid processing and production of anti-FMDV antibodies. Previous work by Moraes and coworkers demonstrated that vaccination of swine with Ad5A24 (Ad5-FMDV-B) resulted in the generation of a protective anti-FMDV antibody response (35). Mutation of the 3C protease resulting in an amino acid change (C163S) is known to inhibit processing of P1 into mature capsid proteins (21). A second adenovirus construct with the same P1 polyprotein derived from FMDV strain A24 and the mutated 3C protease was made, which we designate Ad5-FMDV-T. This construct was predicted to provide enhanced stimulation of a CTL response based on previous work that demonstrated a lack of antibody response to a similar construct made for FMDV strain A12 (31).

To demonstrate the reduced activity of 3C in this new construct, IBRS2 cells were infected with Ad5-FMDV-B, Ad5-FMDV-T, Ad5-Empty, or FMDV strain A24. Cell lysates were analyzed by SDS-PAGE followed by Western blotting. We detected the presence of P1 cleavage products VP0, VP3, and VP1 in the lysates of cells infected with either FMDV or Ad5-FMDV-B, with little or no P1 precursor (Fig. 1). In contrast, cells infected with Ad5-FMDV-T showed a significant decrease in the presence of VP0, VP3, and VP1, with significantly more unprocessed P1 (Fig. 1), suggesting a reduction of $3C^{pro}$ activity and consequent accumulation of P1.

Sera from pigs vaccinated and boosted with Ad5-FMDV-B and Ad5-FMDV-T were tested for FMDV neutralizing antibodies as described in Materials and Methods. All three pigs vaccinated with Ad5-FMDV-B generated neutralizing antibodies following priming, with elevated titers following two rounds of boosting (Fig. 2). In contrast, of the three pigs vaccinated with Ad5-FMDV-T, only p945 generated detectable neutralizing antibodies following priming. Both p945 and p947 had serum neutralizing antibodies following boost, with the titer for p947 remaining relatively low. Neutralizing antibodies were

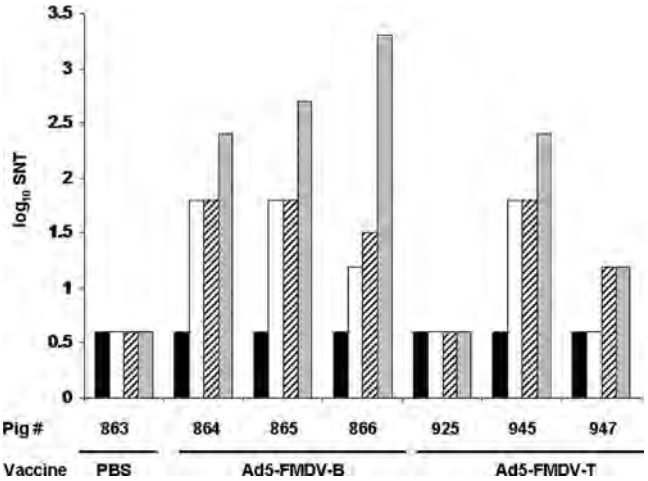

FIG. 2. Reduced neutralizing antibodies in Ad5-FMDV-T-vaccinated animals. Serum samples from pigs vaccinated with PBS, Ad5-FMDV-B, or Ad5-FMDV-T were collected before and following priming and boosting (solid bars, prevaccination; open bars, preboost; striped bars, pre-second boost; shaded bars, final). Serum neutralizing titers (SNT) were determined as described in Materials and Methods. Final serum was collected 26 days or more postboost.

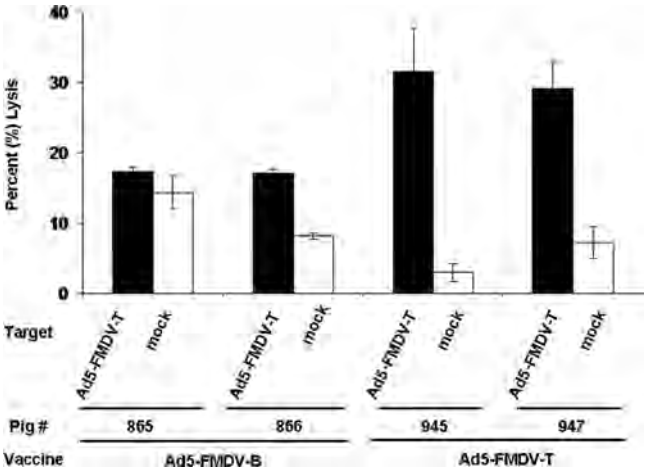

FIG. 3. Elevated CTL activity in Ad5-FMDV-T-vaccinated animals. PBMCs were isolated from vaccinated animals, stimulated in the presence of Ad5-FMDV-T-infected PK(15) cells for 5 days, and then assayed for cytolytic activity against Ad5-FMDV-T-infected or mock-infected target cells, as described in Materials and Methods. The mean and standard deviation of results for duplicate wells of an E:T ratio of 50:1 are shown.

never detected in serum from p925 or from the PBS negative control, p863. These data suggest that Ad5-FMDV-T is less effective than Ad5-FMDV-B at eliciting neutralizing antibodies, presumably due to less efficient processing of P1 by $3C^{pro}$.

Establishment of an FMDV CTL assay. The animals vaccinated with either Ad5-FMDV-T, Ad5-FMDV-B, or PBS were boosted 12 weeks postvaccination and analyzed. PBMCs were collected 24 days postboost and stimulated for 5 days in the presence of PK(15) cells infected with Ad5-FMDV-T and then tested for cytolytic activity against target cells that were either mock infected or infected with Ad5-FMDV-T. Consistent with our hypothesis, we observed the greatest antigen-specific cytolysis by cells derived from pigs vaccinated with Ad5-FMDV-T (Fig. 3). Due to low effector cell numbers, we were unable to evaluate the response against mock-infected cells for p864 and p925, and thus we omitted data from these two animals from Fig. 3. In spite of the observed elevation of antigen-specific cytolysis, with the exception of p945 we observed poor titration of target cell killing across several effector-to-target-cell (E:T) ratios (data not shown). A similar lack of titration of cytolysis by bulk PBMCs was previously observed by Denyer and coworkers, who demonstrated that purification of $CD6^+$ cells by positive selection prior to incubating with target cells resulted in titration of cytolytic activity across E:T ratios (11).

Based on this information, we enriched for $CD6^+$ cells following antigen stimulation in all further experiments, as described in Materials and Methods. PBMCs isolated from pigs vaccinated with Ad5-FMDV-T and enriched for CD6 expression exhibited enhanced cytolysis of Ad5-FMDV-T-infected target cells that titrated across E:T ratios, compared to mock or Ad5-Empty-infected cells (Fig. 4). The level of killing detected by this refined assay was slightly reduced, likely due to the elimination of NK cells from the responding population. The animal vaccinated with PBS showed no specific killing (see Fig. S1 in the supplemental material). In subsequent figures, we show the data for the 50:1 E:T ratio only, with the cytolysis of Ad5-Empty-infected cells subtracted from each group, though all four E:T ratios were always run. Finally, with this new assay, we tested the kinetics of the expansion culture over the course of 5 days and found that the highest level of killing,

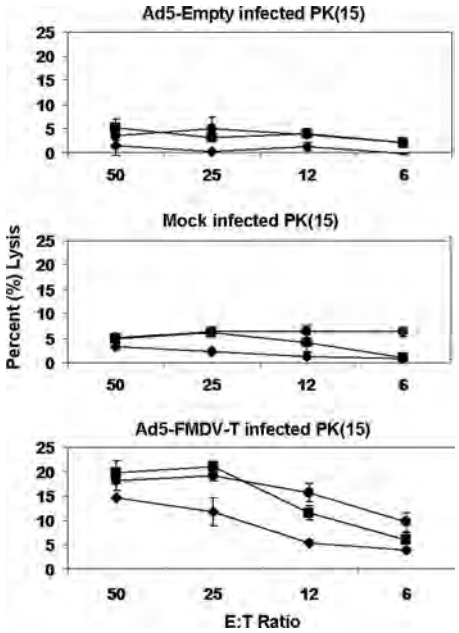

FIG. 4. Titration of $CD6^+$ CTL activity. PBMCs isolated from pigs vaccinated with Ad5-FMDV-T (◆, p925; ■, p945; ●, p947) were stimulated with Ad5-FMDV-T-infected cells for 3 days and then enriched for CD6 surface expression. Effector and target cells were mixed at E:T ratios of 50:1, 25:1, 12:1, and 6:1. Cytolysis of target cells was measured by flow cytometry with target cells mock infected or infected with Ad5-Empty or Ad5-FMDV-T. All error bars represent the standard deviation of results for duplicate wells.

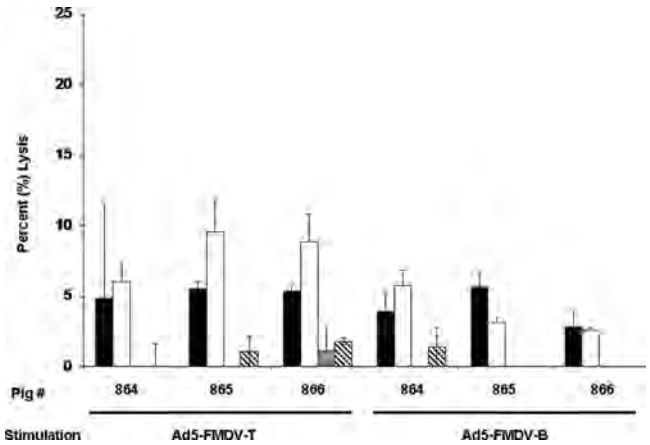

FIG. 5. Differential antigen stimulation. PBMCs from Ad5-FMDV-B-vaccinated pigs were isolated 4 days postboost and stimulated for 3 days with PK(15) cells infected with either Ad5-FMDV-T or Ad5-FMDV-B. Following $CD6^+$ purification, effector cells were assayed for cytolysis of target cells infected with Ad5-FMDV-T (solid bars), Ad5-FMDV-B (open bars), or Ad5-VSV-G (shaded bars) or mock-infected (striped bars) cells. Data are shown relative to Ad5-Empty. The mean and standard deviation of results for triplicate wells are indicated.

with minimum background, was detected on day 3 (data not shown), so all expansion cultures were done for 3 days.

Specificity of CTL cytolytic activity for FMDV P1. The development of this CTL assay was achieved with these vaccinated and boosted animals such that by 9 months following the first boost, antigen-specific cytolysis was difficult to detect, suggesting a waning of CTL immunity. Therefore, all pigs were boosted a second time with the vaccinating virus construct (p864 to p866 with Ad5-FMDV-B and p925, p945, and p947 with Ad5-FMDV-T) and activation of CTL killing was tracked using the assay.

In order to test the ability of the different adenovirus constructs to stimulate CTLs, PBMCs isolated from pigs 4 days after boosting with Ad5-FMDV-B were stimulated with cells infected with either Ad5-FMDV-B or Ad5-FMDV-T. Following enrichment for $CD6^+$ cells, the effector cells were mixed with target cells infected with either Ad5-FMDV-B, Ad5-FMDV-T, Ad5-Empty, or Ad5-VSV-G or mock infected. Ad5-VSV-G contains the gene for glycoprotein G of vesicular stomatitis virus, strain New Jersey (VSV-NJ), and served as a negative-control antigen. $CD6^+$ cells stimulated with Ad5-FMDV-B, compared to those stimulated with Ad5-FMDV-T, appeared to show less overall target cell killing, but there was no statistical difference between these groups (Fig. 5). Specificity for FMDV P1 was shown by little or no cytolysis of target cells infected with Ad5-VSV-G or mock infected (Fig. 5), eliminating the possibility that expression of any adenovirus-delivered transgene may contribute to cell lysis independent of antigen specificity.

Vaccine targeting T cells induces an enhanced CTL response. Following the second boost of animals, PBMCs were obtained on days 4, 8, 14, and 28 and CTL killing activity for each pig was assayed. Antigen-specific cytolysis remained low throughout the experiment in animals vaccinated with the Ad5-FMDV-B construct. In Fig. 6, a small rise in antigen-specific killing on day 4 after the second boost is apparent. Low CTL lysis was observed when analyzing cells taken the day of the boost or 8 and 14 days following the boost. These data suggest that Ad5-FMDV-B is a poor inducer of CTLs. Data from all time points are shown relative to Ad5-Empty, except for data for day 14, which are shown relative to Ad5-VSV-G because small numbers of effector cells isolated necessitated omitting mock and Ad5-Empty target groups.

PBMCs from pigs boosted with Ad5-FMDV-T and stimulated *ex vivo* with Ad5-FMDV-T-infected cells were tested for lysis of target cells infected with Ad5-FMDV-T, Ad5-Empty, or mock infected. In contrast to cells from the Ad5-FMDV-B-vaccinated animals, here we observed increased levels of antigen-specific cytolysis 4 days following the boost that waned to preboost levels by day 14 or 28 (Fig. 6). In all three animals, a high level of specific killing of PK(15) cells infected with the Ad5-FMDV-T construct was observed. Pig 925 had little killing activity 8 days after vaccine boost but good activity (equivalent to the day 4 result) when assayed 14 days after boost. This may indicate a technical problem with that day's sample from that animal (day 8, p925). As shown above, this animal (p925) showed no detectable neutralizing antibody either (Fig. 2). With that exception, the Ad5-FMDV-T-vaccinated animals had higher levels of target cell killing than the Ad5-FMDV-B-vaccinated animals through 14 days following the boost.

Animals vaccinated and boosted with Ad5-FMDV-T showed elevated mean killing of Ad5-FMDV-T-infected target cells compared to that of those vaccinated and boosted with Ad5-FMDV-B when cells isolated 4 days after the respective final boost were analyzed. This enhanced killing was statistically significant ($P < 0.05$; Fig. 7). As described above, the animal that was given PBS as a negative control never showed antigen-specific killing of target cells (see Fig. S1 in the supplemental material).

CTL cytolytic activity correlates with staining by an FMDV peptide-SLA-1 tetramer. In order to further characterize the effector cells, we developed pMHC tetramers for the SLA-1 molecule expressed by these animals, SLA-1*0401. The MHC class I peptide binding prediction algorithm NetMHCpan has previously been demonstrated to accurately capture the binding specificity of SLA class I molecules (25). Using this prediction method, we identified a peptide (MTAHITVPY) derived from the P1 capsid precursor of FMDV strain A24 that binds SLA-1*0401 at a low K_d (dissociation constant) (L. E. Pedersen, M. N. Harndahl, K. Lamberth, W. T. Golde, O. Lund, M. Nielsen, and S. Buus, unpublished data). As a negative control, a second SLA-1 tetramer was used that contained a peptide (ATAAATEAY) derived from the Ebola virus VP35 protein, which also binds to SLA-1*0401. For the experiments associated with the second boost (Fig. 6), a portion of the PBMCs isolated for evaluation of CTL killing were set aside for SLA-1 tetramer staining.

Cells were stained with antibodies specific for CD4 and CD8 and with the tetramers. Analysis by flow cytometry was carried out by gating on the $CD4^-/CD8^+$ cell population and determining the percentage of tetramer-positive ($tetramer^+$) cells (see Fig. S2 in the supplemental material). For pigs vaccinated with Ad5-FMDV-B, little tetramer staining was detected from samples taken 4 days following the second boost (Fig. 8) or thereafter (data not shown). This result correlated with low levels of cytolytic activity from the same samples (Fig. 6). In contrast, pigs vaccinated with Ad5-FMDV-T had a 2- to 3-fold

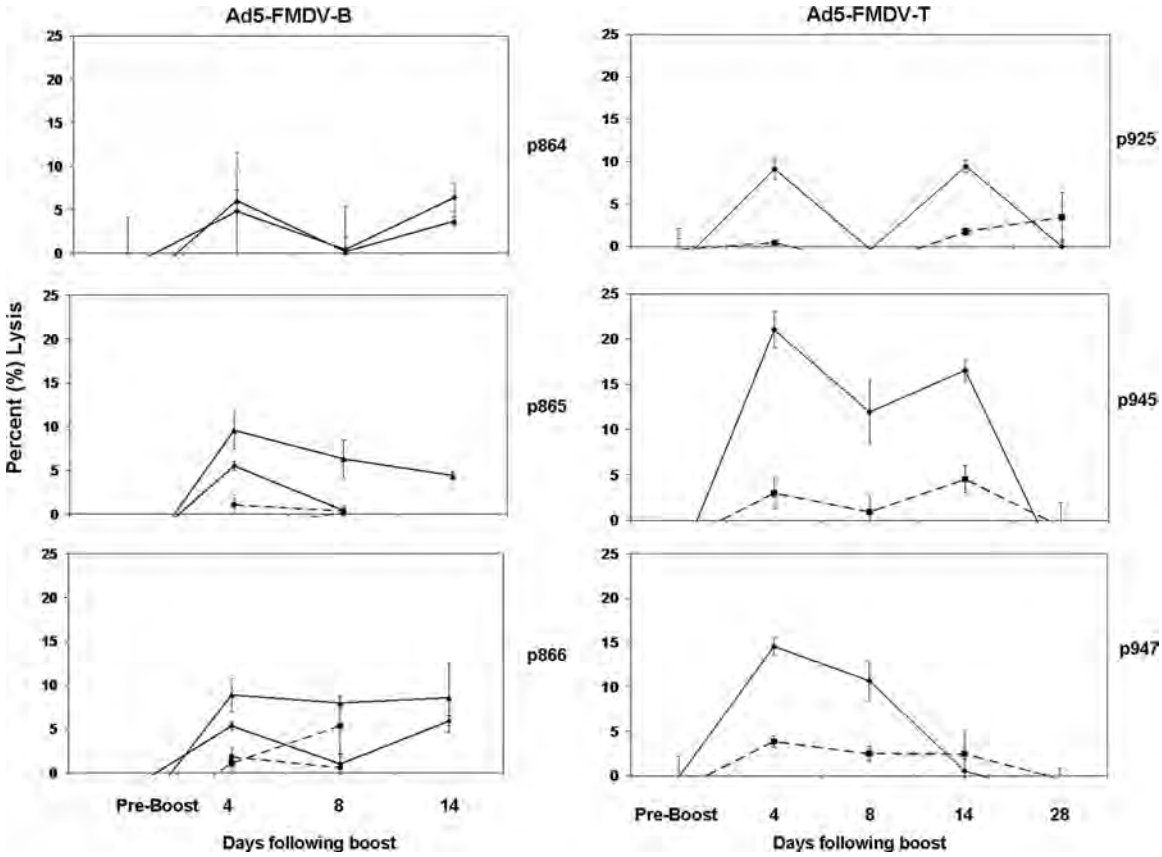

FIG. 6. CD6$^+$ CTL activity generated following second boost. PBMCs from Ad5-FMDV-B (p864, p865, and p866)- or Ad5-FMDV-T (p925, p945 and p947)-vaccinated pigs were isolated before and following boosting and treated as described in the legend to Fig. 5, except that only data from Ad5-FMDV-T secondary antigen stimulation are shown. Target cells: ◆, Ad5-FMDV-T; ■, mock. Additionally, pigs 864, 865, and 866 were tested on target cells: ▲, Ad5-FMDV-B; ●, Ad5-VSV-G. Data from all time points are shown relative to Ad5-Empty. Error bars represent the standard deviation of results for duplicate wells.

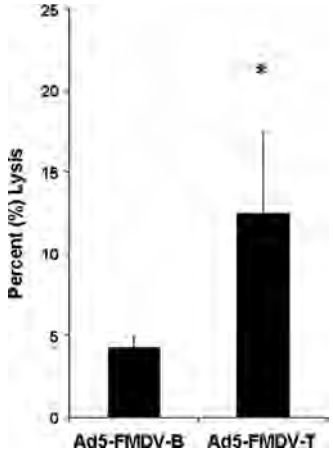

FIG. 7. CD6$^+$ CTL activity generated by Ad5-FMDV-T vaccination. Mean CTL cytolysis activity on day 4 following final boost of pigs vaccinated with Ad5-FMDV-B or Ad5-FMDV-T was determined after subtracting percentage of lysis of the highest negative-control target cell group from that of Ad5-FMDV-T target cells for each individual pig. Error bars represent the standard deviations ($n = 3$), and the asterisk indicates statistical significance ($P < 0.05$) as determined by an unpaired, one-tailed Student's t test with unequal variances.

increase, compared to preboost levels, in tetramer staining of the CD8$^+$ population isolated 4 days following the second boost (Fig. 8). Increased tetramer staining was correlated with peak cytolysis (compare Fig. 6 and Fig. 8) and was no longer detectable by 8 days after the boost. These data show that FMDV peptide MTAHITVPY is presented by SLA-1*0401 molecules *in vivo* and serve as confirmation of the presence of anti-FMDV, MHC-restricted CTLs.

A number of other peptides from the viral capsid precursor protein, P1, were also shown to bind the SLA-1 MHC class I protein. In addition, more peptides were identified that bind SLA-2 of these Yucatan pigs (SLA-2*040201) (L. E. Pedersen, M. Harndahl, M. Nielsen, J. R. Patch, G. Jungersen, S. Buus, and W. T. Golde, unpublished data). We also prepared tetramers from these combinations and tested them on CD8$^+$ T cells from the vaccinated pigs in this study. All of these tetramers were negative for staining cells taken 4 days after boost and all other time points tested. Only the SLA-1*0401/MTAHITVPY tetramer stained cells from the Ad5-FMDV-T vaccinated animals.

DISCUSSION

In the present study, we tested the ability of two nonreplicating adenovirus vectors to generate a CTL cytolytic response

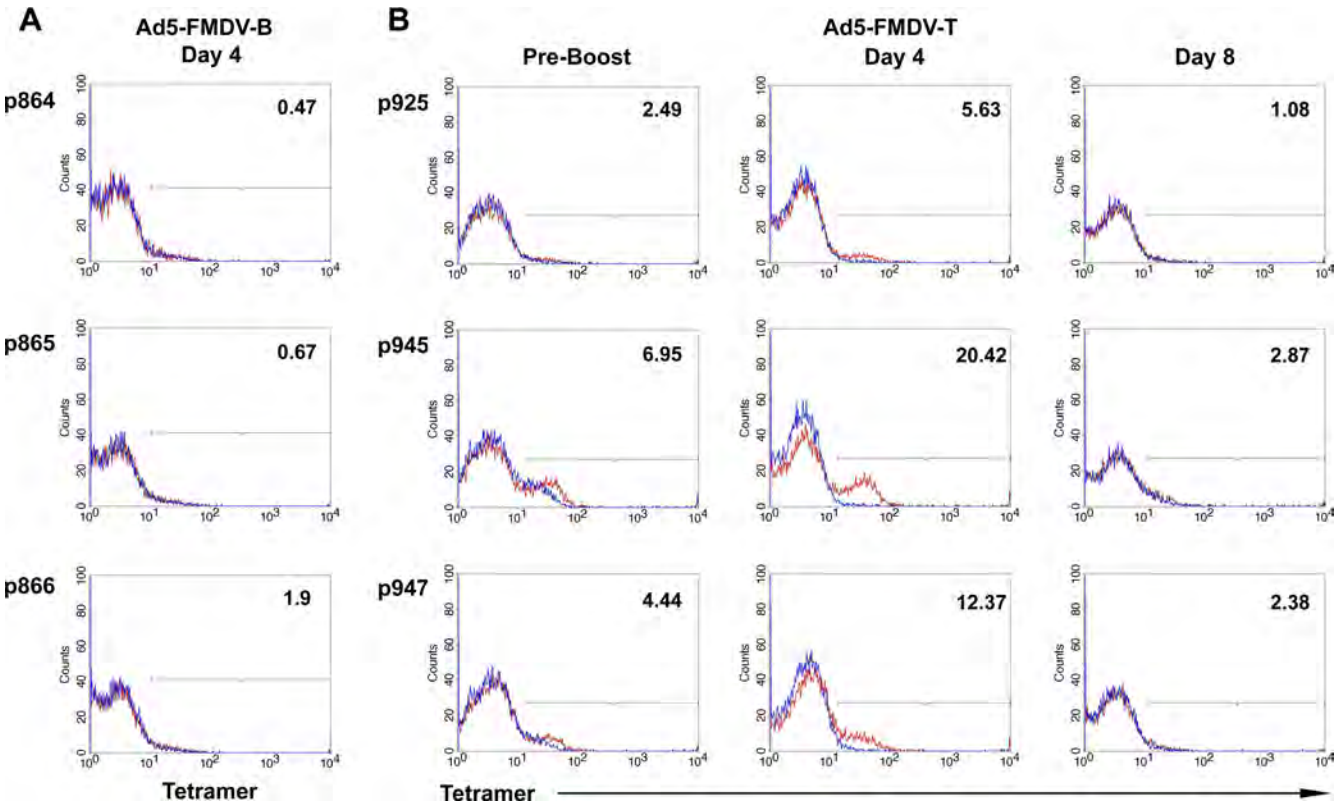

FIG. 8. Tetramer staining of $CD4^-/CD8^+$ CTLs. For time points shown in Fig. 6, PBMCs were stained with antibodies against CD4 and CD8 and costained with SLA-1*0401 tetramers bound to either an FMDV (red) or Ebola virus (blue) peptide. Histograms of tetramer-positive CTLs from Ad5-FMDV-B (A)- or Ad5-FMDV-T (B)-vaccinated animals are shown. Numbers indicate percent FMDV tetramer-positive cells from the $CD8^+$, $CD4^-$ gate and were calculated by subtracting the percentage of cells stained with the negative-control tetramer.

in swine to FMDV epitopes. The ability of this vaccine platform to induce the production of neutralizing antibodies to empty FMDV capsids in both swine (30, 31, 35, 43) and cattle (39) is well established. However, its ability to stimulate an antigen-specific CTL response has not been addressed. We hypothesized that the parental adenovirus-based empty capsid vaccine would provide relatively poor stimulation of CTLs because of the efficiency of capsid processing and formation. We reasoned that inhibited processing of FMDV P1 would enhance CTL activation, presumably as a result of enhanced degradation of P1 and subsequent loading of peptides onto class I MHC molecules. Swine were vaccinated with a T cell-directed adenovirus-based vaccine for FMDV (Ad5-FMDV-T), which contained a mutant $3C^{pro}$ that processes P1 poorly. The decreased processing of P1 by Ad5-FMDV-T was evident in Western blot analysis, as well as in the titers of virus-neutralizing antibodies in serum.

Whole PBMCs isolated from vaccinated animals demonstrated cytolytic activity against appropriate target cells. However, enriching for $CD6^+$ cells before mixing the effector and target cells improved the analysis of CTL activity. These results are in agreement with those of Denyer and coworkers, who demonstrated improved titration of antigen-specific, MHC-restricted cytolysis by enriching for the $CD6^+$ population of swine T cells (11). The $CD6^+$ cell population has been characterized as consisting of MHC-restricted CTLs and helper T cells, including $CD4^+/CD8^-$, $CD4^-/CD8^+$, and $CD4^+/CD8^+$ populations (8, 11, 41, 42, 49). Thus, an additional advantage of positively selecting for $CD6^+$ cells is the elimination of non-MHC-restricted cytolytic cells such as NK cells and γδ T cells, which do not express CD6 (44).

The MHC restriction of this CTL response was strongly supported by SLA-1 tetramer staining of cells derived from these animals. When peripheral blood cells derived from pigs vaccinated with Ad5-FMDV-T were analyzed, an increase in $tetramer^+/CD4^-/CD8^+$ cells was detected. The increase in tetramer-positive cells correlated with the peak of cytolytic activity, consistent with MHC-restricted cytolytic activity. We also noted that for pigs vaccinated with Ad5-FMDV-T, the relative tetramer staining of samples taken 4 days after the second boost correlates with the relative level of CTL killing activity. However, given the small number of animals in the group, this correlation is not statistically significant.

The enhanced cytolysis and tetramer staining of cells derived from pigs vaccinated with Ad5-FMDV-T relative to those vaccinated with Ad5-FMDV-B are supportive of our hypothesis that decreased capsid processing enhances CTL activity. However, it is possible that the precursor and mature capsid proteins are cleaved differently by the proteasome, resulting in a lack of MTAHITVPY peptides displayed in MHC molecules of the Ad5-FMDV-B-infected cells. This could explain why few tetramer-positive cells were detected in animals vaccinated with Ad5-FMDV-B. At minimum, this peptide from the C-terminal half of the VP2 region of P1 is not near the cleavage site at the

junction of VP2 and VP3. Differential tetramer staining cannot, therefore, be attributed to a loss of this peptide due to precursor cleavage by $3C^{pro}$.

Although CTLs play a central role in the adaptive immune response to intracellular antigens, their role in FMDV immunity has received little attention, in part because of the technical difficulties in establishing appropriate assays with which to measure their activity. In addition to the requirement for genetic matching of MHC class I alleles between effector and target cells, the cytopathic nature of FMDV has hampered development of a cytolytic assay. As a consequence, investigators have measured other biological properties of $CD8^+$ cells, such as proliferation (6, 26, 47, 50) or IFN-γ secretion (22), as proxies for CTL killing activity. The present report describes an alternative strategy for directly assessing CTL killing in response to FMDV antigens and relies on recombinant expression of FMDV proteins. In an earlier report, Li and coworkers described vaccination of swine with an attenuated pseudorabies virus containing a gene for the FMDV P1 capsid precursor that was effective at generating CTL killing activity (29). However, these investigators did not address MHC restriction, characterization of the cells responsible for killing, or antigen specificity of the reported CTL activity. In contrast, we now present data describing the first description of using MHC tetramers to identify antigen-specific CTLs in swine in general and the first time that anti-FMDV CTL killing in any livestock species has been correlated with tetramer staining.

Whether CTLs can play a significant role in FMDV protection and vaccination strategies remains to be determined. Previous work in swine found that a complete lack of capsid processing resulted in a failure to generate neutralizing antibodies, and challenged animals developed clinical signs comparable to those of unvaccinated animals (30, 31). However, the dose of vaccine used was more than a log lower than the dose used here. Reports using higher doses of a P1-based vaccine in swine resulted in partial protection in the absence of FMDV-specific antibodies (50). Similar results were obtained in cattle by the same investigators (52).

In vitro, FMDV inhibition of MHC class I surface expression begins in as little as 30 min postinfection (51). Thus, even under ideal conditions where anti-FMDV CTLs are primed and expanded, control of infection by CTLs depends on host cells infected by wild-type virus *in vivo* processing and displaying FMDV peptides leading to the CTL response before inhibition of new MHC class I protein expression is blocked by viral proteases. Although neutralizing antibodies are likely to remain the principal focus of FMDV vaccination, CTLs may be able to contribute to cross-serotype and subtype immunity because potential T cell epitopes are not limited to the structural proteins, where most of the immune-driven diversity is located (4). Whereas antibody epitopes are large and three dimensional, the T cell epitopes are linear and extremely small (8 to 12 amino acids) and have the potential to be highly conserved over the different serotypes of FMDV. Further work directed toward refining the induction and measuring of the CTL response, as well as evaluating protection from challenge, is needed to determine if induction of CTL immunity can yield protection across serotypes in diverse breeds of pigs and other species of interest, especially cattle.

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Characterization of the temporal and spatial distribution and reproductive ratio of vesicular stomatitis outbreaks in Mexico in 2008

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Objective—To characterize the temporal and spatial distribution and reproductive ratio of vesicular stomatitis (VS) outbreaks reported in Mexico in 2008.

Animals—Bovine herds in Mexico in which VS outbreaks were officially reported and confirmed from January 1 through December 31, 2008.

Procedures—The Poisson model of the space-time scan statistic was used to identify periods and geographical locations at highest risk for VS in Mexico in 2008. The herd reproductive ratio (R_h) of the epidemic was computed by use of the doubling-time method.

Results—1 significant space-time cluster of VS was detected in the state of Michoacan from September 4 through December 10, 2008. The temporal extent of the VS outbreaks and the value and pattern of decrease of the R_h were different in the endemic zone of Tabasco and Chiapas, compared with findings in the region included in the space-time cluster.

Conclusions and Clinical Relevance—The large number of VS outbreaks reported in Mexico in 2008 was associated with the spread of the disease from the endemic zone in southern Mexico to areas sporadically affected by the disease. Results suggested that implementation of a surveillance system in the endemic zone of Mexico aimed at early detection of changes in the value of R_h and space-time clustering of the disease could help predict occurrence of future VS outbreaks originating from this endemic zone. This information will help prevent VS spread into regions of Mexico and neighboring countries that are only sporadically affected by the disease. (*Am J Vet Res* 2011;72:233–238)

Vesicular stomatitis is a disease of livestock caused by the VSV of the family *Rhabodviridae*, genus *Vesiculovirus*, for which 2 serotypes, New Jersey and Indiana, have been described for North America.[1,2] The disease is characterized by severe vesiculation and ulceration of tongue, oral tissues, feet, and teats.[2,3] The VSV infects various hosts including horses, cattle, and pigs. In certain conditions, humans and some wildlife species may also become infected. Infection with the VSV is rarely fatal, but it typically has an important impact on the economy because of the direct losses that it causes in infected herds and the imposition of quarantine restrictions that affect animal trade.[4,5] Vesicular stomatitis is also important in the differential diagnosis of vesicular diseases because its clinical signs in cattle and pigs are indistinguishable from those caused by the infection with the foot-and-mouth disease virus.

Abbreviations

EADC	Exotic Animal Disease Commission
R_h	Herd reproductive ratio
VS	Vesicular stomatitis
VSIV	Vesicular stomatitis virus, serotype Indiana
VSNJV	Vesicular stomatitis virus, serotype New Jersey
VSV	Vesicular stomatitis virus

The United States is regularly affected by VSV incursions, and genetic evidence suggests that viruses associated with such incursions likely originate from enzootic areas of Mexico.[6,7] Diagnosis of VS became part of the passive surveillance system for vesicular diseases in Mexico in 1949.[8,a] It has been suggested that VS is enzootic in certain parts of the Mexican states of Veracruz, Oaxaca, Tabasco, and Chiapas, in which the disease is most common during the rainy season.[8] The enzootic area is characterized by tropical weather, with high levels of precipitation, and mean temperatures of 25°C.[8] Other parts of the country such as the states of Jalisco, Michoacan, Hidalgo, Guerrero, and Colima are sporadically affected by epizootics of the disease.[8] Unlike the enzootic zone, the epizootic area is character-

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ized by arid or semiarid weather with mean temperatures of 15° to 20°C.[8]

Most of the VS outbreaks reported annually in Mexico are caused by infection with VSNJV. The number of outbreaks usually increases during the rainy season, from June through September, reaching a peak at the end of the season.[a] Such a pattern has not been observed for VSIV, which is typically responsible for sporadic outbreaks separated by several years of inactivity.[8] Outbreaks of VS often appear more or less simultaneously within a broad area, and the seasonal nature of the outbreaks and the scattered distribution of the disease have suggested that VSV is transmitted by insect vectors such as black flies (*Simulium vittatum*) and the biting midge *Culicoides sonorensis* (Diptera: Ceratopogonidae).[5,8] However, despite vector-borne transmission being considered the main form of spread, direct transmission of VSNJV through contact with infected animals may also occur.[7,9,10]

Reporting of vesicular diseases is mandatory in Mexico. Since 1954, surveillance and outbreak investigation of vesicular diseases such as VS are carried out by the EADC of the Secretaria de Agricultura, Ganaderia, Desarrollo Rural, Pesca y Alimentacion. The EADC is a government branch that is also responsible for investigating foreign animal diseases in Mexico. Following notification of a suspected case of vesicular disease, EADC veterinarians carry out an outbreak investigation in the suspected herds. As part of the investigation, samples of vesicular liquid, blood serum, and epithelium are collected from animals with clinical signs of disease. Samples are tested at the level 3 National Biosecurity Laboratory of the EADC in Mexico City via complement fixation, serum neutralization, competitive ELISA, or a combination of these assays. Cases of infection are ultimately confirmed via virus isolation in cell culture, PCR analysis for genotyping, or both. Preventive control measures implemented before the suspect case is confirmed include restrictions on the movement of people, animals, and vehicles.

After 2 years of no reports of VS outbreaks in the United States, serologically positive cases of the disease, with no virus isolation, were identified in Texas and New Mexico between June and July 2009.[11] Unlike previous VSV outbreaks reported in the southwestern United States, the outbreak was restricted to only 5 cases.[12] Interestingly, this reemergence of VSV in the United States was preceded by an unusual increase in the number of cases and distribution of the disease in Mexico in 2008. In 2008, 321 VS outbreaks were reported in 20 Mexican states. Because < 100 cases are reported annually in Mexico, such increase in the number of cases suggests that changes in the epidemiological dynamics of the disease, including, for example, the rate at which the disease is transmitted or the space-time distribution of cases, may have occurred. Furthermore, one may also hypothesize that the increase in the number of cases reported in 2008 in Mexico may be somehow associated with the reemergence of the disease in the United States in 2009. The purpose of the study reported here was to characterize the temporal and spatial distribution and reproductive ratio of VS outbreaks reported in Mexico in 2008.

Materials and Methods

Animals—A database containing the laboratory results for all VS outbreaks confirmed in 2008 was provided by the EADC. Outbreaks were defined as bovine herds with at least 1 animal with a confirmed positive test result for VSV. The information collected for each outbreak included the location (municipality), date of first identification of VS-like lesions, date of first veterinary visit to the herd, date of diagnosis, number of susceptible animals, number of animals with VS-like lesions, animal species, number of samples collected, and presumptive and final diagnosis.

Outbreaks—Outbreaks were georeferenced by use of the centroid of the municipality in which the outbreak was located. The centroid of each municipality was obtained from databases provided by the Instituto Nacional de Estadistica y Geografia. There are 2,438 municipalities in Mexico, grouped in 31 states and a Federal District (Mexico City). Most (n = 321) of the outbreaks were caused by VSNJV, whereas 4 outbreaks were associated with VSIV infection. Only outbreaks caused by VSNJV were used in the analyses reported here.

General approach—The temporal and spatial distribution of VS outbreaks in Mexico in 2008 was assessed through use of the scan statistic, which is a technique for the detection of space-time clusters of disease. The basic R_h, which is the mean number of secondary outbreaks caused by 1 infectious herd during its entire infectious period,[13] was estimated for each of the space-time clusters detected with the scan statistic and for the enzootic area that includes the states of Tabasco and Chiapas.

Specific approach—The Poisson model of the space-time scan statistic was used to identify significant space-time clusters of the disease. The null hypothesis was that the ratio between number of outbreaks and number of cattle per municipality was evenly distributed throughout the country. In other words, if no clustering was present, the number of outbreaks in a given municipality would be proportional to the size of the cattle population of the municipality. Therefore, and because a 1-sided statistical test was applied, rejection of the null hypothesis for a particular region and period led to the identification of space-time clusters in which the number of reported outbreaks was larger than expected on the basis of the size of the cattle population of the region.

Outbreaks were assumed to have started at the date when VS-like clinical signs were first observed in the herd. Outbreaks were aggregated into 7-day bins to account for intraweek variations in the reporting of cases, which is a type of reporting bias that has been referred to as the Monday effect. The space-time scan statistic defines cylindrical windows with the circular geographic base centered around each municipality centroid and with height corresponding to time.[14] The base and height of the cylinder (ie, the radius of the spatial window and the duration of the cluster) were set up to vary up to a maximum of 50% of the population at risk. The expected number of outbreaks per municipality under

the null hypothesis of homogeneous distribution of the disease was assumed to be proportional to the number of cattle in the municipality. The space-time scan statistic compares the observed-to-expected ratio of outbreaks within each candidate space-time cluster with the observed-to-expected ratio of outbreaks outside the candidate cluster.[14]

A Monte Carlo simulation process was used to test for significance of identified clusters. A large number (n = 999) of random replications of the data set were generated under the null hypothesis of outbreaks being proportional to the size of the cattle population within the candidate cluster. The maximum likelihood of the observed (n = 1) and randomly generated data (999) was then computed. Subsequently, a likelihood ratio test was used to compute, for each candidate cluster, the rank (R) of the maximum likelihood obtained from the real data set compared with the maximum likelihoods from the random data sets. The *P* value of the candidate cluster was computed as $P = R/(1 + 999)$. Candidate clusters in which the condition $P < 0.05$ was estimated were assumed to represent true (significant) clusters of disease. The space-time scan statistic was calulated by use of an open-access statistical program.[14,15,b] Outbreaks were mapped by use of spatial analysis software.[c]

Herd reproductive ratio (R_h)—The R_h is an estimate of the number of herds that became infected through direct or indirect contact with an infectious herd. Thus, $R_h > 1$ indicates that the number of outbreaks is increasing; $R_h = 1$ suggests that the number of outbreaks remains constant; and $R_h < 1$ reflects a decrease in the number of outbreaks.[16] In the present study, R_h was calculated with the epizootic doubling-time method, so that $R_h = 1 + [(\ln 2D)/t_d]$,[16] in which D is the duration (in days) of the infectiousness period of the herd and t_d is the period (in days) in which the number of outbreaks doubled. The value of D was approximated by consideration of the expert opinions of 12 veterinarians responsible for VS surveillance in Mexico. Minimum, most likely, and maximum values of D equal to 15, 30, and 45 days and *D* equal to 7, 10, and 20 days were estimated for the epizootic and enzootic areas, respectively. Because t_d cannot be computed for the interval between the last day by which the number of outbreaks doubled and the end of the epizootic, a value of R_h equal to 0 was assumed at the end of the epizootic.[16]

Results

From January 6, 2008, through December 16, 2008, there were 321 confirmed outbreaks of VSV in Mexico. Three outbreaks were excluded from the analysis because the information on geographic location of the affected herds was missing. Thus, 318 outbreaks were included in the study. The states of Jalisco, Michoacan, and Guerrero accounted for 75% of the total number of outbreaks (**Table 1**). There was an increase in the number of outbreaks starting in September that continued through October and November, in which > 100 cases were reported (**Figure 1**).

One significant ($P < 0.001$) space-time cluster of VS outbreaks was detected by use of the spatial scan statistic. The cluster, which was centered in the municipality of Aquila, state of Michoacan, at 18.36°N, 103.19°W, lasted from September 4 through December 10, 2008, and had a radius of 403.3 km. There were 246 outbreaks confirmed within the cluster, whereas 20.5 outbreaks were expected under the null hypothesis of homogeneous distribution of VS outbreaks in Mexico (**Figure 2**).

The value of R_h in the endemic zone that included the states of Tabasco and Chiapas increased gradually, with the peak taking place at the beginning of August, at which point the most likely value of R_h was estimated

Table 1—Distribution of outbreaks of VS per state confirmed in Mexico in 2008.

State	No. of VS outbreaks	No. of municipalities with VS outbreaks	Proportion of municipalities with VS outbreaks	Percentage of VS outbreaks per state
Hidalgo	1	1	0.01	0.3
Morelos	1	1	0.03	0.3
Oaxaca	1	1	0.00	0.3
Queretaro	1	1	0.06	0.3
Zacatecas	1	1	0.02	0.3
Puebla	2	1	0.00	0.6
Yucatan	2	2	0.02	0.6
Aguascalientes	4	3	0.33	1.3
Colima	4	2	0.20	1.3
Nayarit	5	4	0.20	1.6
Campeche	6	1	0.09	1.9
Mexico	7	2	0.02	2.2
Guanajuato	7	7	0.15	2.2
Veracruz	8	7	0.03	2.5
Chiapas	17	11	0.10	5.3
Tabasco	18	10	0.59	5.7
Guerrero	29	10	0.13	9.1
Michoacan	64	24	0.21	20.1
Jalisco	140	33	0.27	44.0
Total	**318**	**122**	—	**100.0**

— = Not applicable.

to be 10 (**Figure 3**). By the beginning of September, the number of outbreaks in the endemic area decreased, so that the number of outbreaks did not double, and R_h approximated a value of 0 by October. The shape of the R_h pattern within the significant space-time cluster centered in the state of Michoacan was different, compared with that observed in the endemic zone. Within the Michoacan space-time cluster, outbreaks started in September (ie, approx at the time when the number of outbreaks decreased in the endemic zone). At that time, the maximum value of R_h was estimated as 6 within the cluster. The value of R_h gradually decreased and approximated a value of 0 by the end of November.

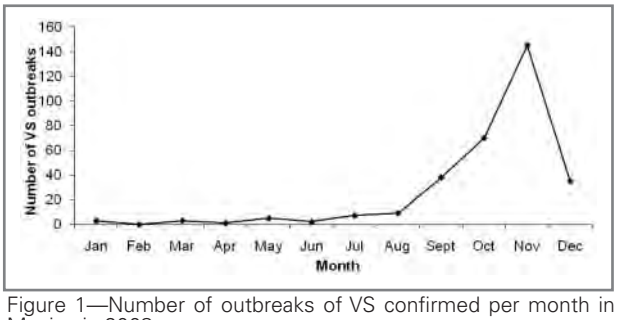

Figure 1—Number of outbreaks of VS confirmed per month in Mexico in 2008.

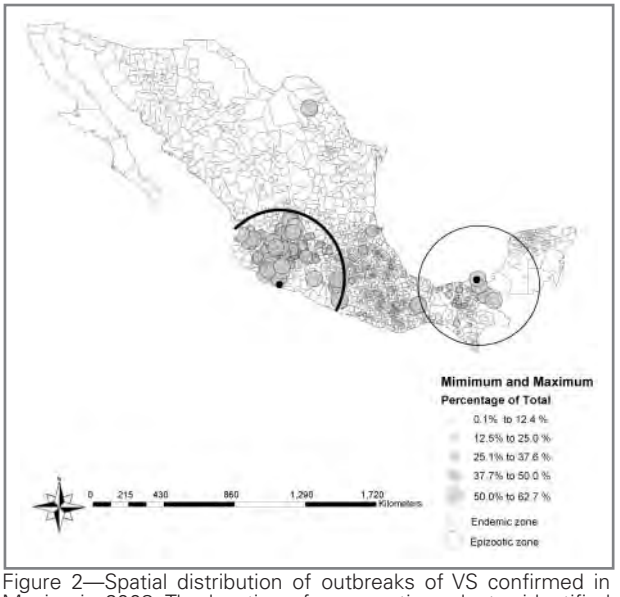

Figure 2—Spatial distribution of outbreaks of VS confirmed in Mexico in 2008. The location of a space-time cluster identified through a scan algorithm (black circle) and the VS-endemic area of Mexico (gray circle) are indicated.

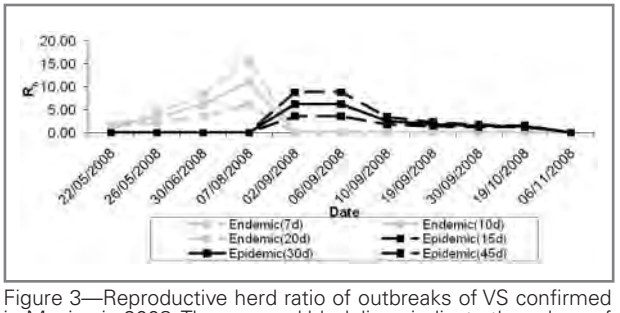

Figure 3—Reproductive herd ratio of outbreaks of VS confirmed in Mexico in 2008. The gray and black lines indicate the values of R_h estimated for the enzootic and epizootic zone and the space-time cluster, respectively (solid line, most likely value; dashed lines, 95% confidence interval with their respective value regarding the duration in days) for different (minimum, most likely, maximum) durations of herd infectiousness, which are indicated (in days) in parentheses.

Discussion

The study reported here revealed that the increase in the number of VS outbreaks reported during 2008 in Mexico, compared with numbers in previous years, was associated with a significant space-time cluster of the disease in a region of central and northern Mexico, where VS does not occur every year (epizootic region) as it does in the southern region of Mexico, where VS is considered enzootic. The significant cluster of the disease, which was detected in a geographic region and time of the year different than those in which the disease typically occurs, was preceded by an increase in the R_h of the disease in the endemic area of Mexico. Implementation of real-time monitoring of space-time clustering of VS and of the R_h value as part of the surveillance system for the disease in Mexico may help to establish an early warning system for VS. It may also ultimately allow epizootic preventive measures to be established in nonendemic regions of Mexico.

A significant cluster of VS outbreaks was identified in the central part of Mexico in 2008. The cluster extended over a large area of the country (511,009 km^2), which is typically considered at low risk for the disease.[8] Additionally, the cluster existed from September through December and immediately after the reporting of cases in the endemic region decreased (Figure 3). These findings suggested that environmental and epidemiological factors may have acted together to favor VS spread from the endemic area into a region in which conditions are usually adverse for the transmission of the disease. The value of R_h in the endemic area gradually increased until it peaked in September and then dramatically decreased afterward. This observation is compatible with the coexistence of favorable conditions for disease transmission that were immediately followed by an adverse change in those conditions. Such favorable conditions, which resulted in the peak of the value of R_h in the endemic region in September, may have led to the spread of the disease into the epizootic region of the country by that time of the year. This hypothesis is supported by the observation that the disease spread into the epizootic region coincided with the peak in the R_h value in the endemic zone.

In the epizootic region, the disease behaved as an epidemic, with an approximate constant value of $R_h > 1$ through September that was followed by a gradual decrease of the transmission rate. Such a pattern in the value of R_h led to an approximately bell-shaped, short-tailed epidemic curve in the epizootic zone, with most of the outbreaks taking place immediately after the introduction of the disease (Figure 1). This observation may be associated with initial favorable conditions for disease spread followed by a change in those conditions that resulted in a short tail for the epizootic curve and in the extinction of the outbreaks by December. Taken together, our findings suggested that by September 2008, there may have been changes in the epidemiological or environmental conditions of Mexico that have

favored the transmission of VSV in the endemic area, as suggested by the increase in the value of R_h in this region and viral spread into areas that are usually not affected by the disease.

Although the factors that may have favored the spread of the disease are still to be determined, an explanation is that climatic forces may have affected the ecology of vectors involved in the spread of the disease. For example, if there were climatic conditions that resulted in an unusually high abundance of disease vectors in the endemic area, then it is also possible that, by the end of the season, the influence of those climatic conditions strong enough to favor the spread of the vectors into areas in which they are usually absent. Results of laboratory experiments have shown that minor differences in environmental factors such as light and humidity influence the feeding behavior of VS vector species.[17] If this experimental finding holds true in natural conditions, then mechanisms for vector competence and distribution might play a role in the spread of the disease and on the selection of specific viral strains associated with specific species of vectors. Consequently, climatic changes may influence not only the abundance of the vectors in the field but also the mechanisms for competition and selection of vectors and virus strains, which may lead to different patterns of disease spread.

Results presented here may have important applications for disease surveillance in Mexico. Real-time surveillance of the disease and monitoring of the value of R_h in the endemic region may lead to the identification of threshold values for R_h that could suggest that VS spread into the epizootic region is likely to occur. Such a surveillance system could take the form of epidemiological alarms to alert the veterinary services about higher than typical risk of disease spread into regions that are usually unaffected by the disease. Although such a system may primarily help to prevent and control VS in Mexico, it could also have some potential implications for prevention of VS epizootics in the United States. In 2009, VS outbreaks were reported in Texas and New Mexico. This was the first time in 5 years that VS was reported in these regions.[11] Interestingly, and even though the origin of the outbreak is unknown, emergence of VS in the United States was preceded by an unusual increase in the number of affected animals and distribution of the disease in central and northern Mexico. If the outbreak in the United States were associated with spread of the disease from the endemic areas of Mexico into the epizootic areas of the country in 2008, then a real-time surveillance system aimed at monitoring variations of the transmission rate in the endemic area of Mexico may help to warn of the development of the disease both in the epizootic area of Mexico and in the United States.

In the present study, certain potentially useful epidemiological data were not available for analysis. For example, information from weather stations collected during or immediately before the outbreaks could have been used to assess the relationship between disease spread and expected behavior of the vectors. Abundance and dispersion of black flies is known to be influenced by the environmental conditions in the vector's microhabitat. However, not all the outbreaks in our study were located close to weather stations, and data from stations that were located nearby were not available to us.

For the calculation of the duration of infectiousness, expert opinion was considered more appropriate than any other source of information because official reports and the literature contained no reliable information regarding the expected infectiousness of an infected herd and on the extent and nature of the influence of epidemiological variables on the variable. For example, it was not clear to us whether the duration of herd infectiousness, which is a factor that could be influenced by disease-dependent and -independent demographic and ecological conditions, would differ between the epidemic and endemic zones. The experts consulted had > 10 years of experience in VS surveillance, and their opinion was supported by evaluation of the clinical histories of outbreaks within each region. For these reasons, the opinion of those with extended access to empirical information in the field was assumed the most accurate approximation of the true duration of infectiousness that could be obtained under the circumstances.

The study reported here showed that the large number of VS outbreaks reported in Mexico in 2008 was associated with an unusual temporal and spatial distribution of the disease in the country. Consequently, implementation of techniques for real-time detection of space-time clusters and computation of the R_h as part of the surveillance program for vesicular diseases in the endemic area of Mexico may help prevent the spread of VS outbreaks into regions of Mexico and neighboring countries that are only sporadically affected by the disease.

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Foot-and-mouth disease virus utilizes an autophagic pathway during viral replication

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is the type species of the *Aphthovirus* genus within the *Picornaviridae* family. Infection of cells with positive-strand RNA viruses results in a rearrangement of intracellular membranes into viral replication complexes. The origin of these membranes remains unknown; however induction of the cellular process of autophagy is beneficial for the replication of poliovirus, suggesting that it might be advantageous for other picornaviruses. By using confocal microscopy we showed in FMDV-infected cells co-localization of non-structural viral proteins 2B, 2C and 3A with LC3 (an autophagosome marker) and viral structural protein VP1 with Atg5 (autophagy-related protein), and LC3 with LAMP-1. Importantly, treatment of FMDV-infected cell with autophagy inducer rapamycin, increased viral yield, and inhibition of autophagosomal pathway by 3-methyladenine or small-interfering RNAs, decreased viral replication. Altogether, these studies strongly suggest that autophagy may play an important role during the replication of FMDV.

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals caused by foot-and-mouth disease virus (FMDV), the type species of the *Aphthovirus* genus within the *Picornaviridae* family (Grubman and Baxt, 2004). The positive-stranded RNA virus contains a genome of approximately 8400 nucleotides (Grubman and Baxt, 2004).

Infection with positive-strand RNA viruses results in a number of changes in the cell, including the rearrangement of intracellular membranes into vesicular structures where viral genome replication takes place (Armer et al., 2008; Bienz, et al., 1973; Bienz et al., 1987; Egger et al., 2002; Monaghan et al., 2004; Pedersen et al., 1999; Schwartz et al., 2004). Poliovirus, the most extensively studied picornavirus, induces a large number of membranous vesicles, most of them surrounded by double lipid bilayers (Dales et al., 1965; Jackson et al., 2005; Suhy et al., 2000). Characterization of these membranes modified during poliovirus infection demonstrated that they contained cellular markers from the endoplasmic reticulum (ER) as well as membranes from other origins, including lysosomes and the Golgi (Cho et al., 1994; Datta and Dasgupta, 1994; Schlegel et al., 1996; Suhy et al., 2000). Other studies have suggested that several features displayed by the vesicles induced during poliovirus infection, such as double-membrane morphology, vesicular content, and apparent ER origin, are all consistent with an autophagic origin (Schlegel et al., 1996; Suhy et al., 2000; Taylor and Kirkegaard, 2007). Autophagy is a cell-regulated pathway designed to degrade and recycle long-lived proteins and cellular components, an important aspect of organelle turnover and management of starvation (Levine and Klionsky, 2004). Autophagy was first characterized morphologically in mammalian cells; however the molecular components of autophagy were first described in yeast (Wang and Klionsky, 2003). A number of signals can induce autophagy in mammalian cells including starvation, differentiation, development, specific drugs, and infection with intracellular pathogens. During autophagy a double-membrane vesicle, the autophagosome, forms in the cytoplasm sequestering cytoplasmic components or small organelles. The formation and maturation of the autophagosome requires the modification of two ubiquitin-like molecules, microtubule-associated protein light-chain kinase 3 (LC3) and Atg12 (autophagy-related protein) (Espert et al., 2007). Autophagosomes fuse with lysosomes to generate single-membrane vesicles named autophagolysosomes, in which the cargo is degraded. Initial studies provided evidence that the source of the membranes forming this structure was both the Golgi and the ER. However, recent studies suggest that the source of these membranes is primarily from the ER (Dunn, 1990; Espert et al., 2007; Juhasz and Neufeld, 2006). More recently, Hailey et al. (2010) suggested that the mitochondrial outer membrane contributes to their biogenesis during

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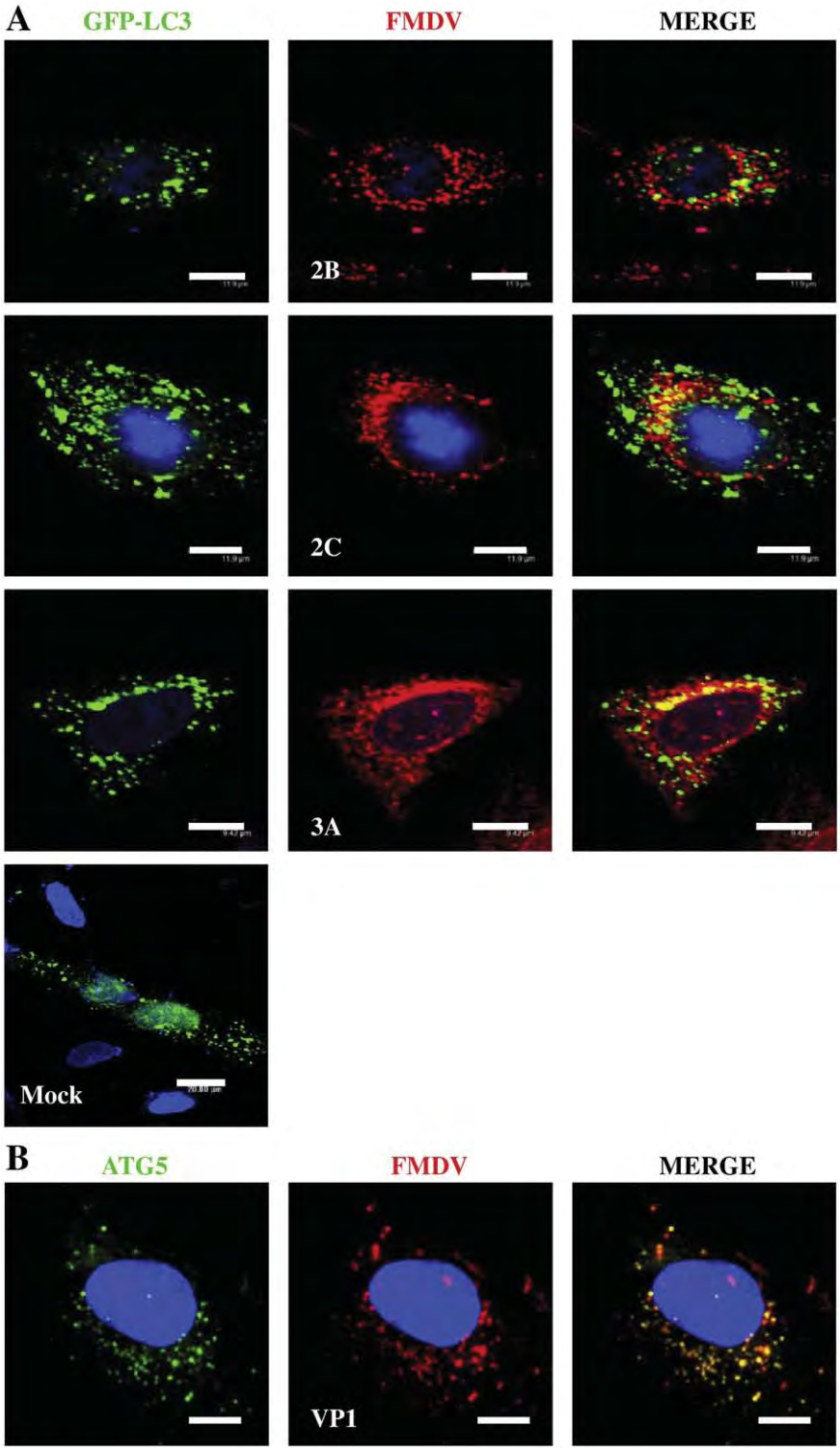

Fig. 1. Analysis of redistribution of autophagy marker GFP–LC3 and FMDV proteins in MCF-10A cells. A. MCF-10A cells were transfected with a plasmid that expresses a GFP–LC3 (green) fusion protein as indicated. Forty-eight hours after transfection, cells were infected with FMDV O_1Campos or mock infected and processed for immunofluorescence staining as described in Materials and methods. FMDV non-structural proteins were detected with specific MAbs and visualized with Alexa Fluor 594 (red). B. MCF-10A cells were infected with FMDV O_1Campos and processed for immunofluorescence staining as describe in Materials and methods. FMDV viral capsid protein VP1, was detected with a MAb and visualized with Alexa Fluor 594 (red); Atg5 was detected with a rabbit antibody and visualized with Alexa Fluor 488 (green).

starvation. The autophagic pathway is also used to degrade intracellular pathogens including bacteria and viruses and may be a component of the innate and adaptive immune responses (Deretic, 2010, Kirkegaard et al., 2004, Lin et al., 2010; Sumpter and Levine, 2010).

Interestingly, recent studies have shown that certain intracellular bacteria and a number of positive-strand RNA viruses, including picornaviruses, induce the formation of autophagic membranes and inhibit either their final maturation or their ability to fuse with lysosomes (Egger et al., 2002; Jackson et al., 2005; Lee et al., 2008;

Pedersen et al., 1999; Schlegel et al., 1996; Suhy et al., 2000). Furthermore the effect of specific autophagy inducers and inhibitors on virus yield supports the idea that autophagosome structures may provide a scaffold for RNA replication by RNA viruses (Dreux et al., 2009; Jackson et al., 2005; Taylor and Kirkegaard, 2007; Wong et al., 2008). These studies also suggested that these double-membrane vesicles, late in infection, may provide a pathway for the non-lytic release of virus (Jackson et al., 2005; Taylor and Kirkegaard, 2007), which would be essential in the establishment of persistent infections, such as that established by FMDV (Alexandersen et al., 2002; Salt, 1993).

In this study, we analyzed the role of autophagy during FMDV replication by using confocal microscopy to monitor the co-localization of specific autophagosome-associated proteins and viral proteins in infected cells. In addition, we explored the effect of inducing or inhibiting the autophagy pathway on FMDV replication. Our results indicate that FMDV non-structural proteins 2B, 2C and 3A, co-localize with the autophagosome marker LC3. Additionally, co-localization of the viral capsid protein VP1 with Atg5 (autophagy-related marker) and LC3 with LAMP-1 in FMDV-infected cells was observed. Furthermore, chemical stimulation or inhibition of the autophagy process directly correlated with increase or decrease in virus production, respectively. Finally, knockdown of LC3 and Atg12, two proteins required to initiate the process of autophagy, led to decreased virus replication. Altogether, these results strongly suggest that autophagy plays an important role in the replication of FMDV.

Results

Redistribution of autophagic markers in FMDV-infected cells

To study if FMDV replication complexes contain constituents of autophagosomes, we used confocal microscopy to monitor the localization of both LC3, a specific marker of autophagosomes, and viral non-structural proteins that have been characterized as critical components of picornavirus replication complex (Bienz et al., 1987; Cho et al., 1994; Jackson et al., 2005; Moffat et al., 2005; Suhy et al., 2000). For most of our experiments we utilized MCF-10A epithelial cells (O'Donnell et al., 2005), unless otherwise indicated. To visualize autophagosomes we expressed LC3 as a fusion product with green fluorescent protein (GFP). In uninfected cells, GFP–LC3 was detected as small discrete foci distributed throughout the cell cytoplasm (Fig. 1A). Upon infection, GFP–LC3 co-localized with FMDV non-structural proteins 2B, 2C, and 3A, by 5 hours post-infection (hpi), adopting a perinuclear pattern (Fig. 1A). By 5 hpi we also show co-localization of the viral capsid protein VP1 with Atg5, an autophagy-related protein that is essential for autophagosome formation (Espert et al., 2007; Matsushita et al., 2007) (Fig. 1B).

To examine the role of autophagy in a system applicable to FMDV pathogenesis studies, we used primary cell cultures derived from bovine pharynx, a site where FMDV primary replication occurs (Arzt et al., 2010; Pacheco et al., 2010a). These cultures, known to be negative for FMDV by virus isolation, RT-PCR and immunostaining (data not shown), were infected with FMDV type O_1Campos at a multiplicity of infection (MOI) of 10. After infection, distribution of viral non-structural protein 3A was compared to the distribution of LC3 by using double-label immunofluorescence and confocal microscopy. Fig. 2 shows a clear co-localization of LC3 and FMDV 3A proteins, with both of the proteins showing a small punctuated distribution pattern.

It has been demonstrated that both LC3 and lysosomal membrane protein 1 (LAMP-1) are components of the mature autophagosome membrane (Espert et al., 2007; Kirkegaard and Jackson, 2005). In order to confirm that autophagy is induced during FMDV replication, we examined the distribution of these two markers in uninfected and infected cells, as well as in the presence of rapamycin, a well-known autophagy inducer. LC3 and LAMP-1 do not appear to co-localize in uninfected untreated cells. Conversely, both in FMDV-infected and in rapamycin-treated cells, LC3 and LAMP-1 proteins clearly co-localize with a perinuclear distribution (Fig. 3), confirming that autophagy occurs in cells infected with FMDV.

Modulation of autophagy activity influences FMDV replication

Well characterized autophagy modulators were used to assess the role of autophagy during FMDV replication in infected cells. FMD viral

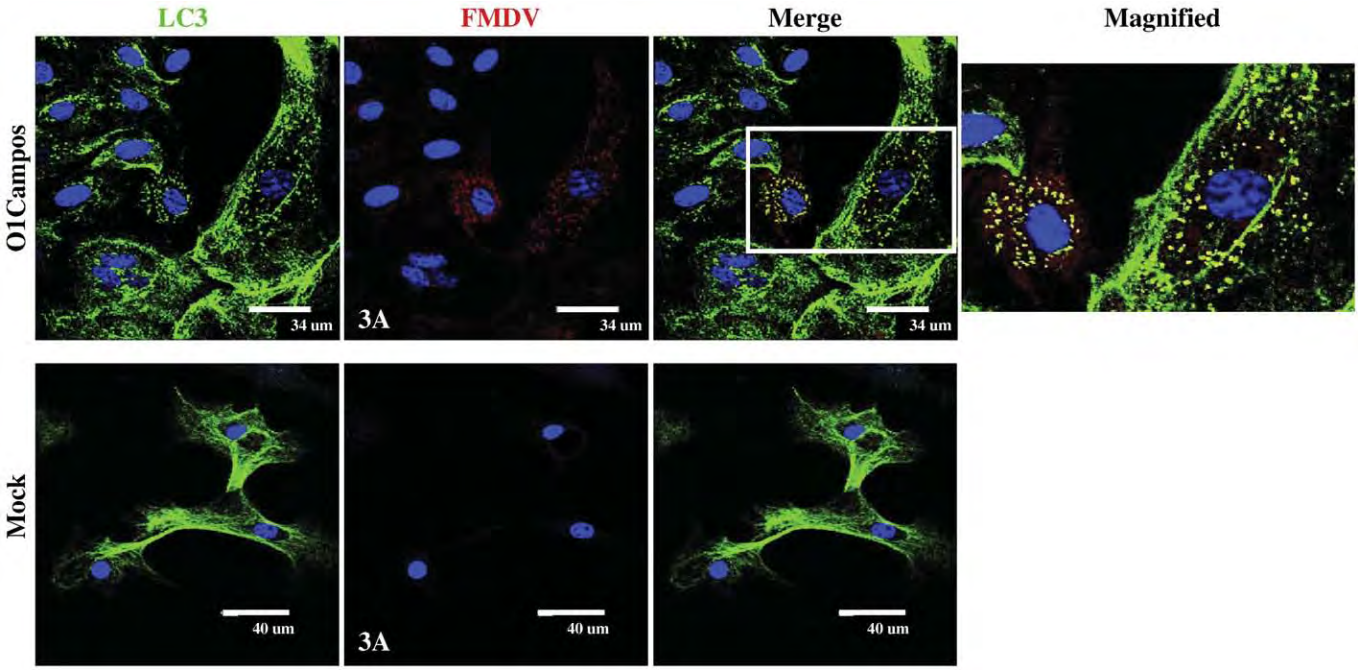

Fig. 2. Analysis of redistribution of autophagy marker LC3 and non-structural FMDV protein 3A in bovine pharynx epithelial primary cultures. Primary cultures of bovine pharynx were infected with FMDV O_1Campos or mock infected and processed for immunofluorescence staining as described in Materials and methods. FMDV non-structural protein 3A was detected with a MAb and visualized with Alexa Fluor 594 (red). LC3 was detected with a rabbit antibody and visualized with Alexa Fluor 488 (green).

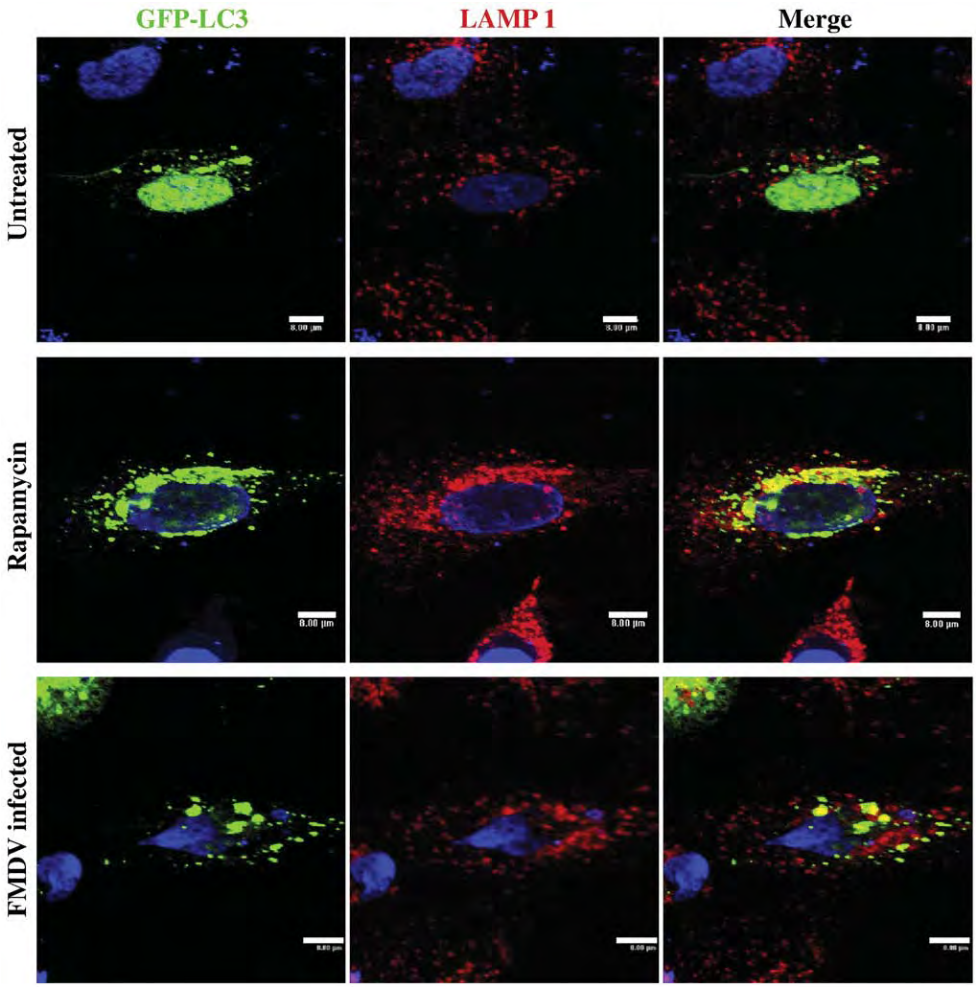

Fig. 3. Detection of GFP–LC3 and LAMP-1 in FMDV-infected cells and in the presence of an autophagy inducer, rapamycin. MCF-10A cells were transfected with a plasmid that expresses a GFP–LC3 (green) fusion protein as indicated. Forty-eight hours after transfection, cells were mock infected or infected with FMDV O_1Campos, or treated with rapamycin. Cells were processed for immunofluorescence staining as described in Materials and methods. LAMP-1 was detected with a MAb and visualized with Alexa Fluor 594 (red).

yield was analyzed in the presence of the autophagy inducer rapamycin or the autophagy inhibitor 3-methyladenine (3-MA). MCF-10A cell cultures were pre-treated either with 50 nM of rapamycin or 20 mM of 3-MA, 2 h prior to infection with FMDV O_1Campos (MOI of 1). Assessment of virus yield was performed at 5 hpi. Yield of virus increased approximately 2 logs when cells were pre-treated with rapamycin (Fig. 4A). Similar results were obtained when cells were infected in the presence of rapamycin and examined by using immunofluorescence and confocal microscopy. At 5 hpi, it was evident that twice the number of rapamycin-treated cells was FMDV-positive when compared to untreated infected cells (data not shown). Conversely, when infected cells were pre-treated with 3-MA,

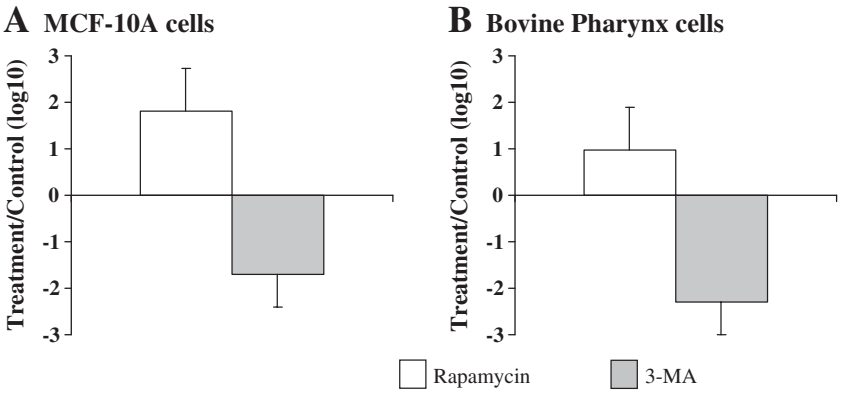

Fig. 4. Viral yields from FMDV-infected cells in the presence of the inducer of autophagy, rapamycin, or the inhibitor of autophagy, 3-MA. MCF-10A cells (A) or bovine pharynx epithelial primary cultures (B) were pre-treated with rapamycin (50 nM) or 3-MA (20 mM) for 2 h before infection with FMDV O_1Campos at an MOI 1, as described in Materials and methods. Viral yields were determined by plaque assay in BHK-21 cells and represented as the ratio of plaque titers of treated and untreated infected cells at 5 hpi. Values are averages and standard deviations representative of two independent experiments.

an autophagy inhibitor, virus yield decreased by nearly 2 logs (Fig. 4A). Moreover, we evaluated the effect of these two compounds in bovine pharynx epithelial primary cultures infected with FMDV O_1Campos. We found that, as observed in MCF-10A cells, when autophagy is induced by rapamycin, viral yield increases up to 1 log. When the FMDV-infected bovine pharynx epithelial cells were treated with 3-MA, viral yield was markedly reduced, more than 2 logs (Fig. 4B). These results suggest that the induction of autophagy during FMDV infection promotes virus replication.

siRNA silencing of the autophagy proteins Atg12 and LC3 results in a reduction of FMDV replication

To confirm the role of autophagy during virus replication, we analyzed the effect of knockdown on transcription levels of autophagy-related proteins LC3 and Atg12. Both proteins have been demonstrated to be critical in the autophagy process (Jounai et al., 2007; Kirkegaard et al., 2004; Kirkegaard and Jackson, 2005; Wang and Klionsky, 2003). When cells were transfected with siRNAs targeting LC3 prior to infection with FMDV O_1Campos, an approximately 3.8-fold and 4.5-fold inhibition in extra- and intracellular viral yield, respectively, was observed (Fig. 5A). Furthermore, an even stronger inhibitory effect on viral replication (greater than 3 logs) was observed when cells were transfected with siRNA targeting Atg12 (Fig. 5B). These results reinforce the hypothesis that autophagy is actively involved in the process of virus replication in cells infected with FMDV.

Increase of cytoplasmic single- and double-membrane vesicles in FMDV-infected MCF-10A cells

Upon induction of autophagy, a membrane cisterna (fold of membrane) known as the isolation membrane (sometimes referred as the phagophore in mammalian cells) appears and curves around part of the cytoplasm. Sealing of the edges of the isolation membrane results in a unique double-membrane vesicle, the autophagosome (Juhasz and Neufeld, 2006). Ultrastructural analysis studies of cells infected with picornaviruses have revealed the presence of virions and other cytoplasmic material within the lumen of double-membrane vesicles (Kirkegaard et al., 2004; Miller and Krijnse-Locker, 2008; Schlegel et al., 1996). To determine whether formation of double-membrane vesicles is linked to autophagy during FMDV infection, electron microscopy was performed on mock and FMDV-infected cells. Quantitative analysis demonstrated an increase in the quantity of the single- and double-membrane vesicles in the FMDV-infected cells (single membrane 160.2 ± 108.2, double membrane 8.1 ± 4.1, and size 0.15 ± 0.04 μm) compared to uninfected cells (single membrane 1.2 ± 3.03, double membrane 0.28 ± 0.89, and size 0.46 ± 0.15 μm) (Fig. 6A). Examination by immunoelectron microscopy (IEM) also showed an increased expression of autophagy-related protein LAMP-1 during virus replication in virus-infected cells compared to uninfected cells (Fig. 6B). Interestingly, IEM also showed a close association of non-structural viral protein 2B with the membranes associated with FMDV infection within the infected cells (Fig. 6B). Therefore, FMDV-infected cells showed evidence of an autophagy-related process, which is absent in mock-infected cells, associated with the expression of non-structural viral proteins.

Discussion

Picornaviruses, as with other positive-strand RNA viruses, generally induce the rearrangement of intracellular membranes to allow the assembly of virus replication complexes (Bienz et al., 1987; Mackenzie, 2005). Co-localization of viral RNA replication complexes with single- or double-membrane vesicles in infected cells has been described several times for various positive-stranded RNA viruses (Bienz et al., 1987; Suhy, et al. 2000; Wong et al, 2008). The double-membrane

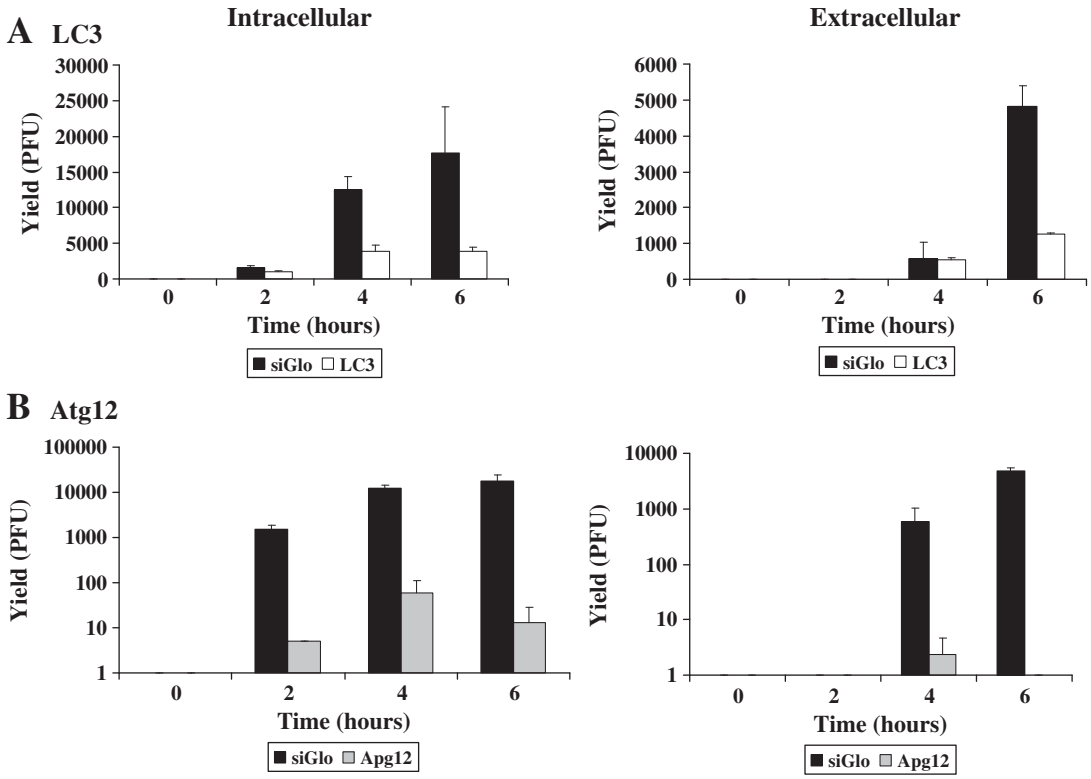

Fig. 5. Viral yields from FMDV infections in MCF-10A cells treated with siRNA to reduce the intracellular concentration of LC3 and Atg12. MCF-10A cells were transfected with four RNA duplexes targeted to LC3 (A), Atg12 (B) or with a control siRNA, siGlo for 48 h at 37 °C as described in Materials and methods. After transfection triplicate plates were infected with FMDV O_1Campos at an MOI of 1 PFU/cell for the indicated times. Plaque titers were determined in BHK-21 cells and expressed as PFU/ml for the intra- and extracellular virus.

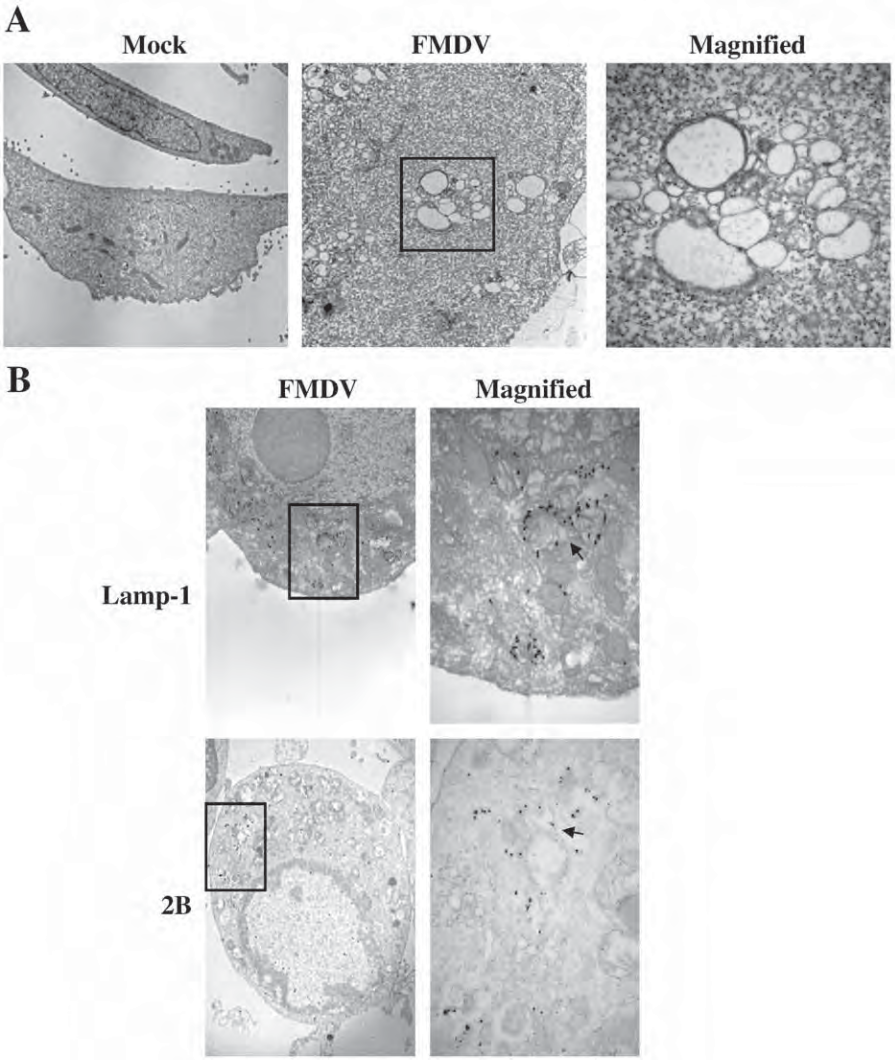

Fig. 6. A — Electron micrographs of mock- and FMDV-infected MCF-10A cells. B — Immunogold detection of LAMP-1 and FMDV 2B non-structural protein individually. LAMP-1 and FMDV 2B proteins were visualized with specific MAb and detected with anti-mouse conjugated with ultra-small gold particles. Note that both proteins are found associated with the infection associated-membranes in the infected cells.

vesicles induced during poliovirus infection shared several features with cellular structures termed autophagosomes, double-membrane vesicles that ultimately mature to degrade their cytoplasmic contents (Dales et al., 1965; Schlegel et al., 1996). Induction of autophagy has been shown to be beneficial for the replication of several viruses including poliovirus, rhinovirus, equine arterivirus, dengue virus, coxsackievirus, hepatitis C virus, and severe acute respiratory syndrome, although the detailed mechanisms remain elusive (Egger et al., 2002; Espert et al., 2007; Jackson et al., 2005; Lee et al., 2008; Pedersen et al., 1999; Wileman, 2006; Wong et al., 2008). Based on these previous findings, it was hypothesized that autophagy may play a role in FMD viral replication. The findings in this report demonstrate that FMDV triggers the autophagy machinery, and this enhances viral replication. This observation is supported by the redistribution of autophagic hallmarks, notably LC3 and LAMP-1, along with virus antigens in FMDV-infected cells, the effect of autophagy modulating compounds on viral yield, and the inhibition of intra- and extracellular virus replication by knockdown of autophagy-related genes.

To understand how autophagy plays a role during FMDV replication, we analyzed the localization of autophagosome markers with viral proteins after infection. We monitored the localization of LC3 with FMD non-structural viral proteins by confocal microscopy, demonstrating that LC3 co-localizes with viral non-structural proteins 2B, 2C and 3A. We also evaluated autophagy in a primary cell culture derived from bovine pharynx, a relevant bovine tissue as a primary site of virus replication (Arzt et al., 2010; Burrows et al., 1981; Pacheco et al., 2010a). Studies performed using bovine pharynx epithelial cell cultures showed a clear co-localization of LC3 and 3A proteins by 5 hpi. For poliovirus the expression of 2BC and 3A in combination, but not separately, induces the formation of double-membrane vesicles that display biochemical markers and fractionate similarly to the vesicles induced during poliovirus infection (Jackson et al., 2005; Suhy et al., 2000). Taylor and Kirkegaard (2007) showed that the expression of the individual poliovirus protein 2BC induces lipid modification of LC3. Conjugation of LC3 to phosphatidylethanolamine (PE) is one of the early steps of autophagosome formation and, it is thought, of vesicle formation during poliovirus infection. In agreement with those results observed for poliovirus infection, within 4 h of FMDV infection, LC3 and LAMP-1, two components of the mature autophagosome membrane, co-localized in both FMDV-infected cells and in rapamycin-treated cells, whereas in uninfected cells no co-localization was seen. Additionally, co-localization of viral capsid protein VP1 with the autophagy-related protein Atg5, was observed (Fig. 1B). Co-localization of autophagosome markers, Atg5 and LC3, with viral replication complexes suggested that at least some RNA replication complexes are present on or near autophagosome-like vesicle.

Co-localization of autophagosome markers and viral proteins could have two explanations. First, the virus could succumb to autophagy as a destructive innate immune response. This has been shown to occur in several viruses and bacteria, including HSV-1, Sindbis Virus, GAS, and TB (Lin et al., 2010; Xu and Eissa, 2010). Some pathogens, however, subvert the cellular autophagy pathway to promote their own replication, including Enterovirus 71 and *Anaplasma phagocytophilum* (Huang et al., 2009; Niu et al., 2008). Wong et al. (2008) have shown that inhibition of signaling pathways or autophagy genes critical for autophagosome formation reduces coxsackievirus B3 (CVB3) viral protein expression and the viral progeny titer. Conversely, increasing the cellular number of autophagosomes prior to viral infection by cellular starvation, rapamycin treatment, or lysosomal inhibition promotes viral replication, suggesting that the host double-membrane autophagosome is likely utilized by CVB3 to facilitate its own replication (Wong et al., 2008).

Poliovirus also subverts the autophagy pathway, inducing structures resembling cellular autophagosomes on which viral RNA replication complexes are assembled. Treatment with either rapamycin or tamoxifen, both inducers of autophagy, increased poliovirus yield 3–5-fold (Jackson et al., 2005). Therefore, autophagosome formation increases poliovirus load suggesting that the autophagic machinery serves a valuable role in viral replication (Jackson et al., 2005).

Accordingly, in our study, viral loads increased when MCF-10A cells as well as the bovine pharynx epithelial primary cells were infected with FMDV in the presence of the autophagy inducer rapamycin (Figs. 4A–B). The increase of viral yield was manifest to a greater extent in the extracellular viral yield as opposed to intracellular virus in MCF-10A cells (data not shown). Our results clearly indicate that FMDV-induced autophagic machinery is favorable for FMDV replication. In contrast, when autophagy is blocked in both cell types by 3-MA, viral loads are reduced (Figs. 4A–B). Interestingly, when autophagy is inhibited by 3-MA, FMDV replication still continues at a reduced level, suggesting that the autophagy machinery may play a promoting role in the replication of FMDV rather than being a critical factor. Based on these results, it is therefore likely that the autophagic machinery does not have a destructive role during FMDV infection and instead contributes to the formation of viral replication complexes.

To further examine the role of autophagy, we tested the effect of LC3 and Atg12 knockdown on FMDV replication. By using siRNA to reduce the amount of intracellular autophagy proteins LC3 and Atg12, an inhibitory effect on viral replication was detected, which was more significant in extracellular than in intracellular viral yields, indicating that the expression of autophagy-related proteins is important and necessary for the virus. Jackson et al. (2005) also reported a larger effect on extracellular than intracellular virus yield when the abundance of autophagy proteins Atg12 and LC3 was reduced by siRNA. These authors suggest that the reduction in autophagosome machinery decreased cell lysis early in infection, or that reduced abundance of autophagosomal machinery decreased non-lytic viral escape, contributing to the decrease in extracellular virions. We show here a similar effect on FMDV, indicating that inhibition of autophagy has a stronger effect on extracellular than intracellular viral yields.

EM analyses of cells infected with poliovirus showed the presence of large numbers of membranous vesicles with diameters of 200–400 nm, which due to the 'cytoplasmic matrix' present in the lumen of the vesicles, were postulated to develop by a mechanism comparable to that of the formation of "autolytic vesicles" (Dales et al., 1965; Schlegel et al., 1996). As mentioned above, positive-strand RNA viruses, such as poliovirus, equine arterivirus and murine hepatitis virus, all require membrane surfaces on which to assemble their RNA replication complexes (Mackenzie, 2005). For instance, in poliovirus-infected cells the membranes that are induced during infection resemble autophagosomes due to the double-membrane-bound morphology that is present even early during the infection, their specific labeling with anti-LAMP-1 antibodies, and their low buoyant densities (Jackson et al., 2005; Suhy et al., 2000). These facts obtained using other RNA viruses support our observations regarding membranous vesicles that resemble autophagosomes and their role during FMDV replication. MCF-10A cells infected with FMDV demonstrated an increase of both single- and double-membrane vesicles, as shown in Fig. 6, which are associated with the autophagy-related protein LAMP-1 as well as FMDV non-structural protein 2B. It has been reported that 2BC expression leads to the accumulation of large single-membrane vesicles (Cho et al., 1994; Suhy et al., 2000) with buoyant densities similar to those of the vesicles induced during poliovirus infection (Suhy et al., 2000). Further investigation of the ultrastructure and biochemistry of these vesicles formed during autophagy and the expression of individual viral proteins may reveal additional steps to the lipid sequestration, cytosolic wrapping, and maturation of these unique intracellular compartments. A hypothesis for the presence of autophagosome-like membranes in poliovirus-infected cells is that these membranes are induced as a component of the innate immune response (Jackson et al., 2005). Autophagy is increasingly appreciated as a functional pathway in the innate immune response to several viruses, intracellular bacteria, and parasites. The specific mechanism by which the autophagosome is utilized during replication in host cells has not yet been elucidated, however it is likely that the autophagosome provides a physical scaffold where the virus complex may reside as has been suggested for poliovirus and coxsackievirus (Jackson et al., 2005; Taylor and Kirkegaard, 2007; Wong et al., 2008).

This report constitutes the first demonstration that autophagy appears to be an important part of the FMDV replication cycle, although the mechanism by which FMDV infection induces the activation of autophagic machinery remains to be determined. The correlation between the autophagy pathway and increase of viral yield supports this observation. It could be speculated that autophagy may be a possible mechanism FMDV uses to maintain a persistent infection. A number of studies have shown the importance of the pharyngeal area tissues during persistent FMDV infection (Burrows et al., 1971, 1981; Prato Murphy et al., 1994; Zhang and Kitching, 2001). The evidence of autophagic machinery activation in FMDV-infected bovine pharynx epithelial primary cultures, a relevant tissue during persistent infection in the natural host, may indicate that under certain circumstances, autophagy enables the non-lytic release of the virion from persistently infected cells.

Materials and methods

Cell lines, virus and plasmid

Human mammary gland epithelial cells (MCF-10A) were obtained from ATCC (catalogue no. CRL-10317) and maintained in a mixture of Dulbecco's minimal essential medium (DMEM, Invitrogen, CA) and F12 Ham media (1:1, Invitrogen) containing 5% heat-inactivated fetal bovine serum (HI-FBS, HyClone), 20 ng/ml epidermal growth factor (Sigma, St.Lois, MO), 100 ng/ml cholera toxin (Sigma), 10 μg/ml insulin (Sigma), and 500 ng/ml hydrocortisone (Sigma).

FMDV type O_1 strain Campos (O_1Campos) 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 determined by plaque assay on (BHK) cells using standard techniques (Pacheco et al., 2010b). Plasmid designed to express GFP–LC3 fusion protein was produced and described elsewhere (Jackson et al., 2005). Bovine pharynx epithelial primary cultures were produced by using a modification of preparing keratinocyte cultures and described elsewhere (Barlow and Pye, 1989; O'Donnell et al., 2001).

Antibodies and reagents

Monoclonal antibody (MAb) 12FB, directed against the FMDV type O_1 structural protein VP1, has been previously described (Stave et al.,

1986). Monoclonal antibodies to the non-structural FMDV proteins 2B, 2C and 3A, were obtained from Dr. E. Brocchi, Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna, Brescia, Italy. Rabbit antibodies to LC3 (AnaSpec) and Atg5 (Santa Cruz) were used as autophagy markers. Anti-LAMP-1 monoclonal antibody (BD Biosciences) was used to detect LAMP-1, a marker of late endosomes and lysosomes. An autophagy inducer, rapamycin (Cell Signaling Technology, Beverly, MA), prepared as a 100 μM stock solution in methanol, was used at a 50 nM concentration diluted in MEM media, and the inhibitor 3-methyladenine (3-MA, Sigma, St Louis), was used at a 20 mM concentration diluted in MEM medium.

Infection and transfection of cells and confocal microscopy

Sub-confluent monolayers of MCF-10A cells or bovine pharynx epithelial primary cultures grown on 12 mm glass coverslips in 24-well tissue culture dishes were infected with FMDV O_1Campos at an MOI of 10 PFU/cell, for 1 h at 4 °C in MEM containing 0.5% HI-FBS and 25 mM HEPES, pH 7.4. After the 1 h adsorption period the supernatant was removed and the cells rinsed with ice-cold 2-morpholinoethanesulfonic acid (MES) buffered saline (25 mM MES, pH 5.5, 145 mM NaCl) to remove unadsorbed virus. The cells were washed once with media before fresh media was added, then incubated at 37 °C. To express GFP–LC3 protein, monolayers of MCF-10A cells were transfected with 1 μg of plasmid DNA by using FuGene (Roche, Mannheim, Germany), following the manufacturer's recommendations. At 24 h post-transfection the cells were infected as described above, fixed with 4% paraformaldehyde (EMS, Hatfield, PA) at the appropriate times and analyzed by confocal microscopy. To study whether rapamycin (50 nM) or 3-MA (20 mM) affected viral replication, cells were pre-incubated with each compound for 2 h at 37 °C, then subsequently infected with FMDV O_1Campos (MOI 1). Immunofluorescence and confocal microscopy using the antibodies listed above were performed as previously described (O'Donnell et al., 2005). In order to get a representative image for each experiment, we analyzed 5 to 10 independent fields examining 15–20 cells/field at a low magnification (40×) and an additional observation at higher magnification (63×) where variable numbers of fields with 2–3 cells/field were examined. Of note, each of the experiments shown in Figs. 1, 2, 3 and 6 was run at least 3–4 independent times.

Viral replication in the presence of compounds

MCF-10A cells or bovine pharynx epithelial primary cultures were incubated with the compounds at the concentrations listed above for 2 h at 37 °C prior to infection. The cells were then infected with FMDV O_1Campos at an MOI of 1 in the presence of either rapamycin or 3-MA. At the end of the adsorption period the supernatant was removed, followed by a single rinse with ice-cold MES buffered saline to inactivate unadsorbed virus. The cells were washed once with media, before fresh media with or without the rapamycin or 3-MA was added. One set of cultures was immediately frozen at −70 °C, and the other set of plates was incubated for an additional 4 h at 37 °C, and then removed to −70 °C. The plates were then thawed and cell debris was removed by centrifugation and viral titer was determined by plaque assay on BHK-21 cell monolayers.

Knockdown of autophagy genes by RNA interference

siRNA SMARTpools consisting of four RNA duplexes targeting the gene of interest (Jackson et al., 2005) and a control siRNA (siRNA Glo) were purchased from Dharmacon (Lafayette, CO). For LC3, both LC3A and LC3B were targeted, bringing the total number of transfected duplexes to eight. MCF-10A cells were grown to densities of 1.0×10^5 cells per well in 24-well tissue culture dishes in 1 ml of media without antibiotics, and transfected using Lipofectamine 2000 (Invitrogen) as previously described (Jackson et al., 2005). Forty-eight hours after transfection, the cells were infected with FMDV O_1Campos 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, rinsed with MEM containing 1% FBS and 25 mM HEPES, pH 7.4, then incubated at 37 °C. At appropriate times post-infection, the cells were frozen at −70 °C, and the thawed lysates were used to determine titers by plaque assay on BHK-21 cell monolayers.

Electron and immunoelectron microscopy

Sub-confluent monolayers of MCF-10A cells grown on tissue culture dishes (MatTek, Ashland, MA) were infected with FMDV O_1Campos, at an MOI of 10 PFU/cell for 1 h at 4 °C in MEM containing 0.5% FBS and 25 mM HEPES, pH 7.4. At the end of the adsorption period the supernatant was removed and the cells were rinsed once with ice-cold MES to remove unadsorbed virus. The cells were then washed once with media followed by addition of fresh media, and incubated at 37 °C for 4 h. At this time, the media was removed and the cells were fixed with a solution containing 2.5% glutaraldehyde, 100 mM NaCl, and 2 mM calcium chloride, in HEPES buffer, pH 7.4 for 1 h at room temperature (RT). The cells were rinsed twice with a solution containing 100 mM NaCl, 2 mM calcium chloride, and 3.5% sucrose in HEPES buffer, pH 7.4 and post-fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide. The cells were stained *en bloc* with 2% aqueous uranyl acetate overnight, dehydrated with ethanol and infiltrated with EmBed 812 resin (EMS). To view sequential organization of the replication complexes and other membrane vesicles, ribbons of 70 Angstrom sections were picked up on slot grids and grid stained with methanolic uranyl acetate and lead citrate. The sections were examined with a Hitachi T-7600 electron microscope operated at 80 kV. Digital images were captured with an Advanced Microscopy Techniques camera (Danvers, MA). For *in situ* IEM analysis, FMDV-infected and mock-infected MCF-10 cell monolayers were fixed in the MatTek dishes using a protocol described elsewhere and ultra-small gold and other reagents from Aurion (Wageningen, Netherlands) were used. Briefly, the cell monolayers were fixed using a solution containing 1% lysine, 4% paraformaldehyde (electron microscopy grade 16%) (EMS), 1% sodium meta-periodate and 0.1% glutaraldehyde in HEPES buffer, pH 7.4 for 1 h at RT. Primary antibodies were incubated overnight at 4 °C. The cell monolayers were rinsed extensively in binding buffer and incubated with an anti-mouse conjugated to ultra-small gold (dil 1/300, Aurion, EMS) for 1 h at RT. The cell monolayers were rinsed 5 times with binding buffer, 2 times with Dulbecco's Phosphate buffered saline (PBS) and fixed with 2% glutaraldehyde in PBS. After extensive washing with MilliQ water (Millipore), the ultra-small gold was enlarged to approximately 10 nm using Gold En-hance (Aurion) for 20 min at RT. The cell monolayers were incubated with a solution of 0.5% osmium tetroxide for 30 min at 4 °C on wet ice, rinsed with water, dehydrated with ethanol and embedded in Spurr's Resin per manufacturer's instructions.

Acknowledgments

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Identification of an NTPase motif in classical swine fever virus NS4B protein

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ABSTRACT

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine caused by CSF virus (CSFV), a positive-sense single-stranded RNA virus within the *Pestivirus* genus of the *Flaviviridae* family. Here, we have identified conserved sequence elements observed in nucleotide-binding motifs (NBM) that hydrolyze NTPs within the CSFV non-structural (NS) protein NS4B. Expressed NS4B protein hydrolyzes both ATP and GTP. Substitutions of critical residues within the identified NS4B NBM Walker A and B motifs significantly impair the ATPase and GTPase activities of expressed proteins. Similar mutations introduced into the genetic backbone of a full-length cDNA copy of CSFV strain Brescia rendered no infectious viruses or viruses with impaired replication capabilities, suggesting that this NTPase activity is critical for the CSFV cycle. Recovered mutant viruses retained a virulent phenotype, as parental strain Brescia, in infected swine. These results have important implications for developing novel antiviral strategies against CSFV infection.

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Introduction

CSF is a highly contagious and often fatal disease that affects swine throughout various regions of the world. The etiological agent, CSFV, is an enveloped virus that belongs to the genus *Pestivirus* within the family *Flaviviridae* (Fauquet et al., 2005). The CSFV genome is a positive-sense single-stranded RNA that encodes a single polyprotein precursor that is co- and post-translationally processed by cellular and virus-encoded proteases to produce four structural (C, E^{rns}, E1, and E2) and 8 non-structural (NS) proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Rumenapf et al., 1993; Lindenbach et al., 2007; Thiel et al., 1996). Replication of Pestivirus viral genome is suggested to occur in close association with cytoplasmic membranes through the synthesis of a negative-stranded full-length genome (Gong et al., 1996; Lindenbach et al., 2007). This function is mediated by the activity of NS proteins. NS5B is a viral RNA-dependant RNA polymerase (Steffens et al., 1999; Xiao et al., 2002, 2006); its activity *in vitro* is enhanced by the presence of NS3 protein (Wang et al., 2010). NS3 has multiple functions and is essential for virus replication (Xu et al., 1997). The serine proteinase activity of NS3 is responsible for cleavage of NS4A, 4B, 5A, and 5B and requires the 64 amino acid NS4A protein as a cofactor (Xu et al., 1997; Tautz et al., 1997; Moulin et al., 2007). Additionally, NS3 possesses both nucleoside triphosphatase (NTPase) and RNA helicase activities (Suzich et al., 1993; Tamura et al., 1993; Wen et al., 2007, 2009). The RNA helicase-active portion of NS3 contains a nucleotide-binding motif (NBM) characteristic of all helicases and numerous NTPases (Walker et al., 1982; Gorbalenya and Koonin, 1989). The role of NS2 and NS5A in CSFV replication is less understood. *In vitro* studies using CSFV RNA replicons showed that NS2 is not essential for replication although its presence increased the persistence of RNA replicons in transfected cells (Moser et al., 1999). The role of NS5A in replication may involve at least two different activities: the stimulation of virus replication via formation of multisubunit replication complexes and the inhibition of virus translation (Xiao et al., 2009; Sheng et al., 2010).

The role of NS4B in CSFV replication remains unclear. In Hepatitis C Virus (HCV) and other related flaviviruses, NS4B was found to be an integral hydrophobic membrane protein required for the assembly of "membranous webs" that are derived from endoplasmic reticulum (ER) or the Golgi apparatus and are important for RNA replication (Hugle et al., 2001; Egger et al., 2002; Kim et al., 2004; Miller et al., 2006; Weiskircher et al., 2009). Einav et al. (2004) have shown that HCV NS4B contains a NBM which is conserved among HCV genotypes. This structural motif binds and hydrolyzes ATP, GTP and GDP and also possesses adenylate kinase activity (Einav et al., 2004; Thompson et al., 2009). Amino acid substitutions in the NBM of HCV NS4B impaired NTP binding and hydrolysis and resulted in decreased NTPase and adenylate

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kinase activity (Einav et al., 2004; Thompson et al., 2009). Furthermore, the NBM was shown to be critical for the role of NS4B during *in vitro* HCV replication (Einav et al., 2004; Lindstrom et al., 2006) and in cellular transformation and tumor formation (Einav et al., 2008).

Conserved sequence elements in NBM-containing proteins that possess NTPase activity harbor characteristic Walker A and Walker B motifs. The Walker A motif consists of a G-rich phosphate-binding loop with a consensus sequence G/AXXXXGKS/T (where X could be any residue) that is involved in binding of β- and γ-phosphates of NTPs. The Walker B motif consists of an Asp residue preceded by a stretch of hydrophobic amino acids (h) hhhhD or hhhhDD/E, that chelates the Mg^{2+} of the Mg–NTP complex (Walker et al., 1982; Gorbalenya and Koonin, 1989; Mimura et al., 1991; la Cour et al., 1985; Pai et al., 1989). In this study, data is presented demonstrating that CSFV NS4B has NTPase activity. *E. coli* expressed His-tagged CSFV NS4B purified protein hydrolyzes both ATP and GTP. Conserved Walker A and B motifs, characteristic of NBMs in CSFV NS4B (residues 209–216 and 335–342, respectively), are shown to be highly conserved in NS4B proteins of the other pestiviruses, Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV). It is also demonstrated that the enzymatic activity of the protein is affected by specific residue substitutions within identified Walker A and B motifs. Additionally, we have observed that NS4B of pestiviruses lacks the universally conserved K residue in the GKS/T signature of A motifs, but a highly conserved K_{206} residue is located at the N-terminal end of the canonical motif that has an effect on the NTPase activity of the protein. Additional results suggest that specific substitutions within the canonical Walker A and/or Walker B motifs in NS4B are deleterious for CSFV, while a CSFV Walker A revertant virus or viruses harboring mutations at K206 were viable and retained a virulent phenotype in infected swine.

Results

Identification of a nucleotide-binding motif in NS4B

Conserved sequence elements in NBM-containing viral proteins include both Walker A and Walker B motifs (Walker et al., 1982; Gorbalenya and Koonin, 1989) (Fig. 1). Amino acid sequence analysis of NS4B proteins showed that pestiviruses possess sequences resembling canonical Walker A (G/AXXXXGKS/T) and Walker B (hhhhD or hhhhDD/E) motifs (Figs. 2 and 3A). These sequences are located towards the C-terminal portion of CSFV NS4B, encompassing amino acid residues 209–216 and 335–342 of the protein, for A and B motifs respectively. Furthermore it was observed that the putative Walker A motif in pestivirus NS4B lacks the universally conserved K residue in the GKS/T signature sequence, where K has been substituted to V or I (Figs. 2 and 3A). Instead, NS4B of these viruses has a conserved K residue (K206 in CSFV) at the N-terminal end of the Walker A motif, resembling the position of the K residue in deviant Walker A motifs found in ATPase catalytic centers of phage terminases (Mitchell and Rao, 2004) and some prokaryotic and yeast DNA-dependent ATPases or GTPases (Koonin, 1993a, 1993b) (Fig. 3B). The putative Walker B motif in CSFV NS4B, $LLGVDSEG_{335–342}$, concurs with the consensus hhhhD (Fig. 3A). The motif is also conserved among other pestiviruses (Fig. 3A). The sequence $DSEG_{339–342}$ within the B motif matches the consensus DXXG found in highly conserved GTP-binding protein families including elongation factors, ras p21, phosphoenolpyruvate carboxykinase and guanine nucleotide-binding proteins of adenylate cyclase (G proteins) (Dever et al., 1987) (data not shown).

CSFV NS4B has NTPase activity

His-tagged CSFV strain Brescia NS4B protein was expressed in *E. coli* (Fig. 4A), and purified to near homogeneity using cobalt immobilized affinity chromatography (IMAC) resins. Protein purity was verified by Coomassie blue staining (Fig. 4B) and Western blot using an anti-poly-His monoclonal antibody (data not shown). Assessment of the ATPase and GTPase activities of wild-type purified CSFV NS4B was performed by evaluating the release of inorganic phosphate using a colorimetric assay as described in Materials and Methods. Consistent with the sequence analysis, purified wild-type NS4B catalyzed the hydrolysis of ATP and GTP (Fig. 4C). Time course analysis of ATP and GTP hydrolysis by wild-type NS4B revealed that the enzymatic activity achieved maximum kinetics after 2 h of incubation with ATP or GTP (Fig. 4C). Data suggest a

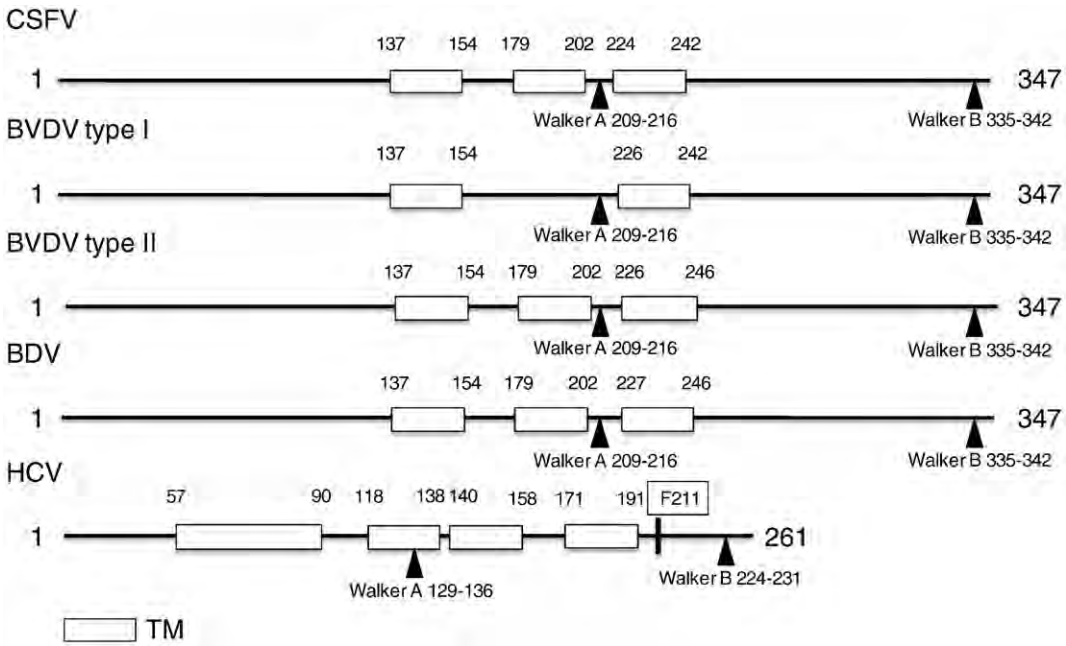

Fig. 1. Schematic representation of NS4B protein from CSFV, BVDV types I and II, BDV, and HCV genotype 1b showing predicted membrane-spanning regions (TM) according to Hofmann and Stoffel, 1993 (TMpred program). Arrowheads indicate locations of Walker A and B motifs. For HCV F211 is the nucleotide binding motif G (Thompson et al., 2009). Numbers indicate amino acid residues.

Accession #	CSFV Strain	Walker A motif	Walker B motif
AY578687	BICv	KSDGLLGTGVS$_{206-216}$	LLGVDSEG$_{335-342}$
X96550	CAP	.....	.....
X87939	Alfort/187	.....	.....
AY578688	CSICv	.....	.....
AY554397	96TD	.....	.....
AY568569	0406/CH/01/TWN	.....	.....
AY775178	Shimen/HVRI	.....	.....
AY805221	C/HVRI	.....	.....
AY259122	Riems	.....	.....
AF326963	Eystrup	.....	.....
AY367767	GXWZ02	.....	.....
AF531433	HCLV	.....	.....
AF407339	39	.....	.....
AF333000	cF114	.....	.....
J04358	HCV	.....	.....
AF091507	HCLV	.....M...	.....
U90951	Alfort A19	.....I...	.....
U45478	Glentorf	.....I...	.....

Fig. 2. Sequence alignment of identified Walker A (amino acid positions 206 to 216) and Walker B (amino acid positions 335 to 342) within CSFV NS4B protein. The Walker A motif consists of a Gly-rich phosphate-binding loop with consensus sequence G/AXXXXGKS/T (where X is any residue). The Walker B motif consists of Asp preceded by a stretch of hydrophobic amino acids hhhhD or hhhhDD/E (where h is a hydrophobic residue) (Walker et al., 1982; Gorbalenya and Koonin, 1989; Mimura et al., 1991; la Cour et al., 1985 and Pai et al., 1989). Notice that the putative Walker A motif indentified within CSFV NS4B lacks the universally conserved Lys residue in the GKS/T signature sequence, where K has been substituted to V.

higher rate of ATP hydrolysis over GTP hydrolysis (30 μM Pi vs. 20 μM Pi, respectively) (Fig. 4C).

Mutations in Walker A and B motifs affect NS4B NTPase activity

To further confirm CSFV NS4B NTPase activity, specific residue substitutions were introduced into the identified Walker A (GLLGTGVS$_{209–216}$) and Walker B (DSEG$_{339–342}$) motifs (Fig. 5), and mutant proteins were tested for their ability to hydrolyze ATP and GTP. Walker A (G209A, L211N, and G214A), Walker B (D339A and G342A), and Walker A/B (G209A, L211N, G214A, D339A, and G342A) mutant proteins demonstrated decreased ATPase and GTPase activities relative to wild-type NS4B (Figs. 6A and B). Overall, mutations in the A motif seem to have a more significant effect on NS4B NTPase activity than mutations introduced in the B motif. To further assess the role of specific residues in the NTPase activity of CSFV NS4B protein, we constructed a Walker A revertant protein harboring an N211L substitution (X_2 position in the canonical A motif) within the backbone of the Walker A mutant (Fig. 5). Interestingly, both ATPase and GTPase activity were partially restored when N211 was reverted back to L in CSFV NS4B protein (Figs. 6C and D). Similar mutations have been shown to affect the NTPase activities of HCV NS4B or Poliovirus 2C proteins (Mirzayan and Wimmer, 1992; Einav et al., 2004). Overall, data confirmed the ATPase and GTPase function of the NS4B protein.

A deviant Walker A motif in CSFV NS4B

Since the identified Walker A motif in CSFV NS4B (GLLGxGVS) deviates from the canonical P-loop sequence (GxxxxGKS/T) by lacking the important phosphate-binding K residue (Figs. 2 and 3B), we substituted conserved K_{206} residues, K206A or K206R, at the N-terminal end of the motif (Fig. 5) and assessed the NTPase activity of the modified proteins. An R residue instead of a K residue is observed at analogous position in NS4B of some BDV isolates (Fig. 3B). Both mutant proteins showed significantly reduced ATPase and GTPase activities relative to wild-type protein (t-test, $P<0.01$). Only K206R mutant protein retained both ATPase and GTPase activities (Figs. 6E and F). These results suggest that in CSFV NS4B the highly conserved K_{206} in the Walker A motif plays a role in NTP hydrolysis. Similar deviations from canonical Walker A motifs have been observed in bacteriophage terminases (Mitchell and Rao, 2004; Tsay et al., 2009) (Fig. 3B).

Effects of NS4B Walker A and Walker B motif disruption on CSFV replication

To assess the role of the Walker A and B motifs in the replication of CSFV a set of mutant viruses was constructed containing amino acid substitutions within the NS4B protein of the CSFV strain Brescia full-length cDNA clone, pBIC. *In vitro* transcribed RNA derived from wild-type and mutant cDNA clones was used to transfect CSFV permissive SK6 cells. Supernatants were harvested and used to infect naive SK6 cell monolayers. CSFV was detected only in cells infected with supernatants derived from wild-type, Walker A revertant, Walker A deviant K206R, and Walker A deviant K206A transfected cells. SK6 cells transfected with RNA transcribed from Walker A mutant, Walker B mutant, and Walker A/B mutant DNA constructs did not yield infectious viruses in three independent attempts (data not shown). Mutations introduced into NS4B were maintained in rescued viruses.

To assess the ability of rescued mutant viruses to replicate *in vitro*, their growth characteristics were evaluated in a multistep growth curve

A

Virus/Protein	Walker A motif								(n)	Walker B motif							
Consensus	*G*	X_1	X_2	X_3	X_4	*G*	*K*	*S/T*	X	H	H	H	H	*D*	X	X	*G*
CSFV/NS4B	*G*	L	L	G	X	*G*	*V*	*S*	(119)	L	L	G	V	*D*	S	E	*G*
BVDV/NS4B	*G*	L	L	G	X	*G*	*I*	*S*	(123)	L	L	G	M	*D*	S	E	*G*
BDV/NS4B	*G*	L	L	G	T	*G*	*V*	*S*	(119)	L	L	G	V	*D*	S	E	*G*
HCV1b/NS4B	*G*	S	I	G	L	*G*	*K*	*V*	(95)	V	P	E	S	*D*	A	A	*A*
HCV3/NS4B	*G*	S	I	G	L	*G*	*R*	*V*	(88)	V	P	E	S	*D*	A	A	*A*
HAV/2C	*G*	K	R	G	G	*G*	*K*	*S*	(41)	V	C	I	I	*D*	D	I	*G*
Polio/2C	*G*	S	P	G	T	*G*	*K*	*S*	(34)	V	V	I	M	*D*	D	L	*N*
FMDV/2C	*G*	K	S	G	Q	*G*	*K*	*S*	(39)	V	V	V	M	*D*	D	L	*G*

B

Virus/Protein	Deviant Walker A motifs										
VHML/gp22	*K*	S	M	R	V	*G*	*Y*	*T*			
Lambda/gpA	*K*	S	A	R	V	*G*	*Y*	*S*			
RM_378/gpPhiN315_66	*K*	P	R	Q	M	*G*	*V*	*T*			
CSFV/NS4B	*K*	S	D	G	L	L	G	X	*G*	*V*	*S*
BVDV/NS4B	*K*	S	D	G	L	L	G	X	*G*	*I*	*S*
BDV/NS4B	*R/K*	S	D	G	L	L	G	T	*G*	*V*	*S*

Fig. 3. (A) Identified Walker A and B motifs within CSFV NS4B are highly conserved among Pestiviruses. Consensus indicates a set of conserved amino acids found in both the GTP-binding members of the G protein superfamily, as well as several viral proteins with nucleotide binding domains. CSFV: classical swine fever virus; BVDV: Bovine Viral Diarrhea Virus; BDV: Border Disease Virus; HCV: Hepatitis C Virus; Polio/2C: Poliovirus 2C protein; HAV2C: Hepatitis A Virus 2C protein; FMDV2C: Foot and Mouth Disease Virus 2C protein. In parenthesis (n) is the number of omitted residues. (B) Alignment of Pestiviruses' Walker A motif with Walker A deviant I motifs (Mitchell and Rao, 2004) found in some bacteriophage proteins where the conserved Lys residue in the GKS/T signature sequence is absent (shaded) and shifted to the beginning of the motif (shaded). VHML: *Vibrio harveyi* bacteriophage; Lambda/gpA: *E. coli* Lambda phage; RM_378gpPhiN315_66: *R* RM_378 *R. marinus* phage.

and compared with parental BICv. All mutant viruses demonstrated growth kinetics similar to BICv although virus titers were at least 0.5 to 1 log lower than the parental virus (Fig. 7). A single substitution (N211L) in a Walker A mutant full-length cDNA clone yielded a Walker A revertant virus, restoring the production of CSFV progeny in SK6 cells. Similarly, substitutions of K206 to A or R, although reducing the NS4B NTPase activity (Figs. 6E and F), do not appear to significantly alter the ability of these viruses to replicate *in vitro*.

Interestingly, these viruses retained a virulent phenotype in infected swine. Animals intramuscularly (IM) inoculated with 10^5 $TCID_{50}$ of any of the mutant viruses (Walker A revertant, Walker A deviant K206R, and Walker A deviant K206A) progressed at the same rate as animals infected with parental BICv in terms of time of death, onset and duration of fever (Table 2), and viremia (Table 3). Viruses isolated from infected pigs retained mutations introduced into NS4B.

Discussion

CSFV non-structural proteins regulate virus replication (Lindenbach et al., 2007; Xu et al., 1997; Moser et al., 1999; Steffens et al., 1999; Xiao et al., 2002, 2006, 2009; Moulin et al., 2007; Sheng et al., 2010). The function of CSFV NS4B protein in viral replication and pathogenesis is poorly understood. Previously, it was found that CSFV NS4B is involved in virulence by modulating the host immune response (Fernandez-Sainz et al., 2010). Here we have shown that CSFV NS4B has ATPase and GTPase activities, and that this activity is mediated by conserved Walker A and Walker B motifs found in the NBM of NTPases (Walker et al., 1982; Gorbalenya and Koonin, 1989). Furthermore, since the identified Walker A motif lacks the universally conserved K residue in the GKS/T signature sequence, we have shown that a highly conserved K_{206} in CSFV NS4B plays *in vitro* a role in ATP and GTP hydrolysis. In that regard, the CSFV NS4B Walker A motif resembles the previously described deviant I Walker A motifs (Mitchell and Rao, 2004).

CSFV NS4B readily hydrolyzes ATP over GTP. Incubation of purified CSFV NS4B with GTP resulted in lower concentrations of Pi relative to the incubation with ATP (Fig. 4C). Similarly, it has been observed that purified His-tagged HCV NS4B hydrolyzes both ATP and GTP, but the protein displayed a higher affinity for ATP and 25-fold faster conversion to ADP than conversion of GTP to GDP (Thompson et al., 2009).

To confirm that ATP and GTP hydrolysis was associated with recombinant CSFV NS4B, a set of mutant proteins was constructed by

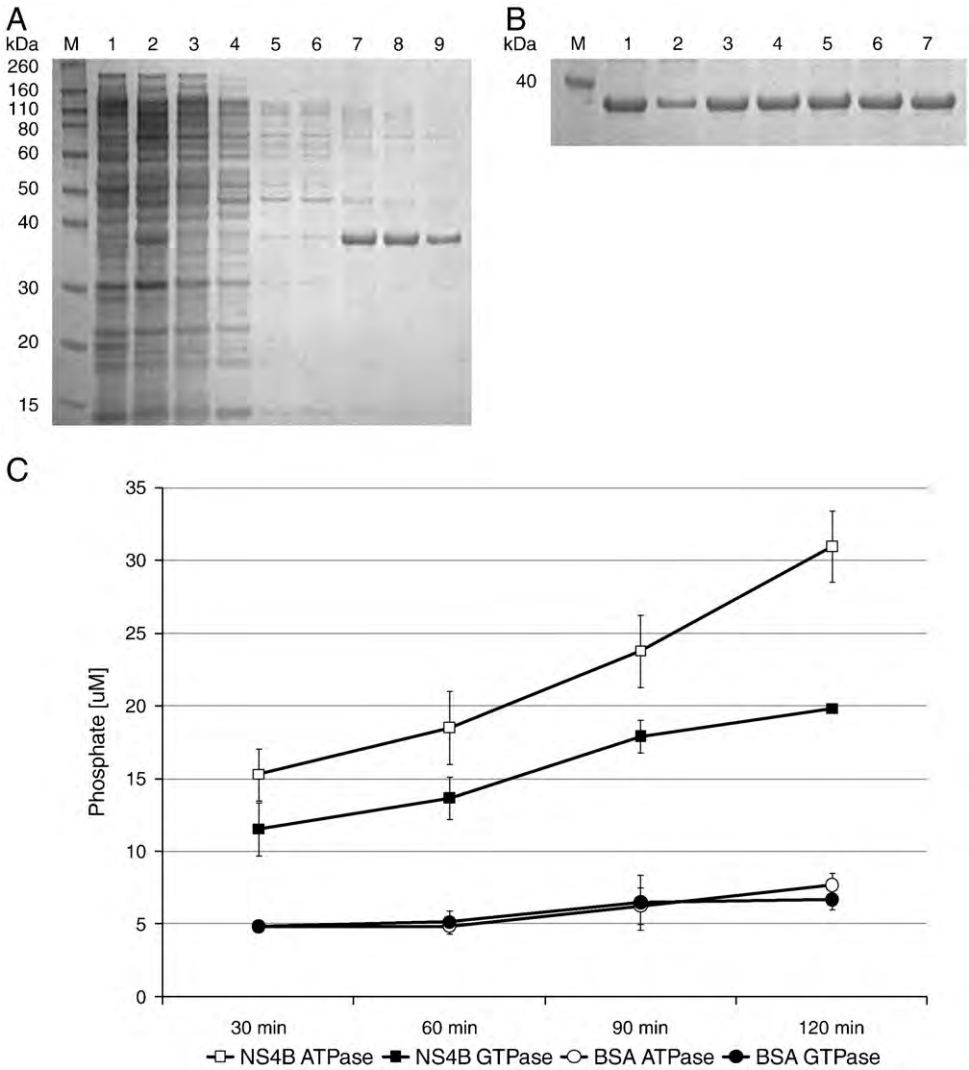

Fig. 4. Purification of *E. coli*-expressed wild-type and mutant CSFV NS4B proteins by immobilized metal ion affinity chromatography (IMAC). (A) Coomassie blue staining of 10% SDS-PAGE showing purification of *E. coli*-expressed wild-type NS4B using HisPur Cobalt Spin Columns. Lanes: M, molecular weight marker (in kDa); 1, cell lysate of BL21(DE3)pLysS transformed with pRSETA vector; 2, cell lysate of BL21(DE3)pLysS transformed with pRSETA encoding wild-type NS4B; 3–6, wash fractions; 7–9, elution fractions containing purified protein. (B) Coomassie blue staining of 10% SDS-PAGE showing purified *E. coli*-expressed wild-type (lane 1) and mutant (lanes 2–7) NS4B. Lanes: M, molecular weight marker (in kDa); 1, wild-type NS4B; 2, Walker A; 3, Walker A revertant; 4, Walker B; 5, Walker A/B; 6, K206R; 7, K206A mutant. (C) ATPase and GTPase activity of wild-type NS4B protein relative to bovine serum albumin (BSA). Proteins were incubated at 30 °C in the presence of Pi-free ATP or GTP and release of Pi (means ± standard deviations from 3 independent experiments) was detected at indicated time points.

introducing amino acid substitutions into the identified A and B motifs. These motifs are involved with binding of phosphate and purine rings of NTP substrates. A significant decrease in NS4B enzymatic activity was observed with Walker A, Walker B, and Walker A/B mutants, demonstrating the NTPase function of the protein (Figs. 6A and B). Further confirmation of the NTPase activity

Virus/Protein	Walker A motif	Walker B motif
CSFV Brescia	^{206}K S D G L L G T G V S^{216}	^{335}L L G V D S E G^{342}
Walker A mutant	K S D A L N G T A V S	L L G V D S E G
Walker A revertant	K S D A L L G T A V S	L L G V D S E G
Walker B mutant	K S D G L L G T G V S	L L G V A S E A
Walker A/B mutant	K S D A L N G T A V S	L L G V A S E A
Walker A deviant K206R	R S D G L L G T G V S	L L G V D S E G
Walker A deviant K206A	A S D G L L G T G V S	L L G V D S E G

Fig. 5. Set of CSFV NS4B Walker A and Walker B mutant proteins and viruses produced in this study. Underlined are amino acid substitutions.

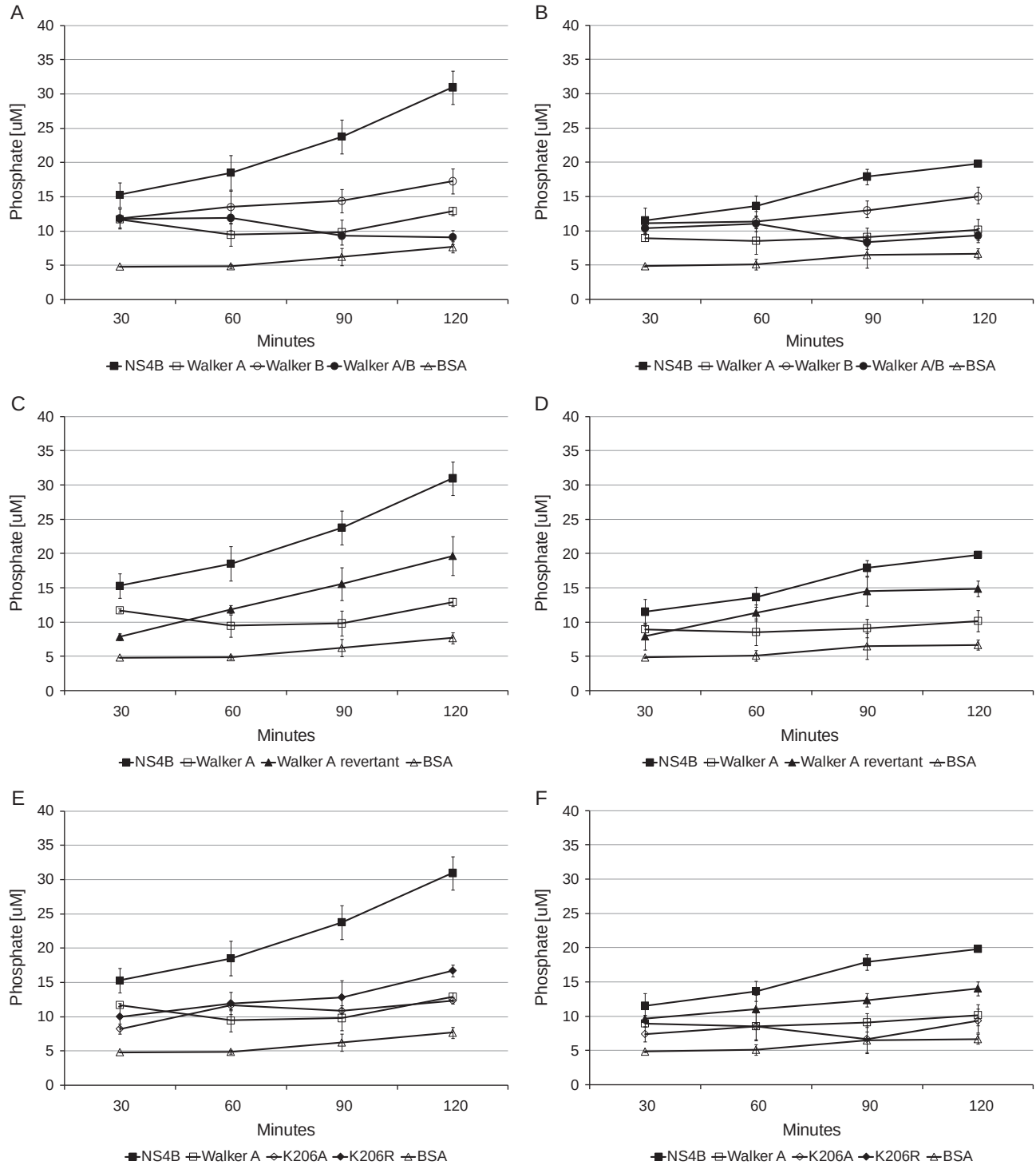

Fig. 6. ATPase (A, C, and E) and GTPase (B, D, and F) activities of wild-type and mutant CSFV NS4B proteins. ATP and GTP hydrolysis of wild-type NS4B relative to Walker A, Walker B, Walker A/B mutants, and BSA (A and B); Walker A revertant NS4B protein (C and D), and Walker A K206A and K206R NS4B mutant proteins (E and F). Assays were performed at 30 °C in the presence of Pi-free ATP or GTP and release of Pi (means ± standard deviations from 3 independent experiments) was detected at 30, 60, 90, and 120 min of incubation.

of NS4B was derived from the Walker A revertant mutant protein. Reversion of N_{211} to wild-type L_{211}, while preserving G209A and G214A mutations, partially restored hydrolysis of ATP and GTP (Figs. 6C and D). The hydrophobic residue at the X_2 position of the A motif appears to have an important effect on NTPase activity, since its mutation in HCV NS4B nearly abolishes GTP hydrolysis (Einav et al., 2004). Furthermore, mutation at an analogous position in poliovirus 2C protein NBM rendered a non-infectious virus (Mirzayan and Wimmer, 1992). The observed changes in CSFV NS4B NTPase activity could be the result of decreased binding and/or decreased hydrolysis of ATP or GTP. In that regard, a double Walker A and B mutant of HCV NS4B was shown to bind both ATP and GTP but with significantly lower affinity while a quintuple mutant was unable to bind either ATP or GTP (Thompson et al., 2009). In the same study, K_{cat} measurements for this double HCV NS4B mutant displayed insignificant changes relative to wild-type, suggesting that these mutations had a strong effect on binding of NTP but little or no effect on the turnover rate.

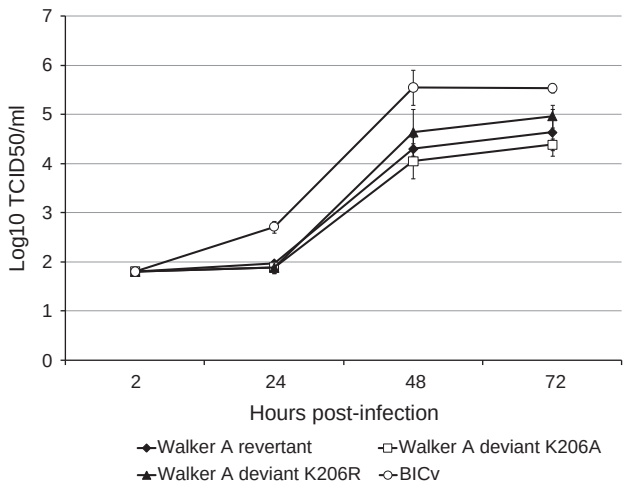

Fig. 7. *In vitro* growth characteristics of NS4B mutants and parental BICv. SK6 cell cultures were infected (MOI = 0.01) with each of the mutants or BICv and virus yield titrated at times post infection in SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: $\geq \log_{10}$ 1.8 TCID50/ml.

The Walker A motif is one of the most common and highly conserved motifs found in genomes (Koonin, 1993a). The identified A motif in CSFV NS4B as well as in other pestiviruses lacks the universally conserved K residue in the GKS/T signature. In the signature sequence, the K residue interacts with the negatively charged β- and γ-phosphates of NTP (la Cour et al., 1985; Pai et al., 1989, 1990). Large terminase proteins (subunits of DNA packing machines in large DNA viruses), including the well-characterized λ phage gpA and SPP1 G2P, lack a canonical Walker A motif. However, a conserved K is consistently located at the N-terminal end of the putative Walker A motifs of all these phage terminases (Mitchell and Rao, 2004). These non-canonical Walker A motifs are known as deviant I Walker A motifs. Here we confirmed that K_{206} is important for NS4B hydrolysis of NTPs, suggesting that pestiviruses may harbor a deviant I Walker A motif in NS4B. However, viruses harboring substitutions of the conserved K_{206} were able to replicate in swine macrophages and retained their virulent phenotype in infected swine, suggesting that in the context of the virus infection this residue is not critical for replication. Nonetheless, this finding may have important implications for developing novel antiviral compounds against CSFV.

We have observed that disruption of predicted Walker A and B motifs in CSFV NS4B significantly affected virus replication. However, replication of the mutant Walker A virus was rescued by introducing an N211L substitution. This virus retained the capability to cause disease in swine, suggesting that L_{211} is critical for the NTPase activity of the virus, and that the NTPase activity of NS4B plays a key role in virus viability and virulence. Binding and hydrolysis of nucleotides by viral proteins mediates a variety of events involved with virus replication including signaling, membrane trafficking, and membrane fusion. For instance, Poliovirus nonstructural 2C protein is an ATPase found in abundance in the membranous replication complex where viral RNA synthesis occurs (Cho et al., 1994; Teterina et al., 1992). Analogous to picornaviral 2C proteins, HCV NS4B possesses NTPase activity and is involved in the reorganization of intracellular membranes and virus replication (Pfister and Wimmer, 1999; Samuilova et al., 2006; Thompson et al., 2009). Furthermore, mutations in the NBM of poliovirus 2C and HCV NS4B impaired viral replication (Teterina et al., 1992; Mirzayan and Wimmer, 1992; Einav et al., 2004).

In summary, we predicted, detected, and mapped residues that confer NTPase activity to CSFV NS4B protein. Furthermore, we showed that residues outside the canonical Walker A motif are important for the *in vitro* enzymatic activity of NS4B, as in described deviant type I Walker A motifs. The NTPase activity of this protein is required for CSFV replication. The defined motifs may represent specific targets for anti-CSFV compounds.

Table 1
Sequences of oligonucleotide primers used for generation of wild-type (wt) and mutant CSFV NS4B protein and mutant viruses.

Primer	Sequence (5′→3′)
wt NS4B for	GGATCCGCTCAGGGGGATGTGCAGAGATGT
wt NS4B rev	CCATGGTTATAGCTGGCGGATCTTTCCTTC
Walker A for	GAAAAAGCGATGCTTTGAACGGCACAGCGGTTAGTGCGGCTATGG
Walker A rev	CCATAGCCGCACTAACCGCTGTGCCGTTCAAAGCATCGCTTTTTC
Walker A revertant for	GAAAAAGCGATGCTTTGCTAGGCACAGCGGTTAGTGCGGCTATGG
Walker A revertant rev	CCATAGCCGCACTAACCGCTGTGCCTAGCAAAGCATCGCTTTTTC
Walker B for	GAACTACTGGGAGTAGCTAGCGAAGCAAAGATCCGCCAGCTA
Walker B rev	TAGCTGGCGGATCTTTGCTTCGCTAGCTACTCCCAGTAGTTC
K206R for	TCAATCAGGCGCGGAAGAAGCGATGCTTTG
K206R rev	CAAAGCATCGCTTCTTCCGCGCCTGATTGA
K206A for	TCAATCAGGCGCGGAGCAAGCGATGCTTTG
K206A rev	CAAAGCATCGCTTGCTCCGCGCCTGATTGA

Codon changes are underlined; for, forward primer; rev, reverse primer.

Materials and methods

Cloning, expression, purification, and detection of wild-type and mutant NS4B proteins

The gene encoding for CSFV strain Brescia NS4B protein (1041 bp) was amplified by polymerase chain reaction (PCR) using specific primers (Table 1). The resulting PCR fragment was cloned into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA). The plasmid was subsequently digested with *BamH*I and *Nco*I and the expected 1041 bp NS4B fragment was directionally cloned into the pRSETA expression plasmid (Invitrogen). Site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA) was utilized to generate mutant NS4B genes using primers described in Table 1. Complete sequences of all DNA constructs were verified by automated sequencing.

One Shot BL21 (DE3) pLysS *E. coli* (Invitrogen) cells were transformed with 10 ng of pRSETA plasmid encoding wild-type or mutant NS4B, cultured in MagicMedia (Invitrogen) for 21 h at 37 °C with shaking, and harvested by centrifugation at 3200 ×g for 15 min. Cell pellets were then treated with CelLytic buffer (Sigma Aldrich, St. Louis, MO) according to the manufacturer's large scale extraction protocol. For His-tagged protein purification, cell lysates were applied to HisPur Cobalt Spin Columns (Thermo Fisher Scientific, Rockford, IL) and the collected fractions were analyzed on a 10% NuPage Novex Bis–Tris gel (Invitrogen) using a discontinuous SDS-PAGE system. Western blots were performed using polyvinylidene fluoride (PVDF) membranes (Invitrogen) and a His-Tag antibody (Novagen, EMD Biosciences, Madison, WI). Reactions were developed using alkaline phosphatase labeled goat anti-mouse IgG antibody with the WesternBreeze Chemiluminescent Detection Kit (Invitrogen). The purified proteins were desalted twice using 7K MWCO Zebra Spin Desalting Columns (Thermo Fisher Scientific). Protein concentration was determined by BCA Protein Assay (Thermo Fisher Scientific) against known standards at OD of 595 nm using a NanoDrop instrument (Thermo Scientific NanoDrop Products, Wilmington, DE).

NTPase assays

The ATPase and GTPase activities of recombinant wild-type and mutant CSFV NS4B proteins were determined using the Bioassay Systems colorimetric ATPase/GTPase assay system according to the

Table 2
Swine survival and fever response following infection with CSFV NS4B mutants and parental BICv.

Virus	No. of survivors/ total no.	Mean time to death (days ± SD)	Fever: No. of days to onset (days ± SD)	Fever: Duration no. of days (days ± SD)	Fever: Max daily temperature (°F ± SD)
Walker A revertant	0/3	7.7 (0.6)	3.7 (0.6)	4.7 (0.6)	105.2 (0.8)
Walker A deviant K206A	0/3	9 (1)	3.5 (0.6)	5.7 (1.2)	106.5 (1.2)
Walker A deviant K206R	0/3	8.7 (0.6)	3 (0)	4.7 (0.6)	105.9 (0.8)
BICv	0/3	9 (1)	3 (0)	6 (1)	107.0 (0.8)

manufacturer's recommendations (QuantiChrom ATPase/GTPase Assay Kit, BioAssay Systems, Hayward, CA). Briefly, NTPase assays were performed with 1.5 μg of protein in 20 μl of Assay Buffer (40 mM Tris, 80 mM NaCl, 8 mM $MgAc_2$, and 1 mM EDTA, pH 7.5). Forty mM of ATP or GTP was added to each well and the reaction was incubated for 30, 60, 90, or 120 min at 30 °C. The assay contained purified inorganic phosphate (Pi)-free NTP substrate to ensure lowest possible background signals. Following incubation with ATP or GTP, 200 μl of BioAssay Systems Reagent were added and the reaction mixtures were developed for 30 min. Samples were read at 635 nm on a Synergy HT Microplate reader (BioTek, Winooski, VT). Phosphate standards provided with the kit were used to generate a standard curve and to calculate the enzymatic activity of wild-type and mutant NS4B proteins. Purified Pi-free Bovine Serum Albumin (Sigma, St. Louis, MO) was used in the assays as a negative control. The results were determined by calculating the amount of Pi produced by the enzyme after incubation with the substrate for the indicated period of time.

Construction of CSFV mutants

A full-length cDNA infectious clone (IC) of virulent CSFV strain Brescia (pBIC) (Risatti et al., 2005) was used as a template in which putative NBM sites in the NS4B gene were mutated. Mutations were introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) performed per manufacturer's instructions and using the primers described in Table 1. Full-length genomic clones were linearized with *Srf*I and *in vitro* transcribed using the T7 Megascript system (Ambion, Austin, TX). RNA was precipitated with LiCl and transfected into SK6 cells (Terpstra et al., 1990) by electroporation at 500 V, 720 Ω, and 100 W with a BTX 630 electroporator (BTX, San Diego, CA). Cells were cultured in Dulbecco's minimal essential media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO), seeded in 6-well plates and incubated for 4 days at 37 °C and 5% CO_2. Effectiveness of the transfection was assessed by immunoperoxidase staining using CSFV E2 monoclonal antibody WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Transfected cultures were harvested at day 4 post-transfection and the presence of infectious virus in the supernatant was detected by seeding the material on fresh SK6 cell cultures. After 4 days, cells were fixed and the presence of E2 expression was assessed by immunoperoxidase staining as described above. Virus growth characteristics were evaluated in multistep growth curves. SK6 cells were infected at a MOI = 0.01 $TCID_{50}$ and virus adsorbed for 1 h (time zero). Samples were then collected at times post-infection through 72 h, and virus yields titrated on SK6 cells. Virus titers were calculated using the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was ≥1.8 $TCID_{50}$/ml.

Table 3
Viremia in pigs inoculated with parental BICv and NS4B mutant viruses.

	Days post infection: 4	6	8	9
Walker A rev virus				
Pig 1	3.97	5.63	7.1	D
Pig 2	3.80	5.80	6.80	D
K206R virus				
Pig 1	3.80	5.97	6.97	D
Pig 2	3.63	5.63	6.63	D
K206A virus				
Pig 1	3.63	5.80	6.97	D
Pig 2	3.80	5.97	7.10	D
BICv				
Pig 1	3.80	5.80	7.10	D
Pig 2	4.00	5.97	6.97	D

Values expressed as $\log_{10}$ TCID50/ml. D: deceased.

DNA sequencing and analysis

All DNA clones were completely sequenced with CSFV-specific primers by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA sequencer (Applied Biosystems). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Mutant viruses isolated from infected animals were sequenced in the region where changes were introduced.

Animal experiments

Mutant viruses recovered from transfected SK6 cells were screened for their virulence phenotype in swine relative to the virulent Brescia strain. Swine used in these studies were 10 to 12 week old, forty-pound commercial breed pigs inoculated intramuscularly (IM) with 10^5 $TCID_{50}$ of mutant viruses or wild-type parental virus (BICv). Clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the 21-day experiment.

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Expert Reviews

Development of vaccines toward the global control and eradication of foot-and-mouth disease

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Foot-and-mouth disease (FMD) is one of the most economically and socially devastating diseases affecting animal agriculture throughout the world. Although mortality is usually low in adult animals, millions of animals have been killed in efforts to rapidly control and eradicate FMD. The causing virus, FMD virus (FMDV), is a highly variable RNA virus occurring in seven serotypes (A, O, C, Asia 1, Sat 1, Sat 2 and Sat 3) and a large number of subtypes. FMDV is one of the most infectious agents known, affecting cloven-hoofed animals with significant variations in infectivity and virus transmission. Although inactivated FMD vaccines have been available for decades, there is little or no cross-protection across serotypes and subtypes, requiring vaccines that are matched to circulating field strains. Current inactivated vaccines require growth of virulent virus, posing a threat of escape from manufacturing sites, have limited shelf life and require re-vaccination every 4–12 months. These vaccines have aided in the eradication of FMD from Europe and the control of clinical disease in many parts of the world, albeit at a very high cost. However, FMDV persists in endemic regions impacting millions of people dependent on livestock for food and their livelihood. Usually associated with developing countries that lack the resources to control it, FMD is a global problem and the World Organization for Animal Health and the United Nations' Food Agriculture Organization have called for its global control and eradication. One of the main limitations to FMDV eradication is the lack of vaccines designed for this purpose, vaccines that not only protect against clinical signs but that can actually prevent infection and effectively interrupt the natural transmission cycle. These vaccines should be safely and inexpensively produced, be easy to deliver, and also be capable of inducing lifelong immunity against multiple serotypes and subtypes. Furthermore, there is a need for better integrated strategies that fit the specific needs of endemic regions. Availability of these critical components will greatly enhance the chances for the global control and eradication of FMDV.

Keywords: DIVA test • foot-and-mouth disease control • inactivated vaccine • modified live vaccine • molecular vaccine • recombinant vector vaccine

More than 113 years ago, Friedrich Loeffler, together with his colleague Paul Frosch from the Robert Koch's Institute of Infectious Diseases in Berlin, reported that foot-and-mouth disease (FMD) was caused by a filterable agent, and this is considered the first description of an animal disease caused by a virus [1]. The report was from the Research Commission on FMD, headed by Loeffler and Frosch, and set up the previous year by the Prussian Ministry of Culture and tasked, among other things, with developing a vaccine against the devastating disease causing "severe economic damage to the country's agriculture" [2]. Inactivated FMD vaccines have been available since the early 1900s and have been instrumental in eradicating FMD from some parts of the world and repressing clinical disease in others. However, despite the use of billions of doses of inactivated FMD vaccines every year, the disease remains active, affecting millions of animals around the globe and causing economic hardship in some of the poorest regions. Today, FMD remains the main sanitary barrier to the commerce of animals and animal products. The characteristic clinical signs of FMD, which has high morbidity but low mortality, include high fever, anorexia, reduction in milk production and vesicular lesions that

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appear in the mouth and feet of affected animals 2–4 days after infection [3,4]. Inactivated FMD vaccines are effective in preventing clinical disease but do not necessarily prevent viral replication at the primary sites of infection, which can result in persistent infections in approximately 50% of naive and vaccinated animals exposed to FMD virus (FMDV) [5]. Although the role of these persistent carrier animals on the FMD transmission cycle remains unclear, its occurrence is one of the main deterrents to using vaccination in the rapid control and eradication of disease outbreaks in FMD-free countries. Another problem with inactivated vaccines is the presence of nonstructural proteins in some vaccine formulations, which interferes with our ability to serologically distinguish infected from vaccinated animals (for details see 'differentiating infected from vaccinated animals' section to follow).

There is a clear need for a new generation of FMD vaccines with an improved profile, capable of not only preventing clinical signs but that can also prevent infection and have the necessary antigenic markers to allow differentiating infected from vaccinated animals (DIVA) testing. These new vaccines need to be fit-for-purpose to address the inadequate short duration of immunity provided by commercial inactivated FMD vaccines, and importantly, their narrow range of antigenic coverage. This is especially important for endemic areas where several FMD viral subtypes may be circulating, complicating the logistics of implementing an effective vaccination program when resources are scarce. Other attributes include the need to accelerate the onset of immunity (currently 7 days) [6] and provide negative markers to effectively distinguish infected from vaccinated animals. In this article we will provide a broad overview of existing FMD vaccines and promising new technologies in the pipeline that might become available in the coming years, and provide vaccine characteristics currently lacking in commercial FMD vaccines. Based on this knowledge, we will discuss the feasibility of global strategies for the control and, when feasible, eradication of FMDV using existing and novel vaccines.

Inactivated FMD vaccines

Industrial production of inactivated vaccines began in the 1950s using the Frenkel method of culturing tongue epithelium collected from healthy slaughtered animals and infecting these cultures with FMDV, followed by collection of the virus-containing medium and virus inactivation using formaldehyde [7]. As one can imagine, the method of using primary cells limited large-scale production and was prone to contamination with adventitious agents. In addition, formaldehyde inactivation was often incomplete, resulting in infective vaccines. In the 1960s, cell-suspension cultures replaced tongue epithelium and later on ethylene imines replaced formaldehyde inactivation [8,9]. Current commercial FMD vaccines consist of inactivated purified antigen (killed virus) devoid of nonstructural viral proteins, usually by chromatographic purification, and formulated with various proprietary adjuvant formulations. These vaccine formulations have proven effective in reducing clinical disease in FMD-endemic areas and have been successfully used as an adjunct treatment in disease eradication programs in Africa, South America and Europe [2].

Foot-and-mouth disease vaccines traditionally represent the largest share of the veterinary vaccine market worldwide in terms of sales, with 26.4% of the entire livestock biological business [10]. Significant steps have been made to improve the quality of FMD vaccines, including changes in the manufacturing process to enable the differentiation of infected from vaccinated animals, but there are important variations between different manufacturers, and between vaccines distributed for FMD-endemic regions versus FMD-free countries. Owing to the high variability of FMDV serotypes and subtypes, the antigen composition of FMD vaccines is tailored for specific world regions and in many cases to specific countries or regions within them. The use of the vaccine in FMD-endemic regions requires an in-depth investigation of the epidemiology of disease and vaccine matching studies to determine whether the vaccine will be effective against the strain(s) circulating in the target area and to ensure the actual profile of the vaccine is suitable for control and eradication [11]. There are primarily three formulations that represent the large majority of all commercial inactivated FMD vaccines worldwide:

- High-potency vaccines (emergency use)
- Oil-emulsion conventional vaccines (routine control)
- Aluminum hydroxide vaccines (for cattle)

Oil-emulsion vaccines are currently prepared as either double oil-emulsion or water-in-oil single emulsion formulations with the former providing higher and longer antibody responses [12–14]. Potency is a major concern with FMD vaccines. The latest revisions of the World Organization for Animal Health Manual of diagnostic tests and vaccines for terrestrial animals states that six protective dose 50 (PD_{50}) per cattle dose is preferred [15]. However, this is not an absolute requirement owing to the acceptance that this would significantly reduce the number of vaccine doses in internationally established FMD vaccine banks. The case for using higher potency vaccines is clear, including greater protection against heterogeneous strains, a quicker onset of immunity, and increased protection from viral shedding and transmission [16–18].

Emergency use (high-potency) FMD vaccines

Due to the instability of the formulated product (12–18 months), vaccines for emergency use are usually stored as frozen antigen concentrates in vaccine banks by FMD-free countries or groups of countries (for review see [19]). For example, the USA, in cooperation with Canada and Mexico, has a tripartite 'North American Foot and Mouth Disease Vaccine Bank'. Vaccines formulated from frozen antigen banks contain at least six PD_{50}, provide an onset of protection from challenge within 4–7 days post-vaccination (dpv; partial immunity as early as 2 dpv) in cattle, swine and sheep, and some provide wider antigenic coverage and protection for heterologous FMDV subtypes within a serotype [6,18–20]. Duration of protective immunity after single vaccination is limited, requiring re-vaccination after 6 months [11,21,22]. Vaccines decrease clinical disease, virus amplification (shed and spread) and reduce the number of persistent infections in vaccinated ruminants challenged with FMDV [18,23,24]. Bulk antigens stored in

vaccine banks require at least 3–4 days for formulation and finishing of vaccine from frozen antigen concentrates, and 2–3 additional days for shipping resulting in deployment delays. This does not take into account the time required for completion of quality control tests to demonstrate purity, safety and potency, which might be a requirement of some biological regulatory agencies.

Conventional (ready-to-use) oil-emulsion FMD vaccines

Oil-adjuvanted vaccines formulated with a potency of at least 3 PD_{50} have been shown to provide an onset of protective immunity within 7 days in cattle, swine and sheep [6,19,25]. Vaccines decrease clinical disease, and virus amplification (shed and spread), but do not prevent the establishment of persistent infections in up to 50% of ruminants vaccinated and later challenged with wild-type FMDV [5,6,24]. Differences in efficacy and potency have been reported between double oil-emulsion versus water-in-oil single-emulsion formulations with a higher antibody response reported for double oil-emulsion [12,13,26]. Enhancement of the immune response induced by the inclusion of saponin in oil-adjuvanted vaccines has been reported [27]. Oil-adjuvanted vaccines are the most used worldwide but, in order to maintain sufficient levels of immunity to suppress occurrence of clinical disease, revaccination must be carried out every 6 months [15]. After multiple doses of vaccines in older animals, vaccination frequency could be decreased to once a year, provided that no new strains not covered by the vaccine formulation emerge or are introduced [28–31].

One of the shortcomings of the current conventional vaccine is the short shelf life once it is formulated either as double-oil emulsion or aluminum gel (for details see [32]).

Aluminum hydroxide-adjuvanted vaccines

The aluminum hydroxide–saponin formulation originally developed for FMD vaccines has some advantages such as ease of production and antigen concentration by adsorption to aluminum hydroxide gels [32]. However, aluminium hydroxide-adjuvanted vaccines have several disadvantages over oil-emulsion vaccines, including the fact that they cause granulomas at the inoculation site [33] and are not as effective in swine as oil-emulsion vaccines [34,35], have a shorter shelf life than oil-emulsion vaccines, are less potent per microgram of antigen, and produce a shorter duration of immunity [32]. However, these vaccines continue to be produced around the world for use mostly in ruminants.

New experimental vaccine platforms

Since structural proteins are the main antigens responsible for inducing protective responses [36,37], several attempts have been made to improve current inactivated FMD vaccines by utilizing cloned capsid proteins expressed by rDNA technology. However, the subunit vaccines produced in *Escherichia coli* and peptide vaccines induce narrow immune responses that the virus easily gets around through antigenic drift mechanisms [38]. Recently, significant improvements in rDNA-based vaccines have been made offering improvements in efficacy, safety and use in disease control and eradication [39]. Most of these improvements consist of introducing mechanisms for proteinase processing of the viral capsid proteins that result in structurally more complex antigens. Some of these new approaches are described in the following sections [40].

DNA vaccines

Vaccination using plasmid DNA containing FMDV sequences has been reported as an efficient way to induce protective immunity in the mouse model [41,42]. However, protection by DNA vaccination in farm animals such as cattle, sheep and pigs has proven more challenging and requires multiple doses and addition of adjuvants and cytokines (e.g., GM-CSF, IL-2) to induce only partial or in some cases full protection [42,43]. In most DNA vaccines the antibody response, which is critical in protection against FMDV, is both limited and short lived [44]. Despite these shortcomings, DNA vaccines are appealing because plasmid DNA does not require high containment facilities for manufacture, is relatively stable for storage, allows for the rapid incorporation of emerging field strain sequences and allows discrimination between infected and vaccinated animals [41,43]. Another interesting feature of DNA vaccines is that they have been reported to induce protection not only against clinical disease but also prevention of the carrier state in sheep [43]. Other applications of DNA vaccines include a prime–boost approach using inactivated vaccines to improve their efficacy [45]. Using priming with a plasmid DNA-containing capsid and some of the nonstructural proteins of FMDV followed by boost with inactivated vaccine and recombinant 3D protein resulted in high antibody titers and protection of swine not only against homologous but also against heterologous challenge. Although not a practical approach at this time, this promising research should continue.

FMD peptides

In laboratory animal models (e.g., mice, guinea pig), several FMD capsid-based peptide vaccine candidates have been shown to induce peptide-specific anti-FMDV serum-neutralizing (SN) antibody titers, and in some instances have been shown to confer protection against FMDV challenge [46,47]. Unfortunately, these positive results in laboratory animal models have not been consistently reproduced in cattle and pigs [25]. Although early studies in cattle showed promise [48], in a large-scale synthetic-peptide vaccination study in 138 cattle using four different FMDV serotype C VP1 G-H loop-based peptides, none of the peptides, tested at several doses and vaccination schedules, conferred protection in above 40% of the vaccinated animals [38]. Notably, several mutant FMDV strains were isolated from vaccinated cattle, suggesting that peptide vaccination induced the rapid generation and selection of FMDV antigenic variants *in vivo*.

Efforts to improve and broaden VP1 G-H loop peptide immunogenicity through the incorporation of T helper (Th) sites and consensus residues into the hypervariable positions (UBI peptide) resulted in a high level of protection in swine following FMDV 01 Taiwan challenge [49]. A subsequent pilot study in cattle showed that the UBI peptide induced peptide-specific antibodies but relatively low SN titers, and failed to protect cattle following FMDV type O challenge at 3 weeks post-vaccination [25].

However, a peptide vaccine is now commercially available [202]. FMDV peptide vaccine adjuvanted with cholera toxin and administered transcutaneously elicited antipeptide antibodies with enhanced virus neutralizing activity in mice [50]. However, further experiments demonstrating efficacy in target species are still required. Recent studies in swine utilizing nontoxic *Pseudomonas aeruginosa* exotoxin A expressing the FMDV VP1 G-H loop failed to induce protective immune responses [51].

The recent development of dendrimeric peptides containing one copy of an FMDV T-cell epitope branching out into four copies of a B-cell epitope provides potential improvements over the conventional linear peptide [52]. Pigs vaccinated with a dendrimeric peptide and subsequently challenged with FMDV did not develop significant clinical signs, appear to have abrogated systemic and mucosal FMDV replication, and did not transmit the virus to contact controls. The dendrimeric peptide used in this experiment elicited an immune response comparable to that found for control of FMDV-infected pigs. Dendrimeric designs for other FMDV serotypes and subtypes need to be developed and tested, but this new technology provides substantial promise for peptide-subunit vaccine development.

Virus-like particles

Foot-and-mouth disease virus-like particles (VLPs) are nonreplicating, nonpathogenic particles that have structural characteristics and antigenicity similar to the parental virus. They are similar in conformation to intact virions and are formed by the self-assembly of processed capsid proteins. A critical component of VLP experimental vaccines is the ability to process the viral capsid polyprotein (P1) into cleaved products that can then assemble into VLPs. There are different protein-processing paths that have been pursued by the inclusion of nonstructural viral proteins 2A, 2B and 3C proteinase [37,53,54]. There are several expression systems for the production of VLPs, including:

- Various mammalian cell lines, either transiently or stably transfected or transduced with viral expression vectors [55,56]
- The baculovirus/insect cell and larvae systems [57–59]
- Various species of yeast including *Saccharomyces cerevisiae* and *Pichia pastori* [60]
- *E. coli* and other bacteria [61–63]

A yeast-derived VLP experimental FMD vaccine was initially described in 2003 [60]. The capsid from a serotype O strain induced SN and ELISA titers in guinea pigs and these animals were protected against homologous challenge. More recently, co-expression of either recombinant bovine IFN-γ [64], IL-18 [65] or HSP-70 [66] and VP1 [64,65,67] constructs has been shown to enhance SN and cell-mediated immune responses in mice; however, no livestock vaccine efficacy studies have been reported.

Baculovirus-derived VLP experimental FMD vaccines have been shown to provide some protection against clinical disease in swine, but fail to protect against viral replication. Similar results using an *E. coli*-derived VLP experimental vaccine were also reported [37]. Recent reports have shown improvements by using baculovirus and silkworm larvae to express FMDV P1 and including protein 2A at strategic sites to facilitate processing of VP1–VP2 and VP0 [59]. The authors provide electron microscopy evidence of VLP assembled in the larvae lysates. Furthermore, vaccines prepared in this fashion for serotypes Asia and O conferred protection when used to immunize cattle [57]. Further testing is necessary to determine the feasibility of this approach for large-scale production of FMD vaccines for livestock.

Hepatitis B virus core (HBc) particles self-assemble into capsid particles and are extremely immunogenic. However, formation of VLPs can be restricted by size and structure of heterologous antigens. The first report of the use of the HBc system for expression of amino acids 141–160 of the VP1 protein of FMDV was made over 20 years ago, and the immunogenicity of the VLP structures was reportedly similar to that of intact FMD particles [68]. Very recently, the formation of VLP in mammalian cells by modified HBc fused with specified FMDV multi-epitopes was studied. Complete VLP structures with one construct was confirmed by electron microscopy and induced both humoral (peptide- and FMDV-specific antibody) and cell-mediated immunity responses in mice [69].

The generation of VLP experimental FMD vaccines using transgenic plants has also shown some laboratory success. *Arabidopsis thaliana*-transformed plant extracts expressing the FMDV VP1 gene were shown to provide protection against FMDV challenge in mice [70]. Similar studies have also been reported using transgenic potato plants [71] or alfalfa plants [72] as immunogens in serology and challenge studies in mice. Related studies using HBc to express a VP1 capsid epitope in transgenic tobacco has also been reported [73]. To date, none of these transgenic plant-derived VLP experimental vaccine candidates have been tested for efficacy and safety in cattle or swine, and the regulatory and manufacturing path for transgenic plant-derived vaccines is not well defined.

Viral-vectored FMD vaccine platforms

Viral vectors have been successfully used to deliver sequences coding for FMDV capsid proteins into animals. There are several examples including herpesvirus (pseudorabies), poxviruses and α-virus vectors. However, the best-documented and most effective platform uses human defective adenovirus 5 (hAd5). Unlike experimental DNA vaccines that mainly target the expression of the specific antigens to be presented to the immune system on the surface of transfected cells, the purpose of this platform is to provide the genetic information to express and process all the FMDV structural proteins, presumably resulting in the formation of virus-like particles in the hAd5-infected cells; although direct evidence of this has not been clearly shown [74]. The hAd5–FMD vaccines are the most extensively tested and were shown to be as effective as inactivated vaccines against FMD. Complete protection has been shown both in swine and cattle receiving one vaccine dose and challenged as early as 7 dpv [75–77]. The hAd5–FMD vaccine platform contains all FMDV capsid proteins and nonstructural proteins 2A and 3C, but lacks other nonstructural proteins such as 3B and 3D. These two proteins are the targets of serological tests aimed at differentiating infected from vaccinated animals, making the hAd5–FMD

platform fully compatible with existing DIVA serological tests. Importantly, the hAd5–FMD platform allows for safe vaccine production without the need for high biological containment facilities, as the process does not involve the use of live FMDV. In addition, this vaccine platform does not require adaptation of field strains to vaccine production cells and precludes potential antigenic changes resulting from cell adaptation and virus replication during the vaccine manufacturing process. A hAd5–FMD serotype A vaccine is the most advanced product candidate in development. To date, this vaccine has been tested in over 150 cattle and shown to provide protection against generalized FMD disease and viremia following a single immunization and intra-dermal lingual (tongue) or contact challenge at 7 or 21 dpv [77]. Similarly to the commercially inactivated vaccines, protection as early as 4 dpv has been demonstrated in some, but not all animals [76]. These new molecular FMD vaccine candidates are currently being manufactured in experimental batches and tested in cattle in the US mainland (for the first time in US history) as part of the veterinary licensing process [77].

cDNA-derived inactivated FMDV vaccine platform

The utilization of cDNA-derived FMDV as a safe candidate platform for production of inactivated vaccines was first reported utilizing leader-deleted FMDV [78–80]. Attenuation of FMDV was achieved by manipulating the genome to eliminate the L^{pro} coding sequence, which is known to be involved in FMDV pathogenesis *in vivo*. In a pilot study, this virus platform was shown to be completely attenuated in cattle based on absence of tongue lesions following direct inoculation of susceptible cattle [79]. Once inactivated, the antigenic properties of the cDNA-derived FMDV vaccine are expected to be those obtained from inactivated wild-type virus. More recently, this platform has been improved by adding unique restriction enzyme sites for rapid swapping of capsid sequences and also by deleting specific epitopes in nonstructural proteins (NSPs) 3B and 3D allowing for serological DIVA testing [Rieder E. Pers. Comm.]. Serum from animals inoculated with this leaderless marker virus can be readily distinguished from parental FMDV-infected animals utilizing DIVA serological tests such as competitive ELISA for NSP. Accordingly, unlike conventional inactivated FMD vaccines, this platform eliminates the need to remove NSP during the manufacturing process, and since the vaccine virus is attenuated, eliminates the concern associated with the manufacture of live FMDV if the vaccine virus escapes from the manufacturing facility.

Modified live vaccines

In the past few years, development of live-attenuated FMD vaccines has shown limited success owing to unstable phenotype, differences in attenuation among various livestock species (e.g., attenuated in cattle but not in swine) or vaccines that were incapable of consistently inducing protective immune responses [81–85]. Some of these vaccines relied on the use of viruses selected in cell culture or in laboratory animals showing attenuated phenotypes, but the mechanisms of attenuation were largely unknown and attenuation was often incomplete and reversible [81]. Other vaccines engineered to contain attenuating mutations or gene deletions were better characterized but were too attenuated and did not consistently induce protection [85]. Owing to these problems and concerns over reversion to virulence through mutation or recombination with field viruses, live FMD vaccines have not been developed. However, new technologies such as the development of reverse-genetic cDNA systems for FMDV provides new opportunities for identifying virulence determinants in the FMDV genome. As new virulence determinants are identified, new possibilities for attenuated vaccines will arise. Translating this knowledge into vaccine candidates will require detailed understanding of the virus–host interaction and mechanisms of pathogenesis in order to address concerns about complete attenuation in all susceptible species, and even eliminating the possibility of reversion to virulence. All this is now possible given the current availability of well-established infectious cDNA FMDV systems and the ability to engineer multiple specific mutations in critical elements that regulate the virulence of the virus [86–90]. Importantly, this methodology also allows the engineering of negative antigenic markers to include DIVA capability.

Adjuvants & biotherapeutics

The FMDV incubation period can be as short as 2 days and animals can shed virus prior to signs of generalized disease [3,4]. Since FMD vaccines generally require at least 4–7 days for protective adaptive immunity to develop, it is critical that FMD control programs address the gap in the onset of immunity provided by current vaccines to limit and control disease spread. With recent breakthroughs in our understanding of antiviral innate defense mechanisms, biotherapeutics or immunomodulators offer the potential for their use as an emergency tool to stop viral shed and spread within 24 h after administration [91–93]. When used in combination with rapid-acting vaccines. it may now be possible to elicit early short-term anti-FMDV effects until the onset of vaccine-induced protective immunity.

It has been previously shown that FMDV replication is inhibited by type I interferons (IFN-α/β) [94,95]. At least two IFN-α/β-stimulated genes, double-stranded-RNA-dependent protein kinase and 2',5'oligoadenylate synthetase/RNase L, are involved in this process [95]. It has been demonstrated that an Ad5 vector containing the porcine IFN (pIFN)-α gene (Ad5–pIFN-α) induces high levels of biologically active interferon when injected in swine. Furthermore, swine inoculated with a single dose of Ad5-pIFN-α were completely protected when challenged with FMDV 1 day later [74]. The level of protection correlated with the Ad5-pIFN-α dose and the level of plasma IFN-α. Additional studies demonstrated that Ad5–pIFN-α treatment alone can protect swine from challenge for 3–5 days and can reduce viremia, virus shedding and disease severity when administered 1 day post challenge. Importantly, a combination of Ad5–pIFN-α and Ad5–FMD vaccination provided both immediate and long-term protection in swine [96,97]. Type II pIFN (pIFN-γ) also has antiviral activity against FMDV and, in combination with pIFN-α, has a synergistic antiviral effect [92]. The results indicate that the combination of type I and II interferons act synergistically to inhibit FMDV replication *in vivo* and confer protection against challenge. Similar studies in cattle have only shown partial but not complete protection against FMDV challenge [91].

Differentiating infected from vaccinated animals

Exposure to structural FMDV capsid proteins as well as NSPs such a RNA polymerase through infection or vaccination will induce the production of antibodies to these proteins. The removal of NSPs from FMD vaccines enables the differential detection of antibodies to the NSPs in FMDV-infected animals. Thus, current DIVA strategies for FMD are based on the use of a diagnostic test that can differentiate the detection of antibodies to NSPs in infected versus vaccinated animals. For detailed information on DIVA tests and their role in control and eradication please see [98–100].

Although the application of current DIVA strategies has been successfully implemented on a herd basis, there is still some concern of vaccinated animals becoming asymptomatic virus carriers without positive reaction to NSP serological tests [101]. Of particular concern is the fact that current vaccines may have residual NSPs (depending on the manufacturing process) that could result in the detection of NSP antibodies in vaccinated animals if multiple doses of vaccine are applied annually [102–104]. In addition, the application of the current DIVA strategy is dependent on diagnostic tests originally developed to determine FMDV infection and used primarily for surveillance and not necessarily as companion tests to vaccines [100,105].

Since the ultimate goal of a DIVA strategy is to 'vaccinate to live,' it is paramount that vaccinated animals exposed to FMDV will not transmit virus. There is therefore a critical need for new and improved FMD vaccines and companion diagnostics specifically designed for DIVA and validated for the purpose intended. It is expected that the next generation of FMDV countermeasures will not only include vaccines designed with negative markers consisting of deletions of NSP epitopes, but also include companion antibody detection assays to determine exposure to FMDV, and direct antigen- or nucleic-acid detecting assays to verify that a vaccinated animal exposed to FMDV is not infected.

Table 1. Current and ideal profile of foot-and-mouth disease vaccines for global eradication.

Characteristic	Current status	Ideal	Ref.
Prevents infection	No	Yes	[5,6,24]
Onset of protective immunity (dpv)	7†	1	[6,19,25]
Broad cross-protection	Only within some serotypes	Across all 7 serotypes	[2]
Duration of immunity	4–12 months‡	Lifelong	[11,21,22]
Shelf life (years)	1	>4§	[32]
Requirement for high biosafety containment	Yes, growth of large amounts of infectious virus	No, noninfectious or attenuated vaccine virus production platform	[32]
DIVA compatible	Requires antigen purification	Negative marker engineered into vaccine platform	[105]
Ability to rapidly incorporate emerging viral strains	Requires adaptation of field strains	Allows rapid production of new antigens	[32]
Short withdrawal period for food consumption (days)	21–60¶	≤21	[32]
Thermal stability	Requires refrigeration	No refrigeration required	[32]
Cost	Moderate	Low	[32]

†Vaccines containing high antigen loads can induce partial protection at 4 dpv.
‡Dependent on vaccine formulation and species.
§Recommendation from Global Roadmap for Improving Tools to control FMD, Agra, India 2006 [106].
¶Varies from country to country.
DIVA: Differentiating infected from vaccinated animals; dpv: Days post-vaccination.

Expert commentary

Global control and eradication of FMD has been proposed for many years. Recently in the Paraguay declaration of 2009, the World Organization for Animal Health, the Food Agriculture Organization and other international agencies and numerous governments set the goal for global eradication of FMD by the year 2030 [201]. Although such declarations are important and eradication seems within reach, particularly in regions where FMD is largely controlled by vaccination, in other regions, where FMD is rampant and FMD control competes with other basic needs such as food, human health and education, such an eradication goal seems unlikely. There is a clear need for alternative control methods, particularly vaccines that can address the major shortcomings of current and even upcoming vaccines. These fit-for-purpose vaccines accompanied by appropriate diagnostics and control strategies should address the needs of each region and particularly induce long-term immunity sufficient to break the endemic transmission cycles that currently maintain the virus circulating in these areas [106]. Since funding for such vaccines, diagnostics and control strategies is limited worldwide, it is important to leverage international capabilities into aligned efforts toward common achievable goals. Such an effort requires international alliances such as the Global Foot and Mouth Disease Research Alliance [203], launched in 2003 as a worldwide association of animal research organizations, that are involved in combating FMD. Its aim is to build a global alliance of partners to generate and share knowledge – in a virtual FMD laboratory – to develop tools that can better combat the threat of the disease. Collaborative research is central to the fulfillment of FMD global eradication.

Five-year view

To achieve the goal of a global effort for FMD control and eradication, the next 5 years are critical. Large parts of the world such as South America are at the brink of eradicating FMD with currently available tools, while others will require new tools

and sustainable approaches that are relevant to their economy and methods of livestock production to achieve the goal of eradication. Without continued effort being placed in the necessary research for the development of better control strategies and appropriate tools, the goal of eradication will unlikely to be realized. The focus of the research should be centered on goals that will address the major problems limiting control and eradication, including understanding of the ecology, pathogenesis and transmission of FMDV in relevant species, and importantly, the development of new and cost-effective technologies designed to provide broad-range and long-lasting protective immunity after vaccination. The information derived from this research effort should lead to vaccines that have the necessary characteristics (listed in Table 1) that will allow the global control and eventual eradication of FMD.

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Key issues

- Foot-and-mouth disease is a devastating disease of farm animals that negatively affects the livelihoods of millions of people around the world.
- Control programs based on test-and-slaughter policies without vaccination are not feasible in endemic countries, most of which are developing countries highly dependent on livestock for their economy and the health of their citizens.
- Success in eradicating the disease in Europe and in most of South America was achieved after sustained and expensive mass vaccination efforts, which are required to attain herd immunity.
- Global eradication using current mass vaccination strategies may not be feasible for impoverished regions that need it the most and can afford it the least.
- Existing foot-and-mouth vaccines are not suitable for global eradication because they:
  - Induce short-term protection requiring re-vaccination every 6 months or less
  - Do not cross-protect against the multiple viral serotypes (seven) and subtypes (dozens)
  - Have a short shelf life and are heat labile requiring cold chain from production to delivery
  - Require expensive biosafety level 3 facilities for growing live virus during vaccine production
- There is a need for vaccines capable of preventing infection and inducing life-long immunity against multiple serotypes and subtypes.
- These vaccines should be safely and inexpensively produced, and easy to deliver.
- There is a need for better integrated strategies that fit the specific needs of endemic regions.
- Only when these critical components are available will the global eradication of foot-and-mouth disease virus be possible.

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Interaction between Core protein of classical swine fever virus with cellular IQGAP1 protein appears essential for virulence in swine

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ABSTRACT

Here we show that IQGAP1, a cellular protein that plays a pivotal role as a regulator of the cytoskeleton interacts with Classical Swine Fever Virus (CSFV) Core protein. Sequence analyses identified residues within CSFV Core protein (designated as areas I, II, III and IV) that maintain homology to regions within the matrix protein of Moloney Murine Leukemia Virus (MMLV) that mediate binding to IQGAP1 [EMBO J, 2006 25:2155]. Alanine-substitution within Core regions I, II, III and IV identified residues that specifically mediate the Core-IQGAP1 interaction. Recombinant CSFV viruses harboring alanine substitutions at residues ^{207}ATI^{209} (I), ^{210}VVE^{212} (II), ^{213}GVK^{215} (III), or ^{232}GLYHN^{236} (IV) have defective growth in primary swine macrophage cultures. *In vivo*, substitutions of residues in areas I and III yielded viruses that were completely attenuated in swine. These data shows that the interaction of Core with an integral component of cytoskeletal regulation plays a role in the CSFV cycle.

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Introduction

Classical swine fever virus (CSFV) is a small, enveloped virus with a positive, single-stranded RNA genome that causes classical swine fever (CSF), a highly contagious disease of swine. CSFV, along with Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV), are members of the genus *Pestivirus* within the family *Flaviviridae* (Fauquet et al., 2005). The CSFV genome is approximately 12.3 kb and contains a single open reading frame encoding a polyprotein of 3898-amino-acids. Co- and post-translational processing of the polyprotein by cellular and viral proteases ultimately yields 11 to 12 final cleavage products (NH_2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) (Rice, 1996). Structural components of the CSFV virion include the glycoproteins E^{rns}, E1, E2, and a nucleocapsid of unknown symmetry, the Core protein.

Within the *Flaviviridae* family, Core is encoded as the second product of the polyprotein. The Core protein of pestiviruses is a small, highly basic polypeptide that is cleaved at its N-terminus by Npro, an event critical for infectious particle production. The Core protein of BVDV has additionally been characterized as lacking significant secondary structures (Murray et al., 2008). It is known that the C-terminal of Core is cleaved by signal peptide peptidase. (Heimann et al., 2006; Rümenapf et al., 1998; Meyers et al., 1989), and it has been suggested that the CSFV Core protein influences regulation of cellular transcription (Liu et al., 1998) and also interacts with host SUMOylation proteins (Gladue et al., 2010).

Analysis of the Core protein of Hepatitis C Virus (HCV), another member of the *Flaviviridae* family, provides further insight into the possible functions of the Core protein of CSFV. HCV Core self-assembles into nucleocapsid-like particles in the presence of nucleic acids (Kunkel et al., 2001) and can directly interact with HCV RNA (Fan et al., 1999; Shimoike et al., 1999; Tanaka et al., 2000). Core can bind other HCV proteins such as NS5A and E1 (Goh et al., 2001; Lo et al., 1996; Masaki et al., 2008) and interacts with host cellular proteins (Jin et al., 2000; Mamiya and Worman., 1999; Otsuka et al., 2000; Yoshida et al., 2002; You et al., 1999), influencing HCV pathogenesis by modulation of signaling pathways, cell transformation and proliferation, regulation of cellular and viral gene expression, apoptosis, and alteration of lipid metabolism. (Giannini and Brechot, 2003; Levrero, 2006; Tellinghuisen and Rice, 2002; Lai and Ware., 2000; McLauchlan, 2000; Ray and Ray, 2001). HCV Core protein is capable of impairing the host's immune response, by interacting with cell molecules that results in suppression of IL-12 synthesis in human macrophages (Eisen-Vandervelde et al., 2004), T cell dysfunction (Yao

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et al., 2007), and inhibition of T-lymphocyte activation and proliferation (Chen et al., 1994; Kittlesen et al., 2000; Yao et al., 2001).

Although the role of CSFV structural glycoproteins in virus virulence has been studied in detail (Meyers et al., 1999; Risatti et al., 2005a, 2005b, 2006, 2007a, 2007b; van Rijn et al., 1994; van Gennip et al., 2004), knowledge about the role of Core protein on the outcome of CSFV infection in swine is limited. Recently, we have shown that CSFV Core protein interacts with proteins of the cellular SUMOylation pathway, SUMO-1 (small ubiquitin-like modifier) and UBC9, a SUMO-1 conjugating enzyme (Gladue et al., 2010). Substitution of Core residues in CSFV involved with binding to SUMO1 and UBC9 resulted in virus attenuation in swine. To further elucidate the role of Core protein in CSFV virulence, we expanded this previous study to identify additional host factors interacting with the Core protein during virus infection. Here we report that the cellular IQGAP1 protein interacts specifically with the CSFV Core protein. IQGAP1 is involved in a diverse set of protein–protein interactions and is a prominent regulator of the cytoskeleton (for a review, see Noritake et al., 2005; Brandt and Grosse, 2007). This protein binds monomeric G proteins: Cdc42 and Rac, likely mediating their effects in reorganizing the cytoskeleton (Fukata et al., 2002; Watanabe et al., 2004).

Specific interaction of IQGAP1 with the Moloney murine leukemia virus (MMLV) matrix (M) protein suggests its involvement in intracellular trafficking of the virus, an interaction that is essential for virus replication (Leung et al., 2006). Mutational studies performed in that report defined residues of MMLV M protein, critical for its interaction with IQGAP1. Based on sites within the MMLV M protein that recognize IQGAP1, four areas (I–IV) within the CSFV Core protein that potentially recognize IQGAP1 were identified. Substitution of Core native amino acid residues with alanine in areas I and III completely abolished the Core–IQGAP1 protein–protein binding whereas substitutions in areas II and IV only partially affected the interaction. CSFV mutants harboring substitutions in these four areas were developed to assess the importance of the Core–IQGAP1 protein interaction for virulence in swine. Remarkably, substitutions in areas I and III of the Core protein, significantly affecting IQGAP1 recognition, correlate with complete absence of virulence in swine. Therefore, the ability of CSFV Core protein to bind cellular IQGAP1 protein during infection plays a critical role in virus virulence within the swine host.

Results

CSFV structural Core protein binds swine IQGAP1 protein

To identify host cellular proteins that interact with CSFV Core protein, we constructed an N-terminal fusion of the Gal4 protein binding domain to the Core protein as 'bait' for the yeast two-hybrid system. Approximately 1×10^7 independent yeast colonies derived from a swine primary macrophage cDNA library containing 3×10^6 independent clones were screened. These colonies were selected for growth using -Leu/-Trp/-His/-Ade media. Plasmids were isolated from positive colonies and sequenced. In-frame proteins were retested for specificity to the Core protein. As a negative control proteins were tested for binding the Lam-BD protein. Several proteins were identified as specific binding partners for the CSFV Core protein (data not shown). One of these proteins, IQGAP1, was selected for further study since IQGAP1 functions as a prominent regulator of the cytoskeleton which likely plays a role in viral pathogenesis. IQGAP1 specifically bound Core protein when compared to binding of Lam-BD protein (Fig. 1).

Mapping areas of the CSFV Core protein critical for IQGAP1 recognition

Previous studies have described the binding of Moloney murine leukemia virus (MMLV) matrix (M) protein with IQGAP1 protein (Leung et al., 2006). Mutational studies defined residues of the MMLV M protein critical for interaction with IQGAP1, revealing a correlation between binding and virus replication. Based on the sites of MMLV M protein that recognize IQGAP1 (Leung et al., 2006), four (I–IV) homologous areas (Fig. 2) were identified in CSFV Core protein using the ClustalW software program (Thompson et al., 1994). CSFV mutants harboring alanine substitutions in place of native amino acid residues within these four areas were developed to assess whether these regions are important for IQGAP1 binding (Figs. 1 and 2). We produced the following mutant proteins containing Ala substitutions within the Core protein: CoreΔIQ.I (^{207}ATI^{209}), CoreΔIQ.II (^{210}VVE^{212}), CoreΔIQ.III (^{213}GVK^{215}), and CoreΔIQ.IV (^{232}GLYHN^{236}) (Table 1). These CoreΔIQ proteins were tested in the yeast two-hybrid system against the swine IQGAP1 protein. Interestingly, substitutions within areas I and III resulted in loss of ability to bind IQGAP1 (Fig. 1). In contrast, substitutions in areas II and IV only caused a decrease in the ability of the Core protein to bind the swine IQGAP1 protein. All CoreΔIQ mutated proteins maintained their ability to bind swine clathrin in the yeast two-hybrid at similar levels (data not shown), indicating that these mutated areas within the CoreΔIQ mutated proteins are areas specific for IQGAP1 binding.

Sequence analysis of the 100 amino acid residues of Core protein from geographically and temporally different CSFV isolates revealed a high degree of sequence similarity and conservancy at putative IQGAP1 target sites (Fig. 2), suggesting these sites play a critical role in the biology of CSFV. Additionally, the areas predicted in CSFV to bind IQGAP1 are highly conserved in BVDV and BDV, further suggesting a critical role for these residues in other pestiviruses (data not shown).

Replication of CoreΔIQ mutant viruses in vitro

To further evaluate the role of CSFV Core IQGAP1 binding sites in the biology of the virus, recombinant viruses based on virulent strain Brescia (BICv) were constructed, containing alanine substitutions in the previously described four critical IQGAP1 binding sites of the Core protein. Mutant viruses referred to as CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, and CoreΔIQ.IVv represent each of the four putative IQGAP1 binding sites within the CSFV Core protein (Table 1 and Fig. 2). Viruses were rescued from transfected cells by 4 dpi (days post-infection). Nucleotide sequences of viable rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at predicted mutated sites were reflected in rescued viruses.

In vitro growth characteristics of these mutant viruses were evaluated relative to parental BICv in a single-step growth curve. Primary swine macrophage cell cultures were infected at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time zero), and samples were collected at 72 h post-infection (hpi). All mutant viruses exhibited about one log10 decrease in titer when compared with parental BICv (Fig. 3), suggesting that all mutant viruses have an *in vitro* growth defect when compared to parental BICv.

Effect of Core–IQGAP interactions on the cell cytoskeleton

The effects of viral infection on γ-tubulin and vimentin were examined to compare the distribution of microtubules and intermediate filaments in cells infected with BICv, CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, CoreΔIQ.IVv, to that seen in uninfected cells (Fig. 5). In uninfected cells, the microtubules (visualized with antibodies recognizing γ-tubulin) as well as the intermediate filaments (visualized with antibodies recognizing vimentin), were arranged in a filamentous network running throughout the cytoplasm. In BICv-infected cells, γ-tubulin was rearranged into a ring surrounding the nucleus. When vimentin was examined a similar rearrangement was observed in BICv infected cells. Examining the appearance of γ-tubulin and vimentin in infected cells also revealed differences between the effects of BICv and CoreIQ viruses. Infection with CoreΔIQ.IIv and CoreΔIQ.IVv led to a rearrangement of both markers, γ-tubulin and vimentin, into a ring

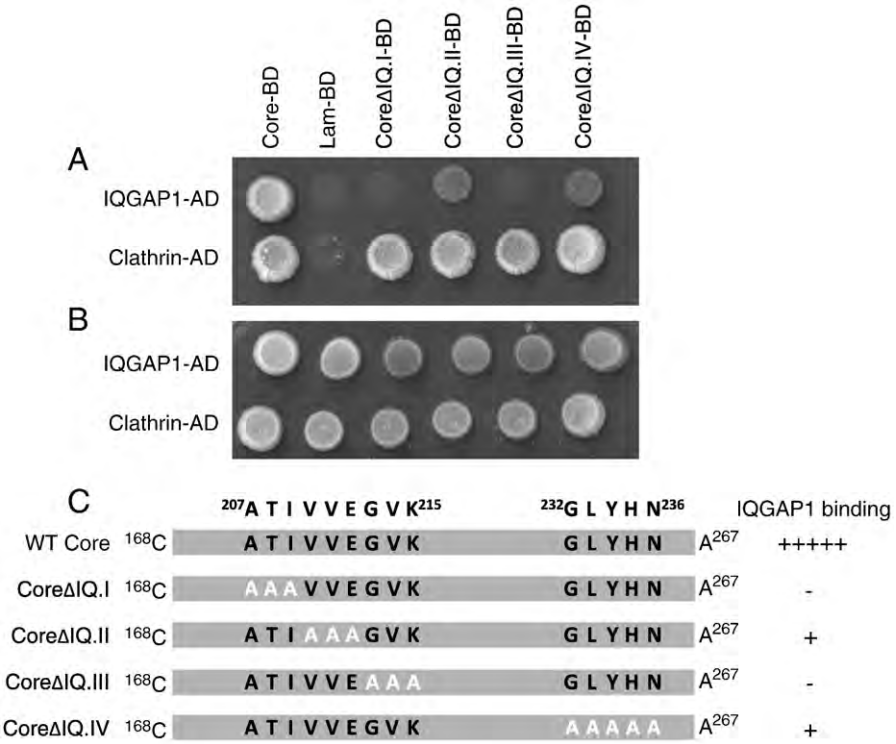

Fig. 1. Reactivity of CSFV wild-type and mutant Core proteins with IQGAP1 protein or Clathrin protein (positive control) in the yeast two-hybrid system. Wild-type Core and mutants I, II, III and IV proteins were tested for their ability to bind IQGAP1 protein. Yeast growth on selective SD-Ade-His-Leu-Trp media (A), and growth on non-selective SD-Leu-Trp media (B). Schematic representation of putative IQGAP1 binding motifs found in CSFV Core protein (C).

surrounding the nucleus similar to the one observed with BICv. In contrast, when CoreΔIQ.Iv and CoreΔIQ.IIIv were studied, both proteins, γ-tubulin and vimentin, retained a radial pattern as seen in uninfected cells (Fig. 5).

Evaluation of the role of CSFV Core IQGAP1 binding sites in CSFV virulence in swine

To examine the *in vitro* effects of deletion on Core protein interaction with IQGAP1 protein, all four CoreΔIQv mutants were intranasally (IN) inoculated into naïve swine, at doses of 10^5 $TCID_{50}$. After inoculation, survival of pigs inoculated with mutant viruses was assessed relative to lethal exposure of control animals infected with virulent BICv. The swine were monitored daily for clinical disease. BICv exhibited a characteristic virulent phenotype (Table 2); none of the control pigs survived the infection, dying or being euthanized around 8 dpi. Interestingly, viruses CoreΔIQ.Iv and CoreΔIQ.IIIv were completely attenuated in swine. Animals survived the infection and remained clinically normal throughout the observation period (21 days) with only one animal out of five infected with CoreΔIQ.IIIv presenting a transient rise in body

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BICv AY578687          CSDDGASASKEKKPDRINKGKLKIAPKEHEKDSRTKPPDATIVVEGVKYQVKKKGKVKGKNTQDGLYHNKNKPPESRKKLEKALLAWAVIAIMLYQPVAA
                                                                I  II III                 IV
Alfort 187 X87939      .......G.......................................................................................E.
LOM EU789580           .......G.......................................................................................E.
cF114 AF333000         .......G.......................................................................................E.
Alfort A19 U90951      .......G.......................................................................................E.
GPE- D49533            .......G.......................................................................................E.
Shimen AY775178        .......G.....................................................................T.......E.
JL1(06)EU497410        .......G.....................................................................T.......E.
SWH DQ127910           .......G.....................................................................T.......E.
Thiverval EU490425     .......G.............................................R..............................T...............E.
Eystrup NC002657       .......G.....................................................S.....................................E.
ALD D49532             .......G.....................................................S.....................................E.
CAP X96550             .......G...................................................R...............................V.....E.
Glentorf U45478        .......G...................................................R...............................V.....E.
HCLV AF531433          .......G.........................R.............................................................E.
Strain C AY805221      .......G.........................R.............................................................E.
Riems AY259122         .......G.........................R.............................................................E.
CS AY578688            .......G...............T.......................................................................
GXWZ02 AY367767        ......GG..D.....M................K.......................................................VT.V.......
virus 39 AF407339      .......G..D.....M................K..S...............................R...................V..V.......
Spain01 FJ265020       .......GN.D.....M................K................I.................................V.......
96TD AY554397          ....S.GG..D.....MS...............K.................N.V...............................T.V.......
0406Taiwan AY568569    ......GG..D.....M................K.............................................K...........V.....V.
Paderborn AY072924     ......GG.E......M................K.......................................................T.V.......
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Fig. 2. Multiple alignments of CSFV Core proteins revealed the presence of highly conserved putative IQGAP1 sites I, II, III, and IV (bold underlined). Shaded are Lys residues involved with Core-SUMO-1 and Core-UBC9 protein–protein interactions as described by Gladue et al. (2010). Shown here is a comparison between geographically and temporally separated viruses.

Table 1
Set of CSFV CoreΔIQ mutant viruses constructed in this study.

Core wild type sequence	[a]Core mutant sequence	Mutant virus
^{207}ATI^{209}	^{207}AAA^{209}	CoreΔIQ.Iv
^{210}VVE^{212}	^{210}AAA^{212}	CoreΔIQ.IIv
^{213}GVK^{215}	^{213}AAA^{215}	CoreΔIQ.IIIv
^{232}GLYHN^{236}	^{232}AAAAA^{236}	CoreΔIQ.IVv

[a] Amino acid position relative to CSFV BICv polyprotein.

temperature. In contrast, animals infected with mutant CoreΔIQ.IIv or CoreΔIQ.IVv, although presenting less severe CSF symptoms than those infected with BICv, died around 12 and 14 dpi, respectively (Table 2). Therefore, there appears to be a close correlation between disrupting Core–IQGAP1 protein–protein binding as detected in the yeast two-hybrid system and the induction of virus attenuation.

Virus shedding (detected in tonsil scrapings and nasal swabs) and viremia in CoreΔIQ.Iv and CoreΔIQ.IIIv-inoculated animals were almost undetectable (sensitivity of detection ≥ 1.8 $TCID_{50}$/ml) at all time points sampled (Fig. 4). Interestingly, high virus loads in blood, nasal swabs, and tonsil scrapings were observed in animals infected with CoreΔIQ.IIv or CoreΔIQ.IVv although those titers were detected at a later time during the infection when compared to BICv-infected pigs (Fig. 4). Similarly, a delayed onset of disease was observed in animals inoculated with these mutant viruses relative to wild-type infected animals.

Animals that survive inoculation with CoreΔIQ.Iv and CoreΔIQ.IIIv when challenged with virulent BICv by 28 dpi were not protected, succumbing to the infection as naïve pigs (data not shown). Antibodies against E2 and E^{rns} were not detected in these animals by the time of challenge as measured by ELISA tests (CSF SERO ELISA and CHEKIT-CSF-MARKER Kits, Idexx Laboratories, Westbrook, ME, USA).

Discussion

Here we describe a specific interaction between IQGAP1 protein, a major cytoskeleton regulator, with CSFV Core protein. Substitution of residues ^{207}ATI^{209} or ^{213}GVK^{215} within Core, disrupting Core–IQGAP1 protein–protein interaction completely, correlates with the induction of virus attenuation *in vivo*. Partial disruption of Core–IQGAP1 binding by substituting Core residues ^{210}VVE^{212} or ^{232}GLYHN^{236} resulted in viruses inducing a delayed onset of CSF, suggesting that the degree of attenuation closely correlates with the ability of the Core protein to interact with IQGAP1, and that this swine–host protein interaction plays an important role in CSFV pathogenesis.

CoreΔIQ viruses showed a slight growth defect in cultured primary swine macrophages. Impaired replication observed *in vitro* usually correlates with virus attenuation *in vivo*, with animals overcoming the infection, likely because of poor virus replication *in vivo* and a rapid clearance of mutant viruses mediated by the host immune response. In fact, CoreΔIQ.Iv and CoreΔIQ.IIIv mutants, presenting the highest extent of attenuation, demonstrated decreased replication during infection in animals. However, CoreΔIQ.IIv and CoreΔIQ.IVv mutants exhibited more limited replication than parental BICv in cultured swine macrophages, but replicated almost as efficiently *in vivo* inducing disease similar to wild-type virus, although with a delayed progression. These two recombinant viruses likely do not contain sufficient mutations within the Core–IQGAP1 interaction to limit CSFV tropism *in vivo*, with viruses retaining the ability to replicate in multiple tissues and cause disease.

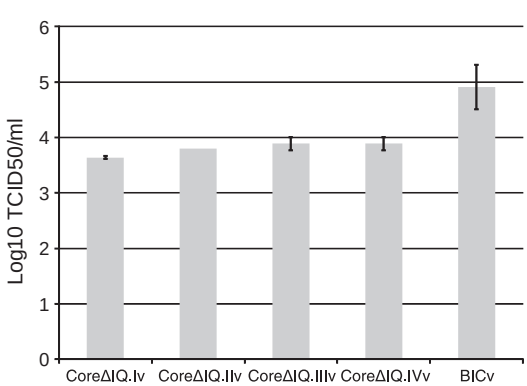

Fig. 3. *In vitro* growth characteristics of CoreΔIQv mutants and parental BICv. Primary swine macrophage cell cultures were infected (MOI = 0.01) with each of the mutants or BICv and virus yield titrated at 72 h post-infection in SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: ≥log10 1.8 TCID50/ml.

The observed interaction of Core with the cell's cytoskeleton may inhibit *in vivo* host immune cell migration, hampering viral CSFV clearance. *Salmonella,* a facultative intracellular pathogen, releases a bacterial effector protein, SseI (also known as or SrfH), that is able to block the migration of host immune cells (McLaughlin et al., 2009). The mechanism involves the interaction of SseI with IQGAP1, reducing dendritic cell (DC) migration *in vivo* that in turn correlates with a reduction in the number of DC and CD4+ T cells in spleens of *Salmonella*-infected mice. *Salmonella* invasion promotes the interaction of IQGAP1 with Rho GTPases Rac1 and Cdc42 to induce actin polymerization (Brown et al., 2007). *In vitro*, knockdown of IQGAP1 significantly reduces *Salmonella* invasion and abrogates activation of Cdc42 and Rac1 by *Salmonella*. Similarly, Ibe, an effector protein of enteropathogenic and enterohemorrhagic *Escherichia coli* interacts with IQGAP1 (Buss et al., 2009). This factor co-localizes with IQGAP1 in *E. coli*-induced pedestals and actin-rich membrane ruffles. Interestingly, these pathogens use common effector mechanisms to increase infectivity. Experiments presented here demonstrated the existence of alterations in the distribution of the cytoskeleton of cells infected with CSFV. Actually, those alterations appear to be mediated by Core protein since mutations introduced in CoreΔIQ.Iv and CoreΔIQ.IIIv appear to disrupt the induction of those cytoskeleton alterations (Fig. 5).

CSFV Core protein seems to play an important role in virus virulence. In a previous study (Gladue et al., 2010) we observed that Core interacts with the cellular SUMOylation pathway proteins, SUMO-1 (small ubiquitin-like modifier) and UBC9, a SUMO-1 conjugating enzyme. In that study, specific residues in Core responsible for binding were shown important for CSFV pathogenesis since mutant viruses harboring substitutions at those positions were attenuated in swine. Interactions of viral proteins with the SUMOylation pathway seems to be important for viral infectivity, as shown for Ebola Virus Zaire VP35, Adenovirus CELO Gam1, Dengue Virus envelope protein, Human Herpesvirus 6 IE2, and Human Cytomegalovirus IE2, either preventing or inducing SUMO conjugation of target proteins (Ahn et al., 2001; Chang et al., 2009; Chiocca, 2007; Tomoiu et al., 2006). Here we extended the analysis of host factors interacting with CSFV proteins, specifically identifying IQGAP1 as a swine host protein binding partner for the CSFV Core protein. We also observed that mutations in the binding site in Core protein can completely abrogate CSFV virulence, demonstrating that acquisition of attenuation correlates with loss of binding to IQGAP1. These studies are providing interesting insights into the pathogenesis of CSFV by elucidating what appear to be multiple roles for Core in the CSFV cycle. Identifying cellular pathways and the viral proteins involved in infectivity could be important for developing better countermeasures to control virus infection in swine.

Materials and methods

Viruses and cells

Swine kidney cells (SK6) (Terpstra et al., 1990), free of BVDV, were cultured in Dulbecco's Minimal Essential Media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort

Table 2
Swine survival and fever response following infection with CSFV IQGAP1 mutants and parental BICv.

Virus	No. of survivors/total no.	Mean time to death (days ± SD)	No. of days to onset (days ± SD)	Fever	
				Duration no. of days (days ± SD)	Max daily temperature (± SD)
CoreΔIQ.Iv	6/6	No	No	No	102.8 (0.6)
CoreΔIQ.IIv	0/7	12 (0.5)	5.9 (2.5)	6.2 (2.7)	106.2 (1.1)
CoreΔIQ.IIIv	5/5	No	4.5 (0.7)[a]	1 (0)	103.8 (0.7)
CoreΔIQ.IVv	1/5	14 (2.4)	4 (0)	6.8 (3.6)	105 (1.2)
BICv	0/6	8.5 (1.9)	3.5 (0.6)	5 (1.6)	106.2 (0.7)

[a] Only one animal had a transient raised body temperature.

Collins, CO). Virulent CSFV Brescia strain was propagated in SK6 cells and used for the construction of an infectious cDNA clone (pBIC) (Risatti et al., 2005a). Growth kinetics was assessed on primary swine macrophage cell cultures prepared as described (Zsak et al., 1996). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). Viral infectivity was detected, after 4 days in culture, by an immunoperoxidase assay using the CSFV monoclonal antibody WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated using the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was ≥1.8 $TCID_{50}$/ml.

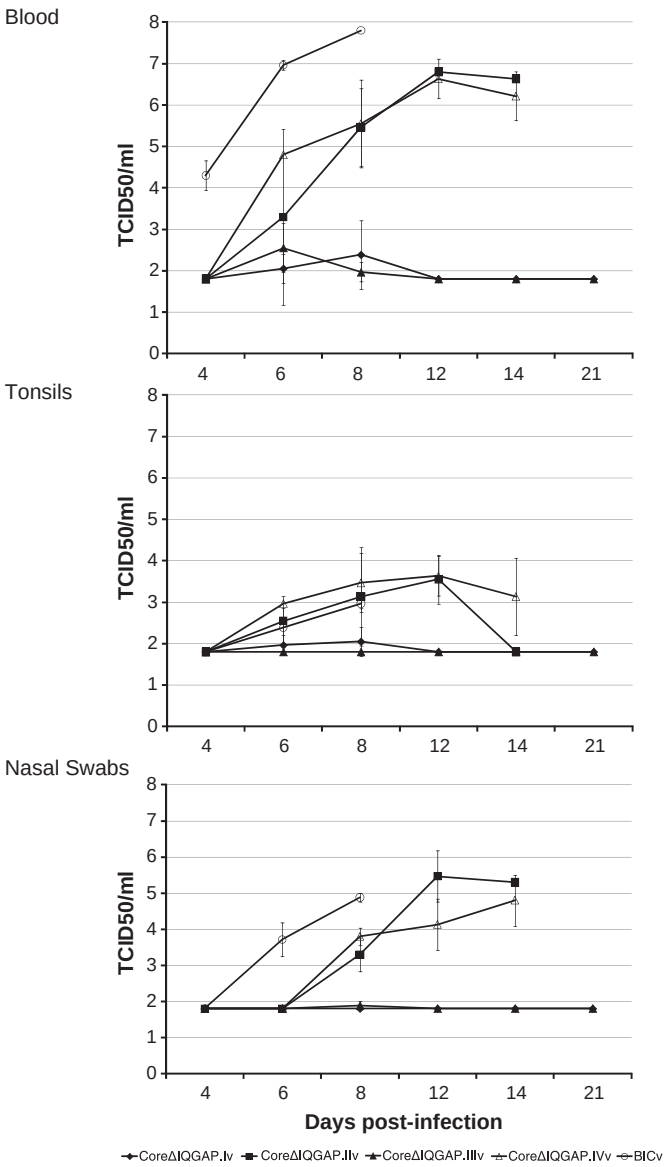

Fig. 4. Virus titers in clinical samples (blood, tonsil scrapings, and nasal swabs) from pigs infected with CoreΔIQ mutants and parental BICv. Each point represents the mean log10 TCID50/ml and standard deviations from at least two animals. Sensitivity of virus detection: ≥log 10 1.8 TCID50/ml.

Construction of CSFV CoreΔIQGAP (CΔIQ) mutants

Full-length pBIC was used as a template in which putative IQGAP1 binding sites in the Core protein were mutated. IQGAP1 binding sites were predicted using the Clustal W Analysis Program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using regions of the MMLV M protein that bind IQGAP1 as a template (Leung et al., 2006). Amino acids in the predicted binding regions were substituted with alanine, introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) performed per manufacturer's instructions. Primers were designed using the Stratagene Primer Mutagenesis program.

In vitro rescue of CSFV Brescia and CΔIQ 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 lithium chloride and transfected into SK6 cells by electroporation at 500 V, 720 Ω, 100 W with a BTX 630 electroporator (BTX, San Diego, CA). Cells were seeded in 12-well plates (Costar, Cambridge, MA) and incubated for 4 days at 37 °C and 5% CO_2. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at −70 °C.

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 (Sanger et al., 1977). Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on an ABI PRISM 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Development of the cDNA library

A porcine primary macrophage cDNA expression library was constructed (Clontech, Mountain View, CA) using monocytes/macrophages obtained from healthy CSFV-free swine exactly as previously described (Gladue et al., 2010). Macrophage cultures were prepared from defibrinated swine blood. Total RNA were extracted from adherent cells using an RNeasy Mini kit (Qiagen, Valencia, 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 porcine β-actin gene. RNA quality was assessed using RNA

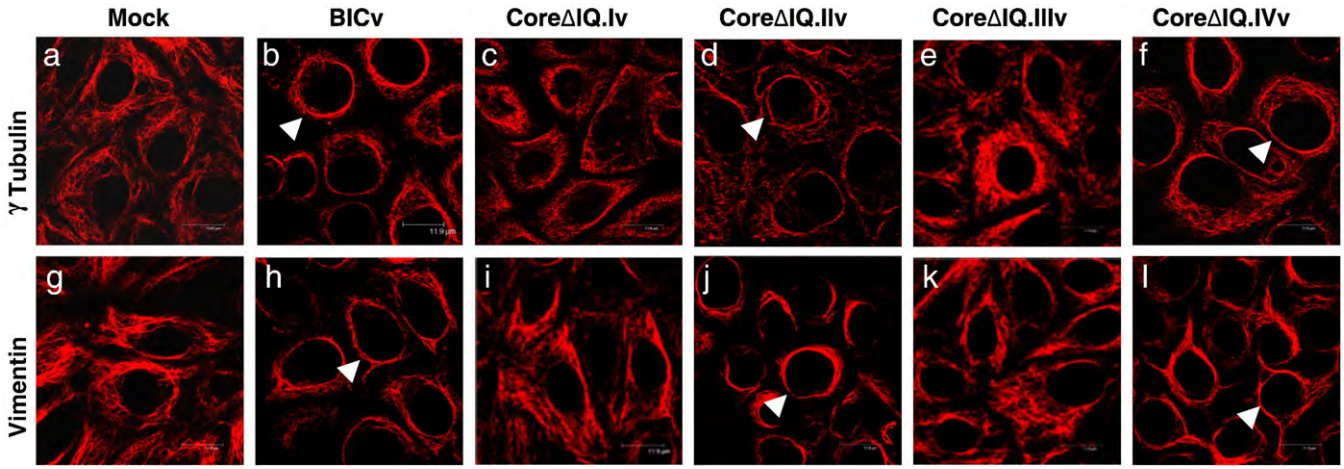

Fig. 5. Effect of BICv and CoreΔIQ viruses infection on host cell microtubules and intermediate filaments. Monolayers of SK6 cells were infected with BIC and CoreΔIQ viruses or mock infected, fixed with paraformaldehyde at 48 h post-infection, and processed for IF staining and confocal microscopy as described in Materials and methods. The microtubule was immunolabeled with mouse anti-γ-tubulin (a–f), and intermediate filaments with mouse-anti-vimentin (g–l). AlexaFluor 594-conjugate antibodies were used as secondary antibodies (red).

Nano Chips on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Cellular proteins were expressed as GAL4-AD fusion proteins while CSFV Core was expressed as GAL4-BD fusion proteins.

Library screening

The GAL4-based yeast two-hybrid system was used for this study (Chien et al., 1991; Fields and Song, 1989). The 'bait' protein, CSFV Brescia Core protein (amino acid residues 168–268 of the CSFV polyprotein), was expressed with an N-terminus fusion to the GAL4 Binding Domain (BD). As 'prey', the previously described swine macrophage cDNA library containing proteins fused to the GAL4 Activation Domain (AD) was used. Screening was done as previously described (Gladue et al., 2010). The IQGAP1 recovered from the library contained amino acids (502–704) of *homo sapiens* IQGAP1 (NCBI Reference Sequence: AAI39732) amino terminal fused to the GAL4-AD.

Infection of cells and confocal microscopy

Sub-confluent monolayers of SK6 cells grown on 12 mm glass coverslips in 24-well tissue culture dishes, were infected with BICv, CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, CoreΔIQ.IVv at a multiplicity of infection (MOI) of 1–5 TCID50/cell, or mock infected for 48 h in Dulbecco's minimum essential medium (DMEM, Invitrogen, CA) containing 1% heat inactivated fetal bovine serum and 1% antibiotics. At forty-eight hours after infection the cells were fixed with 4% paraformaldehyde (EMS, Hatfield, PA) and analyzed by confocal microscopy. Immunofluorescence and confocal microscopy using the antibodies listed below were performed as previously described (O'Donnell et al., 2005). Monoclonal antibodies against γ-tubulin (Sigma) and vimentin (Sigma), at a 1/100 dilution, were used as markers to identify microtubules and intermediate filaments respectively.

Animal infections

Each of the IQGAPv mutants was initially screened for their virulence phenotype in swine relative to the virulent Brescia strain. Swine used in these studies were 10 to 12 weeks old, forty-pound commercial breed pigs inoculated intranasally with 10^5 $TCID_{50}$ of either mutant or wild-type parental virus (BICv). Clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the 21-day experiment. Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman, Coulter, CA).

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Custom-engineered chimeric foot-and-mouth disease vaccine elicits protective immune responses in pigs

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Chimeric foot-and-mouth disease viruses (FMDV) of which the antigenic properties can be readily manipulated is a potentially powerful approach in the control of foot-and-mouth disease (FMD) in sub-Saharan Africa. FMD vaccine application is complicated by the extensive variability of the South African Territories (SAT) type viruses, which exist as distinct genetic and antigenic variants in different geographical regions. A cross-serotype chimeric virus, vKNP/SAT2, was engineered by replacing the external capsid-encoding region (1B-1D/2A) of an infectious cDNA clone of the SAT2 vaccine strain, ZIM/7/83, with that of SAT1 virus KNP/196/91. The vKNP/SAT2 virus exhibited comparable infection kinetics, virion stability and antigenic profiles to the KNP/196/91 parental virus, thus indicating that the functions provided by the capsid can be readily exchanged between serotypes. As these qualities are necessary for vaccine manufacturing, high titres of stable chimeric virus were obtained. Chemically inactivated vaccines, formulated as double-oil-in-water emulsions, were produced from intact 146S virion particles of both the chimeric and parental viruses. Inoculation of guinea pigs with the respective vaccines induced similar antibody responses. In order to show compliance with commercial vaccine requirements, the vaccines were evaluated in a full potency test. Pigs vaccinated with the chimeric vaccine produced neutralizing antibodies and showed protection against homologous FMDV challenge, albeit not to the same extent as for the vaccine prepared from the parental virus. These results provide support that chimeric vaccines containing the external capsid of field isolates can be successfully produced and that they induce protective immune responses in FMD host species.

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INTRODUCTION

Most of Europe, North America and some countries in South America have eradicated foot-and-mouth disease (FMD) through the administration of inactivated-FMD vaccines since the 1960s (Ward *et al.*, 2007). Despite international regulations limiting routine vaccination in many parts of the world, it remains an important strategy for disease control in countries where FMD is endemic. In sub-Saharan Africa, control of FMD is complicated by the African buffalo that serves as a reservoir of FMD virus (FMDV) and contributes to the spread of FMD to other wildlife species and livestock. Moreover, genetic and antigenic characterization of FMDV field isolates have revealed that these viruses evolve rapidly in different geographical areas (Esterhuysen, 1994; Vosloo *et al.*, 1995; Bastos *et al.*, 2001, 2003). This has led to the available vaccines being unable to adequately cover the extent of antigenic variation within the South African Territories (SAT) types of FMDV (Reeve *et al.*, 2010). Thus, for vaccination to be effective in sub-Saharan Africa, it requires the incorporation of vaccine strains representative of viruses circulating in that geographical area and that such strains should be available for specific regions. However, adaptation of wild-type SAT viruses in cell culture to produce high yields of stable antigen is an intricate and time-consuming process that is often associated with a low success rate.

Infectious cDNA clone technology (Zibert *et al.*, 1990; Rieder *et al.*, 1993, 1994) for FMDV SAT viruses has

provided a valuable tool for genetic manipulation and biological characterization of field and laboratory strains (van Rensburg *et al.*, 2004; Storey *et al.*, 2007; Maree *et al.*, 2010). Chimeras, containing the external capsid-encoding region (1B-1D/2A) of another FMDV, retain the replication machinery of the backbone as the P1, 2A and 3C proteins are required for FMD viral capsid assembly (Beard *et al.*, 1999) whilst acquiring the antigenic properties of the parental virus, including its ability to bind to cellular receptors that allow host-cell internalization (Maree *et al.*, 2010). This technology may therefore be utilized towards the production of custom-made vaccines specific to geographical regions or in cases of sudden FMDV outbreak. For such a chimera-derived vaccine to be a viable alternative to currently used vaccines, it should have high titre replication in cell culture, stability of the virus particle and antigen, recovery of high 146S antigen mass following chemical inactivation, appropriate immunological specificity and the ability to elicit a protective immune response in animals (Rweyemamu, 1978; Doel, 2003).

In this study, the feasibility of using a chimeric vaccine was assessed by determining its immunogenicity and protective ability following the immunization of pigs. The vaccine was prepared from a chimeric virus containing the external capsid-encoding region of a rapidly replicating, cell-adapted SAT1 vaccine strain, KNP/196/91, that was exchanged into the genetic background of an SAT2 infectious clone. The recovered chimeric virus exhibited comparable plaque morphologies, infection kinetics, virion stability and antigenic profiles to the KNP/196/91 parental virus. In a full potency test of the chimeric vaccine in pigs, good humoral immune responses were elicited and the majority of the animals were protected against homologous FMDV challenge and passed the 50 % protective dose (PD_{50}) requirement of the Office International des Epizooties for emergency vaccine use. These results suggest that custom-engineered chimeric FMD vaccines can be produced and applied in a fashion similar to the current inactivated vaccines.

RESULTS

Parental viral properties are retained in the recovered vKNP/SAT2

The FMDV SAT1 isolate KNP/196/91 has been proven to be an efficient vaccine strain displaying the required qualities for a good vaccine candidate (see Introduction). Hence, KNP/196/91 virus that displayed broad antigenic coverage (Reeve *et al.*, 2010) was utilized to investigate the prospect of engineering chimeric vaccines to provide protection against FMD for specific Southern African regions affected by circulating SAT1 viruses.

Plaque morphologies for vKNP/SAT2, KNP/196/91 and vSAT2 viruses were compared on BHK-21, IB-RS-2 and CHO-K1 cell lines (Fig. 1a). As shown in Fig. 1(a) vKNP/SAT2 and KNP/196/91 viruses formed micro (<1 mm) to medium (3–5 mm) or small (1–2 mm) to large (6–7 mm) plaques on BHK-21 cells, respectively. On IB-RS-2 cells the plaques were micro to small and micro to medium for the vKNP/SAT2 and KNP/196/91 viruses, respectively. vKNP/SAT2, KNP/196/91 and vSAT2 viruses each formed clear plaques on CHO-K1 cells, which is characteristic of viruses that use heparan sulphate proteoglycans (HSPG) as receptors. The comparable ability to infect cultured cells originating from different species is indicative that the vKNP/SAT2 capsid retained the characteristics of the KNP/196/91 virus. The deduced amino acid sequences of the external capsid-encoding region for vKNP/SAT2 and KNP/196/91 viruses were identical following their transfection and recovery in BHK-21 cells (data not shown).

In growth curves performed in BHK-21 cells (Fig. 1b), vKNP/SAT2 displayed similar replicative ability in BHK-21 cells to KNP/196/91 up to 6 h post-infection. At 12 h post-infection KNP/196/91 and vKNP/SAT2 had peak infectivity titres of 8.9×10^8 and 8.2×10^8 p.f.u. ml^{-1}, respectively. For vaccine purposes the optimal harvest time for both vKNP/SAT2 and KNP/196/91 is 12 h post-infection. This compares favourably to the time balance for stable particle survival resulting from the processes of virus assembly, rate of inactivation and the effect of proteolytic enzymes released into virus-infected cultures (Doel & Collen, 1983). The rate of production of infectious vSAT2 particles was lower than for the SAT1 viruses from 4 to 12 h.

To determine the antigenic profiles of vKNP/SAT2 and KNP/196/91, a virus neutralization test (VNT) was performed against a panel of SAT1 and SAT2 pig and cattle antisera (Fig. 2). The data showed similar neutralization values for the wild-type (passage history: PK_1RS_4) and cell culture-adapted (passage history: B_1BHK_9) isolates indicating that the amino acid variation with adaptation to BHK-21 cells did not significantly influence the major antigenic determinants. As expected, SAT2 antisera showed no cross neutralization with either of the SAT1 isolates (Fig. 2).

Antigen yield is determined by the biophysical properties of FMDV

Biophysical stability of the infectious virus or antigen has been correlated with the protective nature of FMD vaccines (Doel & Baccarini, 1981). With this in mind, vKNP/SAT2 and KNP/196/91 were compared in terms of their biophysical properties following treatment at different temperatures, pH values and salt concentrations (Fig. 3). Following virus exposure to temperatures of 25, 37, 45 and 55 °C (Fig. 3a), viable virus particles were detected at decreased concentrations for vKNP/SAT2, similar to those of KNP/196/91. After 30 min at 55 °C the infectious particles of vKNP/SAT2 and KNP/196/91 had been completely inactivated. After extended incubation at 4 °C both viruses were present up to 28 days (Fig. 3b) and the infectious titres observed for vKNP/SAT2 and KNP/196/91

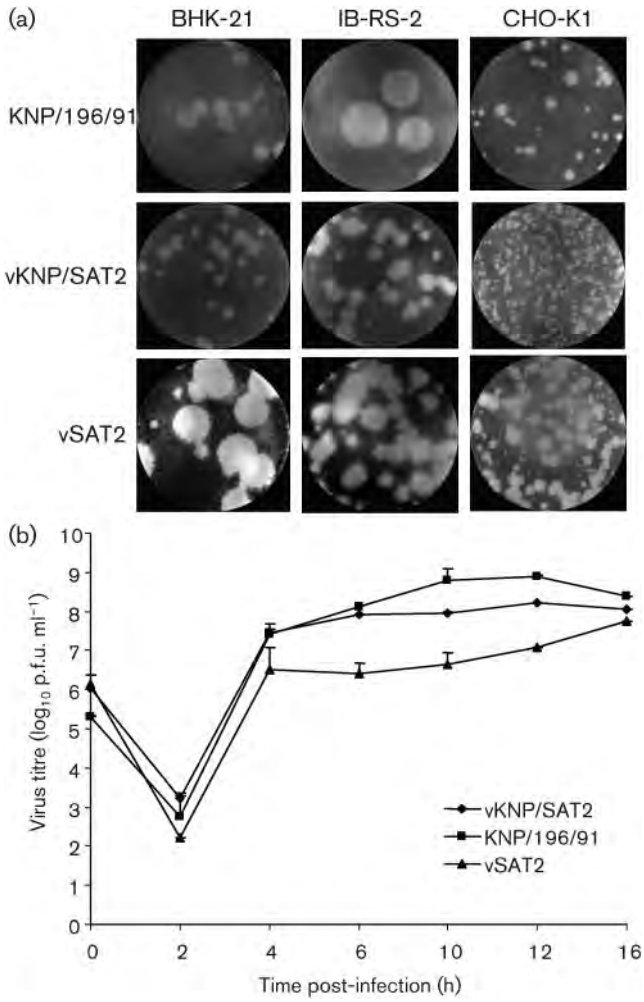

Fig. 1. (a) FMDV replication in BHK-21, IB-RS-2 and CHO-K1 cell cultures expressing diverse cellular receptors utilized by FMDV for cell entry. Morphology of plaques for the KNP/196/91 parental virus, vKNP/SAT2 chimera and vSAT2 were determined by plaque assay at 48 h post-infection. (b) Growth curves for the KNP/196/91 parental virus, vKNP/SAT2 chimera and vSAT2. BHK-21 cells were infected at an m.o.i. of 2 for 1 h and subsequently washed with MES-buffered saline (pH 5.5). Samples were frozen at selected time intervals and titrated on BHK-21 cells. Each data point represents the mean (±SD) of at least two wells.

were 2.6×10^3 and 5.7×10^3 p.f.u. ml^{-1}, respectively, under these conditions. Moreover, no significant variation in thermal stability was observed between the two viruses when they were incubated at 25 °C for 14 days (Fig. 3b). However, no infectious particles were detected at 28 days under these conditions.

The stability of the viruses was tested at pH values ranging from 6.5 to 9.0 (Fig. 3c). Treatment of vKNP/SAT2 and KNP/196/91 at pH 6.5 yielded a slight decrease in the titre of infectious particles after 30 min. The titre of infectious particles for both viruses was similar following treatment with buffers at alkaline pH for 30 min (Fig. 3c). The capsid stability of vKNP/SAT2 was comparable to that of KNP/196/91 at various NaCl concentrations ranging from 50 mM to 1.5 M (Fig. 3d). Taken together, these results did not indicate any differences in the biophysical properties of the parental and chimeric viruses.

Guinea pig antibody titres in relation to vaccine dose

Two separate formulations incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were used to assess the antibody response to immunization in guinea pigs (Fig. 4). Following animal immunization, serum samples were collected at weekly intervals and tested by using a sandwich ELISA specific for KNP/196/91. Seroconversion occurred at approximately 7 days post-vaccination (d.p.v.) and serological responses varied with time ($P<0.001$), dose ($P<0.001$) and vaccine ($P=0.001$; Fig. 4). High antibody titres were obtained for guinea pigs vaccinated with all three of the vKNP/SAT2 vaccine doses and titres peaked between 21 and 28 d.p.v. (Fig. 4a). In comparison, the antibody responses elicited by the KNP/196/91 vaccine were similar to those of the same dose of the vKNP/SAT2 vaccine and peaked at 28 d.p.v. (Fig. 4b).

FMDV-specific antibody responses induced by vKNP/SAT2 and KNP/196/91 vaccines in pigs

To investigate vKNP/SAT2 as a potential chimeric vaccine, a 6 μg dose was taken as the full dose in a decreasing dose potency trial in pigs. Two separate double oil emulsions incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were prepared and used for vaccination in a full potency trial (European Pharmacopoeia, 2006; Office International des Epizooties, 2009). The antibody response elicited in pigs varied with time ($P<0.001$), dose (full, 6.0 μg; quarter, 1.5 μg; one-sixteenth, 0.375 μg; $P=0.003$) and vaccine ($P=0.011$) when monitored at weekly intervals by using a KNP/196/91-specific solid phase competition ELISA (SPCE) (Fig. 5). The full and quarter doses of the vKNP/SAT2 vaccine (Fig. 5a, b) elicited an antibody response comparable to the equivalent doses of the KNP/196/91 vaccine (Fig. 5d, e) up to 21 d.p.v., whereafter the titres for vKNP/SAT2 remained similar until the day of challenge. Most animals vaccinated with the vKNP/SAT2 one-sixteenth dose (Fig. 5c) were borderline positive at the time of challenge. However, the results from the SPCE indicated that the KNP/196/91 vaccine elicited positive antibody responses for all vaccine doses (Fig. 5f).

Comparison of neutralization titres and protection

Serum neutralizing-antibody responses were measured by a VNT at the day of challenge for the vaccinated and control animals (Table 1). All of the pigs were negative for FMDV-specific neutralizing antibody at the start of the study (data not shown). Positive neutralizing-antibody titres were

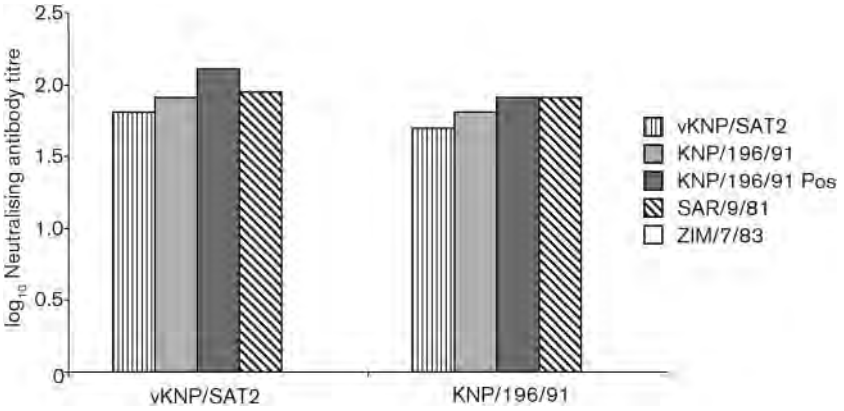

Fig. 2. Antigenic profiles of FMD viruses vKNP/SAT2 (B_1BHK_9) and KNP/196/91 (PK_1RS_4), which has not been cell-culture adapted in BHK-21 cells, tested against FMDV SAT1 antisera (vKNP/SAT2 chimera, KNP/196/91 parental and SAR/9/81) and SAT2 antisera (ZIM/7/83) in a VNT. Pig antisera vKNP/SAT2 and KNP/196/91 were from the vaccinated animals in this potency trial, whereas all other antisera originated from cattle. A positive-control serum of high titre (KNP/196/91 Pos) was included. Serum titres $\geqslant 1.7$ were regarded as positive.

induced for the full doses of both vaccines. For the quarter dose of the vKNP/SAT2 and KNP/196/91 vaccines, three and four animals, respectively, were positive for neutralization of the KNP/196/91 virus. Whilst four pigs vaccinated with the KNP/196/91 one-sixteenth dose had positive neutralizing antibody titres, the entire vKNP/SAT2 one-sixteenth group had no detectable neutralizing virus titre. Statistical analysis indicated that a significant

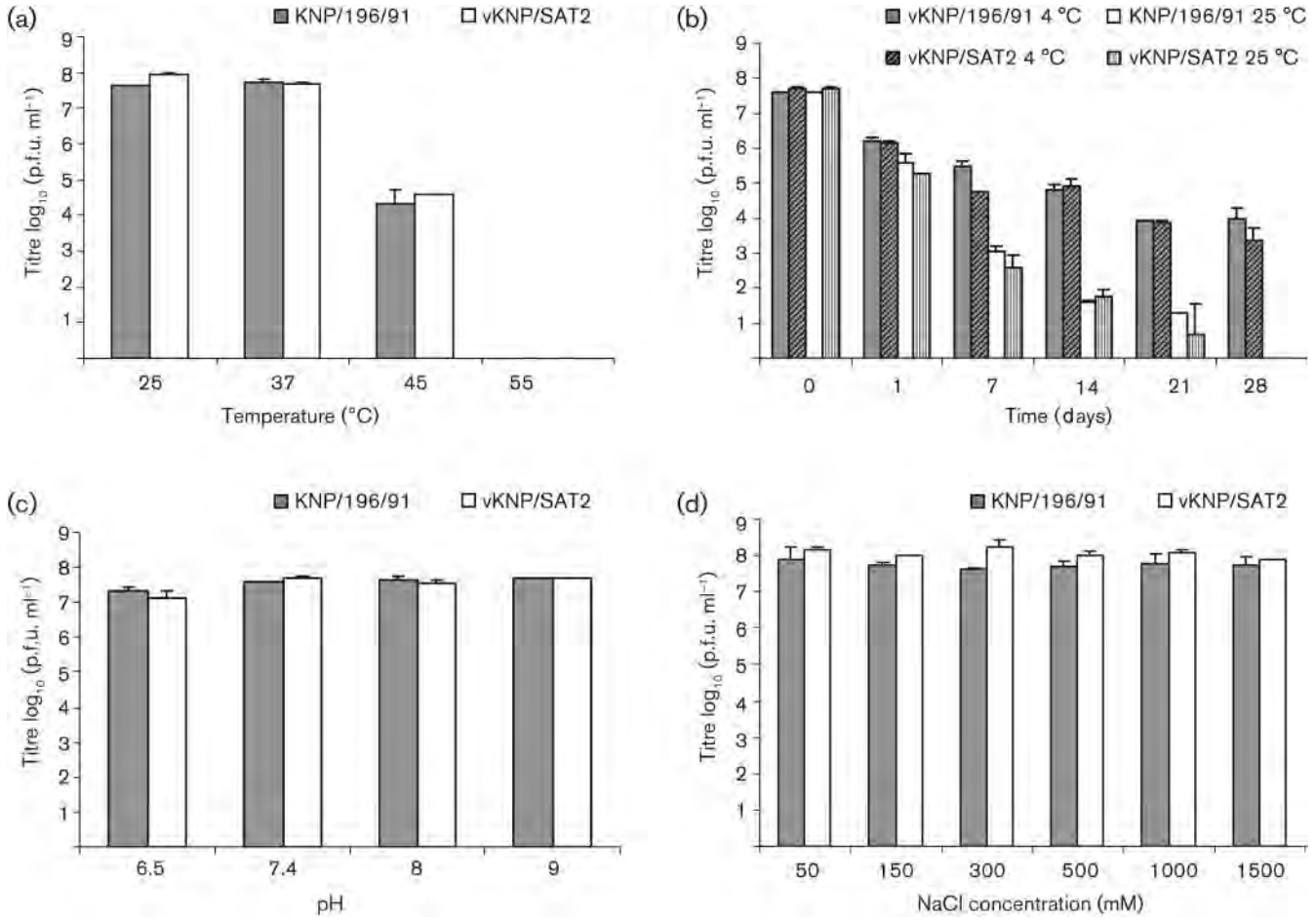

Fig. 3. Biophysical stability of 146S virion particles of KNP/196/91 and the recovered vKNP/SAT2 chimera at different temperatures (a, b), pH (c) and NaCl concentrations (d). Purified virion particles were tested for temperature stability by incubation at the indicated temperatures for 30 min (a), and at 4 °C and 25 °C over a period of 4 weeks (b). All samples were titrated on BHK-21 cells.

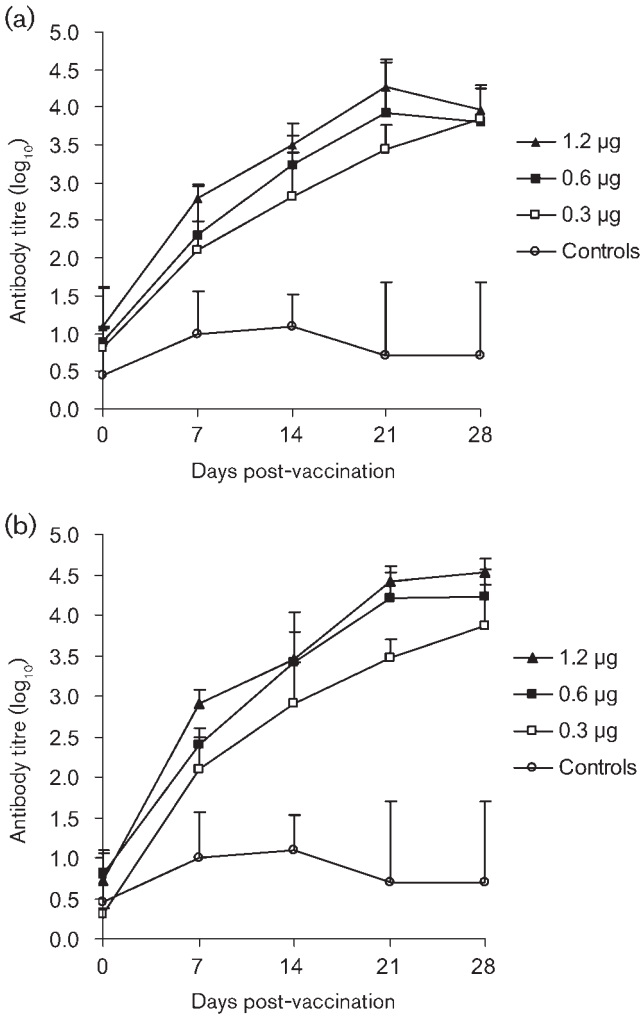

Fig. 4. Antibody responses elicited in guinea pigs for varying antigen payloads (0.3, 0.6 and 1.2 µg) of the vKNP/SAT2 (a) and KNP/196/91 (b) vaccines. Serum samples were tested by using a sandwich ELISA specific for KNP/196/91 and mean titres per group of 10 animals for each vaccine dose were determined at weekly intervals. Bars indicate the mean (±SD) of each group.

difference in neutralizing antibody response was induced by the chimeric and parental vaccines for the one-sixteenth dose (P=0.02), but not for the full and quarter doses. Comparisons between the immune responses for the three doses of the respective vaccines indicated no difference for KNP/196/91, suggesting that the one-sixteenth dose for the parental vaccine already gives a protective response and that it appears to be as efficient as the full dose under these conditions. For vKNP/SAT2 the neutralizing antibody response conferred by the full and quarter dose was not different. The response against the one-sixteenth dose was significantly lower compared with the full (P=<0.02) and the quarter dose (P=0.03). Similar antibody response profiles at day 28 were observed using both the SPCE (Fig. 5) and VNT (Table 1) for animals from all the groups and vaccinated with both antigens.

The mean neutralizing-antibody titres for the animals protected by the vKNP/SAT2 full and quarter dose were 1.98 (SD=0.09) and 1.97 (SD=0.19) log titre, respectively, and ranged between 1.7 and 2.1 for individual pigs. Despite the fact that a mean 1.8 (SD=0.06) log titre for the two unprotected animals in the vKNP/SAT2 full-dose group was similar to titres observed for protected animals and that it is considered positive for SAT types in the VNT, these animals developed FMD lesions. The three animals of the vKNP/SAT2 one-sixteenth dose that were protected had a lower mean titre of 1.09 (SD=0.25) compared with the unprotected animals in the quarter dose, which had mean antibody levels of 1.49 (SD=0.06) log titre. Following challenge, 60 % of the animals receiving the chimeric vaccine were fully protected against disease (Table 1). The onset of FMD lesions in animals with clinical disease was delayed and restricted in distribution compared with the control animals, indicative of partial protection in these animals. For most of the vaccinated pigs increased body temperatures were observed for 1 day only, whereafter temperatures decreased (data not shown). In comparison, the KNP/196/91 vaccine elicited mean titres ranging from 2.21 (SD=0.39) for the full dose, 1.9 (SD=0.27) for the quarter dose and 1.81 (SD, 0.34) for the one-sixteenth dose. Notwithstanding the absence of measurable positive neutralizing-antibody titres for two animals that received the KNP/196/91 vaccine, all animals were protected against challenge. The PD_{50} for the vKNP/SAT2 and parental vaccine were >6.4 and >39.4, respectively, as calculated by the Kärber method (Kärber, 1931). The vaccine potency is expressed as the number at which 50 % of the animals used for the challenge experiments were protected.

DISCUSSION

FMD vaccine candidates should be closely related to field strains (Doel, 1996) and induce an immune response with a broad immunological cross-reactivity (Esterhuysen *et al.*, 1988) for appropriate protection against subtypes (Pay, 1983). This is especially relevant in sub-Saharan Africa where the high degree of antigenic variability in FMDV and the presence of several serotypes, including several subtypes, have important implications for vaccine strain selection. In the present study, by engineering a chimeric virus, a possible alternative to the conventional inactivated-FMD vaccine production for the SAT type viruses was investigated for the development of custom-engineered inactivated-FMD vaccines.

A cross-serotype chimeric virus, vKNP/SAT2, replicated stably in both FMD host- and non-host-species-derived cell lines and the ability to produce plaques was similar for vKNP/SAT2 and KNP/196/91. Although BHK-21 cells are derived from a non-host species, the integrin cellular receptors expressed by these cells while growing in monolayers are recognized by FMDV in an RGD (arginine–glycine–aspartic acid)-dependent manner (Fox *et al.*, 1989; Mason *et al.*, 1994; DiCara *et al.*, 2008 and

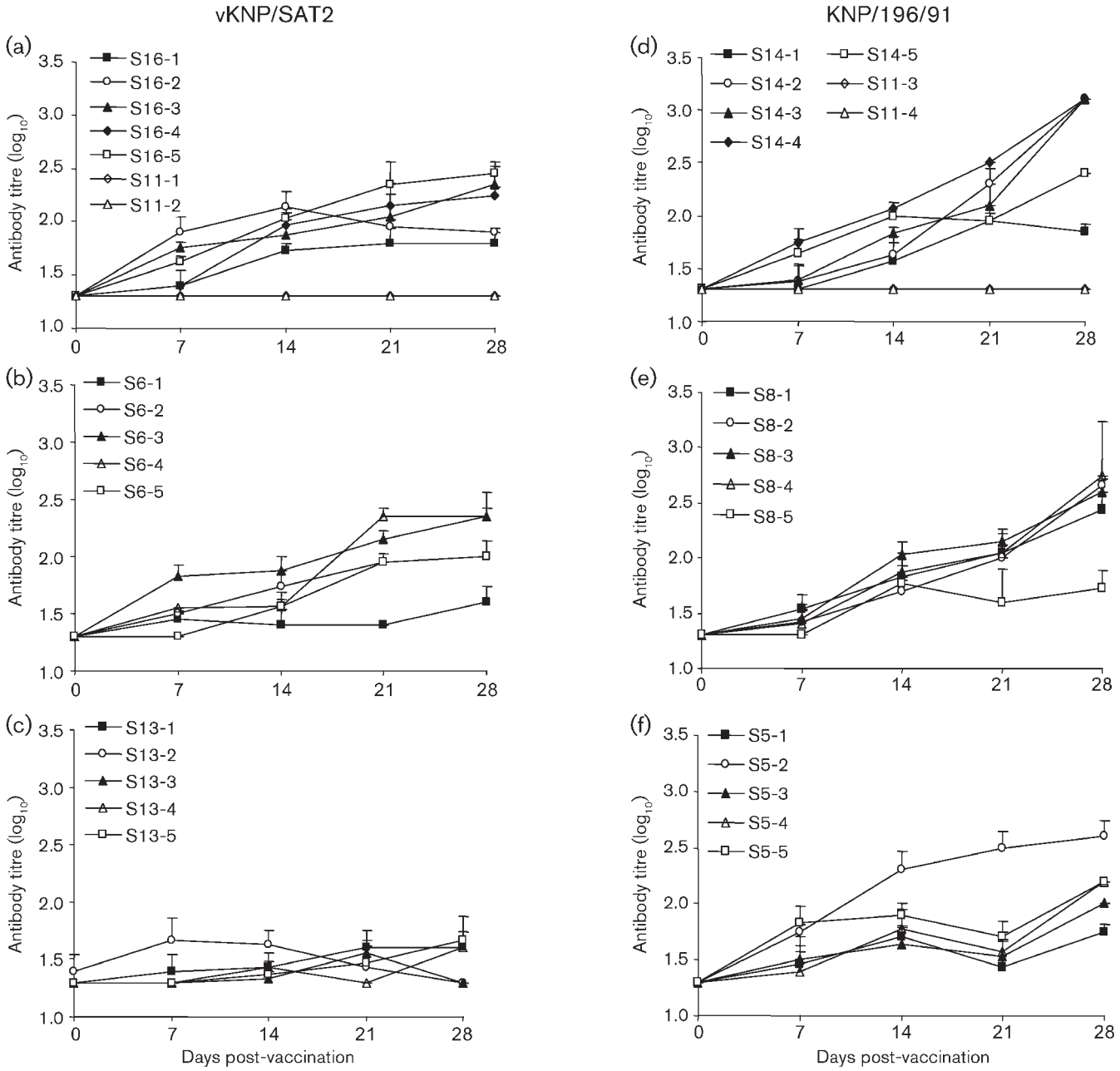

Fig. 5. Antibody responses elicited in pigs by full (6.0 μg), quarter (1.5 μg) and one-sixteenth (0.375 μg) doses of vKNP/SAT2 (a–c) and KNP/196/91 (d–f) vaccines, respectively. Two separate double oil emulsions incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were prepared and used for vaccination. Serum samples collected were tested by using a KNP/196/91-specific SPCE and mean titres per group of five animals for each vaccine dose were determined at weekly intervals. Means were determined from at least two replicates. Animals of the control group (S11 animals) were inoculated with a placebo formulation without antigen.

references therein). The porcine cell line IB-RS-2 was utilized for virus isolation and the VNT, and expresses mainly $\alpha v\beta 8$ on the cell surface (Burman *et al.*, 2006). In contrast, CHO-K1 cells lack the integrin cellular receptors used by FMDV for cell entry and express HSPG, which is used as an alternative receptor by cell culture-adapted FMDV (reviewed by Jackson *et al.*, 2003). Notably, the presence of the external capsid proteins of an SAT1 virus in the genetic background of an SAT2 virus did not alter the biological properties of the chimera markedly, suggesting that it is a method that leads to the design of good vaccine candidates. In fact, vKNP/SAT2 retained the rapid infection kinetics of KNP/196/91 in BHK-21 cells that is imperative for production of a high yield of inactivated

Table 1. Neutralizing antibody responses and post-challenge clinical data for pigs vaccinated with vKNP/SAT2 and KNP/196/91

Vaccines were administered as a single inoculation at day 0. Neutralizing antibody titres were determined on day 28 post-vaccination and are the mean values of three replicates of the VNT. Titres of $\geqslant \log_{10}$ 1.7 were regarded as positive. The maximum temperature observed for each animal after challenge is shown. Temperatures of $\geqslant$39.6 °C were regarded as positive for fever. The degree of disease post-challenge was defined as: none, no lesions on any feet; mild, lesions on only one foot; severe, lesions on two to three feet. Clinical signs of FMD were regarded as lesions on any foot, other than the limb used for challenge, and elevated body temperatures of $\geqslant$39.6 °C. Total protection was considered as a complete absence of lesions.

Vaccine	Animal	Neutralizing antibody titre	Post-challenge clinical data	
			Temperature (°C)	Degree of disease
vKNP/SAT2				
6 μg	16-1	1.7	39.9	Mild
	16-2	1.8	39.7	Mild
	16-3	1.9	39.2	None
	16-4	2.0	39.8	None
	16-5	2.0	39.2	None
1.5 μg	6-1	1.4	39.8	Mild
	6-2	1.5	39.6	Mild
	6-3	2.1	39.3	None
	6-4	2.1	39.3	None
	6-5	1.7	39.2	None
0.375 μg	13-1	0.8	40.4	None
	13-2	0.4	40.6	Severe
	13-3	0	41.0	Mild
	13-4	1.2	39.8	None
	13-5	1.2	39.9	None
KNP/196/91				
6 μg	14-1	1.7	39.2	None
	14-2	2.5	39.9	None
	14-3	2.5	39.3	None
	14-4	2.5	39.0	None
	14-5	1.9	39.6	None
1.5 μg	8-1	2.0	39.3	None
	8-2	2.2	39.0	None
	8-3	1.9	39.1	None
	8-4	1.8	39.1	None
	8-5	1.5	39.4	None
0.375 μg	5-1	1.3	39.9	None
	5-2	2.2	39.5	None
	5-3	1.7	39.6	None
	5-4	1.9	39.4	None
	5-5	1.8	39.4	None
Controls				
Placebo*	11-1	0	39.9	Severe
	11-2	0	40.1	Severe
	11-3	0	41.0	Severe
	11-4	0	40.2	Severe

*Control animals received a placebo formulation containing Tris/KCl buffer.

146S particles to be used as FMD vaccine. Both vKNP/SAT2 and KNP/196/91 displayed the cell culture-adaptation phenotype (Maree *et al.*, 2010), possibly because of interactions with HSPG receptors (Jackson *et al.*, 1996, 2003; Sa-Carvalho *et al.*, 1997; Fry *et al.*, 1999). Such alteration in FMD viruses' ability to utilize HSPG is important as it is associated with more rapid replication in BHK-21 cells, a change in cell tropism, a more effective cell killing capacity and could contribute to improved production of FMD vaccines in suspension cultures (Amadori *et al.*, 1994; Sevilla *et al.*, 1996; Baranowski, *et al.*, 1998).

The potential of vKNP/SAT2 as a vaccine strain producing high yields of stable antigen was emphasized by its comparable biophysical properties to those of KNP/196/91. Virion stability is of importance during the vaccine manufacturing process as the maintenance of intact 146S particles is relevant to the amount of immunogenicity induced by antigens and to vaccine efficacy (Doel & Baccarini, 1981; Doel, 2003). In the present study, a comparable decrease in infectivity was observed for the respective viruses at room temperature and above. The similar capsid stabilities of vKNP/SAT2 and KNP/196/91 observed at various NaCl concentrations is applicable to the range of ionic strengths that might exist during the purification of 146S particles in the vaccine production process. Unlike most other picornaviruses, FMDV is susceptible to low-pH-induced capsid disassembly, a characteristic of great importance to its pathogenicity (King, 2000). Both KNP/196/91 virus and its derivative were stable throughout a range of acidic to basic pH treatments. In addition to the above-mentioned qualities necessary for successful vaccine manufacturing, KNP/196/91 also displays good *r* values, which are an indication of the probability of protection for vaccinated animals against circulating variant viruses in the field (Esterhuysen, 1994; Reeve *et al.*, 2010). Moreover, comparable neutralization profiles were obtained for the respective viruses tested against other SAT1 and 2 antisera, confirming that vKNP/SAT2 retained the antigenic properties of KNP/196/91.

The advantages of using oil formulations for FMD vaccines have been well established and the Montanide ISA 206B (water/oil/water) has proven to be an efficient adjuvant (Barnett *et al.*, 1996) for the SAT types. Long-lasting immune responses were observed for cattle following immunization with vaccine containing KNP/196/91 antigen formulated in Montanide ISA 206B oil-based adjuvant (Hunter, 1996; Cloete *et al.*, 2008). The vKNP/SAT2 and KNP/196/91 vaccines elicited good humoral immune responses in guinea pigs and pigs. The majority of the pigs vaccinated with vKNP/SAT2 were protected against live-virus challenge. In addition, the onset of disease was delayed for most of the vKNP/SAT2-vaccinated pigs when compared with the control animals, and the clinical signs were less severe. This is indeed promising as antigen doses of 6 μg correlate with emergency FMD vaccines ($PD_{50} \geqslant 6$) that induce rapid protective immune responses (Barnett & Carabin, 2002).

Similar neutralizing antibody titres were elicited in pigs vaccinated with the vKNP/SAT2 and KNP/196/91 vaccines, as were observed for SAT type vaccines that conferred protection *in vivo* by Barnett *et al.* (2003). However, for some of the animals it is difficult to find a correlation between protection and neutralizing-antibody response. Protection was observed for three animals of the vKNP/SAT2 one-sixteenth group, which showed no detectable titre in the VNT. Although higher antigen doses elicited more neutralizing antibodies for most animals vaccinated with vKNP/SAT2 and KNP/196/91, even at low antigen concentrations protective immunity was induced. This phenomenon may be explained by additional neutralizing mechanisms that exist in the host, such as complement-enhanced neutralization. Another explanation might be the presence of a cell-mediated immunity (CMI) component contributing to the extent of protection, which could have a different response depending on the difference in non-capsid proteins in the virion. CMI parameters were not measured in this study. Previous studies have indicated that even though antibodies were non-neutralizing *in vitro* and/or at low concentrations, these were in fact protective *in vivo* (Anderson *et al.*, 1971; McCullough *et al.*, 1992; Vianna Filho *et al.*, 1993; Brehm *et al.*, 2008).

The antigen payload, the integrity of the FMDV particle and the conformation of the viral epitopes, are important factors to consider for FMD vaccine efficacy. The reason for the lesser extent of protection in pigs conferred by the vKNP/SAT2 chimeric vaccine is not clear. This matter could be addressed by characterization with well defined mAbs directed against SAT1 FMDV or revaccination of animals to determine the stability of the formulated antigen. To refine the construction of SAT type chimeras, the external capsid-encoding region (1B-1D/2A) can be further manipulated to make such viruses more specific in their ability to infect and replicate in the cells most commonly used for vaccine production. It would also be possible to design SAT type FMD vaccines where regions of the genome are engineered to optimize epitope representation. An additional benefit of using this recombinant DNA technology is that reverse genetics allows for modifications that could be incorporated to support serological differentiation of infected from vaccinated animals for surveillance of FMD in sub-Saharan Africa. The potential now exists to generate more effective new-generation chemically inactivated-FMD vaccines for this highly infectious and economically important disease, which are custom-engineered and specifically produced for geographical areas.

METHODS

Cell lines, viruses and plasmids. Baby hamster kidney-21 cells, clone 13 (BHK-21, ATCC CCL-10), Instituto Biologico Renal Suino-2 cells (IB-RS-2) and Chinese hamster ovary cells strain K1 (CHO-K1, ATCC CCL-61) were propagated as described previously (Storey *et al.*, 2007). Primary pig kidney cells (PK) were maintained in RPMI medium (Sigma-Aldrich) supplemented with 10 % FCS (Delta Bioproducts). The SAT1 virus KNP/196/91 (passage history: PK_1RS_4; subscript indicates the number of passages) was originally isolated from a buffalo in the Kruger National Park, South Africa, and was passaged in cattle (indicated as B) and BHK-21 cells (passage history: $PK_1RS_4B_1BHK_4$). Homologous challenge virus was prepared by three passages of KNP/196/91 in pigs (passage history: $PK_1RS_4B_1BHK_4P_3$). The external capsid-encoding region (1B-1D/2A) of plasmid pSAT2, a genome-length infectious cDNA clone of SAT2/ZIM/7/83 (van Rensburg *et al.*, 2004), was replaced with that of KNP/196/91 to yield plasmid pKNP/SAT2 (constructed by H.G. van Rensburg). In constructing the recombinant clone, pSAT was digested

with endonucleases *Ssp*I and *Xma*I to excise the approximately 2 kb external capsid-encoding region from the pSAT2 clone to facilitate cloning of the corresponding KNP/196/91 amplicon containing the same restriction endonuclease sites.

RNA synthesis, transfection and virus recovery. For RNA synthesis, plasmids pKNP/SAT2 and pSAT2 were linearized with *Swa*I and *Not*I, respectively, and *in vitro* transcribed using a MEGAscript T7 kit (Ambion). *In vitro*-transcribed RNA was transfected into BHK-21 cells using Lipofectamine 2000 reagent (Invitrogen). The cells were maintained at 37 °C for 48 h in virus growth medium (VGM; Eagle's basal medium supplemented with 1 % FCS and 1 % HEPES) and passaged as described previously (van Rensburg *et al.*, 2004). The viruses derived from the genome-length cDNA, designated vKNP/SAT2 and vSAT2, were confirmed by nucleotide sequencing and used in all subsequent experiments. Viral amplification was performed in BHK-21 cell monolayers for chimeric (vKNP/SAT2), parental (KNP/196/91) and vSAT2 viruses. Viruses were harvested at 90 % cytopathic effect at passage five.

Plaque and growth-kinetics assays. BHK-21 cell monolayers were infected with KNP/196/91, vKNP/SAT2 and vSAT2 at an m.o.i. of 2. After 1 h of adsorption, cells were washed with MBS (MES-buffered saline, pH 5.5) and then incubated with VGM at 37 °C. At several time points post-infection, cells and culture medium were frozen at −80 °C. Virus titres were determined by plaque assays (Rieder *et al.*, 1993). Briefly, cells were infected with virus for 1 h, followed by the addition of 2 ml tragacanth overlay and incubation for 48 h. The cells were then stained with 1 % (w/v) methylene blue in 10 % ethanol and 10 % formaldehyde, prepared with PBS (pH 7.4).

Virus purification and stability. vKNP/SAT2 and KNP/196/91 viruses were concentrated using 8 % PEG-8000 (Sigma-Aldrich) and purified on sucrose density gradients (10–50 %), prepared in TNE buffer [50 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl], as described by Knipe *et al.* (1997). Following fractionation, peak fractions corresponding to 146S virion particles [extinction coefficient E_{259}, (1 %)=79.9; Doel & Mowat, 1985] were pooled, the amount of antigen calculated from the peak area, analysed by SDS-PAGE and titrated on BHK-21 cells. The stability of the purified virion particles at different temperatures was determined by incubating the virion particles, diluted 1 : 50 in 2× TNE buffer [100 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl], at 25, 37, 45 and 55 °C for 30 min, and at 4 and 25 °C over a period of 4 weeks. Following cooling on ice, the viruses were titrated on BHK-21 cells. The stabilities of the purified virion particles at different pH (pH 6.5–9.0) and NaCl concentrations (50–1500 mM) were determined by diluting the virion particles 1 : 50 in TNE buffer and incubating at room temperature for 30 min. After pH neutralization with buffer [1M Tris (pH 7.4), 150 mM NaCl], the viruses were titrated on BHK-21 cells.

Antigen preparation and vaccine formulation. The vKNP/SAT2 and KNP/196/91 viruses harvested from infected BHK-21 cell monolayers were inactivated with 5 mM binary ethyleneimine for 26 h at 26 °C, concentrated and purified as described above. Two separate vaccine formulations, incorporating vKNP/SAT2 and KNP/196/91 inactivated 146S antigens as double oil emulsions with Montanide ISA 206B (Seppic), were prepared. The antigen was diluted in Tris/KCl buffer (0.1 M Tris, 0.3 M KCl, pH 7.5) to the required concentration. The oil adjuvant was subsequently mixed into the aqueous antigen phase (1 : 1) at 30 °C for 15 min and stored at 4 °C for 24 h. This was followed by a second brief mixing cycle for 5 min. A placebo vaccine was formulated that contained all the components except antigen.

Immunization of guinea pigs. The vaccines, formulated as described above, were initially tested in guinea pigs to determine the immunogenicity of the respective antigens. Six groups of 20 female guinea pigs (400–800 g each) were immunized intramuscularly with vaccines containing 0.3, 0.6 and 1.2 μg of the inactivated vKNP/SAT2 or KNP/196/91 antigen. Control animals received the placebo vaccine formulation. Ten animals from each group were bled alternately on 0, 7, 14, 21 and 28 d.p.v. Animals were anaesthetized intra-muscularly with a combination of xylazine and ketamine.

Vaccination and viral challenge of pigs. Thirty-four FMD-seronegative pigs (3–4 months of age and weighing 25–30 kg) were divided randomly into six groups of five animals each, and one control group of four animals. Each group was housed in a separate room in the high-containment animal facility of the Onderstepoort Veterinary Institute (ARC-OVI). Subsequent to an initial acclimatization period, the pigs were vaccinated by the intramuscular route immediately caudal to the ear with 2 ml (full dose), 0.5 ml (quarter dose) and 0.125 ml (one-sixteenth dose) of 3 μg ml^{-1} of either vKNP/SAT2 or KNP/196/91 vaccine. Control animals were vaccinated with a placebo vaccine formulation that lacked viral antigen. Serum samples were collected at 0, 7, 14, 21 and 28 d.p.v. for serological assays. At 28 d.p.v. the pigs were inoculated intra-epidermally in the coronary band of the left hind heel bulb with 10^4 $TCID_{50}$ KNP/196/91 challenge virus. During each of these procedures the pigs were sedated using azaperone. The animals were examined daily for fever and clinical signs. Body temperatures of ⩾39.6 and ⩾40 °C were considered as mild and severe fever, respectively. Upon observation of generalization of clinical lesions to other sites (e.g. to the snout and other legs), pigs were removed from the experiment and euthanized. At day 10 post-infection, the remainder of the animals were euthanized.

Antibody detection in guinea pigs. A SAT1 KNP/196/91-specific sandwich ELISA was performed on guinea pig sera to measure the amount of anti-KNP/196/91 antibodies present in each sample. The KNP/196/91 virus was added to a 96-well microplate (Nunc Maxisorp) coated with rabbit anti-KNP/196/91 antiserum. After incubation overnight at 4 °C the plates were washed with 1× PBS containing 0.05 % Tween 80 (PBS-T80). One hundred microlitres of each sample was added to normal bovine serum followed by diluting 1 : 1000 in 1× PBS containing 0.5 % (w/v) casein (PBS-casein). The dilution was added to the plates in triplicate and diluted 1 : 1 in PBS-casein. The plates were then incubated for 1 h at 37 °C and washed with PBS-T80. HRP-conjugated rabbit anti-guinea pig IgG (Sigma-Aldrich), diluted 1 : 100 in PBS-casein, was added and the plates incubated for 1 h at 37 °C. The plates were washed with PBS-T80, followed by addition of the substrate solution consisting of 1 ml (1 % w/v) 3,3′.5,5′ tetramethylbenzidine, sodium phosphate/citric acid buffer and 30 ml (30 % w/v) H_2O_2. After incubating for 15 min at room temperature, the reactions were stopped with 1 M H_2SO_4 and OD_{492} was measured with a Labsystems Multiscan Plus Photometer. The titre was determined from the $\log_{10}$ reciprocal antibody dilution giving 1.0 OD_{492} unit. Sera were considered positive when the titre was equal or greater than twice the value of the negative control sera.

Antibody detection in pigs. Antibodies in pig sera against KNP/196/91 were detected by an SAT1 KNP/196/91-specific SPCE. The SPCE was essentially carried out as described above. Briefly, the KNP/196/91 virus was trapped by rabbit anti-KNP/196/91 antiserum. After incubation overnight at 4 °C the plates were washed with PBS-T80. One hundred microlitres of a 1 : 20 dilution in PBS-casein of each sample was added in triplicate and diluted 1 : 1 in 50 μl of PBS-casein across the plate. Guinea pig anti-KNP/196/91 antiserum diluted 1 : 6000 in PBS-casein was added to the wells then incubated and washed. The addition of anti-species conjugate and the subsequent detection steps were as described above. Antibody titres were determined at the dilution where 50 % inhibition was observed

between the pig sera and the guinea pig anti-KNP/196/91 antisera. A cut-off value of $\log_{10}$ 1.7 was considered positive.

VNT. Neutralizing antibodies against SAT1 KNP/196/91 in serum samples collected at 28 d.p.v. from pigs were measured with a VNT, according to the method described in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* by Office International des Epizooties (2009) using IB-RS-2 cells in microtitre plates. The antibody titres were calculated as $\log_{10}$ of the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of virus in 50 % of the wells.

Data analysis. Antibody titres were presented on a $\log_{10}$ scale as means and SD. Repeated measures ANOVA analyses were performed to estimate the effects of vaccination strain and dose on ELISA antibody titres over time. VNT titres were compared using Student's *t*-test with Bonferroni adjustment for multiple comparisons. Statistical analyses were performed using commercially available software (SPSS version 17.0 for Windows; SPSS) and results interpreted at the 5 % level of significance.

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Rapid and Transient Activation of γδ T Cells to IFN-γ Production, NK Cell-Like Killing, and Antigen Processing during Acute Virus Infection

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γδ T cells are the majority peripheral blood T cells in young cattle. The role of γδ T cells in innate responses against infection with foot-and-mouth disease virus was analyzed on consecutive 5 d following infection. Before infection, bovine WC1$^+$ γδ T cells expressed a nonactivated phenotype relative to CD62L, CD45RO, and CD25 expression and did not produce IFN-γ ex vivo. Additionally, CD335 expression was lacking and no spontaneous target cell lysis could be detected in vitro, although perforin was detectable at a very low level. MHC class II and CD13 expression were also lacking. Following infection with foot-and-mouth disease virus, expression of CD62L and CD45RO was greatly reduced on WC1$^+$ γδ T cells, and unexpectedly, CD45RO expression did not recover. A transient increase in expression of CD25 correlated with production of IFN-γ. Expression of CD335 and production of perforin were detected on a subset of γδ T cells, and this correlated with an increased spontaneous killing of xenogeneic target cells. Furthermore, increased MHC class II expression was detected on WC1$^+$ γδ T cells, and these cells processed protein Ags. These activities are rapidly induced, within 3 d, and wane by 5 d following infection. All of these functions, NK-like killing, Ag processing, and IFN-γ production, have been demonstrated for these cells in various species. However, these results are unique in that all these functions are detected in the same samples of WC1$^+$ γδ T cells, suggesting a pivotal role of these cells in controlling virus infection. ***The Journal of Immunology*****, 2011, 186: 4853–4861.**

There are two major types of T lymphocytes in vertebrates, those that express the αβ TCR and those that express the γδ TCR on their cell surface. In young ruminants, the percentage of cells expressing the γδ TCR is considerably higher (50–60% of PBLs) compared with other mammalian species. What human neonates lack in number of circulating γδ T cells compared with calves may be made up by their activation state (1). However, these cells reduce in number as the animals age and may ultimately account for 5–25% of circulating T lymphocytes (2), more similar to humans (1–20%) (3). The role of these cells in the response to the acute infection caused by foot-and-mouth disease virus (FMDV) is poorly understood.

γδ T cells in the bovine can be divided into two major subsets by expression of the WC1 molecule, a member of the scavenger receptor cystein-rich family of proteins. These subsets express either WC1$^+$/CD3$^+$/CD5$^+$/CD2$^-$/CD6$^-$/CD8$^-$ cell surface proteins or WC1$^-$/CD3$^+$/CD5$^+$/CD2$^+$/CD6$^+$/CD8$^+$ (4, 5). Within the WC1$^+$ population of γδ T cells, there are two subpopulations expressing different isoforms of the WC1 molecule, as follows: WC1.1 and WC1.2. The WC1.1$^+$ γδ T cells appeared to be the main producers of IFN-γ, whereas the WC1.2$^+$ γδ T cells have been reported to proliferate in response to stimulation with mitogens (6, 7). Although the two populations can be distinguished, there is little information to conclude that they differ functionally. However, several reports have pointed out differences in tissue distribution among γδ T cells of the skin and the intestinal mucosa (8, 9). γδ T cells express the CD3 molecule, but stimulation with anti-CD3 Ab showed only a small population that responded by proliferation, further suggesting a difference in subpopulations (10).

Although human and mouse γδ T cells do not share phenotypic properties with bovine γδ T cells, such as expression of WC1 molecule, studies of human γδ T cells show those cells expressing Vγ2 were capable of lysing HSV type 1 (HSV-1) or vaccinia virus–infected Daudi cells (11). Interestingly, blocking CD3 or the γδ TCR with specific Abs led to abrogation of cytotoxicity, although their cytotoxicity was reported to be independent of MHC I or II restriction, indicating this subset of γδ T cells may function like NK cells. In cattle, studies have shown a dramatic increase in γδ T cells in the peripheral blood of cattle within the first days of infection with bovine herpesvirus type I. Furthermore, cattle vaccinated against FMDV were reported to have increased numbers of γδ T cells that adopted a NK-like phenotype and, like human cells, were capable of lysing virus-infected target cells (12). Similarly, in bovine viral diarrhea virus infection, γδ T cells increased 100-fold (13).

A protective role of γδ T cells was demonstrated by Sciammas et al. (14) in a mouse model using HSV-1 infection as γδ-deficient mice succumbed to infection. An interesting observation in these studies was demonstration of Ag-specific reactivity of γδ T cells, indicating that the responses observed were a result of direct recognition of unprocessed HSV glycoprotein. Recently, following extensive description of CD4$^+$/CD25^{high}/Foxp3$^+$ regulatory T cells in mice and humans, a similar function has now been ascribed to subpopulations of bovine WC1$^+$ γδ T cells as opposed to bovine CD4$^+$/CD25^{high}/Foxp3$^+$ cells (15). Taken together, γδ

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Abbreviation used in this article: FMDV, foot-and-mouth disease virus.

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T cells have functions that are well described in other T cell subsets. However, γδ T cells are largely ignored in considerations of vaccine design. Understanding how these functions are executed in γδ T cells may indicate immunological potential of these cells in vaccination against various pathogens.

Foot-and-mouth disease, caused by FMDV, is a devastating disease of domesticated ruminants with large economic consequence. Although vaccination is available, there are drawbacks, including the lack of tests to distinguish between vaccinated and infected animals and short duration of immunity. Different vaccination strategies could be developed, but this requires a thorough understanding of the immune response against FMDV in cattle. In our studies, we have initially aimed at understanding the response of bovine $WC1^+$ γδ T cells during the early phase of infection with FMDV. In a series of experiments, we now show that the γδ T cell subpopulation $WC1^+$ is activated shortly postinfection with FMDV, strain O1 Manisa. Interestingly, these cells show an increased production of IFN-γ and were capable of lysing a tumor cell line in spontaneous cytotoxicity assays. Furthermore, a small subpopulation of these cells activates MHC class II proteins and is capable of processing protein Ags. All of these activated phenotypes are detectable only during acute infection, days 2–4 following inoculation of virus. Therefore, there may be multiple roles for γδ T cells in the acute-phase infection with FMDV, including induction of both innate and adaptive immune responses. Whether such activation status translates into a protective function of γδ T cells during infection with FMDV is now of great interest.

Materials and Methods

Animals

Holstein cattle aged 3–5 mo were purchased from Thomas Morris (Springfield, PA) and acclimated for at least 7 d before use in the experiments. Animals were tested in groups of three and were age, weight, and gender matched. Before infection, baseline analysis was performed. All animal protocols were reviewed and procedures were approved by the Institutional Animal Care and Use Committee.

Virus

The O1 Manisa strain of FMDV was used in all studies described. The O1 Manisa was initially harvested from vesicles of infected cattle, titrated, and kept at −70°C until use. Animals were first sedated, and 1×10^4 50% tissue culture-infective dose was used to infect each animal. The dose was divided into four parts and inoculated in four spots intradermal lingually, at the base of the tongue. Animals were examined daily for development of lesions.

Blood sampling

Peripheral blood was drawn into vacutainer tubes from the jugular vein. Blood samples for cell separation were drawn into tubes containing sodium heparin. For cell separation, blood was diluted 1:1 with PBS in 50-ml conical tubes, underlayed with Lymphoprep (Axis-Shield, Oslo, Norway), and centrifuged at $1000 \times g$ for 20 min. PBMCs were collected and washed at least three times in PBS to remove all platelets and finally resuspended in RPMI 1640 supplemented with 10% FBS and used immediately or were resuspended in sorting buffer (0.5% BSA, 2 mM EDTA in PBS [pH 7.2]) for cell separation.

Cell separation

In assays requiring isolated populations of $WC1^+$ cells, PBMCs were incubated with anti-WC1 mAb (IL-A29; VMRD, Pullman, WA) for 20 min on ice, followed by a single wash in sorting buffer, and later incubated with anti-mouse IgG1 microbeads (Miltenyi Biotec, Gladbach, Germany) for another 20 min on ice. Finally, the cells were washed three times in sorting buffer and sorted through MACS columns. Cells were washed once in PBS and resuspended in RPMI 1640. In some assays, positively sorted cells were rested for 18 h before use.

Cell surface marker staining

To enumerate the γδ TCR^+ cells in total PBMC, cells were labeled with anti-TCR δ-chain Ab (TcR1-N24, GB21A; VMRD), followed by anti-mouse IgG2b (BD Biosciences, San Jose, CA). To assess the expression of CD13, CD25, CD45RO, CD62L, CD335, and MHC II, cells were first labeled with primary Abs, anti-CD13, anti-CD62L (IgG1; AbD Serotec, Oxford, U.K.), anti-CD25, anti-CD45RO (IgG1; VMRD), or anti-CD335–Alexa Fluor 488 (AbD Serotec) for 20 min on ice, and then counterstained

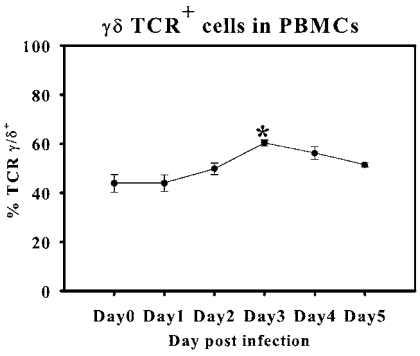

FIGURE 1. Average percentage of γδ TCR^+-expressing cells in nine animals over a period of 5 d postinfection with O1 Manisa FMDV. The percentage of γδ TCR^+ cells was measured on day 0 (before infection) and days 1–5 postinfection with FMDV. $*p = 0.016$ compared with values on day 0.

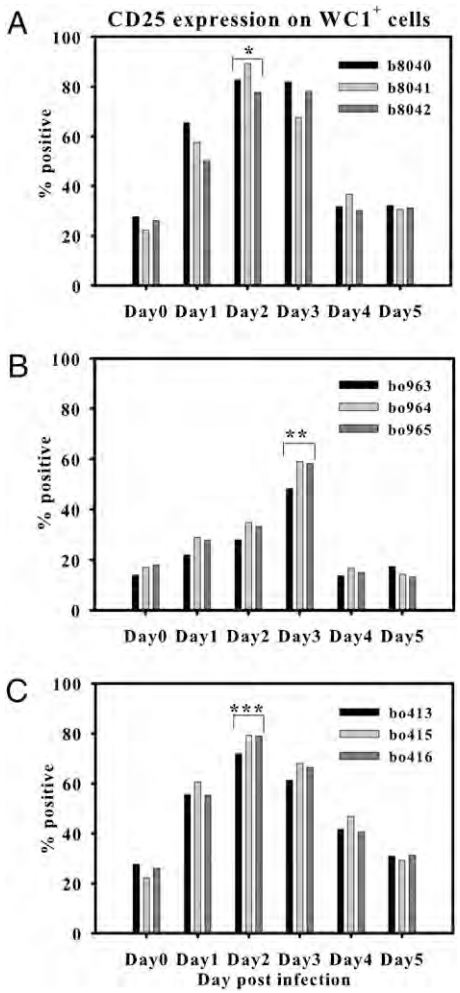

FIGURE 2. Expression of CD25 on $WC1^+$ cells from control (day 0) and O1 Manisa FMDV-infected animals. *A*, Experiment 1; *B*, experiment 2; *C*, experiment 3. $WC1^+$ cells were sorted, as described in *Materials and Methods*, and stained for CD25 together with WC1. The data show three separate experiments performed. $*p = 0.0106$ compared with day 0, experiment 1; $**p = 0.02$ compared with day 0, experiment 2; $***p = 0.012$ compared with day 0, experiment 3.

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with fluorochrome-conjugated anti-Ig. Cells were finally fixed in 2% paraformaldehyde for 20 min on ice, washed, and resuspended in FACS buffer (0.3% BSA, 0.9% sodium azide, PBS). One hundred thousand events were acquired using the FACSCalibur and analyzed in CellQuest (BD Biosciences, San Jose, CA).

Intracellular cytokine measurement

Sorted WC1$^+$ cells were incubated with 3 μg/ml brefeldin A for 5–6 h in RPMI 1640. Later, the cells were stained for IFN-γ or perforin using a protocol described previously (16), with the exception that anti-bovine IFN-γ (MCA1783PE; AbD Serotec) was used. Anti-human perforin Ab was used to detect bovine perforin (clone δG9; BD Biosciences). One hundred thousand events were acquired using the FACSCalibur and analyzed in CellQuest (BD Biosciences).

Spontaneous killing assay

To learn whether WC1$^+$ cells were capable of spontaneously lysing a tumor cell target, we plated sorted WC1$^+$ cells with K562-GFP cells (NK cell-sensitive tumor cell line) in 96-well plates together with 7-amino actinomycin D (live/dead discriminating dye; BD Biosciences) at E:T ratio of 50:1. PBMC isolated from the same animals and stimulated in vitro with human IL-2 and IL-12, a method previously reported to activate NK cells (17), were included as positive controls for K562-GFP killing. Cells were incubated for 4 h at 37°C. Later, 100,000 events were acquired using a FACSCalibur and corrected, as described earlier (16).

Proliferation test

WC1$^+$ cells were tested for their capability to respond to a mitogen during infection with FMDV. Sorted WC1$^+$ cells were labeled with 5 μM CFSE for 15 min at room temperature, followed by addition of an equal volume of FBS to stop the labeling, and washed twice with PBS. A total of 1×10^4 cells was incubated with 5 μg/ml Con A for 72 h. The degree of proliferation was measured by flow cytometry (FACSCalibur; BD Biosciences) and analyzed in WinMDI version 2.9 (Joseph Trotter, http://facs/scripps.edu/software.html). One hundred thousand events were acquired and analyzed.

DQ-OVA assay

The capacity of WC1$^+$ cells to ingest and process Ag was tested on sorted cells. DQ-OVA (Invitrogen, Carlsbad, CA) was added at 2 μg/ml to 10^6 cells and incubated at 37°C for 2 h. Control cells from both infected and noninfected animals were incubated on ice for 2 h. Cells were washed with PBS and resuspended in FACS buffer, and data were acquired in FACSCalibur (BD Biosciences) and analyzed in WinMDI version 2.9 (Joseph Trotter, http://facs/scripps.edu/software.html). One hundred thousand events were acquired and analyzed.

Statistical evaluation

Where appropriate, the data were evaluated statistically with Student *t* test, and *p* values ≤0.05 were regarded as significant.

Results

Percentage of γδ TCR-expressing cells in peripheral blood of cattle following infection with FMDV O1 Manisa

To examine the effect of virus infection on bovine γδ T cells, we initially looked at the number of γδ TCR-expressing cells as a percentage of total PBMCs. To this effect, cells were labeled with γδ TCR and analyzed by flow cytometry. Generally, there were only slight differences in the percentage of γδ TCR$^+$ cells within the first 2 d following infection (Fig. 1). The only significant increase in γδ TCR$^+$ cells was observed on day 3, and was statistically different ($p = 0.016$) from values generated on day 0 (before infection). This increase in cell number partially reflects the increase in activation of bovine γδ TCR$^+$ cells in the early phase of infection, which is shown in the following analyses. No peripheral blood lymphopenia is observed during FMDV

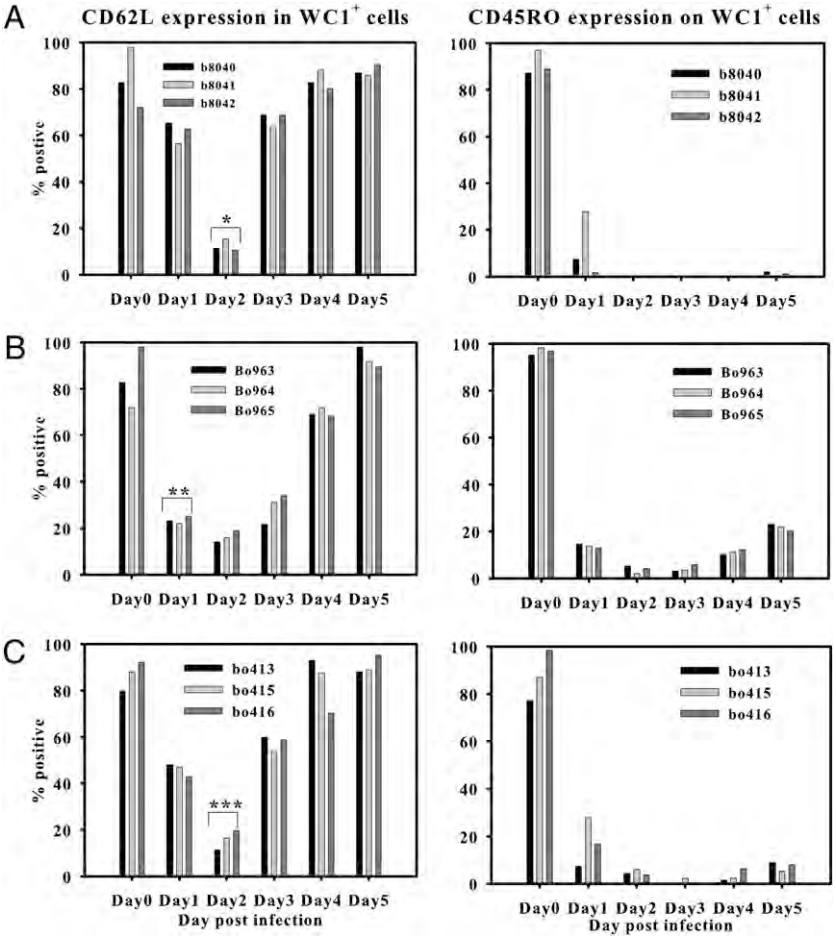

FIGURE 3. Surface expression of CD62L on WC1$^+$ cells from control (day 0) and O1 Manisa FMDV-infected animals. *A*, Experiment 1; *B*, experiment 2; *C*, experiment 3. WC1 cells were sorted, as described earlier, and stained for CD62L and WC1. Data show three separate experiments performed. *$p = 0.040$ compared with day 0, experiment 1; **$p = 0.032$ compared with day 0, experiment 2; ***$p = 0.037$ compared with day 0, experiment 3. Expression of CD45RO on WC1$^+$ cells from control (day 0) and O1 Manisa FMDV-infected animals. WC1$^+$ cells were sorted, as described in *Materials and Methods*, and stained for CD45RO together with WC1. Three separate experiments were performed.

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infection in cattle such that this is not due to a change in total-lymphocyte counts. Contrarily, there is a marked lymphopenia in swine during the acute stage of infection, as we have previously reported (18).

Analysis of the proliferative response of $WC1^+$ cells was also assessed. $WC1^+$ cells isolated from the peripheral blood of cattle infected with FMDV were still responsive to the mitogen, Con A, showing normal in vitro proliferation equivalent to blood samples taken before infection (Supplemental Fig. 1).

Expression of selected cell surface markers following infection with 01 Manisa FMDV

We sought to determine any changes that FMDV may induce regarding expression of surface proteins associated with activation of $WC1^+$ γδ T cells. Cells were enriched by positive selection for WC1 expression, and subsequent populations analyzed expressed both γδ TcR and WC1 on 96–99% of the cells (Supplemental Fig. 2). The cells were labeled with Abs against CD25, CD62, and CD45RO. As opposed to other T cells, there was a constitutive background staining of CD25 on $WC1^+$ cells. Expression of CD25 (Fig. 2) on day 0 was at least 15–25%, but an increase was observed on day 1–3 postinfection. The increase, particularly for day 2, was statistically significant compared with cells on day 0 ($p = 0.0106$ in the first experiment; $p = 0.02$ in second experiment [day 3]; and $p = 0.012$ in the third). Increased expression of CD25 usually indicates activation of T cells.

CD62L is highly expressed on freshly isolated $WC1.1^+$ γδ T cells (Fig. 3) (19). Following infection with FMDV, expression of CD62L was reduced on days 1–3 (Fig. 3), reaching the lowest expression level on day 2 in all three experiments that were done. This change in expression of CD62L, particularly on day 2, was statistically significant in comparison with the cell surface expression of CD62L on $WC1^+$ cells on day 0 ($p = 0.04$, experiment 1; $p = 0.32$, experiment 2; and $p = 0.02$, experiment 3). By day 4, the expression levels had reached those on day 0 before infection with O1 Manisa FMDV. A similar downregulation pattern of CD62L expression has been reported in other species following virus infection.

CD45RO had a rather surprising expression pattern. $WC1^+$ from noninfected animals expressed well over 80% of CD45RO. $WC1^+$ cells from FMDV-infected animals drastically reduced expression of CD45RO (Fig. 3). Moreover, on days 2–5, virtually no cells expressed CD45RO. Recovery of CD45RO resumed by day 4 in experiments 2 and 3, but did not reach the levels observed before infection in the first trial. These data agree with previous reports analyzing bovine γδ T cells in viral infection, although we only followed expression for 1 wk, whereas these authors analyzed the development of a γδ T cell memory phenotype over many weeks (13). In contrast, in vitro results reported by Bembridge et al. (20) show a reduction of CD45RO on activated T cells in animals, but expression on $WC1^+$ was intact in these secondary in vitro activation assays.

Activation to IFN-γ production

The percentage of $WC1^+$ cells staining intracellularly for IFN-γ increased during acute infection with FMDV, peaking 2–3 d postinfection (Fig. 4). There were large variations in individual animal responses, and statistical significance could be demonstrated only in experiment 1 (Fig. 4, $p = 0.031$, day 2). Remarkably, $WC1^+$ cells expressing the antiviral cytokine are not detected 5 d postinfection, indicating these cells go through a very rapid, but short-lived activation. Alternatively, activated cells may be migrating out of the circulation by 4–5 d postinfection.

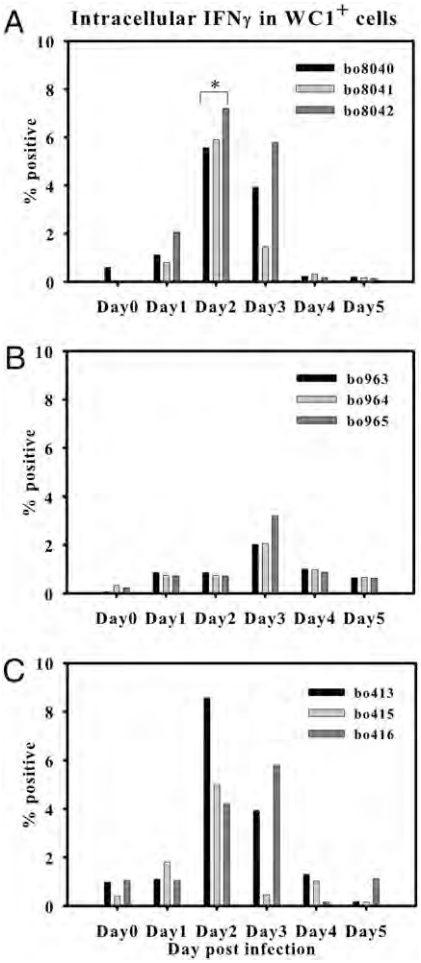

FIGURE 4. Intracellular staining for IFN-γ in $WC1^+$ cells from infected or noninfected animals. *A*, Experiment 1; *B*, experiment 2; *C*, experiment 3. Cells sorted, as described in *Materials and Methods*, and subjected to intracellular staining for IFN-γ; the data represent three separate experiments. $p = 0.031$ compared with day 0, experiment 1.

NK cell-like cytolytic activity of bovine γδ T cells

NKp46 (CD335) is an activating receptor expressed on NK cells. These innate responding cells kill virus-infected and neoplastic cells in an Ag-independent manner via these receptor molecules. We tested for expression of NKp46 by $WC1^+$ cells during the course of acute infection with FMDV. In the 5 d analyzed, a small population of these γδ T cells expressed the CD335 receptor, between 4 and 6% of $WC1^+$ cells. Detection of CD335-expressing cells was very transient, on days 2 and 3, returning to preinfection levels by day 5 postinfection (Fig. 5). Production of the killer cell protein, perforin was also detected in a similar sized population concurrent with the detection of CD335 expression (Fig. 5). Gating on the $WC1^+$ cells and analyzing CD335 and perforin expression shows that the cells expressing CD335 and perforin are the same population when we compare preinfection to 2 d following infection (Fig. 6). This is the peak of expression for both proteins, as shown in Fig. 5. This is a strong indication that these cells are capable of NK-like killing.

Given these results, we tested for killing activity against a tumor cell line, K562. In this study, we show sorted $WC1^+$ cells' capacity for killing K562, stably transfected with GFP (K562-GFP). In the two experiments, γδ T cells from all animals developed the capacity to kill target cells. The killing capability was highest on the second day following infection, and the difference in killing

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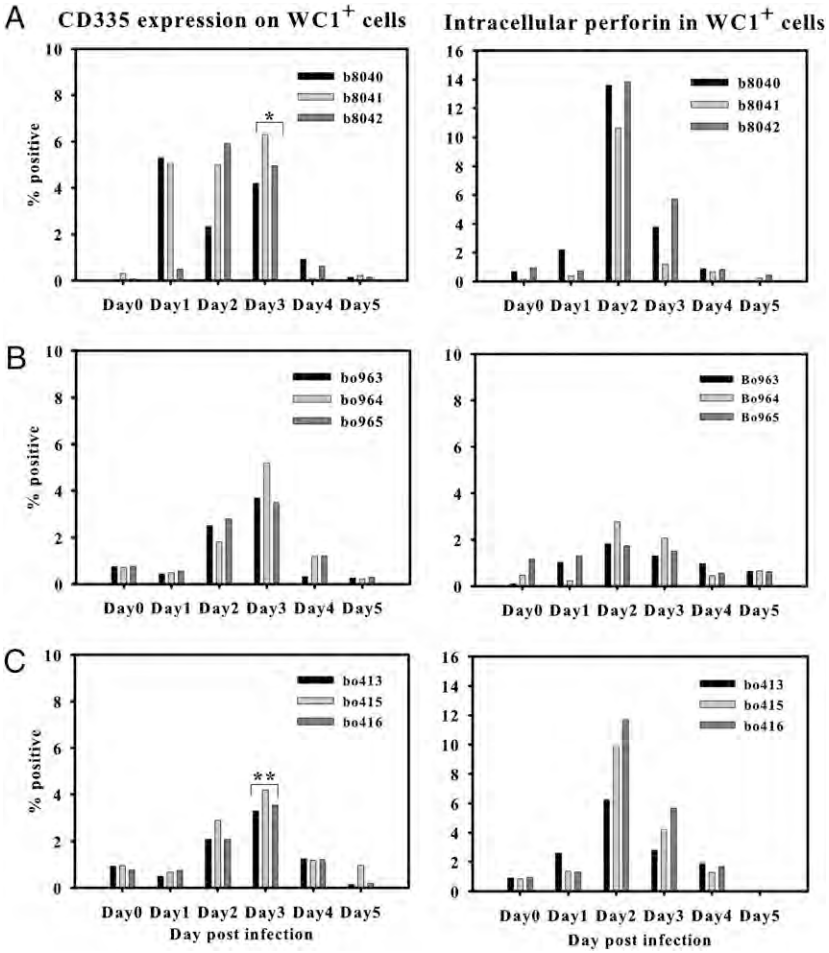

FIGURE 5. Expression of CD335 and perforin on WC1$^+$ cells isolated from noninfected or infected animals. *A*, Experiment 1; *B*, experiment 2; *C*, experiment 3. After sorting, cells were labeled with anti-CD335 or intracellularly with anti-perforin Abs together with anti-WC1$^+$ Ab. *$p = 0.019$, experiment 1; **$p = 0.022$, experiment 3. Three separate experiments were performed.

activity on day 2 differed significantly from activity observed before infection ($p = 0.019$, $p = 0.013$, and $p = 0.041$ for experiments 1, 2, and 3, respectively; Fig. 7). Attempts to develop a killing assay for foot-and-mouth disease virus-infected cells have to date been unsuccessful as even attenuated isolates of the virus were shown to be cytopathic in any bovine cell line we have analyzed.

Expression of cell surface proteins associated with Ag presentation

We analyzed the expression of MHC class II and show a baseline staining of 1–1.5% evident on day 0 that increased appreciably between days 1 and 3 postinfection with O1 Manisa FMDV (Fig. 8). This was significantly higher compared with day 0 ($p = 0.04$ in experiments 1 and 2). The expression gradually reached the

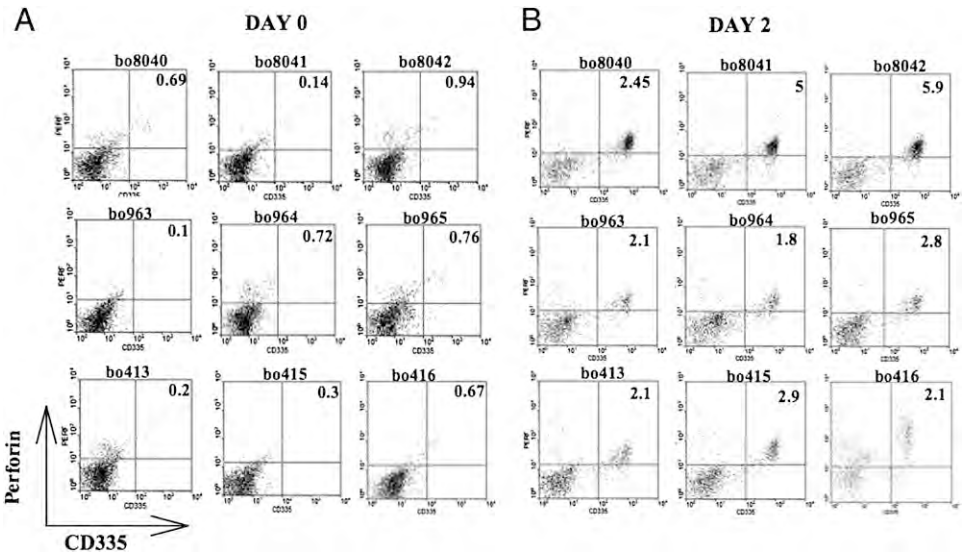

FIGURE 6. WC1$^+$ γδ T cells were prepared, as described in *Materials and Methods*. *A*, Day 0; *B*, day 2. WC1$^+$ γδ T cells prepared from noninfected control animals. Cells were surface stained with anti-CD335, followed by intracellular staining with anti-human perforin.

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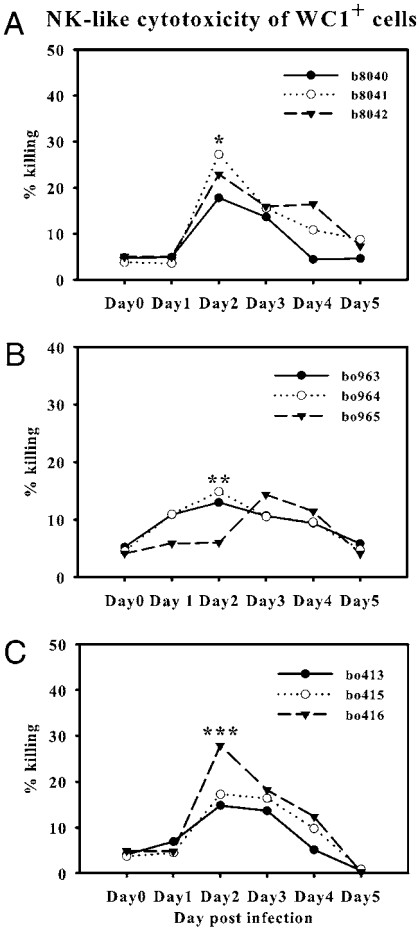

FIGURE 7. Lytic activity of WC1$^+$ cells from FMDV-infected or noninfected animals against K562-GFP. *A*, Experiment 1; *B*, experiment 2; *C*, experiment 3. WC1$^+$ cells were incubated with K562-GFP cells together with 7-amino actinomycin D for 4 h, followed by data acquisition by flow cytometry. Each panel represents a separate experiment of three performed. E:T ratios of 50:1 are compared. *p = 0.019 compared with day 0, experiment 1; ***p = 0.013 compared with day 0, experiment 2; ***p = 0.041 compared with day 0, experiment 3.

preinfection levels by day 5. This increase in MHC class II expression indicated an activated status of WC1$^+$ cells during infection with FMDV. Another unusual observation was the appearance of CD13 on a small population of WC1$^+$ γδ T cells postinfection with O1 Manisa FMDV (Fig. 8). A considerably high level of expression was observed on consecutive 3 d beginning from day 1, although the expression level was not uniform in all animals, particularly in experiment 1. The expression reduced to background levels or was completely absent beginning from day 4. We do not know the function of CD13 on bovine WC1$^+$ γδ T cells, but this molecule is expressed on splenic dendritic cells in cattle (21).

Ag processing by bovine γδ T cells during infection

In other species, γδ T cells have been reported to have functions of Ag processing and presentation (22–24). Data shown in Fig. 8 led us to examine this function by adding self-quenched DQ-OVA to bovine WC1$^+$ cells and measuring the fluorescence resulting from cleavage of DQ-OVA by WC1$^+$ cells. The DQ-OVA was successfully cleaved, resulting in fluorescence of the BODIPY FL dye. We observed a slight increase in fluorescence intensity of cells from noninfected animals on day 0 (Fig. 9), but the highest increase in fluorescence intensity was demonstrated by WC1$^+$ cells originating from animals sampled 3 d following infection with FMDV (Fig. 9).

Discussion

The relative abundance of γδ T cells in young ruminants has been well documented, although the role of these cells in viral infections remains poorly defined. In this study, we show that these cells expand and are activated during infection of Holstein calves with FMDV, strain O1 Manisa. In previous studies of pigs (18, 25), infection with FMDV caused immunosuppression such that lymphocytes isolated from such animals failed to respond to mitogens. We tested this aspect in bovine WC1$^+$ cells and did not observe any profound changes in the proliferative capacity of γδ T cells from infected animals, besides a slightly increased reactivity to Con A on the second or third day of infection. This may suggest activation of γδ T cells following infection with O1 Manisa FMDV. A clearly activated status of bovine γδ T cells was shown by Amadori et al. (12) in their experiments with bovine herpes virus, in which a sharp increase of γδ T cells was detected on the first day postinfection. We show increase in the relative percentage of γδ TCR-expressing cells only on day 3 postinfection with FMDV relative to preinfection.

Analysis of cell surface molecule expression on these cells revealed low-level constitutive expression of CD25 on nonstimulated cells expressing WC1 isolated from PBMC of naive animals, as was reported in studies by Rogers et al. (7). These investigators showed an increased expression of CD25 following stimulation. In our analysis of animals infected with FMDV, we show a similar finding, WC1$^+$ cells increased CD25 expression, indicating activation of these cells in vivo following viral infection.

In this study, we also report a detected loss of CD45RO on γδ T cells beginning from the second day following infection with FMDV. These results are similar to those reported for bovine viral diarrhea virus, although with different kinetics (13). Because currently we do not know whether the γδ TCR recognizes any foot-and-mouth disease viral Ags, we cannot speculate on the Ag-specific stimulation of γδ T cells in this study. However, antigenic stimulation of αβ T cells has been reported to lead to downregulation of CD45RO (20).

CD62L is a homing receptor that directs T cell trafficking into lymph nodes. In the current study, we show a reduction in CD62L expression that is most pronounced 2 d following infection, followed by a rapid recovery of CD62L expression, similar to what has been reported during bovine leukemia virus (26) or *Mycobacterium bovis* (27) infection. In contrast to our finding in this viral infection, Walcheck and Jutila (28) found a much slower downregulation of L-selectin following short-term activation of γδ T cells in vitro with PMA. In that model, they demonstrated that bovine γδ T cells do not appreciably extravagate from blood into parenchyma of lymph nodes. Our results indicate a transient activation of γδ T cells during acute infection with FMDV, and are remarkable not only for the rapid return to CD62L high expression, but that a majority of the circulating WC1$^+$, γδ T cells show this phenotype. Likewise, the rapid increase in CD25 expression involves most WC1$^+$ cells and all correlating with the peak of viremia. However, as these surface molecules are predicted to reflect activation state of the cells, it is interesting that only a small percentage (4–8%) of these γδ T cells are shown to express intracellular IFN-γ, albeit the kinetics of expression correlate to CD25 expression and inversely to CD62L.

CD2 is not normally found on WC1$^+$ cells; however, Daubenberger et al. (29) reported that CD2 could be detected when WC1$^+$ cells were cultured in the presence of *Theileria parva*. This

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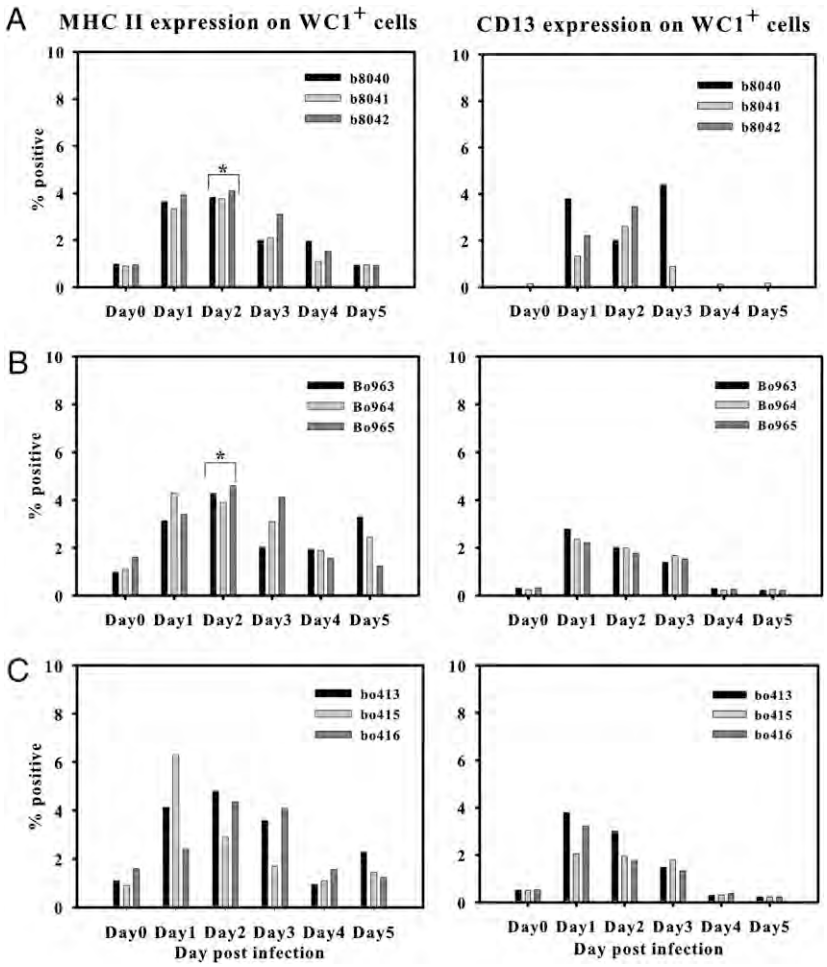

FIGURE 8. Expression of MHC II and CD13 on WC1$^+$ cells isolated from noninfected or infected animals. *A*, Experiment 1; *B*, experiment 2; *C*, experiment 3. After sorting, cells were labeled with anti-MHC II or anti-CD13 Abs together with anti-WC1$^+$ Ab. Data represent three separate experiments performed. For MHC II on day 2, $*p = 0.04$ for experiments 1 and 2.

was concordant with WC1$^+$ cells acquiring a lytic phenotype and capacity to destroy *T. parva*– infected target cells. The role of CD2 in the target cell recognition was not defined. CD335 is a typical marker expressed on NK cells, including bovine NK cells, where it functions as a natural cytotoxicity-triggering molecule (30, 31). Therefore, expression of certain markers may be a result of the stimulus present in the environment that probably contributes to the activation status of cells involved (32). We did not detect CD335 on WC1$^+$ cells from healthy animals. Following infection with O1 Manisa FMDV, a transient expression of CD335 was induced on WC1$^+$ γδ T cells. In addition, we detected expression of intracellular perforin in these same cells with nearly identical kinetics and further show expression of these proteins is by the same subset of WC1$^+$ cells. Increased expression of CD335 may have contributed to acquisition of the cytolytic function of WC1$^+$ γδ T cells following infection with FMDV in our studies, and the capacity to kill tumor target cells peaked 2 d postinfection and was lost by day 5 after inoculation, identical kinetics to CD335/perforin expression.

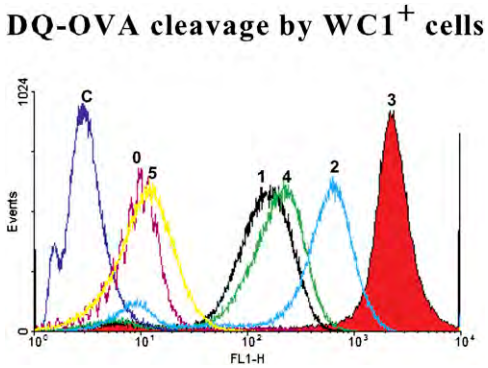

FIGURE 9. Fluorescence intensity of WC1$^+$ cells from noninfected or FMDV-infected animals treated with DQ-OVA for 2 h at 37°C. The histogram represents a single animal from a single experiment of three performed. C, control WC1$^+$ cells from both infected and noninfected incubated at 4°C; 0, day 0 (before infection); labels 1–5, days postinfection.

The NK-like cytotoxic phenotype of γδ T cells in bovine was earlier reported by Brown et al. (33) in studies of infection with *Babesia bovis*. They showed that spontaneous killing was exhibited when parasite-stimulated WC1$^+$ cells were presented with a xenogeneic NK-sensitive target cell line. More relevant to the current study, Amadori et al. (12) showed activation of bovine γδ T lymphocytes following vaccination of calves against FMDV. In those studies, γδ T cells were capable of lysing target cells in a manner similar to NK cells, indicating that either a spontaneous killing capability develops in this population of T cells or their reactivity could be driven by specific viral Ag recognition. The increase in killing capability observed in WC1$^+$ cells in our study coincided with the presence of intracellular perforin, which indicated that the killing mechanism most likely involved perforin, as previously shown by Alvarez et al. (34) in SCID-bo mice. Perforin-bearing γδ T cells have also been reported in American bison with experimental sheep-associated malignant catarrhal fever (35).

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Other viruses have been reported to use CD13 as a cell entry receptor (36, 37). Although CD13 is a receptor for porcine endemic diarrhea virus, permissiveness of porcine cell lines expressing CD13 is low (38). In the present studies, CD13 is not expressed on $WC1^+$ cells, but is activated during acute infection with FMDV. CD13 has not been reported as a receptor for FMDV, and it is unlikely that induction of CD13 leads to γδ T cell infection, as only 2–4% of $WC1^+$ cells express CD13. We did not test the $WC1^+$ cells for the presence of newly synthesized viral proteins, but previous studies have shown FMDV does not infect lymphocytes in vivo. Conversely, CD13 is expressed on splenic dendritic cells in cattle, although no role in dendritic cell function such as Ag presentation was investigated (21).

Our observation that 5% of $WC1^+$ cells also activated expression of class II MHC led to the hypothesis that these cells may be capable of Ag processing. Although all $WC1^+$ cells from infected and noninfected animals ingested DQ-OVA in an Ag-processing assay, the fluorescence intensity was significantly more in cells originating from infected animals on day 3. This may indicate that this function does not require prior activation of γδ T cells and that virus infection does not adversely affect this function of γδ T cells, but rather enhances the cleavage of DQ-OVA, predicting enhanced Ag-processing capacity. The property of ingesting particulate matter was demonstrated for dermal γδ T cells in cattle (39). However, those γδ T cells could not be induced to produce IFN-γ, nor did they express MHC II or CD25. The γδ T cells may also be involved in Ag presentation independently from dendritic cells. In work performed by Howard et al. (40), $WC1^+$ cells could not be stimulated to proliferate by dendritic cells, implying the lack of ligands for γδ T cells on allogeneic dendritic cells or a lack of costimulatory molecules for γδ T cells.

Juleff et al. (41) recently reported infection of two cattle with FMDV following depletion of $WC1^+$ cells in vivo with Ab administered i.v. The authors showed lack of $WC1^+$ cells in the peripheral blood did not have a measurable effect on the course of infection, concluding that $WC1^+$ do not have a major role in the control of FMDV. However, in our studies, we performed functional analysis of $WC1^+$ cells and found evidence of activation of this population of γδ T cells following infection with FMDV. These results are most likely not contradictory, as in gene knockout studies, if the $WC1^+$ cells were eliminated, the breadth of immune responses to virus often compensates for lacking a particular function. The Juleff study did not include multiple Ab treatments to address what happens when multiple responding cell populations are blocked/eliminated in the same animal, most likely due to the very high cost of such a study. We performed no pretreatment of the young, healthy steers used in the current study, so this is a direct assessment of how FMDV infection alters the function of $WC1^+$, γδ T cells.

Others have reported that it has been difficult to generate Ag-specific γδ T cells following vaccination (42). Reports of γδ T cell memory induction in the bovine (13, 43) may indicate that, in this species, specific Ag-dependent stimulation of γδ T cells may exist. It cannot be ruled out that γδ T cell responses at such an early stage as reported in this study result from nonspecific stimulation by endogenously derived ligands. Furthermore, data presented in this work raise the possibility that the γδ T cells can function as APCs. If confirmed, these cells could provide very early and efficient activation of CD4 T cell responses, initiating the classic adaptive immune response. Further studies are needed for clarification of whether such a response is linked to generation of adaptive and later memory immune responses against FMDV.

This rapid response to viral infection suggests that γδ T cells have a role in the innate immune response to viral infections and should be investigated further to understand the basis of this stimulatory effect. In FMDV infection in particular, viral clearance is most likely mediated by many aspects of innate and adaptive immune responses. Minimally, there is a significant type I IFN response mediated by various dendritic cell populations (44, 45), an early Ab response (41, 46, 47), and the γδ T cell response reported in this study. We suggest that all of these responses can work concurrently, consistent with the observation that the high viral load is cleared so early and effectively. Manipulation of these lymphocyte populations may be a way to develop biotherapeutics for the protection of animals against FMDV infection in advance of the development of adaptive immunity following vaccination.

Finally, previous studies in various species have shown the ability of γδ T cells to exert all of the functions we have characterized in this work, including expression of an activated cell phenotype, production of IFN-γ, expression of NK cell phenotype, production of perforin, Ag-nonspecific target cell killing, expression of MHC class II proteins, and Ag processing. This is the first demonstration of all of those functions ex vivo from single individuals and consistent over three trials and nine cattle. All of these activities of peripheral blood γδ T cells from FMDV-infected cattle occurred rapidly following infection, within 3 d, and were all no longer detected by 5 d postinfection in the circulation. This significant finding indicates a prominent role for activated γδ T cells among the various innate and adaptive immune responses that rapidly control this acute viral infection of livestock.

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Disclosures

The authors have no financial conflicts of interest.

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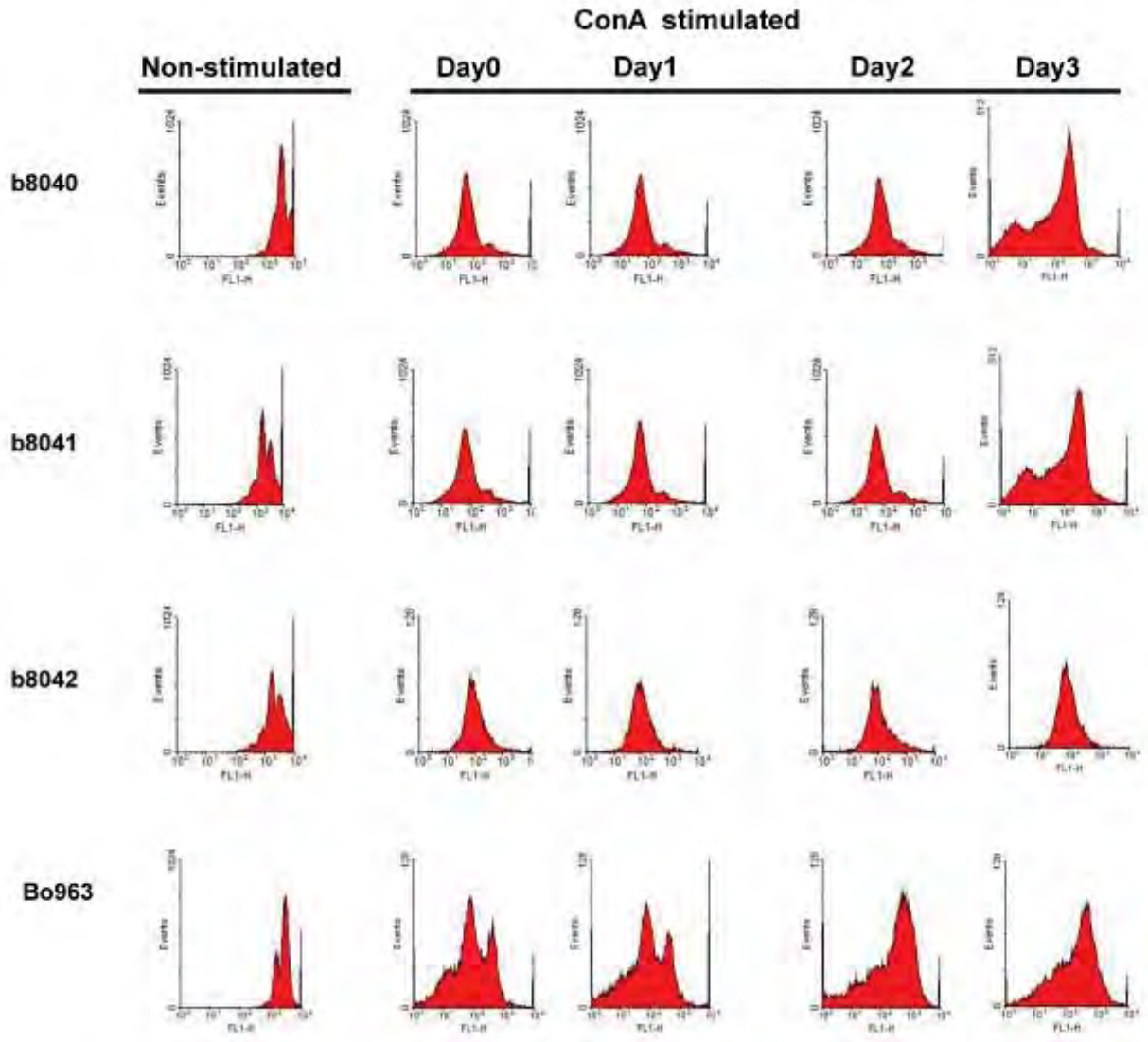
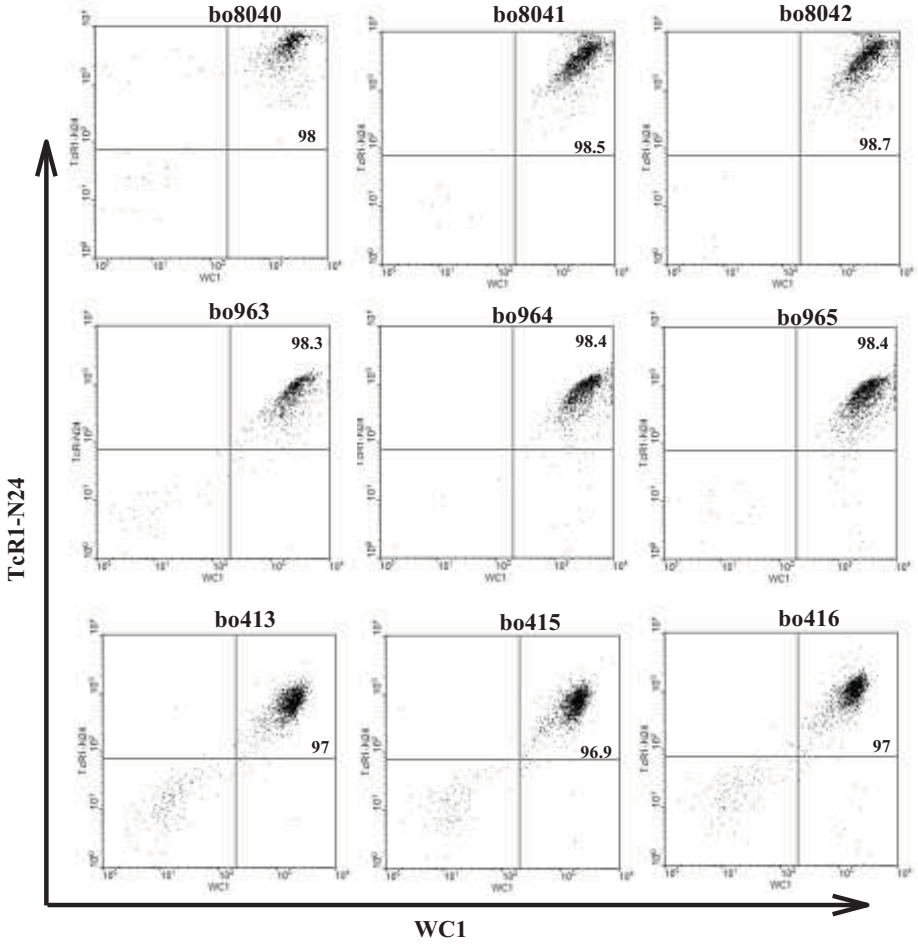


Supplemental Figure 1. $WC1^{+}$ cells were prepared as described in material and methods, and stained with CFSE then incubated for 72 h. A representative figure of CFSE dilution is shown for 4 animals followed for a period of 3 days post infection.

A. Day 0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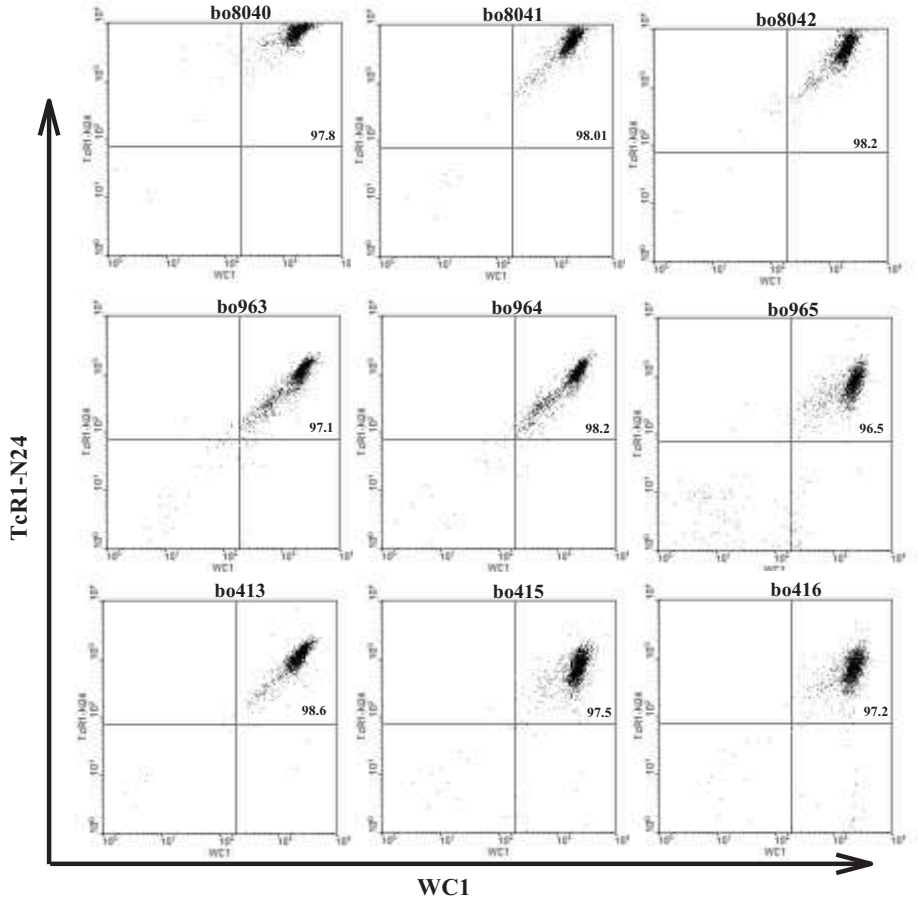
TcR1-N24
WC1

B. Day 2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bo413
98.6
bo415
97.5
bo416
97.2
TcR1-N24
WC1

Supplemental Figure 2. Immunophenotype of $WC1^{+}$ γδ T cells isolated from non-infected or FMDV-infected cattle. Data shown are from Day 0 (control) **(A)**, and Day 2 post infection **(B)**. Cells were separated by MACS system as described in Material and Methods and stained with anti-bovine WC1 and TcR1-N24 antibodies. Data were acquired and analyzed in Becton Dickinson FACSCalibur and CellQuest pro, respectively.

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Lesion Development and Replication Kinetics During Early Infection in Cattle Inoculated With *Vesicular Stomatitis New Jersey Virus* Via Scarification and Black Fly (*Simulium vittatum*) Bite

J. L. Reis Jr[1,2], L. L. Rodriguez[3], D. G. Mead[1], G. Smoliga[3], and C. C. Brown[1]

Abstract

Vesicular stomatitis viruses are the causative agents of vesicular stomatitis, an economically important contagious disease of livestock that occurs in North, Central, and South America. Little is known regarding the early stages of infection in natural hosts. Twelve adult Holstein steers were inoculated with *Vesicular stomatitis New Jersey virus* (VSNJV) on the coronary bands (CB) of the feet via scarification (SC) or by VSNJV-infected black fly *(Simulium vittatum)* bite (FB). Three additional animals were inoculated on the neck skin using FB. Clinical disease and lesion development were assessed daily, and animals were euthanatized from 12 hours post inoculation (HPI) through 120 HPI. The animals inoculated in the neck failed to develop any clinical signs or gross lesions, and VSNJV was detected neither by in situ hybridization (ISH) nor by immunohistochemistry (IHC). Lesions on the CB were more severe in the animals infected by FB than by SC. In both groups, peak VSNJV replication occurred between 24 and 48 HPI in keratinocytes of the CB, as evidenced by ISH and IHC. There was evidence of viral replication limited to the first 24 HPI in the local draining lymph nodes, as seen through ISH. Successful infection via FB required logarithmically less virus than with the SC technique, suggesting that components in black fly saliva may facilitate VSNJV transmission and infection in cattle. The lack of lesion development in the neck with the same method of inoculation used in the CB suggests that specific characteristics of the CB epithelium may facilitate VSNJV infection.

Keywords

vesicular stomatitis virus, livestock, pathogenesis, early infection, black fly

Vesicular stomatitis New Jersey virus (VSNJV) is an arthropod-borne virus (arbovirus) in the family *Rhabdoviridae*, genus *Vesiculovirus.* Geographic distribution of VSNJV is restricted to the Americas, occurring from South America to the western United States,[11] and is one of the causative agents of vesicular stomatitis (VS), a contagious and vector-borne disease of livestock. Although VSNJV infects a wide range of species including wildlife[19] and laboratory and domestic animals,[11] the natural disease is only reported in cattle, pigs, and horses. The disease is clinically indistinguishable from other viral vesicular diseases, especially foot-and-mouth disease, when cattle and pigs are affected.

Vesicular stomatitis is characterized by fever from 48 to 72 hours post inoculation (HPI) with noticeable vesicle formation between 48 and 72 HPI. Vesicles can occur in the oral cavity (dorsal tongue epithelium, hard palate, and gums), muzzle, snout, lip, coronary bands (CB) of the feet, teats, and prepuce.[11,13,15] Vesicles can rupture, with consequent viral shedding and potential animal-to-animal contact transmission.[17] Lesions tend to heal around the second week after vesicle formation. Transmission also occurs by actively infected biting insects, such as black flies (*Simulium* spp.),[6] sand flies (*Lutzomyia* spp.),[18] and biting midges (*Culicoides* spp.).[12] VSNJV has been isolated from nonbiting insects during disease outbreaks,[3] and it is believed they might play a role in mechanical transmission of the virus during epizootics.

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Figure 1. Coronary band. Inoculation via scarification, using bifurcated needle. Inset: close-up view of the bifurcated edge of the needle. **Figure 2.** Coronary band. Inoculation via fly bite, with VSNJV-inoculated black flies in cage.

The detailed pathogenesis of VSNJV infection is unclear. Clinical disease can be consistently reproduced experimentally by application of at least 10^7 $TCID_{50}$ per animal to scarified regions where lesions naturally occur.[14] When VSNJV is inoculated in haired skin of the neck,[14] flank,[14] or ear,[5] lesions do not occur but animals may seroconvert and/or shed virus through the tonsils (subclinical infection). Even though no lesions develop in those haired skin sites, it has been demonstrated that there can be limited viral replication.[8] Also, noninfected black flies physically separated from VSNJV-infected black flies became infected while cofeeding at these sites on a nonviremic host.[10] However, none of the previous studies used in situ hybridization (ISH) or immunohistochemistry (IHC) to detect virus in those areas.

Compared with inoculation via scarification (SC), feeding of VSNJV-infected black flies at sites where vesicle formation normally occurs will reproduce the disease,[6] although the amount of virus contained within the feeding flies is far less than what is required to reproduce disease via SC.[14] Previous studies investigating the pathogenesis of VSNJV in experimentally infected livestock have focused on the clinical course and late stages of the disease[4,9] following infection via SC or intradermal injection. However, the early events of experimental or natural VSNJV infection in livestock, including virus replication kinetics and distribution in tissues, have not been documented.

The present study was designed to sequentially assess and describe lesion development, virus distribution, and replication kinetics in cattle tissues during the early phases of VSNJV infection. Additional objectives were to compare insect bite transmission (VSNJV-infected black fly *(Simulium vittatum)* bite [FB]) to direct inoculation (SC) and to assess the presence of virus inoculated in susceptible versus nonsusceptible regions of skin via FB.

Materials and Methods

All experimental infections and all methods using nonfixed samples were conducted at the USDA-ARS Plum Island Animal Disease Center under biosafety level (BSL)-3Ag conditions. Tissues fixed in formalin were transferred to the Department of Pathology at the University of Georgia, for histological processing, immunohistochemistry, and in situ hybridization studies.

The virus used was a VSNJV field strain (95COB) isolated from a cow during an outbreak in Colorado in 1995. This virus was responsible for a large VS outbreak in 1995 and has been used previously to successfully reproduce clinical disease in cattle.[14]

Fifteen 150- to 250-kg Holstein steers were used in this experiment. Scarification inoculation was done on 7 animals, 6 scarified with virus and 1 with cell culture media only. Eight animals were inoculated by FB: 7 with infected flies and 1 with noninfected flies. Three steers had flies feed on the neck skin, whereas for the other 5 FB animals, inoculation was on the CB. All animals had hair from the area of inoculation removed with clippers followed by shaving 24 hours prior to the inoculation time. They were sedated with an intramuscular injection of 0.3 mg/kg xylazine prior to the inoculation, and sedation was reversed after inoculation by intravenous injection of 2 mg/kg tolazoline.

Inoculation by Scarification

A plastic bifurcated needle (Duotip Tests, Lincoln Diagnostics, Decatur, IL) served as the inoculating tool and was used as previously described.[14] The line of scarification was approximately 8 cm long and located on the lateral aspect of the limbs, on the haired skin of the CB immediately proximal to the thick nonhaired skin (Fig. 1). The inoculum contained 10^7

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$TCID_{50}$ of VSVNJ in 50 µl of cell culture media with 1% antibiotic/antimycotic as previously described;[14] virus-free medium was used as the negative control. The scarification site was created with approximately 25 skin pricks, and then the inoculum or virus-free medium was applied with a pipette. The procedure was repeated, with 25 additional skin pricks and application of additional medium. In experimental animals, the viral inoculum was applied to the right front and rear CB, whereas the left front and rear CB were scarified and treated with virus-free medium. In the mock-inoculated animal, the right front and rear CB were scarified and treated with virus-free medium, whereas the left front and rear CB were left intact. The mock-inoculated animal was euthanatized at 24 HPI, and the virus-infected animals were euthanatized at 12, 24, 48, 72, 96, and 120 HPI.

Inoculation by Fly Bite

Inoculation by fly bite was done as in previous studies.[8] Briefly, black flies were intrathoracically injected, as previously described,[7] with approximately 1 µl containing 10^7 $TCID_{50}$ of VSNJV. Infected black flies were allowed to feed on the designated CB of 4 animals and on sites on the necks of 3 animals. For the CB animals, 30 caged VSNJV-infected black flies were allowed to feed for approximately 20 minutes at the lateral aspect of the right front and rear CB (Fig. 2). Cages were placed in a position to allow flies to access either haired or glabrous CB skin. In general, flies probed or bit in the haired skin, approximately 1 cm proximal to the thick glabrous CB skin. The same number of uninfected black flies was allowed to feed for approximately 20 minutes at the lateral aspect of the left front and rear CB. These 4 FB CB experimental animals were euthanatized at 24, 48, 72, and 96 HPI. One mock-inoculated animal was treated with uninfected black flies on the right CB, with no treatment at the left CB, and was euthanatized at 24 HPI. The same method was used in the group of the 3 neck-inoculated animals. Infected black flies were allowed to feed on the right side of the neck, and uninfected black flies were allowed to feed on the left side of the neck. Of these 3 steers inoculated in the neck, 1 animal was euthanatized at 24 HPI and 2 animals at 72 HPI.

Analysis of Black Flies

As a quality control for the fly bite inoculation, all flies were tested for the presence or absence of mammalian blood by SYBR-Green real-time polymerase chain reaction (RT-PCR) for mammalian DNA (Power SYBR Green PCR Master Mix ABI 4367659) after feeding on cattle. Briefly, nucleic acid was extracted from each individual fly as described below for tissues. RT-PCR was conducted using primers designed specifically to target the 16s mammalian recombinant DNA gene. Forward primer sequence was 5′ CCTGTTTACCAAAAACATCAC 3′ and reverse primer sequence 5′ AYTGTCGATAKGRACTCTWRARTAG 3′ (Invitrogen, Carlsbad, CA). Primers were used at a final concentration of 300 nM per 25 µl reaction. The reaction was performed using 8 µl (per 25 µl reaction) of Power SYBR Green PCR Master Mix (Applied Biosystems 4367659). The amount of nucleic acid template was 2.5 µl per reaction, which was performed on an ABI 7000 sequence detection system using the following cycling conditions: 94°C for 2.5 minutes, and 40 cycles of 94°C for 45 seconds, 50°C for 45 seconds, 72°C for 60 seconds, and a final 72°C hold for 5 minutes. After the RT-PCR, a melt curve was performed to detect the Tm of SYBR Green-bound DNA products. Products with a Tm of approximately 80°C were considered positive for mammalian DNA.

The blood meal analysis was done in all flies used in this study and determined that approximately 20% of the flies (which corresponds to about 6 flies) fed during the period of mock and viral inoculation (data not shown).

Samples

Samples collected immediately after euthanasia included lateral aspect of all 4 CB and potential lesions extending from the original site of the inoculation, right and left neck skin, and local draining lymph nodes (LN) including prescapular, axillary, popliteal, and prefemoral. Spleen, tongue, and tonsils were also collected. Approximately 30 mg of collected tissues were quick-frozen in liquid nitrogen for later transfer to –70°C for virus isolation (VI) and reverse transcriptase RT-PCR (rRT-PCR) for detection of live virus and viral nucleic acid respectively. Additionally, serum was collected just prior to euthanasia for VI and rRT-PCR. Sections <1 cm thick of the remaining tissues were fixed in a 10% buffered formalin solution, for histopathology, immunohistochemistry, and in situ hybridization. The whole neck and CB skin at and around fly cage location and scarified areas were collected and trimmed for histopathologic evaluation. For each of these sites, 4 tissue cassettes were collected, each with 3 samples of epidermis. All 4 hematoxylin and eosin (HE)–stained slides from these blocks (equaling at least 12 sections from each site) were examined; a minimum of 2 slides (6 sections) were selected, and corresponding sections were analyzed by ISH and IHC. A semiquantitative score was attributed to the ISH and IHC slides: negative (N) was assigned for no signal, + for minimal to mild or faint signal, ++ for a moderately discrete but localized signal, and +++ for a prominent and diffuse signal.

Real Time Reverse Transcriptase PCR (rRT-PCR)

Tissues were thawed and immediately macerated by adding two 5-mm stainless steel beads (Qiagen, Catalog No. 69989) and 0.9 ml of MEM-25mM HEPES and then were shaken in a TissueLyser bead beater (Qiagen) for 2 minutes at a frequency of 22 per second. After maceration, 50 µl of sample was transferred to 96-well plates (King Fisher No. 97002540) containing 150 µl of lysis/binding solution. RNA was then extracted using Ambion's MagMax-96 Viral RNA Isolation Kit (Ambion, catalog No. 1836) on a King Fisher-96 Magnetic Particle Processor (Thermo Electron Corp.). Briefly, after the

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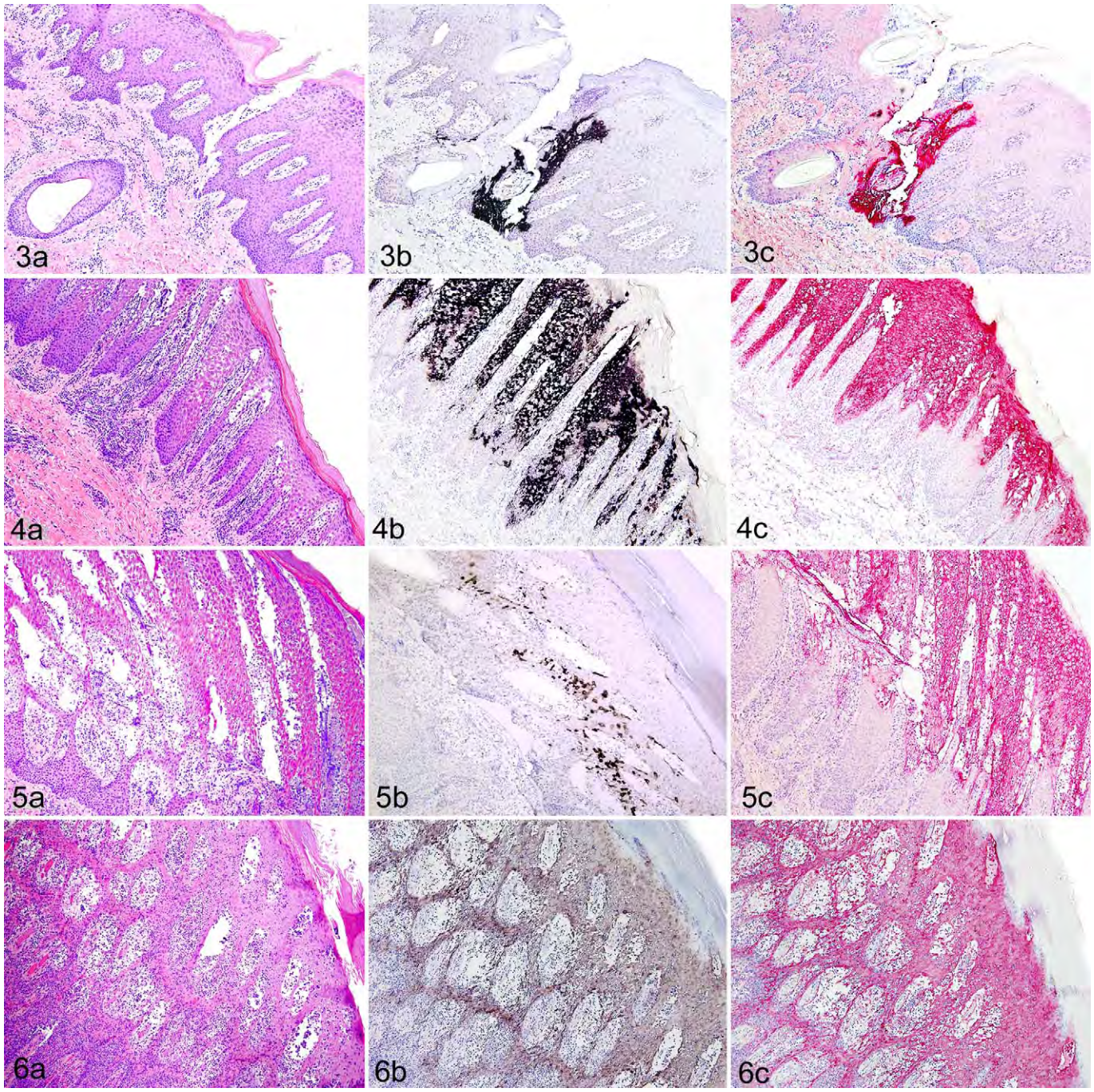

Figures 3–6. Coronary band. Histologic sections of the coronary bands from cattle experimentally infected with VSNJV, 12–72 hours post infection (HPI), (a) with HE stain, (b) in situ hybridization for presence of viral replication (negative sense digoxigenin-labeled riboprobe, anti-digoxigenin antibody, alkaline phosphatase, NBT/BCIP), and (c) immunohistochemistry for viral protein (anti-VSNJV polyclonal antibody, alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain). **Figure 3.** 12 HPI, SC. (a) Focal area of epidermal disruption due to scarification. Discrete and intense focus of ISH (b) and IHC (c) signal. **Fig. 4.** 24 HPI, SC. (a) Focally extensive spongiosis of the stratum spinosum with scattered individual loss of keratinocytes with initial vesicle formation. Intense and diffuse ISH (b) and IHC (c) signal in the cells of stratum spinosum. **Fig. 5.** 48 HPI, SC. (a) Marked epidermal disruption, with necrosis, large cavitations and loss of the stratum spinosum. Note that the stratum basale is spared. (b) Localized ISH signal within necrotic stratum spinosum. (c) Intense and diffuse IHC signal of the stratum spinosum. **Fig. 6.** 72 HPI, SC. (a) Marked epidermal disruption, with diffuse coagulative necrosis of the stratum spinosum. (b) Faint and diffuse ISH signal in necrotic stratum spinosum. (c) Intense and diffuse IHC signal of the stratum spinosum.

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initial 5-minute lysis/binding step, the RNA sample underwent a series of 4 washing steps, a drying step, and a final elution. 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. The extracted RNA was analyzed by rRT-PCR using 2.5 µl of RNA on the ABI 7000 as described below. The remaining macerated tissue was clarified (1000 g for 2 minutes at 4°C), and the supernatant was cleared of possible bacterial contamination using centrifuge tube filters (Spin-X, Costar). Samples were then stored at –70°C for VI. The rRT-PCR was performed using primers and probe designed specifically for the nucleocapsid region of isolate used for the inoculations (95COB). Forward primer sequence was 5′ GCACTTCCTGATGGGAAATCA 3′ and reverse primer sequence 5′ GGGAAGCCATTTATCATCCTCA 3′ (Invitrogen, Carlsbad, CA), and a 6-carboxyfluorescein (6-FAM)–labeled probe 5′ ACCCTGACCGTTCTG 3′ (Applied Biosystems, Foster City, CA) was used. Primers were used at a final concentration of 300 nM, and probes were used at 100 nM per 25 µl reaction. The reaction was performed using the Taqman EZ RT-PCR Core Reagents (Applied Biosystems N808-0236) at the following volumes/concentrations per 25 µL reaction: 5 µl of 5× buffer, 3 µl of 25 mM Mn $(OAc)_2$, 3 µl of dNTPs (combined 1:1:1:1 by volume), 1 µl of rTth, 0.25 µl of Amperase, and 9.7 µl of H_20. The amount of RNA template was 2.5 µl per reaction, which was performed on an ABI 7000 sequence detection system using the following cycling conditions: 60°C for 25 minutes, 95°C for 2 minutes, and 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. Results were expressed as cycle threshold (CT) values. A CT value <40 was considered positive for the presence of virus. Negative control consisted of all rRT-PCR reagents and water in place of template.

Virus Isolation

A 250-µl aliquot of the supernatant described above was placed in individual T-25 tissue-culture flasks containing a monolayer of VERO cells. The virus was allowed to adsorb for 1 hour at 37°C on a rocker plate, followed by addition of 5 ml of maintenance media with 3 subsequent days of incubation at the same temperature. Flasks were examined under an inverted microscope to observe cytopathic effect (CPE) at 24, 48, and 72 HPI. After 72 HPI, CPE-positive samples were stored at –70°C for rRT-PCR, and 250 µl of supernatant from each CPE-negative sample was transferred to a new culture flask (second passage). After 72 HPI, all flasks (CPE positive or negative) from the second passage and all CPE-positive materials from the first passage were processed for rRT-PCR.

Histopathology and Immunohistochemistry

All formalin fixed samples were routinely embedded in paraffin, cut at 3- to 4-µm thickness, and stained with HE for histological examination to assess inflammatory reaction, extent of microvesicle formation, and any other pathologic changes.

For IHC, deparaffinized sections were subjected to antigen retrieval with 80 µg/ml proteinase K for 15 minutes at 37°C, followed by blocking of nonspecific epitopes. Then samples were incubated overnight at 4°C with primary antibody, anti-VSNJV polyclonal, a mouse hyperimmune ascitic fluid (kindly provided by Dr. Robert Tesh, Department of Pathology, Medical Branch, University of Texas, Galveston), at a dilution of 1:1800. Primary antibody was followed by incubation with an alkaline phosphatase-linked polymer system (LabVision). The reaction was revealed using Vector Red chromogen. The slides were lightly counterstained with Mayer's hematoxylin and then cover-slipped using Permount for permanent record. For each IHC protocol, anti-VSV antibody was applied to a left (mock-inoculated side) CB or neck skin and popliteal or prefemoral lymph nodes for negative control. Additionally, each IHC protocol had a slide containing a section of CB skin with a recognizable histological vesicular lesion or a lymph node draining a CB with lesion. For these sections, normal mouse serum was used instead of the anti-VSV antibody. Selected lymph node sections were additionally subjected to an IHC protocol to highlight dendritic cells, using the same procedure outlined above, except that the primary antibody was anti-CD1b (monoclonal ascites fluid antibody; VMRD catalog No. TH97A) at a dilution of 1:100.

In Situ Hybridization

Negative sense riboprobes were used to detect positive-stranded (replication) viral RNA. The riboprobes consisted of 410 bases corresponding to the nucleoprotein (N) gene using methods previously described.[2] The following primers were used to produce the N gene construct: 5′-GAA GAT GGT CTT GAC TTC TTT G-3′ (forward) and 5′-CGA GTT GAT CTT AGC AAG AGT G-3′ (reverse). The N gene segment was cloned and amplified in a pGEM-4Z vector (Promega, Madison, WI). The vector was digested with BglII restriction enzyme to create a template for the negative sense probe, and digoxigenin-labeled nucleotides were added during transcription of the probe. Incorporation of the digoxigenin was verified by dot blot. Sections from tissues that were positive either on VI or on rRT-PCR were processed for ISH as previously described.[1] Tissue sections were treated with 100 µg/ml proteinase K solution and then incubated with the negative sense VSNJV riboprobe (corresponding to the N gene) at 42°C for 12 hours, followed by incubation with antidigoxigenin conjugated to alkaline phosphatase (1:300). Negative controls included noninfected tissues. The reaction was visualized using tetrazolium-based (NBT-BCIP) chromogen. The slides were lightly counterstained with Mayer's hematoxylin then cover-slipped with Permount for permanent record.

Results

Animals Inoculated by FB on the Neck

None of the animals inoculated in the neck skin developed fever or any other clinical sign of disease. They had only

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multiple small (approximately 1 mm in diameter) focal areas of hyperemia at the site of FB in both right and left (infected and uninfected FB) neck skin.

Histologically, the superficial dermis had minimal to mild foci of inflammatory infiltrates in the right and left neck skin of all 3 steers. Dermal infiltrates were slightly more severe in the animal euthanatized at 24 HPI and were composed of small numbers of macrophages with fewer neutrophils. There were no lesions in the epidermis, except for the animal euthanatized at 24 HPI that had scant small (approximately 0.5 mm in diameter) focal areas of epidermal necrosis within the FB region in both right and left sides.

There were very limited positive results by any of the techniques used to detect virus in these tissues. Inoculation site skin in the 24 HPI animals was positive by both VI and rRT-PCR on the right (infected) side. At 72 HPI, 1 of the 2 euthanatized animals at this time had inoculated neck skin positive on rRT-PCR only, with no viral detection with VI, ISH, or IHC. At no time were the tissues of any of these 3 animals positive by IHC or ISH, nor was any evidence of epidermal disruption seen on HE. All other tissues as well as serum collected from these animals were consistently negative for the presence of virus by all techniques (data not shown).

Clinical and Gross Findings in Animals Inoculated in CB

All experimentally infected animals in this group developed fever (>39.2°C) and mild depression between 24 and 48 HPI. No other clinical abnormalities were noted. In the case of the SC-inoculated animals, at 12 HPI, the only lesion was a mild hyperemia along the line of inoculation. Subtle gross lesions, including mild edema and hyperemia, were present at the site of inoculation at 24 HPI in both SC- and FB-inoculated animals. At 48 HPI there was moderate edema and hyperemia restricted to the site of SC. However, the FB-inoculated steer euthanatized at the same time had much more prominent hyperemia and swelling of the right (infected) rear CB, with a few 1- to 2-cm diameter, dark and circumscribed areas of ulcerations that coincided with the sites where the flies were feeding. In addition, in a region beyond where the cage was positioned, in the right rear interdigital space, this animal had marked vesicular formation, characterized by a focally extensive white and elevated area that exuded serous fluid when cut. No gross lesions were present at 48 HPI in the right (infected) front FB-inoculated CB, and analysis of fly blood meals revealed that no flies fed on this foot. At 72 and 96 HPI, the right CB infected by SC had moderate hyperemia and edema, multifocal areas of hemorrhage, and multiple 2- to 3-mm ulcerations corresponding to zones of SC. A serous fluid frequently oozed from the small ulcers. However, at the same time points (72 and 96 HPI), the lesions in the FB-inoculated animals were more severe, with diffuse edema, hyperemia, and a few dark 2- to 3-cm diameter ulcerated areas corresponding to where fly cages were positioned at the time of inoculation. In these FB-inoculated animals, lesions extended far beyond the site of FB with vesicles reaching the interdigital space. At 120 HPI, the SC lesions were similar to or slightly more severe than those seen in the 96 HPI SC steer. In the mock-inoculated animals, CBs developed a mild hyperemia at the site of SC or FB at 24 HPI and occasionally at 48 HPI. The 96 HPI FB and the 120 HPI SC had moderately to markedly enlarged popliteal and prescapular LNs.

Histological Findings in Animals Inoculated in CB

Only 1 SC-inoculated steer was euthanatized at 12 HPI. In this animal in both right and left (virus inoculated and mock inoculated, respectively) CB, small areas of serocellular crusting with underlying epidermal disruption developed, characterized by poorly defined focal eosinophilic areas with loss of definition of cellular borders in the upper strata spinosum and granulosum. There was focal loss of the epidermis at the areas of scarification (Fig. 3a). There was mild to moderate focal neutrophilic exocytosis in the epidermis. The superficial dermis had focal perivascular infiltration of numerous neutrophils and fewer macrophages.

At 24 HPI, both the SC- and FB-inoculated animals presented large coalescing areas of mild to moderate intercellular edema (spongiosis) with stretching of intercellular bridges and shrunken keratinocytes in the deep stratum spinosum (Fig. 4a). Cells from the strata basale and granulosum were consistently spared at this time. There was mild neutrophilic exocytosis throughout the affected epidermis, with moderate numbers of neutrophils and macrophages around perivascular areas of the superficial dermis.

At 48 HPI in the SC-inoculated animal, the narrow zone of inoculated haired CB skin had necrosis of epithelium of the epidermis and hair follicles with accompanying neutrophilic infiltration; the thicker nonhaired skin closer to the hoof had severe intercellular edema and hypereosinophilic, pyknotic, and shrunken keratinocytes limited to the stratum spinosum (Fig. 5a). Multifocal areas of this thick glabrous skin formed cavitations partially filled with numerous neutrophils, fewer macrophages, cell debris, and acantholytic cells. Similar to what was observed at 24 HPI, the strata basale and granulosum were consistently spared. The superficial dermis had moderate perivascular to interstitial infiltration of numerous macrophages and neutrophils, with fewer lymphocytes.

The FB-inoculated steer at 48 HPI had minimal lesions in the right front CB, whereas the right rear CB had severe epidermal disruption affecting a much larger zone of haired skin. These lesions were more severe than those observed in the SC-inoculated animal at this time. Hair follicles were frequently effaced by marked epidermal necrosis and accumulation of degenerated neutrophils. In the nonhaired thick skin (located immediately distal the haired skin but not in contact with the flies), there were extensive areas of full-thickness necrosis from the stratum basale to the stratum granulosum, with much greater damage than seen in the SC-inoculated animal at this time point. Within the stratum spinosum were multiple large coalescing cavitations filled with numerous neutrophils, few macrophages, cell debris, and occasional

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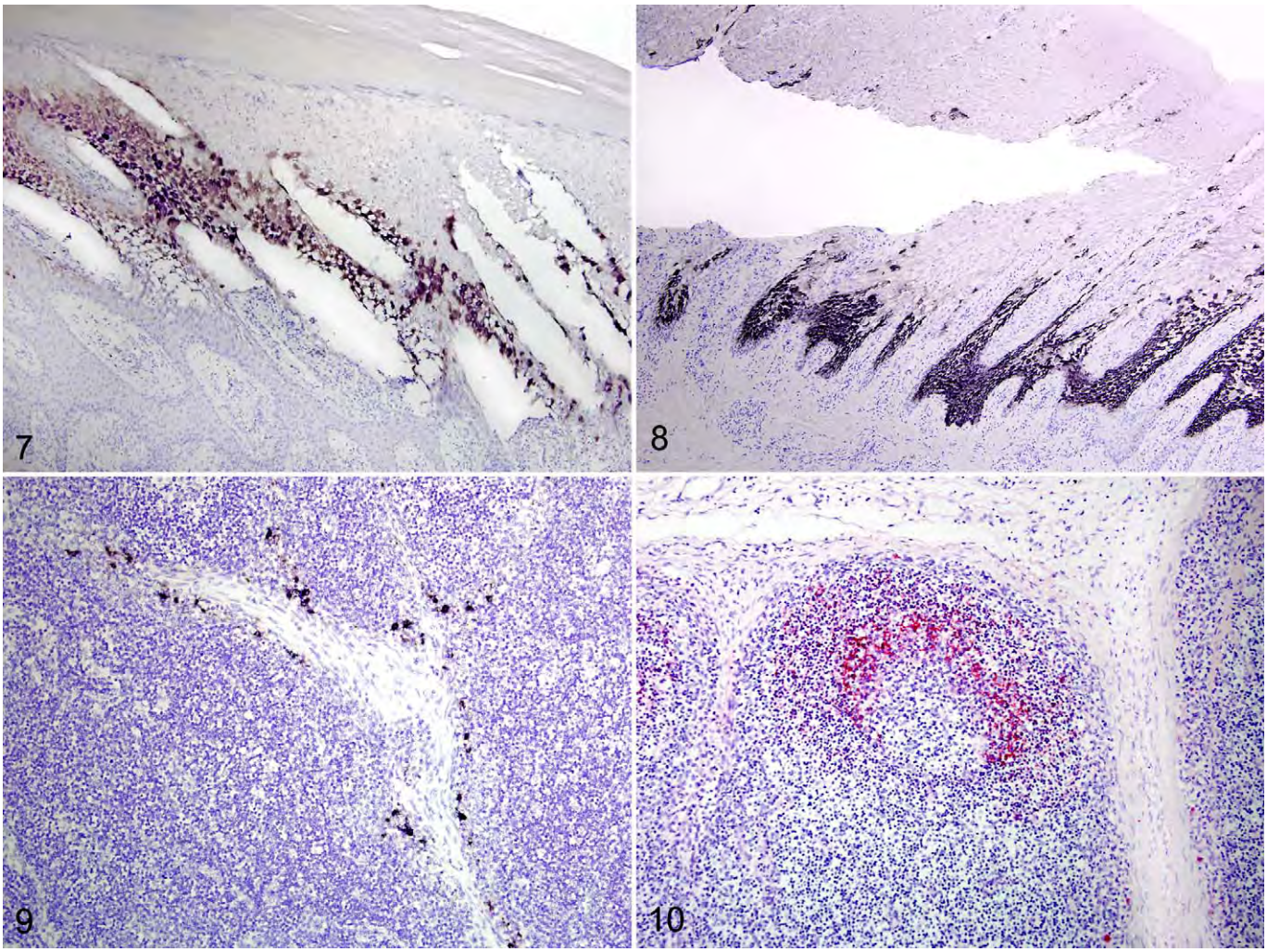

Figure 7. 48 HPI, SC: Coronary band. In situ hybridization localizes replicating virus to the upper stratum spinosum, with sparing of the stratum basale (negative sense digoxigenin-labeled riboprobe, antidigoxigenin-alkaline phosphatase, NBT/BCIP). **Figure 8.** 48 HPI, FB: Interdigital skin. In situ hybridization localizes replicating virus predominantly in the deeper stratum spinosum and stratum basale (negative sense digoxigenin-labeled riboprobe, antidigoxigenin-alkaline phosphatase, NBT/BCIP). **Figure 9.** 24 HPI, FB: Popliteal lymph node. In situ hybridization localizes replicating virus within inflammatory cells in subcapsular areas (negative sense digoxigenin-labeled riboprobe, antidigoxigenin-alkaline phosphatase, NBT/BCIP). **Figure 10.** 72 HPI, SC: Popliteal lymph node. Immunohistochemistry localizes viral protein to the marginal zone of the lymphoid follicles (alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain).

acantholytic cells. There was cleft formation at the stratum basale, with separation of this layer from the stratum spinosum. The dermis had moderate to severe perivascular to interstitial infiltration of numerous macrophages and neutrophils, with fewer lymphocytes and infrequent mast cells.

By 72 HPI, there was extensive necrosis of the thick nonhaired skin at the CB of the SC-inoculated animal, characterized by large pale eosinophilic areas, with loss of cellular detail (Fig. 6a). Areas of necrosis were centered in the stratum spinosum, spared the stratum basale, and extended to but did not efface the stratum granulosum. Numerous neutrophils infiltrated through the intercellular spaces around necrotic keratinocytes and filled areas where there was loss of keratinocytes. The dermis had moderate to severe perivascular to interstitial infiltration of numerous macrophages and neutrophils, with fewer lymphocytes. The animal inoculated by FB had lesions similar to those observed in the FB-inoculated steer at 48 HPI, with more extensive areas of necrosis and loss of epidermis.

At 96 HPI, epidermal lesions in the animals inoculated by either method were similar. There were full-thickness necrosis and cleft formations at the level under the stratum basale, with frequent loss of the entire epidermis of the CB (ulceration).

At 120 HPI, lesions were similar to those observed at 96 HPI but showed evidence of initial epidermal regeneration, in which the basal layer contained numerous and prominent digitiform projections composed of hyperplastic epithelial cells extending into the dermis.

The lymph nodes draining the right (inoculated) CBs presented with nonspecific reactive changes. No difference was observed between SC- and FB-inoculated animals. Mild

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paracortical hyperplasia started at 24 HPI, with gradual development of more paracortical and also medullary cord hyperplasia from 72 to 120 HPI.

Detection of Virus in Animals Inoculated in the Coronary Bands

All results from animals inoculated in the CBs are shown online in a supplementary table (to view online supplement, please go to http://www.vet.sagepub.com/supplement). Detection of VSNJV by VI and rRT-PCR was restricted to the site of inoculation and draining LNs. All samples from mock-inoculated animals and left mock-inoculated CB (on the left side), tongue, tonsil, spleen, and serum from experimental animals were consistently negative by these techniques in all animals using either method of inoculation. For the right CBs (inoculated side), positive VI and low rRT-PCR CT values were positive at all times post inoculation, from 12 to 120 HPI, in both FB- and SC-inoculated groups of animals. However, the right front CB from a steer sampled at 48 HPI, where inoculation with infected black flies was attempted, was negative on VI and only weakly positive (high CT value) on the rRT-PCR. Flies from this site were negative for mammalian DNA, indicating that flies fed very poorly, if at all. For the draining lymph nodes of inoculated animals, live virus was isolated as early as 12 HPI in a scarified animal and no later than 72 HPI in both animal groups. The rRT-PCR results from the draining lymph nodes tended to have much higher CT values (lesser amount of virus) than the inoculated CBs.

In Situ Hybridization for Animals Inoculated in CB

Using the negative sense probe, which is complementary to the viral messenger RNA (mRNA) and antigenome strand (which are both sense/positive strands), for the N gene resulted in segmentally diffuse staining of the keratinocyte cytoplasm in the inoculated CBs. The SC-inoculated steer at 12 HPI had ISH signal from the stratum basale extending up into the midstratum spinosum (Fig. 3b). Signal distribution and intensity varied from darkly distinct small foci at 12 HPI to extensive coalescing large areas at 24 HPI (Fig. 4b) for both methods of inoculation. The signal had the same intensity at 48 HPI, but the positive keratinocytes were scattered within (Fig. 5b) or located around the periphery of necrotic areas (vesicle) for both SC and FB animals. At 72 HPI, both SC- and FB-inoculated animals had positive keratinocytes with fainter but diffuse signal throughout the cytoplasm (Fig. 6b). When we compared the 2 methods of inoculation at 48 HPI, the location of ISH labeling was distinct between SC- (Fig. 7) and FB-inoculated (Fig. 8) animals. For both, the ISH signal was scattered within the stratum spinosum and restricted to the CB epithelium. However, in the FB-inoculated steer, ISH signal was deeper, occurring in the stratum basale of the CB and expanding beyond the fly-feeding area into the interdigital region, with cleft formation under the level of the stratum basale of both CB and interdigital skin (Fig. 8). ISH-positive keratinocytes were present throughout the necrotic epidermis. At 96 HPI, SC- and FB-inoculated animals had faint diffuse signal in the cytoplasm of keratinocytes, as did the SC-inoculated animal at 120 HPI. Positivity was multifocal and located more frequently at the periphery of necrotic areas.

Use of a negative sense probe to detect replicating virus in the draining LNs highlighted scattered large cells with abundant cytoplasm, morphology suggestive of dendritic cells or macrophages, in subcapsular areas of cortex and paracortex regions at 24 HPI only in both SC- and FB-inoculated animals (Fig. 9).

Immunohistochemistry in Animals Inoculated in the Coronary Bands

There was signal representing viral protein in the cytoplasm and in the plasma membrane of keratinocytes in the inoculated CB. This signal was consistently restricted to the epidermis. Epidermal location of the signal followed in a similar fashion with the development of the histological lesion described above, except for the 12 HPI scarified steer, which had IHC signal from the stratum basale up through the midstratum spinosum (similar to the ISH staining). The IHC signal was present at later time points than ISH signal but was otherwise similar in the epidermis, yet it had many similarities to that seen with negative sense riboprobe ISH. That is, staining intensity varied from focal and intense, being restricted to small foci at 12 HPI (Fig. 3c) to more extensive, affecting large coalescing areas from 24 until 72 HPI (Figs. 4c, 5c, and 6c), after which it became fainter and more localized at 96 and 120 HPI. However, the patterns of IHC and ISH diverged in the lymph nodes. In contrast to what was observed with ISH, there was IHC positivity until 120 HPI in the draining lymph nodes, with positive cells in the cortical subcapsular regions at 24 HPI and in perifollicular and follicular regions at 48, 72 (Fig. 10), 96, and 120 HPI. On sections of 24 HPI lymph nodes, IHC specific for dendritic cells (anti-CD1b antibody) highlighted the same types of cells in the same areas as were positive by ISH at 24 HPI (data not shown).

Discussion

In this study, acute clinical disease and typical lesion development were successfully reproduced in cattle experimentally infected with VSNJV by application of virus to a scarified region on the CB and by feeding of VSNJV-infected black flies at the CB. The kinetics of viral replication and dissemination that occur during acute stages of VSNJV infection were analyzed using IHC for detection of viral protein, VI, rRT-PCR, and ISH for detection of viral replication (negative sense riboprobe ISH). Intense ISH signal was observed in CB keratinocytes from 24 to 48 HPI using the negative-sense riboprobe, which recognizes mRNA and replicative intermediate RNA, indicating that viral replication is taking place in these cells. In the same tissues, IHC labeling of viral proteins was observed beginning at 12 HPI and increasing through

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Table 1. Detection of *Vesicular stomatitis New Jersey virus* (VSNJV) by Virus Isolation (VI), Reverse Transcriptase Real Time PCR (rRT-PCR), In Situ Hybridization (ISH), and Immunohistochemistry (IHC) in Steers Inoculated by Scarification or Fly Bite in the Coronary Bands (CB) and Euthanatized at Different Times Post Inoculation (HPI).

Time	12 HPI				24 HPI				48 HPI				72 HPI				96 HPI				120 HPI			
Tissue	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC
Scarification																								
Right front CB	P	29	+++	+++	P	21	+++	+++	P	17	++	++	P	23	+	++	P	16	N	++	P	18	N	+
Right axillary	N	0	N	N	N	33	+	+	P	33	N	+	N	0	N	N	N	0	N	N	N	36	N	N
Right prescapular	P	37	N	N	P	39	N	++	P	28	N	+	N	0	N	N	N	34	N	N	N	33	N	+++
Right rear CB	P	23	+++	+++	P	25	+++	+++	P	17	++	++	P	17	+	++	P	16	+	N	P	18	+	+
Right popliteal	P	28	N	N	P	30	++	++	N	0	N	+	P	22	N	++	N	31	N	++	N	34	N	+++
Right prefemoral	N	0	N	N	N	0	N	N	N	0	N	N	N	0	N	N	N	0	N	+	N	0	N	N
Right prefemoral	N	0	N	N	N	0	N	N	N	0	N	N	N	0	N	N	N	0	N	+	N	0	N	N
Fly bite																								
Right front CB	NA	N A	NA	NA	P	29	+++	+++	N	33	N	+++	P	36	N	N	P	19	N	+	NA	N A	NA	NA
Right axillary	NA	N A	NA	NA	N	0	N	N	N	0	N	N	N	39	N	N	N	37	N	N	NA	N A	NA	NA
Right prescapular	NA	N A	NA	NA	P	33	+	++	N	0	N	N	N	0	N	N	N	28	N	+++	NA	N A	NA	NA
Right rear CB	NA	N A	NA	NA	P	29	+++	+++	P	16	+++	+++	P	18	+	+	P	19	+	+	NA	N A	NA	NA
Right popliteal	NA	N A	NA	NA	P	34	+++	+++	P	32	N	+++	P	39	N	N	N	33	N	+++	NA	N A	NA	NA
Right prefemoral	NA	N A	NA	NA	P	33	+	N	N	0	N	N	N	0	N	N	N	0	N	N	NA	N A	NA	NA
Right prefemoral	NA	N A	NA	NA	P	33	+	N	N	0	N	N	N	0	N	N	N	0	N	N	NA	N A	NA	NA

N, negative; NA, not applicable (there were no animals in those time points); NP, not processed; P, positive. rRT-PCR results expressed as cycle threshold value (CT value <40 was considered positive for the presence of virus).

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72 HPI, with the signal becoming more localized and faint from 96 to 120 HPI. Therefore, these results demonstrate for the first time that the in vivo peak of viral replication occurs between 24 and 48 HPI and demonstrate clearly that the keratinocytes from the CB are permissive cells for early viral replication. The fact that the intensity of IHC signal persists until 72 HPI, with faint ISH labeling at that time, is likely associated with the natural cycle of viral replication, with virus replicating early and viral protein remaining in the cells longer. The presence of virus and therefore potential viral shedding from CBs persisted to the end of the experiment, 120 HPI, as shown by VI and rRT-PCR.

In the local draining lymph nodes of animals inoculated in the CBs, whether cattle were infected by SC or FB, replicating VSNJV was detected for the first time via ISH (negative sense riboprobe) and was restricted to the 24 HPI time point. ISH signal was scattered and distributed predominantly in the subcapsular areas within individual cells with morphology suggestive of macrophages or dendritic cells. On adjacent sections, anti-CD1b antibody highlighted cells with similar morphology and distribution of those containing replicating virus at 24 HPI. Morphologic features and anti-CD1b labeling in subcapsular sinuses strongly suggest that these cells are dendritic cells. With the negative sense riboprobe, any positive signal represents the presence of positive sense RNA, which would be either a replicative intermediate of the virus or viral mRNA. In either case, positive ISH signal is an indication of active viral replication in the lymph node. In the LNs, live virus or its nucleic acid was detectable by VI and/or rRT-PCR for up to 72 or 120 HPI, respectively. However, neither of these parameters provides definitive evidence that the virus is replicating there, as virus could be passively carried to the lymph node. So this study is the first to show VSV replication beyond the site of the vesicle in cattle.

Both SC and FB inoculation methods resulted in disease when introduced at the CB, and the character and time course of infection had some definite similarities. However, there were also notable differences, with more severe and extensive gross and histological lesions in the FB-inoculated animals.

Feeding of infected black flies at the CBs could reproduce the disease, although the amount of virus contained within the total FB inoculum is far less than with SC. Previous pilot studies demonstrated that inoculation of virus via SC with smaller amounts than 10^7 $TCID_{50}$ per animal, as used in this study, did not consistently produce clinical disease in cattle (L. Rodriguez, personal communication) or pigs.[16] The amount of VSNJV shed in saliva when a black fly is probing and blood feeding is not known; however, in previous studies that used an in vitro method to collect fly saliva,[9] VSNJV titers between $10^{1.5}$ and $10^{2.34}$ plaque-forming units (pfu)/ml were found in the saliva of black flies infected in the same manner as in our experiment. Flies probably cannot inoculate much more than 10–30 µl of saliva each. In this experiment, blood meal analysis revealed that only 6 flies out of 30 per cage participated in feeding. Consequently the total amount of inoculated fly saliva is very low. Although it is technically not feasible to quantitatively compare pfu with $TCID_{50}$, it would seem that a total dose of less than 200 pfu is considerably less than 10^7 $TCID_{50}$. Even with this lower dose, the lesion severity increased earlier and more rapidly in the FB compared with the SC animals. Therefore, it appears that infection by FB can be successful with markedly decreased amounts of virus, compared with SC, which suggests that there may be a facilitating factor supplied by the insect inoculation.

In this study, we also inoculated virus into neck skin using FB and examined the tissues using VI, HE, IHC, and ISH. Previous VSNJV studies in cattle[14] with SC of the CBs and flank skin could produce lesions only in the inoculated CBs, with no lesion development in the flank or in any other tissue.[14] However, it has been shown that noninfected black flies physically separated from VSNJV-infected black flies became infected while cofeeding at these nonvesicular sites on the same nonviremic host.[10] Our findings confirmed the lack of gross lesion development and demonstrated no histological viral-associated lesions. In addition, none of the multiple examined sections were positive either by IHC or ISH, although there was limited positivity using VI and rRT-PCR. Therefore, presence of the virus in this type of tissue is probably short and transient, although enough to be transmitted to other flies, with no resultant vesicle development or detectable virus by ISH or IHC.

Lesions developed only when the inoculation was in the CB, and they were restricted to the site of inoculation (CB), with viral replication and dissemination to the draining lymph node. These data indicate that the characteristics of the epithelium may well play a pivotal role in susceptibility to the virus infection.

The epidermis of the CBs and the epidermis of other areas where vesicular lesions develop, such as oral cavity (tongue, hard palate and gums), muzzle, teats, and prepuce, share similar histological and physiological features. These areas are unhaired, have thicker epidermis with a prominent stratum spinosum, and are more subjected to mechanical stress. These areas require higher amounts and more specialized intercellular adhesion molecules for proper tissue cohesiveness. In contrast, the neck and the flank skin are haired and have a markedly thinner epidermis compared with those areas where VSNJV replicates and forms vesicles, with less mechanical stress and therefore decreased concentrations of intercellular bridges. Comparing the morphophysiological features of the anatomical locations where VSNJV lesions develop to those sites where vesicles do not form, it seems plausible that the virus uses cell membrane receptors (perhaps intercellular junction molecules) for viral entry and/or uses part of specialized cell pathways for viral replication.

In summary, our study demonstrates that VSNJV replicates successfully and extensively in the keratinocytes from specific anatomical locations with thick unhaired skin. Vesicular lesions were limited to the site of inoculation, with peak of viral replication between 24 and 48 HPI in the CB. Replicating virus was detected in draining lymph nodes but only at 24 HPI. Lesion development was markedly more severe and extensive when the virus was transmitted via fly bite, which suggests that components in the saliva facilitate VSNJV infection.

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Declaration of Conflicting Interests

The authors declared that they had no conflicts of interest with respect to their authorship or the publication of this article.

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Antiviral activity of bovine type III interferon against foot-and-mouth disease virus

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ABSTRACT

Foot-and-mouth disease (FMD) is one of the most serious threats to the livestock industry. Despite the availability of a vaccine, recent outbreaks in disease-free countries have demonstrated that development of novel FMD control strategies is imperative. Here we report the identification and characterization of bovine (bo) interferon lambda 3 (IFN-λ3), a member of the type III IFN family. Expression of boIFN-λ3 using a replication-defective human adenovirus type 5 vector (Ad5-boIFN-λ3) yielded a glycosylated secreted protein with antiviral activity against FMD virus (FMDV) and vesicular stomatitis virus in bovine cell culture. Inoculation of cattle with Ad5-boIFN-λ3 induced systemic antiviral activity and up-regulation of IFN stimulated gene expression in multiple tissues susceptible to FMDV infection. Our results demonstrate that the type III IFN family is conserved in bovines and boIFN-λ3 has potential for further development as a biotherapeutic candidate to inhibit FMDV or other viruses in cattle.

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Introduction

Foot-and-mouth disease virus (FMDV) is the etiologic agent of one of the most devastating diseases that can affect cloven-hoofed livestock. Infection with FMDV causes an acute disease that spreads very rapidly and is characterized by fever, lameness and vesicular lesions on the feet, tongue, snout and teats, with high morbidity but low mortality (Grubman and Baxt, 2004). With the exception of North America, Western Europe, New Zealand and Australia, FMD is enzootic in the rest of the world and disease control is achieved by inhibition of animal movement, slaughter of infected and in contact animals, disinfection of contaminated premises and vaccination with an inactivated whole virus antigen. However, use of this vaccine is not recommended in FMD free-countries due to technical limitations in differentiating vaccinated from infected animals, the need of expensive high containment facilities to produce the antigen and the more severe trade restrictions established by the international organization of animal health for animals or animal products derived from countries that use this vaccine (OIE, 2007). Furthermore, since the current vaccine does not confer protection until at least five to seven days post-vaccination, vaccinated animals are susceptible to FMD prior to this time. Thus, in recent years the OIE has recognized that to be successful, FMD control programs should include the use of antivirals and/or immunomodulatory molecules that, in addition to newly developed marker vaccines would rapidly control the disease before the onset of the vaccine-induced adaptive immune response (Scudamore and Harris, 2002).

In all vertebrates, expression of interferons (IFN) constitute the first step in the immune response against viral infection and, indeed, administration of IFNs as biotherapeutics has been effective in controlling several viral infections (Basler and García-Sastre, 2002; Stark et al., 1998; Fensterl and Sen, 2009). In the case of FMDV, we have previously demonstrated that treatment of bovine, porcine and ovine cells with type I or type II IFNs dramatically inhibits viral replication (Chinsangaram et al., 1999, 2001; Moraes et al., 2007). Furthermore, swine inoculated with a replication-defective human adenovirus 5 vector (Ad5) that delivers porcine (po) IFN-α were sterilely protected when challenged with FMDV 24 h and up to 3–5 days post-inoculation (Chinsangaram et al., 2003; Dias et al., 2011; Moraes et al., 2003). Studies to understand the mechanism by which type I and II IFNs protect swine against FMD have shown that at least some IFN stimulated genes (ISGs) and migration of immune cells to the sites of FMDV infection play a significant role in controlling viral replication (Chinsangaram et al., 1999; de los Santos et al., 2006; Diaz-San Segundo et al., 2010; Moraes et al., 2007). However, a similar approach in cattle has only shown delayed disease and reduced clinical signs (Wu et al., 2003).

Recently, a new family of IFNs has been described, type III IFN or IFN-λ (Kotenko et al., 2003; Sheppard et al., 2003). These IFNs are related to the type I IFN gene family and also to the interleukin (IL) 10

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family of ligands. Within the type III IFN family, three structurally related members have been identified in humans, mice and chickens: IFN-λ1 (IL29), IFN-λ2 (IL28A) and IFN-λ3 (IL28B) (Karpala et al., 2008; Kotenko et al., 2003; Sheppard et al., 2003) and two members, IFN-λ1 (IL29) and IFN-λ3 (IL28B) have been recently identified in swine (Sang et al., 2010a, 2010b). Similar to type I IFN, expression of type III IFN is induced in response to recognition of pathogen associated molecular patterns (PAMPs) and activation of transcription factors, such as nuclear factor κB (NF-κB) and interferon regulatory factors (IRF)-3 and -7 (Iversen et al., 2010). Type III IFN signals through a heterodimeric cellular receptor that is composed of IL28-Rα, a type III IFN specific subunit, and IL10-Rβ, a subunit shared by other IL10 related cytokines. Despite the fact that type I and type III IFNs act on different receptors, they trigger strikingly similar responses through the activation of multiple members of the signal transducer and activator of transcription (STAT) family (Zhou et al., 2007). However, expression of the type III IFN receptor in a tissue specific manner, mainly in epithelia, has been proposed as one of the mechanisms evolved by different organisms to possibly prevent and protect themselves from viral invasion through the skin and mucosal surfaces (Sommereyns et al., 2008) especially with pathogens like FMDV, whose first site of replication is the mucosa (Arzt et al., 2010). Although not strictly robust, IFN-λ has been shown to induce protection against several viruses in cell culture, as well as in animal models, including herpes simplex virus type 2 (HSV-2) (Ank et al., 2006), hepatitis B and hepatitis C viruses (Marcello et al., 2006; Robek et al., 2005), influenza virus and other human pneumotropic viruses (Mordstein et al., 2010). IFN-λ has also been described as an inducer of antiproliferative activity by triggering host antitumor mechanisms (Maher et al., 2008). Furthermore, a role in the adaptive immune response, as well as modulation of the balance of Th1/Th2 immune responses has been recently proposed for IFN-λ1 biasing toward a stronger block of Th2 responses (Jordan et al., 2007; Morrow et al., 2010).

So far, no member of the type III IFN family has been described in bovines and sequencing of the bovine genome has not provided evidence of predictive sequences for this type of IFN (The Bovine Genome Sequencing and analysis consortium et al., 2009). Very recently a predictive sequence of an IL28B-like mRNA has been deposited in GenBank but no related literature is available.

Here we report the identification, cloning and characterization of a member of the bovine (bo) type III IFN family, boIFN-λ3 or boIL28B. Nucleotide and protein sequence analyses indicated that the cloned boIFN-λ3 displays significant homology with respect to previously identified porcine, human and mouse IFN-λ3 sequences, and to the predicted dog, chicken, rat and monkey sequences. Using an Ad5 vector we have expressed boIFN-λ3 protein in tissue culture and tested for biological antiviral activity against FMDV and vesicular stomatitis virus (VSV). Consistently, ISG expression was up-regulated in bovine cells after treatment with boIFN-λ3. Furthermore, inoculation of cattle with Ad5-boIFN-λ3 resulted in systemic antiviral activity and induction of ISG mRNA and protein expression in several tissues, including those of the upper respiratory tract and skin.

These results indicate that bovine type III IFN behaves similarly to other species orthologs and has the potential of being developed as a biotherapeutic to limit FMDV infection, replication and/or spreading.

Results

Identification of boIFN-λ3

In previous studies we used DNA microarrays to evaluate transcription profiles of bovine cells infected with FMDV (Zhu et al., 2010). Although bovine type III IFNs sequences had not been identified when we initiated this project, we included in the microarray several probes with sequence homology to human type III IFNs. Interestingly, we observed that upon FMDV infection there was significant up-regulation of the mRNA detected by the human IFN-λ3 probes. Therefore to better understand if type III IFN plays any role in controlling FMDV infection, we amplified the full length coding sequence of boIFN-λ3 by RT-PCR of mRNA extracted from primary embryonic bovine kidney (EBK) cells. Analyses of 3 independent clones allowed us to identify a fragment of 585 nucleotides within an 818 nucleotide region, corresponding to a full length open reading frame of 195 amino acids that displayed significant sequence homology to the previously identified IFN-λ3 (IL28B) sequences in *Sus scrofa* (po) (NM_001166490), *Homo sapiens* (hu) (NM_172139) *Mus musculus* (mu) (NM_177396) and an IFN-λ3 predicted *Bos taurus* (bo) sequence recently deposited in GenBank (XM_002695050) (Fig. 1). The closest homology was observed with respect to the *S. scrofa* counterpart with values of 85% for the DNA and 76% for the protein sequences. Comparison of protein sequences across species and localization of the putative signal sequence, suggested that a previously deposited boIFN-λ3 sequence (predictive) likely contains an aberrant N-terminal extension. To this end the identified boIFN-λ3 sequence encodes for a protein of MW = 21587.6 with a pI of 8.20, a predicted signal peptide between amino acids 1 and 23 (Bendtsen et al., 2004) and a putative N-linked glycosylation sequence between amino acids 112 and 115 (NetNGlyc 1.0 Server, Gupta et al., 2004) (Fig. 1B).

boIFN-λ3 displays antiviral activity in vitro

Expression of boIFN-λ3

To corroborate the predicted biochemical properties and determine if the identified boIFN-λ3 sequence encoded for a biologically active IFN product, we expressed the protein using a replication-defective human Ad5 vector containing the cytomegalovirus (CMV) immediate early promoter to drive transcription of the gene (Moraes et al., 2001). Porcine IBRS2 cells, which do not express endogenous type I IFNs (Chinsangaram et al., 2001), were infected with the Ad5-boIFN-λ3 vector and recombinant protein expression was analyzed in cell extracts and supernatants. A protein with multiple bands between 21 and 34 kDa was detected by western blot analysis in both fractions using a specific rabbit polyclonal antibody produced in our laboratory (Fig. 2A). Infection of IBRS2 cells with a control Ad5 vector did not result in the induction of a comparable protein. Addition of tunicamycin, an inhibitor of N-linked glycosylation, to the Ad5-boIFN-λ3 infected IBRS2 cells, resulted in a protein with a discrete MW of approximately 21 kDa in both, the cell extract and the supernatant, indicating that boIFN-λ3 protein was glycosylated at Asn residues.

Detection of antiviral activity in vitro

The biological activity of the expressed boIFN-λ3 protein was tested in EBK and Madin–Darby bovine kidney (MDBK) cells which were pretreated for 24 h with filtered supernatants from Ad5-boIFN-λ3 infected IBRS2 cells and challenged with FMDV and VSV respectively. Fig. 2B shows that the supernatants from Ad5-boIFN-λ3 infected IBRS2 cells contained between 32,000 and 64,000 U/ml of antiviral activity against FMDV. A stronger response 51,200 to 102,400 U/ml was observed when the same supernatant was tested in MDBK cells challenged with VSV (Fig. 2C). The specificity of the response was determined by incubating the same supernatants with rabbit anti boIFN-λ3 serum prior to the assay. Most of the antiviral activity, 80 to 90%, was neutralized by addition of the specific polyclonal antibody (Fig. 2D). No background antiviral activity was detected in supernatants from uninfected or control Ad5-Blue infected porcine IBRS2 cells and no neutralization of antiviral activity was observed by addition of preimmune rabbit serum (Fig. 2D, arrow), or anti-bovine IFN-α antibody (Fig. 2D, arrowhead). These results indicated that the

Fig. 1. DNA and amino acid sequence analysis of boIFN-λ3 (boIL28B). Nucleotide (A) and deduced amino acid (B) sequences were aligned to known homologous sequences. pred.: predicted; hu: human; mu: murine; po: porcine; aa: amino acid; cds: coding sequence. Predicted signal peptide: amino acids 1–23 (boxed in red). Putative N-glycosylation site: amino acids 112–115 (boxed in blue).

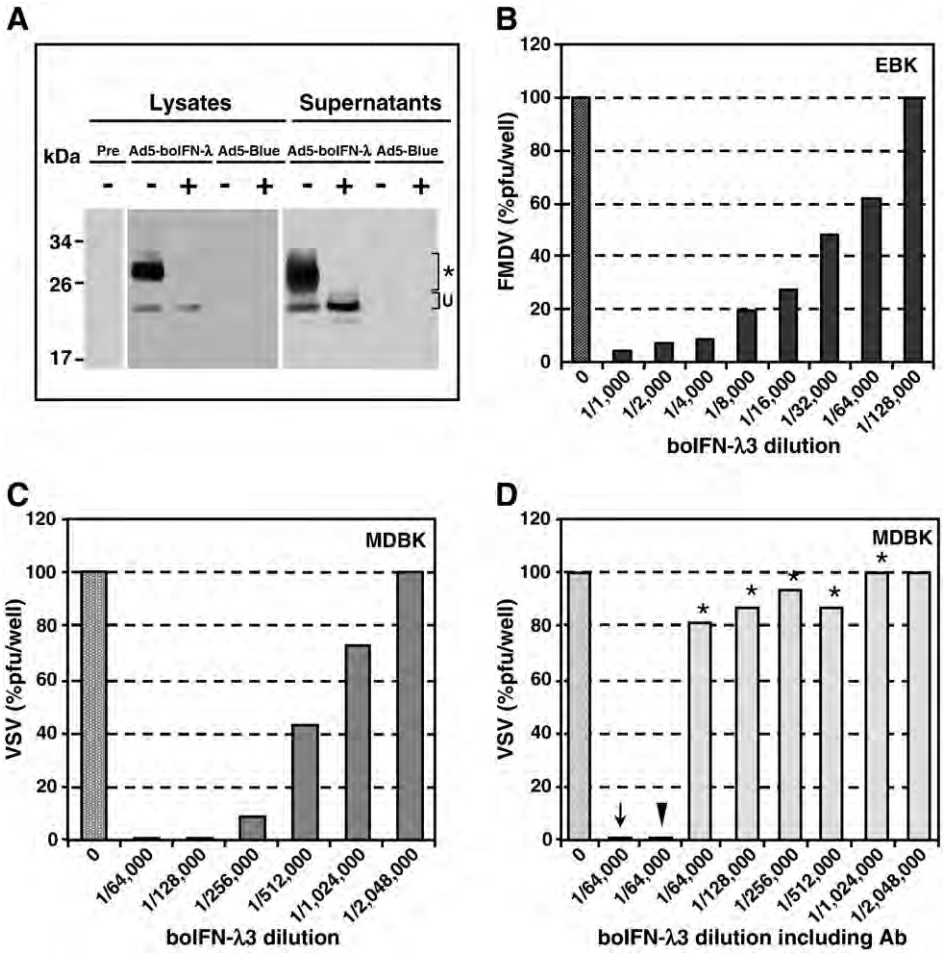

Fig. 2. Characterization of boIFN-λ3 biochemical activity. IBRS2 cells were infected with Ad5-boIFN-λ3 or Ad5-Blue for 24 h in the presence (+) or absence (−) of tunicamycin. Cell lysates and supernatants were evaluated for the presence of recombinant boIFN-λ3 protein by western blot analysis using a polyclonal rabbit antibody. Pre: Cell lysate of Ad5-boIFN-λ3 infected cells probed with rabbit pre-immune serum. *: Glycosylated species of boIFN-λ3. U: Un-glycosylated form (A). Antiviral activity of the recombinant boIFN-λ3 protein expressed in IBRS2 cells was evaluated in primary EBK cells challenged with FMDV (B) and in MDBK cells challenged with VSV (C). Neutralization of antiviral activity with anti-boIFN-λ3 polyclonal antibody in MDBK cells challenged with VSV. Arrow and arrowhead represent neutralization in the presence of rabbit pre-immune serum and polyclonal anti-bovine IFN-α, respectively. Asterisk (*) denotes neutralized antiviral activity (D).

expressed boIFN-λ3 displays antiviral activity in bovine epithelial cells.

It has been reported that although type I and type III IFN bind distinct receptors, they induce similar cellular responses (Kotenko et al., 2003; Sheppard et al., 2003; Zhou et al., 2007; Dumoutier et al., 2004). We therefore assayed the antiviral activity in MDBK or EBK cells treated with boIFN-α and boIFN-λ3 or a combination of both IFNs against VSV and FMDV, respectively. We also incubated the cells with poIFN-α, which we have demonstrated to exhibit antiviral activity in bovines (Wu et al., 2003). Overall, treatment with different

Table 1
Bovine oligonucleotide primer and probe sequences for real-time RT-PCR.

Gene	Accession #	Forward primer	TaqMan probe	Reverse primer
CCL2	EU276059	GCTACTCACAGTAGCTGCCTTCAG-30	CCGAGGTGCTCGCTCAGCCAG-56	GCGACTTGGGAGTTAATTGCA-98
CCL3	AY077840	AGCCAGGTCTTCTCGGCAC-124	ATTTGGCGCTGACACC-144	AGAAGCAGCAGGCCGTTG-178
CCL20	NM_174263	CCAGTATTCTTGTGGGCTTCACA-166	AGCAGCTGGCCAATGAAGCCTGTG-190	GGTGTAAAAGACAACTGCATTGATG-239
CXCL10	EU276062	GTCATTCCTGCAAGTCAATCCTG-142	CCACGTGTCGAGATTA-166	CCCATTCTTTTTCATTGTGGC-204
GAPDH	NM_001034034	GCATCGTGGAGGGACTTATGA-572	CACTGTCCACGCCATCACTGCCA-594	GGGCCATCCACAGTCTTCTG-638
IFN-α	XM_001250455	AGCACACCTTCCAGCTTTTCA-319	CAGAGGGCTCGGCC-341	GGAGGCTCTTGTCCCACACA-376
IFN-β	M15477	CTACAGCTTGCTTCGATTCCAA-278	ACGTCAGAGCCTTAAA-302	CTGCCC AGAG TT TGAC-341
IL10-Rβ	NM_001076975	TTTGACAAACTGAGCGTCATCA-913	AAGTGTCTGAAAGCTGCAA-938	CGGCCCCAGGGTTCA-973
IL28-Rα	XM_868941	CCAGCTGCCGCATTGTCT-435	CCCCAACCAGATATG-454	TCCTTCCAGAAATTCACCTCATAGT-494
IFN-λ3	HQ317919	ACTCATCCCTGGGCCACA-335	CCTGGAGCAGCCCCTTCTCACG-354	GCTTGGAGTGGATGTTCTGCA-397
IRF7	BC151518	GGACTGTGACACGCCCATCT-1535	ACTTCGGCACCTTCT-1558	CCCGGAACTCCAGCAGTTC-1596
ISG15	BC102318	GCGTGTACAAGCGGACCAGT-409	CTGGCTGTCTTTTGAAGGGAGGCCC-430	AGCGGGTGCTCATCATCC-474
MDA5	XM_615590	AGGAGTCAAAGCCCACCATCT-2541	ATTGGCGCTGGACACA-2563	TTCTGTGTCATGGGCTTGAACT-2606
MX1	AY251193S10	CGTCCGGAGCACGAAGAA-595	CGTCCGGAGCACGAAGAA-614	CGTCCGGAGCACGAAGAA-650
OAS1	AY243505	CCAAAGTTGTGAAGGGTGGC-161	CTCAGGCAAAGGC-183	TGATCGTCCCCTGAGGGTC-216
PKR	BC126646	TGCCAAACTGGCTTATGAAAAG-545	CAGAAACAATGAGAGATGG-574	TCACCACACGCAGCACTGA-613
RIGI	XM_580928	GACTCATTGCCCCAGGTCATT-1192	CTGACTGCCTCAGTTGGCGTTGGA-1216	TCGGCTGTGTTTTTGGCAT-1259

doses of type I and type III IFNs in combination resulted in enhanced antiviral activity, as expected and previously reported for other species (Supp. Fig. 1).

Analysis of gene expression in bovine cells treated with boIFN-λ3

To better understand the mechanism of antiviral activity elicited by boIFN-λ3 in bovine cells, we measured the levels of RNA by quantitative real time reverse transcription PCR (qRT-PCR) of IFNs and several ISGs whose expression has been shown to be up-regulated by type III IFN in other species (Ank et al., 2006). For the analysis of gene expression we focused on IFN-α, IFN-β, IFN-λ3 and ISGs, including chemokines CCL2, CCL3, CCL20, CXCL10, activators of the IFN pathway such as IRF-7, RIG I and MDA5 and some genes with known antiviral activity, ISG15, Mx1, OAS1 and PKR (Table 1). We also measured the expression of type III IFN receptors, IL28-Rα and IL10-Rβ. Little or no induction of type I or type III IFN mRNA was observed in these cells after treatment with boIFN-λ3, boIFN-α or poIFN-α. However, a dose response effect was observed for several ISGs when the cells were incubated with each IFN independently (Fig. 3) and 50 U of each IFN were sufficient to reach the saturation level. We did not detect any enhanced effect when boIFN-λ3 was used in combination with either poIFN-α or boIFN-α. Analysis of the expression of the type III IFN receptors (IL28B-Rα and IL10-Rβ) showed considerable basal levels of RNA with ct values of 24–28 as compared to ct values of 20–21 for the housekeeping gene GAPDH, in samples containing 5 ng of total RNA. Expression of such levels of IL28B-Rα and IL10-Rβ is consistent with the sensitivity of EBK cells to type III IFN.

Ad5-boIFN-λ3 induces antiviral activity and ISG up-regulation in cattle

In order to test the response of cattle to inoculation of Ad5-boIFN-λ3, a pilot experiment was performed with 4 animals that were inoculated with Ad5 vectors expressing IFNs as transgenes or empty vector control Ad5-Blue (Moraes et al., 2001). We chose a dose of 10^{11} pfu of Ad5-IFN/animal based on previous studies with type I IFN, in which 10^{10} pfu/animal was required to induce any measurable antiviral activity in serum, and treatment with Ad5-poIFN-α was evaluated at the same time because this vector displayed the highest antiviral activity despite the species difference (Wu et al., 2003). The systemic IFN biological antiviral activity one day after administration of the vectors was 25 U/ml for the animal inoculated with Ad5-Blue, 50 U/ml for the animals receiving each IFN alone and 160 U/ml for the animal receiving the combination of IFNs, indicating that all Ad5 IFN constructs were expressing the recombinant proteins.

Expression of ISGs in tissues

Twenty-four hours after Ad5 inoculation, animals were euthanized, necropsies were performed and tissues of the respiratory tract, skin and lymphoid organs were sampled based on previously reported studies about the pathogenesis of FMDV (Arzt et al., 2010). Peripheral blood mononuclear cells (PBMCs) were also extracted before and after treatment. The expression of several genes including IFN and ISGs was measured by qRT-PCR and an induction of 2 fold or more was considered as a "hit" and was represented in red. While treatment with either Ad5-IFN had 95 and 98 hits respectively, treatment with the combination of Ad5-IFNs displayed 139 hits in comparison to the Ad5-Blue control animal (Fig. 4). Approximately 48% of these hits

Treatment	IFNα	IFNβ	IFNλ3	CCL2	CCL3	CCL20	CXCL10	IRF7	RIGI	MDA5	ISG15	MX1	OAS1	PKR	IL28Rα	IL10Rβ
5U boIFN-λ3	2.5	0.5	1.0	1.1	1.0	0.8	4.7	4.6	19.8	18.2	735.1	53.6	334.3	8.4	1.1	1.0
10U boIFN-λ3	1.3	0.6	0.1	1.3	0.9	1.1	6.6	5.8	31.1	26.2	1114.0	67.4	394.8	10.4	0.8	0.9
50U boIFN-λ3	3.2	2.5	1.7	1.7	0.8	1.5	8.5	8.5	56.4	61.2	2073.5	103.1	758.6	16.1	0.9	1.2
100U boIFN-λ3	0.9	0.4	1.5	1.9	1.0	2.6	8.6	9.8	64.8	68.6	2513.7	111.6	761.4	16.9	1.2	1.2
5U poIFN-α	3.0	1.0	1.6	2.0	1.1	1.7	5.8	8.4	20.7	53.2	2189.6	111.4	735.0	16.2	0.9	1.2
10U poIFN-α	4.2	0.7	0.4	2.0	1.1	1.2	8.1	9.7	32.7	79.3	3114.6	126.0	860.9	18.8	1.0	1.2
50U poIFN-α	4.9	0.8	1.6	4.4	1.2	2.8	121.5	14.7	54.8	132.7	4469.5	145.2	1090.4	23.0	1.2	1.2
100U poIFN-α	1.2	1.1	0.8	4.6	1.2	5.2	178.7	15.1	56.9	127.9	5081.6	137.9	1022.8	23.1	1.1	1.4
5U boIFN-α	1.6	1.1	0.8	0.9	0.8	0.8	4.9	3.6	49.8	13.3	516.2	43.2	308.8	6.7	0.8	0.7
10U boIFN-α	0.9	0.7	0.8	1.1	0.8	1.9	10.0	4.8	73.7	24.1	881.1	56.6	430.4	9.5	0.9	0.7
50U boIFN-α	1.5	1.7	1.1	1.9	0.8	2.0	9.3	7.2	96.7	48.8	1721.1	80.2	638.4	12.3	0.9	0.9
100U boIFN-α	1.9	1.7	0.8	1.7	0.8	2.3	27.8	7.2	99.3	53.6	1740.1	69.6	675.7	13.6	0.9	0.9
5U boIFN-λ3 + 5U poIFN-α	3.4	0.5	0.9	1.4	0.8	1.5	2.8	5.9	46.3	33.4	1237.3	73.0	519.2	9.7	0.9	0.8
5U boIFN-λ3 + 5U boIFN-α	3.6	0.9	0.5	1.1	0.8	1.8	2.9	3.9	26.5	16.0	656.6	39.9	328.4	7.5	0.7	0.8
50U boIFN-λ3 + 50U poIFN-α	1.6	0.8	0.6	3.0	0.8	4.0	53.8	11.0	96.4	94.0	3031.4	99.1	905.1	18.3	0.9	1.0
50U boIFN-λ3 + 50U boIFN-α	2.9	0.2	0.7	1.4	0.6	0.8	8.3	8.4	69.5	44.6	1906.5	64.7	532.0	11.4	0.6	0.7

Fold induction ≥ 2 with boIFN-λ3 treatment
Fold induction ≥ 2 with poIFN-α treatment
Fold induction ≥ 2 with boIFN-α treatment
Fold induction ≥ 2 with boIFN-λ3 and boIFN-α treatment

Fig. 3. Examination of gene expression in bovine cells treated with IFNs. EBK cells were treated for 24 h with varying concentrations of boIFN-λ3, poIFN-α or boIFN-α alone or in combination, as indicated. RNA was isolated and gene expression was analyzed by qRT-PCR. Primers and probes are described in Table 1. Results are expressed as relative fold induction of cells treated with IFN with respect to cells treated with medium (mock). Shaded colored areas represent induction relative to mock treated cells. Color coding indicates gene induction according to each treatment.

(marked with an E) displayed enhanced expression (equivalent to the addition of each independent effect or higher) suggesting that combination treatment results in a stronger IFN response. The expression of IRF-7 was highly up-regulated by the combination treatment in all examined tissues. It was also noticeable that in PBMCs, Ad5-poIFN-α induced twice the number of up-regulated genes as compared to Ad5-boIFN-λ3. In parallel, the levels of mRNA for type III IFN receptor subunits were also measured to evaluate the sensitivity of each analyzed tissue to this type of IFN. Constitutive basal expression of type III IFN receptors (IL28-Rα and IL10-Rβ) was detected in all analyzed tissues indicating that these tissues could be susceptible to type III IFN (Fig. 5). Interestingly, expression of both receptor subunits, and particularly IL28-Rα, was up-regulated after IFN treatment in most of the analyzed tissues including the naso- and oropharynx (Fig. 4), tissues recently reported as the initial site of virus replication after aerosolization of FMDV (Arzt et al., 2010).

Detection of Mx1 protein

Visualization of Mx1 protein by immunohistochemistry (IHC) is a common technique to evaluate *in situ*, the effects of type I and type III IFNs (Jung and Chae, 2006; Sommereyns et al., 2008). One day after inoculation, animals treated with Ad5-boIFN-λ3 showed increased Mx1 protein signal in the epithelium of the dorsal soft palate, other mucosal epithelia of the upper airways and different locations of skin including coronary band or interdigital skin as compared to Ad5-Blue inoculated animals (Fig. 6A, compare panels c and g with a and e and Table 2). With the exception of the proximal lung, the Mx1 signal in epithelial tissues was always weaker in the animal treated with Ad5-poIFN-α than in the animal treated with Ad5-boIFN-λ3 (Fig. 6A, panels b and f, and Table 2). In contrast, the animal treated with the combination of Ad5-IFNs showed the highest Mx1 signal in most of the tissues (Fig. 6A, panels d and h and Table 2). No or almost undetectable positive signal was seen in the control animal (Fig. 6A, panels a and e). The levels of Mx1 protein

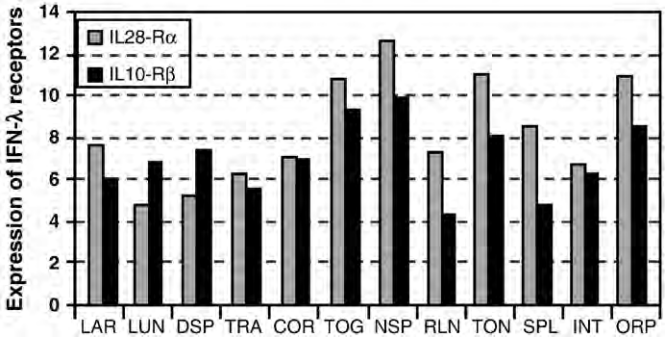

Fig. 5. Expression of type III IFN receptors, IL28-Rα and IL10-Rβ, in bovine tissues. Gene expression was analyzed by qRT-PCR in RNA samples extracted from the bovine tissue of the control animal (larynx [LAR], proximal lung [LUN], dorsal soft palate [DSP], trachea [TRA], coronary band skin [COR], tongue [TOG], nasopharynx [NSP], retro-pharyngeal lymph node [RLN], palatine tonsil [TON], spleen [SPL], interdigital skin [INT] and oropharynx [ORP]) and normalized against GAPDH.

detected by IHC correlated with the up-regulation in the expression of mRNA analyzed by qRT-PCR (Fig. 6B and Table 2). Interestingly, the protein signal in lymphoid tissue, *i.e.*, palatine tonsil, retropharyngeal lymph node or spleen in the animal inoculated with Ad5-poIFN-α was stronger than the signal observed in the same organs of the animal treated with Ad5-boIFN-λ3 (Table 2).

Taken together, our results indicated that boIFN-λ3 has antiviral activity in cattle *in vivo* and induces the expression of several ISGs when used alone or in combination with IFN-α.

Discussion

Members of the type III IFN family, also known as IL28A, IL28B and IL29, have been recently identified in several species including

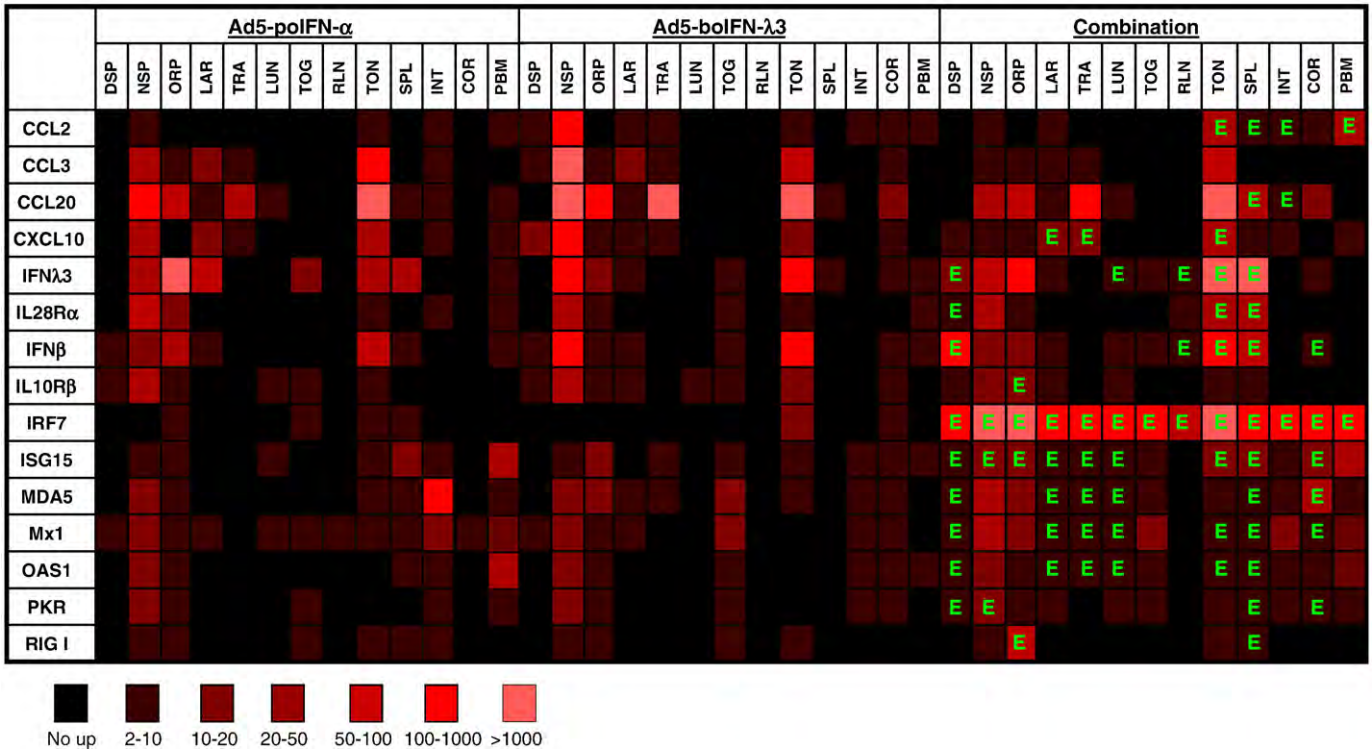

Fig. 4. Examination of gene expression in tissues and PBMCs isolated from bovines treated with Ad5-boIFN-λ3, Ad5-poIFN-α, or a combination of both IFNs. Gene expression was measured by qRT-PCR in RNA samples extracted from the listed bovine tissues (dorsal soft palate [DSP], nasopharynx [NSP], oropharynx [ORP], larynx [LAR], trachea [TRA], proximal lung [LUN], tongue [TOG], retropharyngeal lymph node [RLN], palatine tonsil [TON], spleen [SPL], interdigital skin [INT], coronary band skin [COR] and PBMCs [PBM]). Results are expressed as relative fold induction values of Ad5-IFN treated with respect to Ad5-Blue-control treated animals. Relative gene induction is represented by red color indexing [brightest red (greater than 1000 fold) to black (lower than 2 fold)]. E denotes enhanced up-regulation with the combination treatment.

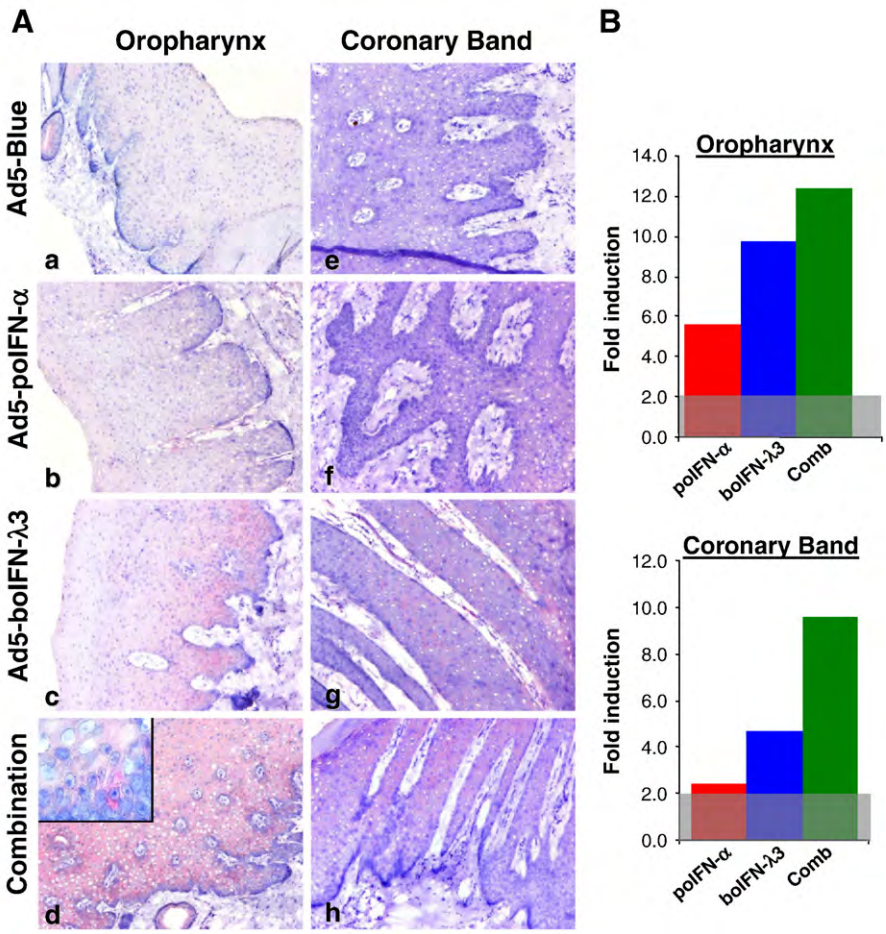

Fig. 6. Detection of Mx1 expression by immunohistochemistry (IHC) and real time RT-PCR. A. Tissues from oropharynx (panels a to d) and from coronary band skin (panels e to h) were harvested from bovine necropsies 24 hpi with Ad5-Blue (control), Ad5-poIFN-α, Ad5-boIFN-λ3 or a combination of Ad5-boIFN-λ3 and Ad5-poIFN-α (combination). Mx1 protein was detected by staining with mouse mAb against human Mx1, and a biotinylated anti mouse/avidin–peroxidase complex developed with Fast Red TR/Naphthol. Positivity is shown in bright purple. Sections were counterstained with Harry's hematoxylin (blue). Magnification, ×10. Insert in panel d shows a higher magnification (×60) of the oropharynx basal epithelium layer in the indicated region. B. Relative expression of Mx1 mRNA analyzed by real time RT-PCR in oropharynx and skin from coronary band. Primers and probes are described in Table 1. Results are expressed as relative fold induction of mRNA in tissue from cows treated with Ad5-IFNs with respect to mRNA in tissue from a cow treated with Ad5-Blue (control). Relative mRNA levels were determined by comparative cycle threshold analysis and a relative fold induction of 2 or less was considered as background which is depicted as a light gray area across the graphs. Expression of GAPDH mRNA was used as normalizer.

human, mouse and swine (Kotenko et al., 2003; Sang et al., 2010a, 2010b; Sheppard et al., 2003). These IFNs are expressed in response to virus infections and mediate the induction of antiviral activity (Ank et al., 2006; Kotenko et al., 2003; Meager et al., 2005; Robek et al., 2005). However, no sequences for type III IFN are available in the published bovine genome (The Bovine Genome Sequencing and Analysis Consortium et al., 2009). By using PCR primers homologous to human sequences, we amplified the mRNA coding for one member of the bovine type III IFN family, boIFN-λ3 (boIL28B). Although the same sequence was obtained in several independent clones, the presence of allelic polymorphism, a common feature within IFN families, cannot be ruled out (van Pesch et al., 2004; Sang et al., 2010a, 2010b). More studies are required to demonstrate this hypothesis. Cloning of the identified boIFN-λ3 gene and expression in mammalian cells resulted in the synthesis of an N-linked glycosylated protein of approximately 21–34 kDa that displayed specific antiviral activity against FMDV and VSV as examined by plaque or virus titer reduction assays in bovine tissue cultures. Analysis of gene expression in cells treated with boIFN-λ3 showed patterns similar to those displayed by treatment with bovine or porcine IFN-α. Induced genes included PKR and OAS1, which have antiviral activity against FMDV (Chinsangaram et al., 1999; de los Santos et al., 2006), and CXCL10 which has been proposed to play a role in immune cell infiltration to the sites of FMDV replication (Diaz-San Segundo et al., 2010). Expression of ISG15 was also significantly up-regulated in bovine cells treated with IFNs. Recently we have shown that in bovine cells infected with FMDV there is an up-regulation of ISG15 (Zhu et al., 2010). Interestingly, the levels of ISG15 are higher when the infection is carried out with an attenuated strain of FMDV, leaderless virus, as compared to wild-type

Table 2
Expression of Mx1 in different tissues in animals treated with Ad5-poIFNα, Ad5-boIFNλ or the combination of the two.

	Ad5-poIFNα	Ad5-boIFNλ3	Combination
Dorsal soft palate	+[a]/2.9[b]	++/2.1	+++/6.9
Oropharynx	±/5.7	++/9.7	+++/12.4
Nasopharynx	+/20.5	++/15.0	+++/28.6
Proximal lung	++/2.3	±/1.0	+++/4.2
Tongue	±/3.1	++/13.4	+++/11.2
Retro LN	++/2.3	+/1.1	++/1.7
Palatine tonsil	+++/2.4	+/1.7	+++/4.9
Spleen	++/3.9	±/0.7	++/5.9
Skin interdigital	±/15.9	+/7.2	++/11.9
Skin coronary band	±/2.4	+/4.7	++/9.6

[a] Semi-quantitative analysis of Mx1 protein signal in tissue sections detected by IHC: − is negative and +++ maximum signal (±<+<++<+++).
[b] Level of fold induction of mRNA detected in each tissue by real time RT-PCR, compared with the same tissue in a non-treated animal. Only animals with an expression ≥2 are considered up-regulated.

FMDV, however further work is required to demonstrate if this gene has a role in controlling FMD.

To determine if boIFN-λ3 had activity *in vivo*, we performed an initial experiment inoculating one cow each with Ad5 vectors delivering poIFN-α, boIFN-λ3, a combination of both, or an empty control (Ad5-Blue). We chose poIFN-α instead of boIFN-α because in previous experiments we had observed a good antiviral response of this IFN despite the species difference (Wu et al., 2003). We inoculated the animals with a relatively high dose of Ad5-vector (10^{11} pfu/animal) to have a measurable response without FMDV challenge. Despite these high Ad5 doses, the levels of antiviral activity in serum were rather low, however significant variation had been previously observed in cattle and swine inoculated with type I IFN alone (Wu et al., 2003; Dias et al., 2011) and protection against FMD has still been observed (Dias et al., 2011). Inoculation of cattle with Ad5-boIFN-λ3 alone or in combination with Ad5-poIFN-α induced a systemic antiviral activity and up-regulation of specific gene expression in multiple tissues, particularly in the upper respiratory tract, the initial site of virus infection, and in the skin, the main site of FMDV replication (Arzt et al., 2010). FMDV is highly sensitive to the actions of type I and type II IFNs *in vitro* and *in vivo* (Chinsangaram et al., 1999, 2001; Dias et al., 2011; Moraes et al., 2007), however, since treatment with IFN-α only conferred partial protection in cattle (Wu et al., 2003), there is an active interest in developing and testing new antivirals with proven efficacy in this species.

Similar to the results *in vitro*, expression of several genes was induced in the tissues and PBMCs of cows treated with type III IFN. Although most of the analyzed genes were also induced by IFN-α, there is evidence in other species suggesting that selective expression of the type III IFN receptor in epithelial cells contributes to a better response to this type of IFN and prevents undesired side effects (Ank et al., 2006; Sommereyns et al., 2008). In support of this hypothesis, Ank et al. (2006) have shown that type III IFN treatment is effective in controlling herpes simplex virus 2 infection in epithelial tissue in vaginal mucosa. It has been reported that upon aerosolization of FMDV there is a pre-viremic phase when the virus mainly targets epithelial cells of the upper respiratory tract; once viremia is established, the virus preferentially infects epithelial cells of the skin and mouth (Arzt et al., 2010; Pacheco et al., 2010). Therefore, targeting antivirals to epithelial cells of the upper respiratory tract could potentially help control FMDV amplification at the initial site of infection. Indeed, expression of type III IFN receptors, IL28-Rα and IL10-Rβ was detected in most of the bovine tissues analyzed, but up-regulation, especially for IL28-Rα, was highest in the oro-, nasopharynx, tongue and palatine tonsil, all tissues present in the upper airways. The strongest and broadest up-regulation in gene expression in response to IFN treatment was observed for IRF-7, with highly enhanced up-regulation for the combination treatment of type I and type III IFNs, particularly in the oro-, nasopharynx and palatine tonsil. Expression of IRF-7 is induced by IFNs and mediates a positive feedback loop that controls the expression of most subtypes of IFN-α and other immunomodulatory molecules (Honda et al., 2005; Marie et al., 1998). Although we did measure, by qRT-PCR, the levels of boIFN-α we did not detect its induction with any treatment (data not shown). However, since there are at least 13 predicted IFN-α gene sequences within the bovine genome (The Bovine Genome Sequencing and Analysis Consortium et al., 2009) it is possible that other subtypes were induced but not detected with the available reagents. Interestingly, in PBMCs, the number of up-regulated genes induced by Ad5-poIFN-α was almost double as compared to Ad5-boIFN-λ3 treatment, indicating that blood cells are not so susceptible to type III IFN. In support of this observation, Lasfar et al. have reported that bone marrow-derived macrophages and splenocytes showed a very weak activation in response to type III IFN treatment (Lasfar et al., 2006). Overall, up-regulation of gene expression indicated a systemic response to type I and type III IFN treatment.

Our results suggest that, as previously reported in other species, boIFN-λ3 is involved in establishing an antiviral state in specific bovine tissues such as those present in the upper airways and the skin which are the preferred sites for FMDV replication. Therefore, use of boIFNλ3 has potential for development as an antiviral strategy to control FMD or other bovine diseases that target similar organs.

Material and methods

Cell lines and viruses

Human 293 cells (ATCC CRL-1573) were used to generate and propagate recombinant adenovirus. EBK cells were obtained from the Animal Plant and Health Inspection Service (APHIS), Ames, Iowa and porcine kidney cells (IBRS2) were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the Plum Island Animal Disease Center (PIADC), Greenport, NY. EBK cells were used for determination of IFN bioactivity against FMDV and IBRS2 cells were used to express IFN proteins from Ad5-IFN constructs. MDBK cells (ATCC CCL-22) were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and used for determination of IFN bioactivity against VSV. All cells were maintained in Eagle's minimal essential medium (EMEM, GIBCO BRL, Invitrogen, Carlsbad, CA) containing either 10% calf serum or 10% fetal bovine serum (FBS) supplemented with antibiotics. Baby hamster kidney cells (BHK-21, ATCC CCL-10) were used to propagate and titer FMDV. These cells were maintained in EMEM containing 10% calf serum and 10% tryptose phosphate broth supplemented with antibiotics. FMDV subtype A12 was generated from the full-length infectious clone pRMC35 (Rieder et al., 1993) and used for the biologic assay of IFN. VSV serotype New Jersey (VSVNJ) was provided by FADDL at PIADC. Recombinant Ad5 viruses including Ad5-boIFN-λ3, Ad5-poIFN-α and Ad5-Blue were obtained as previously described (Moraes et al., 2003).

Identification, cloning and expression of boIFN-λ3

RNA was isolated from EBK cells infected, at a multiplicity of infection (MOI) of 1 for 6 h, with an attenuated FMDV serotype A12 lacking the leader protein coding region (leaderless virus), and using an RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's directions. Approximately 1 μg of RNA was treated with DNase I (Sigma, St. Louis, MO) and was used to synthesize cDNA with M-MLV reverse transcriptase (Invitrogen) and random hexamers following the manufacturer's directions. Two oligonucleotides: 5′ ***ATCGAT***ATGGCCCCGGGCT GCACGCT 3′ (FW) and 5′ ***TCTAGA***TTAGACACACTGGTCTCCGCTGGC 3′ (RW) containing ClaI and XbaI restriction sites, respectively, were designed to amplify cDNA containing the putative IFN-λ3 (IL28B) sequence using bovine cDNA as template and PfuUltra™ high-fidelity DNA polymerase (Agilent Technologies, Santa Clara, CA). The amplified PCR fragment was cloned in pPCR4 blunt TOPO (Invitrogen) and the sequence of 3 independent clones was determined by standard DNA sequencing in an ABI 3730 XL system (Applied Biosystems, Foster City, CA). The sequence of boIFN-λ3 was identical in all 3 clones and it was submitted to GenBank, and assigned accession number HQ317919. One of the clones was digested with ClaI/XbaI and the resulting 588 bp boIFN-λ3 DNA fragment was ligated to ClaI/XbaI digested pAd5-Blue (Moraes et al., 2001) for expression in mammalian systems. Recombinant Ad5 viruses, including Ad5-boIFN-λ3, Ad5-poIFN-α and Ad5-Blue, were produced by transfection of 293 cells with previously PacI-digested pAd5 plasmids. Viruses were plaque-isolated, propagated in 293 cells and purified by CsCl gradient centrifugation. Viral titer was determined by the method of tissue culture infectious dose 50 ($TCID_{50}$) and converted to plaque-forming units (pfu)/ml. To analyze the protein expressed by Ad5-boIFN-λ3, IBRS2 cells were infected with the Ad5-boIFN-λ3 vector and cellular extracts and supernatants were collected

24 h post-infection and subjected to SDS-PAGE followed by Western blot analysis. A polyclonal antibody was obtained by inoculation of rabbits with the same Ad5-boIFN-λ3 vector. When indicated tunicamycin (10 μg/ml) was added during the infection to examine for the presence of N-linked glycosylation.

Determination of IFN biological activity

Plaque reduction assay

IBRS2 cells were infected with the Ad5 vectors and 24 hpi the supernatants were removed, centrifuged to pellet cell debris, and filtered through a Centricon 100 at 4000 rpm for 10 min to remove Ad5 vector. EBK and MDBK cells were pre-treated with 2-fold serial dilutions of the filtered supernatants. When required, a neutralizing polyclonal antibody was used (1 μl rabbit immune serum per ml of cell supernatant). Twenty-four hours later, supernatants were removed, and cells were washed and challenged with approximately 100 pfu of FMDV (EBK cells) or VSV (MDBK cells) for 1 h followed by overlay with gum tragacanth and incubation for 24–48 h. Plaques were visualized by staining with crystal violet. Antiviral activity was determined 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 untreated cells. Results were expressed as units of antiviral activity/ml of sample. A similar technique was used to measure antiviral activity in plasma derived from inoculated cattle.

Virus yield reduction assay

EBK cells were treated as previously described with 2-fold serial dilutions of filtered IBRS2 supernatants infected with Ad5-IFNs. Twenty-four hours later, treated cells were washed with PBS followed by infection with FMDV at a MOI of 1. One hour after the infection, cells were washed with 2-(N-morpholino) ethanesulfonic acid buffer (MES) pH = 6 followed by incubation in cell culture media for an additional 7 h (total incubation period is 8 h). Supernatants were then collected and virus yield was measured on BHK-21 cells by standard plaque assay (Chinsangaram et al., 1999). Results were expressed as $\log_{10}$/ml of sample.

Animal experiment

An animal experiment was performed under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Plum Island Animal Disease Center and animals were kept under strict controlled conditions in the PIADC biosafety level 3 animal facilities. Four male Holstein cows, of about 400 lb each, were inoculated intramuscularly (IM) with 2×10^{11} pfu of Ad5 vectors encoding different IFNs or Ad5-Blue control. One animal was inoculated with 2×10^{11} pfu of Ad5-Blue (control), one animal with 10^{11} pfu of Ad5-poIFN-α and 10^{11} pfu of Ad5-Blue, one animal with 10^{11} pfu of Ad5-boIFN-λ3 and 10^{11} pfu of Ad5-Blue and one animal was inoculated with a combination of 10^{11} pfu of Ad5-poIFN-α and 10^{11} pfu of Ad5-boIFN-λ3. Twenty-four hours after Ad5 inoculation, animals were euthanized and necropsies were performed to isolate multiple tissues and analyze the expression of several genes by qRT-PCR. Animals were bled before and after the treatment for measurement of IFN biological antiviral activity in plasma, as described above and PBMCs were extracted from heparinized blood to obtain mRNA.

Analysis of mRNA

A qRT-PCR assay was standardized and used to evaluate the mRNA levels of multiple genes in monolayers of EBK cells or bovine tissues and PBMCs exposed to different IFNs and control Ad5-Blue vector. RNA and cDNA were prepared as described above. An aliquot (1/40) of the cDNA was used as template for qRT-PCR using TaqMan Universal PCR Master Mix (Applied Biosystems). Primers and TaqMan minor groove binding (MGB) probes were designed with Primer Express™ software v.1.5 (Applied Biosystems). Forward and reverse primers were purchased from Invitrogen and the FAM-labeled TaqMan MGB probes from Applied Biosystems. Bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control to normalize the values for each sample. The sequences for the primers and probes are listed in Table 1. Reactions were performed in an ABI Prism 7900 Sequence detection system (Applied Biosystems).

IHC for detection of Mx1 protein in bovine tissues

Frozen 4 μm-sections of multiple bovine tissues obtained after necropsy were mounted onto electrostatically charged glass slides (SuperFrost Plus, Fisher Scientific, Worcester, MA) and fixed for 10 min in acetone at −20 °C. Thereafter, the slides were kept at −70 °C for up to 8 wk, until they were stained. For immunostaining, the slides were incubated with the primary antibody mouse mAb anti-human Mx1, kindly provided by Dr. Otto Haller, University of Freiburg. The bound primary antibodies was detected by the avidin–biotin–peroxidase complex technique (Vectastain ABC Kit Elite; Vector, Burlingame, CA) according to the manufacturer's instructions and developed with Fast Red TR/naphthol (Sigma, St. Louis, MO). Slides were counterstained with Harry's hematoxylin and coverslipped by using routine methods. To control the specificity of antibody binding, a duplicate negative control serial section treated with nonspecific primary antibody was used.

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Substitution of Specific Cysteine Residues in the E1 Glycoprotein of Classical Swine Fever Virus Strain Brescia Affects Formation of E1-E2 Heterodimers and Alters Virulence in Swine[▽]

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E1, along with E^{rns} and E2, is one of the three envelope glycoproteins of classical swine fever virus (CSFV). E1 and E2 are anchored to the virus envelope at their carboxyl termini, and E^{rns} loosely associates with the viral envelope. In infected cells, E2 forms homodimers and heterodimers with E1 mediated by disulfide bridges between cysteine residues. The E1 protein of CSFV strain Brescia contains six cysteine residues at positions 5, 20, 24, 94, 123, and 171. The role of these residues in the formation of E1-E2 heterodimers and their effect on CSFV viability *in vitro* and *in vivo* remain unclear. Here we observed that recombinant viruses harboring individual cysteine-to-serine substitutions within the E1 envelope protein still have formation of E1-E2 heterodimers which are functional in terms of allowing efficient virus progeny yields in infected primary swine cells. Additionally, these single cysteine mutant viruses were virulent in infected swine. However, a double mutant harboring Cys24Ser and Cys94Ser substitutions within the E1 protein altered formation of E1-E2 heterodimers in infected cells. This recombinant virus, E1ΔCys24/94v, showed delayed growth kinetics in primary swine macrophage cultures and was attenuated in swine. Furthermore, despite the observed diminished growth *in vitro*, infection with E1ΔCys24/94v protected swine from challenge with virulent CSFV strain Brescia at 3 and 28 days postinfection.

Classical swine fever (CSF) is a highly contagious disease of swine. The etiological agent, *Classical swine fever 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* (2). The 12.3-kb CSFV genome contains a single open reading frame that encodes a 3,898-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 posttranslational processing of the polyprotein by cellular and viral proteases (5). 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 E^{rns} loosely associates with the viral envelope (22, 23). E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor (19). E1 has been implicated (21), along with E^{rns} and E2 (4), in viral adsorption to host cells. Importantly, different modifications introduced into these glycoproteins appear to have an important effect on CSFV virulence (3, 6, 9, 10, 11, 12, 15, 20).

In lysates of CSFV-infected cells, E2 glycoprotein forms homodimers and heterodimers with E1 glycoprotein (14, 22) via disulfide bridges between cysteine residues. Formation of these disulfide-linked heterodimers also occurs in CSFV virions (19), suggesting that the interaction between envelope proteins plays an important role in virus assembly, virion maturity, and consequently in efficiency of viral infection. The E1 envelope protein of CSFV strain Brescia contains six cysteine residues at E1 positions 5, 20, 24, 94, 123, and 171. E1 residues that contribute to the formation of disulfide bridges with E2 glycoprotein are yet to be identified. Furthermore, the biological significance of E1-E2 heterodimer formation during infection and the role of E1 Cys residues during CSFV replication in target cells or in infection of swine are not known.

In this study, we have used oligonucleotide site-directed mutagenesis to construct a panel of single and double recombinant mutant viruses by substituting cysteine to serine residues within CSFV glycoprotein E1. These mutants were used to investigate whether substitutions of each of these Cys residues could affect formation of E1-E2 heterodimers in infected primary swine macrophages and virus virulence in swine. We observed that individual substitutions of these residues did not have an effect on the formation of E1-E2 heterodimers. Growth kinetics was not affected, nor was the ability to replicate or cause disease in swine. Interestingly, 11 out of 15 possible constructs containing double Cys-to-Ser substitutions were deleterious for the virus. A partial disruption of E1-E2 heterodimer formation was observed in cells infected with a virus harboring Cys24Ser and Cys94Ser substitutions in E1 protein. The virus, E1ΔCys24/94v, showed a decreased growth rate in primary swine macrophages and was attenuated in swine. The remaining constructs harboring double cysteine-to-

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serine mutations retained the ability to produce virus progeny, formed heterodimers in infected cells, and were virulent in swine. Data presented here suggest that specific E1 cysteine residues are critical for virus viability and virus growth; most probably, these residues play a role in E1-E2 interactions, affecting assembly of mature viral particles.

MATERIALS AND METHODS

Viruses and cells. Swine kidney cells (SK6) (17), free of BVDV, were cultured in Dulbecco's minimal essential medium (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO). CSFV strain Brescia was propagated in SK6 cells and used for the construction of an infectious cDNA clone (IC) (9). Growth kinetics was assessed on primary swine macrophage cell cultures prepared as described by Zsak et al. (24). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). Presence of viral antigen was detected, after 4 days in culture, by an immunoperoxidase assay using the CSFV monoclonal antibody (MAb) WH303 (1) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated using the method of Reed and Muench (8) and expressed as 50% tissue culture infective doses ($TCID_{50}$)/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 MAb WH303.

Construction of CSFV E1 cysteine mutant viruses (E1ΔCys). A full-length IC of the virulent CSFV strain Brescia (pBIC) (9) was used as a template in which Cys residues in the E1 glycoprotein were substituted with Ser residues. Cys-to-Ser amino acid substitutions (E1ΔCys) were introduced by site-directed mutagenesis using the QuikChange XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) performed per the manufacturer's instructions using the following primers (only forward primer sequences are shown; nucleotide changes compared with the native sequence are in boldface): C5, 5′ GCCTATGCCCT ATCACCTTAT**AGT**AATGTGACAAGCAAAATAGGG 3′; C20, 5′ TACATA TGGTACACTAACAAC**AGT**ACCCCGGCTTGCCTCCCCAAA 3′; C24, 5′ ACTAACAACTGTACCCCGGCT**AGC**CTCCCCAAAAATACAAAGATA 3′; C94, 5′ TCCCATGAAGAACCTGAAGGC**AGT**GACACAAACCAGCTGAA TTTA 3′; C123, 5′ TGGAATGTTGGCAAATATGTG**AGT**GTTAGACCAGA CTGGTGGCCA 3′; C171, 5′ TCAACCACGGCATTCCTCATC**AGC**TTGAT AAAAGTATTAAGAGGA 3′.

***In vitro* rescue of CSFV Brescia and E1ΔCys recombinant mutant viruses.** Full-length genomic clones were linearized with SrfI and *in vitro* transcribed using the MEGAscript T7 system (Ambion, Austin, TX). RNA was precipitated with LiCl and transfected into SK6 cells by electroporation at 500 V, 720 Ω, 100 W, with a BTX 630 electroporator (BTX, San Diego, CA). Cells were seeded in 12-well plates and incubated for 4 days at 37°C and 5% CO_2. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at ≤−70°C.

DNA sequencing and analysis. Full-length clones were completely sequenced with CSFV-specific primers by the dideoxynucleotide chain-termination method (16). *In vitro* rescued viruses and viruses recovered from infected animals were sequenced in the mutated region, whereas the E1ΔCys24/94v genome was sequenced completely. Sequencing reactions were prepared with the Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on an ABI Prism 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA). The final DNA consensus sequence represented an average 5-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Comparison of predicted alpha-helices and beta strands between parental BICv E1, double mutant E1ΔCys24/94 E1, and quadruple mutant E1ΔCys24/94/123/171 E1 proteins were done with NetSurfP ver. 1.1 (7).

Western blot analysis. Formation of E1-E2 heterodimers by BICv and the mutant viruses was analyzed in lysates of SK6-infected cells by Western immunoblot assays. CSFV E1 was detected with an anti-E1 serum produced in rabbits against CSFV strain Brescia E1 glycoprotein expressed in *Escherichia coli* (3). E2 detection was performed using anti-E2 monoclonal antibody WH303 (1). SK6 monolayers were infected (multiplicity of infection [MOI] = 1) with BICv or E1ΔCysv 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 either reducing or nonreducing conditions in precast NuPAGE 12% Bis-Tris acrylamide gels (Invitrogen). Western immunoblot assays were performed using the WesternBreeze chemiluminescent immunodetection system (Invitrogen).

Animal infections. Each of the E1ΔCys mutants was initially screened for its virulence phenotype relative to the virulent Brescia strain using 40-pound commercial-breed swine that were 10 to 12 weeks old. Pigs were inoculated intranasally with 10^5 $TCID_{50}$ of either mutant or wild-type virus. For screening, 22 pigs were randomly allocated into 11 groups of 2 animals each, and pigs in each group were inoculated with either BICv or one of the E1ΔCysv mutants. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea, and cough) and changes in body temperature were recorded daily throughout the experiment.

For protection studies, pigs were randomly allocated into 3 groups. Pigs in groups 1 and 2 (n = 4) were inoculated with E1ΔCys24/94v, while pigs in group 3 (n = 4) were mock infected. At 3 days postinfection (dpi) (group 1) or 28 dpi (groups 2 and 3), animals were intranasally challenged with BICv. 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 postchallenge, with blood obtained from the anterior vena cava in EDTA-containing tubes (Vacutainer). Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman, Coulter, CA).

RESULTS

Viability of CSFV E1ΔCys recombinant mutant viruses. Highly conserved Cys residues within E1 protein of CSFV strain Brescia (Fig. 1) were targeted for mutagenesis in the context of a full-length cDNA clone. Single Cys-to-Ser substitutions in E1 protein yielded viruses E1ΔCys5v, E1ΔCys20v, E1ΔCys24v, E1ΔCys94v, E1ΔCys123v, and E1ΔCys171v, while double Cys-to-Ser substitutions yielded only viruses E1ΔCys5/171v, E1ΔCys20/171v, E1ΔCys94/171v, and E1ΔCys24/94v (Table 1). Partial nucleotide sequences of viable rescued virus genomes were identical to parental DNA plasmids, confirming the fidelity of the RNA sequence in rescued viruses. Double substitutions within E1 protein that included combinations of residues 5/20, 5/24, 5/94, 5/123, 20/24, 20/94, 20/123, 24/123, 24/171, 94/123, and 123/171 did not yield infectious viral progeny after three independent transfection experiments using SK6 cells (Table 1). These data suggest that more than one Cys residue in E1 is necessary for proper assembly of infectious virus particles, particularly Cys20 and Cys123, since 8 of 11 combined substitutions that yielded nonviable viruses involved these two residues.

Replication of CSFV E1ΔCys mutants in primary swine macrophages. *In vitro* growth characteristics of rescued single and double mutant viruses were evaluated in primary swine macrophage cell cultures, these being primary CSFV target cells during infection of swine, and compared to parental BICv in a multistep growth curve (Fig. 2A and B). All recombinant viruses, with the exception of E1ΔCys24/94v, displayed growth kinetics indistinguishable from parental BICv (Fig. 2A and B). Growth of the double mutant E1ΔCys24/94v was significantly slower than that of BICv, exhibiting titers 10- to 15-fold lower than those of parental virus. Additionally, E1ΔCys24/94v showed reduced-size foci of infection on SK6 cells relative to those of parental BICv (Fig. 2C). Although these substitutions in E1 were not lethal like mutations observed with the majority of double mutants (Table 1), substitutions of E1 Cys24 and Cys94 significantly affected virus growth in macrophages and virus spreading in SK6 cells. The complete genome sequence of E1ΔCys24/94v confirmed that the introduced Cys-to-Ser substitutions are the only deviations from the original parental BICv genomic sequence. Double mutants that did not affect

	CSFV E1 protein residues											
	1-30			91-130						161-180		
BICv	LSPY**C**NVTSK	IGYIWYTNN**C**	TPA**C**LPKNTK	PEG**C**DTNQLN	LTVELRTEDV	IPSSVWNVGK	YV**C**VRPDWWP	NSASTTAFLI	**C**LIKVLRGQV
ALD	.....	.....	.....	.....	.....	.....	.....	.....	I
Eystrup	.....	.....	.....	.....	.....	.....	.....	.....	I
Alfort/187	.....	.....	.....	.....	.....	.....	.....	.....	I
Alfort A19	.....	.....	.....	.....	.....	.....	.....	.....	I
Alfort/Tuebingen	.....R.	.....	.....	.....	...K.....	V.....I..	.....	.....	.....
GPE-	.....	.....	.....	.....	.....	.....	.....	.....M....	I
Chinese strain	.....	.....	.....	.....	.....	.....	.....	.....I....	I
Thiverval	.....	.....	.....	L.....	.....	.....	.....	.....	I
Riems	.....	.....	.....	.....	.....	.....	.....	.....I....	I
CS	.....	...V.....	.....	.K.....	.....	.....R	.....	.....	I
Paderborn	.....R.	.....	.....	.....	...G.....	V.....I..	.....	.....	....I.....
Borken	.....R.	.....	.....	.....	...K.....	V.....I..	.....	.....	.....
Sp01	.....R.	.....	.....	.....	...K.....	V.....I..	.....	.....	.....
India	.....	.....	.....	.....	.....	.....	.....	.....	..V.....I
BVDV NADL	A....D.DR.	.....K..	.....	VMD..KT...	.....T.AE.	..G....L..	...I..N...	.A.T.....V	..V.IV...M
BVDV VEDEVAC	A....E.ERR	L.....K..	.....	IHD..K....	.....T.AE.	..G....L..	...I.....	.A.T.....V	..V..A....
BVDV CP7	A....E.ER.	L.....K..	.....R...	IQD..K....	.....T.AE.	..G....L..	.....	TA.T.....V	..V..V....
BVDV Osloss	A....E.ERR	L.....K..	.....	IHD..K....	...G.T.AE.	..G....L..	...I.....	TA.T.....V	..V..V....
BVDV Nose	A....D.DR.	L....F.K..	.....	IMD..KT...	.....T.A..	..G....L..	...I.....	.A.T.....V	..V.MV...M
BVDV NY'93	A....G.ER.	...V...K..	.....R..R	VDT..K....	...ATTVAE.	..GT...L..	...I.....	.A.T.....	F.V.A....L
BVDV Oregon C24v	A....D.DR.	L....F.K..	.....	ITD..KT...	.....T.A..	..G....L..	...I.....	.A.T.....V	..V.MV...M
BVDV 890	A....G.ER.	...V...K..	.....R...	VDT..K....	...ATTVAE.	..GT...L..	...I.....	.A.T.....	F.V.A....L
BVDV SD1	A....D.DR.	.....F.K..	.....	ITD..KT...	..I..T.A..	..G....L..	...I.....	.A.T.....V	....MV....
BDV C413	A....G.EK.	...V...K..	.....R...	VDT..K....	...ATTVAE.	..G....L..	...I.....	YA.T.....	F.VIA....L
BD78	A....G.EK.	...V...K..	.....R...	VDT..K....	...ATTVAE.	..R....L..	...I.....	.P.T.....	F.V.A....L
R2727	A....D.DR.	L....F.K..	.....	IMD..KTK..	.....T.A..	..G....L..	...I.....	.A.T.....V	..V.MI...M
H2121 Chamois-1	Q.....K.	...V...S..	.....	.DD.....	...S.KV...	.....LE.	...I..Y...	.....	..V.L....I
Gifhor	Q.....R.	.....	.....G...	.DQ..M....	...D.KV...	.....L..	...I.....	S.....	..V.....
X818	Q.....R.	...V.....	.....	.SV.....	...S..VD..	.....L..	.....	.....	..V.....
BD31	Q.....R.	.....	.....	.SE.....	...G..VD..	.....L..	.....	.....	..A.....
L83/84	.....R.	.....	.....	.SE.....	...G..VD..	.....L..	.....	.....	..V.....
BM01	Q.....R.	.....	.....R...	.....L....	...G.....	V.....	...I.....	.....	..V.....

FIG. 1. A multiple alignment of CSFV core proteins revealed the presence of highly conserved putative Cys residues (bold and underlined) among pestiviruses (CSFV, classical swine fever virus; BVDV, bovine viral diarrhea virus; BDV, border disease virus).

TABLE 1. CSFV E1 Cys-to-Ser mutant viruses rescued from SK6 cells and their virulence phenotype in infected swine

Rescued mutant virus	Substituted Cys residue(s)	Phenotype
E1ΔCys5v	5	Virulent
E1ΔCys20v	20	Virulent
E1ΔCys24v	24	Virulent
E1ΔCys94v	94	Virulent
E1ΔCys123v	123	Virulent
E1ΔCys171v	171	Virulent
–[a]	5/20	–
–	5/24	–
–	5/94	–
–	5/123	–
E1ΔCys5/171v	5/171	Virulent
–	20/24	–
–	20/94	–
–	20/123	–
E1ΔCys20/171v	20/171	Virulent
E1ΔCys24/94v	24/94	Attenuated
–	24/123	–
–	24/171	–
–	94/123	–
E1ΔCys94/171v	94/171	Virulent
–	123/171	–

[a] –, no virus rescued.

virus growth in swine macrophages included Cys171, suggesting that this residue plays a minor role in the process of formation of infectious viral particles.

Detection of E1-E2 heterodimers in infected SK6 cells. Since formation of E1-E2 heterodimers mediated by disulfide bridges is observed in lysates from cells infected with CSFV (22) and in mature CSFV virions (19), we tested for the presence of these dimers in SK6 cells infected with parental or recombinant viruses. Under nonreducing conditions, Western immunoblot assays using anti-E2 MAb WH303 showed that single or double Cys-to-Ser substitutions in E1 generally did not affect formation of E2 homodimers or E1-E2 heterodimers in infected SK6 cells, since the complexes are still formed and appear partially functional (Fig. 3A). Only lysates from cells infected with recombinant E1ΔCys24/94v showed changes in the proportions of homo-/heterodimers expressed. A similar pattern was observed when formation of homo- and heterodimers was detected with a polyclonal anti-E1 antibody (Fig. 3B). Infection with E1ΔCys24/94v leads to a shift in the ratio of homo-/heterodimers relative to parental virus infections, with an increased accumulation of E2 homodimers over E1-E2 heterodimers while proportions of E2 monomers remained unchanged. Additionally, it was observed that electrophoretic mobility of E1ΔCys24/94v E1-E2 heterodimers is slower than that of any of the other mutants or parental BICv.

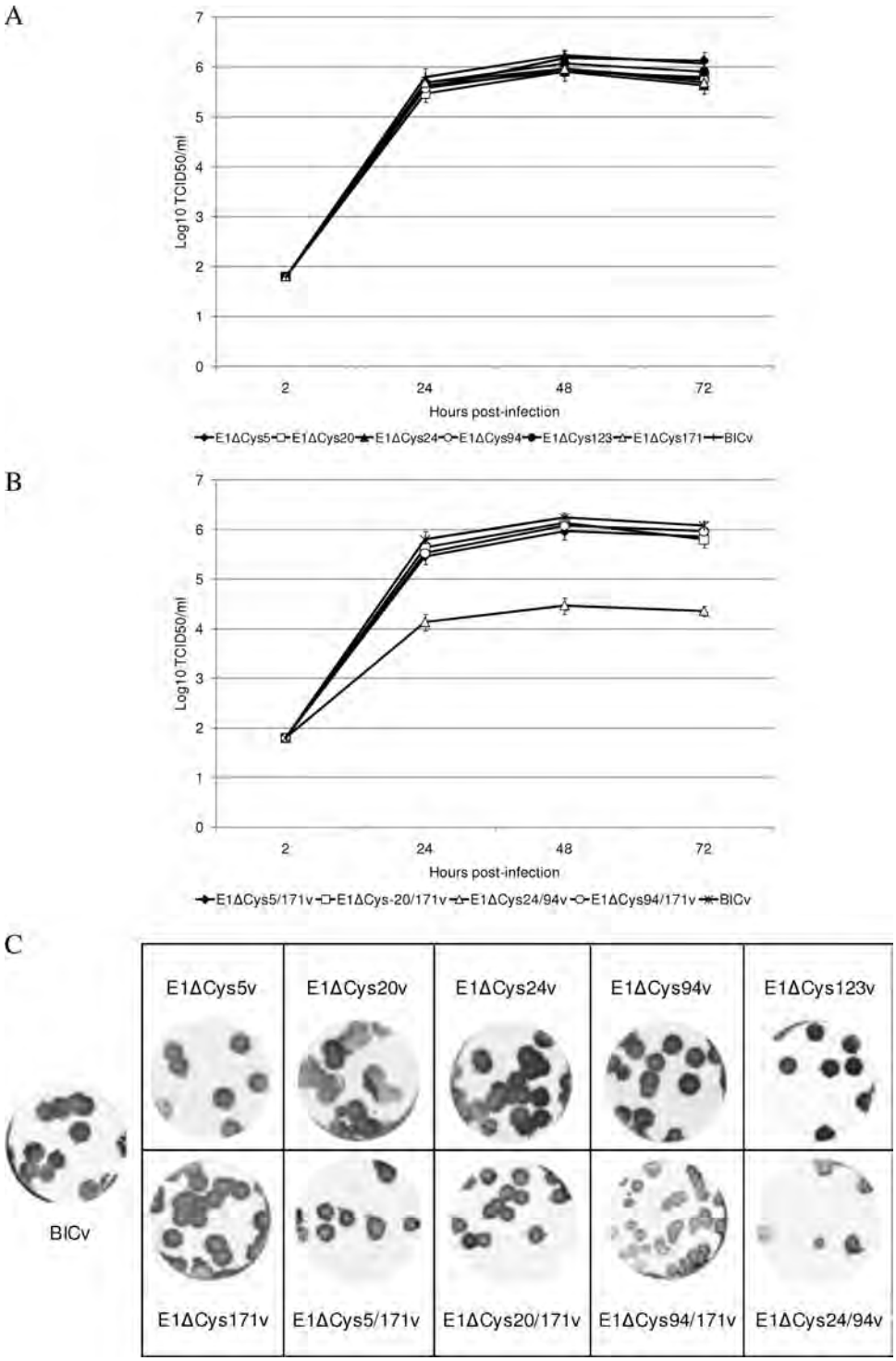

FIG. 2. *In vitro* growth characteristics of E1ΔCys mutant viruses relative to parental BICv. (A and B) Primary swine macrophage cell cultures were infected (MOI = 0.01) with each of the single (A) and double (B) E1ΔCys mutant viruses or BICv. Virus yields were calculated by titration at times after infection of SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection, $\geq \log_{10}$ 1.8 $TCID_{50}$/ml. (C) Foci of infection of single and double E1ΔCys mutant viruses and parental BICv were detected on SK6 cell monolayers by immunoperoxidase staining using anti-E2 MAb WH303.

This may happen with the E1-E2 heterodimer in the absence of critical disulfide bonds, causing its configuration to become less compact. We observed minor changes in the levels of expression of E1 and E2 proteins in SK6 cells infected with E1ΔCys24/94v relative to levels of expression observed in cells infected with BICv (Fig. 3C and D). Data confirm that Cys residues 24 and 94 are required for the proper formation of E1-E2 heterodimers.

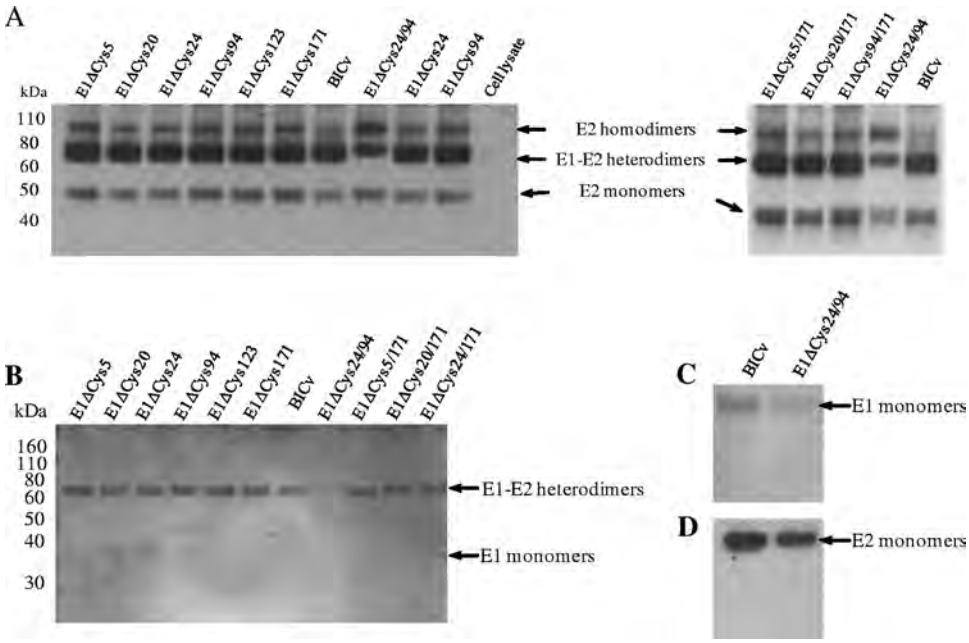

FIG. 3. Western blot analysis of SK6 cell lysates infected with E1ΔCys single and double mutant viruses. Detection of E1-E2 heterodimers with anti-E2 MAb WH303 (A) or with an anti-E1 rabbit anti serum (B) and monomeric forms of E1 (C) and E2 (D). SDS-PAGE was performed under nonreducing (A and B) or reducing (C and D) conditions.

Substitutions of Cys24 and Cys94 residues in E1 lead to virus attenuation. *In vivo* infections using viable single and double mutant E1 recombinant viruses (Table 1) were assessed in swine. Naïve pigs were intranasally inoculated and monitored for clinical disease using either BICv or a mutant virus. Single and double mutant viruses, with the exception of E1ΔCys24/94v, induced a clinical disease indistinguishable from disease induced by parental BICv (Table 2). Pigs infected with these viruses presented clinical signs of CSF starting at 3 to 5 dpi, with clinical presentation and severity similar to those observed in animals inoculated with BICv. Viremia, viral loads in nasal swabs and tonsils, and white blood cell counts were similar in animals inoculated with parental or recombinant viruses containing single (data not shown) or double (Fig. 4) Cys-to-Ser substitutions. Interestingly, pigs ($n = 8$) infected with E1ΔCys24/94v survived the infection and remained normal throughout the observation period (21 days) (Table 2 and Fig. 4). In all cases, partial nucleotide sequences of E1 protein from viruses recovered from infected animals were identical to those of stock viruses used for inoculation (data not shown). Viral loads in these animals were significantly lower than those observed in pigs inoculated with parental virus, indicating a rather limited replication of this recombinant virus harboring substitutions in E1 at Cys24 and Cys94, resulting in an attenuated phenotype. Additionally, it appears that Cys171 plays a considerable role in pathogenesis since animals infected with double mutant viruses E1ΔCys5/171v and E1ΔCys20/171v survived longer (3 and 7 days, respectively) than did animals infected with parental BICv (Table 2).

E1ΔCys24/94v induces protection against lethal CSFV challenge. The limited *in vivo* replication kinetics shown by

TABLE 2. Swine survival and fever response following infection with CSFV E1ΔCys mutants and parental BICv

Virus	No. of survivors/total no.	Mean time to death, days (SD)	Fever		
			No. of days to onset (SD)	Duration, days (SD)	Maximum daily temp, °C (SD)
BICv	0/6[a]	9.5 (1.3)	3.5 (0.57)	5.75 (0.95)	41.3 (0.18)
E1ΔC5v	0/2	9.5 (2.1)	3.5 (0.7)	6 (1.4)	41.3 (0.08)
E1ΔC20v	0/2	7.5 (0.7)	3.5 (0.7)	4 (0)	41.2 (0.54)
E1C24v	0/2	13.5 (0.7)	3.5 (0.7)	10 (1.4)	41.2 (0.07)
E1ΔC94v	0/2	9.5 (3.5)	3.5 (0.7)	6 (2.8)	41.2 (0.15)
E1ΔC123v	0/2	9.5 (0.7)	3.5 (0.7)	6 (0)	41.1 (0.07)
E1ΔC171v	0/2	9.5 (4.9)	5 (1.4)	4.5 (3.5)	41.1 (0.16)
E1ΔC5/171v	0/3	12.5 (0.7)	4 (0)	8.5 (0.7)	41.7 (0.71)
E1ΔC20/171v	0/3	16.5 (0.7)	5 (1.4)	11.5 (0.7)	41.1 (0.08)
E1ΔC94/171v	0/3	11 (0)	4 (0)	7 (0)	41.4 (0.22)
E1ΔC24/94v	8/8[a]				39.8 (0.26)

[a] Values represent two different but identical experiments.

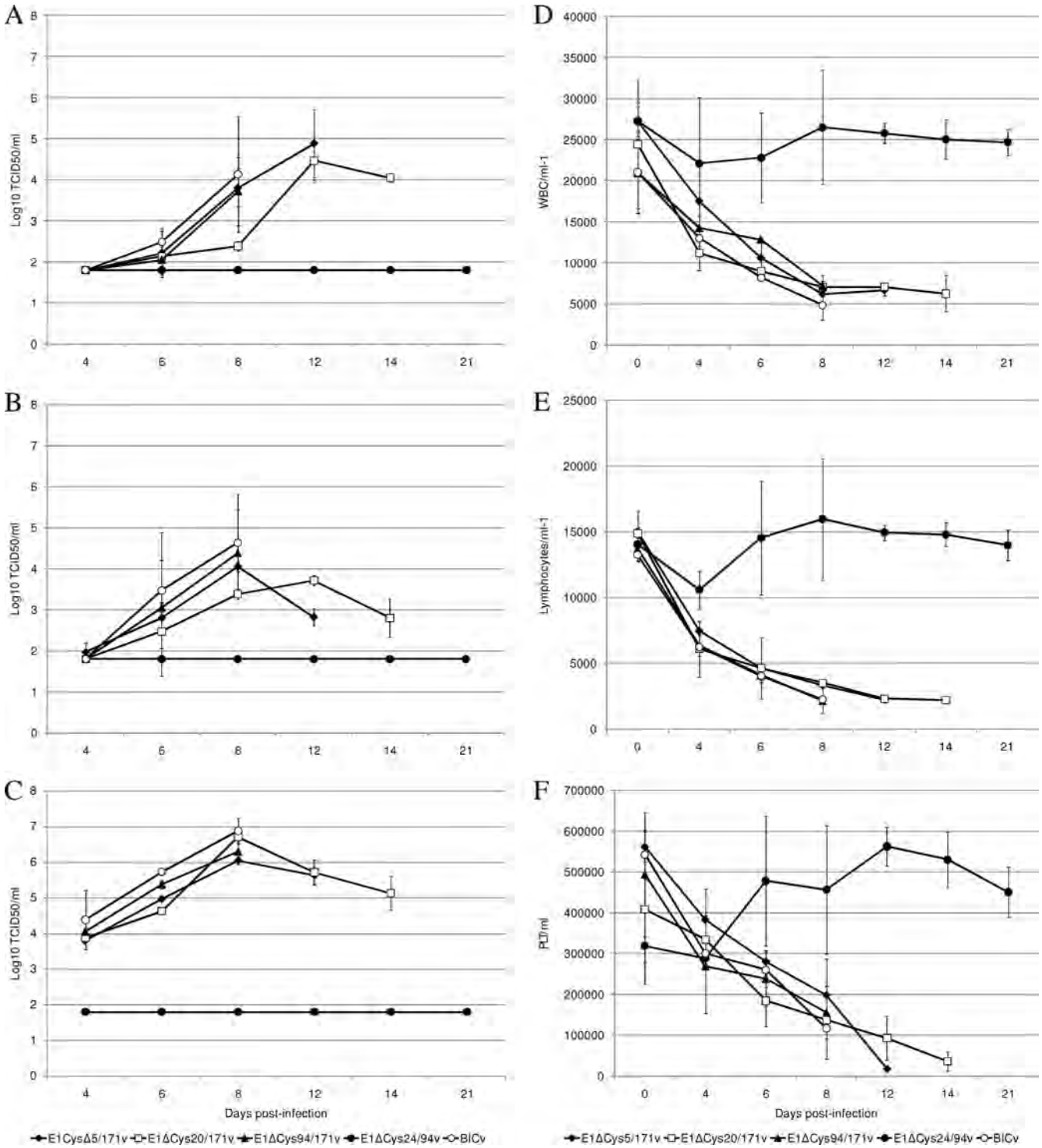

FIG. 4. Viral loads and hematologic changes detected in pigs inoculated with double E1ΔCys mutant and parental viruses. (A to C) Virus titers detected in nasal swabs (A), tonsil scrapings (B), and blood (C) after infection with E1ΔCys5/171v, E1ΔCys20/171v, E1ΔCys94/171v, E1ΔCys24/94v, or parental BICv. Each point represents the mean $\log_{10}$ $TCID_{50}$/ml and standard deviations from at least two animals. Sensitivity of virus detection, $\geq\log_{10}$ 1.8 $TCID_{50}$/ml. (D to F) White blood cell (WBC) (D), lymphocyte (E), and platelet (PLT) (F) counts after infection.

E1ΔCys24/94v is similar to that observed with CSICv (9), a live-attenuated vaccine virus that induces protection against BICv. In order to assess the ability of this double mutant to induce protection against CSFV, pigs were inoculated with E1ΔCys24/94v and challenged at 3 dpi (early) and at 28 dpi (late) with virulent BICv. Pigs were protected against an early and late challenge with BICv (Table 3 and Fig. 5). Mock-vaccinated control pigs developed anorexia, depres-

TABLE 3. Swine survival and fever response in E1ΔCys24/94v-infected animals after the challenge with parental virulent BICv

Challenge group	No. of survivors/total no.	Mean time to death, days (SD)	Fever		
			No. of days to onset (SD)	Duration, days (SD)	Maximum daily temp, °C (SD)
Mock	0/4	10.5 (1.5)	4.5 (0.57)	5.75 (0.95)	41.2 (0.19)
E1ΔCys24/94v, 3 dpi	3/4	10[a]	4[a]	6[a]	40.3 (0.57)
E1ΔCys24/94v, 28 dpi	7/7	0	0	0	39.7 (0.51)

[a] Data correspond to the only animal out of the four inoculated that presented CSFV-related symptoms.

sion, and fever by 4 days postchallenge (dpc) and a marked reduction in circulating leukocytes and platelets by 4 dpc (Fig. 5). Pigs died or were euthanized *in extremis* by 9 dpc (Table 2). Pigs inoculated with E1ΔCys24/94v exhibited partial protection by 3 dpi. Three out of four pigs survived infection and remained clinically normal, without significant changes in their hematological values (Fig. 5). All animals challenged at 28 days post-E1ΔCys24/94v infection were also protected, remaining clinically normal, without alterations of hematological profiles (Fig. 5). Virus was detected in low titers from blood, nasal swabs, and tonsil scrapings at 4, 6, and 8 dpc in some animals infected with E1ΔCys24/94v and challenged at 3 dpi (Fig. 5). BICv was undetectable in clinical samples obtained from any E1ΔCys24/94v-infected pigs that were challenged at 28 dpi.

Altogether, these results suggest that Cys24 and Cys94 are not critical for E1-E2 heterodimer formation in infected cells but that they have an effect on the overall rate of formation of these dimers that ultimately affects virus growth *in vitro* and *in vivo*, leading to a complete attenuation of the parental virus.

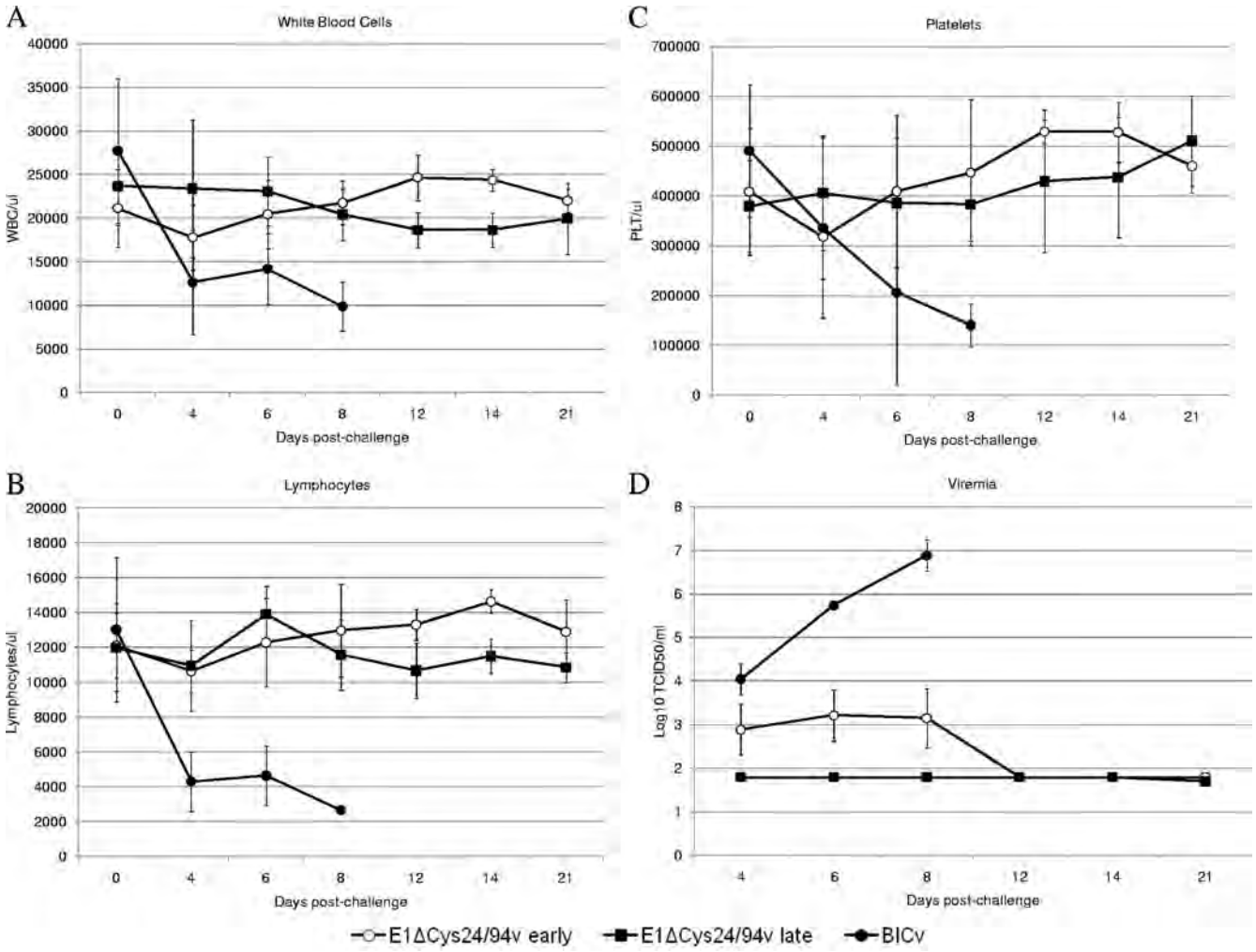

FIG. 5. Hematologic changes (A, B, and C) and viremia (D) detected in pigs inoculated with attenuated E1ΔCys24/94v and challenged with virulent BICv at 3 (early) or 28 (late) days postinfection. Values from four animals at each time point.

DISCUSSION

Cysteine bridges seem to play an important role in the formation of E1-E2 heterodimers (19, 22) and consequently impact the functioning of these glycoproteins during infection. We therefore performed a mutational analysis of the six cysteine residues in the E1 envelope protein within the context of a full-length infectious CSFV cDNA clone and analyzed infectivity of rescued recombinant viruses in primary swine macrophage cultures and in swine.

Substitution of each cysteine residue at positions 5, 20, 24, 94, 123, and 171 to serine in BICv E1 yielded viable mutant viruses. Introduced mutations did not affect formation of E1-E2 heterodimers in infected cells or virus infectivity either *in vitro* or *in vivo*, suggesting that more than one disulfide link may contribute to the formation of E1 and E2 heterodimers and to the functions of these proteins. A similar observation has been made with other viruses; a single substitution of conserved Cys171 to Ser (Fig. 1), located in the putative transmembrane domain of E1 protein, in the context of a BVDV E1- and E2-pseudotyped vesicular stomatitis virus, did not preclude formation of heterodimers or virus infectivity (13). It is possible that in these single-site mutants other interactions may contribute to formation of heterodimers and overall efficiency of the viral infection. Ronecker et al. (13) observed that substitutions of conserved Lys174Ala and Arg177Ala in BVDV E1 (Fig. 1) significantly reduced the formation of heterodimers in cells infected with pseudotyped viruses, indicating that these charged amino acids in the transmembrane domains of BVDV E1 also contribute to the interaction with E2.

The precise mapping of E1 Cys residues involved with active CSFV infection could not be determined in this study since 11 out of 15 possible combinations of double substitutions to Ser (Table 3) were deleterious, indicating that all these changes most probably affected morphogenesis of the virion. However, we also observed that three out of four double Cys mutant viruses rescued (E1ΔCys24/94, E1ΔCys5/171, E1ΔCys20/171, and E1ΔCys94/171) involve substitutions at Cys171, suggesting that this residue is not critical for virus infection. When inoculated into swine, these mutants retained the same capability of causing severe disease in swine as did parental BICv, showing that *in vivo* E1 functions are retained and not influenced by the lack of Cys residues at position 171. Conversely, no viral progeny was obtained when combined substitution involved Cys123, suggesting a critical role of this residue in the formation of mature virions.

The combined substitutions of Cys24 and Cys94 were not deleterious but significantly reduced BICv infectivity of swine macrophages and the ability of the virus to spread in SK6 cells. The decreased efficiency of viral infection by E1ΔCys24/94v corresponds with an impaired ability of mutated E1 to form heterodimers with E2 in infected cells, where a shift toward the accumulation of homodimers over heterodimers was observed. This finding suggests a contribution of these residues to form disulfide bonds with E2 that when modified may exert subtle conformational changes within E1 that impact active viral infection. Significant secondary structure changes in E1 due to Cys substitutions were not predicted (data not shown).

Our results suggest that Cys24 and Cys94 mutations affect BICv infectivity in swine. Different from the acute fatal disease induced by virulent BICv, infections caused by E1ΔCys24/94v were subclinical in swine and characterized by decreased viral loads in target organs and reduced virus shedding. No reversions to wild-type genotype were observed in viruses isolated from these animals. Attenuation of E1Cys24/94v in pigs could conceivably involve some aspect of virus attachment and/or efficiency of entry into critical target cells *in vivo* or altered trafficking of the virus within infected host cells. The effective protective immunity elicited by E1Cys24/94v suggests that modified E1 could be used for the development of live-attenuated vaccines. Similar studies have demonstrated that mutation or deletion of Cys171, one out of nine cysteine residues found in CSFV E^{rns} protein that is involved in the formation of homodimers via disulfide bonds, yielded infectious viruses lacking the capability to form homodimers in infected SK6 cells (18). These CSFV E^{rns} mutants were attenuated in pigs and induced a significant neutralizing antibody response in these animals.

In summary, our studies determined that substitutions of individual Cys residues in glycoprotein E1 are not essential for *in vivo* infectivity, exhibiting virulence characteristics similar to those of parental virus in the natural host. Data suggest that Cys24, Cys94, and Cys123 play major roles in E1 function, likely affecting virion morphogenesis by interacting with E2 protein. An improved understanding of the genetic basis and mechanisms of virus infection and virus virulence will permit rational design of efficacious biological tools for controlling CSF. Additionally, double Cys-to-Ser substitutions at positions 24 and 94 produce complete attenuation of the virulent Brescia isolate.

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Chemical disinfection of high-consequence transboundary animal disease viruses on nonporous surfaces

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ABSTRACT

Disinfection is a critical part of the response to transboundary animal disease virus (TADV) outbreaks by inactivating viruses on fomites to help control infection. To model the inactivation of TADV on fomites, we tested selected chemicals to inactivate Foot and Mouth Disease virus (FMDV), African Swine Fever virus (ASFV), and Classical Swine Fever virus (CSFV) dried on steel and plastic surfaces. For each of these viruses, we observed a 2 to 3 log reduction of infectivity due to drying alone. We applied a modified surface disinfection method to determine the efficacy of selected disinfectants to inactivate surface-dried high-titer stocks of these three structurally different TADV. ASFV and FMDV were susceptible to sodium hypochlorite (500 and 1000 ppm, respectively) and citric acid (1%) resulting in complete disinfection. Sodium carbonate (4%), while able to reduce FMDV infectivity by greater than 4-log units, only reduced ASFV by 3 logs. Citric acid (2%) did not totally inactivate dried CSFV, suggesting it may not be completely effective for disinfection in the field. Based on these data we recommend disinfectants be formulated with a minimum of 1000 ppm sodium hypochlorite for ASFV and CSFV disinfection, and a minimum of 1% citric acid for FMDV disinfection.

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1. Introduction

TADV are high-consequence pathogens that can cause high morbidity and mortality in livestock resulting in severe economic losses stemming from quarantines and loss of export business [1]. While the prevention of an accidental or intentional release of these viruses is most important, once an outbreak has occurred, disinfection is critical both to stop further dissemination of infection and to bring contaminated facilities back into production. Despite the importance of this work little information is available about the efficacy of disinfectants to inactivate TADV, especially on surfaces such as those to be encountered in farm settings (e.g. walls, floors, farm equipment, etc.).

Foot and mouth disease virus (FMDV) is one of the most contagious infectious agents known due to its rapid replication cycle and its stability in the environment [2]. FMDV is non-enveloped and it maintains infectivity in the environment long after drying (reviewed in [3]), thus fomites are a major mechanism of virus spread. For example, the difficulties associated with contamination of fomites in affected premises during and after the 2001 FMDV outbreak in the UK have been well documented [4,5]. CSFV and ASFV are both enveloped viruses and although they are less stable in the environment than FMDV, in both cases fomites are potential mechanisms of virus spread (reviewed in [6,7]). Indeed, contaminated transport vehicles are thought to have transmitted CSFV to an uninfected swine herd during the Netherlands outbreak in 1997 [8].

FMDV is known to be very sensitive to low pH [9] and thus acid-containing disinfectants have been used successfully to clean up after FMDV outbreaks; 0.2% Citric Acid is recommended by the World Organization for Animal Health (OIE) for use against FMDV [10]. In contrast ASFV is quite stable and will survive over a wide range of pH. Various disinfectants have been used during ASFV and CSFV outbreaks in recent decades, including 2% sodium hydroxide [11,12].

Most of the published disinfection data for these viruses has been generated in a liquid format. Disinfection of viruses in liquids can provide basic information about virus sensitivity to various chemicals. However, in the field virus is shed into the environment, thus disinfectants are applied to surfaces potentially contaminated with dried virus [13]. Quantitative carrier tests (QCT) have been

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used as a model to determine the efficacy of disinfectants for various microbes dried on a wide range of surfaces; this type of assay has been described in ASTM E1053 [14] and by Sattar and coworkers [15]. However, methods for modeling enveloped virus disinfection on nonporous surfaces vary, resulting in data that is difficult to compare (reviewed in [16]). A key problem with surface disinfection is that many enveloped viruses tend to lose significant infectivity during the drying process under both environmental and laboratory conditions, resulting in a very small difference between the virus that remains intact after drying and the overall limit of virus detection. The addition of calf serum or other proteins can be used to stabilize virus in the inoculum during drying and the use of higher titer stocks can increase the resultant titer after drying [17]. While potentially solving issues specific to a particular virus, modifications to the composition of the inoculum may change the parameters of the assay, leading to an inaccurate assessment of disinfectant efficacy (reviewed in [18]).

Numerous commercial disinfectants for some high-consequence animal disease viruses have been approved by the US Environmental Protection Agency (EPA); however, there is a lack of published carrier test chemical disinfection data for many TADV. In this manuscript, we present a method for nonporous surface disinfection that takes into account the problems of disinfection with enveloped viruses. This method allows for large inoculum volume but limits the downstream volumes of disinfectant and neutralizer to prevent the loss of detectable virus due to dilution. Here we applied this method to determine the efficacy of selected disinfectants to inactivate high-titer stocks of three structurally different high-consequence TADV.

2. Methods

2.1. Cells and viruses

FMDV strain A24 stocks were generated in BHK-21 cells (ATCC# CCL-10). CSFV strain Brescia and the Swine Kidney cell line SK6 were obtained from Dr. Manuel Borca (PIADC). ASFV strain BA71/v was obtained from the PIADC virus repository and grown in Vero cells (ATCC# CCL-81).

2.2. Virus stock production

Briefly, cells were infected at an MOI of 0.01 PFU/cell in 850 cm^2 roller bottles and incubated at 37 °C until either 100% cytopathic effect was observed (FMDV and ASFV) or 5 days post infection (CSFV). To harvest the FMDV stocks, the infected cell supernatants were clarified by centrifugation, aliquoted and stored at −70 °C prior to use. For CSFV and ASFV stocks, the infected cells were scraped from the roller bottles and centrifuged at low speed to remove the medium. The cell pellets were resuspended in 5 ml of fresh media and subjected to 2 cycles of freezing at −70 °C and thawing at 37 °C, then sonicated 3 times for 30 s each on ice. The cell debris was removed by clarification and the supernatants were aliquoted and stored at −70 °C prior to use.

2.3. Disinfectants and neutralizers

All tested concentrations of sodium hypochlorite (Baker) were neutralized with Fluid Thioglycolate Medium (FTM, Difco). All concentrations of citric acid (Acros Organics) were neutralized with sodium bicarbonate (Invitrogen) except 2% citric acid, which was neutralized with 1.25 M sodium hydroxide (Ricca Chemical). 4% Sodium carbonate (Ricca Chemical) was neutralized with 0.025 M sodium citrate pH 1.1. All disinfectants were diluted in 400 ppm calcium carbonate to simulate hard water conditions. A control consisting of a 1:1 mixture of disinfectant and neutralizer was tested for cytotoxic effects in each disinfection assay described in section 2.4.

2.4. Disinfection assay

This protocol is a modification of ASTM E1053: Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces [14]. Briefly, virus stocks were diluted in 1X phosphate buffered saline (PBS). The final concentration of calf serum in the virus inoculum was 1%. 100 μl of this mixture was pipetted on the surface coupons, either stainless steel base molds (Fisher Scientific #15182505C) or non-tissue culture treated polystyrene 6-well plates (Falcon #351146). ASFV and FMDV were dried at 30 °C in a static temperature incubator (Incufridge, Revolutionary Science). CSFV was dried at ambient temperature (20 °C–24 °C) in a biosafety cabinet with the lights off. Once dried, virus was exposed to 500 μl of the disinfectant for the indicated contact time at 22 °C in the Incufridge. At the end of the contact time, 500 μl of the appropriate neutralizer was added and the dried virus was scraped into the mixture, which was then added to 1 ml of cell culture media. In each experiment, one control coupon with dried virus was exposed to 500 μl of a 1:1 mixture of the disinfectant and neutralizer (recovery control) and one coupon without dried virus was exposed to 500 μl of cell culture media (surface cytotoxicity control). All control coupons were incubated at 22 °C for the maximum indicated contact time simultaneously with the coupons exposed to disinfectant. After the contact time was complete, the recovery control received another 500 μl of the neutralizer:disinfectant mixture prior to scraping, and then the entire mixture was added to 1 ml of cell culture media after scraping.

These post-disinfection samples were serially diluted in the appropriate media and titrated on susceptible cells in 96-well plates. FMDV infection was identified by the presence of destructive cytopathic effects 2 or 3 days post infection. ASFV was identified by the formation of plaques after 5–7 days post infection. CSFV was detected by fixing the cells 3–5 days after infection in 50% acetone/50% methanol and immunohistochemical staining with a monoclonal antibody to CSFV as described in Risatti et al. [19]. The titer of the recovered virus was calculated using the Spearmann-Karber endpoint titration method [20]. Because of virus dilution and the number of replicate wells infected per dilution, the lower limit of detection in this assay is 0.8 $\log_{10}$ $CCID_{50}$.

3. Results

3.1. Recovery of dried viruses from nonporous surfaces

In order to be registered as a disinfectant, EPA requires at minimum a 4-log reduction in virus titer in treated samples [21]. Since the lower limit of virus detection in our assay is 0.8 $\log_{10}$ $CCID_{50}$, it was necessary to confirm that after drying, a minimum of 4.8 $\log_{10}$ $CCID_{50}$ could be recovered in the assay controls. High-titer stocks of FMDV, CSFV and ASFV were diluted in PBS then dried on stainless steel and plastic surfaces, according to the methods. After drying, the virus was resuspended in PBS then diluted in cell culture media and titrated in parallel with an aliquot of the undiluted inoculum. For FMDV and ASFV approximately 2 logs of virus infectivity were lost due to drying on either steel or plastic surfaces (Fig. 1). Considerably more virus was lost due to drying in the case of CSFV, with almost a 3-log reduction observed on both surfaces. This occasionally resulted in titers of less than 4.8 $\log_{10}$ $CCID_{50}$ for CSFV that invalidated the results of some experiments due to insufficient virus recovery (Fig. 1).

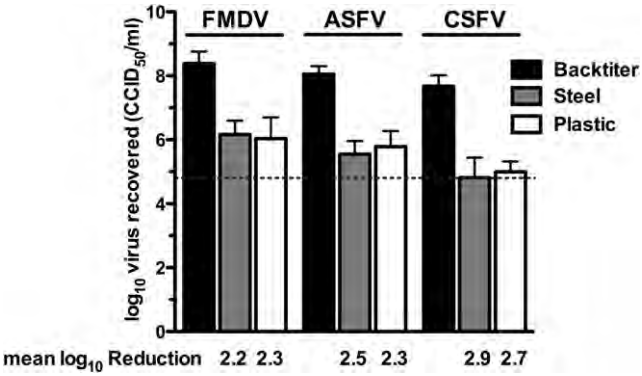

Fig. 1. Recovery of test viruses from steel and plastic surfaces. Virus was dried on the on either steel or plastic surfaces, resuspended and titrated. The backtiter was a direct titration of an aliquot of the virus inoculum that was not dried. The mean $\log_{10}$ reduction is the comparison of the virus recovery on each surface relative to that of the backtiter. Error bars indicate the mean of at least 5 experiments, ± SD. The dashed line at 4.8 indicates the minimum recovery required to achieve a 4-log reduction by disinfectant.

3.2. Dose response of disinfection

Dose-response assays were conducted to determine effective concentrations of disinfectant for each dried virus on the test surfaces. Various concentrations of disinfectants were applied to the dried virus preparations for a 10-min contact time and the titer of remaining infectious virus after neutralization was determined. Although all three viruses were effectively inactivated by sodium hypoclorite, ASFV was completely inactivated at 4–5 fold lower concentrations than FMDV or CSFV (Fig. 2). Significant differences between the surfaces were not apparent with any of the viruses tested. Interestingly, while CSFV was the least stable due to drying (Fig. 1), the infectious portion of the dried virus preparation had similar resistance to sodium hypochlorite as FMDV.

3.3. Comparison between sodium hypoclorite and citric acid

Since citric acid has been used in the field for FMDV disinfection, we wanted to compare the required effective hypochlorite concentration with that of citric acid. Disinfection assays were carried out on multiple days with multiple replicates to ensure consistency of complete high-titer stock disinfection. Concentrations were included based on those from dose–response experiments (Fig. 2) that ensured complete disinfection in 10 min. Table 1 shows the mean log reduction between recovered dried virus and the disinfection samples for each virus on each surface with each concentration of disinfectant. For FMDV and ASFV, similar concentrations of citric acid were capable of reducing virus by greater than 4-log units on both plastic and steel surfaces. However citric acid was unable meet this criterion for CSFV on either surface even at 2% concentration. ASFV was completely inactivated by 500 ppm hypochlorite, but 1000 ppm hypochlorite was required for CSFV and FMDV.

3.4. Time-course of disinfection

Ten minutes has been a standard contact time in many disinfection protocols, but in many cases this is in excess and may lead to corrosion of surfaces. To determine the kinetics of sodium hypochlorite disinfection, assays were performed with various contact times. Sodium hypochlorite concentrations were used based on those from Table 1 that ensured complete disinfection in 10 min Fig. 3 demonstrates that 1000 ppm sodium hypochlorite completely disinfected FMDV on both surfaces by 4 min. Similarly, 500 ppm hypochlorite completely disinfected ASFV on both surfaces by 6 min. While 1000 ppm hypochlorite was able to completely disinfect CSFV on steel surfaces by 6 min, the full 10 min contact time was required on the plastic surface for complete CSFV disinfection.

Sodium carbonate (soda ash) has commonly been used as a disinfectant for transport vehicles moving between FMDV-endemic and non-endemic countries. While it is not registered by the EPA for virus disinfection, it has been given an exemption by USDA [22] and is recommended by the AUSVETPLAN [23] and FAO [24]for FMDV disinfection. To our knowledge, published data demonstrating the effectiveness of sodium carbonate against FMDV dried on surfaces does not exist. Because sodium carbonate has been demonstrated not to be highly effective against surface-dried avian influenza [25] and ASFV has been shown to be resistant to high pH [26], ASFV was included as a negative control in our experiments. While a greater than 4-log reduction of infectious FMDV was observed after sodium carbonate disinfection, the treatment did not completely disinfect the dried inoculum (Fig. 4).

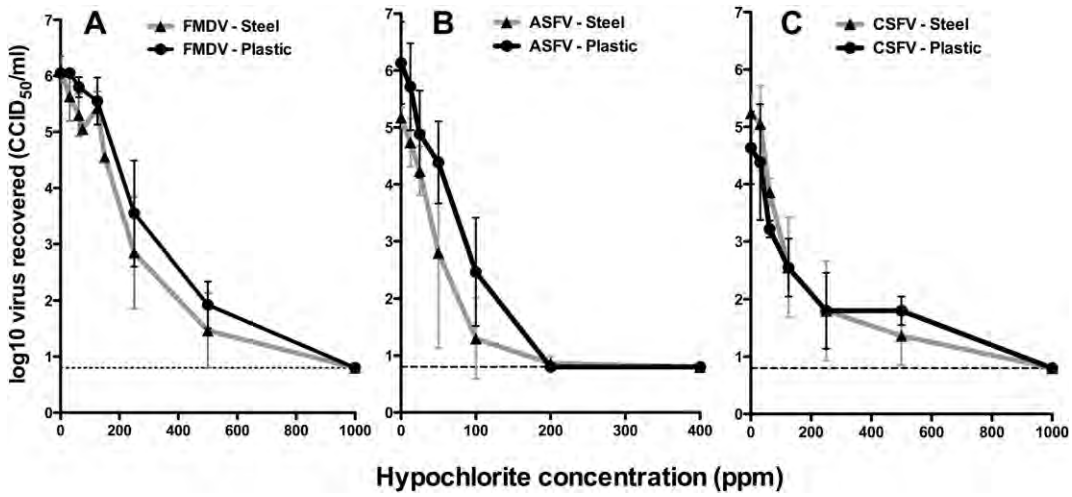

Fig. 2. Effects of disinfectant concentration on dried virus recovery. FMDV (A), ASFV (B), CSFV (C) were dried on stainless steel or plastic coupons, exposed to the indicated concentration of sodium hypochlorite for 10 min, followed by neutralization with FTM and virus titration. Each data point is the mean of at least 3 experiments, ± SD. Dashed line indicates lower limit of virus detection.

Table 1
Log_{10} Reduction of Viruses by Chemical Disinfectants[a].

Virus	Surface	500 ppm Hypochlorite	1000 ppm Hypochlorite	0.5% Citric Acid	1.0% Citric Acid	2.0% Citric Acid
FMDV	Steel	2.63 ± 0.53 $n = 2(4)$	5.55 ± 0.47 $n = 8(12)$	4.7 ± 0.88[b] $n = 2(4)$	5.0 ± 0.35 $n = 5(10)$	n.d.
	Plastic	2.38 ± 0.18 $n = 2(4)$	5.6 ± 0.8 $n = 5(10)$	4.13 ± 0.72[b] $n = 2(4)$	5.1 ± 0.49 $n = 7(10)$	n.d.
ASFV	Steel	4.8 ± 0.46 $n = 9(9)$	n.d.	n.d.	4.8 ± 0.11 $n = 5(9)$	n.d.
	Plastic	4.75 ± 0.61 $n = 6(9)$	n.d.	n.d.	4.88 ± 0.38[b] $n = 4(8)$	5.13 ± 0.42 $n = 8(9)$
CSFV	Steel	n.d.	4.4 ± 0.35 $n = 8(8)$	n.d.	2.38 ± 0.35 $n = 2(4)$	3.25 ± 0.35 $n = 2(4)$
	Plastic	n.d.	4.25 ± 0.18 $n = 5(8)$	n.d.	n.d.	3.38 ± 0.53 $n = 2(4)$

n.d.: Not done.
[a] Log_{10} reduction by indicated disinfectant ± standard deviation. Contact time was 10 min n = number of individual disinfection assays (total number of disinfection replicates).
[b] Disinfection did not reduce virus to undetectable levels.

As expected, 4% sodium carbonate was not able to disinfect dried ASFV by the 4-log standard.

4. Discussion

We present here a modified quantitative carrier test assay to test the efficacy of disinfectants against three high-consequence TADV. Because of the greater loss due to drying of enveloped viruses, we kept inoculum volumes high and volumes of disinfectant and neutralizer low to increase the lower limit of detection. The crux of this modification is effective neutralization that ensured neither virucidal nor cytotoxic effects from the disinfectant/neutralizer mixture. High-titer stocks were used to increase the virus concentration to enhance virus stability in lieu of the addition of stabilizers such as bovine serum. While this was mostly successful for both enveloped viruses in this study, in the case of CSFV the data from some disinfection assays was rejected because less than 4.8 logs of virus was recovered in the controls.

Using this method, neutralization was achieved with a volume equal to that of the disinfectant. Maintaining the virus in a small volume increases recovery after drying, but effective neutralization of highly concentrated disinfectants becomes more difficult. Therefore, this method might not be appropriate for testing classes of disinfectants that require neutralization by large dilutions. For instance, disinfectant trials of a surfactant using this method resulted in incomplete neutralization and considerable cytotoxicity in downstream cell culture detection assays (data not shown).

To our knowledge, this is the first study reporting ASFV surface decontamination with liquid chemicals. Heckert and coworkers [27] reported results using vapor phase hydrogen peroxide to disinfect ASFV and CSFV in liquid suspension and dried on glass and steel surfaces. They observed less than a two-log loss of infectivity by drying ASFV and CSFV without disinfection. While this loss was slightly lower than that observed in our experiments, the inoculum used in their studies had a greater concentration of calf serum (5% vs. 1% in this manuscript).

One remarkable finding was the fact that 2% citric acid was unable to completely disinfect dried CSFV in our experiments. Unlike FMDV, which is very unstable in low pH conditions, it is known that CSFV is less susceptible to acid treatment [28]. It has been suggested by Krey and coworkers [29] that this may be due to the possibility that acid-induced conformational changes in the pestivirus glycoproteins that occur during fusion are reversible and therefore virus regains infectivity when it is incubated at physiological pH with susceptible cells.

Sodium carbonate is widely recommended as a disinfectant for FMDV-contaminated surfaces in situations that citric acid might damage sensitive equipment. While greater than a 4-log reduction was observed with sodium carbonate against FMDV, in half of the experiments with this chemical we detected infectious virus after the disinfection process. The observation that ASFV was not completely disinfected by sodium carbonate is not particularly surprising since ASFV has been shown to be stable at high pH [26] and avian influenza was also resistant to sodium carbonate [25]. Because sodium carbonate is not recommended by livestock health agencies for CSFV surface disinfection, we did not test sodium carbonate against CSFV.

The data presented here extend published liquid-format disinfection data for high-consequence TADV to surface disinfection. One area of contention is that we were unable to achieve complete

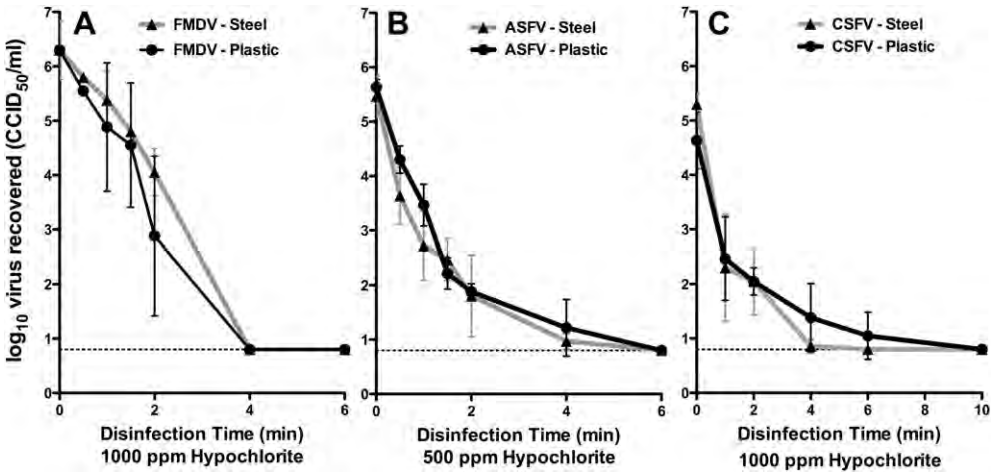

Fig. 3. Effects of shortened contact time on dried virus disinfection. FMDV (A), ASFV (B) or CSFV (C) were dried on stainless steel or plastic coupons then exposed to the 1000 ppm (A and C) or 500 ppm (B) sodium hypochlorite for the indicated contact time, neutralized with FTM and titrated. Each data point is the mean from at least 3 experiments, ± SD. Dashed line indicates lower limit of virus detection.

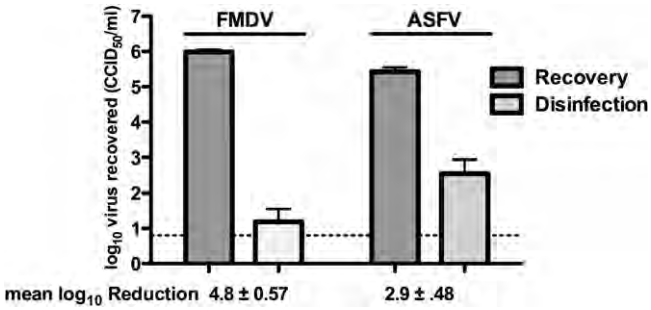

Fig. 4. Sodium carbonate disinfection of FMDV and ASFV. The indicated viruses were dried on steel coupons then either recovered in a 1:1 mixture of sodium carbonate and citrate buffer or disinfected with 4% sodium carbonate for 10 min at 22 °C. Error bars indicate the mean of 8 replicates in 4 experiments (FMDV) or 4 replicates in 2 experiments (ASFV). The mean log reduction due to disinfection compared to the recovery is indicated ± SD. Dashed line indicates lower limit of virus detection.

disinfection of dried FMDV with 0.5% citric acid (Table 1), indicating that the 0.2% citric acid recommended by OIE [9] may not always be sufficient to completely disinfect dried FMDV in the field. Our data indicates that the use of citric acid is not effective for CSFV disinfection and that low concentrations of sodium hypochlorite are effective for ASFV disinfection.

There are many hurdles to successful disinfection testing of enveloped viruses; each virus will have its own specific difficulties in optimizing high-titer stock production and structural differences among virus families will affect survival after drying. Once these issues are resolved, this disinfection system can be used for these viruses and other chemicals. This system can also be easily modified to use other surfaces such as wood, glass or rubber. A future goal of this work is to more closely model the fomites found in the environment by using FMDV-infected cow secretions and ASFV-infected pig blood as inocula to dry and disinfect.

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REVIEW

Evidence of Activation and Suppression during the Early Immune Response to Foot-and-Mouth Disease Virus

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Summary

Foot-and-mouth disease virus causes a serious disease of livestock species, threatening free global trade and food security. The disease spreads rapidly between animals, and to ensure a window of opportunity for such spread, the virus has evolved multiple mechanisms to subvert the early immune response. The cycle of infection in the individual animal is very short, infection is initiated, disseminated throughout the body and infectious virus produced in <7 days. Foot-and-mouth disease virus has been shown to disrupt the innate response *in vitro* and also interacts directly with antigen-presenting cells and their precursors. This interaction results in suboptimal immune function, favouring viral replication and the delayed onset of specific adaptive T-cell responses. Detailed understanding of this cycle is crucial to effectively control disease in livestock populations. Knowledge-based vaccine design would specifically target and induce the immunological mechanisms of early protection and of robust memory induction. Specifically, information on the contribution of cytokines and interferon, innate immune cells as well as humoral and cellular immunity can be employed to design vaccines promoting such responses. Furthermore, understanding of viral escape mechanisms of immunity can be used to create attenuated viruses that could be used to develop novel vaccines and to study viral pathogenesis.

Introduction

Infection of susceptible livestock species with foot-and-mouth disease virus (FMDV) is characterized by the rapid onset of clinical signs within 2–5 days of exposure. Clinical signs of disease include fever, malaise and the development of vesicles on the coronary bands of the feet, in the mouth and on the tongue and teats. Viraemia is detectable in the same time frame and resolves very quickly, as do clinical signs, by which time an infected individual can have secreted enormous amounts of virus into the environment and infected countless other animals. To achieve such a rapid takeover of the host, FMDV must manipulate the early immune response to ensure a window of opportunity in which to replicate and spread, before the onset of effective adaptive immunity.

Understanding the host/pathogen interaction and the contributions of innate versus adaptive immune responses has become a central topic in FMDV research. An improved knowledge of the role of the immune response in disease progression and pathology is key to both understanding transmission between animals and the rational design of intervention strategies.

In this review, we will summarize current knowledge of the interplay between FMDV and the host immune system early in infection, i.e. within 48 h of onset of viraemia. We will aim to identify the key viral and cellular components of this interaction, and areas for further

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research and of potential interest for vaccine development.

FMDV Manipulates Target Cells to Subvert Effective Immunity

The interaction of FMDV with the host begins via its infection of epithelial cells. Following binding to its cellular receptor, FMDV is endocytosed, and its genetic material enters the cell cytoplasm. The single strand of viral RNA genome encodes the capsid proteins and a number of non-structural (NS) proteins. The NS proteins are required not only for translation and replication of the genome but also for hijacking the cellular machinery of the host cell to synthesize progeny virus and subvert the immune response.

A number of FMDV proteins have specific functions that include inhibition of the innate response of infected cells. The leader protein (L^{pro}) is a papain-like proteinase, which initially functions to cleave itself from the viral polyprotein (Strebel and Beck, 1986; Kleina and Grubman, 1992). It then cleaves the cellular translation initiation factor eIF4G, causing shut-off of host cap-dependent mRNA translation while leaving intact the IRES-dependent translation mechanisms used by the virus (Devaney et al., 1988; Kirchweger et al., 1994). Further studies revealed that L^{pro} regulates type I interferon (IFN) production, not only at the level of translation (Chinsangaram et al., 1999) but also by limiting transcription of IFN-β (de Los Santos et al., 2006). *In vitro* studies revealed that FMDV infection results in L^{pro}-dependent degradation of nuclear factor κ-B (NF-κB), which affects transcription of IFN and many other factors involved in the innate immune response (de Los Santos et al., 2007; Zhu et al., 2010). While the precise mechanism of L^{pro}'s effects on NF-κB is undefined, nuclear localization and retention of L^{pro} is required; mutation of a conserved motif affecting L^{pro} nuclear retention rendered an FMDV strain attenuated both *in vitro* and *in vivo* (de los Santos et al., 2009). L^{pro} has also been shown to degrade IFN-regulatory factor 3/7 *in vitro* (Wang et al., 2010), which could potentially synergize with its effects on NF-κB in terms of immune disruption, although whether this mechanism operates during FMDV infection is unknown.

Cytotoxic T lymphocytes (CTL) are able to kill virus-infected cells following recognition of viral peptides bound to major histocompatibility complex (MHC) class I molecules on the infected cell surface. The existence of a CTL response to FMDV has only recently been uncovered (Guzman et al., 2008), most likely because FMDV devotes considerable resources to avoid stimulating just such a response. *In vitro* studies show that FMDV NS proteins 2B and 2C acting together, or their precursor 2BC, inhibit the secretory pathway (Moffat et al., 2005), paralleling the function of other picornavirus NS proteins, such as coxsackievirus and rhinovirus 2Bs (de Jong et al., 2008) and poliovirus 2B and 3A (Deitz et al., 2000). During poliovirus infection, these effects substantially reduce MHC class I expression at the cell surface to a level that protects cells from CTL-mediated lysis *in vitro* (Deitz et al., 2000). This intracellular alteration in protein trafficking also results in limiting the secretion of proinflammatory cytokines, for example IL-6, IL-8 and IFN-β (Dodd et al., 2001). A similar down-regulation of MHC class I at the cell surface has also been observed in FMDV-infected cell lines (Sanz-Parra et al., 1998). Down-regulation of MHC class I is a common strategy in viral infection and cancer, used to avoid CTL killing. The immune system, however, has evolved a coping strategy, using natural killer (NK) cells to destroy low MHC class I-expressing cells. Interestingly, during FMDV infection, a reduced capacity of NK cells to lyse target cells and secrete IFN-γ has been demonstrated (Toka et al., 2009).

Other NS proteins contribute to FMDV virulence, although their mechanisms of action remain unclear. For example, $3C^{pro}$-dependent histone H3 cleavage occurs in infected cells (Grigera and Tisminetzky, 1984) and is followed by a decrease in host RNA synthesis (Falk et al., 1990). Additionally, $3C^{pro}$ has been implicated in cleaving the translation factors eIF4A and eIF4G, thus exacerbating the shut-off of host cell translation (Belsham et al., 2000). A global effect on host cell transcription and translation should contribute to modulate the host response to viral infection.

FMDV Interacts with Professional Antigen-Presenting Cells

Antigen-presenting cells (APC) are the master regulators of immune responses, so perhaps it is no surprise that FMDV has evolved to prevent them functioning optimally. The virus is likely to first come into contact with APC as a result of its lytic infection of epithelial cells. This results in local tissue damage, causing inflammation and the release of so-called 'danger signals' (Gallucci and Matzinger, 2001) to alert the immune system to the attack. Inflammatory mediators cause local vasodilation and increased vascular permeability, enabling the recruitment of mononuclear cells to infection sites (Goldsby et al., 2000). These mononuclear cells then respond to the inflammatory microenvironment, differentiating towards macrophage or dendritic cell (DC)-like phenotypes.

Phagocytosis of FMDV by porcine macrophages has been demonstrated *in vitro* (Rigden et al., 2002), although following uptake the cells do not become productively infected (Baxt and Mason, 1995). These observations

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support the idea that porcine macrophages play a key role in resolution of disease via phagocytosis and destruction of antibody-opsonized FMDV (McCullough et al., 1992).

While the majority of studies on APC interactions with FMDV have been carried out in pigs, mice can be experimentally infected and have yielded potentially interesting data. Mouse bone marrow-derived DCs were infected by FMDV *in vitro* without detectable cytopathic effect or live virus production. FMDV caused down-regulation of MHC class II and CD40 in infected DC cultures, resulting in a decreased ability of the cells to stimulate T-cell proliferation to either allogeneic or FMDV antigens (Ostrowski et al., 2005). DC exposed to live, compared to inactivated FMDV, induced the potentially immunosuppressive cytokine, IL-10 in T cells incubated with infected DCs (Ostrowski et al., 2005). Moreover, repeating the experiment with DC–splenocyte co-cultures, the authors detected a threefold higher titre of T-independent neutralizing IgM when DCs were exposed to live FMDV compared to inactivated virus. They proposed that this was attributed to the induction of IL-6 in DC, which subsequently induced IL-10 secretion, but this time from B lymphocytes in the co-culture (Ostrowski et al., 2007).

The interpretation of these experiments deserves caution because of the species used; however, the work has recently been given significant support from a study in pigs (Diaz-San Segundo et al., 2009). Porcine monocytes infected *in vitro* were differentiated into DCs, but these infected cells produced APCs that secreted high amounts of IL-10 (as opposed to IFN-γ) in allostimulatory T-cell co-cultures. This may indeed be physiologically relevant as the amount of IL-10 in serum from FMDV-infected swine was higher than in naïve animals. Together, these studies propose a scenario whereby FMDV induces high levels of IL-10 production, either directly or indirectly through DCs. While this cytokine supports T-cell-independent antibody responses, it may suppress T-cell activation during acute infection.

The interaction of fully differentiated porcine DCs and FMDV is less clear. Early studies reported that 10% of skin DCs were infected *in vitro* by a wild-type FMDV isolate and were dying as a result (Gregg et al., 1995). Although the experimental conditions used were not explicitly defined, it is plausible that direct loss of a proportion of the APC population during infection could adversely affect the immune response to FMDV. In contrast, Rigden et al. (2002) reported, as unpublished data, similar findings in DCs as in pulmonary macrophages in which almost all cells exposed to wild-type FMDV subsequently expressed NS proteins. In contrast to the study by Gregg et al., Rigden et al. (2002) detected no evidence of cell death caused by the virus.

A later re-examination of the effects of FMDV exposure on porcine skin DCs [identified as Langerhans cells (Nfon et al., 2008)] indicated a complete lack of viral replication (Bautista et al., 2005). However, exposure of DCs to live, but not UV-inactivated FMDV, did induce the production of substantial quantities of type I IFNs. Despite being stimulated to produce IFNs, FMDV-exposed DCs surprisingly did not modulate their antigen uptake or surface molecule expression (Bautista et al., 2005), indicating that APC function remains intact following exposure to the virus. The situation appears to be slightly more complicated when considering skin DCs isolated during infection, as these cells exhibit defective IFN responses up until 42–50 days post-infection, despite not being infected themselves (Nfon et al., 2008). These Langerhans cells show stable to slightly increased expression of APC-related proteins, including MHC class II and CD80/CD86 and antigen-processing function, implying FMDV mediates maturation of these cells.

Monocyte-derived DCs (MoDC) are an *in vitro* model for the DC, which differentiate *in vivo* at sites of inflammation, such as FMDV lesions, and are generating increasing interest in the field. As in porcine skin DCs, MoDC also exhibit an IFN-α response to a range of FMDV strains *in vitro* (Nfon et al., 2008). The cells appear not to be infected by the virus either *in vitro* or *in vivo*, but as noted above, the virus is able to infect their CD172a$^+$ progenitors and interfere with DC development (Diaz-San Segundo et al., 2009). Monocytes taken from pigs during the acute stages of FMDV infection also fail to differentiate into DCs capable of responding to IFN-inducing toll-like receptor (TLR) ligands *in vitro* (Nfon et al., 2008). However, like the Langerhans cells, monocytes from FMDV-infected pigs differentiate into DCs with stable expression of MHC class II and CD80/CD86 and process antigen, indicating that differentiation into APCs occurs. In another study, MoDCs from infected pigs did not stimulate a mixed lymphocyte reaction (MLR), indicating a compromise of DC functions (unpublished). One of the mechanisms by which FMDV directly disrupts IFN responses might be by selectively inhibiting the transcription of specific genes involved in this function (Rodríguez-Calvo et al., unpublished observation), although other possibilities also warrant investigation.

Plasmacytoid DCs (pDC) are distinct in lineage and function from the DC populations mentioned above as their main role is in the production of large quantities of IFNs in response to viral infection. Porcine pDCs are infected by FMDV–antibody complexes and express NS proteins and secrete IFN-α. In contrast, they do not become infected or produce IFN-α in response to virus alone (Guzylack-Piriou et al., 2006). In acutely infected swine, the blood pDC population is depleted, and the

remaining pDC are less able to produce IFN-α in response to TLR ligands or FMDV, although they recover this ability by day 7–9 post-infection (Nfon et al., 2010). Further studies are required to determine how pDC secrete IFN in response to FMDV immune complexes despite detectable production of viral proteins.

Stimulation of the Early Antibody Response by FMDV

The early B-cell response to FMDV infection is typified by a strong neutralizing antibody response, with serum IgM detectable as soon as 3–4 days post-infection in cattle, followed by IgA and then IgG peaking 1–2 weeks later (Collen et al., 1989; Salt et al., 1996; Juleff et al., 2009). In infected swine, a similar response is detected (Pacheco et al., 2010).

Early work in nude mice demonstrated that anti-FMDV IgM production occurred in the absence of T-cell help (Borca et al., 1986; Collen et al., 1989). This result has recently been confirmed in cattle by Juleff and colleagues, who went on to show that during infection, antibody isotype switching to high-affinity IgG can also occur in the absence of detectable T-cell help (Juleff et al., 2009). While some T-independent antibody responses may be initiated without DC help (Pape et al., 2007; Scandella et al., 2007), DC-derived factors such as B-cell-activating factor of the TNF family (BAFF) are likely to be required to induce class switching in naïve B cells (Litinskiy et al., 2002). While this has yet to be studied *in vivo* for FMDV, in a porcine *in vitro* model, both BAFF and IL-2 were essential for anti-FMDV recall IgG responses (Bergamin et al., 2007b). In this model, BAFF was DC derived, while IL-2 was added exogenously to represent the contribution of T-cell help. However, in some situations, DC may also secrete IL-2 (Granucci et al., 2001), which could potentially maintain the T independence of the antibody response. It should be noted, however, that expression of BAFF in the context of DNA vaccination of pigs did not improve antibody responses (Bergamin et al., 2007a). Although a role for DC has yet to be explicitly proven in the early B-cell response to FMDV, it is likely that stimulation of T-cell responses by DCs will be required for protection following vaccination to achieve induction of memory B cells.

FMDV Stimulation and Manipulation of the T-cell Response

In swine, there is some evidence that T-cell function may be broadly affected during acute FMDV infection. Peaking at day 2 post-infection, a transient lymphopenia is observed in infected animals, mainly affecting $CD8^+$ T cells, but beginning to resolve from day 4. However, even after resolution of the lymphopenia, T cells responded poorly to the mitogen concanavalin A, demonstrating an ongoing impairment in function (Bautista et al., 2003). This work was partly supported by a study of Diaz-San Segundo et al. (2006) who showed that C serotype FMDV could productively infect both T and B lymphocytes to a high level (30% and 60%, respectively), resulting in lymphopenia, again most profoundly affecting $CD8^+$ T cells and inducing mitogen unresponsiveness (Diaz-San Segundo et al., 2006). However, neither study addressed the mechanism of lymphopenia, for example Diaz-San Segundo et al. (2006) documented productive infection of lymphocytes without cell death, raising the possibility that the lymphopenia was not related to virus-mediated killing. In fact, it now seems that the observed lymphopenia is likely to represent IFN-induced lymphocyte egress from the blood into the affected sites or lymphoid tissues. Support for this hypothesis comes from murine studies in which administration of IFN-α *in vivo* results in a broad lymphopenia, which is not observed in IFNα/β receptor knockout mice (Kamphuis et al., 2006), as well as the detection of high levels of type I IFNs in the blood of infected swine (Nfon et al., 2010). It is important to note that a transient virus-induced IFN-mediated lymphopenia in the peripheral blood may not be a sign of immunosuppression but rather can be physiological and potentially beneficial to the host (Schattner et al., 1983; Kamphuis et al., 2006). In fact, the rapid recovery from lymphopenia detected in FMDV-infected swine is consistent with altered migration patterns in response to cytokine signals rather than loss and subsequent repopulation of lymphocytes.

Joshi and colleagues also detected infection of bovine lymphocytes exposed to FMDV *in vitro* and found an inhibited response to mitogen activation (Joshi et al., 2009). However, the situation is less clear-cut, as contact-challenged cattle exhibit normal lymphocyte numbers and subpopulations (Windsor et al., 2008). Moreover even during acute infection, T cells from infected cattle proliferate well to stimulation with either mitogens or non-FMDV recall antigens (Windsor et al., 2008). Interestingly, although proliferation assays were carried out up to day 19 post-infection, only a modest specific T-cell response to FMDV antigen was ever observed, although serum IL-10 levels remained low.

Overall, it is currently not possible to make clear statements about the nature and consequences of the interactions between FMDV and T cells. As detailed earlier, there are clear differences between results obtained *in vitro* and *in vivo* in cattle and between studies in pigs when different isolates are used. Clearly, quite different results could be obtained if the challenge viruses were

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harvested directly from animals or had been adapted to grow in cells which did not express the integrin molecules that act as viral receptors. There may be additional uncharacterized mutations that confer altered tropism *in vitro* and *in vivo*, which could account for the different experimental observations. This puzzle will not be resolved easily, and systematic studies to correlate virus genotype and phenotype will have to be performed *in vitro* and *in vivo*.

These studies serve to highlight the gap in our understanding of the importance and precise function of the T-cell response during FMDV infection. For example, while immunity to FMDV during infection is initially dominated by T-independent antibody production, in vaccinated cattle, antibody titres are not always predictive of protection, and in carrier animals, live virus persists in the face of high titres of neutralizing antibody (DiMarchi et al., 1986; McCullough et al., 1992; Juleff et al., 2008). In fact, T-cell responses to vaccination in cattle in combination with antibody responses may better predict protection from challenge (Glass and Millar, 1994; Hohlich et al., 2003), for example detection of established T-cell responses may predict rapid development of a protective memory immune response after rechallenge.

T-cell responses to FMDV further have the advantage of being serotype cross-reactive, making them attractive targets for vaccination strategies (Collen et al., 1998b). However, the success of subunit vaccines incorporating T-cell epitopes has been limited by a lack of fundamental understanding of T-cell responses to FMDV infection. T-cell epitopes have been identified within both structural and NS proteins during infection and vaccination (van Lierop et al., 1992; Collen et al., 1998b), but the use of such epitopes in vaccine strategies has met with, at best, limited success. The T-cell response in mice following DNA vaccination with epitopes from FMDV structural and NS proteins did not induce specific antibodies and yet was sufficient for protection from disease (Borrego et al., 2006). However, only partial protection of swine could be achieved by vaccination with recombinant vaccinia virus expressing the FMDV 3D protein (Garcia-Briones et al., 2004). A similar phenomenon was observed in cattle, where a range of peptide vaccines were tested but none induced protection in more than 40% of challenged animals (Taboga et al., 1997; Rodriguez et al., 2003). T cells are likely to be key to the development of long-term protective immune responses to FMDV by supporting and maintaining T-cell-dependent antibody responses. The studies described above suggest these T-cell responses are serotype cross-reactive, which may provide opportunities to develop vaccines to the different serotypes using promiscuous T-cell epitopes combined with specific B-cell epitopes.

Conclusion

In summary, in the early stages of infection, FMDV initially interacts with components of the innate immune system. These components are likely to include host mononuclear phagocytes, macrophages and multiple DC subsets. The initial response of these cells is production of type I IFNs, although the virus possesses several mechanisms to slow or inhibit this process. The innate interaction progresses to initiate the adaptive immune response, most likely beginning with the induction of T-cell-independent B-cell responses. The classical helper T-cell response is stimulated later in infection, and the subsequent induction of $CD8^+$ lymphocytes is still poorly understood (Collen et al., 1998a; Childerstone et al., 1999; Guzman et al., 2008).

Many details of FMDV's interaction with the early immune response remain unknown. There is accumulating evidence for a complex interaction of the virus with host APCs. This is especially important to understand because the ability of the virus to hijack DC functions early in infection represents the equivalent of storming the control room of the immune system. For example, it seems that DC-derived factors may be required for the induction of the T-independent antibody response (Bergamin et al., 2007b), but may also be suppressing T-lymphocyte activation (Ostrowski et al., 2005; Diaz-San Segundo et al., 2009). Moreover, it appears that the virus has evolved ways of subverting the innate, type I IFN response (Nfon et al., 2008) or differentiation of inflammatory APCs from their precursors (Diaz-San Segundo et al., 2009) or both. Given the highly acute and contagious nature of this infection, the evolution of this virus to disrupt APC functions may provide just the temporary escape that it needs to spread to the next host. Dissection of the FMDV–APC relationship will likely be critical to understanding immunity to FMDV and also to rational vaccine design for the future.

Outbreaks of FMDV in naïve populations of livestock resemble the spread of a wildfire in dry woodlands driven by hot winds. Immune evasion needs only be short lived, but must be immediate to allow FMDV to be a successful pathogen. Therefore, a unique characteristic of new vaccines being developed for outbreak responses should address the need to induce a rapid, innate response as well as the classical T-cell-dependent, adaptive B-cell response. Such vaccine formulations are not simple to design, as innate immune responses in mammals have evolved to be down-regulated by numerous mechanisms to avoid unnecessary inflammatory damage to the host. How to activate these responses well enough to protect against infection of livestock with FMDV is the subject of intense research at this time.

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REVIEW

The Pathogenesis of Foot-and-Mouth Disease I: Viral Pathways in Cattle

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Summary

In 1898, foot-and-mouth disease (FMD) earned a place in history as the first disease of animals shown to be caused by a virus. Yet, despite over a century of active investigation and elucidation of many aspects of FMD pathogenesis, critical knowledge about the virus–host interactions is still lacking. The aim of this review is to provide a comprehensive overview of FMD pathogenesis in cattle spanning from the earliest studies to recently acquired insights emphasizing works which describe animals infected by methodologies most closely resembling natural infection (predominantly aerosol or direct/indirect contact). The three basic phases of FMD pathogenesis *in vivo* will be dissected and characterized as: (i) pre-viraemia characterized by infection and replication at the primary replication site(s), (ii) sustained viraemia with generalization and vesiculation at secondary infection sites and (iii) post-viraemia/convalescence including resolution of clinical disease that may result in long-term persistent infection. Critical evaluation of the current status of understanding will be used to identify knowledge gaps to guide future research efforts.

Introduction

Foot-and-mouth disease virus (FMDV; family *Picornaviridae*; genus *Aphthovirus*) causes an acute disease of cloven-hoofed animals characterized by fever, lameness and vesicular lesions of the feet, tongue, snout and teats. These debilitating effects, rather than high mortality rates, are responsible for severe productivity losses associated with foot-and-mouth disease (FMD). The highly contagious nature of the virus and severity of economic impacts associated with the disease determine FMD's status as the most important disease limiting trade of animals and animal products throughout the world.

The earliest recognition of the clinical entity of FMD is generally credited to Fracastorius's observations in the 16th century (Fracastorius, 1546); however, the first step towards understanding the pathogenesis of FMD was Loeffler and Frosch's landmark demonstration that the disease was caused by a filterable agent (i.e. virus) (Loeffler and Frosch, 1897). In the years since that discovery, investigation of the pathogenesis of FMD has been conducted in various manners and has been reviewed relatively recently (Grubman and Baxt, 2004; Alexandersen and Mowat, 2005). However, progress in understanding the mechanisms of this important disease has been impaired by two substantial obstacles: (i) the biosecurity requirements for working with the virus have limited research to only a handful of institutions worldwide and (ii) the seven serotypes and myriad strains of the virus have proven to often be as dissimilar as they are similar, thus limiting the ability to extrapolate findings from many studies to understanding of FMD in general.

It is generally accepted that FMDV spreads predominantly by direct or indirect contact with infected animals, their secretions or contaminated food products. It is also known that under certain circumstances, the virus travels over extensive distances to cause incursions at previously virus-free premises [reviewed in (Alexandersen et al., 2003b)]. Although airborne dissemination of infectious aerosols is often implicated, the contributory roles of humans (fomites), wildlife and waterborne spread are often not easily discerned. Conventional wisdom regarding FMD pathogenesis asserts that natural infection of cattle and sheep occurs via the respiratory route by aerosolized virus

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(Donaldson et al., 1987), whereas pigs are more commonly infected by consumption of virus-contaminated food or through skin lesions while in contact with infected animals or their secretions (Alexandersen et al., 2003a). However, despite many years of research, the basic events and mechanisms utilized by the virus to infect, cause disease within one animal, and disseminate to other animals remain incompletely elucidated.

Much of the basic knowledge about FMDV virus–host interactions is derived from *in vitro* studies under controlled cell culture conditions. These studies, while important, cannot fully address the complexity of the virus–host interaction at the tissue, organ and systemic levels in the natural hosts. Of particular interest is the identification and characterization of sites of primary viral infection in the host and the pathways, mechanisms, host factors and viral factors that determine generalization (i.e. viraemia) and shedding of the virus into the environment.

Improved understanding of FMD pathogenesis has substantial basic science value, but also has translational relevance by providing requisite data for rational design of the next generation of vaccines and biotherapeutic agents. These tools may impair or prevent the following closely related, yet distinct phases of FMD: primary viral infection, generalization (viraemia), clinical disease and viral persistence in ruminants. In this sequence of events, persistence has a unique significance. The infectivity of persistently infected ruminants to naïve animals is believed to be low under most circumstances; yet, it is not demonstrably negligible. The perceived threat of contagion from carriers is one of the factors that dictate trade policies of FMD-free nations. And it is these policies which drive the cost–benefit analyses that determine FMD response strategies; most models indicate that without a 'vaccinate-to-live' policy, the economically favoured control strategy in most scenarios is 'stamping out' (i.e. mass depopulation) (Bates et al., 2003; Ward et al., 2009). Because persistence is a consequence of some or all of the other stages of FMDV infection, it follows that improved understanding and mitigation of the various stages of FMD (i.e. pathogenesis), ultimately, is a requisite path to decreasing the need for mass depopulations.

The goals of this review are to provide a comprehensive overview of the field of FMDV pathogenesis and to identify prioritized knowledge gaps that remain in the understanding of this important disease. As pathogenesis in cattle has been most thoroughly investigated, this body of work will be described at length herein; an accompanying (companion) article will review pathogenesis in other species, specific, atypical clinical FMD syndromes and the molecular mechanisms characterized *in vitro* which suggest explanations of pathogenesis in live animals.

Stages of FMD Pathogenesis in Cattle

The terms 'pre-viraemia', 'viraemia' and 'post-viraemia' have generally accepted definitions in the description of stages of FMD pathogenesis. However, other terms such as 'early' and 'persistent' have been used variably in the published literature and have led to some confusion. While authors will, likely, continue to use terminology to suit their needs and preferences, for the purposes of this review, the stages of FMD pathogenesis are defined as:

1. *Pre-viraemia*: the period from when an animal is first infected with FMDV until virus is first detected within the intravascular (i.e. blood) compartment with a sustained and quantitatively increasing trend (determined by virus isolation (VI) or detection of viral RNA). Pre-viraemia may include a passive and transient presence of virus in the blood in the period immediately following first exposure to FMDV.
2. *Establishment of viraemia*: temporally occurs within *pre-viraemia*. However, because of the importance of this transition, it will be treated as a distinct event.
3. *Viraemia*: the period during which FMDV can be detected within the intravascular compartment with kinetics suggestive of active viral replication (determined by VI or detection of viral RNA). This period typically coincides with the clinical phase of disease. Viraemia may be undetectable in some infected animals even when they develop lesions at secondary sites of replication. However, if replication has occurred at secondary sites, viraemia must have occurred, and failure of detection in such instances may be attributed to insufficient frequency of sampling and/or inadequate detection sensitivity.
4. *Post-viraemia*: the period following viraemia starting with the first negative assay on blood (determined by VI or detection of viral RNA) which includes:
   - (i) Resolution of clinical signs
   - (ii) Short-term persistence of infectious virus, antigen and/or RNA in specific tissues
   - (iii) Persistent infection (carrier state): the period after 28 dpi in which infectious FMDV may be detected on at least one of multiple oesophageal–pharyngeal (OP) samples (as defined by OIE)
   - (iv) Chronic long-term sequelae including hirsutism, heat-intolerance (panting) and thyroid dysfunction, which have been reported in recovered cattle (discussed in companion article)

Pre-viraemia and Primary Sites of Infection in Cattle

FMD pathogenesis has been most thoroughly investigated in cattle, less so in swine and minimally in other species.

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Yet despite several decades of investigation of the bovine pathogenesis, a clear consensus still does not exist regarding many basic aspects of virus–host interaction. Basic events of early infection, such as tissue sites and cellular characteristics of primary infection and replication, are not clearly established. Contradictions exist in the scientific literature that are likely due to multifactorial differences across studies including virus strains used, methods of virus detection, techniques used for animal exposure, tissues examined and variability amongst small numbers of experimental animals. Despite the relevance of all of these factors, strain-specific viral properties have likely created the most substantial challenge to generating a consensus understanding of the pathogenesis of FMD. Amongst the myriad strains of FMDV, there surely are aspects of tropism and virulence which are strain specific; however, conclusions regarding these factors can not be adequately documented unless different strains are examined *in vivo* in studies in which all other experimental conditions remain constant. Such studies are rarely performed because of constraints of cost and logistics, and as such, the conclusions from most pathogenesis studies are only directly applicable to a single FMDV strain. Overall, upon examination of the complete breadth of the FMD pathogenesis literature, one is best advised to accept that many of the seemingly contradictory conclusions across studies may reflect intrinsic differences in experimental design.

Early investigators considered the natural routes of infection by FMDV to be via the upper gastrointestinal tract. However, as early as 1952, the susceptibility of cattle to respiratory tract inoculation was experimentally investigated (Henderson, 1952). Korn's, 1957 work is often cited as the earliest demonstration that the respiratory tract provides the site of primary infection; however, in that work, the author cites two earlier studies that described infection of cattle via aerosol (Fredorf, 1949) or intranasal deposition (Mohlmann, 1954). The remainder of the following discussion of pre-viraemia will focus upon contact and direct respiratory tract inoculation as these are considered to closely simulate natural exposure in cattle; however, it is acknowledged that cattle may sometimes be infected by direct transepithelial penetration and by ingestion, and this might result in different primary replication sites and distinct patterns of viral dissemination.

Korn (1957) infected cattle with FMDV by moist gauze deposition on the nasal planum and euthanized animals at various times after inoculation. By performing VI on various tissues, this work demonstrated that in pre-viraemic animals, FMDV was more frequently retrieved from nasal mucosae and nasopharynx than stratified squamous epithelial sites within the oral cavity. This work is also noteworthy in providing the only documentation of microvesiculation at a primary replication site (nasal mucosa).

Following Korn's tissue-specific work, much of the investigation of FMD pathogenesis was conducted in studies utilizing VI from various clinical samples with an emphasis on OP fluid ('probang samples') and/or serum as a means of detecting 'pharyngeal' FMDV replication and viraemia, respectively (Burrows, 1968a; Sellers et al., 1968; McVicar et al., 1970; Burrows et al., 1971; McVicar and Sutmoller, 1976). Although these early works are informative, they clearly have limitations. The probang cup, while useful as a diagnostic instrument, provides a crude specimen for pathogenesis investigation. Although a VI-positive OP sample indicates the presence of infectious virus, it is insufficient to discern the origin of virus from amongst oesophagus, pharynx, oral cavity, lung or nasal cavity; each, individually, containing numerous distinct tissue and cell types. Regardless, in 1968, Burrows and colleagues demonstrated the presence of FMDV in OP samples prior to development of vesicles (Burrows, 1968a) and somewhat later confirmed pre-viraemic 'pharyngeal' replication by demonstrating that OP VI positivity occurred prior to detection of virus in blood subsequent to contact exposure (Sellers et al., 1968; Burrows et al., 1971). However, within approximately the same time period, Eskildsen (1969) brought into question the notion that VI-positive probang samples were necessarily indicative of pharyngeal FMDV replication by demonstrating that deposition of FMDV directly into the lung via tracheostomy consistently resulted in generalized disease. Thus, the relative contributions of upper and lower respiratory tract to FMD pathogenesis were already in question.

The pre-viraemic viral dynamics in the bovine respiratory tract were more precisely defined in subsequent experiments which demonstrated that primary replication of FMDV could be detected in OP fluid within 2–6 h of intranasal deposition depending upon viral strain and dose administered (McVicar et al., 1970; McVicar and Sutmoller, 1976). These experiments also demonstrated that the onset of viraemia occurred at or near the peak of FMDV detection from OP samples [16–72 h post-inoculation (hpi)] and that the lag (eclipse) time between inoculation and OP positivity was inversely related to the FMDV dose inoculated.

In 1981, the first comprehensive examination of distribution of FMDV in numerous distinct tissues during acute infection of cattle was reported (Burrows et al., 1981). By returning to the strategy of dissecting and screening tissues individually, this work provided compelling evidence that in the pre-viraemic phase, FMDV-positive OP samples likely represented pharyngeally

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replicated FMDV. Additionally, the use of several routes of exposure and three different viruses broadened the implications of this work. Overall, amongst pre-viraemic cattle, dorsal soft palate, pharynx (precise region not indicated) and retropharyngeal lymph node were the tissues most frequently found to be VI positive and had the highest mean FMDV titres. These experiments found other tissues including lungs, tonsils and nasal mucosae to be involved in pre-viraemic FMD with lower frequency and at generally lower viral titres.

In theory, microscopic localization of FMDV during pre-viraemic infection should provide the most definitive information regarding the primary site(s) of infection; however, the combinatorial data from a handful of studies is less than completely congruous. Microscopic localization of pre-viraemic FMDV has been performed by *in situ* hybridization (ISH) (Brown et al., 1992, 1996; Hofner, 1995) and immunohistochemistry (Arzt et al., 2010; Pacheco et al., 2010). Brown and colleagues inoculated cattle using an improvised aerosol chamber and examined several tissues by ISH at various times post-exposure. The overall conclusion was that the lungs were the most likely primary infection site based on localization of FMDV RNA to respiratory bronchioles in (presumed) pre-viraemic steers. However, viewed retrospectively, both works from this group have perplexing findings. In the earlier work (Brown et al., 1992), the strongest ISH signal at 6 hpi was detected at the lesion predilection sites of the feet with weaker signal seen in tongue and carpus (all unexpected in pre-viraemic animals). This could be explained either by the occurrence of a transient, low grade, undetected viraemia or by pre-existing (non-FMD-related) lesions that became FMDV contaminated at the time of aerosol inoculation. The latter hypothesis is unlikely because the finding was similarly repeated in several animals within the study, and the former cannot be addressed as blood was not collected from the animals euthanized at early time points (discussed further below). Additionally, it is unfortunate that tissues of the pharynx and nasal cavity were not examined within that study (Brown et al., 1992) to address the earlier indications that such tissues were important primary replication sites (Korn, 1957; Burrows et al., 1981). The later work from this group did examine the soft palate that was FMDV ISH negative at 24 hpi; however, only a single (presumed) pre-viraemic animal was included for each virus used in this study (Brown et al., 1996).

Two recent works have investigated early pathogenesis in an aerosol inoculation model using trimodal (VI, rRT-PCR, immunomicroscopy) detection systems for FMDV (Arzt et al., 2010; Pacheco et al., 2010). These studies screened a large quantity of tissues from animals spanning the pre-viraemic period; however, the works have the similar limitation of examining only a single serotype of FMDV. These authors demonstrated that within this system, bovine FMDV infection initiated (6 hpi) at the crypt regions of the follicle-associated epithelia at mucosa-associated lymphoid tissue (MALT) regions of the nasopharynx (Figs 1 and 2). Shortly thereafter (12 hpi), FMDV replication (i.e. immunoreactivity for non-structural proteins) was detected within the pulmonary alveolar septa. At both pharyngeal and pulmonary sites, FMDV colocalized with cytokeratin indicating that the infected cells were of epithelial histogenesis. Furthermore, these works demonstrated a trend that as viraemia approached, FMDV became more prominent within the lungs and less apparent in the pharyngeal tissues as determined by all three modalities of detection. It is noteworthy that lymph nodes from pre-viraemic cattle were nearly uniformly FMDV negative.

Lastly, various studies have directly demonstrated or indirectly suggested that FMDV may enter the systemic circulation and disseminate to distant sites during the pre-viraemic period. This leads to the semantic issue of whether it is appropriate to define primary and secondary viraemias in the pathogenesis of FMD. Distinct primary viraemia has been identified in the pathogenesis of morbilliviruses (Auwaerter et al., 1999; von Messling et al., 2004) and herpesviruses (Cohen, et al., 2007). There are sporadic reports of detection of transient, low titre (trace) viraemia in cattle following contact infection or exposure to FMDV aerosols (Hofner, 1995; McVicar and Eisner, 1983; Sutmoller and McVicar, 1976b). Additionally, in calves exposed to aerosolized virus, the detection of FMDV RNA in pedal and oral epithelia at six hpi, before the onset of 'true' viraemia and clinical signs is best explained by haematogenous spread (Brown et al., 1992). FMDV has also been isolated from the pharynx in cattle as early as four hours after virus was instilled into mammary tissue, before the onset of viraemia (Burrows et al., 1971). It is feasible that low titres of cell-free or cell-associated virus were present in the circulation in these animals but were undetectable by current methodologies because of the large volume of the circulatory system (Sutmoller and McVicar, 1976a). Overall, the consistency and the mechanisms by which this putative primary low-level viraemia occurs remain to be elucidated, and the significance to overall understanding of FMD pathogenesis remains unknown.

Establishment of Viraemia in Cattle

Understanding the mechanisms by which FMDV enters the systemic circulation and maintains high-titre viraemia is of critical importance to basic scientific understanding of the pathogenesis, but also to control efforts. Effective abrogation of viraemia markedly decreases severity of

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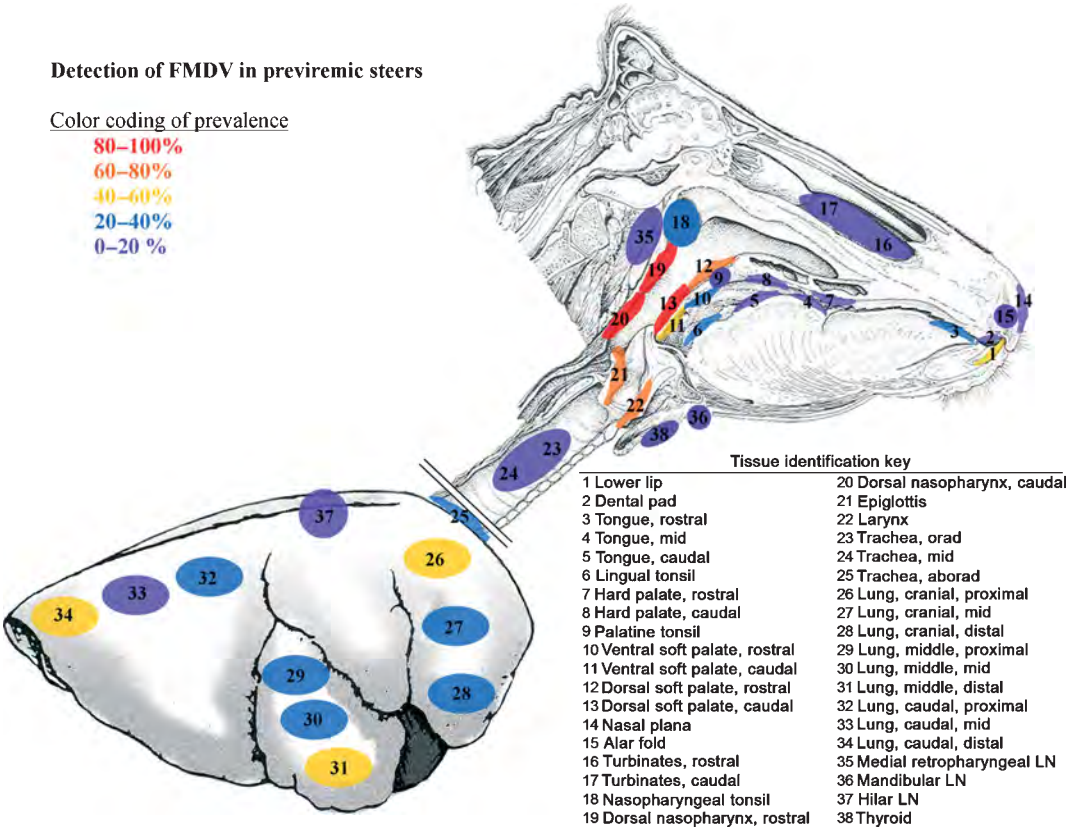

Fig. 1. Tissue-specific detection of foot-and-mouth disease virus (FMDV) or viral RNA by virus isolation (VI) or rRT-PCR in pre-viraemic steers inoculated via aerosol with 10^7 BID_{50} of FMDV-O1-Manisa 3–24 h prior. Only epithelia of the nasopharynx and larynx occupy the highest stratum of 80–100% indicating these tissues as the most consistent sites of primary infection. Prevalence values were calculated as number of animals in which a tissue was determined positive/number of animals in which that tissue was assayed. Inclusion criterion was negative VI on serum at the time of euthanasia. Data adapted from Arzt et al., Veterinary Pathology 2010.

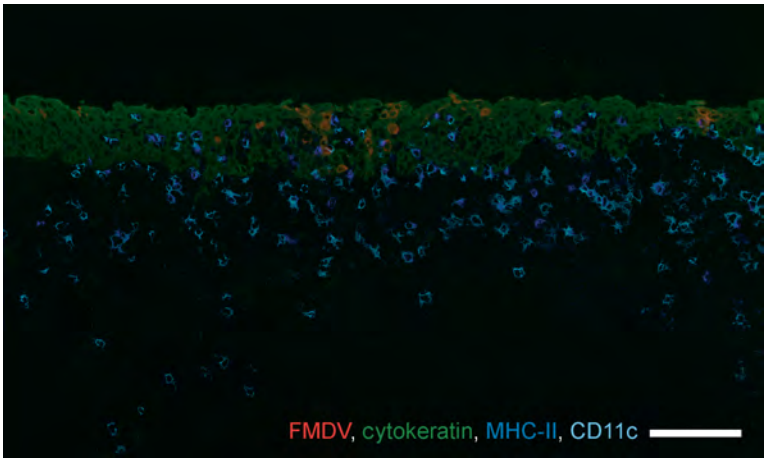

Fig. 2. Primary infection of epithelial cells of the nasopharynx is the earliest microscopically detectable event in foot-and-mouth disease in cattle. Multichannel immunofluorescent technique was applied to bovine dorsal soft palate 12 h after aerosol inoculation with 10^7 BID_{50} of foot-and-mouth disease virus (FMDV)-O1-Manisa. Regional orange staining represents colocalization of signal from FMDV capsid (red) and pancytokeratin (green). CD11c-positive (light blue) and MHC-II-positive (dark blue) cells are present individually and in clusters in close proximity to, but with no specific relationship to FMDV-positive cells. Scale bar, 100 μm.

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clinical disease and extent of shedding, thus the economic impact of incursion into naïve herds or flocks. Indeed, current vaccines likely function by preventing viraemia not primary infection. Despite the importance of this issue, very few pathogenesis studies have specifically addressed how viraemia is established, and the conventional wisdom on the subject is derived almost entirely from speculation. To suggest that an organ or tissue may serve as a 'portal' for establishment of viraemia, certain requirements should be met individually or, ideally, in combination: (1) FMDV should be detected in that tissue prior to detection of viraemia, (2) experimental exposure of that tissue (in isolation) should be shown to result in viraemia and (3) a cellular/molecular mechanism for movement of FMDV from the interstitium to the intravascular compartment should be demonstrated (or sensibly projected).

Although requisite 3 is the most elusive at present given the incomplete elucidation of FMDV–host molecular interactions *in vivo* (discussed in the companion manuscript), several studies have met requisites 1 and 2. As discussed earlier (previraemia section), several studies have demonstrated the presence of FMDV in the respiratory tract prior to viraemia (Arzt et al., 2010; Burrows, 1968a; Burrows et al., 1981; Korn, 1957; McVicar et al., 1970; Sutmoller and McVicar, 1976b). However, defining the viraemia portal (upper versus lower respiratory tract) is complicated by the fact that these putative sites are interconnected, and in live animals, air flow (and hence aerogenous FMDV) passes freely between the external environment, nasal cavity, pharynx and all levels of lungs. This complexity was elegantly addressed by Sutmoller and McVicar (1976b) by placement of tracheostomy tubes in steers as a means of isolating the upper and lower respiratory tracts for examination of viral dissemination under variable exposure conditions. By demonstrating similar FMDV kinetics in blood between tracheostomized cattle inoculated either intranasally or by contact, this work concluded that either the upper or lower respiratory tract may individually serve as portals of FMDV entrance to the systemic circulation. Further evidence supporting the importance of the nasopharynx in establishing viraemia came from Burrows' work, which demonstrated that lungs of aerogenously infected cattle were often (but not exclusively) VI negative unless the animals were viraemic; this suggested that the virus had to reach some other site (presumably nasopharynx) to establish viraemia which then could be detected in lung. However, more recent work has shown that in the period in which viraemia is established, substantially greater quantities of FMDV are detectable in the lungs when compared to the pharynx suggesting a pulmonary portal (Arzt et al., 2010). This finding has been consistent across several animals aerosol inoculated with FMDV serotypes O or A and has been confirmed by VI, rRT-PCR and immunomicroscopy specific for FMDV structural and non-structural proteins.

The notion that FMDV viraemia is established at regional lymph nodes draining sites of primary replication is intuitively reasonable, but has not been supported by the primary literature. At least two important pathogenesis studies have suggested this mechanism while acknowledging that it was speculative in nature (Korn, 1957; Sutmoller and McVicar, 1976b). Korn's data by demonstrating absence of FMDV in lymph nodes of pre-viraemic cattle contradicted this notion. Conversely, pre-viraemic cattle in Burrows' work had relatively high prevalence and titre of FMDV in retropharyngeal lymph nodes, yet this author did not speculate viraemia was established by the lymphoid route (Burrows et al., 1981). A more recent study concluded that lymphoid organs were not the portal for viraemia based upon low frequency of detection of FMDV in tonsils and lymph nodes of pre-viraemic cattle (Arzt et al., 2010). Although there surely is room for FMDV strain specificity as an explanation, it should be noted that afferent lymphatics draining the pharynx and lungs pass through the highly vascular regional MALT prior to reaching primary lymph nodes. As such, if one chose to favour establishment of viraemia via lymphoid tissue, the MALT/BALT must be included either as precedent to lymph node involvement or as a direct route to the systemic circulation.

Viraemia

Experimental contact challenge experiments in cattle, sheep and pigs have demonstrated that viraemia, as determined by VI or qRT-PCR, is readily detectable from 1 to 2 days prior to the onset of pyrexia or other clinical signs (Aggarwal et al., 2002; Alexandersen et al., 2002b, 2003a; Arzt et al., 2010). Viraemia results in widespread distribution of FMDV to various tissues and organs, including epithelia, visceral organs and across the blood–brain barrier (Fogedby, 1962; Burrows et al., 1981). Furthermore, all excretions and secretions can contain virus during the viraemic and clinical phases of disease (Alexandersen et al., 2003b). The viraemic phase is characterized by the onset of the FMD hallmarks of vesiculation and erosion of various epithelial sites including the mouth, feet, teats, prepuce and pillars of the rumen (Seibold, 1963; Alexandersen and Mowat, 2005; Arzt et al., 2009). Progression of vesiculation often extends beyond the period of viraemia. Infrequently, gross lesions are associated with other secondary predilection sites including the myocardium (Gulbahar et al., 2007; Ryan et al., 2008; Tunca et al., 2008). Additionally, during the viraemic period, there

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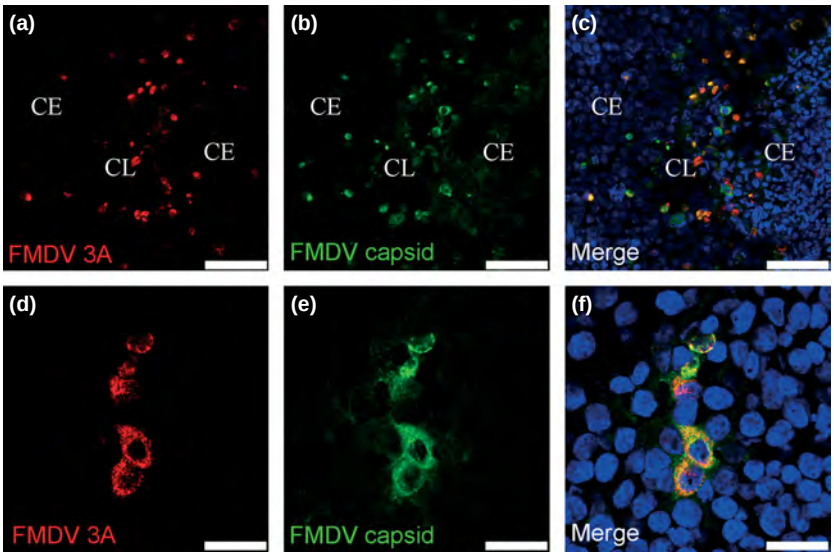

Fig. 3. Foot-and-mouth disease virus (FMDV) replicates in the palatine tonsil crypt epithelium during viraemia. (a) to (f) Palatine tonsil cryosections, 4 days post-intradermolingual challenge with $1 \times 10^{5.7}$ $TCID_{50}$ of FMDV O UKG 34/2001. (a) FMDV 3A protein (red) and (b) FMDV capsid (green) were detected in the palatine tonsil crypt epithelium (CE) and crypt lumen space (CL). (c) Merge images of (a) and (b) demonstrating colocalization of capsid and 3A, nuclei stained blue (DAPI). (d) to (f) Higher power images highlighting the cytoplasmic pattern and colocalization of FMDV 3A (red) and FMDV capsid (green) within the crypt epithelium. (f) Merge images of (d) and (e) nuclei stained blue (DAPI). Scale bars: (a) to (c), 50 μm; (d) to (f), 20 μm.

may be extensive replication in the palatine tonsils (Fig. 3) and lungs (Arzt et al., 2010).

The anatomical and cellular source of the high titres of virus detected in the blood during viraemia remains one of the important knowledge gaps regarding FMD. Typically, only vesicles have greater quantities of FMDV than viraemic blood which suggests a vesicular source, except that viraemia can precede vesicular lesions for several hours or days and in some cases viraemia is present in the absence of grossly detected vesicular lesions. Also, it is unlikely that several minute vesicles could maintain the high viraemic titres of FMDV given the large bovine blood volume. However, microscopic vesicles have been described in the tongue and haired skin of cattle in the absence of macroscopic lesions (Seibold, 1963; Gailiunas, 1968; Yilma, 1980). Despite the absence of gross lesions, it has been shown that bovine haired skin can contain relatively high titres of virus, approaching but not exceeding the amount detected in the blood at the peak of viraemia (Gailiunas and Cottral, 1966). Thus, virus replicating within non-lesional epithelial sites (i.e. skin) could contribute to the high-titre viraemia detected in animals in the absence of macroscopic lesions (Brown et al., 1995; Murphy et al., 2010). Additionally, it has recently been suggested that FMDV replication in the lungs of cattle may substantially contribute to maintenance of high-titre viraemia (Arzt et al., 2010). Detection of high quantities of viral RNA, antigen and infectious virus in pulmonary tissues starting at the onset of viraemia combined with the large mass of the lungs supports this notion.

Several studies have reported tissue-specific viral loads in viraemic cattle (Burrows et al., 1981; Zhang and Alexandersen, 2004; Arzt et al., 2010). However, such data must be reviewed cautiously as it is impossible to accurately determine the contribution to a tissue's 'viral load' from bloodborne virus. The common approach to correct this complication by subtracting the blood titre from the tissue titre is an imperfect solution as it falsely assumes similar blood volumes in all tissues examined. Bearing this limitation in mind, it is still noteworthy that the highest viral loads reported in these studies (excluding vesicles) were lymph nodes and myocardium (Burrows et al., 1981), lungs (Arzt et al., 2010) and vesicle-free lesion predilection sites (Zhang and Alexandersen, 2004).

There is limited and contradictory evidence regarding replication or transport of FMDV in peripheral blood mononuclear cells (PBMC) in cattle (Alexandersen et al., 2002a; Zhang and Alexandersen, 2004). By contrast, a transient lymphopenia has been detected early after infection in swine which may be a consequence of infection of T cells (Bautista et al., 2003; Diaz-San Segundo et al., 2006). However, the susceptibility of porcine PBMC to FMDV infection may be dependent on the serotype of challenge virus, for example; PBMC isolated from serotype C-infected swine were shown to be infected during

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viraemia, coinciding with depletion of T cells in lymph nodes and the spleen (Diaz-San Segundo et al., 2006). In contrast, PBMC isolated from serotype O-infected swine were not infected, and the transient lymphopenia may be the consequence of recruitment of lymphocytes from blood to sites of infection and inflammation (Bautista et al., 2003; Toka et al., 2009). There are no reports in the literature documenting significant lymphopenia or generalized immunosuppression in cattle as a consequence of FMDV infection (Juleff et al., 2009). Although it is apparent that FMDV can interact with bovine lymphocyte populations *in vitro* (Harwood et al., 2008; Joshi et al., 2009; Summerfield et al., 2009), the role of these interactions during viraemia, and on FMD pathogenesis in general, remains to be determined.

Dendritic cells (DCs) have a central role in the induction of innate and adaptive immune responses, yet their role in FMD pathogenesis is poorly understood. The various subpopulations of DCs and their precursors are functionally unusual in their ability to move from tissues into the (lymphatic) vasculature and, additionally, from the blood into tissues. It has been demonstrated that the presence of anti-FMDV antibodies may lead to a shift in affinity and uptake of antibody-opsinized virus by Fc receptor–expressing cells such as DCs (Summerfield et al., 2009). This shift in affinity may be responsible in part for the early localization of FMDV to lymphoid follicles, which develop into germinal centres (GCs) following antigen exposure. FMDV has been detected in mandibular lymph node follicles as early as 3 days post-intradermolingual challenge (Juleff et al., 2008). GCs are an important component of the humoral immune response, and B cells in the GC macroenvironment undergo intense proliferation, selection, maturation and death during antibody responses (McHeyzer-Williams and McHeyzer-Williams, 2005). The precise influence of FMDV localization within GCs on the immune response remains to be determined; however, it is likely that at this stage critical events transpire which initiate the early phase of the adaptive immune response.

An effective immune response against FMDV, leading to clearance of viraemia and tissue viral loads, is characterized by the rapid induction of specific antibody and is thought to be dependent on the interaction between antibody–virus complexes and the phagocytic cells of the reticuloendothelial system (McCullough et al., 1986, 1988, 1992; Juleff et al., 2009). These interactions may account for the accumulation of FMDV in the spleen, liver and lymph nodes during viraemia reported in some studies (Zhang and Alexandersen, 2004). Although FMDV structural and non-structural proteins have been detected in lymph nodes of cattle during viraemia, FMDV proteins have not been detected in the spleen or liver, and it is not clear if FMDV undergoes productive replication at these sites (Juleff et al., 2008).

Post-Viraemia

It is well established that in the convalescent period, subsequent to clearance of viraemia, FMDV continues to be present in very high titres at the lesion predilection sites. Additionally, after clearance of virus from lesion sites, FMDV persists in certain tissues for prolonged times and a subset of FMDV-infected ruminants develop chronic asymptomatic infection referred to as persistence or the carrier state (Alexandersen et al., 2002a; Salt, 2004a). Although various trends have been described regarding tissues affected, species susceptibility and viral genomic alterations associated with persistence, much remains unknown. A noteworthy knowledge gap is the poor understanding of the extent of the threat posed by carriers to naïve animals. However, global trade policy is intimately tied to FMD persistence and two inescapable realities are (i) that the concern of transmission from asymptomatic carriers is the main reason why FMD-free nations restrict imports of live hoof stock from enzootic regions or regions with freedom with vaccination and (ii) one of the main reasons that FMD-free nations depopulate when confronted with FMDV incursion is because of failure of vaccination to prevent the carrier state.

The historic recognition of FMDV persistence in ruminants has been reviewed extensively (Salt, 1993; Alexandersen et al., 2002a, 2003b; Grubman and Baxt, 2004). Briefly, the recovery of infectious FMDV from convalescent cattle was first convincingly demonstrated by Van Bekkum et al. (1959). Reports of similar findings from various investigators established the notion that FMD persistence (defined as recovery of live virus at more than 28 dpi) is a common sequel to infection of ruminants and that roughly 50% (with substantial variability across studies) of ruminants become persistent carriers for a variable period of time. Another landmark was Burrows' demonstration of tissue-specific localization of persistence with dorsal soft palate and dorsal pharynx implicated as the most frequent sites of recovery of FMDV from post-viraemic cattle (Burrows, 1966, 1968b). Yet, the anatomical and cellular sites where FMDV persists and the origin of virus detected by probang sampling still remain incompletely elucidated. Burrows had additionally recovered FMDV from several other tissues of carrier animals including the oesophagus, ventral soft palate, pharynx, glosso-epiglottic space and tonsillar sinuses. These early studies were somewhat limited in that they detected FMDV by VI, a technique which may be compromised in carrier animals by the presence of high titres of neutralizing antibody. Work by Donn et al. (1994) highlighted the

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limitations of applying conventional VI techniques for detecting the carrier state in tissue samples and the benefits of detecting viral RNA by the polymerase chain reaction method. In that study, viral RNA was detected in the tonsil, ventral and dorsal soft palate and cranial oesophagus of contact challenged cattle; however, all the tonsillar and oesophageal samples were negative by VI. Although this may indicate a greater sensitivity of RT-PCR relative to VI, the molecular approach to detection has a distinct limitation in that it gives no indication whether or not detected RNA is associated with infectious virus.

More recently, detailed time course experiments have characterized clearance of FMDV RNA from tissues during the immediate post-acute period in cattle (Zhang and Alexandersen, 2004). This work demonstrated that viral RNA was detectable in various tissues for up to two days after cessation of viraemia, but was largely cleared from most tissues of cattle at 14 days post-challenge. However, the same study demonstrated FMDV RNA in pharyngeal tissues (carrier and non-carriers) and lymph nodes (carriers only) up to 72 dpi. Notably, only one tissue (dorsal soft palate) contained viral RNA in every animal from which the probang specimen was positive by VI. This correlation strongly suggested a dorsal palatal source of virus detected via probang. Other works have similarly described that viral RNA is detectable in pharyngeal tissues beyond 28 dpi (Salt, 1993; Alexandersen et al., 2002a). Similarly, in sheep, viral RNA has been detected in tonsil, dorsal soft palate and nasopharynx up to 43 days post-needle or contact challenge (Horsington and Zhang, 2007). A recent study in sheep showed that by 10 dpi, the only tissues where FMDV still replicated were the tonsil and soft palate (Ryan et al., 2008).

Microscopy studies utilizing ISH in bovine tissues have provided further support for the importance of pharyngeal tissue in the convalescent (5–17 dpi) (Prato Murphy et al., 1999) and persistent (42–82 dpi) (Zhang and Kitching, 2001) periods. Both of these studies described intraepithelial localization of FMDV RNA within dorsal and ventral soft palate and pharynx with the strongest ISH signal detected in the basal and deep spinous regions. Although the phenotypes of infected cells were not investigated in these works, the signal distribution was most consistent with epithelial cells. Strong ISH signal was also identified within the palatine tonsil at 14 dpi with a signal distribution suggestive of lymphoid follicle association (Prato Murphy et al., 1999). Microscopic localization of FMDV antigens in these regions from carrier animals has not been published previously; however, recent work has suggested that FMDV structural and non-structural antigens are present in pharyngeal tissues with a more limited distribution compared to that described by ISH (Fig. 4, Pacheco et al., manuscript in progress). The detection of non-structural proteins supports the concept that virus is replicating at these sites during persistence. Yet, the histogenesis of cells involved remains elusive with morphologic and phenotypic evidence suggesting roles for dendritic cells and epithelial cells.

Additional insights on tissues involved in persistence has come from a recent study that examined pharyngeal tissue samples harvested from cattle 38 days post-contact challenge using a combination of different techniques to detect viral RNA and proteins (Juleff et al., 2008). These investigators readily detected FMDV genome, using laser capture microdissection and qRT-PCR, in GCs within the dorsal soft palate, pharyngeal tonsil, palatine tonsil, lateral retropharyngeal lymph node and mandibular lymph node. In contrast to earlier ISH studies, viral RNA was not identified within the overlying epithelia. These findings were confirmed by ISH studies and by immunohistochemistry using a monoclonal antibody specific for conformational, non-neutralizing epitopes on the FMDV capsid. FMDV capsid antigen (but not non-structural proteins) was detected in mandibular lymph node GCs of 22 animals examined between 29 and 38 days post-contact infection, irrespective of the carrier status at the time of euthanasia. These novel findings have provided evidence for a previously undescribed stage of FMD in cattle in which it is presumed that the virus persists in a non-replicative form. Because this condition was observed in carriers and non-carriers, it was proposed that it may occur as a common sequel to infection regardless of whether virus replication was persisting at other sites. However, it remains to be elucidated whether FMDV detected in lymphoid tissue GCs is replication competent. Furthermore, it is unclear if these virus depots contribute to viral repopulation of other cells in the pharynx with subsequent replication and release of FMDV (i.e. probang-positive carriers). Future studies in a number of species, including the African buffalo and possibly appropriate mouse models, will answer these questions.

Various mechanisms have been proposed to explain the establishment and maintenance of FMDV persistence [reviewed in (Alexandersen et al., 2002a; Salt, 2004b)]; however, further elucidation is clearly required. The hypothesis that immune mechanisms play a role in persistent infection in ruminants is supported by the early observations that infected vaccinated cattle become persistently infected more consistently than unvaccinated ones (McVicar and Sutmoller, 1969) and observations by Ilott et al. (1997) that dexamethasone treatment decreases viral shedding from persistent cattle. One study compared cytokine and toll-like receptor RNA levels in dorsal soft palate lymphoid tissues between carrier and non-carrier cattle at 64 dpi with only TNF-α having significantly elevated levels in persistently infected animals ($P = 0.027$, $n = 2$) (Zhang et al., 2006). By contrast, all other genes

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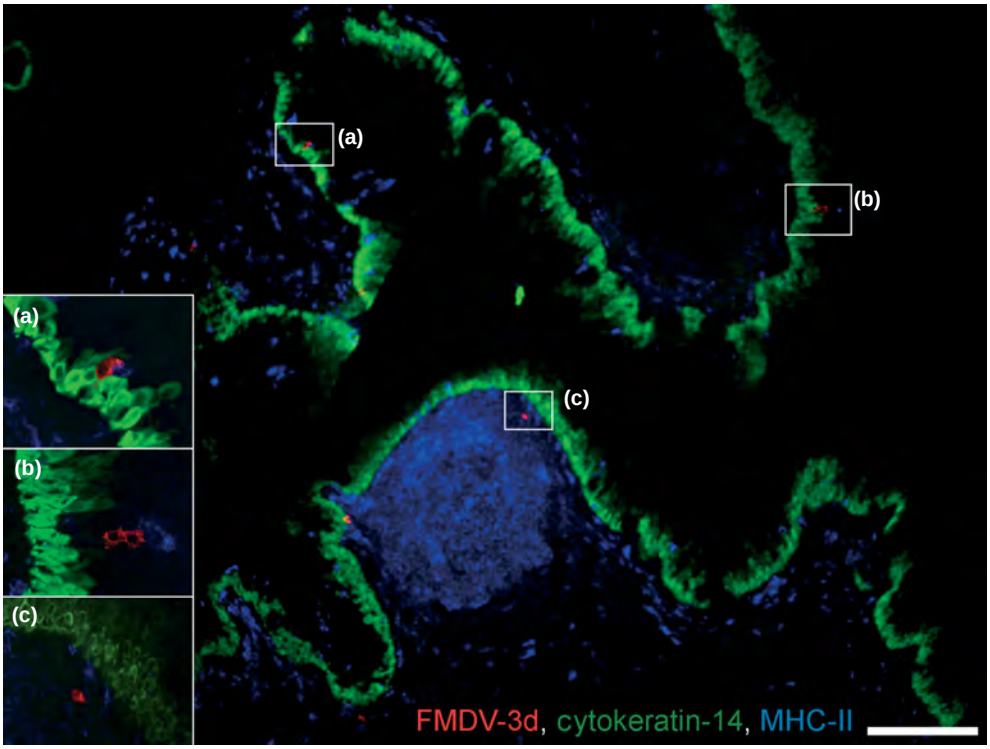

Fig. 4. During persistent foot-and-mouth disease in cattle, rare cells containing foot-and-mouth disease virus (FMDV) antigens may be detected in nasopharyngeal tissues within epithelium (a, b) and subjacent lymphoid tissue (c). Cells containing FMDV-3D (red) are negative for pancytokeratin (green) and MHC-II (dark blue). Dorsal soft palate, 37 days post-contact challenge with FMDV-O1-Manisa. Multichannel immunofluorescent technique. Scale bar, 200 μm.

investigated (IFNα, β, γ, IL1a, 2, TLR-3, 4) had similar expression between carrier and non-carrier tissues. In a separate report, it was described that IFN-γ treatment of persistently FMDV-infected cells resulted in marked decrease in detection of viral antigens and RNA suggesting that this cytokine may play a role in clearance of infection *in vivo* (Zhang et al., 2002).

Few studies have demonstrated that FMDV capsid protein mutations and associated antigenic variation occur during persistent infection *in vitro* (Diez et al., 1990; Martin Hernandez et al., 1994; Holguin et al., 1997) and *in vivo* (Gebauer et al., 1988; Salt, 1993; Jangra et al., 2005). It has been suggested that the failure of high levels of IgA in OP fluid to clear FMDV in carrier cattle may be partly due to such antigenic variation. Recently, it was demonstrated that a substitution change in the B-C loop of VP2 may be associated with persistent FMDV infection in cattle (Horsington and Zhang, 2007). Antigenic sites of FMDV VP2 are of high immunological importance, and mutations within these sites have been shown to affect antigenicity (Kitson et al., 1990). Although such works raise intriguing questions regarding mechanisms of persistence, much work remains to be performed.

Conclusions

Cure, prevention and eradication are the ultimate goals of the study of any disease. Even basic research feeds into a cumulative effort directed towards disease control and eradication. Considering these goals in the context of FMD, the modern investigator may take solace from the accrual of some major accomplishments over the last 100 years; however, much work clearly remains. Various high priority knowledge gaps have been mentioned throughout the preceding sections and are reiterated in Table 1; the bridging of these gaps will, ultimately, contribute to FMD control and eradication.

It may be asserted that the biggest problem overshadowing all of FMD research is that (based upon recent history) incursion of FMDV into FMD-free nations still results in massive depopulations of uninfected but 'high-risk' animals. This practice is based more upon policy than science and is closely related to the inability of currently available vaccines to prevent primary infection and persistence. The rationales behind use of depopulation as the means of control are (i) eliminating any infected, acute shedders of large quantities of virus (or susceptible

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Table 1. High priority knowledge gaps in understanding of FMD pathogenesis

What are the sites of primary infection (tissue and cellular understanding; multiple serotypes/strains and host species)?
What are the host (cellular and molecular) determinants of tropism beyond integrins?
What innate and adaptive immune factors contribute to tropism?
Which innate and adaptive immune factors are subverted by FMDV and by what mechanisms?
What are the roles of dendritic cells, NK cells and $\gamma\delta$-T cells during the various stages of infection?
What are the mechanisms of shedding of virus into secretions and naturally generated aerosols?
How can understanding of tropism and primary infection be manipulated to generate 'rationally designed' vaccines?
What are the sites and mechanisms of establishment of viraemia?
How common is primary, passive viraemia and what is the relevance to pathogenesis?
What are the sites of replication responsible for maintenance of viraemia?
What are the viral and host determinants of establishing persistence at the species and individual animal levels? (How) does vaccination affect persistence?
What are the anatomical and cellular sites of persistence in different ruminant species?
Why are ruminants, but not swine susceptible to persistent FMDV infection?
To what extent are persistently infected ruminants a threat to naïve animals?
What unique pathogenesis events occur in gravid females and embryos/foetuses?
What are the specific virus–host interactions which determine (cardio)myotropism?
What are the most appropriate challenge models in the different species to study FMDV pathogenesis?
Does cell cycle status have a role in determining permissiveness to FMDV replication and persistence?

FMDV, foot-and-mouth disease virus; FMD, foot-and-mouth disease.

at risk animals) and (ii) eliminating potential infectivity amongst carrier (persistent) animals. Thus, development of means to prevent or cure primary infection and the carrier state in ruminants are of utmost importance. However, these lofty goals do not exist in isolation, but rather in a web of interdependence of sources and resolutions of individual contributory knowledge gaps. As such, it seems advantageous for investigators to consider pathogenesis research as the investigation of a continuum rather than a series of isolated events.

Central within the complex web of FMD virus–host interactions lies the ruminant nasopharynx including the dorsal soft palate, nasopharyngeal walls/ceiling and associated MALT. The last 50 years of FMD research have repeatedly implicated these tissues for their importance in primary infection and in persistence. Given that nasopharyngeal tissues have well-documented roles in acute and chronic stages of infection, it seems likely (though has not been demonstrated) that a continuum of events occurs in these tissues between these stages of disease. Interestingly, infection of nasopharyngeal tissues is also morphologically quite distinct from the more dramatic lesions at secondary replication sites. Unlike the classic pathological changes seen in vesicles, FMDV infection in the nasopharynx is predominantly non-vesicular, induces minimal inflammation and does not induce acantholytic degeneration. Yet, by still unknown mechanisms, these epithelial tissues are uniquely susceptible to primary and persistent FMDV infection. It is a natural corollary to question what it is about these tissues that determines their differential FMDV–host interactions. Foremost amongst numerous potential explanations are the exceedingly broad categories of unique cellular characteristics and immunological (innate and/or adaptive) factors. A more thorough understanding of these processes ultimately may lead to effective modulation of such cellular and immune responses for the benefit of preventing or resolving infection.

Recent advances in FMD pathogenesis have highlighted the benefits of integrative approaches to answering such questions rather than viewing these subjects distinctly as acute disease, persistent disease, immunology and vaccinology. Similarly, successful research groups have demonstrated the advantages of drawing upon the complementary contributions of teams of scientists with distinct skill sets including clinical veterinary medicine, immunology, molecular biology, pathology, bioinformatics and vaccinology. It seems likely that the next generation of 'rationally designed' FMD vaccines and biotherapeutics will have to more directly integrate recent and ongoing advances in pathogenesis and immunology to affect implementation of major improvements in FMD control.

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Transboundary and Emerging Diseases

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REVIEW

The Pathogenesis of Foot-and-Mouth Disease II: Viral Pathways in Swine, Small Ruminants, and Wildlife; Myotropism, Chronic Syndromes, and Molecular Virus–Host Interactions

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Summary

Investigation into the pathogenesis of foot-and-mouth disease (FMD) has focused on the study of the disease in cattle with less emphasis on pigs, small ruminants and wildlife. 'Atypical' FMD-associated syndromes such as myocarditis, reproductive losses and chronic heat intolerance have also received little attention. Yet, all of these manifestations of FMD are reflections of distinct pathogenesis events. For example, naturally occurring porcinophilic strains and unique virus–host combinations that result in high-mortality outbreaks surely have their basis in molecular-, cellular- and tissue-level interactions between host and virus (i.e. pathogenesis). The goal of this review is to emphasize how the less commonly studied FMD syndromes and host species contribute to the overall understanding of pathogenesis and how extensive *in vitro* studies have contributed to our understanding of disease processes in live animals.

Introduction

Preventing the introduction of foot-and-mouth disease virus (FMDV; family *Picornaviridae*; genus *Aphthovirus*) is generally believed to be the most important constraint to international trade of cloven-hoofed livestock and wildlife and their derived products. Nations which are free of FMDV expend considerable resources to prevent incursions and to be prepared for the eventuality of introduction of FMDV. There is also substantial investment in research on the virus and mechanisms of disease transmission and pathogenesis. Investigation into FMD pathogenesis has focused primarily on virus–host interactions in cattle with the work predominantly funded by a handful of FMD-free nations with high-value beef and bovine dairy production industries. Numerous studies have described pathogenesis in swine, sheep, goats and wildlife. Yet, overall, the greatest depth of understanding of FMD pathogenesis comes from bovine models, whereas the breadth of understanding comes from study of other host species. Although this research paradigm did not arise as a globally coordinated effort, it has proven to be a reasonable approach to the overall goal of greater understanding of FMD virus–host interactions. However, as FMD research progresses, ultimately, a greater depth of investigation will need to be applied to hosts other than cattle to understand similar and unique aspects of pathogenesis in these species.

Most aspects of FMD are similar across host species; in all species, it is predominantly an acute, febrile infirmity with the hallmark characteristic of vesicle formation within the stratified squamous epithelia of the oral cavity, feet, teats, ruminal pillars (ruminants) and other sites. However, several aspects of FMD pathogenesis are variably species-specific and determined by unique pathogenesis events. For instance, it is often stated that pigs are the supreme generators of aerosolized FMDV, whereas cattle aerosolize less virus yet are more susceptible to infection with aerosolized FMDV (reviewed in Grubman and Baxt, 2004). Yet, the physiological bases for the differences in aerosol shedding and susceptibility remain unknown.

One of the most commonly cited examples of species-specific FMD traits is the existence of FMDV strains with limited host ranges. The best known and most clearly elucidated example is the 1997 Taiwan strain which only affected swine and was not virulent to cattle. For this virus, it was demonstrated that a deletion in the FMDV-3A non-structural (NS) coding region determined the porcinophilia (Beard and Mason, 2000). Other examples include the South Korean 2002 serotype O that was porcinophilic (Oem et al., 2008) and 2010 South Korean serotype A strain that was apparently porcinophobic for commercial pigs yet induced seroconversion of farm raised boars under field conditions (K.-N. Lee, personal communication). In general, infection of domestic species other than cattle with serotypes other than O is relatively uncommon (D. Paton, personal communication). However, this may reflect strain-specific, host range determining factors or simply the facts that serotype O is most prevalent globally and cattle are the most commonly diagnosed host.

Other FMD syndromes that likely involve unique virus–host interactions are persistent infection of ruminants, myocarditis which may have higher incidence among young animals, and reproductive losses which have been reported mostly in small ruminants. For each example, there is intrinsic value to elucidation for the sake of characterizing the critical aspects of the individual syndromes. But the greater benefit may come from a comparative pathogenesis approach (i.e. breadth in combination with depth). For instance, understanding why persistent infection only occurs in ruminants may require thorough understanding of why it does not occur in pigs. Similar logic could be applied to the investigation into the association of FMDV with abortion or high-mortality outbreaks in wildlife species. In each case, the manifestation is likely dependent on virus–host interactions that are specific to a particular strain of virus (determinants of virulence) and/or host characteristics (species, breed, age, sex, reproductive status, nutritional status, etc.).

Investigation into FMDV virus–host interactions *in vitro* has delivered many critical contributions to the current understanding of interaction between host cells and the virus (reviewed in Grubman et al., 2008). The most thoroughly developed bodies of work are those describing the importance of α_V integrins as viral receptors and the manners in which FMDV modulates host cell function via selective proteolytic cleavage of various cellular proteins. Although *in vitro* data have overarching relevance to pathogenesis in all species, defining the direct connection to findings in live animals is often challenging.

The purpose of this review is to provide a comprehensive summary of the current understanding of (i) FMD pathogenesis in hosts other than domestic cattle, (ii) pathogenesis of atypical FMD syndromes and (iii) investigations into molecular pathogenesis conducted *in vitro*. This work will complement two companion articles that will review pathogenesis in cattle and immune responses to FMDV. The goal of these collective works is to identify critical knowledge gaps to be addressed through future work on FMD.

Pathogenesis in Swine

Pigs are the second most commonly studied subjects for FMD pathogenesis research. Recent and historic reviews have supported the notions that compared to cattle, pigs are more refractory to aerogenous infection, more susceptible to infection via the gastrointestinal route and generate greater quantities of aerosolized virus (Sellers, 1971; Alexandersen and Mowat, 2005; Sellers and Gloster, 2008). Yet, the pathophysiological explanations for these differences remain poorly defined. The main explanation for this knowledge gap is that only two reports on porcine FMD pathogenesis have examined tissues from pre-viraemic FMDV-infected pigs (Terpstra, 1972; Murphy et al., 2010). Thus, viral dynamics in tissues and blood during viraemia have been well characterized, but primary sites of replication and mechanisms of establishment of viraemia remain elusive.

In 1972, Terpstra reported experimental infection of pigs with FMDV via 3 distinct routes and found that, in pre-viraemic animals, the tissue-specific distribution of progeny virus was highly dependent upon the route of exposure (Terpstra, 1972). In that study, aerosol inoculation resulted in high-titre FMDV replication in the lungs followed by systemic dissemination at 72 hours post-infection (hpi); by contrast, oral inoculation led to primary infection of the palatine tonsil followed by spread to regional lymph nodes. Additionally, this work, contrary to conventional wisdom, documented a lower minimal infectious dose when FMDV was administered to pigs via aerosol as compared to oral instillation. A more recent

study, which quantified viral loads in tissues in pre-viraemic pigs, reported that at 24 h post-contact infection, viral RNA was detected in coronary band epithelium, liver, tongue, soft palate, pharynx, spleen and mandibular lymph node prior to viraemia which was not detected until 48 hours post-exposure (hpe; Murphy et al., 2010). These authors proposed that virus may have disseminated to these tissues via the lymphatic system; however, such extensive dissemination is inconsistent with solely lymphatic distribution. An alternative (and seemingly inescapable) explanation is that virus entered the intravascular (blood) space prior to 48 hpe, but was below the threshold of detection. A lingering question from this study, and regarding porcine FMD pathogenesis in general, is whether FMDV may enter the vasculature via the respiratory, gastrointestinal and/or transcutaneous (biting) routes.

Subsequent to contact exposure, pigs have been demonstrated to become viraemic at 24–48 hpe and develop vesicles at 48–72 hpe (Alexandersen et al., 2001, 2003a; Murphy et al., 2010). Additionally, it has been suggested that the high quantity of FMDV aerosolized by pigs is generated in the upper respiratory tract. This conclusion was based upon higher quantities of viral RNA recovered from soft palate, trachea and nasal mucosa as compared to lung in one study (Alexandersen et al., 2001). However, an earlier work had shown that FMDV is released from both the upper and the lower respiratory tract of infected pigs and that the relative contributions were dependent upon the stage of disease and route of infection (Donaldson and Ferris, 1980).

In situ hybridization and RT-PCR-based studies have demonstrated that abundant FMDV RNA may be detected in various epithelial sites in viraemic pigs, regardless of the presence of vesicular lesions (Brown et al., 1995; Alexandersen et al., 2001). This suggests that the high-titre viraemia may be maintained by FMDV replication in various peripheral tissues as well as within the respiratory tract. However, direct comparison of FMDV load in tissues versus blood in viraemic pigs indicates that only lesional lingual and pedal epithelia have a substantially higher quantity of viral RNA than blood (Alexandersen et al., 2001; Murphy et al., 2010).

Microscopic localization of FMDV in pigs has been limited to characterization of the viraemic state (Brown et al., 1995; Monaghan et al., 2005b; Durand et al., 2008; Arzt et al., 2009). One study found that FMDV RNA could be localized in all strata of all epithelia examined from viraemic pigs with greatest signal intensity (inconsistently) within the *stratum basale* (Brown et al., 1995). More recent works have characterized FMDV RNA (Durand et al., 2008) or antigen (Monaghan et al., 2005b; Arzt et al., 2009) distribution in developing vesicles and have similarly indicated that lesions are initiated with infection of basal cells overlying dermal papillae or deep dermo-epidermal junctions. Lack of detection of FMDV in dermal cells or vascular endothelial cells suggests that in the morphogenesis of vesiculation, virus transits from the intravascular space to (pre)vesicular epithelium via a cell-independent mechanism. Additionally, Murphy et al. (2010) observed in pigs that vesicle development continued after viral loads in tissue started to decline and suggested that both host response and viral replication play a role in vesicle formation.

Pigs are reported to typically clear FMDV within 3–4 weeks post-infection, and it is generally accepted that persistent infection does not occur. A few reports, however, challenge to that notion. One publication defied conventional wisdom by demonstrating the detection of FMDV RNA in the sera of pigs up to 252 dpi (Mezencio et al., 1999). Additionally, two recent works detected viral RNA in the tonsils, mandibular and cervical lymph nodes from both needle and contact challenged pigs at 28 dpi (Zhang and Bashiruddin, 2009) and up to 36 dpi (Mohamed et al., in press). These studies highlight the delayed clearance of viral RNA from pharyngeal and lymphoid tissues compared to the skin and mouth epithelium, which contained the highest levels of viral RNA during the acute stage of infection. Another report describes the detection of infectious FMDV serotype C and viral RNA from various lymphoid tissues of pigs up to 17 dpi (Rodriguez-Calvo et al., 2011). However, as infectious virus has not been isolated from any pig later than 28 dpi in any of these studies, it remains true that the existence of a FMD carrier state has not been documented in swine. Overall, improved understanding of the ability of pigs to resist persistent infection may suggest strategies to prevent or cure persistence in cattle.

Pathogenesis in Small Ruminants

The relatively scarce published literature on FMD in sheep and goats [small ruminants (SR)] suggest that the pathogenesis is quite similar to that which occurs in cattle with relatively distinct pre-viraemic, viraemic and post-viraemic phases (Arzt et al., 2011). Based on experimental direct contact and intranasal inoculation studies, the incubation period of FMD in sheep and goats is typically between 2 and 8 days, but may be as short as 24 h (Kitching and Hughes, 2002; McVicar and Sutmoller, 1971). As in cattle, infection via inhalation of aerosolized virus is considered the most important natural route in these species with infection of FMDV through the feet not considered a common route under most conditions (Sellers, 1971). Goats and sheep have been demonstrated to be efficiently infected via intranasal deposition of

FMDV with rising pre-viraemic titres of oropharyngeal (OP) fluid strongly suggesting primary pharyngeal replication of virus (McVicar and Sutmoller, 1971; Singh, 1979). These data do not, however, rule out the possibility that virus detected in OP fluid may have been generated in other compartments, such as the nasal cavity or lungs. Indeed, there is limited evidence that primary replication may occur in the nasal mucosa of sheep (Grosso and Osvaldo, 1971). Although the specific sites of primary infection in SR have not been documented, it is likely that distinct phases within the upper and lower respiratory tract may occur as has been demonstrated in cattle (Arzt et al., 2010).

Viraemia has been demonstrated to occur 24–30 h subsequent to intranasal inoculation in both SR species and may last for 1–5 days (Hughes et al., 2002). As in cattle and pigs, fever and vesicles are the hallmarks of clinical FMD in SR and typically occur 12–48 h after the onset of viraemia. In the acute phase of infection of sheep, FMDV has been isolated from OP fluid, nasal secretions, saliva, lachrymal secretions, urine, faeces and milk (Sharma, 1981). Foot-and-mouth disease virus was detected in the milk of does for approximately the same time period as viraemia, but typically with titres 1–3 $\log_{10}$ greater than blood suggesting FMDV replication in mammary tissue and/or concentration of virus in milk. As in cattle, virus may be detected in nasal secretions, OP fluid and exhaled aerosols during the incubation period; however, unlike cattle, aerosol shedding of virus may peak before the onset of clinical signs (Burrows, 1968a; Alexandersen et al., 2002).

There is only one published description of infectious virus titres in ovine tissues during viraemia in sheep (Sharma and Murty, 1981). This work demonstrated the highest quantities of virus in tissues of pharynx, tongue epithelium, nasal mucosa, soft palate and tonsil. Recent work utilizing qRT-PCR and antibodies to structural and NS proteins has demonstrated FMDV replication in epithelial and lymphoid tissues of sheep in both the acute and persistent phases of disease (Waters et al., unpublished data). Evidence suggesting replication in sites other than the respiratory tract and feet in sheep has implicated the mammary gland (McVicar and Sutmoller, 1971), ruminal pillars (Geering, 1967) and teat epithelia (Krumholz, 1963). Heart lesions associated with neonatal mortality have also been described (Khankishiev et al., 1958) and are outlined in the Myotropism section.

Vesicles of the non-haired, stratified squamous epithelia are the most prominent sign of FMD in SR. However, asymptomatic infection is generally considered to be more common in sheep (Cardassis et al., 1966; Gibson and Donaldson, 1986) and goats (Pay, 1988) compared to cattle and swine. Field observations and experimental studies generally agree that in sheep, pedal erosions and vesicles are relatively common, but oral lesions occur substantially less frequently (Littlejohn, 1970). However, some experimental infections of sheep have resulted in oral vesicles, with pedal lesions much less common (Waters, unpublished data). It has been noted that when oral lesions do occur in sheep, they mainly occur on the dental pad, in contrast to cattle, where the tongue is the most common site (Geering, 1967). Oral lesions may occur more commonly in goats relative to sheep with some strains of FMDV (Olah et al., 1976), yet in a field outbreak affecting sheep and goats (presumably with the same virus), clinical signs were reported to be more mild in the latter.

The highly variable manifestations of FMD in SR may lead to misdiagnosis as bluetongue, peste des petites ruminants or foot rot (Kitching and Hughes, 2002). Although the presence and distribution of vesicles is variable, severe lameness is not uncommon with sheep sometimes 'creeping about on their knees', sloughing hooves and developing severe secondary bacterial infections (Bang, 1912; Zaikin, 1959; Geering, 1967). Eroded and ulcerated epithelia may also predispose affected animals to various viral infections. Field reports have also described outbreaks with severe clinical signs in goats (Shukla et al., 1974). Overall, both viral and host factors likely contribute to the variability of clinical manifestations of disease in SR, with virus strain-specific and host pedigree-specific factors similarly contributing to responsibility for determining outcome.

Foot-and-mouth disease virus has been demonstrated to persist in sheep beyond the resolution of typical clinical disease (Burrows, 1968b); however, the duration of persistence was reported to be shorter than in cattle, with virus being isolated from sheep up to 9 months after experimental infection (McVicar and Sutmoller, 1969). Titration of virus from several ovine tissues (soft palate, pharynx and tonsil) indicated that the tonsils may play a significant role in persistence in sheep; however, this finding has not been investigated further (Burrows, 1968b). Recently, FMDV viral antigens have been detected in lymphoid tissue in a similar pattern to that seen in cattle in the persistent phase (Juleff et al., 2008); however, unlike cattle, pre-scapular lymph nodes were consistently positive in sheep up to 42 dpi subsequent to coronary band inoculation (Waters et al., unpublished data). Persistence has been demonstrated to occur in goats with relatively lower prevalence compared to sheep (McVicar and Sutmoller, 1972).

One area which has been investigated more thoroughly in sheep as compared to other species is the effects of FMDV infection on pregnancy. There is substantial anecdotal evidence that upon infection of gravid ewes,

abortion may occur (Littlejohn, 1970). Incidence of abortion documented in field reports has varied widely from 3% to 100% (Oppermann, 1921) and is seemingly independent of stage of gestation. Although the health of the aborting ewes is not generally compromised by the foetal death and abortion, some reports have suggested more severe clinical disease in periparturient ewes compared with non-gravid ones (Brown, 2002; Tyson, 2002).

The only published experimental studies examining FMD during pregnancy showed that FMDV can cross the placenta at 45, 75 and 90 days gestation resulting in foetal death and mummification (Ryan et al., 2007b, 2008). In contrast to field reports, abortion (i.e. foetal expulsion) was not seen in these studies, suggesting that virus-induced death of the foetus may not be followed by luteolysis and expulsion. Another observation from these studies was that higher incidence of trans-placental transmission may occur in dams infected at earlier stages of gestation. More recent work has shown that in instances in which FMDV crossed the placenta, the foetuses were consistently infected and died with resultant autolysis (Waters et al., unpublished data). Infectious virus was not detected in these foetuses and serology indicated no evidence to suggest a foetal immune response to FMDV. However, viral RNA was readily detected in mummified foetal tissues by qRT-PCR up to 42 days after foetal death with the greatest quantities found in cardiac and skeletal muscle. These findings suggest that qRT-PCR [but not virus isolation (VI)] would be an appropriate method of diagnosis of FMDV-induced foetal death.

The broad spectrum of clinical severity of FMD in SR is likely multifactorial including host- (breed, age, physiological state and concurrent disease) and virus-specific factors; however, such determinants of virulence remain poorly described.

FMD in Wildlife

Natural and experimental FMDV infection has been reported in numerous species of free-ranging and captive wildlife (reviewed in Schaftenaar, 2002; Pinto, 2004). Susceptibility has been reported, primarily, among wild members of the order Artiodactyla (even-toed ungulates) in the families Bovidae, Cervidae, Suidae, Tayasuidae, Camelidae, Giraffidae and Antilocapridae; however, infection has been described in several species from other orders including Proboscidea (Hedger and Brooksby, 1976; Bengis and Erasmus, 1988; Table 1). Not surprisingly, the pathogenicity of FMD among wildlife ranges from asymptomatic to fatal. Susceptibility among wildlife species raises several concerns which are closely related to yet distinct from the issues associated with FMD in domestic species.

The greatest concern to FMD-free nations regarding FMD in wild animals is the complication that arises if wild animals are infected during an outbreak as occurred in 1924–1925 in mule deer in California (Keane, 1927). Under such circumstances, response coordinators must consider whether the species involved are competent as intra- and inter-species transmitters of infection, whether these species could become a virus reservoir and whether culling is required. In enzootic regions, eradication of FMD is complicated by the presence of infected wild animals which may serve as a reservoir of reintroduction to domestic herds. An additional concern is whether wild or captive rare or endangered species should be vaccinated and/or culled in an outbreak situation.

Amongst wild Bovidae, more work has been performed on FMD pathogenesis in African (Cape) buffalo (*Syncerus caffer*) than any other species. This is largely because of the susceptibility of this species to persistent infection with South African strains (SAT 1, 2, and 3) of FMDV for up to 5 years in an individual and up to 20 years in an isolated herd. Transmission from buffalo to cattle and impala (*Aepyceros melampus*) has been observed in nature (Dawe et al., 1994a; Bastos et al., 2000) and transmission to cattle experimentally demonstrated (Hedger and Condy, 1985; Dawe et al., 1994b). In natural infections, most observed FMDV-infected buffalo are subclinical (Keet et al., 1996).

Following experimental inoculation in buffalo, viraemia can be detected between 1 and 3 days post-inoculation (dpi), peaks at 5 dpi and is undetectable by 9 dpi (Anderson et al., 1979; Gainaru et al., 1986). As in cattle, the pharynx and dorsal soft palate are thought to be the sites of primary infection (Thomson and Bastos, 2004). Buffalo shed virus in respiratory aerosols, saliva and nasal secretions similar to cattle but for a longer period (Bengis and Erasmus, 1988). Inter-digital and coronary band foot lesions and, less often, dental pad, tongue, lip or hard palate lesions are observed (Hedger et al., 1972; Young et al., 1972; Thomson et al., 2003). Three weeks after infection, most animals are fully recovered (Thomson et al., 2003).

Although FMDV can be isolated from OP samples for months or years following infection, most evidence suggests that inter-species transmission is not common. Additionally, the mechanisms of transmission are remain unclear. It is thought buffalo calves may become infected by carrier animals as they lose colostral immunity and subsequently transmit the infection to other species during the acute phase of their infection (Gainaru et al., 1986; Bengis and Erasmus, 1988). Additionally, it has been suggested that sexual activity may serve as a means of transmission from buffalo to cattle. This theory is supported by seasonal occurrences of transmission to cattle

Table 1. Characteristics of foot-and-mouth disease in selected wildlife species

Family	Species[a]	Clinical disease	Viraemia	Lesions[b]	Mortality	Carrier
Bovidae	**African buffalo**	Mild or subclinical	Yes	None or mild	No	5 years
	Impala	Variable	Yes	Yes, Rm	Young	No
	Wildebeest	None–moderate	Yes	Yes		No
	Kudu	Yes	Yes	Yes	No	140 days
	Eland	Mild	Yes	Yes		No
	Sable antelope	Mild	Yes	Yes	Yes	No
	Saiga antelope	Severe		Yes	High	
	Mountain gazelle	Severe		Yes, Rm, Ht, Pn, SkM, Horn	Mod to high	No
	Dorcas gazelle	Yes		Yes	Young	
	Blackbuck	Yes		Yes	Low	
	Mongolian gazelle	Yes			Low to high	
	European bison	Yes			None to high	
	N. American bison	Moderate to severe	Yes	Yes, Rm, Ht	Low	No
Cervidae	**Red deer**	Mild to subclinical	Yes	Yes	None	28 days
	Roe deer	Severe	Yes	Yes	None	No
	Fallow deer	Mild or subclinical	Yes	Yes	None	28 days
	Sika deer	Mild	Yes	Yes	Low	28 days
	Muntjac deer	Severe	Yes	Yes	High	No
	Reindeer/caribou	Yes			Young	
	Mule deer	Severe	Yes	Yes, Rm, Ht	High	No
	White-tailed deer	Moderate	Yes	Yes, Ht	Moderate in adults	11 weeks
	Elk	Mild or subclinical	Yes	Yes, Rm	None	No
	Sambar	Yes		Yes	Low	
	Spotted deer	Yes		Yes	Low	
Antilocapridae	**Pronghorn**	Severe	Yes	Yes, Rm, Pn	Low	No
Suidae	**Warthog**	Variable	Yes	Yes, Knp	Yes, SAT1	No
	Bush pig	Severe	Yes	Yes	No	No
	Wild boar			Yes		
	White collared peccary	Moderate		Yes	None	

[a]Bold text indicates species in which transmission has been documented.
[b]Yes indicates lesions involving only feet and/or oral cavity, Rm, rumen; Ht, heart; Pn, pancreas; SkM, skeletal muscle; KnP, kneeling pad (warthog).

corresponding with the start of the buffalo breeding season, and the isolation of FMDV from semen and preputial washings of buffalo (Bastos et al., 1999).

Among African antelope species, limited clinical studies and experimental infections involving several species have been reported. In Kruger National Park, numerous outbreaks of FMD, caused by the SAT strains, have occurred in impalas (*Aepyceros melampus*; Thomson et al., 2003). Some outbreaks involving the same virus strain have occurred 6–18 months apart, suggesting the virus may be maintained within the impala population or periodically reintroduced from other species (Vosloo et al., 2009). Experimental infections, however, have failed to demonstrate any carrier state in impalas. Impalas are highly susceptible to aerosol infection (R. G. Bengis and G. R. Thomson, unpublished data in Vosloo et al., 2009). They shed less virus than cattle or buffalo but are still capable of infecting cattle (Bengis and Erasmus, 1988). Following inoculation, impalas may develop typical coronary band, oral and ruminal pillar lesions, depending on the viral strain (Bengis and Erasmus, 1988). In some studies, they became viraemic but did not develop lesions (Anderson et al., 1975). Virus could not be isolated beyond 7 dpi and antibody to FMDV did not persist beyond 300 days (Hedger et al., 1972). The disease is not usually fatal to adult impala but may cause fatality in the young (Meeser, 1962).

Concurrent with FMD outbreaks in domestic animals in Israel, outbreaks have occurred in mountain gazelles (*Gazella gazella*) in two nature reserves in 1985 (Shimshony et al., 1986; Shimshony, 1988) and another in 2007 (Berkowitz et al., 2010). In the larger 1985 outbreak, it was estimated that 2000 gazelles of all ages died which represented a mortality of at least 50% of the population on the reserve, whereas a smaller 1985 outbreak resulted in approximately 10% mortality. The high mortality at one reserve was attributed to increased susceptibility because of historic inbreeding within that population (Shimshony, 1988). In addition to typical vesicles, gross lesions included sloughing of horns and lesions of cardiac,

diaphragmatic, lingual and skeletal muscles. The outbreak in 2007 affected 10–15% of the local population with reportedly high case fatality. Lesions in this outbreak were similar to the 1985 events, but also included pancreatic necrosis and inflammation (Berkowitz et al., 2010).

Subsequent to the 1985 outbreaks, an experimental infection of gazelles resulted in four of eight inoculated animals dying during the first 15 dpi and two additional animals dying at 114 and 117 dpi following the development of inappetence, weakness and cachexia (Perl et al., 1989). The animals that died early had lesions consistent with those seen in the natural infection; whereas the two gazelles that died 114 and 117 dpi had marked pancreatic changes consisting of atrophy of exocrine and endocrine tissue, inter-lobular oedema and acinar necrosis. In one animal, the islets of Langerhans were absent. The authors speculated the two gazelles died from complications of diabetes mellitus as has rarely been described in cattle (Barboni and Manocchio, 1962). Considering the pancreatic lesions described, other syndromes that may have contributed to the fatalities include exocrine pancreatic insufficiency (late stage deaths) and pancreatitis (acute and late stage deaths). Interestingly, severe pancreatic lesions involving both exocrine and endocrine compartments have also been associated with experimental infection of pronghorn (*Antilocapra amercana*; Rhyan et al., unpublished data).

Naturally occurring FMD has been observed in European bison (*Bison bonasus*) on reserves and in zoos, and in North American bison (*Bison bison*) and hybrids in zoos (Folmer, 1939). In natural infections, lesions have included typical vesicles, myocarditis and pneumonia. An experimental contact infection study in yearling North American bison demonstrated intraspecies transmission and transmission from cattle to bison (Rhyan et al., 2008). Clinical signs were observed 2 dpi, and development of lesions paralleled that seen in cattle. Vesicles progressing to erosions on the palate, dental pad and gingiva, especially adjacent to the incisors, were common. Rumen pillar lesions were present in one animal. One bison euthanized 6 dpi had focal myocarditis characterized by mixed mononuclear inflammatory cell infiltrate surrounding and infiltrating one Purkinje fibre with focal necrosis of individual cells in the fibre. Oropharyngeal specimens from three bison taken at 22 dpi were negative by RT-PCR and virus isolation.

Naturally acquired and experimental FMD has been described in several cervid species (reviewed in Pinto, 2004). Natural infection occurred in mule deer (*Odocoileus hemionus*) concurrent with an outbreak in cattle in California in 1924–1925 (Keane, 1927). Disease control efforts included culling of approximately 22 000 deer. Ten per cent of the deer had typical FMD lesions including oral and pedal vesicles, erosions and ulcers with at least some mortality. A recent, experimental contact infection study in mule deer demonstrated a fulminant, high-fatality syndrome with marked oral, pedal and ruminal vesicles, myocardial necrosis and intra- and inter-species transmission with cattle (Fig. 1; Rhyan et al., 2006). Immunohistochemical analysis indicated tropism of FMDV for multiple tissues including muscle (Arzt and Rhyan, unpublished data). Amongst other cervid species, elk are relatively resistant to FMDV (Rhyan et al., 2008), whereas white-tailed deer are relatively susceptible (McVicar et al., 1974).

Among wild Suidae, few field observations and experimental studies have been reported. Warthogs (*Phacochoerus aethiopicus*) are considered highly susceptible to SAT viruses with severity of disease depending largely on the strain of virus present (Bengis and Erasmus, 1988). Viral excretion is less than that seen in cattle, buffalo and domestic pigs. In one study, inoculation of warthogs with SAT 2 produced viraemia, lesions on the feet, accessory digits and kneeling pads, and one oral lesion on the gingiva of one of five warthogs (Hedger et al., 1972). Virus did not persist and warthogs did not become carriers. Two bush pigs (*Potamochoerus porcus*) infected with SAT 2 developed severe vesicular lesions on the feet and accessory digits (Hedger et al., 1972). Viraemia was detected in one animal and one animal developed lesions on the snout. Carrier status was not detected in bush pigs.

Natural infection in wild boar (*Sus scrofa*) has been observed in zoos and wild populations in several areas of the world (Hone, 1990). In Israel, between the years 1986 and 1995, FMDV was isolated from specimens from 8 of 47 wild boar sampled; a serologic survey showed the majority of wild boar from three areas had evidence of viral replication (Yadin and King, unpublished data). The outbreak of FMD in domestic livestock in Bulgaria in 2011 was immediately preceded by the discovery of FMDV in a wild boar with foot lesions located 2 km from the Turkish border (ProMED-mail archive 20110107.0091). Sequence analysis of virus obtained from the boar indicated >99% VP1 nucleotide homology to PanAsia-2 viruses in Turkey and Iran (Valdazo-González et al., 2011). This somewhat circumstantial evidence suggests that incursion may have occurred via movement of infected boar from Turkey and further suggests that wild boar were competent to transmit FMDV to domestic livestock.

Observations of natural and experimental FMD in wildlife species suggest that the pathogenesis of infection in wild ruminants and suids is, in general, similar to that seen in their domestic counterparts. Based on reported studies, several aspects of pathogenesis are seemingly host species dependant including susceptibility to infection,

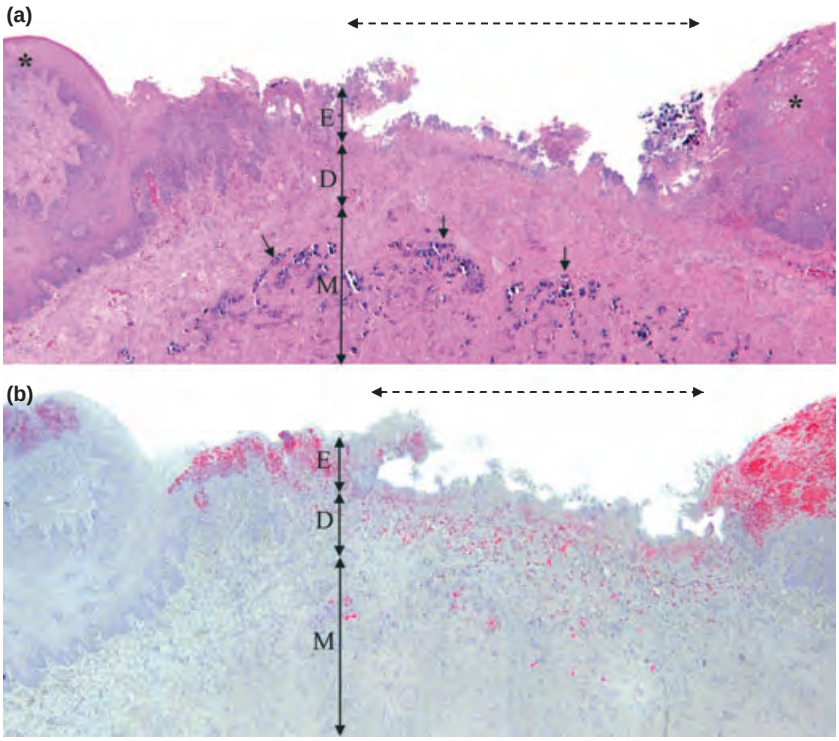

Fig. 1. Multitropic foot-and-mouth disease virus (FMDV) infection of epithelium and muscle. Lingual vesicle from mule deer, 8 days post-contact (bovine) exposure to FMDV-O1-Manisa. Extensive erosion is delineated by horizontal dashed arrow. Vertical arrows delineate extents of epithelium (E), dermis (D) and musculature (M). (a) Microvesiculation is present in epithelium flanking the erosion (*). There is extensive necrosis and mineralization of myocytes (short arrows). H & E stain, 2×. (b) Anti-FMDV VP1 immunohistochemistry applied to serial section of tissue in panel (a). Greatest intensity of virus staining (red) is within epithelium with moderate virus localization within dermis (presumptive phagocytic cells) and myocytes. Images from Rhyan and Arzt, unpublished data.

severity of disease, location of vesicular lesions, predilection for myotropism, ability to transmit infection and competence as persistent carriers. However, as with domestic species, all of these aspects of infection are also dependent upon virus-specific factors and unique interactions between virus and host. The pathogenesis events that result in high case fatality in some wildlife outbreaks are likely variable and multifactorial. Contributory components include myocarditis, pancreatitis and inability to ambulate leading to insufficient food intake and increased susceptibility to predation. Overall, improved understanding of pathogenesis-defining events in wildlife species has the potential to contribute substantially to elucidation of similar and distinct events in domestic species.

Myotropism of FMDV

Classical FMD is generally a syndrome of high morbidity and low mortality with manifestations of infection limited to epithelial sites. However, as early as 1912 there were descriptions of high-mortality FMD outbreaks (Bang, 1912). This relatively rare clinical variant came to be known as 'malignant FMD' and was described as a more common feature among young FMDV-infected animals as compared to adults. Indeed, case fatalities of 50% to 80% were documented among calves, and in piglets under 14 days old mortality approached 100%. There are also reports of high mortality associated with FMDV infection in wild ruminants, such as gazelle (Shimshony et al., 1986) and impala (Hedger et al., 1972). Although fatalities associated with FMD are often attributed to myocarditis, there is relatively little specific investigation into this syndrome and the pathogenic mechanisms remain poorly defined.

One of the first reports indicating the cause of high-mortality FMDV described myocardial lesions in cattle which died shortly after infection, including inflammatory infiltrates and cardiomyocyte degeneration and necrosis (Huguenin, 1924). Similar histological findings were later reported by Epstein (Epstein, 1951). Recently, a phenotypic characterization of naturally occurring myocardial lesions in lambs concluded that infiltrates were mixed (lymphohistiocytic and neutrophilic); integrin expression consisted of α5 expression in myocytes and heterogenous α5 and αV amongst inflammatory cells (Gulbahar et al.,

2007). In fulminant necrotizing myocardial lesions in cattle, FMDV antigens are largely limited to cardiomyocytes (Fig. 2). It has also been suggested that lesions of the conduction system (SA-Node, AV-Node, AV-Bundle and the Purkinje fibres) of the heart were associated with mortality because of FMDV (Martini, 1936). This was supported by the later finding of electrocardiographic abnormalities, including prolonged R–R values in FMDV-infected cattle (Bazhenov, 1971). In addition to young cattle, FMDV-associated myocardial necrosis and myocarditis has been documented in pigs (Potel, 1967), sheep (Salyi, 1939), goats (Bhalla and Sharma, 1965), adult cattle, gazelle (Shimshony, et al., 1986) and mule deer (Rhyan and Arzt, unpublished data).

There is limited evidence of concurrent lesions of skeletal and cardiac muscle in FMDV-infected cattle (Korn and Potel, 1954) and mule deer (Fig. 1). The former study found that lesions of both types of striated muscle were present in many FMDV-infected calves regardless of whether the calves died from infection. Additionally, viral RNA has been detected in the heart and skeletal muscle of both foetal and neonatal lambs via qRT-PCR and *in situ* hybridization after infection with FMDV (Ryan et al., 2007a). Subsequent work demonstrated the presence of structural and NS FMDV proteins *in situ* in heart and skeletal muscle of infected lambs and foetuses regardless of whether the animals died as a result of FMDV infection (Waters et al., unpublished data). Recently, utilization of serum biomarkers in FMDV-infected animals has indicated that subclinical myocardial damage may occur far more frequently than indicated by case fatality alone (Waters et al., unpublished data).

Collectively, these findings suggest that FMDV myotropism occurs via specific virus–host interactions which dictate permissiveness to infection of either or both types of striated myocytes in specific situations. Myotropism may occur with or without concurrent vesicular lesions. The presence of concurrent vesicles defines conditions of expanded multitropism (muscle and epithelium), whereas the absence of vesicles suggests substitution of tropism (muscle, but not epithelium). It is likely that such variations are defined by combinations of viral and host factors and that there are specific interactions which determine the greater susceptibility of developing (i.e. young) animals to these conditions. Distinct expression patterns of αV integrins in certain animals is one potential category of host factors which might define predilection for myotropism; however, this is highly speculative and certainly other host factors may be involved. Additionally there is some evidence that specific strains of FMDV may have a greater predisposition for myotropism regardless of host factors. Overall, the economic impact attributed to high mortality of cardiotropic strains of FMDV and concern regarding potential use as agroterrorism weapons provide justification for additional investigation into the pathogenesis of this poorly understood variation of FMD.

Chronic Sequelae of Foot-and-Mouth Disease

As FMD-free nations typically control incursions by depopulation of affected and at risk animals, there is never a chance to observe FMD-convalescent animals under natural conditions in these regions. Such 'late stage' pathogenesis events have not been investigated in containment laboratories because of the cost associated with long-term maintenance of animals and the perception that chronic FMD sequela are not research priorities. However, in FMD-enzootic regions, animals are allowed to survive and convalesce subsequent to FMD. As such, a distinct group of chronic post-FMD syndromes have been described which are independent of the carrier state (Alexandersen et al., 2003b). With the exception of permanent losses from case fatalities, most animals recover from FMD within 8–15 days. However, there are complications and chronic long-term sequelae to FMD which have been reported as important contributors to significant economic losses (Barasa et al., 2008).

The most common complications after recovery are the results of severe inflammation associated with rupture of vesicles, especially of the feet and teats, which predispose the affected areas to secondary infections. Although sloughing of vesicles typically results in erosion, ulceration occurs less frequently and thus exposes the deeper subepithelia to environmental microbes (Arzt et al., 2009). Severe inflammation of the feet may often cause the horn of the hoof to separate from the digit (more frequent in pigs than ruminants) and in severe cases the hoof may be completely shed. Secondary bacterial infection often leads to under-running of horn tissue, chronic hoof malformations and lameness (Ghanem and Abdel-Hamid, 2010; Orsel et al., 2010). In dairy cattle, it is clear that acute FMD impairs milk production and in some cases milk secretion may stop until the subsequent lactation, and/or remain depressed indefinitely (Minett, 1948). Healing of teat lesions following FMD is often delayed in lactating animals because of milking or nursing. As a consequence, animals are susceptible to recurrent mastitis, particularly associated with causative agents that require primary teat injury to establish infection (Saini et al., 1992). Additionally, failure of milk supply may contribute to the relatively higher mortality rates in calves with FMD compared to adults (Barasa et al., 2008).

Perhaps, the least well-described and most perplexing sequel to FMD is the complex of clinical signs variably referred to as 'heat-intolerance syndrome (HIS)', 'hairy panters', 'asoleadas' (in Argentina) and 'peludas' (in

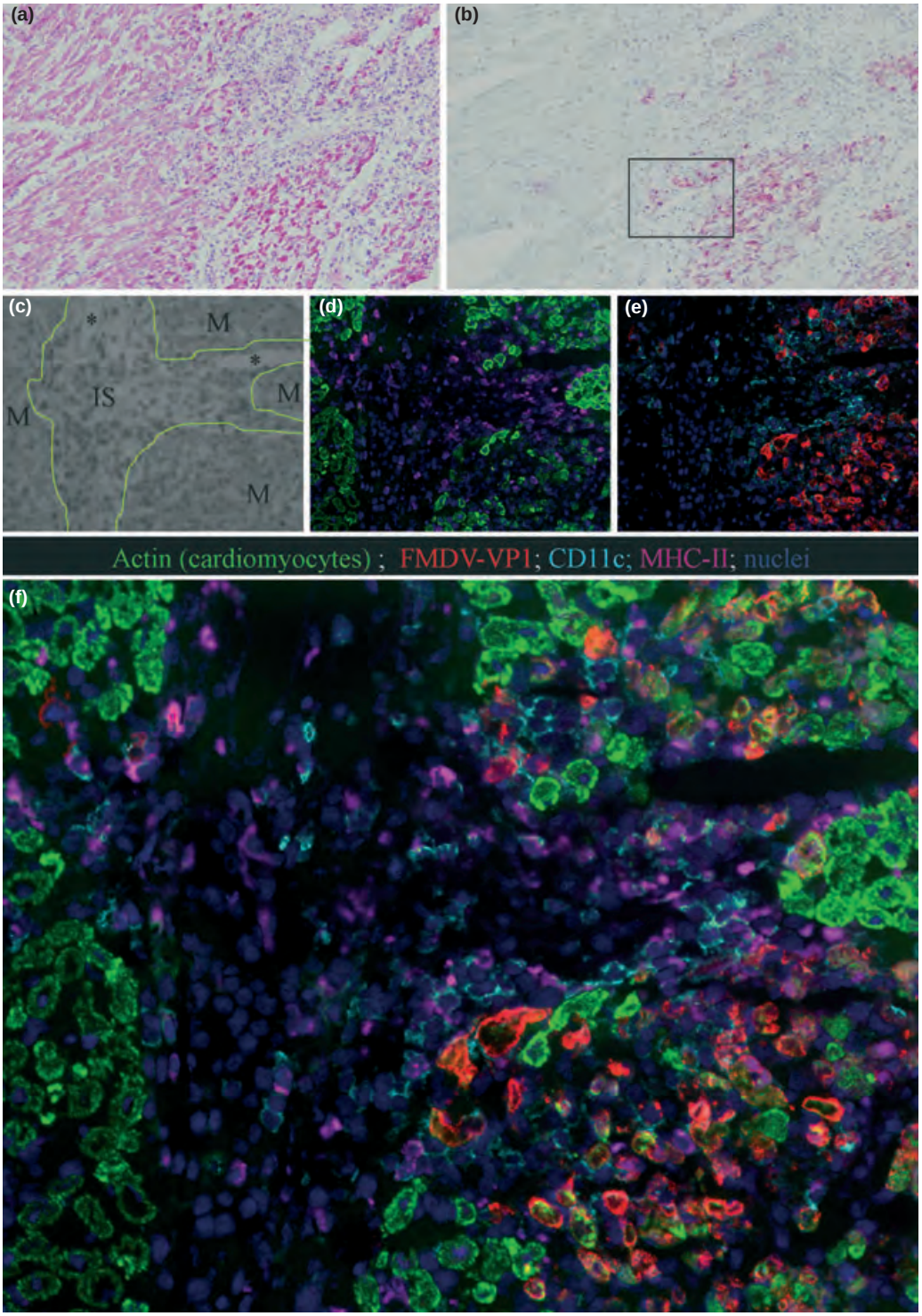

Fig. 2. Foot-and-mouth disease virus (FMDV)-induced necrotizing myocarditis. (a–f) Right atrium from adult steer found dead 7 days after intradermolingual inoculation with FMDV-O1-Manisa. (a) Routine H & E stain demonstrates hypereosinophilic cardiomyocytes and interstitial inflammatory infiltrates. (b) Anti-FMDV-VP1 immunohistochemistry shows localization of FMDV to region of necrosis in (a); box indicates region shown in (c–f). (c) Map of immunofluorescent images: M regions delineated by green lines are cardiomyocyte areas; IS, interstitium; *vessel lumina. (d–f) Simultaneous multichannel immunofluorescence. Monoclonal antibodies: actin = green, FMDV-VP1 = red, CD11c = light blue, MHCII = purple. Foot-and-mouth disease virus colocalizes with actin in cardiomyocytes. MHCII and CD11c positive dendritic cells and macrophages are abundant in interstitium, but also infiltrate and dissect infected myocytes.

Brazil; M. Hugh-Jones, personal communication). These syndromes were first reported as sequelae to FMD prior to the implementation of major control efforts in Europe and South America (reviewed by Minett, 1948). The condition has also been described in India and Pakistan (Mullick, 1949; Maqsood et al., 1958; M.S. Maddur, personal communication); recently, there have been a number of reports in Africa (Catley et al., 2004; Barasa et al., 2008; Ghanem and Abdel-Hamid, 2010) and anecdotal description in Turkey (N. Bulut, personal communication).

The condition is characterized clinically by intolerance to increased environmental temperatures. Pronounced panting during hot weather is the striking feature accompanied by increased body temperature and pulse rate (Minett, 1948; Maqsood et al., 1958). Other typical signs include abnormal hair growth described as either hirsutism (Ghanem and Abdel-Hamid, 2010) or hypertrichosis as a result of failure of cyclic seasonal shedding of hair (Minett, 1948; Maqsood et al., 1958). Affected animals do not thrive and are usually emaciated although an obese form has also been described (Domanski and Fitko, 1959). Milk production is severely reduced (Ghanem and Abdel-Hamid, 2010), and there are often reproductive disturbances including anoestrus, nymphomania, and abortions or birth of dead and weak calves (Minett, 1948; Maqsood et al., 1958; Domanski and Fitko, 1959). The consensus from the literature is that there is no permanent natural recovery with signs recurring during hot weather for the duration of affected animals' life. One report indicates 13% case fatality associated with the condition in a single herd with the deaths attributed to cardiovascular disturbances (Nai, 1936). More commonly, economic losses come from poor productivity and resultant culling (Minett, 1948).

An early investigation into haematologic and biochemical alterations amongst panters demonstrated anaemia, hyperphosphataemia, lactic acidosis, hypoproteinaemia, and hypocalcaemia (Mullick, 1949). These findings have recently been supported by a study of dairy cattle in Egypt with confirmed exposure to FMDV approximately 3 months before signs of HIS (Ghanem and Abdel-Hamid, 2010). In addition to the changes demonstrated by Mullick, serum Na, Cl, Mg, Zn, Fe, albumin, cholesterol, and cortisol were significantly reduced. Serum levels of ALT, AST, and monoaldehyde were significantly increased in affected cows; serum glucose was also significantly increased although hyperglycaemia was considered mild and below the cut-off for the diagnosis of diabetes mellitus in affected cattle.

The pathophysiology of these disturbances and their relation to acute FMD are not clear. Minett examined myocardium, liver, spleen, kidney, pituitary, thyroid and adrenal glands from cattle in India with signs of HIS and detected lesions only in the thyroid (Minett, 1949). The morphology was generally consistent with thyroid hyperplasia and suggestive of hyperthyroidism. Similar changes in thyroid morphology of affected cattle have been reported by other investigators (Maqsood et al., 1958; Domanski and Fitko, 1959). However, several factors confound attempts to deduce thyroid functionality from histological properties (Abbot and Prendergast, 1934; Jelinek et al., 2003). Proper assessment of thyroid function requires evaluation of serum T3/T4 levels in the context of serum TSH and TRH. As such data have never been reported for HIS cattle, the relationship between HIS and thyroid function remains undetermined. Early investigators' efforts to elucidate the functional derangements of HIS have included administration of thyroxin which aggravated the symptoms (Mullick, 1949) and administration of thiouracil which alleviated the symptoms (Maqsood et al., 1958). These findings generally support the hypothesis that there is an interference with the hypothalamic-pituitary-thyroid axis; however, precise interpretation is less than completely straightforward.

Additional histological changes that have been reported in HIS cattle include adrenal cortical atrophy of *zona fasciculata* and either atrophy or hypertrophy of *zona reticularis* (Domanski and Fitko, 1959). Lymphocytic inflammation of the pituitary has been described with *pars anterior* affected more severely than *pars intermedia* and *pars nervosa* (Nai, 1936). Another report describes degranulation of pituitary β-cells and decreased hypopyseal weight amongst HIS cattle (Domanski and Fitko, 1959). Additional evidence for disruption of endocrine function in FMD comes from descriptions of pancreatic necrosis in cattle during clinical FMD and in cattle that have died or completely recovered (Nai, 1940; Manocchio, 1974). Disruption of these endocrine organs could, in part, account for the biochemical changes reported by Ghanem and Abdel-Hamid in heat intolerant cattle and the hyperglycaemia and hypoinsulinaemia detected during clinical FMD (Yeotikar et al., 2003; Ghanem and Abdel-Hamid, 2010; Nahed, 2010).

Foot-and-mouth disease virus has been isolated from the pituitary glands of viraemic and post-viraemic cattle which were needle-challenged with type A, O and C strains of FMDV, providing additional support for the histological observations during HIS (Scott et al., 1965). Foot-and-mouth disease virus was present in the pituitary glands during the early clinical (1–3 dpi), clinical (2–4 dpi) and convalescent (8 dpi) stages of disease. The viral titres in the pituitary during the clinical stage were higher than the corresponding serum titre, which supports FMDV concentration or replication within the pituitary. However, persistent detection of FMDV after the

cessation of viraemia provides more compelling evidence that viral replication may occur at this site.

The published reports describing post-FMD HIS are sufficiently compelling to suggest that distinct syndromes with unique virus–host interactions do exist. Additionally, the various abnormalities of the hypothalamo-pituitary-endocrine axes during acute FMD support the hypothesis that virus-induced effects to these organs may cause permanent damage and metabolic derangement. However, it must be emphasized that all the reported cases and investigations into heat-intolerance syndrome are based on field observations which do not exclude other underlying pathogens or non-infectious pathoaetiologies. Thus, the described syndromes could be the result of infection with FMDV combined with other pathogens or metabolic dyscrasias. The association of HIS with European breeds (and crosses) of cattle in Asia may be significant to the underlying pathogenesis (Minett, 1949; Mullick, 1949; Ghanem and Abdel-Hamid, 2010; M.S. Maddur, personal communication); however, this apparent susceptibility, similarly, may reflect various pathoaetiologies including unique nutritional needs or pathogen vulnerabilities amongst the European breeds.

Although the web of causality has not been elucidated, there is abundant support that HIS is a sequela of FMD in cattle. Ghanem and Abdel-Hamid reported that approximately 7% of the herd showed clinical signs of HIS following FMD (Ghanem and Abdel-Hamid, 2010). Similar figures have been reported by livestock producers in Tanzania (Catley et al., 2004), and HIS has been reported to account for approximately 28% of the total FMD-associated losses in south Sudan (Barasa et al., 2008). However, because of the insidious and inconsistent nature of this syndrome, it is rarely accounted for in economic analyses and modelling studies. Thus, the economic losses associated with HIS are a potentially significant and often overlooked element of the impact of FMD in enzootic regions. Further investigation into the pathogenesis and economic impact of HIS would contribute to better understanding of the significance of this syndrome at the individual animal and population levels.

FMD Molecular Pathogenesis

Numerous studies have been conducted *in vitro* to characterize FMDV molecular pathobiology and virus–host interactions, and this body of work has been thoroughly reviewed (Mason et al., 2003; Grubman et al., 2008; Summerfield et al., 2009). These studies have provided invaluable insights which could not possibly have been achieved by studies in live animals. Some of these results have been confirmed to be relevant *in vivo*; however, as is often the case, translation of *in vitro* findings to improved understanding of natural infection of live animals is often obscure. The *in vitro* studies can be broadly divided into two main topic categories: interaction of viral structural proteins with host receptors and intracellular mechanisms involving viral NS proteins and host factors.

Interaction of viral structural proteins with host receptors

Foot-and-mouth disease virus contains a single-stranded positive-sense RNA genome of approximately 8500 nucleotides surrounded by an icosahedral capsid composed of 60 copies each of four structural proteins VP1, VP2, VP3, and VP4 (also termed 1D, 1B, 1C and 1A; Rueckert and Wimmer, 1984; Grubman and Baxt, 2004). The viral protein VPg (3B) is covalently linked to the 5′ end of the genome (Sangar et al., 1977; Grubman, 1980). The three-dimensional structure of a number of FMDV serotypes has been determined by X-ray crystallography (Lea et al., 1994, 1995; Curry et al., 1996; Fry et al., 1999). The structural proteins VP1, VP2 and VP3 fold into eight-stranded β barrels which fit together to form the majority of the virus particle, whereas VP4 protein is buried on the inside of the capsid (reviewed in Grubman and Baxt, 2004). The β barrel strands of VP1, VP2 and VP3 are connected by loops which form the outer surface of the virus particle. Of particular interest regarding the tropism of FMDV is a prominent surface exposed loop connecting the βG and βH strands (known as the G–H loop) of VP1. This loop contains the highly conserved sequence, arginine-glycine-aspartic acid (RGD), which has been shown to be a recognition sequence for the integrin family of cell surface receptors enabling binding and subsequent virus internalization into acidic endosomes (Pierschbacher and Ruoslahti, 1984a,b; O'Donnell et al., 2005).

Integrins are type I heterodimeric membrane proteins consisting of an α and β subunit and are involved in cell adhesion, signalling, cell migration and thrombosis (Hynes, 2002). Of the 24 known integrins, 8 bind the RGD tripeptide. Of the RGD-binding integrins, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$ and $\alpha v\beta 8$ appear to be the major receptors for FMDV in cell culture (Berinstein et al., 1995; Jackson et al., 2000, 2002, 2004; Neff et al., 2000; Neff and Baxt, 2001; Duque and Baxt, 2003). Additionally, recent studies in cattle and sheep have demonstrated that $\alpha v\beta 6$ is expressed constitutively on the surface of epithelial cells at sites which are normally targeted by FMDV, while $\alpha v\beta 3$ was detected at epithelial sites that are not FMDV-permissive and also found in close association with blood vessels in various tissues (Monaghan et al., 2005a; Brown et al., 2006; O'Donnell et al., 2009). The $\beta 1$ integrin was shown to be present at lesion-predilection sites with distribution limited to basal cells (Monaghan et al., 2005a).

However, the only integrin receptor of FMDV detected on FMDV-positive cells in lingual vesicles was $\alpha_V\beta_6$ (Monaghan et al., 2005a; O'Donnell et al., 2009). Similarly, simultaneous localization of FMDV antigens with $\alpha v\beta 3$ or $\alpha v\beta 6$ integrins at primary infection sites in the nasopharynx indicated that only $\alpha v\beta 6$ is detectable in infected pharyngeal epithelial cells (Arzt, unpublished data). The role of $\alpha_V\beta_8$ has not been assessed because of the lack of availability of an appropriate antibody. These results suggest that $\alpha v\beta 6$ and $\beta 1$ integrins may be requisite receptors for FMDV infection *in vivo* and furthermore that these integrins may be important determinants of cellular tropism.

Research utilizing infectious FMDV cDNA clones with mutated or deleted RGD sequences gave rise to virions that were non-infectious in tissue culture and could not cause disease in susceptible animals (McKenna et al., 1995; Rieder et al., 1996; Leippert et al., 1997). Although the RGD motif is highly conserved in FMDV field isolates, recently, a serotype A virus isolated from cattle was shown to possess a SGD sequence in place of RGD (Rieder et al., 2005). The 'SGD virus' showed strong preference for utilization of the $\alpha_V\beta_6$ receptor and exhibited a selective advantage to initiate infection in cattle relative to the RGD-containing virus counterpart.

Despite strong evidence for the importance of α_V integrins as FMDV receptors, there is also evidence which strongly suggests that in addition to receptor expression, other cell-specific or tissue-specific host factors are essential co-determinants of tropism (Whitton et al., 2005). In particular, it is clear that when an animal becomes infected with FMDV, numerous tissues and cells with high $\alpha v\beta 6$ expression do not support FMDV replication (O'Donnell et al., 2009). This is similarly true for primary (aerogenous) and secondary (haematogenous) phases of exposure to virus. Additionally, because the distribution of $\alpha v\beta 6$ integrin in the epithelia at lesion-predilection sites is diffuse, it is not clear why FMD vesicles are focal and well delineated. It has been proposed that the distribution of initiation of lesions reflects the pattern of exit of virus from the blood at the predilection sites (Yilma, 1980; Arzt et al., 2009). Yet considering the role of integrins in tropism, the real question is 'once the virus initiates a lesion, why doesn't every $\alpha v\beta 6$-positive cell in the region become infected?'. Consideration of this question should include the potential roles of facilitating factors within infected cells as well as resistance-determining factors either in adjacent (uninfected) cells or within the extracellular space. While the tropism-defining role of $\alpha v\beta 3$ is less well defined than that of $\alpha v\beta 6$, the (peri)vascular distribution makes it attractive to suggest that this integrin may contribute to enabling the virus's ability to enter or exit blood (or lymphatic) vessels.

Further evidence to support a role for $\alpha v\beta 6$ in pathogenesis has come from recent studies which showed that the viral integrin-binding site appears most highly adapted for binding to $\alpha v\beta 6$ (Burman et al., 2006). Virus binding to this integrin occurs in a two-step process that involves an initial cation-dependent interaction with the RGD and a second stabilizing or 'synergy' interaction that was resistant to EDTA dissociation (Dicara et al., 2008). The stabilizing interaction was shown to be dependent on two conserved leucine residues in the RGD +1 and +4 positions and the integrity of a helical structure immediately C-terminal to the RGD sequence. In contrast, stable binding of FMDV to $\alpha_V\beta_3$ was not observed (Dicara et al., 2008). In studies of viral entry utilizing both biochemical and confocal microscopic analyses, it was shown that serotype A, O and C viruses enter early endosomes via clathrin-mediated endocytosis, followed by trafficking into recycling endosomes (Berryman et al., 2005; O'Donnell et al., 2005; Martin-Acebes et al., 2007). While it was initially believed that viral RNA was released from recycling endosomes, recent results, using dominant-negative rab protein mutants showed that the viral RNA was released primarily from the early endosomes (Johns et al., 2009). Why the virus was observed moving into later endosomal compartments is not clear at this point. However, $\alpha v\beta 6$ was also shown to traffic through early and recycling endosomes, suggesting that viral pentamers may remain attached to the integrin during the entry process (Berryman et al., 2005). Taken together, these results provide additional support for the role of $\alpha_V\beta_6$ integrin as the major viral receptor utilized in susceptible animals.

Foot-and-mouth disease virus can also utilize non-integrin receptors to initiate infection. Several studies have shown that FMDV can infect cultured cells via F_c receptor-mediated antibody uptake (Mason et al., 1993, 1994; Baxt and Mason, 1995), although it is not known if this occurs *in vivo*. It has recently been demonstrated that immune-complexed FMDV can productively infect and kill bovine monocyte-derived dendritic cells *ex vivo* via a CD32 (FcγR)-mediated mechanism (Robinson et al., 2011). Additionally, multiple passages of type O viruses in cell culture resulted in viruses capable of utilizing the heparan sulphate (HS) receptor and correlated with selection of viruses containing an extra positive charge on the virion surface (Sa-Carvalho et al., 1997; Fry et al., 1999). Inoculation of high doses of HS-utilizing viruses into cattle resulted in clinical lesions but the viruses recovered from these animals could only utilize an integrin and not the HS receptor. These results suggest that HS binding may not play a role in pathogenesis *in vivo*. In contrast to integrin-binding viruses, HS-binding viruses enter cells via a caveola-mediated mechanism with subsequent virus trafficking

to acidic early endosomes. These results have provided novel insights into FMDV endocytosis as they demonstrate that FMDV can use multiple entry mechanisms that lead to acidic endosomes (O'Donnell et al., 2008). Yet the relevance of these mechanisms to naturally occurring FMDV infection *in vivo* remains to be determined.

There is also evidence supporting the existence of an additional, currently unidentified FMDV receptor that is distinct from integrins, HS and immunoglobulin/Fc-mediated mechanisms (Baranowski et al., 1998; Zhao et al., 2003). Only a small number of viruses appear to infect cells in this way and only one such virus has been reported to establish infection in pigs (Zhao et al., 2003). The role of these additional receptors in pathogenesis remains speculative, and further experiments are required to establish their importance *in vivo*.

A biochemical feature of FMDV that may contribute to its tropism is the acid lability of the virion. Below pH 6.5, the virus particle dissociates into pentamers presumably as a result of electrostatic repulsion between a cluster of histidine residues at the interface between VP2 and VP3 that are protonated at low pH (Curry et al., 1995; Ellard et al., 1999). This instability at low pH may be involved in the targeting of productive virus replication to specific tissues and ensures that infectious virus cannot pass beyond the stomach of monogastric hosts or the abomasum of ruminants.

Intracellular molecular pathogenesis

Upon infection of cells, the FMDV RNA genome is released into the cytoplasm and translation begins via an internal ribosome entry site (IRES) located approximately 1300 bases from the 5′ end. The RNA is translated as a single long open reading frame (ORF) into a polyprotein which is processed mainly by viral encoded proteinases to initially generate the leader (L) protein and three precursor proteins P1-2A, P2 and P3. Subsequent processing of the three precursor proteins yields the four viral structural proteins and nine additional NS proteins. As seen in other picornaviruses, some of the intermediate protein products synthesized by FMDV contribute to various viral functions (Leong et al., 2002; Moffat et al., 2005, 2007). However, many of the functions and interactions of FMDV NS proteins with host factors remain unclear. In addition to the ORF, the 5′ and 3′ untranslated regions of the genome also have significant roles in virus translation as well as replication (Mason et al., 2003).

Virus replication occurs in the cytoplasm although some viral NS proteins including L, 3D and precursor 3CD also translocate to the nucleus (Garcia-Briones et al., 2006; de los Santos et al., 2007). The NS proteins perform a number of functions during the virus replication cycle that promote virus production and block or limit host responses. These include (i) processing of the viral polyprotein by L and 3C proteinases and the 18 amino acid, viral peptide 2A, (ii) replication of the viral genome by the viral RNA-dependent RNA polymerase, 3D, a process which also requires NS protein 3B to initiate RNA synthesis and 2C (Saunders and King, 1982), (iii) cleavage of host proteins including translation initiation factor eIF4G by L resulting in marked down-regulation of host protein synthesis (Devaney et al., 1988) and cleavage of histone H3 (Tesar and Marquardt, 1990) and Sam68 (Lawrence and Rieder, personal communication) by the FMDV 3C proteinase, (iv) inhibition of host transcription as a result of nuclear cleavage of transcription factor nuclear factor kappa B (NF-κB; Grigera and Tisminetzky, 1984; Falk et al., 1990; de los Santos et al., 2007), and (v) inhibition of trafficking of proteins through the endoplasmic reticulum/Golgi secretory pathway by 2B and 2C and/or 2BC (Moffat et al., 2005, 2007). The NS proteins 2B, 2C and 3A also play a role in rearrangement of host cell membranes which become the sites for viral RNA replication and capsid assembly (O'Donnell et al., 2001; Knox et al., 2005; Pena et al., 2008).

Although some advance has been made in recent years, viral determinants of virulence for FMDV in natural hosts remain undefined. Among the most thoroughly investigated virulence factors is the L proteinase. Virus constructs lacking the L sequence (leaderless FMDV) have been shown to be avirulent in cattle (Brown et al., 1996) and pigs (Chinsangaram et al., 1998). Cattle exposed to leaderless FMDV by aerosol did not show viraemia or clinical signs of FMD, had minimal quantities of viral RNA detected in lungs by ISH and no viral RNA detected in any other tissue. More recently, FMDV mutants with in-frame insertions in L were shown to be markedly attenuated in cattle (Piccone et al., 2010). These viruses had a more extensive dissemination within the respiratory tract (compared to leaderless FMDV) after aerosol inoculation in cattle; but similar to the leaderless virus, there was neither viraemia nor clinical disease. However, the multifactorial nature of FMDV virulence was suggested by the finding that some leaderless FMDV constructs retained partial virulence (Almeida et al., 1998).

A number of studies suggest that NS protein 3A may also play a role in virus tropism and host range (Giraudo et al., 1990; Beard and Mason, 2000; Nunez et al., 2001). The FMD virus, which caused the 1997 outbreak in Taiwan, was relatively unique in that it only affected swine and was not virulent to cattle. Molecular characterization of the virus revealed a deletion in the 3A coding region, and reverse genetics demonstrated that the mutated 3A was partially responsible for the porcinophilic phenotype of the virus (Beard and Mason, 2000). Interestingly, it

had been previously shown that egg-adapted live vaccine candidates of FMDV containing deletions in 3A of similar size and location to the Taiwan virus were attenuated for cattle but not for swine (Giraudo et al., 1990). Furthermore, it was subsequently shown that a point mutation in 3A, at a distinct site from the 3A deletion, resulted in adaptation of an FMDV isolate to cause disease in guinea pigs (Nunez et al., 2001). Collectively, these data indicate that 3A contains multiple viral virulence determinants.

Tissue culture-based works have indicated that FMDV also utilizes a number of host proteins to promote various aspects of its replication (Pilipenko et al., 2000; Martinez-Salas et al., 2001; Lawrence and Rieder, 2009). Host cellular proteins have been found to interact with both viral RNA and viral proteins and enhance viral translation and replication. It was recently demonstrated that RNA helicase A, a methylated nuclear protein, is redistributed in a non-methylated form to the cytoplasm during FMDV infection and interacts with NS proteins 2C and 3A as well as with the 5′ end of the FMDV genome to promote virus replication (Lawrence and Rieder, 2009). This interesting observation provides a clear example of FMDV targeting a specific host cell protein and altering both its subcellular localization and biochemical composition for its own benefit. Additionally, canonical and non-canonical host translation factors have been shown to be involved in FMDV IRES-mediated translation (reviewed in Pacheco et al., 2009). At present, FMDV's exploitation of host mechanisms for viral replication and translation have not been thoroughly investigated in live animals.

Conclusions

Foot-and-mouth disease is well known for the classic manifestations from which the name of the disease has been derived. It is infrequently associated with high-fatality outbreaks, abortion and chronic wasting syndromes. However, these are all within the range of manifestations of FMD and surely represent definable pathogenesis events at the cellular and molecular levels of virus, host and the interaction between the two. Many aspects of these events have been characterized in live animals and/or in tissue culture-based studies; yet much remains incompletely elucidated.

There are several aspects of the 'lesser' FMD syndromes and species described herein that are of critical importance to FMD research. For example, it is often stated that the disastrous impact of the European FMDV-O-Panasia outbreaks of 2001 was, in part, because of the delay in proper diagnosis of FMD in sheep subsequent to incursion to the United Kingdom (Ferris et al., 2006; Valarcher et al., 2008). Furthermore, the delay is widely described as a result of the propensity of sheep to have subclinical or clinically mild FMD lacking oral lesions. Thus, the atypical (relative to cattle) ovine pathogenesis led directly to critical failures; whereas improved understanding of this aspect of FMD might lead to novel diagnostic and surveillance practices to prevent recurrence. Similarly, it is well established that several transboundary excursions of FMDV have occurred through movements of live sheep and goats (reviewed in Kitching and Hughes, 2002). Movement of small ruminants often follows distinct patterns, and in some cases may escape the levels of stringency applied to trade of cattle. These differences provide further justification for the investigation into comparative aspects of clinical FMD syndromes between small ruminants and cattle.

Another example of the importance of increased focus on FMD in non-bovine species is related to the observation that FMDV is (apparently) incapable of establishing persistent infection in suids. The ability to prevent or cure the FMD carrier state in ruminants is hugely important to acceptance of 'vaccinate-to-live' policies; and it is possible that the keys to prevent ruminant persistence may lie in elucidating how pigs are naturally able to completely eliminate FMDV.

Improving the understanding of the breadth of variations of FMD pathogenesis in wildlife is, also, of substantial importance. Persistent infection in Cape buffalo is one of the major challenges to control and eradicate FMDV from Africa. The role of Asian buffalo in FMDV ecology is not known; however, elucidation of the role of this species could provide great benefit to control efforts in the vast regions where *Bubalus spp.* exist. Additionally, numerous other susceptible wild animals are present in enzootic regions and likely contribute to unknown extents to FMDV population dynamics. Similarly, susceptible wildlife species are present in most of the FMD-free nations of North America and Europe which could add substantial complications if FMDV were introduced to these regions. In each case, the most pressing questions are (i) whether the relevant species can transmit FMDV within, and across species and (ii) is the species competent as a long-term FMDV carrier?

The molecular pathogenesis studies we have described herein provide the most detailed understanding of how FMDV interacts with host cells. Of particular relevance is the deeper understanding of interactions between viral and host factors, including not only interaction between viral capsid proteins and host cell receptors, but also NS proteins and viral RNA. We are just beginning to unravel these complex virus–host interactions that ultimately determine whether virus – host battles favours the virus resulting in disease or the host resulting in abortive or subclinical infection. These types of studies ultimately will be necessary to explain with depth, the full breadth of

Table 2. High priority knowledge gaps in understanding the pathogenesis of atypical FMD syndromes and species other than domestic cattle

1. What are the sites of primary infection in small ruminants, swine and wildlife species?
2. Why is FMD often mild in small ruminants and (how) might that information be useful to control infection in cattle and swine?
3. What are the mechanisms of shedding of virus into secretions and naturally generated aerosols; why do pigs generate greater quantities of aerosolized FMDV?
4. What are the host (cellular and molecular) determinants of tropism beyond integrins?
5. How can understanding of tropism and primary infection be manipulated to generate 'rationally designed' vaccines?
6. What are the sites and mechanisms of establishment of viraemia in swine and small ruminants?
7. What wildlife species are capable of becoming carriers?
8. What wildlife carrier species are capable of intra- and/or inter-species transmission?
9. What is the likelihood that various wildlife species would could play a significant role in a natural incursion of FMDV into previously free regions?
10. Why are suids unable to establish persistent infection and (how) may this be manipulated to control persistence in ruminants?
11. What unique pathogenesis events occur in gravid females and embryos/foetuses?
12. What are the specific virus–host interactions which determine (cardio)myotropism?
13. What pathophysiological events lead to heat-intolerance syndrome?
14. What is the significance of the heat-intolerance syndrome at the individual animal and population levels?
15. How do virus–host interactions and ecological factors contribute to determination of geographical range of distinct serotypes and strains of FMDV?

manifestations of FMDV infection. One of the critical requirements for FMD research to remain relevant will be the ability of investigators to maintain the symbiosis through which *in vivo* and *in vitro* works are mutually beneficial and feed continuous progress.

The ultimate goal of the study of FMDV pathogenesis is translational application of basic science data towards the eradication of FMD from this planet. Although the announcement of the eradication of rinderpest is anticipated to occur in 2011, eradication of FMD, even by optimistic assessment, is still several decades away (Rweyemamu et al., 2008) and will require substantial breakthroughs in vaccinology and/or biotherapeutics development (Rodriguez and Grubman, 2009). It is likely that the products that will facilitate eradication of FMD will originate in rational design processes which have their basis in basic pathogenesis research. Several critical knowledge gaps in the understanding of FMDV–host interactions have been mentioned throughout this work and are listed in Table 2; a more comprehensive list is included in the companion manuscript (Arzt et al., 2011). It is envisioned that a systematic approach to bridging these gaps will gradually and ultimately bring us closer to FMD eradication.

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ORIGINAL ARTICLE

Factors Associated With Within-Herd Transmission of Serotype A Foot-and-Mouth Disease Virus in Cattle, During the 2001 Outbreak in Argentina: A Protective Effect of Vaccination

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Summary

Argentina suffered an extensive foot-and-mouth disease (FMD) epidemic between July 2000 and January 2002, 3 months after obtaining the official FMD-free without vaccination status conferred by the World Organization for Animal Health. This is one of the largest FMD epidemics controlled by implementation of a systematic mass vaccination campaign in an FMD-free country. In 2000, 124 herds were reported as FMD positive, 2394 herds in 2001 and one in January 2002; the total number of cattle herds in the country at that time was approximately 230 000. Estimates of FMD transmission are important to understand the dynamics of disease spread and for estimating the value for the parameterization of disease transmission models, with the ultimate goals of predicting its spread, assessing and designing control strategies, conducting economic analyses and supporting the decision-making process. In this study, the within-herd coefficient of transmission, β, was computed for herds affected in the 2001 FMD epidemic and categorized as low or high based on the median value of β. A logistic regression model was fitted to identify factors significantly associated with high values of β. Results suggested that the odds of having a high within-herd transmission were significantly associated with time from initial herd infection to disease detection, date of report, vaccination, and time from initial herd infection to herd vaccination. Results presented in this study demonstrate, in quantifiable terms, the protective impact of vaccination in reducing FMD transmission in infected herds. These results will be useful for the parameterization of epidemiological models aimed at quantifying the impact of vaccination and for the design and implementation of FMD emergency vaccination strategies in face of an epidemic.

Introduction

Foot-and-mouth disease (FMD), which is considered one of the most contagious animal diseases (World Organization for Animal Health (OIE), 2009), is endemic in certain regions of Asia, Africa, the Middle East and South America. In South America, the disease was first introduced in the 1870s (Saraiva, 2004). Foot-and-mouth disease has an important economic impact because of the direct losses caused to animal production and the costs

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associated with control of the disease and trade limitations (James and Rushton, 2002). Two of the world largest cattle meat producers, Brazil and Argentina, are located in South America. In the year 2000, there were approximately 230 000 cattle herds in Argentina (SENASA, unpublished observation).

Argentina was recognized as FMD-free-with vaccination in 1996 and FMD-free-without vaccination in May 2000 by the OIE. However, 124 herds were infected in August, 2000, with serotypes A (A/Arg/00) and O (O/Arg/00) FMD viruses. Brazil and Uruguay were also affected by this epidemic. Although the epidemic was considered controlled by the end of 2000 (Correa Melo et al., 2002) by using movement restriction and 'stamping out' strategies, early in 2001, a serotype A FMD virus, A/Arg/01, which was more virulent than A/Arg/00 (Konig et al., 2007; Garcia-Nunez et al., 2010), caused a new epidemic that affected 2519 herds throughout the country (Perez et al., 2004a). Movement restrictions were imposed, and two vaccine doses were applied to susceptible herds surrounding an outbreak through early April 2001, when compulsory mass vaccination of bovine herds twice a year was implemented nationwide except in Patagonia region, which remained FMD-free (Perez et al., 2004a). Differences of serotype A strains A/Arg/00 and A/Arg/01 antigenic sites from vaccine reference strain A24/Cruzeiro resulted in two consecutive vaccine reformulations to incorporate strains A/Arg/00 and A/Arg/01 to the vaccine (Mattion et al., 2004; Konig et al., 2007). Although infected herds were not intended to be vaccinated, certain infected herds with no evident clinical sign of disease were vaccinated at the emergency or at the systematic vaccination campaigns. The epidemic lasted through January 2002 (Perez et al., 2004a,b).

Field data collected from outbreaks occurred through 2001 in the FMD epidemic in Argentina provide important information to estimate parameters that are prerequisite for the formulation of disease spread epidemiological models, such as the within-herd transmission rate (β). Noteworthy, much of the information regarding parameterization of FMD virus transmission that is available in the peer-reviewed literature refers to serotype O strains, likely, because most of the epidemics recently reported in FMD-free countries were caused by serotype O FMD viruses and because serotype O is the most prevalent FMD virus serotype in the world (Orsel et al., 2007; Mardones et al., 2010). Subsequently, within-herd transmission of FMD virus has most frequently been assessed using experimental data (Orsel and Bouma, 2009; Orsel et al., 2009) and simulation models (Carpenter et al., 2004; Keeling, 2005), rather than field data.

In this study, the within-herd coefficient of transmission of serotype A FMD virus was computed using data from a large FMD epidemic that affected Argentina in 2001; 2394 herd outbreaks were reported in Argentina in 2001, in what is considered one of the largest epidemics caused by serotype A FMD virus in an FMD-free country that has been controlled by implementation of a systematic mass vaccination campaign (Perez et al., 2004a). Furthermore, the association between the value of β and epidemiological factors, including vaccination status of the herd, was assessed. Results presented in this study will contribute to the parameterization of FMD spread models and to quantify the impact of vaccination campaigns in face of an FMD epidemic.

Materials and Methods

Data source

Official records from the 2394 FMD outbreaks reported in Argentina from February through December 2001 were obtained from the Argentine National Service for Animal Health and Agri-food Quality (SENASA). An FMD outbreak was defined as a herd in which FMD infection was officially recognized by a SENASA local veterinarian, based on the observation of FMD-like clinical signs and lesions (vesicular lesions in tongue, dental pad, interdigital, coronary and teat, lameness, salivation, pyrexia and mortality in young animals) in at least one animal in the herd, the results of the field investigation and/or serological sampling. Mortality was not considered because of the nil mortality rate, and only bovine herds were analysed because 97% of infected animals were cattle (Mattion et al., 2004).

Each record referred to a single outbreak and contained information about herd size, susceptible animals per age category: <1 year, 1–2 years or >2 years (non-diseased cattle in the herd at the beginning of the outbreak), initial and final (cumulative) number of cattle showing FMD-like clinical lesions, report date, serologic test results (available for some herds), estimated date of FMD virus introduction into the herd (based on age of the lesions), vaccination status (vaccinated or unvaccinated herd), date of vaccination, location of the herd recorded as decimal degrees coordinates based on a grid system used by SENASA and duration of the outbreak.

Computation of the within-herd transmission coefficient, β

Within-herd transmission of FMD virus was estimated using a modification of the mass action frequency-dependent principle (McCallum et al., 2001). The coefficient of transmission (β) was defined as the average number of individuals that are newly infected from an infectious individual per unit of time (De Jong, 1995); in lay terms,

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β is a rate used to estimate how many susceptible individuals acquire the infection from an infectious individual in a susceptible population over a given period of time. For this study, we used the number of animals showing FMD-like lesions rather than the true number of infected animals; hence, if the proportion of FMD-infected animals that developed clinical signs remains, on average, the same throughout an outbreak, then estimates of β will remain unbiased. Because duration of the outbreaks was heterogeneous, the number of animals showing FMD-like lesions at the end of the outbreak was adjusted by the duration of the outbreak using $C_a = C/t$, where C is the number of new cases identified in the epidemic (the difference between the cumulative number of cases at the end of the epidemic and the number of cases at the beginning of the epidemic), C_a is the adjusted number of cases, and t is the duration of the outbreak in days; thus, C_a is an estimate of the expected number of cases caused by a single infected animal in 1 day. The value of β (per day) was consequently estimated as

$$\beta = \frac{NC_a}{SI}$$

where N is the number of animals in the herd, which was assumed to be constant through the duration of the outbreak, justified by the nil mortality rate observed in the epidemic and by the prohibition of animal movement imposed at the beginning of the epidemic, I is the number of animals infected at the time of outbreak detection, and S is the number of susceptible animals at the beginning of the outbreak, $S = N - I$.

Outbreaks (n = 1001, 41.8%) with incomplete or insufficient information or for which the *sine qua non*-condition for disease transmission $C > I$ could not be verified from the records were excluded from the analysis to control for potential information bias associated with inaccurate collection or recording of field data.

The frequency-dependent model assumes that the transmission of the disease is not dependent on animal density. This assumption was biologically sound to the authors because animals in free-range systems, which is the most common production system in Argentina, are free to move and contact animals with no imposed restriction. However, one may argue that animals in intensive production systems could have more contacts with more individuals in a day, compared to animals in extensive production systems; thus, a density-dependent model may be considered more appropriate than the frequency-dependent model used in this study. To assess whether results were different depending on whether density or frequency-dependent models were used to fit the data, in addition to the frequency-dependent formulation, the value of β was also computed using a density-dependent approximation of the form $\beta = C_a/SI$, and results were compared with those obtained using the frequency-dependent model using a Spearman's rank correlation.

Statistical analysis

Zero-truncated probability distributions that best fitted the values of β in either and both vaccinated and unvaccinated herd outbreaks were identified using the @RISK® software (Palisade Corporation, New York/London v. 5.0).

Multivariate logistic regression

Herds with β values larger than the median value of β (Mβ) estimated for the epidemic were categorized as cases, whereas herds in which $\beta \leq$ Mβ were categorized as controls. Therefore, a case in this study was defined as an outbreak in which disease transmission was larger than the epidemic's background, which was defined as the median value of β. A multivariate logistic regression model was used to assess the association between herds with values of $\beta >$ Mβ and epidemiological factors hypothesized to influence the value of β. Scatter plots for all possible bivariate associations and correlation coefficient were computed to asses for preliminary potential relationship between the epidemiological factors and the values of β. Candidate epidemiological factors entered into the regression model were geographical location of the herd (to control for potential spatial dependence of the observations), duration of the outbreak in days, days to detection or d_{det} (defined as the difference, in days, between the date of SENASA detection, and the estimated date of FMD virus introduction into the herd), main age group of the herd and vaccination status (unvaccinated, vaccinated 0–4 post or 1–7, 8–14, 15–28 and >28 days before the estimated date of FMD virus introduction into the herd). Records with incomplete information of these variables were excluded from the analysis. Two-way interactions and quadratic terms were considered in all models and tested for significance ($P < 0.05$). Significant terms were retained in the model. Odds ratios and 95% confidence intervals were computed for variables included in the final model. Model fit was assessed using the Pearson chi-square goodness-of-fit statistic. Statistical analyses were performed using Minitab (Minitab Inc., State College, PA, USA).

Results

Summary statistics

A total of 1349 herds (56.4%) records were available for the analysis after removing records with insufficient or inaccurate information (n = 1001 with inaccurate

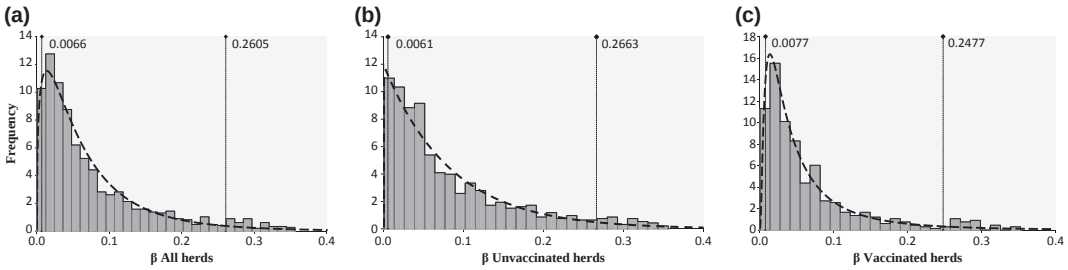

Fig. 1. Observed values (—) and fitted distributions (- - -) for the coefficient of transmission β in all herds (unvaccinated + vaccinated) (a), unvaccinated herds (b) and vaccinated herds (c). Numbers at the top of the graphs represent the 90% probability interval of the distribution.

information and $n = 44$ records with missing outbreaks initial and/or end dates). The mean (median) values of β were estimated as 0.22 (0.06) for all herds, 0.26 (0.06) for unvaccinated herds and 0.17 (0.04) for vaccinated herds. The distribution that best fitted the β values for all herds was Pearson type six with parameters 1.3437, 5.2769 and 0.24513 and a most likely value of 0.077, whereas the distribution of β in vaccinated and unvaccinated herds was best fitted by a log-normal distribution with parameters 0.069158 and 0.09656, and a most likely value of 0.069, and by an exponential distribution with parameter 0.083 and most likely value of 0.083, respectively (Fig. 1). Visually, there was no evident trend of β during the epidemic time period (Fig. 2), whereas the bivariate association between time to detection and within-herd transmission was visually apparent (Fig. 3). Results obtained using the frequency-dependent model were moderately correlated (R-Spearman = 0.633, $P < 0.001$), with those obtained using the density-dependent model.

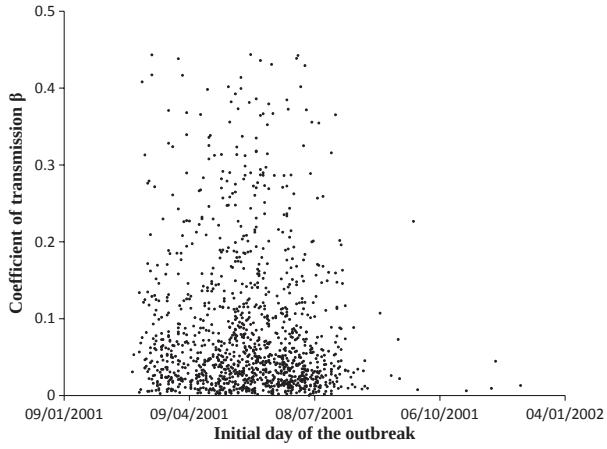

Fig. 2. Bivariate association between the coefficient of transmission, β, of foot-and-mouth disease and starting date of the outbreak during the 2001 Argentine epidemic.

Multivariate logistic regression

A total of 1221 outbreaks were used to fit the regression model, because 128 records in which vaccination date were missing were excluded from the analysis (Table 1).

Significant variables retained in the final multivariate logistic regression model were report date, d_{det} and vaccination status/days to vaccination (Table 1); interestingly, the odds for high disease transmission were lower for those herds in which infection took longer to detect (Fig. 3). The model fitted the data well (Pearson chi-square test, $P = 0.235$).

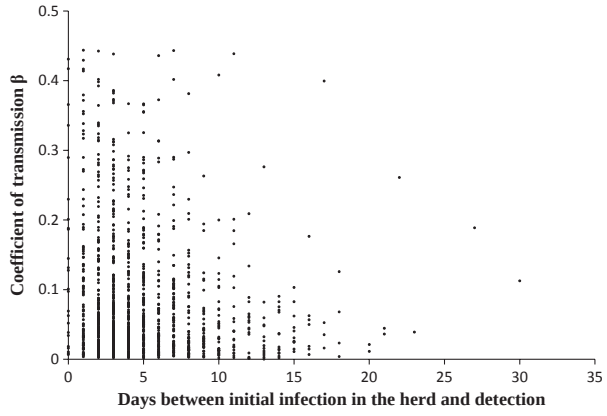

Fig. 3. Bivariate association between the days to detection and β. There is an evident inverse relationship of the values, as the number of days to detection increase, the within-herd transmission is lower.

Discussion

Within-herd transmission of the FMD epidemic reported in Argentina in 2001, which was computed using field data, was significantly associated with d_{det}, date reported, vaccination and time between vaccination and estimated date of virus introduction into the herd. Previous studies regarding FMD transmission have been carried out under experimental conditions (Cox et al., 2007; Orsel and Bouma, 2009), in which the payload of the antigen in the

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Table 1. Multivariate logistic regression association between selected epidemiological factors and foot-and-mouth disease within-herd transmission rates in Argentina, 2001

Variable	Category	*n*	Odds ratios	95% confidence intervals	*P*-value
Report date		1221	1.00	(0.99, 1.00)	0.004
Days to detection[a]		1221	0.94	(0.91, 0.97)	<0.001
Vaccination	Non vaccinated	823	*	*	Reference
	0–4 days after[a]	25	0.60	(0.26, 1.36)	0.218
	1–7 days before[a]	105	0.49	(0.32, 0.75)	0.001
	8–14 days before[a]	56	0.61	(0.35, 1.07)	0.085
	15–28 days before[a]	57	0.79	(0.45, 1.36)	0.391
	>28 days before[a]	156	0.69	(0.47, 1.01)	0.056

Pearson chi-square $P = 0.235$, log-likelihood = −823.690, test that all slopes are zero $G = 45.219$, $P < 0.000$.
[a]Days in relation to the estimated day of first infection in the herd.

vaccine, challenge dose, exposure, age and animal condition, strain used in the vaccine and challenge (normally homologous antigen used), and inoculation techniques is set under controlled conditions, but in which, arguably, epidemiological conditions observed in the field could hardly be replicated. In this study, we used observational data collected from one of the few large serotype A FMD epidemics controlled by vaccination reported in the international peer-reviewed literature.

In this study, the protective effect of the vaccine was evidenced by the association between vaccination and low rate of within-herd transmission. These results are in agreement with early studies, suggesting that emergency vaccination has a protective impact on disease transmission and that there is a decreased transmission rate within the herd even if the vaccine is applied soon before or even few days after initial infection in the herd.(Cox and Barnett, 2009; Orsel and Bouma, 2009). Noteworthy, some authors suggest that there is an increased risk of animals becoming carriers as the time between vaccine and challenge decreases (Barnett and Carabin, 2002), which could not be assessed in this study because of the lack of data on persistently infected animals.

It is known that within- and between-herd disease spread is typically able to be controlled by systematic FMD vaccination with protective vaccines in endemic areas (Bergmann et al., 2004), but emergency vaccination effect in face of an epidemic on within-herd spread has not been previously evaluated in the field. Emergency vaccination has gained consideration as a control strategy during FMD outbreaks in disease-free areas, especially since the 2001 UK outbreak (Hutber et al., 2011). Experimental studies have shown that emergency vaccination can be an effective strategy to control FMD during an outbreak by reducing clinical disease, subclinical infection, excretion and transmission (Cox et al., 2007; Cox and Barnett, 2009; Orsel and Bouma, 2009; Hutber et al., 2011).

Results of this study showing that emergency vaccination in infected herds decreased within-herd transmission even when vaccination was applied soon after the start of the outbreak may have important applications when evaluating whether a 'stamping out' policy or emergency vaccination should be applied, especially because the international OIE ban length was shortened to 6 months when using emergency vaccination 'to live'. Economic analyses of these two control strategies should be performed as well as considering aspects regarding animal welfare concerns, especially brought to attention after the UK 2001 outbreak. Use of emergency vaccination to control FMD epidemics is also likely to be increasingly used in the future because the performance of diagnostic methods capable of differentiating vaccinated from infected animals by detection of antibodies to non-structural proteins had improved and such methods are now officially accepted (Bergmann et al., 2003).

Estimated time between exposure to FMDV serotype O and earliest detection of infection (latent period) ranges from 3.1 to 4.8 days, and time between first positive samples and first clinical signs (subclinical period) ranges from 2 to 2.3 (Mardones et al., 2010). Therefore, it does take a certain period of time until every susceptible animal in the herd is exposed to the virus and, eventually, infected. In a scenario where animals are vaccinated at an early stage of herd infection, individual immunity raises in parallel to within-herd disease spread, so that if the rate of spread is lower than the rate of disease protection conferred by the vaccine, at certain point, at least some animals become immune and disease spread is restrained. During the latent period, there is no transmission to new individuals; additionally, disease transmission is lower in subclinical infected animals than in animals presenting clinical disease (Orsel et al., 2009).

Consistently with the results presented in this study, evidence on the effects of vaccination reducing within-herd transmission was observed in a field study in Laos

People's Democratic Republic in which morbidity, in villages vaccinated less than a month before the beginning of the outbreak, was lower than in villages with unvaccinated herds (Rast et al., 2010).

Argentine cattle have been systematically vaccinated up to 1 year before the beginning of the epidemic with A24/Cruzeiro strain, so it is expected that residual immunity was present in at least some animals older than 1 year.

Almost all vaccinated herds included for this study used a formulation with A/Arg/00. An early analysis of this epidemic reported that vaccines formulated with A/Arg/00 (trivalent O1/C-A24-A2000) induced 50–60% protection when challenged with the heterologous A/Arg/01 strain isolated in the epidemic (Mattion et al., 2004), which might explain, at least in part, the association between vaccination status and transmission rate detected in this study. Assessment of the protection conferred specifically by the homologous A/Arg/01 strain in within-herd transmission was not possible because data required for the analysis were available in only <10 herds vaccinated with A/Arg/01 and because herds vaccinated with the homologous strain (A/Arg/01) were not affected. For those reasons, it is very likely that the protective effect of vaccination may have been underestimated in this study.

Estimates of FMD transmission rates have been computed using experimental data, in which animals are typically more intensively evaluated for the detection of infection and clinical disease, compared to field conditions. In this study, FMD transmission rate was estimated using observational data on clinically diseased animals collected during the epidemic. Subclinically infected animals that went through the outbreak undetected were not accounted in the analysis. If, as expected, the proportion of infected animals that did not develop clinical signs was, on average, relatively constant throughout the duration of the outbreak, then the value of β estimated in this study would still be unbiased. However, values of β computed should be regarded as an estimate of the average within-herd transmission of the outbreak, as one would expect the value of β to decrease for each particular outbreak as the number of susceptible and infected animals decreases, and immunity increases, towards the end of the outbreak.

Long time-to-detection was also significantly associated with low risk of within-herd transmission. A possible explanation is that, for densely populated herds, which are typically managed through intensive production systems, disease might have spread faster and detection may have taken place earlier, compared to extensive production units, in which disease spread and disease detection are expected to be slower. This result is in agreement with early studies of this epidemic, which suggest that FMD may have been more easily detected in intensive production systems, especially at the beginning of the epidemic (Ward and Perez, 2004), where increased contact between animals occur, and higher transmission rates are found. Report date was also associated with disease transmission; initial outbreaks showing, in general, high values of β, likely, because the level of awareness among producers increased with time.

Values of β were independent of the geographical location of the herd, suggesting that the FMDV A/Arg/01 virulence was homogeneous throughout the country. Main age group of the herd was also independent from the value of β; however, it is also possible that age-dependent associations could have been revealed if association with type of production would have been assessed, but such information was not available to the authors.

In conclusion, results presented in this study suggest, in quantitative terms, the effect of vaccination in the reduction of within-herd transmission of FMD virus, reinforcing the impact that emergency vaccination has in controlling FMD epidemics. Because of the transboundary nature of FMD spread, this result is particularly relevant for countries that withdrew FMD vaccination, but that are still exposed to FMD virus incursions from infected regions.

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Predicting antigenic sites on the foot-and-mouth disease virus capsid of the South African Territories types using virus neutralization data

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Foot-and-mouth disease virus (FMDV) outer capsid proteins 1B, 1C and 1D contribute to the virus serotype distribution and antigenic variants that exist within each of the seven serotypes. This study presents phylogenetic, genetic and antigenic analyses of South African Territories (SAT) serotypes prevalent in sub-Saharan Africa. Here, we show that the high levels of genetic diversity in the P1-coding region within the SAT serotypes are reflected in the antigenic properties of these viruses and therefore have implications for the selection of vaccine strains that would provide the best vaccine match against emerging viruses. Interestingly, although SAT1 and SAT2 viruses displayed similar genetic variation within each serotype (32 % variable amino acids), antigenic disparity, as measured by r_1-values, was less pronounced for SAT1 viruses compared with SAT2 viruses within our dataset, emphasizing the high antigenic variation within the SAT2 serotype. Furthermore, we combined amino acid variation and the r_1-values with crystallographic structural data and were able to predict areas on the surface of the FMD virion as antigenically relevant. These sites were mostly consistent with antigenic sites previously determined for types A, O and C using mAbs and escape mutant studies. Our methodology offers a quick alternative to determine antigenic relevant sites for FMDV field strains.

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INTRODUCTION

Foot-and-mouth disease (FMD) is widely considered an economically important disease of livestock, and is a compulsory OIE notifiable disease as it remains a global threat to national and international trade in livestock and livestock products. In 2010, outbreaks have occurred in South America, Asia, the Middle East and Africa (FMD Reference Laboratory Network Report, 2010). FMD is of particular importance in Africa where the disease is endemic and six of the seven immunologically distinct serotypes occur (Thomson, 1994; Vosloo *et al.*, 2002). Although foot-and-mouth disease virus (FMDV) causes a clinically indistinguishable vesicular disease in cloven-hoofed animals, the seven serotypes display different geographical distributions and epidemiology (Samuel & Knowles, 2001; Bastos *et al.*, 2001, 2003a, b; Knowles & Samuel, 2003; Bronsvoort *et al.*, 2004).

The South African Territories (SAT) types 1, 2 and 3 are confined to sub-Saharan Africa, although incursions into the Middle East by SAT1 (1961–1965 and 1970) and SAT2 (1990 and 2000) viruses have been recorded (Ferris & Donaldson, 1992; Bastos *et al.*, 2001; Records of the OIE). The SAT3 serotype has a restricted distribution and essentially occurs only in southern Africa (Bastos *et al.*,

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2003b), Asia-1 is restricted to Asia and the Middle-East (Ansell *et al.*, 1994), while serotypes A and O occur globally (Samuel & Knowles, 2001). The distribution of FMD samples received from Africa from 2000 to 2010 revealed that the majority of outbreaks (41 %) were caused by serotype SAT2 viruses. The prevalence of SAT1 and type O viruses was similar with 19 and 23 %, respectively. Outbreaks caused by type A viruses were identified for 15 % of the reported cases. In contrast, SAT3 viruses caused only two outbreaks during this period in the Democratic Republic of Congo and South Africa, respectively, whereas type C was only detected in Kenya in 2004 (Annual OIE/FAO FMD Reference Laboratory Network Reports; Records of the OIE). Eradication of the disease from the African continent is unlikely due to the presence of large numbers of the free-living maintenance host, the African buffalo (*Syncerus caffer*). These animals provide a potential source of infection for domestic livestock and wildlife (Dawe *et al.*, 1994; Bastos *et al.*, 2000; Vosloo & Thomson, 2004) and pose a constant threat to susceptible livestock (Vosloo & Thomson, 2004) in the rest of the world.

Persistently infected buffalo are the ideal hosts to maintain co-infection of different virus serotypes and to facilitate antigenic and molecular evolution of the virus (Condy *et al.*, 1985; Esterhuysen, 1994; Vosloo *et al.*, 1996, 2006). Genetic variation results from changes to the viral genome as a consequence of the high mutation rate of the virus. Therefore, the genetic diversity will most likely be reflected in antigenic differences. The outer capsid proteins are directly involved in antigenicity since 30–50 % of their residues are exposed on the virion surface, many of which constitute neutralizing epitopes (Acharya *et al.*, 1989; Logan *et al.*, 1993; Lea *et al.*, 1995; Mateu, 1995; Usherwood & Nash 1995). Although several antigenic sites have been identified for A and O serotypes (Xie, *et al.*, 1987; Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; McCahon *et al.*, 1989; Kitson *et al.*, 1990; Saiz *et al.*, 1991; Crowther *et al.*, 1993a), a dearth of knowledge exists for the epitopes of the SAT types. Identification of those residues that comprise the antigenic determinants of the SAT viruses will allow the identification of those changes in outbreak strains that may cause escape from protection afforded by the vaccine. Once such epitopes for the SAT types have been identified, it may be possible to predict the protection afforded by a vaccine against a specific outbreak virus.

The SAT types display appreciably greater intratypic genomic and antigenic variation than the 'Euro-Asian' types (Vosloo *et al.*, 1992, 1995, 1996; Esterhuysen, 1994; Bastos *et al.*, 2001, 2003a, b). Even within a serotype, distinct genetic and antigenic variants exist in different geographical regions. This has implications for the control of the disease by vaccination, since it may render available vaccines less effective (Hunter, 1998). Consequently, the ability to predict vaccine efficacy would be a valuable tool in an effective control strategy as control of FMD in Africa is essentially via strategic vaccination and restriction of animal movements.

Traditionally, the *in vitro* virus neutralization test (VNT) and statistically calculated r_1-values are used to determine antigenic relationships between a vaccine strain and an outbreak virus (Rweyemamu *et al.*, 1978). Meanwhile molecular epidemiological techniques have offered the possibility of more detailed analyses of FMD epidemiology (Vosloo *et al.*, 1992; Samuel & Knowles, 2001; Bastos *et al.*, 2001, 2003a, b; Knowles & Samuel, 2003). We have investigated the genetic variation of the complete capsid-coding region of representative FMDV strains found in sub-Saharan Africa during a 28 year period from 1974 to 2002. Variable regions on the capsid proteins of SAT1 and SAT2 isolates were then combined with structural data and serological relatedness to identify possible epitopes that could be prone to antigenic variation in the SAT viruses.

RESULTS

Phylogenetic relationships and topotype diversity in sub-Saharan Africa

The minimum evolution phylogeny, based on P1 nucleotide sequences of representative SAT viruses that caused outbreaks across the African continent over the past 28 years, is indicated in Fig. 1. The SAT viruses clustered according to serotype with high bootstrap support. The distribution of SAT3 serotype is limited to southern Africa. The five SAT3 isolates represented five of the six previously described topotypes. Three major virus lineages, which have evolved separately, exist within serotypes SAT1 and SAT2. The lineages clustered according to their geographical location (southern, western and eastern Africa) and are in agreement with the FMD topotype concept described for the European and SAT serotypes (Samuel & Knowles, 2001; Bastos *et al.*, 2001, 2003a, b; Knowles & Samuel, 2003). Exceptions, however, were observed for SAT1 and SAT2, where isolates from East Africa, i.e. SAT2/KEN/08/99, SAT1/KEN/05/98 and SAT1/TAN/37/99, clustered in the southern topotypes (Fig. 1), possibly due to historical movement of buffalo and livestock between the two regions (Bastos *et al.*, 2001; Sangare *et al.*, 2003). Similarly, an isolate from Sudan, SAT1/SUD/3/76, demonstrated high sequence similarity, based on the complete P1-coding region, with West African isolates, e.g. SAT1/NIG/05/81, indicating historical spread of the disease between East and West Africa. Using a 16 % nt difference cut-off value (Bastos *et al.*, 2003b), seven of eight previously described distinct lineages could be identified within the SAT1 serotype, while nine of the fourteen SAT2 topotypes could be resolved using the P1 phylogeny (Fig. 1). Within the southern African region, Zimbabwe shared different SAT1 (1, 2 and 3) and SAT2 topotypes (I, II and III) with neighbouring countries. This correlates well with phylogeny based on the 1D sequence only (data not shown). P1 phylogeny did not provide significantly more resolution for

epidemiology purposes, although stronger bootstrap values were observed. An exception was the Angolan SAT2 isolate, ANG/4/74, which grouped separately from other Southern African SAT2 isolates according to 1D phylogeny and was previously assigned to a different topotype (Bastos *et al.*, 2003b; Sangaré *et al.*, 2004). However, using complete capsid sequence data (Fig. 1), this isolate was more closely related to the other South African isolates with good bootstrap support.

The intratypic nucleotide variation of the P1 region for SAT1, 2 and 3 was calculated to be 47.3 (n=20), 48.9 (n=23) and 39.5 % (n=5), respectively, and found to be considerably higher than the intratypic variation reported previously for types A, O and C (<18 %) (van Rensburg & Nel, 1999; Knowles & Samuel, 2003). The nucleotide and amino acid variation in a complete alignment of the SAT P1-coding region is summarized in Table 1. The complete P1 region of the SAT1 viruses was 2232 nt in length, encoding 744 aa, with the exception of the SAT1 isolates NIG/15/75, NIG/06/76 and NIG/08/76 (topotype 8) that consisted of 2229 nt. The corresponding region of the SAT2 viruses was 2220 nt in length, with the exception of a West African isolate, i.e. SAT2/SEN/07/83 (topotype VI). The P1-coding region of this isolate is 2217 nt in length and has a 6 nt deletion in the 1C-coding region and a 3 nt insertion in 1D. The five SAT3 isolates revealed the most variation in sequence length of the P1 region, ranging from 2214 to 2223 nt and encoding a polypeptide of between 738 and 741 aa in length. The maximum number of nucleotide differences observed intratypically in any pairwise alignment of the P1 regions of isolates within SAT1, SAT2 and SAT3 serotypes was 581 (26.1 %), 558 (25.1 %) and 617 (27.74 %), respectively. The average proportion of nucleotide differences was estimated to be 53.6, 55.7 and 57 % over synonymous sites, and 9.4, 9.2 and 11.2 % over non-synonymous sites for the three serotypes, respectively. Therefore, our data suggests an overall ratio of synonymous : non-synonymous changes to be approximately 6 : 1 for SAT 1, 2 and 3.

Analysis of antigenic properties of SAT1 and SAT2 viruses

One-way antigenic relationships (r_1-values) were used to compare SAT1 and SAT2 viruses from various topotypes in sub-Saharan Africa to select reference strains within each serotype. Since SAT3 viruses has the most restricted distribution and are the least frequently recovered from buffalo (Bastos *et al.*, 2003b) these isolates were not included in this study. The virus isolates and the mean r_1-values are summarized in Tables 2 and 3. Sera from cattle vaccinated twice with the two SAT1 viruses, SAR/09/81 and KNP/196/91, both belonging to topotype 1, demonstrated r_1-values that were higher with isolates belonging to the same topotype compared with isolates from other topotypes. Among the 20 SAT1 isolates analysed against the vaccine strains, 15 (n=3) and 15 % (n=3) showed

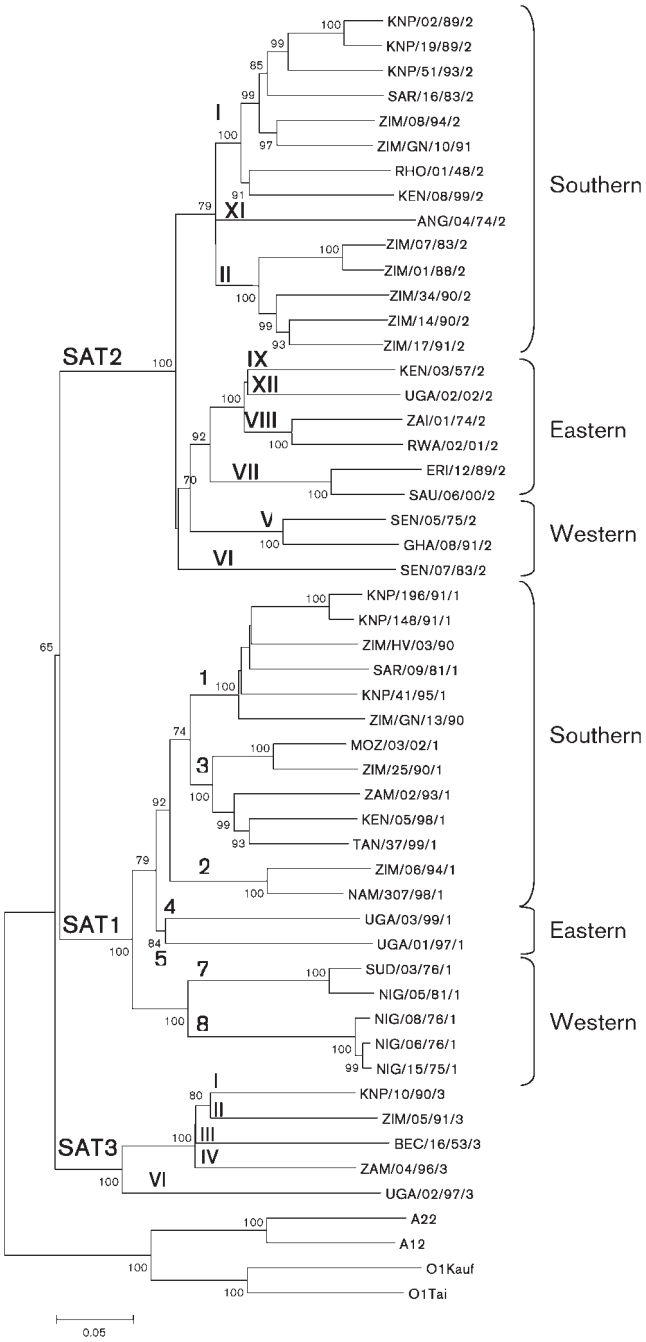

Fig. 1. Minimum evolution tree depicting the gene relationships for the P1-coding regions, respectively, of SAT1, 2 and 3 viruses from South (Kruger National Park, KNP; South Africa, SAR; Zimbabwe, ZIM; Rhodesia, RHO; Mozambique, MOZ; Namibia, NAM; Zambia, ZAM; and Angola, ANG), West (Nigeria, NIG; Senekal, SEN; and Ghana, GHA) and East Africa (Uganda, UGA; Rwanda, RWA; Zaire, ZAI; Kenya, KEN; Tanzania, TAN; Eritrea, ERI; Saudi Arabia, SAU and Sudan, SUD). The major topotypes for SAT1 (labelled 1–8), SAT2 (labelled I–XII) and SAT3 (I–VI) viruses are indicated. No representative isolates of SAT1 topotype 6 and of SAT2 topotypes III, IV, X, XIII and XIV were included. Bootstrap support ⩾65 is based on a 1000 replicates and is indicated next to the relevant node.

Table 1. Variation within the nucleotide and amino acid sequences of the P1 polyprotein in a complete alignment of the SAT1, 2 and 3 viruses

Serotype	Genome region	No. nucleotide positions aligned*	No. variant nucleotide				Variant nucleotide (%)†				No. amino acid positions aligned*	No. variant amino acids				Variant amino acids (%)†			
			All	SAT1	SAT2	SAT3	All	SAT1	SAT2	SAT3		All	SAT1	SAT2	SAT3	All	SAT1	SAT2	SAT3
	1A	258	120	100	106	72	46.5	38.8	41.1	27.9	84	24	11	15	2	28.5	13.1	17.9	2.4
	1B	654	391	285	315	230	59.8	43.6	48.2	35.2	219	125	54	64	48	57.1	24.7	29.2	21.9
	1C	675	413	304	308	231	61.2	45.0	45.6	34.0	224	146	82	68	52	65.5	37.1	30.6	23.4
	1D	677	498	379	379	341	73.6	56.0	56.0	50.4	224	158	105	107	91	70.5	48.0	50.2	42.7
	All capsid proteins	2232	1422				62.8				744	429				57			

*The number of nucleotides and amino acids were based on CLUSTAL_X alignments of the complete P1-coding region of the SAT serotypes.
†The number of variant nucleotides or amino acids for each genomic region or capsid protein relative to the total number of positions was used to estimate the percentage (%) variability.

r_1-values $\geqslant 0.4$ with the SAR/09/81 and KNP/196/91 viruses, respectively, all belonging to topotype 1 (Table 2). Furthermore, 55 (n=11) and 70 % (n=14) of the isolates had r_1-values in the range from 0.2 to 0.39, indicating that a high potency vaccine may still afford protection in animals. The remaining isolates reacted poorly to antisera directed against these vaccine strains, indicating that the vaccines will most probably not protect animals in the field against these strains. Convalescent sera to a West African SAT1 virus, i.e. SAT1/NIG/05/81, showed reasonable cross-protection (r_1-values of 0.2–0.39) against 75 % (n=15) of the SAT1 viruses (Table 2). Only one isolate had an r_1-value of $\geqslant 0.4$, i.e. SUD/3/76, which belongs to the same topotype as the Nigerian isolate. The difference in cross-reaction with the reference sera might be indicative of differences in shared epitopes of the isolate in question to the reference virus.

The SAT2 reference antisera used in the study were from the SAT2 vaccine strains ZIM/07/83 and KNP/19/89 that belong to SAT2 topotypes I and II, respectively, and two convalescent antisera, that of ERI/12/89 and RWA/02/01 from topotypes VII and VIII, respectively (Table 3). The antigenic variation for the SAT2 isolates was more pronounced compared with SAT1 isolates, with r_1-values below 0.2 even when vaccine and field strains from the same topotype were compared. With the exception of the reaction of the ZIM/07/83 isolate to the KNP/19/89 sera, none of the other SAT2 isolates showed r_1-values in the range of 0.4–1.0 against any of the four reference strains. Although none of the SAT2 isolates had r_1-values of 0.2–0.39 against ZIM/7/83 antisera, at least 16.6 % (n=4) of the isolates fell into this range using KNP/19/89 antisera. Furthermore, 28 and 25 % (n=6 and n=5) of isolates had r_1-values of 0.2–0.39 when tested against ERI/12/89 and RWA/02/01 antisera, respectively. From the data in Table 3 the convalescent sera (ERI/12/89 and RWA/02/01) appear to be more cross-reactive than the sera derived from the immunized animals (ZIM/07/83 and KNP19/89). However, the best consensus estimate model described by Reeve *et al.* (2010) did not show a difference for sera prepared by vaccination or collected from infected animals.

Comparison of amino acid variation within the SAT1 and 2 serotypes

We extended our study to investigate potential regions involved in the antigenic variability of SAT1 and SAT2 viruses based on the identification of hypervariable regions and positions of high entropy in the capsid-coding regions for SAT viruses. Hypervariable regions, in which 60 % or more of the residue positions varied (using overlapping windows of 10 aa), were identified in the outer capsid proteins, i.e. 1B, 1C and 1D (see Supplementary material, available in JGV Online). Within hypervariable regions, entropy was used to measure the uncertainty at each amino acid position within the SAT alignment (Schneider & Stephens, 1990). Interestingly, differences were observed

Table 2. The mean r_1-values and number of variable amino acids in the capsid proteins of SAT1 isolates as measured against reference strains (SAR/09/81, KNP/196/91 and NIG/05/81)

−, VNTs were not done.

SAT1 isolates*	Topotype†	SAR/09/81‡		KNP/196/91‡		NIG/05/81§	
		Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value
KNP/196/91‖	**1**	49	0.44	**0**	**1.00**	109	0.21
SAR/09/81‖	**1**	**0**	**1.00**	49	0.44	105	0.34
ZIM/GN/13/91	1	44	0.50	38	0.55	107	0.23
ZIM/HV/03/90	1	42	0.40	31	0.48	100	0.20
KNP/148/91	1	46	0.37	17	0.36	106	0.34
KNP/41/95	1	54	0.25	50	0.36	118	0.23
NAM/307/98	2	73	0.21	67	0.28	109	0.22
ZIM/06/94	2	75	0.23	77	0.33	113	0.17
MOZ/03/02	3	63	0.24	59	0.37	107	0.32
ZIM/25/90	3	68	0.27	62	0.10	109	−
TAN/01/99	3	−	0.13	−	0.28	−	0.25
TAN/37/99	3	55	0.25	55	0.36	105	0.27
ZAM/02/93	3	63	0.16	60	0.14	106	0.10
KEN/05/98	3	56	0.23	58	0.34	101	0.23
UGA/03/99	4	73	0.26	80	0.25	100	0.32
UGA/01/97	5	91	0.24	102	0.27	114	0.29
SUD/03/76	7	103	0.28	107	0.25	17	0.60
NIG/05/81‖	**7**	105	0.19	109	0.28	**0**	**1.00**
NIG/15/75	8	105	0.17	108	0.26	65	0.21
NIG/06/76	8	104	0.10	106	0.12	63	0.17
NIG/08/76	8	103	0.14	106	0.36	63	0.18

*The passage histories and country of origin of the study viruses as well as GenBank accession numbers of the P1 sequences have been described in Reeve *et al.* (2010).

†The topotype designations is based on 1D phylogeny proposed by Bastos *et al.* (2001); Sangare *et al.* (2003); Vosloo *et al.* (2006); Sahle *et al.* (2007a).

‡The reference SAT1 test sera used in the VNTs. The sera were prepared by two consecutive vaccinations on days 0 and 28 with reference SAT1 viruses and subsequently bled on day 38.

§The convalescent test sera were prepared by inoculating cattle intradermolingually with 10^4 $TCID_{50}$ of SAT1/NIG/5/81 virus and collecting blood 21 days post-infection.

‖The homologous viruses used as reference strains. The bold values indicate the homologous reaction in VNTs or alignment with homologous sequence.

between hypervariable sites for SAT1 and 2 isolates obtained from eastern, western and southern Africa. The hydrophilicity and deduced surface exposure were also considered for each residue and highly variable regions correlated with regions of significant hydrophilicity (see Supplementary material). Comparison of the P1 amino acid sequences of SAT1 and 2 with type A and O sequences, revealed that many of the SAT1 and 2 hypervariable regions corresponded or were located in close proximity to previously identified immuno-dominant sites on types O and A (Table 4; Xie *et al.*, 1987; Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; McCahon *et al.*, 1989; Kitson *et al.*, 1990; Saiz *et al.*, 1991; Crowther *et al.*, 1993a). Some hypervariable regions were located within flexible structural loops or downstream of protease cleavage sites.

As expected, the internally located 1A protein was the most conserved of the structural proteins with only 27 % variable positions on amino acid level (only 6 % identified as parsimony-informative) between SAT1 and SAT2 (Table 1). Alignment of the amino acid sequences of the 1A polypeptide revealed no hypervariable regions, but common substitutions that are unique to each serotype were observed. Two amino acids in the 1A protein that are potentially specific for SAT2 and for SAT1 viruses are an Iso or Val at position 76 and Phe at position 80 for SAT2 viruses, while SAT1 viruses present with Phe and Val at the corresponding positions (see Supplementary material).

The 1B protein, 219 aa in length contained 24 and 29 % variable amino acid positions for SAT1 and 2, respectively (57 % in a complete alignment) (Table 1). Within the 1B capsid protein four hypervariable sites were identified within each serotype (Table 4), i.e. βA–βB loop (aa positions 31–45), βB–βC loop (aa 64–82), βC–βD loop (aa 93–101) and βE–βF loop (aa 130–134/141). Only the

Table 3. The mean r_1-values and number of variable amino acids in the capsid proteins of SAT2 isolates as measured against reference strains (ZIM/07/83/2, KNP/19/89/2, ERI/12/89/2 and RWA/02/01/2)

−, VNTs were not done.

SAT2 isolates*	Topotype†	ZIM/07/83‡		KNP/19/89‡		ERI/12/89§		RWA/02/01§	
		Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value
KNP/19/89‖	**I**	59	0.14	**0**	**1.00**	86	0.25	90	0.10
KNP/02/98	I	54	0.09	16	0.11	81	0.12	84	0.19
KNP/51/93	I	56	0.07	33	0.09	81	0.12	83	0.18
SAR/16/83	I	60	0.03	39	0.03	83	0.05	85	0.10
ZIM/08/94	I	52	0.08	39	0.12	83	−	79	−
ZIM/GN/10/91	I	49	0.11	42	0.11	81	0.09	80	0.09
ZIM/07/83‖	**II**	**0**	**1.00**	59	0.41	96	0.20	90	0.19
ZIM/14/90	II	49	0.05	57	0.09	91	0.14	85	0.10
ZIM/17/91	II	51	0.13	56	0.05	90	0.14	88	0.24
ZIM/01/88	II	19	0.18	51	0.13	87	0.16	86	0.13
ZIM/34/90	II	61	0.14	67	0.28	88	−	91	−
RHO/01/48	II	60	0.17	58	0.12	86	0.21	87	0.27
KEN/08/99	IV	68	0.05	53	0.07	86	0.04	86	0.06
GHA/08/91	V	89	0.09	80	0.09	74	0.18	62	0.21
LBR/01/74	V	−	0.03	−	0.05	−	0.12	−	0.16
SEN/05/75	V	100	0.02	90	0.05	82	0.03	71	0.06
SEN/07/83	VI	96	0.10	84	0.20	106	0.25	98	0.35
SAU/06/00	VII	97	0.06	86	0.06	38	0.26	73	−
ERI/12/89‖	**VII**	96	0.14	86	0.31	**0**	**1.00**	70	0.32
RWA/02/01‖	**VIII**	90	0.13	90	0.09	70	0.23	**0**	**1.00**
KEN/03/57	IX	93	−	94	−	87	−	56	−
ANG/04/74	XI	85	0.09	88	0.10	91	0.04	80	0.05
UGA/02/02	XII	93	0.02	92	0.03	84	0.04	57	0.08
ZAI/01/74	XII	92	0.02	86	0.05	66	0.12	33	0.17

*The passage histories and country of origin of the SAT2 viruses and the GenBank accession numbers of the P1 sequences have been described in Reeve *et al.* (2010).

†The SAT2 topotype designations is based on 1D phylogeny proposed by Bastos *et al.* (2003b); Sangaré *et al.* (2004); Sahle *et al.* (2007b).

‡The reference SAT2 test sera used in the VNTs. The sera were prepared by two consecutive vaccinations on days 0 and 28 with reference SAT2 viruses and subsequently bled on day 38.

§The convalescent test sera were prepared by inoculating cattle intradermolingually with 10^4 $TCID_{50}$ of SAT2/ERI/12/89 and SAT2/RWA/02/01 viruses, respectively, and collecting blood 21 days post-infection.

‖The homologous viruses used as SAT2 reference strains. The bold values indicate the homologous reaction in VNTs or alignment with homologous sequence.

βB–βC and βE–βF loops have significant surface exposure in the complete virion, while the N terminus of 1B (βA–βB loop) is located on the inner surface of the capsid in close proximity with the N terminus of 1D.

The 1C protein varied from 221 to 222 aa in length for SAT1 and SAT2 isolates, respectively. The exception was a SAT2 isolate from West Africa, SEN/7/83, which was only 220 aa in length. For 1C, the overall variable amino acid positions was 65 % (37 % variation within SAT1 and 30 % within SAT2) (Table 1). The genetic heterogeneity of 1C was confined to four hypervariable regions (Table 4), i.e. N terminus (aa 30–45), βB–βC loop (aa 63–77), βE–βF loop (aa 125–142) and βG–βH loop (aa 165–183/172). The heterogeneity was more pronounced in SAT1 viruses with some unique amino acids confined to different topotypes. The latter three of these hypervariable regions (consisting of the βB–βC, βE–βF and βG–βH loops) correlated with hydrophilic surface-exposed regions, indicating its possible contribution to antigenic determinants (see Supplementary material).

The 1D protein, the most variable of the outer capsid proteins, varied in length from 213 aa for SAT2 to 219 aa for SAT1 with 71 % overall variable amino acid positions (48 % within SAT1 and 50 % within SAT2) (Table 1). The SAT2 virus, SEN/07/83, was the exception with 214 aa in length. The inserted amino acid of SEN/07/83 is a Trp between residues 28 and 29 within a conserved positively

Table 4. A summary of the hypervariable regions observed in an alignment of the amino acid sequences of SAT1 and two outer capsid proteins

The hypervariable regions were considered in relation with structurally exposed loops and with known antigenic sites in serotype A and O sequences. *Residues within the starred regions have significant surface exposure (Fig. 2) and entropy that could contribute to the changes in antigenicity observed.

	Amino acid hypervariable region†														
	Capsid secondary structure elements														
Capsid	1B	1B	1B	1B	1C	1C	1C	1C	1D	1D	1D	1D	1D	1D	1D
β-Sheets	A–B	B–C	D–D	E–F; H–I	A–B	B–C	E–F	G–H; H–I	N_T	B–C	E–F	F–G	G–H	H–I	C_T
Axis		3 ×		2 ×		3 ×	2 ×			5 ×			2 ×	5 ×	3 ×
SAT1	31–44	62–82*	97–101	130–134*	30–45	59–78*; 83–91	124–141*	165–183	9–35	43–57*	93–102	113–123	135–151; 156–169	176–187	201–222
SAT2	37–43	69–79*	91–100	129–140*	27–47	62–76*	125–137*	165–173	16–42	45–72*	80–92; 100–104	108–118	136–145; 151–166	172–183	193–217
	FMDV antigenic sites†														
Type O		Site 2 70–77/78		Site 2 131–134; T188I		Site 4 56–58			T-cell	Site 3 43–48			Site 1a, 5 144–149, 154		Site 1b 206–207
Type A		Site 3 82–88; 196				Site 5 58–61; 69–70	Site 3 136–139; 195						Site 1 142–157	Site 4 169; 175–178	Site 2 200–212

*The hypervariable regions have been derived from the alignment in the Supplementary material.

†The antigenic sites are a summary of that described in Thomas *et al.* (1988); Baxt *et al.* (1989); Bolwell *et al.* (1989); Saiz *et al.* (1991); Kitson *et al.* (1990); Crowther *et al.* (1993a); Guzman *et al.* (2010).

charged motif (KRRXH for SAT2 or RRXH for SAT1) located in a region similar to a previously identified T-cell epitope of 1D for serotype C and O (Pérez Filgueira *et al.*, 2000; Guzman *et al.*, 2010). At least seven discrete hypervariable regions were identified in 1D of SAT1 and SAT2 viruses (Table 4). These regions corresponded with the N terminus (residues 9–40 in SAT viruses), βB–βC loop (residues 43–62/71 for SAT1 and 2), βE–βF loop (residues 80–103), βF–βG loop (residues 110–122), βG–βH loop (residues 136–167), βH–βI loop (residues 176–187) and the C terminus (residues 199–220/192–212 for SAT1 and 2). With the exception of the N terminus and βF–βG loop, the hypervariable regions coincided with hydrophilic surface-exposed regions.

Amino acid residues situated within regions of hypervariability in the outer capsid proteins, which exhibit high entropy and hydrophilicity, and on structurally exposed loops were regarded as having the potential for involvement in antibody recognition sites. These regions are therefore possibly involved in the antigenicity of the virion (Table 4 and Supplementary material).

Amino acid changes and antigenic types within the SAT serotypes

To obtain information regarding the relationship between genetic distance and mean r_1-values for SAT1 and SAT2 viruses, the mean r_1-values obtained for each field isolate against the reference sera were plotted against the total amino acid changes between the field strain and reference virus (Supplementary Fig. S1, available in JGV Online). For SAT1 viruses, a close genetic relationship with the reference strain, as observed by considerable amino acid identity, did agree with sufficient serological cross-reaction. However, for the SAT2 viruses the observed r_1-values were low, even for viruses with considerable identity with the reference strain. The availability of the deduced amino acid sequences for the P1 regions of the VNT analysed viruses allows for the direct comparison of amino acid residue changes between any two isolates. In addition, residues located in hypervariable regions that are surface-exposed can be identified and counted between any two sequences. The changes specifically corresponding to surface-exposed residues are expected to correlate directly with antigenic variability as it may abrogate antibody binding and neutralization *in vitro*. This can be explained using two examples, one for SAT1 and SAT2, respectively.

In a pairwise alignment of the P1 polypeptide of the SAT1/SAR/9/81 virus and five additional topotype 1 isolates (KNP/196/91, KNP148/91, KNP/41/95, ZIM/GN/13/91 and ZIM/HV/03/90), 17 to 55 variable amino acids were observed (Table 2). The r_1-values for these viruses varied from 0.22 to 0.45, indicating poor but some cross-reaction to good cross-reaction (Table 2 and Supplementary Fig. S1). Since the outer-capsid protein surface-exposed residues are directly involved in antigenicity (Logan *et al.*, 1993; Lea *et al.*, 1995), clusters of variable surface-exposed amino acids may constitute antigenic regions on the virion. Using this approach the putative residues directly involved in antigenicity could be narrowed down compared to amino acid alignment alone. Indeed, residue positions with high entropy and surface exposure were located in the βB–βC (aa 71–74) and βE–βF (aa 133–134) loops and aa 196 of 1B, the βB–βC (aa 64–68) and βE–βF (aa 134–136) loops of 1C, and the 1D βB–βC loop (aa 46–49), residues 142–148 and 157 in βG–βH loop, 177–179 in the βH–βI loop and 208 and 214 in the C terminus.

Within the SAT2 serotype similar observations were made. Comparison of the P1 polypeptide of six SAT2 isolates that belong to topotype 1 (KNP/19/89, KNP/02/89, KNP/51/93, SAR/16/83, ZIM/08/94 and ZIM/GN/10/91) revealed 16 to 42 variable residue positions (Table 3). The r_1-values against SAT2/KNP/19/89 sera varied from 0.04 to 0.39 where the genetically closely related KNP/02/89 with 16/744 variable residues compared to KNP/19/89, had a maximum r_1-value of only 0.17 against KNP/19/89 sera (Table 3 and Supplementary Fig. S1b). These 16 variable residues are likely involved in abolishing the neutralization by SAT2/KNP/19/89 antisera. Using structural data, surface-exposed residues with high entropy could be identified in the βE–βF (aa 130–134) loop of 1B, the βE–βF (aa 129–134) loop of 1C, and βB–βC (aa 63–68), βE–βF (aa 83–85), βF–βG (aa 110–112), βG–βH (aa 136–140 and 156–161), βH–βI (aa 172–176) and C terminus (aa 200–202) of 1D.

Taken together the inability of anti-SAT1/SAR/09/81 or anti-SAT2/KNP/19/89 antiserum from vaccinated animals to neutralize genetically related viruses may be the result of amino acid variation in the above-mentioned surface-exposed β-loops.

Mapping putative antigenic sites on the virus capsid

We modelled the SAT1 and SAT2 capsid structures using O_1BFS (Logan *et al.*, 1993) as template. The model was based on the optimal alignment of the SAT1 virus, SAR/09/81, or the SAT2 virus, ZIM/07/83, P1 sequences to the corresponding sequence of O_1BFS. The homology modelled structure was calculated by the satisfaction of spatial restraints as described by empirical databases. Using this method and mapping the observed variable loops (Table 4) to the structures, we identified several putative antigenic sites on the virus capsid (Fig. 2a, b). In particular, we observed differences between serotypes, but also within topotypes in a given serotype.

Taking all the topotype information into consideration, the variable residues (high entropy) within surface-exposed loops were regarded as immune relevant and were mapped to the SAT1 and SAT2 pentamer structures, respectively (Fig. 2a, b and Table 4). For both serotypes the variable regions outside the 1D βG–βH loop were concentrated around the fivefold and threefold axis of the virion and the C-terminal of 1D. These regions correlated strongly with

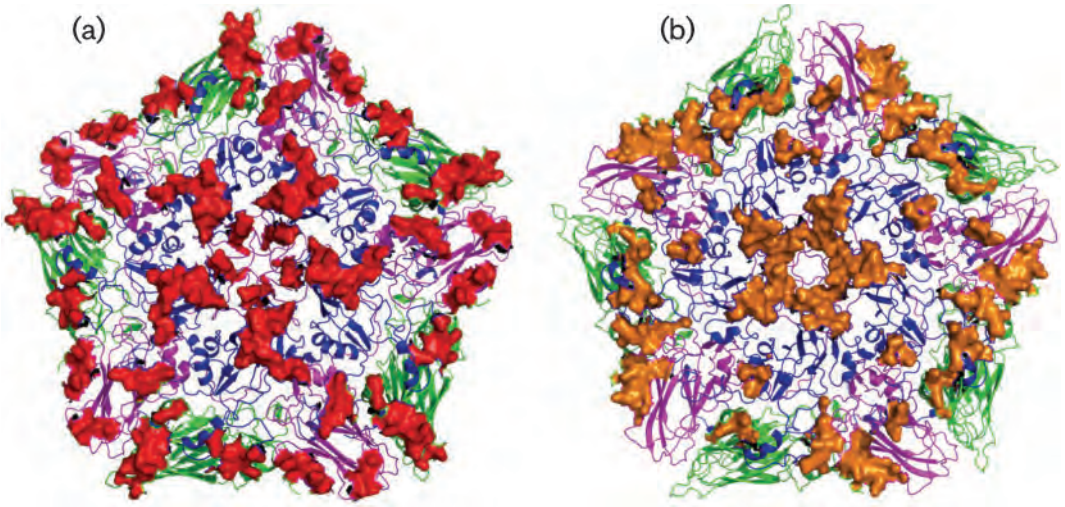

Fig. 2. Three-dimensional structure of (a) SAT1 and (b) SAT2 pentamer, modelled using the O_1BFS co-ordinates (1FOD; Logan *et al.*, 1993), as template. The protein subunits are colour coded: VP1 (blue), VP2 (green) and VP3 (cyan). The position of surface-exposed residues with high entropy is indicated in red (a) or orange (b) on the SAT1 or SAT2 pentamers, respectively. The alignment used for the extraction of data are available as in Supplementary material.

previously identified neutralizing epitopes of types A and O (Table 4). The role of these residues as antigenic determinants can therefore not be excluded. Furthermore, it can be hypothesized that some of the putative epitope regions are probably discontinuous. The close proximity of 1B residues 71–74, 133–134 and 196 or 1B residues 71–74, 196 and 1C residues 64–68 around the threefold axis of the virion or 1D residues 46–49, 84–86 and 177–179 around the fivefold axis of SAT1 viruses are examples (Fig. 2a, b).

DISCUSSION

This study reports on a genetic and phylogenetic analysis of the entire capsid-coding region and its deduced amino acid sequence of the three SAT serotypes of FMDV. We have also consolidated amino acid variation and r_1-values with structural data in order to predict areas on the surface of the FMD virion that are antigenically significant.

Based on the complete P1-coding region the phylogeny of 48 SAT isolates with diverse geographical distribution in Africa revealed the same evolutionary lineages within a serotype as previously described for 1D phylogeny (Bastos *et al.*, 2001, 2003a, b). Phylogenetic analysis of the complete P1- or 1B- and 1C-coding regions of the SAT isolates indicated similar tree topologies compared to 1D phylogeny (data not shown). Phylogeny, based only on the internally located 1A genomic region, revealed similar structuring than that described previously for the Leader and 3C protease-coding regions (van Rensburg *et al.*, 2002), although different from the outer capsid-coding regions. Darwinian selective pressure for the evolution of the non-structural proteins and the internally located 1A, exist to preserve structure and functionality and escape from immune response is less important (van Rensburg *et al.*, 2002).

High levels of genetic diversity in the P1-coding region within the SAT serotypes are reflected antigenically and therefore have implications for the control of the disease through vaccination. Antigenic analysis of field isolates in relation to vaccine strains, based on VNTs, play a significant role in evaluating the suitability of existing vaccine strains (Jangra *et al.*, 2005; Paton *et al.*, 2005; Brehm *et al.*, 2008), despite significant variation having been reported with VNTs (Rweyemamu *et al.*, 1978, 1984). It is generally accepted that r_1-values higher than 0.4 demonstrates a good cross-protection of the vaccine against the field isolate (Samuel *et al.*, 1990). Although SAT1 and SAT2 viruses displayed a similar number of variable amino acids (32 %) in a complete alignment, antigenic variation within SAT1 was less pronounced (73 % of r_1-values >0.2) than for SAT2 viruses (17 % of r_1-values >0.2) within the dataset. The implication for control by vaccination is that a high potency or bi-valent SAT1 vaccine will most likely be effective across topotypes. Indeed, it has been shown for serotype A viruses that a high potency vaccine provides protection against heterologous challenge despite low r_1-values (Brehm *et al.*, 2008). Better antigenic relationships were obtained for the SAT1 viruses belonging to the same topotype of the reference viruses (with less than 16 % aa variation). In the past, the combination of SAT1/SAR/9/81 and SAT1/KNP/196/91 in a tetravalent vaccine, containing also a SAT2 and a SAT3 strain, were able to protect against SAT1 outbreaks in Southern Africa (Hunter, 1998).

The SAT2 reference strains ZIM/7/83 and KNP/19/89, on the other hand, did not have good antigenic relationships

with most SAT2 isolates, even within the same topotype. ZIM/7/83 antisera cross-reacted weakly to the SAT2 viruses in this study and were not able to neutralize the genetically closely related ZIM/1/88 (19 aa differences in a pairwise comparison). From our dataset, it seems unlikely that SAT2/ZIM/7/83 alone will provide protection against the large genetic and antigenic diversity among the SAT2 viruses. Antigenic relationships against KNP/19/89 was better with at least 16 % of the isolates having an r_1-value >0.2, while ERI/12/98 and RWA/02/01 antisera were able to neutralize at least 20 % of the viruses *in vitro*. Similar to the SAT1 viruses, better antigenic relationships to ERI/12/98 and RWA/02/01 were obtained for SAT2 viruses belonging to the same topotypes. Our study suggests that extensive antigenic variation occurs for SAT2 viruses across Africa and has serious implications for vaccine strain selection. A previous serological comparison of SAT2 viruses from Kenya suggested extensive antigenic variation in this country alone (Ndiritu *et al.*, 1983). The poor antigenic coverage of existing vaccine strains against field strains call for urgent development of multiple region-specific or topotype-specific vaccine strains. Additionally, there is a need for a SAT2 vaccine strain that will provide protection against a wide range of antigenic types in the field.

mAbs against FMDV were used in the past to identify antigenic variants and to resolve epitopes that play a role in the neutralization of the virus. The majority of these mAbs were against Euro-Asian serotypes A, O and C (Xie *et al.*, 1987; Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; McCahon *et al.*, 1989; Kitson *et al.*, 1990; Saiz *et al.*, 1991; Crowther *et al.*, 1993a). Only one report described the mapping of epitopes to a SAT2 virus (RHO/1/48) isolated in 1948 (Crowther *et al.*, 1993b), with residues 147–149 and 156 in the flexible and surface-exposed G–H loop of 1D proven to be important in *in vitro* neutralization by a mAb.

We followed a different approach by combining amino acid variation and calculated r_1-values from *in vitro* cross-protection titres in VNTs, together with structural data, to predict areas on the surface of the capsid as antigenically relevant. This modelling approach has identified putative antigenic regions that correlated with cross-neutralization *in vitro* for the SAT1 and SAT2 serotypes, and may contain neutralizing epitopes for each serotype. These antigenic sites were consistent within, but not between serotypes, and were found to match some of the mAbs identified antigenic sites in other serotypes. These are the hypervariable, surface-exposed structural loops observed for SAT1 or SAT2 viruses, i.e. βB–βC and βE–βF loops of 1B, the βB–βC and βE–βF loops of 1C, and the N-terminal, βB–βC, βG–βH, βH–βI loops and C-terminal of 1D. The antigenic sites for SAT2 viruses differed by having no significant variation within the βB–βC loop of 1C, but with additional variation in the βD–βE and βF–βG loops of 1D. Epitopes identified from mAb escape mutants for other serotypes confirms the immune relevance of these structural loops. The βB–βC, βE–βF and βH–βI loops of 1B contains site 2 of serotype O (Kitson *et al.*, 1990; Crowther *et al.*, 1993a), site 3 on A10 (Thomas *et al.*, 1988) and βB–βC also correlates with subsite D2 on C (Mateu *et al.*, 1995). The βB–βC loop of 1C is in agreement with site 3 on A10 (Thomas *et al.*, 1988), while βB–βC, βG–βH, βH–βI loops and C-terminal of 1D all agree with epitopes identified for serotypes A, O and C (Kitson *et al.*, 1990; Crowther *et al.*, 1993a; Thomas *et al.*, 1988; Mateu *et al.*, 1995).

Although the putative antigenic sites need to be confirmed using mAbs and sequencing of virus escape mutants, the methodology employed in this study offers a potentially quick, easy and cheap alternative to the identification of antigenic relevant sites on field FMD strains.

METHODS

Virus isolates, RT-PCR, sequencing and analysis. The viruses included in this study were either supplied by the World Reference Laboratory (WRL) for FMD at the Institute for Animal Health, Pirbright (UK) or form part of the virus bank at the Transboundary Animal Diseases Programme, Onderstepoort (South Africa). The 20 SAT1 and 23 SAT2 FMDV isolates from 17 countries in Africa, selected for genetic characterization, represented a broad geographical distribution. The viruses were propagated in IB-RS-2 cells prior to RNA extraction, cDNA synthesis and PCR amplification. A description of the passage histories, host species and representative topotypes can be found in Reeve *et al.* (2010); Bastos *et al.* (2001, 2003a, b) and Sangare *et al.* (2003). The GenBank accession numbers are as provided in Reeve *et al.* (2010). The SAT3 viruses included in the P1 phylogenetic analysis were SAT3/BEC/01/65 (GenBank accession no. M28719), SAT3/KNP/10/90 (GenBank accession no. AF286347), SAT3/UGA/02/97 (GenBank accession no. AY192556), SAT3/ZAM/04/96 (GenBank accession no. AF023525.1) and SAT3/ZIM/05/91 (GenBank accession no. AY168799.1).

The Leader-P1-2A-coding regions of the isolates were obtained via RT-PCR of viral genomic RNA as described previously (Bastos, 1998; van Rensburg *et al.* 2002). Direct DNA sequencing of amplicons yielded a consensus sequence representing the most probable nucleotide for each position. Sequences of the approximately 2.2 kb P1-coding region were compiled and edited using the BioEdit 5.0.9 software (Hall, 1999). The nucleotide and deduced amino acid sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and phylogenetic trees constructed using the minimum evolution algorithm within MEGA4 software (Tamura *et al.*, 2007). Entropy plots were drawn from the deduced amino acid alignments using the BioEdit 5.0.9 software (Hall, 1999).

Animal sera and VNT. The antigenic diversity of the field isolates was determined using cross-neutralization assays in microtitre plates on IB-RS-2 cells, carried out as described by the OIE (2009). Cattle sera prepared by two consecutive vaccinations (vaccinated at day 0, boosted at day 28 and bled at day 38) or convalescent sera obtained from cattle 21 days post-infection with reference viruses (SAT1: SAR/09/81, KNP/196/91 and NIG/05/81; SAT2: ZIM/7/83, KNP/19/89/2, ERI/12/89 and RWA/02/01) were used. For cattle immunizations oil emulsion vaccines were prepared using binary ethylenimine-inactivated sucrose density gradient purified antigens for strains SAT1/SAR/09/81, SAT1/KNP/196/91, SAT2/ZIM/07/83 and SAT2/KNP/19/89/2, respectively. Each 2 ml dose, containing 4 μg 146S antigen and Montanide ISA 206 (Seppic) as the oil adjuvant, were mixed 50 : 50 with the aqueous antigen phase to produce a water-in-oil-in-water emulsion. Convalescent sera were prepared by inoculating cattle

intradermolingually with 10^4 $TCID_{50}$ of the SAT1/NIG/5/81, ERI/12/89 and RWA/02/01 viruses, respectively.

The end-point titre of the serum against homologous and heterologous viruses was calculated as the reciprocal of the last dilution of serum to neutralize 100 $TCID_{50}$ in 50 % of the wells (Rweyemamu *et al.*, 1978). All neutralization titre determinations were repeated at least twice, each time using sera from two animals. One-way antigenic relationships (r_1-value) of the field isolates and vaccine viruses relative to the reference strains were calculated as the ratio between the heterologous and homologous serum titres and were interpreted as proposed by Samuel *et al.* (1990). Briefly, r_1-values between 0 and 0.19 indicated highly significant antigenic variation from the reference strains; values of 0.20–0.39 showed antigenic relatedness where some protection may be provided by a potent vaccine based on the reference strain; and values of 0.40–1.0 demonstrated that the reference and field strains are sufficiently antigenically similar for the reference strain to provide good protection. The means of the r_1-values were calculated (Tables 1 and 2) and plotted against total amino acid changes between the field strains and reference viruses.

Structural modelling. Homology models of the capsid proteins of SAT1 and SAT2 were built using Modeller 9v3 (Sali & Blundell, 1993) with O_1BFS coordinates (1FOD) as template (Logan *et al.*, 1993), alignments were performed with CLUSTAL_X and modelling scripts were generated with the structural module of FunGIMS. Structures were visualized and the surface-exposed residues identified with PyMol v1.1rc2pre (DeLano Scientific LLC).

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Mapping of amino acid residues responsible for adhesion of cell culture-adapted foot-and-mouth disease SAT type viruses

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ABSTRACT

Foot-and-mouth disease virus (FMDV) infects host cells by adhering to the α_V subgroup of the integrin family of cellular receptors in a Arg–Gly–Asp (RGD) dependent manner. FMD viruses, propagated in non-host cell cultures are reported to acquire the ability to enter cells via alternative cell surface molecules. Sequencing analysis of SAT1 and SAT2 cell culture-adapted variants showed acquisition of positively charged amino acid residues within surface-exposed loops of the outer capsid structural proteins. The fixation of positively charged residues at position 110–112 in the βF–βG loop of VP1 of SAT1 isolates is thought to correlate with the acquisition of the ability to utilise alternative glycosaminoglycan (GAG) molecules for cell entry. Similarly, two SAT2 viruses that adapted readily to BHK-21 cells accumulated positively charged residues at positions 83 and 85 of the βD–βE loop of VP1. Both regions surround the fivefold axis of the virion. Recombinant viruses containing positively charged residues at position 110 and 112 of VP1 were able to infect CHO-K1 cells (that expresses GAG) and demonstrated increased infectivity in BHK-21 cells. Therefore, recombinant SAT viruses engineered to express substitutions that induce GAG-binding could be exploited in the rational design of vaccine seed stocks with improved growth properties in cell cultures.

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1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of cloven-hoofed animals causing significant distress and suffering in animals. Although mortality is usually low (<5%) (Thomson, 1995), morbidity can reach 100% and the impact can be catastrophical when an outbreak occurs in a FMD-free region with immunologically naïve population of livestock. Consequently FMD is classified by the OIE as one of the most important infectious diseases of livestock (Office International des Épizooties Terrestrial Manual, 2009). The economically critical effects to livestock farming due to the high cost of disease control and international trade restrictions (Sellers and Daggupaty, 1990) was evidenced during the 2000–2001 outbreaks in Europe and the virus escape that occurred more recently, in 2007 in the United Kingdom (Samuel and Knowles, 2001; Cottam et al., 2008). In endemic regions, FMD is controlled by restricting animal movement, the implementation of vaccination programmes and biosecurity measures. In disease-free countries where vaccination is normally not applied, ring-vaccination is only used in an emerging outbreak with subsequent slaughtering of vaccinated animals (Müller et al., 2001; Tomassen et al., 2002).

In South Africa, other regions of the African continent, as well as in some Asian and South American countries, regular immunisation is essential for disease control, and in maintaining FMD-free status. Current FMD vaccines are chemically inactivated preparations of concentrated, virus-infected cell culture supernatants (Office International des Épizooties Terrestrial Manual, 2009). Therefore, large-scale vaccine production utilize a suitable cell line, like BHK-21 cells, and requires that the vaccine strain is adapted and propagated in cell culture (Amadori et al., 1994, 1997). However, these cell lines have limited (monolayers) or no (in suspension) expression of the required primary receptor for infection by FMDV

Abbreviations: FMD, foot-and-mouth disease; FMDV, FMD virus; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; SAT, South African Territories.

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(Amadori et al., 1994). In the early 1980s it was noted that viruses of the three SAT serotypes, endemic in Africa, are notorious for their difficulty to adapt to BHK-21 cells (Pay et al., 1978; Preston et al., 1982). It is thought that the cell surface molecules, which may act as virus receptors, exert an important selective pressure on viral RNA quasi-species, thereby enabling adaptation. Studies have shown that repeated passaging of FMDV in cultured cells rapidly gave rise to mutant viruses within the population (Rieder et al., 1994; Herrera et al., 2007). Adaptation of wild-type SAT viruses in cell culture to produce high yields of stable antigen is an intricate and time-consuming process that is often associated with a low success rate.

FMD virus (FMDV), the type species of the *Aphtovirus* genus in the family *Picornaviridae* (Racaniello, 2006), infects epithelial cells by adhering to any of four members of the α_V subgroup of the integrin family of cellular receptors, *i.e.* $\alpha_V\beta1$, $\alpha_V\beta3$, $\alpha_V\beta6$ and $\alpha_V\beta8$ (Berinstein et al., 1995; Neff et al., 1998, 2000; Jackson et al., 1997, 2000, 2002, 2004; Duque and Baxt, 2003). Attachment to the receptors is mediated via a highly conserved Arg–Gly–Asp (RGD) motif (Fox et al., 1989; Baxt and Becker, 1990; Mason et al., 1994; Leippert et al., 1997) located within the structurally disordered βG–βH loop of VP1 (Acharya et al., 1989; Lea et al., 1995; Curry et al., 1997). Following FMDV–receptor interactions, the virus is internalised and the viral genome is released in the cytosol. Adaptation of FMD field isolates to enable efficient replication in cultured cells is accompanied by changes in viral properties, including the acquisition of the ability to bind to alternative cellular receptors such as cell surface glycosaminoglycans (GAGs) (Jackson et al., 1996, 2001; Sa-Carvalho et al., 1997; Zhao et al., 2003). The interactions of a diverse group of ligands, such as growth factors, chemokines, herpes simplex virus (HSV), human immunodeficiency virus, respiratory syncytial virus, alphaviruses, dengue virus, adeno-associated virus and FMDV, to the highly sulfated GAGs (also known as heparan sulfate proteoglycans, HSPG) is typically via a positively charged domain on these proteins (Patel et al., 1993; Gromm et al., 1995; Jackson et al., 1996; Chen et al., 1997; Krusat and Streckert, 1997; Sa-Carvalho et al., 1997; Byrnes and Griffin, 1998; Klimstra et al., 1998; Summerford and Samulski, 1998; Fry et al., 1999, 2005; Zhao et al., 2003). The ability of a type O virus to enter cells following adhesion to HSPG is thought to be dependent on the presence of the positively charged Arg residue at position 56 of VP3 which results in a net gain of positive charge on the virion surface (Sa-Carvalho et al., 1997; Fry et al., 1999).

Replacement of the external capsid-coding sequence of an infectious cDNA clone with the corresponding region of an outbreak virus results in the transfer of surface-exposed epitopes from the aetiological agent to the recombinant virus (Zibert et al., 1990; Rieder et al., 1993; Almeida et al., 1998; Van Rensburg and Mason, 2002; Van Rensburg et al., 2004). The chimeric viruses, produced in this manner, induce a protective immune response in animals similar to that of the outbreak virus. However, co-transferral of undesirable traits, such as capsid instability and poor cell culture adaptation of the field virus may also occur. Therefore, application of reverse genetics technology in FMD vaccinology includes the identification of amino acid sequences associated with the acquisition of HSPG-binding during cell culture adaptation of SAT viruses and the introduction of these changes into chimeric constructs.

In this report we identify novel amino acid residues within the capsid proteins of SAT1 and SAT2 viruses that affect the virus' ability to grow in different cell lines. We demonstrated that cell culture adaptation to BHK-21 cells is acquired following repeated passaging of the field viruses in these cells. Furthermore, we illustrated that this phenotype can be transferred to an infectious cDNA clone of a FMD field virus from which viable cell culture-adapted viruses were recovered.

2. Materials and methods

2.1. Cells, viruses and plasmids

Baby hamster kidney (BHK) cells, strain 21, clone 13 (ATCC CCL-10) were maintained as described by Rieder et al. (1993). Chinese hamster ovary (CHO) cells strain K1 (ATCC CCL-61) were maintained in Ham's F-12 medium (Invitrogen), supplemented with 10% foetal calf serum (Delta Bioproducts). Primary pig kidney (PK) and Instituto Biologico renal suino (IB-RS-2) cells Plaque assays were performed using a tragacanth overlay method and 1% methylene blue staining (Rieder et al., 1993).

Viruses used in this study included four SAT1 viruses, *i.e.* KNP/196/91, SAR/9/81, NAM/307/98 and ZAM/2/93; and four SAT2 isolates, *i.e.* ZIM/14/90, ZIM/5/83, ZAM/7/96, and KNP/19/89. The host species the viruses were isolated from is summarized in Table 1. The SAT2/ZIM/7/83 vaccine strain is a derivative of ZIM/5/83 and is used in inactivated vaccines to assist with control of FMD along the borders of South Africa. The viruses were isolated in primary pig kidney cells (PK) and grown on IB-RS-2 cells (parental viruses) prior to adaptation to BHK-21 cells (vaccine strains). To distinguish the low passage, parental viruses from their BHK-21 culture-adapted derivatives, we will refer to the field isolate by its accession code followed by P (parental) or Vac/BHK (vaccine or BHK-21 cell-adapted strain) superscript. The passage histories of the viruses are summarised in Table 1.

The construction of plasmids pSAT2, pNAM/SAT and pSAU/SAT is described elsewhere (Böhmer, 2004; Van Rensburg et al., 2004; Storey et al., 2007). In short, the pNAM/SAT and pSAU/SAT were constructed by replacing the outer capsid-coding region of pSAT2 with the corresponding region of SAT1/NAM/307/98 and SAT2/SAU/6/00 using the unique restriction sites SspI and XmaI in VP2 and 2A-coding regions, respectively (Böhmer, 2004; Storey et al., 2007).

2.2. RNA extraction, cDNA synthesis, PCR amplification and nucleotide sequencing

RNA was extracted from infected cell lysates using either a guanidium-based nucleic acid extraction method (Bastos, 1998) or TRIzol® reagent (Life Technologies) according to the manufacturer's specifications and used as template for cDNA synthesis. Viral cDNA was synthesised with SuperScript III™ (Life Technologies) and oligonucleotide 2B208R (Bastos et al., 2001). cDNA copies of the *ca.* 3.0 kb Leader/capsid-coding regions of the viral isolates were obtained by PCR amplification using AdvanTaq™ DNA polymerase (Clontech) with specific oligonucleotides (NCR2: 5′-GCTTCTATGCCTGAATAGG and WDA: 5′-GAAGGGCCCAGGGTTGGACTC) following the manufacturer's recommendations. Sequencing of the amplicons was performed using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (PerkinElmer Applied Biosystems). The GeneBank accession numbers of the wild-type (low passage history) virus P1 sequences are as follows: DQ009715 (SAR/9/81); DQ009716 (KNP/196/91); DQ009717 (NAM/307/98); DQ009719 (ZAM/2/93); DQ009726 (ZIM/7/83); DQ009728 (ZIM/14/90); DQ009741 (ZAM/7/96); DQ009735 (KNP/19/89). The differences in deduced amino acid sequences between the wild-type and cell culture-adapted strains are summarised in Table 2.

2.3. Site-directed mutagenesis of cDNA clones

Site-directed mutagenesis of plasmids pNAM/SAT and pSAU/SAT was accomplished by using amplicon overlap site-directed mutagenesis. The forward mutagenesis primer for

Table 1
Summary of SAT1 and SAT2 viruses and their derivatives used in production, field isolate and their derivatives, passage histories, titres prior to and after adaptation and properties in cell culture.

Serotype	Viruses[a]	Host species	Passage history[b]	Vaccine stock[c]	Titre (pfu/ml) in BHK cells	CHO-K1 infectivity[d]
SAT1	KNP/196/91^{P}	Buffalo	PK_1	–	5.2×10^7	1.08×10^{-4}
	KNP/196/91^{Vac}	–	$PK_1RS_4B_1BHK_5$	MSV	1.3×10^8	2.80×10^6
	SAR/9/81^{Epi}	Impala	epithelium	–	9.6×10^7	6.40×10^{-3}
	SAR/9/81^{Vac}	–	$PK_1RS_4BHK_5$	MSV	7.2×10^9	1.10×10^6
	NAM/307/98^{P}	Buffalo	PK_1RS_1	–	–	–
	NAM/307/98^{BHK}	–	$PK_1RS_1BHK_5$	–	1.1×10^7	–
	ZAM/2/93^{P}	Buffalo	BTY_1RS_2	–	8.6×10^6	2.82×10^{-4}
	ZAM/2/93^{BHK}	–	$BTY_1RS_2BHK_5$	–	2.2×10^8	1.06×10^7
SAT2	KNP/19/89^{P}	Buffalo	$PK_1RS_1B_1$	–	2.2×10^5	1.19×10^{-5}
	KNP/19/89^{Vac}	–	$B_1PK_1RS_1BHK_4$	MSV	1.1×10^7	5.06×10^6
	ZIM/5/83^{P}	Bovine	BTY_4RS_1	–	1.8×10^5	5.91×10^{-5}
	ZIM/5/83^{BHK}	–	$BTY_4RS_2BHK_8$	–	3.4×10^7	–
	ZIM/7/83^{Vac}	Bovine	$B_1BHK_4B_1BHK_5$	MSV	1.8×10^7	6.58×10^6
	ZIM/14/90^{P}	Buffalo	BTY_1RS_3	–	1.0×10^5	–
	ZIM/14/90^{BHK}	–	$BTY_1RS_3BHK_8$	–	2.0×10^6	–
	ZAM/7/96^{P}	Buffalo	BTY_1RS_2	–	8.0×10^6	–
	ZAM/7/96^{BHK}	–	$BTY_2RS_2BHK_8$	–	5.6×10^7	–

[a] The viruses used in this study were notated by the superscript P = most primary isolate for that strain defined as the lowest passage history; Vac = vaccine strain adapted on BHK-21 for production purpose; Epi = isolate from the epithelium tissue of the host species; BHK = propagated in BHK-21 monolayers.
[b] The passage history of the primary isolates and their derivates are indicated by cell type followed by the number of passages: B = bovine; PK = primary pig kidney cells; BTY = bovine thyroid cells; RS = IB-RS-2 cells; BHK = baby hamster kidney cells (strain 21, clone 13).
[c] MSV = master seed virus.
[d] The infectivity in CHO-K1 cells is expressed as a titre difference between a 1 and 24 h post-infection of CHO-K1 cells. A negative value is indicative of inability to infect and replicate in CHO-K1 cells, whereas a positive value indicate infection and replication in the same cell line. "–" has not been done.

pNAM/SAT was CAGTCGTCTTCTCCaaacGAcGCACCACTCGCTTCGC (NAMmut4; lower cased letters represent altered bases), replacing the wild-type KGG sequence at position 110–112 of VP1 with KRR. Similarly the mutagenesis primers for pSAU/SAT were GTGGGCGACCACCggcgcGCCTTTTGGCAGCCTAAC (SAUmut1) and GTACGCTGACAGCAaGCACaaactcCCGTCAACCTTC (SAUmut2). The latter primers introduced the E85R and E161K substitutions respectively. Briefly, the first PCR reactions were performed using NAMmut4, SAUmut1 and SAUmut2 as the sense mutagenic and cDNA-2A (CGCCCCGGGGTTGGACTCAACGTCTCC; XmaI site underlined) as the antisense oligonucleotides. The second PCR reactions were performed with the reverse complements of NAMmut4, SAUmut1 and SAUmut2 as antisense mutagenic in combination with 5′-specific sense oligonucleotides, cDNA-NAM (CGGAATATTGACCACCAGCCATGGTACCACCAC; SspI site underlined) or cDNA-SAU (CGGAATATTGACCACACGTCACGGAACCACGAC; SspI site underlined). Cycling conditions for both PCRs were as follows: 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 1 min (20 cycles). The amplicons of the first two reactions were combined in equimolar amounts, denatured at 95 °C for 20 s, extended and enriched by another 25 cycles of amplification with cDNA-NAM or cDNA-SAU and cDNA-2A using TaKaRa Ex Taq™ (Takara). The resulting *ca.* 2.2 kb DNA fragment (containing either pNAM/SAT or pSAU/SAT mutations) was digested with SspI and XmaI and cloned into the corresponding region of pSAT2. The mutations were verified by nucleotide sequencing of the complete P1-coding region using selected oligonucleotides and no unintended site mutations were found.

2.4. *In vitro RNA synthesis, transfection and virus recovery*

Plasmids containing genome-length cDNAs, chimeric cDNA or site-directed mutated cDNA clones were linearised at the SwaI site downstream of the poly-A tract and used as templates for RNA synthesis utilising the MEGAscript™ T7 kit (Ambion). Transcript RNAs were examined in agarose gels for their integrity and quantitated by spectrometry. BHK-21 cell monolayers in 35 mm cell culture plates (Nunc™) were transfected with 2–3 μg of the *in vitro* synthesised RNA using Lipofectamine2000™ (InVitrogen). Transfected monolayers were incubated at 37 °C with a 5% CO_2 influx for 48 h in Eagle's basal medium (BME) containing 1% foetal calf serum (v/v) and 25 mM HEPES. The virus-containing supernatants were used to infect fresh BHK-21 monolayers (35 mm cell culture plates) using 1/10th of clarified infected supernatants and incubated for 48 h at 37 °C. Viruses were subsequently harvested from infected cells by a freeze–thaw cycle and passaged four times on BHK-21 cells, using 10% of the supernatant of the previous passage. Following the recovery of viable viruses, the presence of the mutations was verified once again with automated sequencing.

2.5. *Evaluation of the ability of SAT types of FMDV to infect and replicate in CHO-K1 cells*

Twenty-four hours growth kinetics was performed in CHO-K1 cells which express GAG receptors (Jackson et al., 1996; Sa-Carvalho et al., 1997). Monolayers of CHO-K1 cells in 35 mm cell culture plates were infected with a MOI of 5–10 of the parental and vaccine strains (Table 1) for 1 h or 24 h at 37 °C. Infected monolayers were then frozen at −70 °C and thawed. Viruses from the lysed monolayers were titrated on BHK-21 monolayers and viral growth was calculated by subtracting the 24 h titre results from the 1 h titre results. Positive titers were an indication that the viruses are able to infect and replicate in CHO-K1 cells, suggesting the ability to utilise GAG receptors for cell entry. The nucleotide sequences of the isolates that were able to infect and replicate in CHO-K1 cells within 24 h were determined and compared to those of the parental viruses.

2.6. *Sequence alignments and structural modelling*

Homology models of the capsid proteins (VP1-4) for a representative virus of each of the SAT1 and two serotypes (SAT1/SAR/9/81, SAT2/ZIM/7/83) were built using Modeller 9v3 (Sali and Blundell, 1993) with 1FOD (Logan et al., 1993) as the template. SAR/9/81 and ZIM/7/83 capsid proteins were aligned with that of O1BFS

Table 2
Comparison of the capsid amino acid sequences of the SAT1 and SAT2 primary isolates and their cell culture-adapted derivatives.

Protein	β–β Structure	SAT1 isolate[a]			
		SAR/9/81	KNP/196/91	NAM/307/98	ZAM/2/93
VP2	βB–βC	–	Q2074R[b]	–	–
		–	Q2170H	–	–
		–	S2196N	–	–
VP3	βE–βF	–	D3009V	–	–
	βG–βH	–	–	**E3135K**[b]	–
		–	–	**E3175K**[b]	–
		–	–	–	A3180V
		D3192Y	–	–	–
		–	–	S3203T	–
		S3217I	–	–	–
		–	–	S3219L	–
VP1	βF–βG	–	Y1018H	–	–
	βF–βG	–	–	T1025A	–
		–	–	A1033T	–
		–	R1049K	–	–
		A1069G	–	–	–
		–	–	–	K1086Q
		N1110K[b]	**K1110K**[b]	–	–
		–	–	–	**N1111K**[b]
		G1112R[b]	**G1112R**[b]	G1112D	**G1112R**[b]
		–	–	K1157A	–
		–	–	E1177Q	–
		–	V1179E	–	–
		–	K1206R	–	–
		–	K1210R	–	–

Protein	β–β Structure	SAT2 isolate[a]			
		KNP/19/89	ZIM/5/83 vs. ZIM/7/83	ZAM/7/96	ZIM/14/90
VP2	βG–βH	I2032V	–	–	–
		–	M2077T	–	–
		–	–	–	E2096Q
		–	–	–	**Q2170R**[b]
VP3	βE–βF	H3036	–	–	–
		T3043S	–	–	–
		Q3049E	–	–	–
		–	–	–	**T3129K**[b]
		–	–	–	D3132N
		–	–	E3148K	–
		P3192T	–	–	–
VP1	βD–βE	–	M1028V	–	–
		–	A1064G	–	–
		–	–	**E1083K**[b]	–
	βD–βE	**Q1085R**[b]	–	–	–
		R1098T	–	–	–
	βG–βH	–	**E1161K**[b]	–	–
		–	Y1169H	–	–
		T1171A	–	–	–
		F1194L	–	–	–
		V1207A	–	–	–

[a] The amino acid residues have been numbered independently for each protein. For each residue, the first digit indicates the protein (VP1, VP2 or VP3) and the last three digits the amino acid position in either a SAT1 or SAT2 alignment. The P1 polypeptide of SAT1 viruses is 744 amino acids and that of SAT2 viruses 741 amino acids.
[b] Amino acid changes to a positive charge in surface-exposed loops are shown in bold.

using ClustalX and the sequence similarities for both were above 80%. The homology structure was calculated by the satisfaction of spatial restraints as described by empirical databases. Structural checks of the model were done using WHAT CHECK (Hooft et al., 1996).

The complete P1 sequences of 56 low passage SAT viruses (26 SAT1 and 30 SAT2) from 17 different countries in Africa, available on GenBank, were translated in BioEdit and aligned using ClustalX. The viruses used in the alignment were isolated from clinical material on PK cells and grown 1–4 passages in IBRS-2 cells (Bastos et al., 2001, 2003) and were referred to as wild-type viruses. The P1 sequences of the wild-type viruses were compared to the BHK-21 adapted viruses.

3. Results

3.1. Adaptation of SAT viruses in cell culture selects variants that gain a net positive charge on the virion surface

SAT viruses used for vaccine production are typically adapted to cultured cells following limited passages in BHK-21 cells. In this study, we have investigated the phenotypic and genetic changes associated with the transition of SAT viruses from wild-type to the cell culture-adapted phenotype. The morphology of virus plaques produced on BHK-21 cell monolayers following infection with SAT1 and SAT2 vaccine strains differed from the plaques produced by the parental strains which have not been adapted to BHK-21 cell

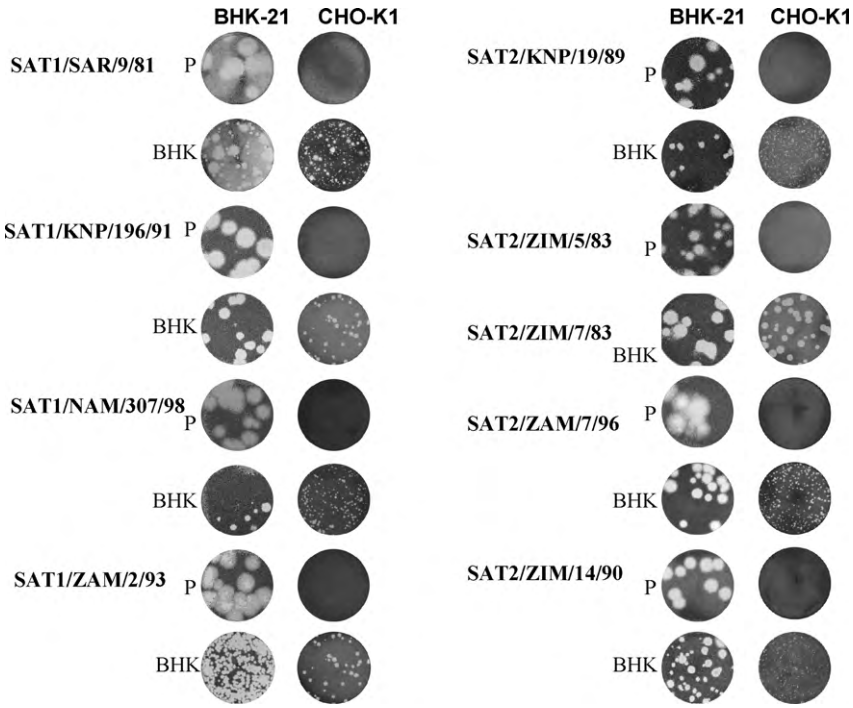

Fig. 1. Plaque morphologies of the parental (P) and cell culture (BHK) derived viruses obtained using monolayers of BHK-21 and CHO-K1 cells. Cells infected with the indicated viral strains were incubated with tragacanth overlay for 40 h prior to staining with 1% methylene blue. Plaques for SAT1 and SAT2 wild-type viruses are generally large with opaque edges and adaptation is accompanied by smaller to medium plaques and clear edges.

culture (Fig. 1). In particular, SAT1/KNP196/91^{P} (isolated on primary pig kidney cells) and SAT1/SAR/9/81^{P} (epithelium of infected impala) viruses displayed large plaques (7–8 mm) with opaque edges. In contrast, plaques produced by the corresponding vaccine derivatives, KNP/196/91^{Vac} and SAR/9/81^{Vac} viruses (high passage), exhibited a mixture of small (1–2 mm), medium (3–4 mm) to large plaques with clearly defined edges. Whereas the parental strains failed to infect CHO-K1 cells, both vaccine strains produced small and clear plaques on CHO-K1 cells (Fig. 1) and reached titers of 2.8×10^6 and 1.1×10^6 pfu/ml after 24 h growth on CHO-K1 cells, respectively (Table 1). Table 2 summarizes the amino acid differences of the low and high passage viruses. Comparison of the amino acid sequence in the outer capsid proteins of the KNP196/91^{P} and KNP/196/91^{Vac}, in particular, revealed 11 amino acid substitutions in the adapted strain, *i.e.* three substitutions in VP2, one in VP3 and seven in VP1.

The structural location of these 11 residues in the capsid proteins was mapped to a SAT1 capsid protomer. Fig. 2 illustrates two substitutions, the first in VP1 (Gly112 → Arg) and the second in VP2 (Gln74 → Arg), of neutral amino acids with positively charged residues which are located within surface-exposed loops that connect β-sheet structures. The Gln74 → Arg substitution in VP2 is located in the βB–βC loop which is characteristically hypervariable (see Table 3 for details). The proximity of this substitution to three other positively charged residues, Lys206 → Arg, Arg208 and Lys210 → Arg, located in the C-terminus of VP1 (Table 2 and Fig. 2) is noteworthy since these residues were reported to form part of the walls of a heparin-binding depression of serotype A viruses (Fry et al., 2005). Positively charged residues were found to be conserved at these positions in a complete alignment of SAT1 isolates (data not shown). The Gly112 → Arg substitution in VP1 forms part of a novel sequence in the βF–βG loop comprising three residues (KGG for KNP/196/91^{P}) where positively charged residues (KGR) accumulated during cell culture adaptation of SAT1 viruses (Table 2). The altered amino acid sequence correlates with the Asn110 → Lys and Gly112 → Arg substitutions in the VP1 protein of SAT1/SAR/9/81^{Vac}. The 3D structural model of a protomeric unit revealed that the positively charged SAT1 substitutions, at positions 110 and 112 in each of the interacting VP1 subunits, surround the pore located at the fivefold axis of the virion (Fig. 2). An interesting observation was that adjacent to this positively charged sequence of the VP1 βF–βG loop a Val was selected for in the place of an Asp at position 9 of VP3 in KNP/196/91^{Vac} (Table 2; Fig. 2) which may increase the local positive charge on the virion surface even more and increase the virus' ability to adhere to negatively charged sulfated polysaccharides.

Similar to KNP/196/91 and SAR/9/81, two other SAT1 field viruses (NAM/307/98 and ZAM/2/93) adapted and acquired the ability to infect CHO-K1 cells upon repeated passages in BHK-21 cells. The parental NAM/307/98 virus, isolated from buffalo (*Syncerus caffer*) in the West Caprivi Game Reserve in Namibia in 1998 (Bastos et al., 2001; Storey et al., 2007) underwent slow adaptation to cell culture, only attaining titers of 10^5 pfu/ml after eight passages in BHK-21 cells, and progeny viruses failed to infect CHO-K1 cells (Fig. 1). Only following repeated passaging in BHK-21 cells, this isolate finally adapted to BHK-21 cells (NAM/307/98^{BHK}) as measured by its ability to infect CHO-K1 cells and by producing titers in excess of 10^7 pfu/ml in BHK-21 cells (Table 1). NAM/307/98^{BHK} yielded clear plaques of small (1–2 mm) and medium (3–5 mm) sizes and acquired the ability to infect CHO-K1 cells (Fig. 1). Sequence analysis revealed nine amino acid substitutions in the capsid proteins of the adapted strain (Table 2). Only two of these were positively charged and located within surface-exposed loops of VP3, *i.e.* residues Glu135 → Lys and Glu175 → Lys. The residues are located around the threefold axis of the pentamer unit (Fig. 2). Another SAT1 field isolate, ZAM/2/93 (Fig. 1), adapted rapidly in BHK-21 cells, only requiring two rounds of passaging, and the progeny viruses acquired two positively charged amino acids at position 111–112 of VP1 (Asn111 → Lys and Gly112 → Arg) (Table 2). This result is in agreement with the substitutions observed for the SAT1 vaccine strains KNP/196/91 and SAR/9/81. Therefore, we have identified a sequence "hotspot"

Table 3
A summary of the amino acid variation in the putative HSPG-binding sites identified for SAT1 and SAT2 BHK-21 adapted strains. The residue variation was obtained from complete P1 alignments of 24 SAT1 and 24 SAT2 field viruses.

	Amino acid variation					
	VP2	VP3		VP1		
	2074[a]	3135	3175	1083–1085	1110–1112	1161
SAT1	Q/R/A/K/E/N/S	E/N/A/D/V/S/T	E		N/K/H/R/A/T-G/N/D-G/N/D	
SAT2				E/D/I/T/K-H-E/R/K/T/S/Q/A		T/A/E/S/Q

/ denotes differences at the same aa position and "-" indicates the next aa.
[a] The first digit indicates the protein (VP1, VP2 or VP3) and the last three digits the amino acid position in either a SAT1 or SAT2 alignment.

(amino acids 110–112 of VP1) for the accumulation of positive charges during cell culture adaptation of SAT1 viruses.

We subsequently investigated whether SAT2 viruses that have acquired BHK-21 cell adaptation, do so by selecting positive charge substitutions in "hotspots" on the external capsid proteins. The SAT2 vaccine strain KNP/19/89^{Vac} (Table 1) was originally isolated from buffalo in the Kruger National Park (KNP/19/89^{P}). Whereas the viral plaques produced by the KNP/19/89^{Vac} strain on BHK-21 monolayers were medium (3–5 mm) in size and clearly defined, those produced on CHO-K1 cells were needle point (<1 mm) to small (1–2 mm) in size (Fig. 1). The KNP/19/89^{Vac} attained a titer of 5×10^6 pfu/ml at 24 h post-infection of CHO-K1 cells (Table 1). Another SAT2 vaccine strain, ZIM/7/83, passaged five times in BHK-21 cells, infected and replicated effectively in CHO-K1 cells, reaching a titer of 6.5×10^6 pfu/ml 24 h post-infection of CHO-K1 cells (Table 1), and produced medium-sized plaques in this cell line. The parental virus ZIM/5/83 failed to grow in CHO-K1 cells. For the two SAT2 vaccine strains (KNP/19/89^{Vac} and ZIM/7/83), only substitutions in VP1, *i.e.* Gln85 → Arg for KNP/19/89 and Glu161 → Lys for ZIM/7/83 (Table 2), effected in significant charge difference on the virion surface (Fig. 2). The latter substitution in VP1 is preceded by two positively charged residues, *i.e.* Lys159–His160, at the C-terminal base of the βG–βH loop. The Gln85 → Arg found in KNP/19/89^{Vac}, is structurally neighbouring the fivefold axis of the virion and forms part of a three positively charged amino acid sequence in the βD–βE loop of VP1. However, since VP1 residue 85 was conserved as Glu in ZIM/7/83, the amino acids at position 159–161 (Lys–His–Glu) of VP1 that changed to Lys–His–Lys in the vaccine strain may be necessary for BHK-21 cell adaptation in this strain.

The role of the three residues at position 83–85 in VP1 in the cell culture adaptation of SAT2 viruses was supported following the adaptation and characterisation of the field virus ZAM/7/96 (Table 1), isolated from buffalo (Bastos et al., 2003). Whereas this parental isolate produced large (6–7 mm) plaques on BHK-21 monolayers, the cell culture-adapted variant produced a mixture of medium (3–5 mm) and large plaques and was able to infect CHO-K1 cells, unlike its parental counterpart (Fig. 1). A Glu → Lys substitution was observed at position 83 of VP1, as well as residue 148 of VP3 (Table 2). Whether the two substitutions contributed synergistically or independently to the cell culture-adapted phenotype is not known. The ability of high passage ZIM/14/90 to infect and replicate in CHO-K1 cells (Fig. 1) was associated with Gln170 → Arg and Thr129 → Lys substitutions in VP2 and VP3,

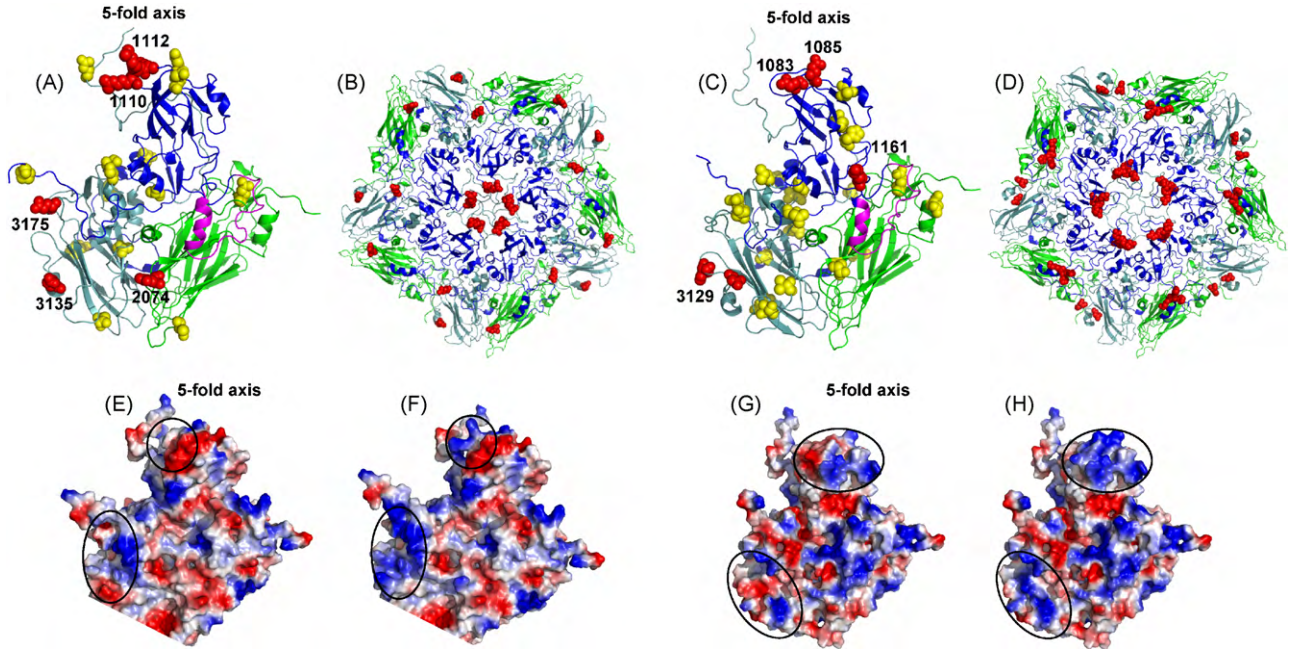

Fig. 2. 3D structure of a SAT1 and SAT2 protomeric subunit (A and C) and pentamer (B and D), modelled using the O1BFS co-ordinates (1FOD) as template. (A and B) The position of surface-exposed positive charge amino acid changes observed in cell culture-adapted SAT1 viruses is indicated in red. KRR, KGR and HRK positively charged residues were observed for SAR/9/81, KNP/196/91 and ZAM/2/93 at residue positions 110–112 of VP1. Lysines were present at residues 135 and 175 of VP3 in NAM/307/98 and an arginine at position 74 of VP2 in KNP/196/91. (C and D) For SAT2 viruses the positively charged residues at VP1 positions 83 and 85 (red) is surrounding the pore at the fivefold axis. The protein subunits and structural features for both models are colour coded: VP1 (blue), VP2 (green) and VP3 (light-teal), the G–H loop of VP1 containing the RGD motif (magenta). Other residue changes observed in SAT1 and SAT2 viruses are shown in yellow (see Table 2 for detail). (E–H) The electrostatic, accessible surface view of the SAT1 and SAT2 modelled biological protomers are shown. The electrostatic potential was coloured with positive charge as blue and negative in red and the scale of colouring was kept constant. The position of the charge change during adaptation is outline in black and is shown from E to F for SAT1 and G to H for SAT2 viruses.

respectively (Table 2). As indicated in Fig. 2, the selection of positively charged residues during adaptation occurred against a background of existing positive charges on the virion surface.

3.2. Genetic characterisation of residues involved in binding to CHO-K1 cells

Examination of the outer capsid protein sequences of 26 SAT1 and 30 SAT2 viruses from our database revealed a high level of variability at the residue positions associated with change during cell culture adaptation of the viruses (Table 3). For instance, residue 74 of VP2 is characterised by high entropy (Hx > 1.5) and forms part of a variable loop extending from residue 60–85, as is evident from a complete alignment of the capsid proteins of SAT1 viruses. At least 30% of the SAT1 viruses ($n = 8$) in the complete capsid protein alignment contained a Lys or Arg residue at position 74. However, the SAT1 isolates ($n = 26$) did not display the ability to infect and replicate in CHO-K1 cells (results not shown). In contrast, residue position 135 of VP3, also characterised by high entropy (Hx > 1.8), does not contain positively charged residues in any of the SAT1 field isolates, while position 175 of VP3 contains a conserved Glu residue in all 26 isolates. The VP1 residues 110–112, located in the short, variable βF–βG loop, display entropy of Hx > 0.7 and the absence of positively charged amino acids at positions 111 and 112. Where a Lys or Arg residue did occur at position 110, it was, however, followed by a negatively charged Asp residue at positions 111 or 112 in 50% of the cases.

A complete amino acid alignment of 30 SAT2 field isolates depicted the βD–βE loop of VP1 as a hypervariable region with residues 83 and 85 displaying high entropy (Hx > 1.3), while the His at position 84 was conserved. Only one isolate, KEN/3/57, an archive vaccine strain, contained a Lys–His–Lys sequence at this position, similar to that observed in a vaccine strain, KNP/19/89^{Vac}, and an adapted strain, ZAM/7/96^{BHK}. An Arg residue was repeatedly observed at position 85 of VP1 during adaptation of KNP/19/89 to BHK-21 cells. Residue 161 of VP1 was found to be variable in SAT2 viruses and a Lys residue was present at this position only in the cell culture-adapted strain ZIM/7/83^{Vac}. Notably, this residue follows two other positively charged residues, Lys159–His160, in VP1 of this virus.

The genetic changes observed in the case of cell culture-adapted SAT2/ZIM/14/90 were more complex and their implication for cell adaptation was less apparent (Table 2). From a structural perspective, the acquired positively charged residues at position 170 of VP2 and 129 of VP3 were neither surface exposed, nor were they located in the small heparin-binding depression (Fry et al., 2005) on the virion surface (Fig. 2). These changes were selected against a background of existing positively charged residues surrounding the threefold axis of the virus. This resulted in an increase in the positive polarity at local regions of the virion. In support of this observation was the finding that selection occurred against a negatively charged Asp residue at position 132 of VP3, which mutated to an Asn. Residues 129 and 132 of VP3 are located within a highly variable region, as observed from the P1 alignment of 30 SAT2 viruses, and the possibility that this area may function as an epitope cannot be excluded.

3.3. Generation and characterisation of recombinant viruses with altered surface charges

To study the effect of individual mutations in a defined genetic background, we constructed recombinant virus mutants in FMDV SAT2 infectious cDNA. Of the 26 SAT1 and 21 SAT2 amino acid changes introduced during cell culture adaptation of the parental virus isolates, we selected the following residue positions in VP1 for the generation of site-directed mutant recombinant viruses (Fig. 3A): (1) RGD to KRR at position 110–112 in a SAT1/SAT2 chimeric construct, *i.e.* pNAM/SAT (Storey et al., 2007); (2) Gln85 → Arg which increased the net positive charge of VP1 surrounding the fivefold axis of SAT2 virions and (3) Glu161 → Lys which was unique in that SAT2/ZIM/7/83 accumulated three positively charged residues at the base of the βG–βH loop. The latter two mutations were constructed in a SAT2/SAT2 chimeric infectious clone, pSAU/SAT, containing the outer capsid-coding region of the SAT2/SAU/6/00 virus in the pSAT2 genetic background (Böhmer, 2004). The SAU/6/00 isolate caused a severe outbreak in dairy herds in Saudi Arabia in 2000. However, production of a SAU/6/00 vaccine is hindered by low 146S antigen yields, a consequence of SAU/6/00 being notoriously difficult to adapt to BHK-21 cells.

The effect of these mutations on surface charge distributions is shown in Fig. 2(E, F and G, H). The SAT1 mutations at position 110–112 (Fig. 2E and F) and the SAT2 mutations at position 83–85 of VP1 (Fig. 2G and H) had a strong effect on the local surface electrostatic potential. A distinct local surface area, neighbouring the fivefold axes, lost its negative charge and gained a predominantly positive charge in both SAT1 and SAT2 viruses, although the amino acid positions were dissimilar. However, the positively charged region spanning residues 159–161 of the SAT2 vaccine strain (ZIM/7/83), was located at the base of the βG–βH loop and did not conform to the region surrounding the fivefold axis or the depression at the VP2/VP3/VP1 border (Fry et al., 2005).

Viable recombinant mutant viruses, designated vNAM(KRR)/SAT, vSAU(E85R)/SAT and vSAU(E161K)/SAT, were recovered from all three mutant clones and passaged in BHK-21 cells until CPE was observed (3–5 passages). Sequencing of the capsid-coding region of the progeny viruses revealed that the introduced KRR mutation was fixed in the population as a KGR sequence, but no other unintentional mutations were introduced in the mutant viruses.

3.4. Plaque phenotypes and relative infectivity titers

We subsequently investigated the ability of the recombinant mutant viruses to infect CHO-K1 cells, and the effect these mutations had on infectivity titers in BHK-21 cells. Confirmation of the role of positively charged residues, located at position 110–112 in VP1 of SAT1 vaccine strains, in the adaptation to BHK-21 cells was obtained by introducing a KRR sequence into the vNAM/SAT interserotype chimeric virus. The vNAM/SAT virus contain the outer capsid-coding region of a Namibian buffalo isolate, *i.e.* NAM/307/98 (Bastos et al., 2001), and displays poor growth and adaptation to BHK-21 cells. Furthermore, vNAM/SAT lacked BHK-21 cell culture adaptation as measured by the inability to infect CHO-K1 cells and poor titers on BHK-21 cells. The plaques in Fig. 3B illustrate the transition of the large, opaque wild-type plaques produced by the vNAM/SAT (wild-type outer capsid) to small, well-defined plaques on BHK-21 cell monolayers of the mutated progeny virus, vNAM(KRR)/SAT. The vNAM(KRR)/SAT also attained titers of 10^6 pfu/ml within 5 passages in BHK-21 cells, while viruses harbouring the corresponding wild-type outer capsid-coding region only produced titers of 10^5 pfu/ml after eight passages. In summary, the mutations in the VP1 βF–βG loop neighbouring the fivefold axis of the SAT1 viruses, a site prone to variation, conferred the ability to adapt rapidly to BHK-21 cells and grow in CHO-K1 cells. This ability of the virus is probably due to utilisation of alternative HSPG receptors for cell binding and entry in cultured cells.

The effect of an Arg at VP1 position 83 or a Lys at 161 of vSAU/SAT is shown in Fig. 3B. No significant differences were observed in the plaque morphologies of the progeny virus populations obtained from the non-mutated vSAU/SAT and vSAU(E85R)/SAT and vSAU(E161K)/SAT mutated viruses (Fig. 3B). On BHK-21 mono-

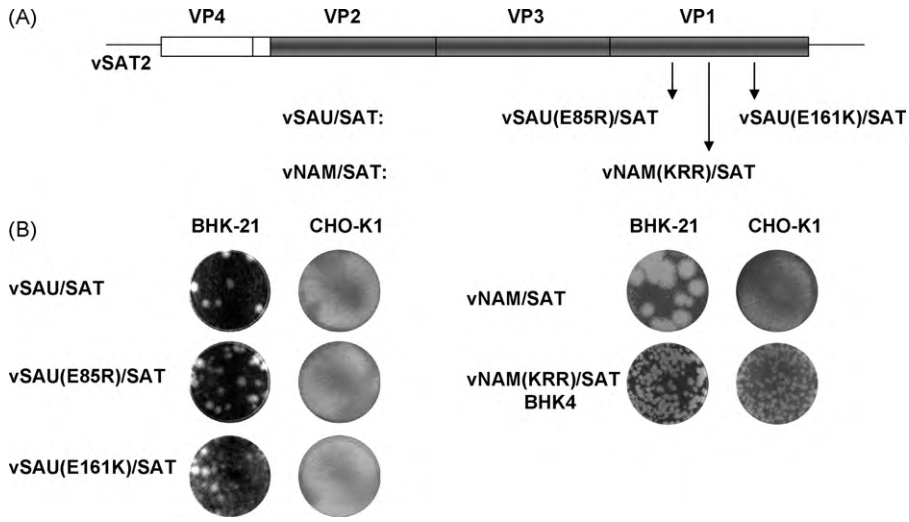

Fig. 3. (A) Schematic representation of the chimeric FMDV constructs and the introduction of mutations described in this study. (B) Plaque morphologies of chimeric viruses containing the wild-type outer capsid proteins of SAT1/NAM/307/98 and SAT2/SAU/6//00 cloned into the genetic background of pSAT2. The change in plaque phenotype on BHK-21 cells and the susceptibility of CHO-K1 cells for infection by the mutant vNAM(KRR)/SAT is shown. The mutant vSAU(E85R)/SAT and vSAU(E161K)/SAT displayed the same plaque morphology than the wild-type chimera and did not grow in CHO-K1 cells.

layers, the virus plaques were opaque, a phenotype commonly associated with viruses which have not been adapted to BHK-21 cells. Furthermore, CHO-K1 cells were not able to sustain infection by the vSAU/SAT or mutated virus populations. The infectivity titers on BHK-21 cells were also comparable for the three virus populations. Neither vSAU/SAT, nor the two mutants, vSAU(E85R)/SAT and vSAU(E161K)/SAT, were able to utilise HSPG for cell entry.

An in-depth analysis of the amino acid sequences neighbouring the introduced mutations in vSAU/SAT revealed that the introduced Lys85 was preceded by an acidic Glu residue at position 83, while the Lys161 was preceded by a DSTH sequence at VP1 residue positions 157–160. In both instances the added positive charge was compromised by acidic residues in its immediate environment, which may explain the absence of interaction with the negatively charged HSPG molecules.

4. Discussion

Despite the success of conventional vaccines in the control of FMD in the developed world, inactivated vaccines are unable to cover the vast antigenic variability within the SAT types in southern Africa (Hunter, 1996). Recombinant inactivated SAT type vaccines, structurally designed to be effective for specific geographic regions, may overcome the limitation of antigenic variation (Van Rensburg et al., 2004). However, the transfer of antigenic determinants during the replacement of the outer capsid proteins is simultaneously accompanied by the transfer of the receptor preferences of the field isolate. This could impact adversely on the use of recombinant viruses as vaccine seed stock due to the lack of cell culture adaptation and the consequent low yields in 146S particles. In addition, adaptation to the BHK-21 production cell line is often a cumbersome, time consuming and expensive process (Rieder et al., 1993; Van Rensburg et al., 2004).

To address the limitation of acquiring this adaptation phenotype by selection, we investigated the accumulated genetic changes of SAT1 and SAT2 viruses which have been adapted to BHK-21 cells, including efficacious vaccine strains. Our study supports the evidence from diverse virus families that cell culture adaptation of viruses (Patel et al., 1993; Chen et al., 1997; Krusat and Streckert, 1997; Byrnes and Griffin, 1998; Klimstra et al., 1998; Summerford and Samulski, 1998), and more specifically, FMDV (Jackson et al., 1996; Sa-Carvalho et al., 1997; Fry et al., 1999), selects from the quasi-species population variants with affinity for HSPG as observed by the ability to infect and replicate in CHO-K1 cells. Comparison of the outer capsid proteins of SAT1 and SAT2 viruses, with and without this phenotype, revealed a pattern of mutations with the common property of increasing the net positive charges on the virion surface, particularly surrounding the fivefold axis of the virion. Adaptation of the viruses in BHK-21 monolayer cells select for positively charged, surface-exposed residues which are most probably involved in the facilitation of cell entry via HSPG molecules.

Binding of viruses to HSPG or other GAGs occurs mainly through electrostatic interactions between positively charged Lys and Arg groups on the virus surface and the negatively charged N and O sulfated groups of the GAG molecules (Gromm et al., 1995; Byrnes and Griffin, 1998, 2000). Similarly to FMDV, repeated passaging of alphaviruses in BHK-21 cells also leads to reduced plaque size and an increased HSPG-binding ability (Marshall et al., 1962; Heydrick et al., 1966; Byrnes and Griffin, 1998). The accumulated positively charged residues and increased affinity to HSPG probably leads to direct interaction between the Arg or Lys and the polysaccharide backbone. In numerous other HSPG-binding proteins the strength of binding to the substrate was affected by the degree of sulfation of the polysaccharide backbone (Byrnes and Griffin, 1998). The selection of positively charged residues during cell-culture adaptation was previously reported for type O viruses (Sa-Carvalho et al., 1997; Jackson et al., 1996, 2001). Adaptation of the O1 Campos virus to cell culture selected viruses with an H → R change at position 56 of VP3 (Jackson et al., 1996; Sa-Carvalho et al., 1997). The 3D structure of the virion revealed that the positively charged residue 56 of VP3 is located within the recessed heparin-binding site, a depression on the virion surface situated at the junction of VP1, VP2 and VP3 (Fry et al., 1999).

Although we did not observe any of these changes in the VP3 proteins of the SAT1 or SAT2 viruses, residue changes at positions 135 and 175 in the VP3 were present in SAT1/NAM/307/98. Both these changes may contribute singly or accumulatively to the adaptation phenotype. The effect of each of these changes individually and accumulatively on cell culture adaptation and possible HSPG-binding is, therefore, not known and needs investigation to fully understand the mechanisms of cell culture adaptation. Furthermore, this is the first report on the fixation of positively charged residues 110–112 in the βF–βG loop of VP1 of SAT1 isolates and

its possible correlation with the ability of SAT1 viruses to replicate in CHO-K1 cells. The importance of this amino acid sequence in adaptation of SAT1 isolates to cultured BHK-21 cells and its possible function as a HSPG-binding site is evident from: (1) the KNP/196/91^{Vac} and SAR/9/81^{Vac} vaccine strains (Fig. 1) with the ability to infect and replicate in CHO-K1 cells; (2) the field isolate ZAM/2/93 adapted rapidly to cell culture as observed by the appearance of small plaques on BHK-21 cells and growth in CHO-K1 cells; (3) variation at the residue positions 110 and 112 of the VP1 capsid protein in an alignment of 26 SAT1 wild-type viruses with the inability to grow on CHO-K1 cells (Table 3); and (4) the introduction of the positively charged residues 110–112 in the vNAM/SAT chimeric virus was consistent with the small plaque phenotype and growth in CHO-K1 cells of the KNP/196/91 and SAR/9/81 vaccine strains. With the exception of SAT1/KNP/196/91, none of the other SAT1 viruses showed genetic changes in VP2 between the primary isolates and cell culture-adapted derivatives. The Gln74 → Arg substitution in VP2 is structurally associated with the shallow depression observed at the junction of the three major capsid proteins described by Fry et al. (2005).

The only mutations in VP3 observed during adaptation of SAT2 viruses were the Glu148 → Lys change of ZAM/7/96 and the Thr129 → Lys substitution of ZIM/14/90, although both residues were not fully exposed on the virion surface and did not correlate with the shallow HSPG-binding depression (Fry et al., 2005). These mutations, however, did not occur in isolation in these viruses and were possibly selected against a background of existing positively charged residues (Fig. 2). In the case of Thr129 → Lys mutation, it was accompanied by an Asp132 → Asn mutation, with the removal of a surface-exposed negative charge. Whether the two substitutions contributed synergistically or independently to the cell culture adaptation phenotype is not known. The ability of ZIM/14/90 to infect and replicate in CHO-K1 cells (Fig. 1) was associated with Gln170 → Arg change in VP2 together with the Thr129 → Lys substitution in VP3, an indication that the accumulative contribution may play a role. Noteworthy is the observation of existing positively charged residues in close approximation of these changes, suggesting that the selection of positively charged residues during adaptation transpired against a background of existing positive charges on the virion surface. The residue changes at position 83–85 of VP1 were observed for both the SAT2 viruses, KNP/19/89 and ZAM/7/96. The role of these residues, protruding from the fivefold axis, in BHK-21 adaptation and possible binding of HSPG is in agreement with observation for SAT1 viruses.

Phenotypic characterisation of the recombinant mutants revealed that the variants that were able to infect CHO-K1 cells shared a small plaque phenotype in BHK-21 cells. The small plaque phenotype produced by the vNAM(KRR)/SAT mutant in BHK-21 cells was consistent with the small plaques observed for the SAT1 vaccine strains SAR/9/81^{Vac} and KNP/196/91^{Vac}. Introduction of the cell culture adaptation phenotype during the construction of a chimeric virus may thus be advantagous for improved infectivity in cultured BHK-21 cells used in the production of FMD vaccines. On the contrary, neither vSAU(E85R)/SAT nor vSAU(E161K)/SAT produced plaques with altered morphology on BHK-21 cells compared to vSAU/SAT with the wild-type outer capsid. A systematic analysis of the sequences adjacent to both mutations revealed the presence of negatively charged residues which may act as a repelling force to the negatively charged sulfated groups of GAG's. Therefore, the role of the VP1 amino acid sequences of SAT2 isolates in cell culture adaptation requires further investigation.

The development of new vaccine strains to protect against emerging SAT viruses relies strongly on the virus yield of the new strain in the production cell line, the yield of 146S particles, stability of the virus and antigen, and a close antigenic relationship to field isolates causing current FMD outbreaks. BHK-21 cells are the cell line of choice for production of inactivated FMD vaccines and SAT viruses are notorious for their difficulty to adapt to BHK-21 cells (as outlined in Section 1). Our results demonstrated that for the purpose of SAT type vaccine production, viruses previously impossible to adapt to cell culture, can be designed with improved growth properties in cell cultures. Introductions of the cell culture adaptation phenotype to chimeric FMDV may be beneficial to the productivity in BHK-21 cells and the production 146S antigen.

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Effects of glycosylation on antigenicity and immunogenicity of classical swine fever virus envelope proteins

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Mutants

ABSTRACT

Classical swine fever virus (CSFV) harbors three envelope glycoproteins (E^{rns}, E1 and E2). Previous studies have demonstrated that removal of specific glycosylation sites within these proteins yielded attenuated and immunogenic CSFV mutants. Here we analyzed the effects of lack of glycosylation of baculovirus-expressed E^{rns}, E1, and E2 proteins on immunogenicity. Interestingly, E^{rns}, E1, and E2 proteins lacking proper post-translational modifications, most noticeable lack of glycosylation, failed to induce a detectable virus neutralizing antibody (NA) response and protection against CSFV. Similarly, no NA or protection was observed in pigs immunized with E1 glycoprotein. Analysis of E^{rns} and E2 proteins with single site glycosylation mutations revealed that detectable antibody responses, but not protection against lethal CSFV challenge is affected by removal of specific glycosylation sites. In addition, it was observed that single administration of purified E^{rns} glycoprotein induced an effective protection against CSFV infection.

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Introduction

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine. The etiological agent, classical swine fever virus (CSFV), is an enveloped virus that is classified as a Pestivirus within the family Flaviviridae (Fauquet et al., 2005). The CSFV genome is a positive sense single-stranded RNA that contains a single open reading frame that encodes an approximately 3900-amino-acid polyprotein. This polyprotein is co- and post-translationally processed by host and viral proteases to yield 11 to 12 final cleavage products (NH_2–Npro–C–E^{rns}–E1–E2–p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH) (Lindenbach et al., 2007). There are three envelope glycoproteins in the CSFV virion, E^{rns}, E1, and E2 (Thiel et al., 1991). E^{rns} is loosely associated with mature virions (Thiel et al., 1991), it possesses ribonuclease activity (Rumenapf et al., 1993), and is heavily glycosylated with carbohydrate moieties at seven glycosylation sites contributing to nearly half of the molecular mass of the protein (Branza-Nichita et al., 2004; Sainz et al., 2008). E1 is a small 33 kDa protein that forms heterodimers with E2 protein (Weiland et al., 1990) and contains three glycosylation sites (Fernandez-Sainz et al., 2009; Thiel et al., 1993). E2 is a major determinant of CSFV virulence (Risatti et al., 2005; Van Gennip et al., 2004) and is involved in virus attachment and entry into target cells (Hulst and Moormann, 1997). E2 protein contains one putative *O*-linked glycosylation site and six *N*-linked glycosylation sites (Risatti et al., 2007; Thiel et al., 1991; van Rijn et al., 1994). E2 is the major CSFV immunogen and has been used to develop subunit vaccines. These vaccines induce protection against CSFV although the onset of immunity is significantly delayed relative to live attenuated vaccines (Moormann et al., 2000).

The effects of glycan moieties found in these envelope proteins on induction of host humoral responses and protection against CSFV are not well understood. For many enveloped viruses (e.g. hepatitis C virus and human immunodeficiency virus) patterns of envelope protein glycosylation have been shown to influence the number of available epitopes and to modulate immune recognition of antigens, affecting the humoral immune response (Fournillier et al., 2001). In the case of hepatitis C virus (HCV), carbohydrate moieties on viral envelope proteins modulate neutralizing activity. Using HCV infectious particles, it was observed that removal of single *N*-linked glycans at sites N1, N2, N4, N6 and N11 from E2 protein rendered mutant viruses highly sensitive to neutralization by antibodies from HCV-seropositive patients (Helle et al., 2010). These data suggest that those glycans reduce the

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accessibility of neutralizing antibodies to cognate epitopes in E2 glycoprotein. Mutation of the fourth glycosylation site in HCV E1, regarded as a poor immunogenic protein, significantly enhanced the anti-E1 humoral response in terms of both seroconversion rates and antibody titers (Fournillier et al., 2001). In the case of human immunodeficiency virus (HIV) gp120 envelope glycoprotein, proper expression of conformational epitopes is dependent on the integrity and presence of carbohydrate moieties (Haigwood et al., 1992), since fully glycosylated gp120 was found to present neutralizing epitopes more effectively than the nonglycosylated form of the envelope protein (Haigwood et al., 1992). Nevertheless, these glycan moieties also have been shown to play a role in the masking of viral neutralizing epitopes (Back et al., 1993), leading to the "glycan shield" concept that implicates carbohydrates in HIV neutralizing antibody resistance (Wei et al., 2003). Similarly, glycosylation of envelope proteins affects immunogenicity of Porcine Reproductive and Respiratory Syndrome virus (PRRSV). Three putative N-linked glycosylation sites (N34, N44, and N51) are located on the glycoprotein 5 (GP5) ectodomain of PRRSV, where a major neutralization epitope also exists. Mutations of residue N44 did not result in infectious progeny, whereas viruses carrying mutations at N34, N51, and N34/51 exhibited enhanced sensitivity to neutralization by wild-type PRRSV-specific antibodies. Furthermore, inoculation of pigs with the mutant viruses induced significantly higher levels of neutralizing antibodies against the mutant as well as the wild-type PRRSV, suggesting that the loss of glycan residues in the ectodomain of GP5 enhances both the sensitivity of these viruses to in vitro neutralization and the immunogenicity of the nearby neutralization epitope (Ansari et al., 2006).

In previous studies, we have observed that changes in the glycosylation patterns of CSFV envelope proteins indeed affected virus virulence and viability (Fernandez-Sainz et al., 2009; Risatti et al., 2007; Sainz et al., 2008). Those studies showed that substitutions of specific N residues to A, in the context of *N*-glycosylation consensus sequence N-X-S/T (Kornfeld and Kornfeld, 1985), within CSFV E^{rns} (N269A), E1 (N594A and N500A/N513A), or E2 (N116L) glycoproteins yielded attenuated viruses that were also immunogenic, inducing an effective protection against challenge with virulent CSFV as early as day 3 post-inoculation. Notably, the rest of the single glycosylation site mutant viruses were virulent in swine, while complete lack of glycosylation of these proteins in the context of an infectious cDNA clone was deleterious for CSFV (Fernandez-Sainz et al., 2009; Risatti et al., 2007; Sainz et al., 2008). Although significant, these observations limited the study of glycosylation and its effect on antigenicity and/or immunogenicity of CSFV envelope proteins. To overcome that limitation, we carried out comparative studies in swine inoculated with baculovirus-expressed CSFV BICv (strain Brescia infectious clone virus; Risatti et al., 2005) envelope proteins to address the role of glycosylation on the induction of humoral immune response and protection against the virus. Data presented here show that glycosylated forms of E^{rns} and E2 proteins induced virus neutralizing antibodies in immunized swine and conferred an effective protection against CSFV. In contrast, animals immunized with glycosylated E1 protein did not develop a detectable NA response and succumbed when exposed to the virulent virus. Interestingly, complete lack of immunogenicity was observed when pigs were immunized with nonglycosylated forms of the envelope proteins resulting from improper post-translational modifications of E^{rns}, E1, and E2 proteins. When assessing the effect on immunogenicity of individual glycosylation sites, it was observed that the removal of single sites from E^{rns} or E2 proteins did not affect their ability to induce a protective immunity against CSFV, although it had an effect on the induction of the NA response. Results described here show that glycosylation plays a major role in the immunogenicity of CSFV envelope proteins, most likely an effect linked to the correct conformation of E^{rns}, E1, and E2 proteins.

Results

Expression of CSFV envelope proteins

Recombinant baculoviruses harboring CSFV envelope protein genes, with and without sequences encoding for signal peptides (Fig. 1), were used to infect Sf9 cells and protein expression was analyzed by

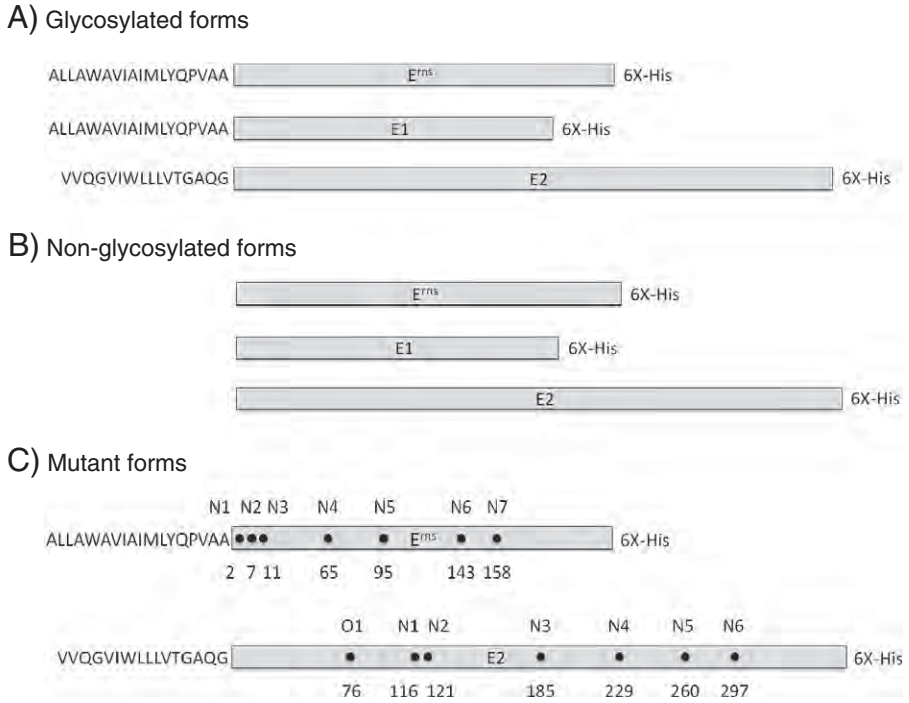

Fig. 1. Schematic representation of classical swine fever virus envelope proteins expressed in baculovirus. Glycosylated forms of the proteins were expressed with signal peptides encompassing the last 18 amino acids of Core protein (E^{rns} and E1) or the last 16 amino acids of E1 protein (E2) (A). Nonglycosylated forms of the proteins were expressed without signal peptides (B). Single site glycosylation mutant forms of E^{rns} and E2 proteins were generated by site-directed mutagenesis using wild-type genes as targets. Mutations of amino acid residues at indicated glycosylation sites within E^{rns} and E2 proteins (N and O) resulted in N to A substitutions in the consensus N-X-T/S (*N*-glycosylation) or P to A (*O*-glycosylation) (C). E2 was expressed without the trans-membrane domain. All proteins were expressed with poly-histidine tags (6× His) for detection and purification purposes.

Western blot. As shown in Fig. 2, glycosylated and nonglycosylated forms of E^{rns}, E1, and E2 were expressed and recognized by the anti-His C-terminus antibody. Glycosylated forms of the proteins showed the expected molecular sizes; E^{rns} 48 kDa, E1 31–32 kDa, and E2 55 kDa (Moser et al., 1996; Ruggli et al., 1995; Rumenapf et al., 1993). For the glycosylated E1 protein a second band of approximately 26 kDa was also detected (Fig. 2) that may represent different degrees of glycosylation (Fournillier et al., 2001). The electrophoretic mobility of E^{rns}, E1, and E2 expressed without signal peptides was found to be compatible with that of deglycosylated forms of the proteins that were expressed with signal peptides and previously treated with glycosidase PNGaseF. The molecular weights of nonglycosylated E^{rns}, E1, and E2 proteins were approximately 25 kDa, 21 kDa, and 41 kDa, respectively, and corresponded to their predicted mass calculated from the amino acid composition, indicating the absence of proper glycosylation (Fig. 2) (Ruggli et al., 1995).

Recombinant baculoviruses harboring E^{rns} and E2 mutated genes encoding for single N to A substitutions at *N*-glycosylation sites (N-X-T/S) and at the predicted *O*-glycosylation site in E2 protein were used to infect Sf9 cells. The differences in electrophoretic mobility observed among expressed E^{rns}, and E2 mutated proteins (Supplementary Fig. S1) corresponded to the differences observed in lysates from SK6 cells infected with glycosylation mutant viruses (Risatti et al., 2007; Sainz et al., 2008).

Immunogenicity of CSFV envelope proteins

To investigate the effect of modified forms of envelope proteins on the immunogenicity and protective efficacy against CSFV, pigs were inoculated with purified glycosylated or nonglycosylated forms of the CSFV envelope proteins. Animals were boosted at days 28, 42, and 56 after the first inoculation, and challenged IN one week after the last inoculation with a virus (BICv) derived from an infectious clone of the highly virulent isolate Brescia (Risatti et al., 2005). Pigs vaccinated with glycosylated E^{rns} and E2 were protected and survived challenge without showing clinical signs of the disease (Table 2). Only a mild and transient increase in body temperature was observed in two out of four animals inoculated with glycosylated E2 protein. Conversely, animals inoculated with nonglycosylated forms of E^{rns} and E2 proteins succumbed to the challenge, and showed signs of the disease indistinguishable from those observed in control animals. These data indicate that lacking proper post-translational modifications, most noticeable lack of glycosylation, significantly affects immunogenicity of E^{rns} and E2 proteins. Along with those observations, viremia in challenged animals immunized with nonglycosylated forms of E^{rns} and E2 was indistinguishable from viremia observed in control pigs (Figs. 3A and B). All these animals succumbed to infection no later than 14 dpc. Immunization with E^{rns} or E2 glycoproteins elicited a response that resulted in virus titers in blood approximately 100-fold lower relative to control pigs (Figs. 3A and B). Clearance of challenging virus was observed by 21 dpc in the E^{rns} glycoprotein group and by 14 to 21 dpc in the E2 glycoprotein group. Overall, data suggest that correct topology and structure of E^{rns} and E2 glycoproteins is necessary for eliciting an effective immune response against CSFV.

Pigs immunized with glycosylated or nonglycosylated form E1 protein were not protected against BICv (Table 2), developing CSF signs characterized by anorexia, depression, and an onset of fever by 3–4 dpc, ultimately succumbing to the infection within 8 to 14 dpc. E1 protein administered as purified protein does not induce protection against CSFV.

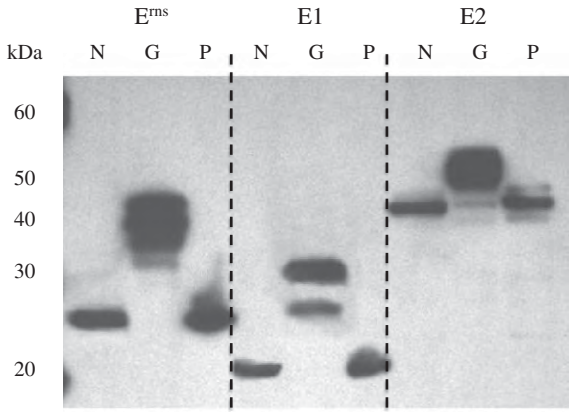

Fig. 2. Western blot analysis of baculovirus-expressed nonglycosylated (N), glycosylated (G), or glycosylated PNGase F-treated lysate (P) forms of CSFV BICv (Risatti et al., 2005) envelope proteins E^{rns}, E1, and E2. Lysates from Sf9 cells inoculated with recombinant baculoviruses were separated under denaturing conditions in 10% Bis–Tris gels followed by detection with anti-his C-term antibody (Invitrogen).

Antibody response in immunized pigs

The ability of glycosylated and nonglycosylated forms of E^{rns} and E2 proteins to induce antibodies in swine was tested by ELISA and seroneutralization following immunization.

Using ELISA, only animals immunized with glycosylated forms of E^{rns} and E2 proteins showed an antibody response (Fig. 4A). Similarly, neutralizing antibodies titers (NA) measured against BICv showed that immunization with nonglycosylated forms of E^{rns} or E2 did not elicit detectable NAs (Fig. 4B), correlating with the lack of immunogenicity induced in swine by these forms of the proteins. ELISA and NA data suggest that lack of proper conformation of E^{rns} and E2 glycoproteins considerably affects the exposure of critical epitopes.

NAs were induced by immunization with glycosylated forms of the proteins (Fig. 4B), correlating with the observed induction of protection against BICv. The NA response induced by glycosylated E2 occurred earlier than the response in pigs immunized with glycosylated E^{rns}. NA titers of 1:500 or higher were detected in serum samples from all animals at day 7 after the second dose of glycosylated E2 (Fig. 4B). A sustained antibody response was observed in this group of pigs until challenge, when NA titers in all the animals were detected above 1:2000. NA titers in pigs immunized with glycosylated E^{rns} were detected in one animal (#79 = 1:128) at day 7 after receiving a second dose of the protein, whereas a uniform response was observed in all pigs only by 14 days post-third immunization (Fig. 4B). Notably, NA titers in this group of animals were 1:64 by the time of challenge, and in spite of rather reduced E^{rns} antigenicity compared with that of E2, the induced antibody response precluded the appearance of CSF signs in these animals.

Immunogenicity of E^{rns} glycoprotein

The finding that purified glycosylated E^{rns} elicited low levels of NA titers but induced protection against challenge with BICv after four immunizations prompted analysis of whether or not this protein was still immunogenic after a fewer number of inoculations. Three different groups of pigs were intramuscularly immunized with one, two or three doses of E^{rns} glycoprotein at 14 day intervals (Fig. 5) and challenged at 21 days after the last inoculation. Interestingly, protection against challenge was achieved even when pigs received only one dose of E^{rns} (Table 3). Neither E^{rns} antibodies nor virus neutralizing antibodies against CSFV were detected in sera collected from these animals (Fig. 5C), although these pigs seroconverted by day 7 after challenge (data not shown). Animals that received 2 or 3 doses of E^{rns} presented low NA titers at challenge (Figs. 5A and B) and were clearly seropositive by ELISA. These data suggest either a low sensitivity of the antibody detection test used to quantify NAs, or additional yet unidentified immune mechanisms (i.e. non-neutralizing antibodies), are contributing to protection against CSFV induced by glycosylated E^{rns}.

Table 1
Sequences of the oligonucleotide primers used for synthesis of glycosylated and nonglycosylated forms of E^{rns}, E1, and E2 proteins.

Primer name	Sequence[a]
E^{rns} nonglycosylated forward	5′ CACCATGGAAAATATAACTCAA 3′
E^{rns} glycosylated forward	5′ CACC*ATGGCCCTATTGGCATGGGCAGTGATAGCAATTATGTTATACCAACCTGTTGCAGCCG*AAAATATAACTCAA 3′
E^{rns} reverse	5′ TTAGTGATGGTGATGGTGATGGGCATAGGCACCAAA 3′
E1 nonglycosylated forward	5′ CACCATGCTATCACCTTATTGT 3′
E1 glycosylated forward	5′ CACC*ATGGCCCTATTGGCATGGGCAGTGATAGCAATTATGTTATACCAACCTGTTGCAGCCC*TATCACCTTATTGT 3′
E1 reverse	5′ TTAGTGATGGTGATGGTGATGGCCTTGTGCCCCAGT 3′
E2 nonglycosylated forward	5′ CACCATGCGGCTAGCCTGCAAG 3′
E2 glycosylated forward	5′ CACC*ATGGTCGTGCAAGGTGTGATATGGCTGTTACTGGTAACTGGGGCACAAGGC* CGGCTAGCCTGCAAG 3′
E2 reverse	5′ TTAGTGATGGTGATGGTGATGTTCTGCGAAGTAATC 3′

[a] Signal peptides shown in italics; his-tag sequences shown underlined.

Antibody response in pigs immunized with single glycosylation site mutated E^{rns} and E2 glycoproteins

Since modified E^{rns} and E2 lacking proper glycosylation abrogated the antibody and the protective response in inoculated pigs, we assessed if any of the individual glycosylation sites present in these proteins were critical in the induction of the antibody response. Animals were immunized with single site glycosylation mutated E^{rns} protein or E2 protein (Supplementary Fig. S1) and challenged with BICv as described above. Sera obtained from these animals were tested for the presence of antibodies.

No detectable NAs were present in sera from pigs that received mutated forms of E^{rns} protein. However, sera from all these animals tested positive by ELISA 7 days after receiving a second dose of the proteins (data not shown).

NAs were detected in sera from pigs immunized with single site glycosylation mutated forms of E2 protein by 7 days after the second inoculation (Fig. 6). Sera from pigs immunized with O1, N1, N2, N3, and N4 exhibited significantly lower NA titers compared to sera from animals immunized with E2 protein (Fig. 6), whereas mutations at N5 and N6 did not affect E2 immunogenicity (Fig. 6). Overall, most of the E2 single glycosylation mutations diminished the antigenicity of E2 protein.

Protection elicited by single glycosylation site mutated E^{rns} and E2 glycoproteins

The contribution of single glycosylation sites in E^{rns} and E2 proteins in conferring protection against CSFV was assessed by challenging immunized pigs (see above) with virulent BICv. While non-immunized control pigs succumbed to the lethal infection, all animals immunized with mutated forms of either glycoprotein survived the challenge and were protected against clinical disease after four inoculations, with the exception of pigs immunized with E^{rns} N3, E^{rns} N7, and E2 N2 proteins; these animals exhibited elevated body temperatures for more than 6 days (Table 4), a temporary loss of appetite and mild depression. Viremia was detected in all groups of immunized animals and for the same approximate length of time. Virus titers in animals immunized with mutated forms of E^{rns} or E2 tended to be higher than virus titers detected with native forms of the proteins. Nonetheless, in all cases, virus titers were significantly lower in animals immunized with different forms of E^{rns} or E2 than in mock-vaccinated animals (Figs. 7A and B).

Recombinant E2 single glycosylation mutant viruses have differential sensitivity to NAs raised against glycosylated E2 protein

The finding that immunization with mutated forms of E^{rns} and E2 elicited differential NA responses against BICv (Fig. 6) prompted the opposite analysis, where the neutralizing activity of sera raised against glycosylated E2 was determined against recombinant E2 glycosylation mutant viruses (Risatti et al., 2007). E2N1v, a mutant virus harboring a N116A substitution in E2, was significantly more resistant to neutralization by sera raised against glycosylated E2 than BICv (NAb titers of 220 vs. 603 respectively, $p<0.05$, *t*-test). Meanwhile E2N4v, a mutant virus harboring a N229A substitution in E2, was significantly more sensitive to neutralization by sera raised against glycosylated E2 than BICv (NAb titers of 1494 vs. 603 respectively, $p<0.05$, *t*-test). The reactivity to neutralization of the rest of the mutant viruses was similar to BICv (data not shown). The data suggest that mutations at these glycosylation sites may lead to conformational changes in E2 protein that either shield or expose important neutralizing epitopes.

Discussion

Glycosylation of viral envelope proteins influences the immunogenicity and the sensitivity of the virus to neutralizing antibodies. By maintaining the appropriate conformation of proteins, oligosaccharides may hinder proteolytic degradation and with that affect T-cell recognition (Li et al., 2008; Sodora et al., 1991). Removal of glycans from viral envelope proteins has been shown to enhance (Doe et al., 1994; Reitter et al., 1998; Wei et al., 2003), interfere with (Sjolander et al., 1996), or have no effect on their immunogenicity (Bolmstedt et al., 1996). In this study we characterized the role of glycosylation of the three CSFV envelope proteins, analyzed its ability

Table 2
Swine survival and fever response following vaccination of swine with glycosylated and nonglycosylated E^{rns}, E1, and E2 proteins.

Protein	No. of survivors/ total no.	Mean time to death (no. of days ± SD[a])	Fever		
			No. of days to onset (± SD)	Duration (no. of days ± SD)	Max temp, °C (± SD)
E^{rns} nonglycosylated	0/6	13 (1.0)	3.5 (0.5)	4.5 (1.5)	42.1 (0.31)
E^{rns} glycosylated	6/6	–[b]	2.67 (1.5)	3.83 (2.6)	40.8 (0.32)
E1 nonglycosylated	0/2	13.5 (0.5)	4 (0.0)	6 (0.0)	41.6 (0.0)
E1 glycosylated	0/2	14.5 (5.5)	3.5 (0.5)	6.5 (0.5)	41.6 (0.35)
E2 nonglycosylated	0/6	13.5 (0.5)	2.5 (1.5)	9 (2.0)	42 (0.27)
E2 glycosylated	6/6	–	7[c] (1.0)	2.5 (0.5)	41.4 (0.62)

[a] SD: standard deviation.
[b] "–" negative.
[c] Only 2 animals out of total of 6 inoculated animals.

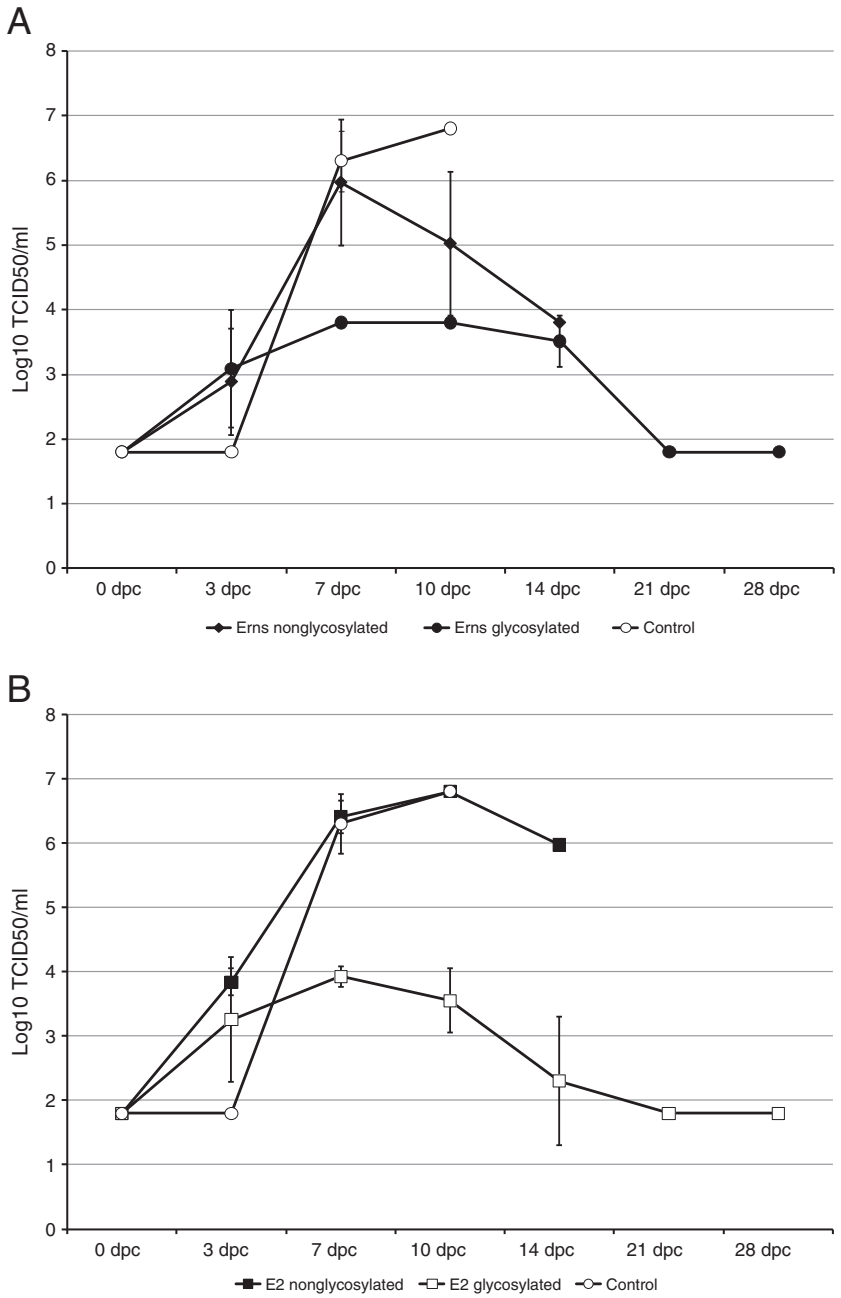

Fig. 3. Means and standard deviations of virus titers in blood from pigs immunized with nonglycosylated (n = 4) and glycosylated (n = 4) forms of E^{rns} (A); and nonglycosylated (n = 4) and glycosylated (n = 4) forms E2 (B) proteins and challenged with BICv. dpc: days post-challenge. Values expressed as log10 $TCID_{50}$/ml. Sensitivity of the assay ≥1.80 log10 $TCID_{50}$/ml.

to induce humoral immune responses, and confirmed its necessity to induce protection in swine against challenge with highly virulent CSFV.

Four conclusions can be drawn from this study: (i) only glycosylated E^{rns} and E2 proteins were able to induce a NA response and protection against CSFV in swine; (ii) E1 glycoprotein was not immunogenic nor protective against CSFV even in its native form and after four immunizations; (iii) purified E^{rns} glycoprotein was immunogenic, inducing an effective protection against CSF in swine even after one immunization; and (iv) the immunogenicity of E^{rns} and E2 glycoproteins is greatly affected by specific glycosylation sites.

(i) Sera from swine inoculated with nonglycosylated forms of the E^{rns} and E2 envelope proteins failed to neutralize CSFV infection of SK6 cells. A similar effect has been previously observed for HIV (Benjouad et al., 1992), where no neutralizing activity was observed in sera from rabbits immunized with deglycosylated forms of major envelope protein gp160. However, in those studies the sera from these rabbits recognized gp160 by RIA; sera from swine immunized with non-glycosylated forms of E^{rns} or E2 did not react with the proteins in competitive ELISA tests. The percentages of inhibition in sera from pigs after four immunizations with nonglycosylated forms of E^{rns} or E2 were similar to those of pre-immune sera. It is likely that modifications induced in these envelope proteins lacking proper post-translational modifications, noticed by complete lack of glycosylation, results in conformational changes that affect exposure of important epitopes that mediate the NA response against CSFV and induce protection against the virus. Here, we found that only glycosylated E^{rns} and

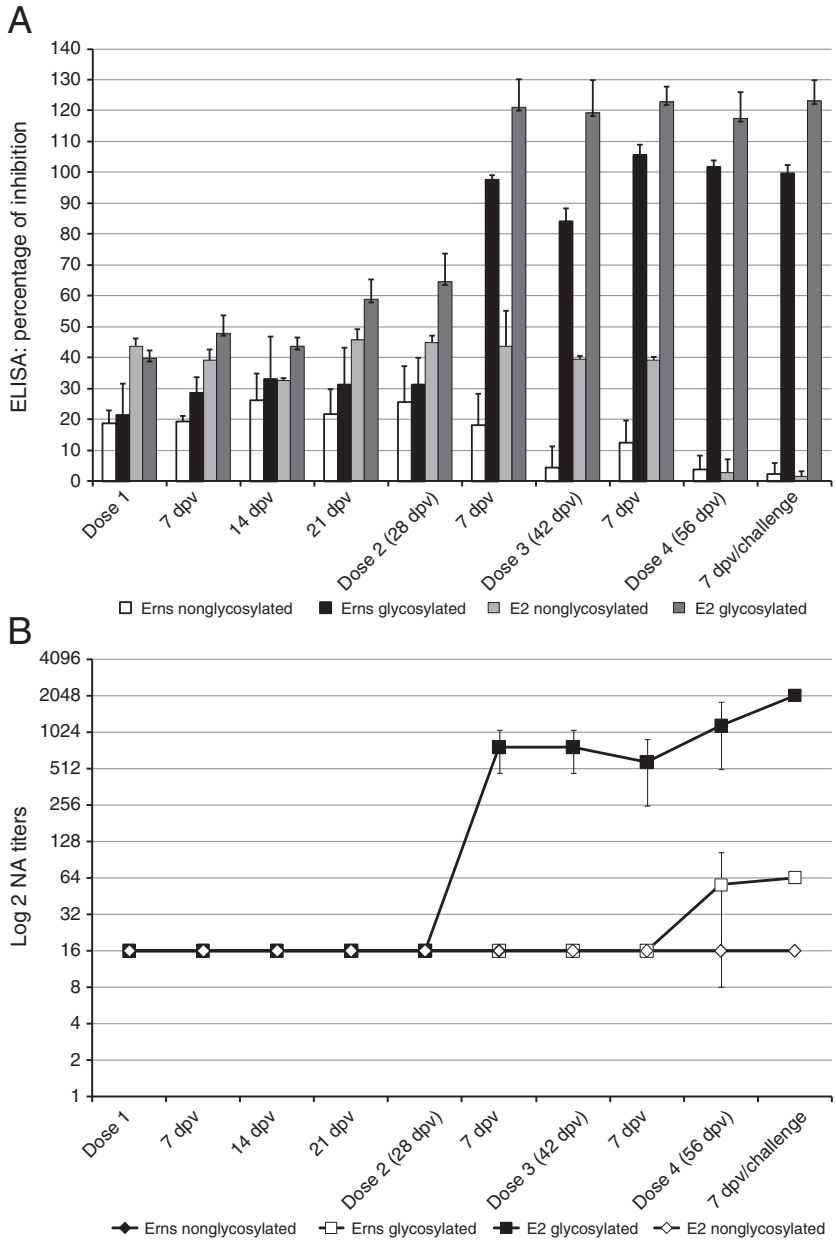

Fig. 4. Antibody response in pigs immunized with nonglycosylated or glycosylated forms of CSFV envelope proteins E^{rns} and E2. Group of pigs, four pigs per group, were inoculated intramuscularly with different forms of the envelope proteins on days 0, 28, 42, and 56; then challenged intranasally with BICv 1 week after last immunization. ELISA antibody titers (A) are expressed as percentage of inhibition (see Materials and methods). NA titers (B) are expressed as log 2 of the reciprocal of the highest serum dilution neutralizing approximately 100 TCID50 of BICV. dpv: days post-vaccination. Error bars correspond to standard deviations.

E2 forms of the proteins can induce the production of antibodies establishing a strong protective response against CSFV.

(ii) The baculovirus-expressed E1 glycoprotein did not induce a detectable NA response in sera from immunized pigs even after administration four times as an oil-in-water emulsion. Under these conditions, E1 did not elicit a protective response since challenged animals succumbed to infection with virulent CSFV. The poor immunogenicity of E1 protein was also observed in animals vaccinated with recombinant competent vaccinia virus that succumbed to CSFV infection when challenged intranasally 5 weeks post-vaccination (Konig et al., 1995). As observed here, those animals did not show detectable antibodies against the protein before challenge, although high antibody titers against the vector were detected. This suggests that E1 protein may not play a significant role in immunity against CSFV.

(iii) Purified E^{rns} protein induced an effective protective response against CSFV. The immunogenicity of E^{rns} was observed first in pigs vaccinated with a replication competent recombinant vaccinia virus that resisted CSFV infection when challenged 5 weeks post-vaccination (Konig et al., 1995). In those animals there were no detectable NA a week before challenge and sera contained only non-neutralizing antibodies detectable by ELISA. Different from the experiments performed here, antigens expressed by recombinant vaccinia viruses are delivered into eukaryotic cells and usually elicit high levels of cytotoxic T lymphocytes (CTL) (Moss, 1991, 1992), leading to effective antiviral activity (Byrne and Oldstone, 1984). However, no CTL epitopes have been reported for E^{rns} protein. We also observed that protected pigs, challenged 21 days after a single vaccination with the E^{rns} protein, did not have detectable NAs or ELISA-detectable

antibodies at the time of exposure to CSFV. Rather, antibodies against E^{rns} were detected by 7 days post-challenge at much higher titers than in mock immunized animals, indicating a boosting effect by the virus infection, whereas NAs were detected later, by 10 days post-challenge. This rapid onset of humoral immunity observed after exposure to CSFV may have played a role in virus clearance and disease progression. The apparent lack of NA titers in protected animals may be the result of the presence of undetectable but still efficacious levels of NAs. Alternatively, protection may be mediated by still undefined immune mechanisms. Nevertheless, these mechanisms are likely to be antibody-mediated (i.e.: antibody-mediated cytotoxicity or antibody-complement-mediated cytotoxicity) since the induction of T-cell mediated mechanisms in animals immunized with purified protein preparations it is less plausible.

(iv) Individual glycosylation sites in E^{rns} and E2 proteins have an effect on the induction of humoral responses after immunization. Glycan moieties may limit the antigenicity of viral envelope proteins and restrict the binding of some antibodies to key epitopes. Contrary to what has been observed for other viruses, removal of individual glycosylation sites from E^{rns} and E2 proteins did not result in enhancement of the antibody response in vaccinated pigs. Rather, vaccination with five of the E2 mutated proteins resulted in lower NA titers (O1, N1, N2, N3, and N4) relative to fully glycosylated protein, suggesting that NA induction depends on the presence of these glycosylation sites. Data from cross-neutralization studies between E2 sera and recombinant glycosylation mutant viruses E2N1v and E2N4v indicate that N1 and N4 sites in the E2 protein affect antigenicity of the protein, an effect compatible with structural changes that affect proper exposure of neutralizing epitopes. Interestingly, N1 and N2 sites are located towards the N-terminus of E2 between two proposed structural units of the proteins, conformed by domains D and A (van Rijn et al., 1994). Both structural units contain neutralizing epitopes. N3 and N4 sites reside in an area of E2 still structurally undefined. Eventually, alteration of glycosylation patterns in E2 might affect exposure of currently unidentified epitopes. Nonetheless all mutated proteins were able to induce a protective immunity after four immunizations. Further mapping of these glycosylation sites will be required to determine what combina-

Table 3
Swine survival and fever response in pigs immunized with different doses of glycosylated Erns proteins and challenged with BICv.

No. of doses (×)	No. of survivors/ total no.	Mean time to death (no. of days ± SD[a])	Fever		
			No. of days to onset (± SD)	Duration (no. of days ± SD)	Max temp, °C (± SD)
3×/challenge at 7 days	2/2	–[b]	3.0 (0.00)	1.0 (0.00)	40.8 (0.77)
3×/challenge at 21 days	2/2	–	–	–	39.7 (0.00)
2×/challenge at 21 days	2/2	–	4.0 (0.00)	4.0 (0.00)	41.6 (0.56)
1×/challenge at 21 days	2/2	–	5.5 (0.71)	2.5 (0.71)	40.7 (0.14)
0×/challenge	0/2	11.5 (2.12)	3.0 (0.00)	9 (1.41)	42.2 (0.35)

[a] SD: standard deviation.
[b] "–" negative.

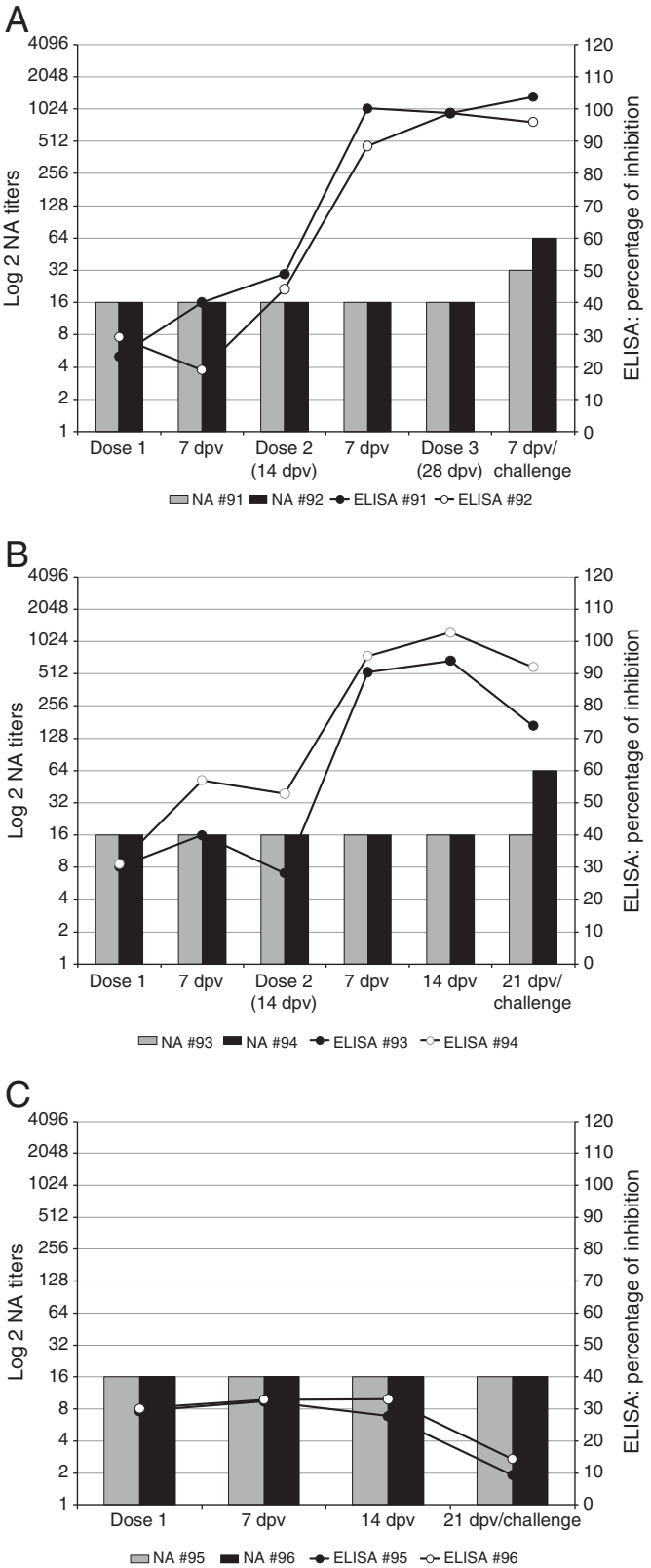

Fig. 5. Antibody response in pigs immunized with different number of doses of glycosylated E^{rns} protein before challenge with BICv. Pigs (n = 2) were inoculated intramuscularly three times at days 0, 14, and 28 and challenged 7 days after last vaccination (A), twice (n = 2) on days 0 and 14 and challenged 21 days after last vaccination (B), or once (n = 2) and challenged 21 days after last vaccination (C). Not shown are animals (n = 2) immunized with 3 doses of E^{rns} challenged at 21 days after last vaccination. Serum samples were collected weekly after the first immunization and antibody titers were detected by ELISA and measured by seroneutralization. NA titers are expressed as log 2 of the reciprocal of the highest serum dilution neutralizing approximately 100 TCID50 of BICv and antibodies detected by ELISA are expressed as percentage of inhibition (see Materials and methods). dpv: days post-vaccination.

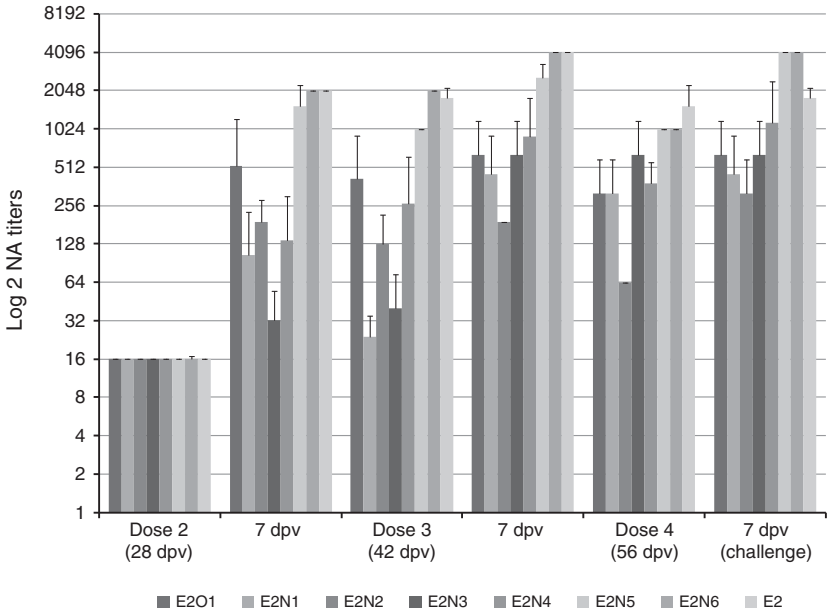

Fig. 6. Development of NAs in pigs immunized with E2 single site glycosylation mutants. Pigs (n = 2 per group) were inoculated intramuscularly with each mutant forms of E2 at days 0, 28, 42, and 56 and challenged 1 week after last immunization. Shown are NA titers from a week previous to seroconversion. NA titers are expressed as log 2 of the reciprocal of the highest serum dilution neutralizing approximately 100 TCID50 of BICv (see Materials and methods). Error bars correspond to standard deviations.

tion of mutations will lead to a complete lack of immunogenicity, as observed here with nonglycosylated forms of the protein. Similarly, vaccination with E^{rns} mutated proteins did not result in enhancement of the antibody response. Removal of individual sites greatly affected the production of NAs, since immunization with glycosylation mutated proteins did not induce detectable NAs, while fully glycosylated E^{rns} induced a NA response detectable by 14 days post-third immunization. In both cases animals were protected against lethal CSFV challenge. Clearly, immunogenicity of E^{rns} is dependent on glycosylation.

Table 4
Swine survival and fever response in pigs immunized with E^{rns} and E2 single site glycosylation mutant forms of the proteins and challenged with BICv.

Protein	No. of survivors/ total no.	Time of death (dpc) (±SD[a])	Time of the onset of fever (dpc) (±SD)	Duration of fever (days) (±SD)	Maximal temperature (°C) (±SD)
E^{rns}	1/1[b]	–	7 (0.00)	1(0.00)	40.1 (0.00)
E^{rns} N1	2/2	–	3 (4.24)	0.5 (0.70)	40 (0.21)
E^{rns} N2	2/2	–	3.5 (4.94)	2 (2.82)	40 (0.28)
E^{rns} N3	2/2	–	3 (2.82)	6.5 (3.58)	41 (1.13)
E^{rns} N4	2/2	–	2.5 (3.53)	1 (1.41)	40 (0.42)
E^{rns} N5	2/2	–	2 (2.82)	3.5 (4.94)	40.4 (0.35)
E^{rns} N6	2/2	–	4.5 (0.70)	3.5 (3.53)	40.3 (0.14)
E^{rns} N7	1/1[b]	–	4 (0.00)	6 (0.00)	41.6 (0.00)
E2	2/2	–	4 (4.24)	3 (2.82)	40.2 (0.21)
E2O1	2/2	–	–	–	39.8 (0.00)
E2N1	2/2	–	2.5 (3.53)	3.5 (4.94)	40.6 (0.91)
E2N2	2/2	–	3.5 (2.12)	8.5 (0.70)	40.8 (0.07)
E2N3	2/2	–	3.5 (4.94)	0.5 (0.70)	40.3 (0.63)
E2N4	2/2	–	6.5 (9.19)	1.5 (2.12)	40 (0.42)
E2N5	2/2	–	7 (9.89)	1.5 (2.12)	40.1 (0.35)
E2N6	1/1[b]	–	–	–	39.6 (0.14)
Control	0/2	11.5 (4.94)	3 (0.00)	9 (4.24)	42 (0.00)

"–" negative.
[a] One of the two animals in each of these groups died at early stages of the immunization period by unrelated causes.
[b] Standard deviation.

In summary, the results presented here indicate that neutralizing epitopes in E^{rns} and E2 proteins are dependent on the presence of glycosylation; most probably, as E2 data suggests, critical glycosylation sites are located within certain domains of this protein (i.e. D/A domains). We show that preventing proper post-translational modification in E^{rns} and E2 that mainly results in lack of glycosylation lead to the synthesis of non-immunogenic proteins that failed to induce protection against CSFV, while removal of single glycosylation sites did not significantly affect the overall immunogenicity of these proteins. We observed that only purified E^{rns} and E2 glycoproteins mediate immunity against CSFV, since glycosylated and nonglycosylated forms of E1 protein failed to induce a detectable antibody response and failed to induce protection in vaccinated pigs. A highly significant observation was that a single dose of purified E^{rns} induces protection against CSFV although the exact mechanism(s) mediating that protection remains uncertain.

It is possible that observations presented here will be useful for designing subunit vaccines against CSFV.

Materials and methods

Cells and viruses

Propagation of recombinant baculoviruses and expression of the envelope proteins were performed in the *Spodoptera frugiperda* derived Sf9 cell line. Sf9 cells were maintained in adherent cultures in Grace's insect cell culture medium (TNM-FH) supplemented with 10% fetal bovine serum and gentamicin (Lonza, Walkersville, MD).

Swine kidney cells (SK6) (Terpstra et al., 1990), free of BVDV, were used to propagate CSFV BICv (Risatti et al., 2005) and glycosylation mutant viruses (Fernandez-Sainz et al., 2009; Risatti et al., 2007; Sainz et al., 2008). SK6 cells were cultured in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Lonza).

For titration of CSFV from clinical samples, SK6 cells in 96-well plates (Corning, Lowell, MA) were infected and incubated at 37 °C and 5% CO_2 for 4 days. Viral infectivity was detected using an immunoperoxidase assay with the CSFV monoclonal antibody (mAb) WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated according to

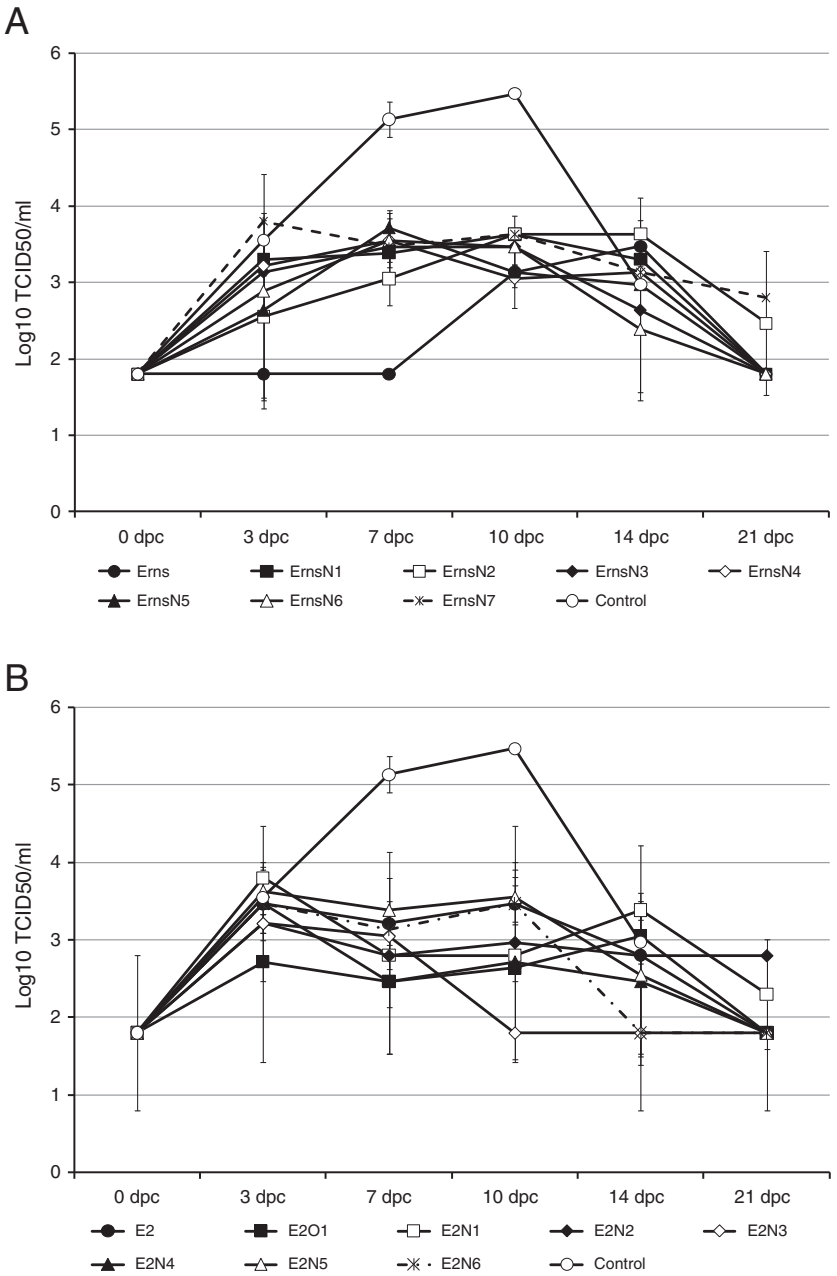

Fig. 7. Average of virus titers in blood from pigs, two pigs per group, immunized with E^{rns} single site glycosylation mutants (A), or E2 single site glycosylation mutants (B) after challenge with virulent CSFV strain Brescia (n = 2). Viremia from one animal only is shown for E^{rns}, E^{rns} N7, and E2N6. dpc: days post-challenge. Values are expressed as log10 $TCID_{50}$/ml. Sensitivity of the assay ≥1.80 log10 $TCID_{50}$/ml. Error bars correspond to standard deviations.

the method of Reed and Muench (1938) and were expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml. As performed, test sensitivity was $\geq \log_{10}$ 1.8 $TCID_{50}$/ml.

Generation of recombinant baculoviruses

Polymerase chain reaction (PCR) was utilized to synthesize CSFV BICv E^{rns}, E1, and E2 genes using specific sets of primers (Table 1). Reverse primers included sequences encoding for six histidine residues (6×-HIS) to allow protein detection and purification by immobilized-metal affinity chromatography. Sets of forward primers with or without sequences encoding for putative CSFV signal peptides (Moser et al., 1996; Ruggli et al., 1995; Rumenapf et al., 1993; van Rijn et al., 1996) were used to synthesize glycosylated and nonglycosylated forms of the three envelope protein genes. The E2 gene was synthesized without its transmembrane region (Hulst et al., 1993) to facilitate its subsequent expression and purification. The resulting PCR fragments were eluted from a 1% agarose gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA), and cloned directionally into pENTR/D-TOPO entry vector (Invitrogen). To confirm that inserted genes were in frame, entry clones were verified by automatic sequencing.

Single site glycosylation mutated E^{rns} and E2 genes were obtained by site-directed mutagenesis (QuikChange Multi Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA) using specific primers (Risatti et al., 2007; Sainz et al., 2008) (Table 1). pENTR/D-TOPO vectors harboring E^{rns} or E2 genes were used as template in those reactions.

Recombinant baculoviruses were produced using the BaculoDirect C-Term expression system (Invitrogen) according to the manufacturer's

instructions. Briefly, recombinant baculoviruses expressing envelope proteins were constructed by an attL-attR recombination reaction between entry clones and BaculoDirect Linear DNA containing the Herpes Simplex Virus thymidine kinase gene located between recombination sites for negative selection with ganciclovir. Sf9 cells were transfected using Cellfectin (Invitrogen) and subsequently propagated as recommended in the protocol provided by the manufacturer. Cells were grown until signs of infection were observed (6–8 days after transfection). The resulting stock (P1) was harvested and amplified once by infecting confluent Sf9 cells to obtain a high-titer viral stock (P2).

Detection and purification of recombinant proteins

Sf9 cells (about 1.5×10^6/ml) were infected at MOI = 5 or 10 with recombinant baculoviruses and cells and culture media were harvested after incubation at 27 °C for 5 to 7 days. Cultures were centrifuged at $2000\times g$ for 5 min at 4 °C and the cell pellet was washed twice with phosphate-buffered saline (PBS) followed by resuspension in RIPA buffer (Sigma Aldrich, St. Louis, MO). After incubation for 10 min on ice, preparations were clarified by centrifugation at $16{,}100\times g$ for 10 min. Expression of the recombinant envelope proteins was verified by Western blot analysis. Briefly, protein lysates were separated under reducing conditions on a 10% NuPage Novex Bis–Tris gel (Invitrogen) by a discontinuous SDS-PAGE system and transferred to polyvinylidene fluoride (PVDF) membrane (Invitrogen). Membranes were blocked, and then incubated overnight with anti-his C-term antibody (Invitrogen) at a dilution of 1:5000. Membranes were washed and incubated with goat anti-mouse IgG antibody conjugated with alkaline phosphatase (WesternBreeze Chemiluminescent Detection Kit, Invitrogen). Envelope proteins were detected using CDP-star chemiluminescent substrate provided with the kit and by exposing to X-ray films (Kodak X-OMAT LS film, Kodak, Rochester, NY).

For purification, protein lysates were applied to HisPur Cobalt Resin Columns (Thermo Fisher Scientific, Rockford, IL) and purified following the manufacturer's instructions. The collected fractions were analyzed by Western blotting as described above and Coomassie blue staining (Simply Blue SafeStain, Invitrogen).

As control for deglycosylation of envelope proteins, glycosylated envelope proteins were treated with peptide N-Glycosidase F (PNGase F, New England Biolabs, Ipswich, MA) following the manufacturer's directions. Briefly, infected cell extracts were denatured at 100 °C for 10 min in glycoprotein denaturing buffer (New England BioLabs). The reaction mixture was placed on ice for 5 min, and then digested with PNGase F for 1 h at 37 °C in the presence of 1% NP-40.

Protein concentration was determined by the BCA Protein Assay (Thermo Fisher Scientific) against bovine serum albumin standard, by measuring OD at 595 nm in a NanoDrop (NanoDrop Technologies, Wilmington, DE).

Animal experiments

Eighteen to twenty kiliograms, 2 to 3 months old, commercial breed pigs were used in all these experiments. In all cases, pigs were inoculated with purified envelope proteins mixed with Sigma Adjuvant System (Sigma-Aldrich, St. Louis, MO) to obtain an oil-in-water emulsion according to instructions given by the manufacturer. One milliliter of each inoculum containing ~50 μg of purified protein was injected via intramuscular route into the neck of animals. Mock-vaccinated animals received adjuvant alone. After immunization pigs were challenged intranasally (IN) with 10^5 $TCID_{50}$ of highly virulent BICv.

For initial screening, pigs (n = 14) were allocated randomly into 6 groups (n = 2). A mock-vaccinated control group (n = 2) was also included. Pigs were inoculated with nonglycosylated or glycosylated forms of E^{rns}, E1, or E2 proteins and boosted with the same dose 28, 42, and 56 days later. Blood samples were collected from each animal weekly starting from the day of first inoculation. Pigs were challenged 1 week after the last inoculation. After challenge, pigs were observed daily for 28 days for manifestation of CSF clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea, and cough). Body temperatures were recorded daily throughout the experiment.

For confirmatory purposes, pigs (n = 18) were randomly allocated into one of four vaccination groups (n = 4) or a mock-vaccination control group (n = 2). Pigs were inoculated with nonglycosylated or glycosylated forms of E^{rns} or E2 proteins (vaccinated groups) or non-vaccinated (control group). Protein concentrations, route of inoculation, adjuvant formulations, vaccination schedule, and challenge were performed as described above. Blood, serum, nasal swabs, and tonsil scrapings were collected from each pig at 0, 3, 7, 10, 14, 21, and 28 days post-challenge (dpc).

The protective efficacy of glycosylated E^{rns} protein was further assessed in swine. Pigs (n = 10) were allocated into 4 vaccine groups (n = 2) and 1 non-vaccinated control group (n = 2). Pigs were inoculated as described above. Two vaccine groups received 3 doses of glycosylated E^{rns} at 2-week intervals (days 0, 14, and 28) and were challenged at 7 and 21 days post-third inoculation, respectively. A third vaccine group received 2 doses of glycosylated E^{rns} at 2-week interval (days 0 and 14) and was challenged at 21 days post-second inoculation. The fourth vaccine group received 1 dose of glycosylated E^{rns} (day 0) and was challenged at 21 days post-inoculation. Serum samples were obtained weekly after vaccination.

To assess the immunogenicity of single site glycosylation E^{rns} and E2 mutated proteins, pigs (n = 34) were randomly allocated into sixteen vaccinated groups (n = 2), and a mock-vaccinated control group (n = 2). Seven vaccinated groups received single site glycosylation mutated E^{rns} proteins (N1, N2, N3, N4, N5, N6, and N7) (Figs. 1 and Supplementary Fig. S1A). Seven vaccinated groups received single site glycosylation mutated E2 proteins (O1, N1, N2, N3, N4, N5, and N6) (Figs. 1 and Supplementary Fig. S1B). Two vaccinated groups received glycosylated E^{rns} and E2 proteins, respectively. The control group was mock vaccinated. Animals were inoculated with these proteins at days 0, 28, 42, and 56 and then challenged 1 week after the last vaccination.

Antibody detection

Serum samples were analyzed for the presence of CSFV E^{rns} specific antibodies using commercial ELISA kits: IDEXX CHEKIT CSF MARKER ELISA Test Kit (Idexx Laboratories, Westbrook, ME) for detection of antibodies against E^{rns} and detection of anti-E2 antibodies was performed with IDEXX CSF SERO Antibody ELISA Test Kit (Idexx Laboratories). ELISA test kits were used according to the protocols provided by the manufacturer.

Seroneutralization and cross-neutralization assays were performed with heat-inactivated serum samples (56 °C for 30 min). Two-fold serial dilutions of serum were prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and mixed with equal volumes of BICv or glycosylation mutant viruses (Risatti et al., 2007; Sainz et al., 2008) containing 10^2 $TCID_{50}$. Serum-virus mixtures were incubated for 1 h at 37 °C and then transferred to 96-well flat-bottom tissue culture plates (Corning) followed by addition of SK6 cells (1×10^4 per well). Plates were incubated at 37 °C and 5% CO_2 for 4 days. Supernatant was then removed from each well and the cells were fixed with methanol–acetone (50% vol./vol.) solution and air-dried. Plates were stained by immunoperoxidase assay using the Vecstatin ABC Kit, Vector Laboratories, Burlingame, CA following manufacturer's instructions. Neutralizing antibody titers were expressed as the reciprocal of the highest two-fold serum dilution neutralizing BICv (Reed and Muench, 1938).

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.08.025.

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ORIGINAL ARTICLE

Genetic and antigenic relationships of vesicular stomatitis viruses from South America

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Abstract Vesicular stomatitis (VS) viruses have been classified into two serotypes: New Jersey (VSNJV) and Indiana (VSIV). Here, we have characterized field isolates causing vesicular stomatitis in Brazil and Argentina over a 35-year span. Cluster analysis based on either serological relatedness, as inferred from virus neutralization and complement fixation assays, or nucleotide sequences of two separate genes (phosphoprotein or glycoprotein) grouped the field isolates into two distinct monophyletic groups within the Indiana serogroup. One group included seven viruses from Brazil and Argentina that were serologically classified as Indiana-2 and Cocal virus (COCV). The other group contained three viruses from Brazil that were serologically classified as Indiana-3 and the prototype of this group, Alagoas virus (VSAV). Interestingly, two vesiculoviruses that were isolated from insects but do not cause disease in animals, one from Brazil (Maraba virus; MARAV) and the other from Colombia (CoAr 171638), grouped into two separate genetic lineages within the Indiana serotype. Our data provide support for the classification of viruses causing clinical VS in livestock in Brazil and Argentina into two distinct groups: Indiana-2 (VSIV-2) and Indiana-3 (VSIV-3). We suggest using nomenclature for these viruses that includes the serotype, year and place of occurrence, and affected host. This nomenclature is consistent with that currently utilized to describe field isolates of VSNJV or VSIV in scientific literature.

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Introduction

Vesicular stomatitis (VS) is a viral disease of cattle, horses and pigs that, in pigs and cattle, is clinically similar to foot-and-mouth disease [25]. The causative agents of VS are viruses belonging to the family *Rhabdoviridae*, genus *Vesiculovirus*, which have been serologically classified into two serotypes: New Jersey (VSNJV) and Indiana (VSIV). These viruses are endemic in northern South America (Peru, Colombia, Ecuador, Venezuela), Central America and southern Mexico and cause sporadic outbreaks in Bolivia, northern Mexico and the southwestern United States. In the rest of South America, VS has only been reported in Brazil, and occasionally in Argentina, where VS cases are caused by viruses related to VSIV that have been serologically classified as either Indiana-2 or Indiana-3 [2, 10]. The prototype species for these serological groups are *Cocal virus* (COCV) and *Vesicular stomatitis Alagoas virus* (VSAV), respectively [9]. Previous studies have described the molecular characterization and epidemiology of VS strains occurring in Central and North America [18–20, 25]. We have previously reported the full-length genomic sequence of the prototype viruses of VSIV-2 (COCV) and VSIV-3 (VSAV); however, there is little

information on the phylogenetic relationships of viruses causing outbreaks in South America [21].

Here, we report the genomic sequences of the glycoprotein (G) and phosphoprotein (P) genes of ten viral strains recovered from outbreaks in Brazil and Argentina between 1963 and 1998, as well as two viral strains isolated from *Lutzomyia sp*., one from Para, Brazil, in 1983 (MARAV) and one from Durania, Colombia, in 1986 (CoAr 171638). Nucleotide sequences were used to reconstruct phylogenetic relationships among these viruses, and these relationships were compared to cluster analyses based on serological cross-reactivity. Our results showed that viruses causing clinical VS in Brazil or Argentina can be serologically and phylogenetically classified into two distinct groups within the Indiana serogroup, namely VSIV-2 and VSIV-3.

Materials and methods

Viral strains and RNA extraction

Table 1 summarizes the information for the viral isolates used in this study. Reference strains COCV and VSAV have been described previously [21]. Strains associated with VSIV-2 outbreaks in Brazil and Argentina and VSIV-3 outbreaks in Brazil were kindly provided by the Pan American Foot-and-Mouth Disease Center (PANAFTOSA). MARAV was obtained from the USDA-APHIS Foreign Animal Disease Diagnostic Laboratory (FADDL), and CoAr 171638 was kindly provided by Dr. R. Tesh [29]. Virus nomenclature follows the VIIIth Report of the International Committee on Taxonomy of Viruses (ICTV) [9]. Serological classification for vesicular stomatitis virus Indiana subtypes Indiana 2 or Indiana 3 has been previously used to describe viruses causing outbreaks in Brazil and Argentina [1].

Viruses were passed once in BHK-21 cells at a multiplicity of infection of 0.01 or less. Total RNA was extracted using TRIzol-LS® (Invitrogen, Carlsbad, CA) as described previously [24]. RNA pellets were resuspended in sterile water and kept at −70°C until tested.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Reverse transcription was performed using random hexamers (Invitrogen) and SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Primers for PCR and sequencing reactions were designed based on the published sequences of VSIV, COCV, and VSAV or based on newly obtained sequences (primer sequences are available from the corresponding author upon request). PCR reactions were performed using Pfu DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Nucleic acid amplifications were performed in a 9600 Perkin-Elmer thermocycler (PE Applied Biosystems, Foster City, CA) as described previously [24]. Products were analyzed by electrophoresis and visualized by ethidium bromide staining. Based on newly acquired sequences, two primer sets (IN2PF 5'-TTACCAAAATCAGGAGGATGA with IN2PR 5'-GCCTCCCACCGAGATG and IN3PF 5'-AGAGCAGCTCCYTCTTATTAT with IN3PR 5'-TCATCATTCCATTTCCTC) were designed to selectively amplify either the VSIV-2 or VSIV-3 hypervariable region of the phosphoprotein gene, which is routinely used for phylogenetic analysis.

DNA sequencing, alignments and phylogenetic analysis

Single-band products were purified directly from the RT-PCR reaction using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). Multiple-band products were separated in agarose gels and extracted using a QIAquick Gel Extraction Kit (QIAGEN). PCR products were sequenced by dideoxy sequencing using a BigDye Terminator™ Sequencing Kit on a 3100 or 3700 automated sequencing instrument (PE Applied Biosystems) as described previously [26]. Sequencher™ v4.1 software (GeneCodes, Ann Arbor, MI) was used to analyze nucleotide sequence fragments and assemble contigs. Consensus sequences were derived from at least two independent forward and reverse sequences, but in most cases, there was extensive sequence overlap, and at least four sequences in each direction were available. Amino acid alignments were performed using MegAlign® (DNAStar Inc., Madison, Wisconsin) and multiple sequence alignments were performed using ClustalX [30]. Parameters and models of nucleotide substitution for each nucleotide dataset were estimated without an outgroup using Modeltest, version 3.7 [22]. Phylogenies were reconstructed using PAUP* 4.0b10 [28]. Neighbor-joining phylogenetic trees based on serological distance (determined by either virus neutralization or complement fixation assays as described below) were resolved using the "Neighbor" program in the PHYLIP phylogeny inference package Ver. 3.6 [11]. All sequences reported here have been deposited in GenBank and assigned accession numbers (JF795507-JF795530).

Complement fixation

Field viruses VSIV-3 95Minas GeraisB, VSIV-2 98ParanaE, VSIV-2 98Sta CatarinaB1, and VSIV-2 98Sta CatarinaB2 were identified by serology at the PANAFTOSA diagnostic laboratory but were unavailable for further

Table 1 Demographic, passage information and nomenclature of viral strains characterized in this study

Nomenclature	Geographical origin	Year of isolation	Host	Passage history
VSIV-2 63SaltoE	Salto, Buenos Aires, Argentina	1963	Equine	BHK p7
VSIV-2 66RanchariaE	Rancharia, Sao Paulo, Brazil	1966	Equine	BHK p5
VSIV-2 86MaipuE	Maipu, Buenos Aires, Argentina	1986	Equine	BHK p8
VSIV-2 79RiberaoE	Riberao Preto, Sao Paulo, Brazil	1979	Equine	BHK p6
VSIV-2 98Sta CatarinaB1	Santa Catarina, Brazil	1998	Bovine	BHK p2
VSIV-2 98Sta CatarinaB2	Santa Catarina, Brazil	1998	Bovine	BHK p2
VSIV-2 98ParanaE	Parana, Brazil	1998	Equine	BHK p2
VSIV-3 77EspinosaB	Espinosa, Minas Gerais, Brazil	1977	Bovine	BHK p9
VSIV-3 84SergipeE	Sergipe, Brazil	1984	Equine	BHK p2
VSIV-3 86Agulhas NegrasB	Agulhas Negras, Rio de Janeiro, Brazil	1986	Bovine	BHK p5
VSIV-3 95 Minas GeraisB	Minas Gerais, Brazil	1995	Bovine	Epithelium
MARAV	Maraba, Para, Brazil	1983	Insects	Vero p1, BHK p4
CoAr 171638	Durania, Colombia	1986	Insects	Vero p3

serological testing in this study. Complement fixation (CF) tests were performed to determine the serological relationships among selected reference viruses. Briefly, twofold dilutions of guinea pig hyperimmune sera were titrated against each virus. Viruses were used in a dilution containing 2.5 CF 50% units. Four hemolytic units of complement were used in all tests. A serological distance matrix obtained as described previously [1] was used for cluster analysis. Complement fixation R-values were calculated as described below.

Virus neutralization

Virus neutralization (VN) titrations were performed using 10[3] infectious doses/mL. Briefly, fourfold dilutions of guinea pig hyperimmune sera that were specific for the different VSV strains used in the study were made and mixed with viral suspensions. Four duplicate wells of 100 µL inoculum/well were incubated for 1 h at 37°C and then kept at 4°C for at least 30 minutes prior to inoculation of 24-hour-old BHK-21 cell monolayers. Plates were incubated for a period of 48 h at 37°C in a humid atmosphere containing 5% CO_2. Plates were fixed and stained, serum neutralization titers were calculated according to the Spearman and Karber method, and the cross-relationships between strains were expressed as a cross-reactivity product $R = 100(r1 \times r2)^{1/2}$, where r is the relationship of the neutralizing activity of serum against the heterologous strain, divided by the activity of serum against the homologous strain. Two-way immunological relationships were established as described previously [1]. A serological distance matrix was obtained and utilized for cluster analysis.

Results

All of the VSIV-2 isolates and COCV showed cross-reactivity, ranging from 57 to 85% by CF, with low cross-reactivity (<30%) with VSIV-3 isolates (Table 2). Likewise, all of the VSIV-3 isolates showed very high CF cross-reactivity (82-96%) within their group. MARAV showed CF titers ranging from 25 to 44% with VSIV-2, 34% with VSIV and only 14-19% with VSIV-3 strains.

Cross-neutralization values ranging from 33 to 47% were observed amongst VSIV-2 viruses, with <5% cross-reactivity with VSIV-3 strains. All of the VSIV-3 isolates showed cross-neutralization values of 21-100%, with no other viruses showing cross-neutralization ≥1% (Table 3). Interestingly, COCV cross-neutralized (24-27%) with all VSIV-2 isolates except 79RiberaoE (a 7% cross-neutralization titer). MARAV virus showed no cross-neutralization with VSIV-3 strains and only marginal cross-neutralizing reactivity with VSIV-2 (2-5%) or VSIV (2%) (Table 3).

Relationships among viruses were inferred from serological distance data based on both CF (Fig. 1a) and VN tests (Fig. 1b). Both tests clearly classified all clinical isolates into VSIV-2 or VSIV-3 with their respective prototype viruses. MARAV grouped with VSIV in the CF-based dendrogram and grouped with VSIV-2 isolates in the dendrogram based on VN. However, MARAV did not cluster closely with COCV as has been reported previously [31].

The nucleotide sequences of the entire open reading frame (ORF) of both the phosphoprotein (P) and glycoprotein (G) were determined for each of the viruses listed in Table 1 with the exception of VSIV-3 84SergipeE (which failed to grow upon arrival at the PIADC and did

Table 2 Complement Fixation R values (%)

	VSNJV (%)	VSIV (%)	2 Sal (%)	2 Rib (%)	2 Ran (%)	2 Mai (%)	COCV (%)	MARAV (%)	VSAV (%)	3 Ser (%)	3 Esp (%)
VSIV	0	–									
2 Sal	0	4	–								
2 Rib	0	18	*83*	–							
2 Ran	0	16	*57*	*85*	–						
2 Mai	0	17	*68*	*62*	*82*	–					
COCV	0	26	*69*	*73*	*63*	*64*	–				
MARAV	0	34	25	31	44	43	30	–			
VSAV	0	10	18	14	18	16	14	17	–		
3 Ser	0	7	16	20	28	17	16	19	*83*	–	
3 Esp	0	11	12	12	15	13	10	14	*82*	*84*	–
3 A N	0	8	16	13	18	14	19	17	*96*	*96*	*92*

Values greater than 50% are given in italics. Strain abbreviations are as follows (2 Sal = VSIV-2 63SaltoE; 2 Rib = VSIV-2 79RiberaoE; 2 Ran = VSIV-2 66RanchariaE; 2 Mai = VSIV-2 86MaipuE; 3 Ser = VSIV-3 84SergipeE; 3 Esp = VSIV-3 77EspinosaB; 3 A N = VSIV-3 86Agulhas NegrasB)

Table 3 Virus Neutralization R values (%)

	VSNJV (%)	VSIV (%)	2 Sal (%)	2 Rib (%)	2 Ran (%)	2 Mai (%)	COCV (%)	MARAV (%)	VSAV (%)	3 Ser (%)	3 Esp (%)
VSIV	1	–									
2 Sal	1	1	–								
2 Rib	1	1	*33*	–							
2 Ran	1	1	*47*	*27*	–						
2 Mai	1	1	*45*	*38*	*38*	–					
COCV	1	1	*27*	7	*24*	*24*	–				
MARAV	1	2	5	4	3	5	5	–			
VSAV	1	1	1	1	1	1	1	1	–		
3 Ser	1	1	1	1	1	1	1	1	*67*	–	
3 Esp	1	1	1	1	1	1	1	1	*21*	*71*	–
3 A N	1	1	1	1	1	1	1	1	*35*	*67*	*100*

Values greater than 20% are given in italics. Strain abbreviations are as follows (2 Sal = VSIV-2 63SaltoE; 2 Rib = VSIV-2 79RiberaoE; 2 Ran = VSIV-2 66RanchariaE; 2 Mai = VSIV-2 86MaipuE; 3 Ser = VSIV-3 84SergipeE; 3 Esp = VSIV-3 77EspinosaB; 3 A N = VSIV-3 86Agulhas NegrasB)

not provide adequate template for PCR and sequencing). All VSIV-2 isolates and MARAV had a G ORF of 1539 nt. The G ORF was shorter for all VSIV-3 isolates (1536 nt) and longer for CoAr 171638 (1542 nt), with the differences in length located in the putative signal and cytoplasmic domains. Residues comprising or adjacent to previously identified glycosylation sites for VSIV [6] were completely conserved among the sequenced viruses listed in Table 1. There were relatively few changes in the predicted fusion domains, both within and across subtypes.

In the case of the P ORF, all VSIV-2 field isolates were identical in length to COCV (795 nt), while field isolates of VSIV-3 possessed a P ORF of 786 nt, three nt longer than that reported for VSAV (783 nt). This difference was at the extreme carboxyl end of the P ORF, where the VSIV-3 field isolates code for an additional amino acid (Asp for VSIV-3 86Agulhas NegrasB and Gly for both VSIV-3 77EspinosaB and VSIV-3 95Minas GeraisB). The lengths of the P ORF for MARAV and CoAr 171639 were 798 nt and 813 nt, respectively.

Two small, highly basic, non-structural proteins, C' and C, are encoded in a second ORF within the P gene of VSIV [16] and VSNJV [27]. Despite the conservation of this feature, no specific function has been attributed to these small proteins, and recombinant VSIV mutants lacking them display similar *in vitro* growth kinetics to wild-type virus [16]. All of the VSIV-2 field isolates showed a conserved ORF in the second frame of the P gene, and these putative proteins have a predicted pI of 11.60 or higher, which is characteristic of the C proteins of VSV [26]. The

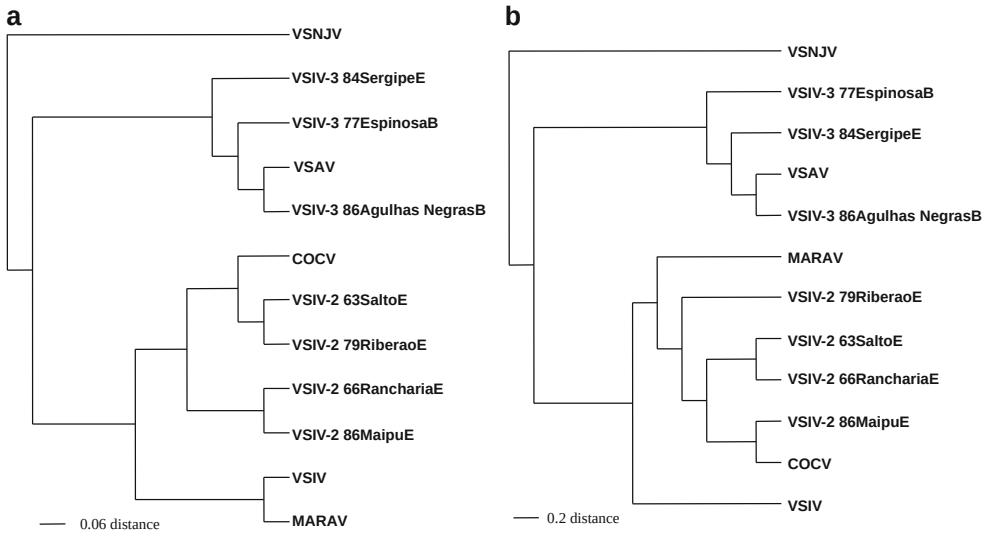

Fig. 1 Neighbor-joining phylogenetic trees based on serological distances obtained by complement fixation (**a**) or virus neutralization (**b**) assays. Trees were created using the Neighbor program in the PHYLIP v3.6 package

putative C'/C ORF in the VSIV-2 86MaipuE genome possessed an additional 10 aa at the carboxy terminus relative to other VSIV-2 isolates (Supplemental Figure 1). This deviation in length of the C proteins for VSIV-2, which does not affect the total length of the P protein, has not been observed previously in either VSIV or VSNJV. As described previously, [21] C proteins were absent in VSAV, and not surprisingly, they were also absent in all VSIV-3 field isolates sequenced. This unique feature provides further support for the taxonomic classification of the VSIV-3 subtype. In MARAV, a second ORF is found that could encode a 49-aa protein with a predicted pI of 12.51, indicating the possible presence of a C protein. There are two non-overlapping ORFs in CoAr 171638, which could encode proteins of 29 aa (pI 12.52) or 35 aa (pI 11.35).

The nucleotide phylogenetic relationships within the Indiana serotype and the two insect viruses (MARAV and CoAr 171638) were determined using either the complete G gene (Fig. 2a) or the hypervariable region of the P gene (Fig. 2b). Both phylogenies showed all clinical isolates separating distinctly into monophyletic VSIV-2 or VSIV-3 lineages. In both phylogenetic trees, the insect viruses MARAV and CoAr 171638 formed separate lineages closer to VSIV than to the other two Indiana subtypes. Within each subtype, the percent amino acid sequence divergence values observed for the glycoprotein were 5.9, 8.6 and 12.3 for VSIV, VSIV-2 and VSIV-3, respectively.

Discussion

Vesicular stomatitis was described over 100 years ago, with reports of vesicular lesions in the mouth and feet of cattle and horses [8, 12]. Two distinct serotypes (New Jersey and Indiana) were identified [7], and subsequently, two additional subtypes of the Indiana serotype (VSIV-2 and VSIV-3) were described [10]. The nomenclature and classification of vesiculoviruses does not clearly demarcate vesiculoviruses causing vesicular stomatitis clinical disease from serologically related viruses that have been isolated from insects. This study shows that field isolates causing VS in Argentina and Brazil fall within two clearly defined groups that are phylogenetically supported by analyses based on both serological and phylogenetic distance. These relationships were initially characterized by two-way serological cross-reactions (R values) based on either complement fixation or neutralization tests using hyperimmune sera generated in guinea pigs [1]. Complement fixation tests allow for more cross-reactions, since they are based on more conserved viral proteins (e.g., N) [1, 5, 13]. Neutralization tests are serotype-specific and reflect differences in the sequence of the viral glycoprotein, since neutralizing epitopes are located exclusively on this protein [4, 15, 17].

As expected, the CF results in this study showed a markedly lower level of specificity than those of VN tests (Tables 2 and 3). Based on the CF test, field isolates clearly grouped into two distinct serological groups that included COCV and VSAV. The same is true for the VN test, with the exception that COCV showed little cross-neutralization (7%) with VSIV-2 79RibeaoE. The insect-derived virus MARAV did not show cross-neutralization with any other viral isolate, nor did it fall into one of the three established Indiana subtypes. Interestingly MARAV displayed a mixed relationship with VSIV and VSIV-2 based on CF (a nucleoprotein-dependent assay) cross-reactivity (Table 2). It would be interesting to see how the sequence of the MARAV nucleoprotein, a highly conserved VSV protein [21, 26], compares to those of VSIV and VSIV-2. However, the sum of our serologic and nucleotide data indicates

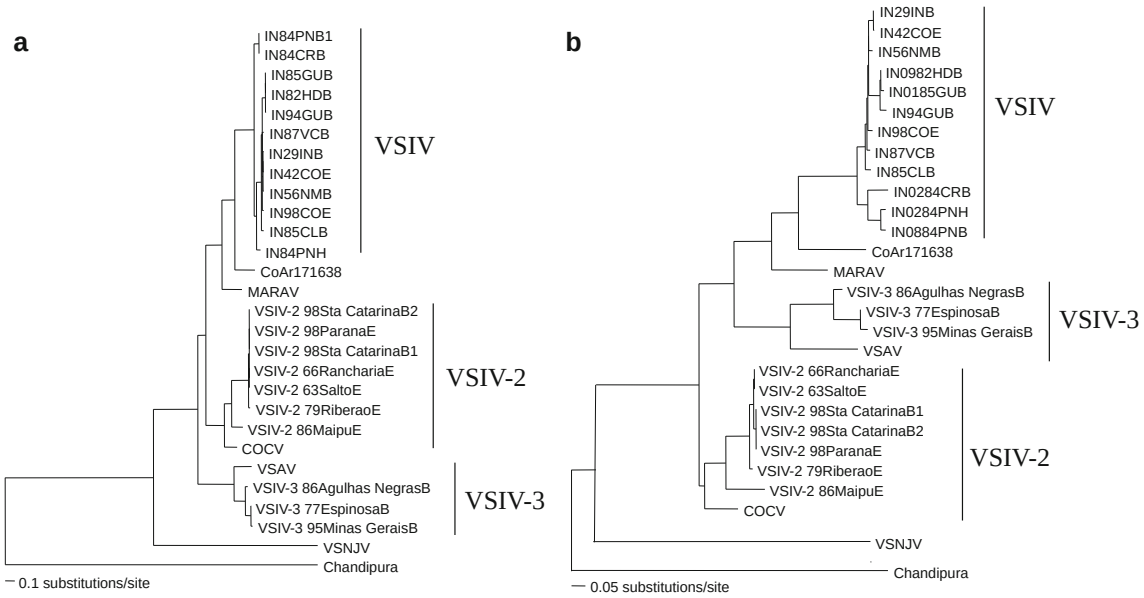

Fig. 2 Maximum-likelihood phylogenetic trees based on the nucleotide sequence of the complete glycoprotein (**a**) or the hypervariable region of the phosphoprotein (**b**). Parameters of nucleotide substitution were estimated without outgroup sequences using Modeltest and selected using the Akaike's Information Criteria (AIC). All VSIV isolates in GenBank with both the P hypervariable region and the complete glycoprotein were included. Both trees are outgroup-rooted with the distantly related Chandipura and VSNJ viruses

that MARAV should not be considered a VSIV-2 isolate as has been previously suggested [31].

Nucleotide sequencing of the P gene evidenced the lack of C proteins in all field isolates of VSIV-3, as had been reported previously only for the prototype strain VSAV. The fact that we now confirm the absence of C proteins in other natural field isolates of VSIV-3 provides strong support for the taxonomic classification of this virus subtype. This is an interesting finding, since the function of these small, highly basic proteins remains unclear. Their absence in all VSIV-3 isolates, along with the fact that recombinant VSIV strains without C proteins grow normally *in vitro*, support the notion that they are dispensable, at least in VSV strains affecting mammals.

Phylogenetic analysis based on partial sequences of the P or the full-length G protein provided strong support for the classification of these VSIV-2 and VSIV-3 isolates (Fig. 2). In this work, we included phylogenetic reconstructions based on the glycoprotein to complement the neutralization test data that serve as the basis for serological classification of these viruses. Unlike the P-gene-based phylogenetic tree, where VSIV-2 is ancestral to VSIV-1 and VSIV-3, the G-based tree showed VSIV-3 ancestral to the other two subtypes. This is consistent with the serology-based cluster analysis, where VSIV-3 is also ancestral to the other two subtypes. However, in all cases, the isolates associated with vesicular disease in Brazil and Argentina grouped monophyletically with their respective prototype virus.

Phylogenetic analysis based on nucleotide sequence included four viruses from VS outbreaks in Brazil, one virus from Minas Gerais (1995), serologically classified as VSIV-3, and three others from the southern states of Parana and Santa Catarina in 1998, serologically classified as VSIV-2. The 1995 isolate from Minas Gerais grouped within the VSIV-3 clade and was most closely related to another virus from the same Brazilian state (Espinosa) isolated in 1977 (Fig. 2). The three 1998 VSIV-2 viruses clustered with a virus isolated in 1966 from the nearby state of Saõ Paulo, Brazil (Rancharia), and a virus isolated in 1963 from Salto, in northeast Argentina (Fig. 2). The relatively low level of genetic variation observed between viruses collected many years apart from areas of geographic proximity is consistent with similar evolutionary constraints previously observed for VSNJV isolates [20, 23]. Additional analysis with an increased number of isolates and further refined geographic information of viruses causing VS in Brazil could help to determine if ecological factors play a role in the evolutionary patterns of VSIV-2 and VSIV-3.

An interesting observation is that VSIV-2 had mainly been reported affecting horses [2]. However, viruses from a VSIV-2 outbreak in 1998 in the states of Santa Catarina and Parana mainly affected cattle. The causative virus was closely related to equine viruses from 1963 and 1979 in both the G and P genes, indicating the capacity of the same viral lineage to affect different host species.

Two vesiculoviruses isolated from insects in South America were also included in our phylogenetic analysis,

one from Pará, Brazil (MARAV), and the other from Durania, Colombia (CoAr 171638). MARAV had been shown previously to share similarities with VSIV and COCV by CF tests and was isolated from the same region as the second isolation of COCV [14, 31]. The CoAr 171638 virus from Colombia was one of five virus isolates obtained from naturally infected phlebotomine sand flies (*Lutzomyia* spp.). These five isolates were reported as indistinguishable from the VSIV-3 prototype (VSAV) by CF and plaque reduction neutralization [29], and in a previous report, the partial G sequence of another of the original five virus isolates, CoAr 171044, was analyzed [3]. These isolates are of particular interest, as they would extend the distribution of VSIV-3 well outside its known area of occurrence of mid- to northeast Brazil. Our data showed that, although clearly related to the Indiana viruses, CoAr 171638 forms a separate genetic lineage from VSIV-2 and VSIV-3 in both P- and G-based phylogenetic trees and should not be classified with either of these virus subtypes (Fig. 2).

The purpose of this work was to serologically and genetically characterize viruses causing vesicular disease in livestock over a 35-year period in Brazil and Argentina. Our results clearly showed that these viruses can be classified as VSIV-2 or VSIV-3. Current VSV taxonomy, as established in the Eighth Report of the ICTV (revised in 2010), defines VSIV, VSAV and COCV as members of the genus *Vesiculovirus*, all of which are within the VS Indiana serotype. Based on the findings reported here, we propose that the nomenclature VSIV-2 or VSIV-3 should be utilized when describing viruses causing vesicular stomatitis. Specific virus nomenclature can follow that of other arboviruses, with the year and place of occurrence and the affected host (e.g., VSIV-2 –79RiberaoE). This nomenclature is similar to that currently utilized to describe field isolates of VSNJV or VSIV and should be applied to additional isolates of VSIV-2 and VSIV-3.

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NOTE

Use of Inactivated *Escherichia coli* Enterotoxins To Enhance Respiratory Mucosal Adjuvanticity during Vaccination in Swine▽

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In order to augment responses to respiratory vaccines in swine, various adjuvants were intranasally coadministered with a foot-and-mouth disease virus (FMDV) antigen to pigs. Detoxified *Escherichia coli* enterotoxins LTK63 and LTR72 enhanced antigen-specific mucosal and systemic immunity, demonstrating their efficacy as adjuvants for nonreplicating antigens upon intranasal immunization in swine.

Most pathogens initiate infections through contact with the mucosal surfaces of their hosts, thereby leading to colonization of the epithelium and/or invasion of tissues. Vaccination strategies that induce the production of mucosal immunity are desirable, as this approach can reduce the contact of pathogens with epithelial cells and possibly prevent dissemination to peripheral sites of the body. This is particularly important in the respiratory tracts of animals, as many of the potent innate barrier defenses present in the digestive system (e.g., low stomach pH, digestive enzymes, etc.) are not found in the airways. Given that the mucosae are inherently toleragenic (1), the use of adjuvants is essential to induce robust responses for nonreplicating mucosal vaccines (4). Unfortunately, the efficacy of many adjuvants in domestic animals (including pigs) has not been thoroughly tested, particularly for vaccines administered via the intranasal (i.n.) route.

The best-characterized mucosal adjuvants are the heat-labile enterotoxin (LT) produced by *Escherichia coli* and the closely related cholera toxin (CT) elaborated by *Vibrio cholerae*. Regrettably, these toxins are unsafe for use in humans (6), which dramatically limits their use in human and veterinary vaccines. To address this issue, detoxified mutants of LT, known as LTR72 and LTK63, were previously generated and have minimal residual toxicity (5, 8) but retain mucosal adjuvant activity in some animal species (10). Recently, several other adjuvant candidates have been developed in an attempt to elicit more potent mucosal immune responses. The interaction between bacterial CpG DNA and Toll-like receptor 9 (TLR 9) has made this motif a promising candidate for boosting both systemic and mucosal immunity. Another adjuvant that has been shown to augment mucosal and systemic immune responses is chitosan, a polysaccharide derived from the exoskeleton of crustaceans. More recently, chitosan nanoparticles harboring CpG motifs induced mucosal and systemic immune responses in mice (3, 11).

In this work, four mucosal adjuvants (wild-type CT [20 μg], LTK63 [100 μg], LTR72 [100 μg], and CpG/chitosan [200 μg] [manufacturer's recommended dosages shown in brackets]) were tested to assess their efficacy upon coadministration with a model foot-and-mouth disease virus (FMDV) antigenic peptide via the intranasal route. Groups of five female Yorkshire pigs (6 weeks old; John Correy, Scotland, CT) received the mucosal adjuvants admixed with a peptide derived from FMDV serotype O_1-BFS VP1 G-H loop (which contains at least one important neutralizing epitope of the virus [9]). This peptide is referred to as the "TCA" peptide, representing a well-conserved VP4 T helper cell epitope, VP1 site C, and VP1 site A epitopes. The animals were intranasally inoculated with 100 μg of TCA peptide (reconstituted in 400 μl of water, the volume given to each animal) (Genemed Synthesis Inc., South San Francisco, CA) at weeks 1, 2, 3, and 5, with a parenteral

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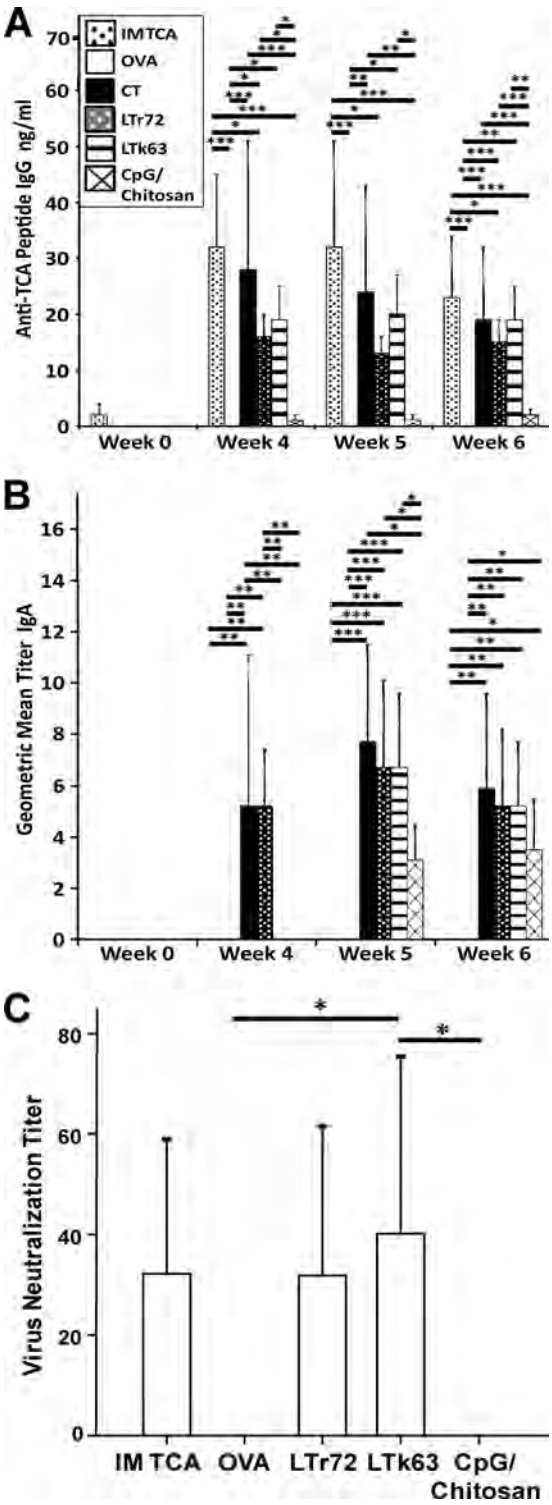

FIG. 1. Antigen-specific humoral immune responses. Anti-FMDV peptide serum IgG (A) and mucosal IgA (B) antibody responses, as measured by ELISA (one animal from the group given CT as an adjuvant died before completion of the study and was not included in any analyses). IgG concentrations were interpolated from a standard curve, and IgA concentrations are expressed as a geometric mean titer (endpoint titer determined to have an absorbance two times higher than background). IM TCA, i.m. TCA peptide. (C) Virus neutralizing titers as measured by plaque reduction assay (CT was excluded from the analysis, as statistics were impeded by the dramatically skewed data distribution of this group [only one animal in this group had measurable VNT, but it was the highest in the study at 1:130]). The bars represent the mean responses, and the error bars indicate 1 standard deviation from the mean. Horizontal black bars indicate pairwise comparisons between groups with significance denoted by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

boost given at week 4 with MPL+TDM+CWS RIBI adjuvant system (100 μg; administered intramuscularly [i.m.]; Sigma-Aldrich, St. Louis, MO). The parenteral boost was included to augment serum antibody and virus neutralization titers, since mucosal immunity alone may not adequately control viral infection/dissemination (7). Additionally, control groups included pigs sham immunized i.n. with chicken ovalbumin (OVA) plus RIBI adjuvant (Sigma-Aldrich, St. Louis, MO) at week 0, with a week 4 i.m. boost of OVA and RIBI or with TCA peptide plus RIBI adjuvant administered i.m. at weeks 0 and 4. Even though CT retains toxicity, it has been shown to be efficacious when administered i.n. to pigs, and we thus utilized this adjuvant as a "gold standard" for comparative purposes.

Serum samples were collected for assessment of anti-TCA peptide IgG responses as measured by an enzyme-linked immunosorbent assay (ELISA) (previously described [2]), and the antibody concentration was calculated by the method of Barrette et al. (2) (statistical significance for all analyses was determined using a one-way analysis of variance [ANOVA] with *post hoc* multiple pairwise comparisons by Fisher's least significant difference [LSD] test). A negligible IgG response was observed in all i.n. immunized groups prior to week 4; however, the i.m. TCA peptide-immunized group did produce a notable response at week 3 (data not shown). Animals i.n. inoculated with CT, LTR72, or LTK63 or i.m. inoculated with the TCA peptide produced statistically significant IgG antipeptide responses by week 4 relative to those inoculated with OVA or CpG/chitosan (Fig. 1A), and this tendency was observed throughout the remainder of the experiment (the responses for LTR72-inoculated animals were not significant at week 5 but the trend could still be observed [$P = 0.1$ compared to the values for OVA- or CpG/chitosan-inoculated animals], and the responses were again significant at week 6). Interestingly, the responses were not different among the groups producing a significant IgG response, with the exception of i.m. TCA peptide and LTR72. Thus, LTR72 and LTK63 appear to induce serum anti-TCA peptide IgG responses as vigorous as those of the group given CT as an adjuvant. It is important to note that the week 4 parenteral booster immunization of mucosally primed animals did not seem to augment immunity, indicating that i.n. immunization alone was primarily responsible for the observed antibody responses. In order to assess the functional capabilities of the humoral antibody responses, FMDV plaque reduction assays were conducted in order to determine virus neutralization titers (VNT). No VNT were observed at week 4 in any group; however, by the end of the study, significantly higher VNT were observed in animals inoculated with LTK63 than in animals inoculated with OVA or CpG/chitosan (Fig. 1C). Also, the results with animals inoculated with LTR72 tended to have a trend toward statistical significance compared to the animals inoculated with OVA

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($P = 0.07$) or CpG/chitosan ($P = 0.05$), and a trend was also observed in animals inoculated i.m. with TCA peptide compared to animals inoculated with OVA ($P = 0.07$) or CpG/chitosan ($P = 0.05$). These data indicate that mucosal immunization was as effective as i.m. inoculation for generating serum IgG antibodies, but i.m. boosting may be necessary to induce serum virus neutralization activity.

Anti-TCA peptide IgA responses (Fig. 1B) were measured by ELISA from nasal wash samples collected from pigs and are reported as the geometric mean titer. Secreted IgA antibodies were evident by week 4 in pigs receiving the CT and LTR72 adjuvants compared to all other groups, and animals inoculated with LTK63 had significant responses compared to those receiving i.m. TCA peptide or OVA at weeks 5 and 6. Animals vaccinated with CpG/chitosan exhibited a trend toward significance at week 5 compared to animals vaccinated with OVA or i.m. TCA peptide ($P = 0.07$ and $P = 0.06$, respectively) and attained statistical significance by week 6, although the responses were notably lower than those observed with the other mucosal adjuvants. No significant IgA responses were detected in animals inoculated with OVA or i.m. TCA peptide at any time points. Antigen-specific IgA antibody-secreting cells (exceeding 50 cells/million in an enzyme-linked immunospot [ELISpot] assay) were observed in the nasal mucosal tissues of some CT- and LTR72-inoculated pigs, indicating that at least some of the mucosal immune responses resulted from plasma cells located in the nasal mucosa (data not shown).

Taken together, the LT mutants used as adjuvants in this study were effective for inducing systemic and mucosal immunity to an antigenic FMDV peptide in the respiratory tracts of swine, whereas CpG/chitosan was less efficient. Even though the LT mutants showed efficacy in this experiment, modification of the approach is necessary to improve the time to immunity and to increase the magnitude of the mucosal response. Based on the delay of roughly 4 weeks, it appears that adjustments in dose, adjuvanticity, delivery, antigen, or frequency of administration may be needed to optimize mucosal immune responses in the respiratory tracts of pigs to pathogens such as FMDV.

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Increased efficacy of an adenovirus-vectored foot-and-mouth disease capsid subunit vaccine expressing nonstructural protein 2B is associated with a specific T cell response

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ABSTRACT

We previously demonstrated that an adenovirus-based foot-and-mouth disease virus (FMDV) serotype A24 capsid subunit vaccine, Ad5-A24, expressed under the control of a cytomegalovirus promoter (CMV) can protect swine and bovines against homologous challenge, but in a similar approach using swine vaccinated with an Ad5-vectored FMDV O1 Campos vaccine, Ad5-O1C, the animals were only partially protected when challenged at 21 days post-vaccination (dpv). Recently, we demonstrated that inclusion of the complete coding region of nonstructural protein 2B in the Ad5-A24 vector resulted in improved immune responses in pigs. We also found that inclusion of a modified CMV promoter (pCI), Ad5-CI-A24-2B, enhanced the efficacy of the vector. To address the limited immunogenicity of Ad5-O1C, we have produced a new set of Ad5 vectors with the complete 2B coding region under the control of either the original or the modified version of the CMV promoter, Ad5-O1C-2B, or Ad5-CI-O1C-2B, respectively. To evaluate the potency and efficacy of the new vectors we performed 2 sets of experiments in cattle. In the first experiment we compared the original vector with vectors containing the pCI promoter and partial or full-length 2B. All groups were challenged, intradermally in the tongue, at 21 dpv with FMDV O1C. We found that in all vaccinated groups 2 of 4 animals were protected from clinical disease. In the second experiment we directly compared the efficacy of vectors with a partial or full-length 2B under the control of the original CMV promoter. While all animals in the control group developed clinical disease, 2 of 4 animals in the group receiving Ad5-O1C vaccine and 3 of 4 animals in the group receiving Ad5-O1C-2B vaccine were completely protected after challenge. We also observed a 100-fold reduction of virus shedding in Ad5-O1C vaccinated animals and the group receiving Ad5-O1C-2B had an additional 10-fold reduction compared with the Ad5-O1C vaccinated group. There was no difference in the level of neutralizing antibodies in the vaccinated groups. However, we detected a significant antigen specific-$CD4^+$ and $CD8^+$ T cell response as early as 1 day post-challenge (dpc) in both Ad5-O1C and Ad5-O1C-2B groups. Interestingly, the group receiving Ad5-O1C-2B had a statistically significant higher antigen specific-$CD4^+$ and $CD8^+$ T cell response at 5 dpc and 3 and 5 dpc, respectively, as compared to the Ad5-O1C inoculated group. These results indicate that inclusion of the complete 2B coding region improves the efficacy of Ad5 vaccines against FMDV serotype O and induces specific-$CD4^+$ and $CD8^+$ T cell responses that correlate with protection.

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1. Introduction

Foot-and-mouth disease (FMD) is considered one of the most contagious and economically devastating diseases affecting cloven-hoofed livestock worldwide [1–4]. FMD is characterized by fever, lameness and the appearance of vesicular lesions on the mouth, tongue, nose, feet and teats [3,4].

The etiologic agent, FMD virus (FMDV), has a positive-sense, single-stranded RNA genome and belongs to the genus *Aphthovirus*

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in the family *Picornaviridae*. FMDV is antigenically variable and consists of seven serotypes (A, O, C, SAT 1–3 and Asia) and numerous strains within each serotype. The viral genome is approximately 8.5 kb and is enclosed within an icosahedral capsid of about 30 nm that comprises 60 copies each of four structural proteins (VP1–4). A single long open reading frame (ORF) encodes a polyprotein which is processed post-translationally to yield both intermediate and mature structural and nonstructural (NS) viral proteins. Viral capsid assembly is dependent on NS protein $3C^{pro}$ cleavage of the structural protein precursor, P1-2A [5].

An FMD outbreak in a previously disease-free country can cause significant economic losses due to high morbidity and the associated trade restrictions imposed on these countries by trade partners based on the standards set by the World Organization for Animal Health (OIE) [4,6]. The most recent examples of re-introduction of FMDV with significant economic and social consequences are outbreaks in Japan and South Korea in 2010 and in South Korea in 2011 [7,8]. Control measures include restriction of movement of susceptible animals, slaughter of infected and FMD-susceptible in-contact animals and decontamination [9]. In addition, countries can utilize FMD emergency vaccines, but current regulations of the OIE 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 by documenting the absence of disease for three months after the last case, while countries that do not slaughter vaccinated animals must wait six months prior to regaining FMD-free status [9].

The currently available commercial vaccine is a chemically inactivated whole virus preparation emulsified with adjuvant [4,10]. The limitations associated with the current vaccine and the rationale for the above OIE policy are the difficulty to unambiguously discriminate infected from vaccinated animals (DIVA) using currently approved diagnostic tests and the observation that vaccinated ruminants can be asymptomatically infected and become virus carriers [4]. To address the former concern some vaccine manufacturers may partially purify the vaccine by chromatography to remove most of the viral NS proteins [11]. An additional concern is that production of the inactivated vaccine requires expensive high-containment manufacturing facilities and there is a risk of escape of infectious virus from these facilities as occurred in Pirbright in 2007 [11–15]. As a result attempts to develop a new generation of vaccines which address some or all of the above limitations have been ongoing for more than 30 years.

It is well characterized that in the natural infection, virus clearance is dependent on the development of a specific neutralizing antibody response [16,17]. Similarly, protection induced by vaccination with an inactivated vaccine correlates with high levels of neutralizing antibodies, although in some protected animals the level of neutralizing antibody is very low [18,19]. The role of cellular immunity in protection of animals against FMDV infection is still controversial, however, T-cell epitopes have been identified within both FMDV structural and NS proteins [20–22], and the existence of a FMDV-specific $CD8^+$ T-cell response has been demonstrated in cattle after infection or vaccination [23,24]. Furthermore, a specific T-cell mediated lymphoproliferative response following vaccination has also been described in animals vaccinated with adenovirus or vaccinia virus vectors expressing the FMDV capsid precursor protein P1 [25]. However, peptide vaccines, vectored vaccines containing NS proteins, or DNA vaccines containing B and/or T cell epitopes have had limited success in protection of susceptible animals against subsequent FMDV challenge [24,26,27].

Thus far, the most successful approach to produce an alternative vaccine for FMD is based on a replication-defective human adenovirus type 5 (Ad5) vector that contains the capsid coding region of FMDV serotype A24 and the complete 3C protease coding region of A12 [28–30]. This vaccine has been efficacious in both swine and cattle [28–30], and currently the U.S. Department of Agriculture, Agricultural Research Service, the U.S. Department of Homeland Security, and GenVec, Inc. are in the process of developing the Ad5-A24 vaccine for USDA, Center for Veterinary Biologics licensure and inclusion in the U.S. National Veterinary Stockpile [28].

FMDV serotype O vaccines are known to require larger amounts of antigenic mass than serotype A vaccines [31,32]. We have constructed an Ad5-O1 Campos (Ad5-O1C) vaccine, but in initial studies in swine it was less effective against homologous challenge than the same dose of Ad5-A24 [33]. Since the addition of the full-length coding region of NS protein 2B to the Ad5-A24 vaccine has been shown to improve protection in pigs [34], we attempted to enhance the efficacy of the Ad5-O1C vaccine by including this coding region and also modifying the promoter driving the expression of the FMD cassette. In this manuscript we compare the new vectors in cattle using homologous challenge at 21 days post-vaccination (dpv). Our results indicate that addition of full-length 2B, improves vaccine efficacy, but the use of a more efficient promoter is not reflected in enhanced protection. We also demonstrate that the increase in protection in the Ad5-O1C-2B vaccinated group correlates with a rapid and significant increase in the percentage of $CD8^+$ and $CD4^+$ T cells expressing interferon gamma (IFNγ) in response to challenge.

2. Materials and methods

2.1. Cells and viruses

Human 293 cells (ATTC CRL-1573) from the American Type Culture Collection (ATCC; Rockville, MD) were used, from passages 24 to 40, to generate, grow and titer all recombinant human Ad5 vectors used in this study [35,36]. The recombinant Ad5 viruses used included: Ad5-Blue, which lacks the FMDV coding regions, was used as a negative control [37], Ad5-O1C, which contains the FMDV O1 Campos P1-2A and partial 2B coding region (94 of the amino-terminal 154 amino acids of 2B) and the 3B and 3C proteinase coding region of FMDV A12 under the control of the cytomegalovirus (CMV) promoter [33], and three novel Ad5 constructs engineered for this study. These vectors are described below (Section 2.2) and include Ad5-CI-O1C, Ad5-CI-O1C-2B, and Ad5-O1C-2B (the latter 2 vectors contain the complete 2B coding region from A12). All Ad5 vectors were purified by CsCl gradient centrifugation and viral titers were determined by standard protocols [29]. Swine kidney cells (IB-RS-2 cells), were utilized to measure FMDV transgene expression following infection by the Ad5 vectors. Plaque reduction neutralization (PRN) assay and FMDV titration from blood and nasal swabs samples were performed on baby hamster kidney (BHK)-21 cells (ATCC CCL-10). BHK-21 cells were also utilized for production of radiolabeled FMDV O1C antigen used in a radioimmunoprecipitation (RIP) assay. FMDV O1C used as a challenge virus was produced by experimental infection of a bovine and a pool of the vesicular fluid was harvested and titered. The bovine infectious dose at 50% (BID_{50}) was determined by titration of the challenge virus via intradermolingual inoculation.

2.2. Construction of recombinant adenoviruses

The construction of Ad5-CI-O1C was performed by digesting pShuttle-P1(O1C)-2AX3C [33] with ClaI and XbaI and ligating the fragment of interest into the pAd5-CI-RL vector [38].

In order to engineer viruses expressing the full-length 2B coding region, a PCR using the plasmid pRMC35 as template [39] with primers 5′CCAAATCGATGCCCTTCTTCTTTGCTGACG3′ and 5′GAGCTTAGCTCTCACTTTCAGAGGTCTCTGACGCTCGAGTTTCTCTG-CTCTCTCGAGGTC3′ was performed and the amplified fragment

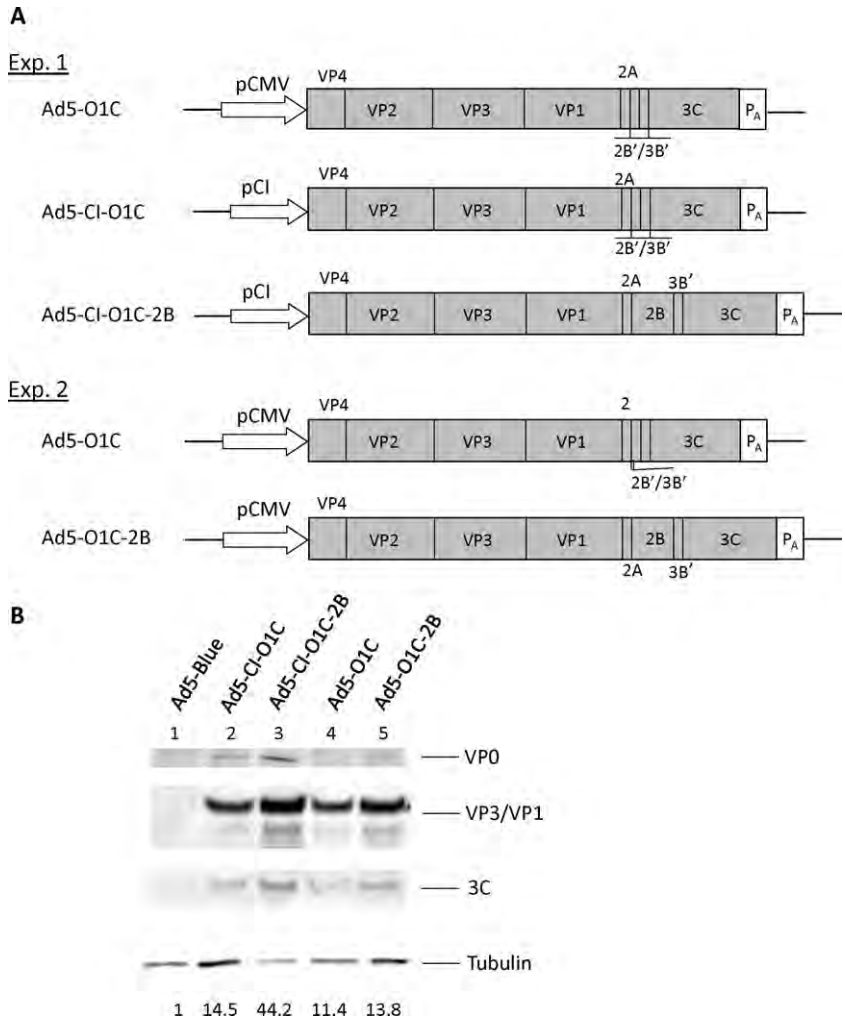

Fig. 1. Adenovirus vectored vaccines. Panel A. Schematic representation of the Ad5-vectored FMD vaccines used in this study. cDNA containing the depicted FMDV O1C and A12 coding regions was incorporated in the E1 region of an E1/E3 deleted Ad5 vector under the control of either the pCMV or pCI promoter. A truncated portion of 2B containing the amino terminal 94 amino acids of this protein (2B′) is present in Ad5-O1C and Ad5-CI-O1C. pCMV, the immediate-early promoter of human CMV; pCI, optimized CMV promoter; P_A, SV40 polyadenylation signal. Panel B. Expression of FMDV structural and NS 3C proteins in IB-RS-2 cells infected with the various Ad5 constructs. Whole cell lysates from IB-RS-2 cells infected with the indicated Ad5 constructs at MOI of 20 were prepared at 24 hpi and analyzed by Western blotting. A mix of rabbit monospecific polyclonal antibodies against each of the FMDV structural proteins at 1:2000 dilution was used to evaluate the expression and processing of capsid proteins. A monoclonal antibody against tubulin was used as loading control. Numbers at the bottom of the blot indicate the relative quantitation of band intensity of VP0, VP3, VP1, and 3C in lanes 2–5 normalized to tubulin in lane 1. Lane 1, Ad5-Blue; lane 2, Ad5-CI-O1C; lane 3, Ad5-CI-O1C-2B; lane 4, Ad5-O1C and lane 5, Ad5-O1C-2B.

was digested with HindIII and BplI and cloned into pKSII-P1(O1C)2AX3C to generate pKSII-P1(O1C)-2ABX3C(A12). The later was double digested with ClaI and XbaI and inserted into the pAd5-Blue and pAd5-CI-RL vectors. The final plasmids were linearized with PacI and transfected into 293 cells using a transfection kit (Transfection MBS Mammalian Trasfection Kit, Stratagene, La Jolla, CA) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Each recombinant virus was harvested 4–10 days post transfection.

The presence of the inserted transgenes was confirmed by restriction digestion analysis and sequencing. A schematic representation of the recombinant viruses is represented in Fig. 1, Panel A.

2.3. Expression of O1C proteins in Ad5-FMD infected cells

IB-RS-2 cell monolayers were grown in 6 well plates and infected overnight with Ad5 vectors at a multiplicity of infection (MOI) of 20. The cells were washed twice with phosphate-buffered saline (PBS), lysed and centrifuged and the cytoplasmic extracts frozen at −70 °C until used. Aliquots were run on 12% polyacrylamide gels (NuPAGE, Invitrogen), transferred to a PVDF membrane (Millipore, Bedford, MA) and examined by Western blot analysis using polyclonal rabbit antibodies against VP1, VP2 and VP3, at a dilution of 1:2000 in PBS/Tween, as primary antibodies. Immunocomplexes were detected using an alkaline-phosphatase-labeled goat anti-rabbit IgG (KPL, Gaithersburg, MD) at the same dilution. Immunoblots were developed using BCIT/NBT phosphatase substrate following the manufacturer's instructions (KPL). The quantification of expression of FMDV structural proteins and 3C was performed with a Kodak GelLogic 440 Imaging System using Kodak Molecular Imaging Software Version 4.0 (Carestream Health, Inc., Rochester, NY).

2.4. Vaccine trials in bovine

Two independent experiments were performed in the high-containment facility of the Plum Island Animal Disease Center following a protocol approved by the Institutional Animal Use and Care Committee. In both experiments, groups of 4 Holstein steers were housed in separate rooms. All animals were vaccinated intramuscularly (IM) in the neck in a total of 2 ml of PBS containing 5×10^9 plaque forming units (pfu) of each Ad5 construct.

In the first experiment, the immunogenicity and efficacy of the two new vectors, Ad5-CI-O1C and Ad5-CI-O1C-2B, were compared to our first generation vector, Ad5-O1C and Ad5-Blue as a negative control. At 21 dpv all bovines were challenged intradermally (ID) in the tongue with 10^4 BID_{50} of FMDV O1C.

In the second study, we performed a head-to-head comparison of the Ad5-O1C and Ad5-O1C-2B vectors. Twelve bovine were divided into three groups, two groups were inoculated with Ad5-O1C or Ad5-O1C-2B and the third group with Ad5-Blue (control group). All animals were challenged at 21 dpv as described above for the first experiment.

2.5. Clinical monitoring and sampling

After challenge, animals were examined and a clinical score was recorded daily for 7 days, at 10 days, and a final clinical examination was performed at 14 days post-challenge (dpc). Clinical scores were determined by the number of feet presenting FMD lesions plus the presence of secondary lesions in the mouth/snout. The maximum score is 5, and lesions restricted to the site of challenge were not counted. Rectal temperature data was monitored on a daily basis throughout the experimental period.

Nasal swabs and serum samples were taken prior to challenge and daily thereafter for seven days to assess virus shedding and viremia, respectively. In both experiments, serum samples were collected weekly, inactivated at 56 °C for 30 min, and stored at −70 °C. Serum samples were also frozen without treatment, and nasal swab specimens were collected in 2.5 ml MEM containing 2% antibiotics, 0.2% bovine serum albumin (BSA) and 20 mM Hepes buffer and clarified for 10 min at 12,600 × *g* prior to freezing at −70 °C.

2.6. Evaluation of humoral immune response

Heated serum samples were tested for the presence of FMDV-specific neutralizing antibodies by a PRN assay as described previously [30]. Neutralizing titers were reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) induced by FMDV O1C in BHK-21 cells.

Sera obtained at the day of challenge were also tested for the presence of antibodies against viral structural and NS proteins. Antigen was prepared by radiolabeling FMDV O1C infected BHK-21 cells with [^{35}S]methionine and a RIP assay was performed as previously described [29,40].

2.7. Virus titration in blood and nasal secretions

Untreated serum samples and nasal swabs were assayed for the presence of virus by plaque titration on BHK-21 cells (passage levels 64–70) as previously described [30]. Virus titers were expressed as $\log_{10}$ pfu per ml of serum or nasal swab.

2.8. Flow cytometric analysis

Blood samples were drawn into heparin-containing vacuum tubes, diluted with PBS 1:1, and layered onto Lymphoprep (Axis-Shield; PoC AS, Oslo, Norway). Cells were centrifuged for 20 min at 20 °C. Peripheral blood mononuclear cells (PBMCs) were collected and washed in PBS twice followed by one wash with RPMI 1640, and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), HEPES, L-glutamine, and antibiotic/antimycotic solutions.

PBMCs were stimulated overnight with FMDV O1C or with a non-specific stimulator (PMA + Ca Ionophore) for 18 h at 37 °C in 5% CO_2. Cells were thereafter incubated with GolgiStop (BD Bioscience, Franklin Lakes, NJ) following the manufacturers recommendation, pelleted and resuspended in staining buffer consisting of PBS containing 10% FBS (vol/vol) for flow cytometry. To analyze the expression of cell surface molecules, we used mouse anti-bovine CD4-FITC and mouse anti-bovine CD8-AF647 (both from AbD Serotec, Raleigh, NC). After staining, cells were fixed and permeabilized with BD Cytoperm/Cytofix (BD Biosciences) for 30 min at 4 °C, washed in BD Perm/Wash three times, and finally stained intracellularly with mouse anti bovine IFNγ-RPE (AbD Serotec). Background levels of RPE fluorescence were determined by staining with the corresponding IgG1-RPE isotype controls (AbD Serotec). 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 Pro software (BD Bioscience). For statistical analysis, Student's *t*-test was performed using Microsoft Excel.

3. Results

3.1. Expression and processing of FMDV proteins

All Ad5 vector constructs produced detectable amounts of FMDV O1C capsid proteins and 3C protease under either the CMV promoter or a modified version of the CMV promoter containing an intron signal (pCI) [38]. Western blot analysis of lysates from cells infected with all listed vectors (Fig. 1, Panel B, lanes 2–5) plus Ad5-Blue was performed with monospecific polyclonal antibodies against the FMDV capsid proteins and 3C. Processed capsid proteins VP0, VP1 and VP3, as well as 3C protease were identified and these proteins were not present in lysates from Ad5-Blue-infected cells (Fig. 1, Panel B, lane 1). In cells infected with vectors containing the full length 2B protein (Ad5-CI-O1C-2B, lane 3 and Ad5-O1C-2B, lane 5) there is enhanced expression of the structural proteins and 3C as compared to the vectors using the same promoters, but containing only a truncated 2B.

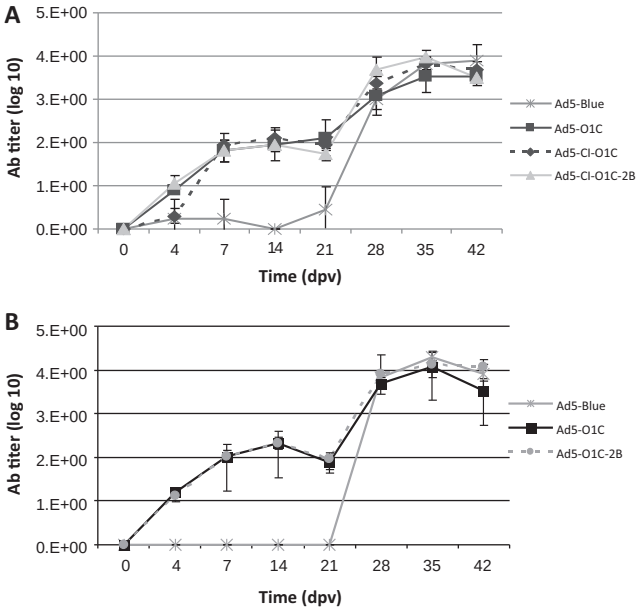

Fig. 2. Kinetics of neutralizing antibodies against FMDV O1 Campos in bovines vaccinated with Ad5 vectors and challenged at 21 dpv with FMDV. Serum samples were collected at 0, 4 dpv, and weekly following vaccination and challenge. Titers are expressed as the inverse dilution of serum yielding a 70% reduction in the number of plaques (PRN_{70}). Panels A and B represent the mean (±S.D.) of each group in experiments 1 and 2, respectively.

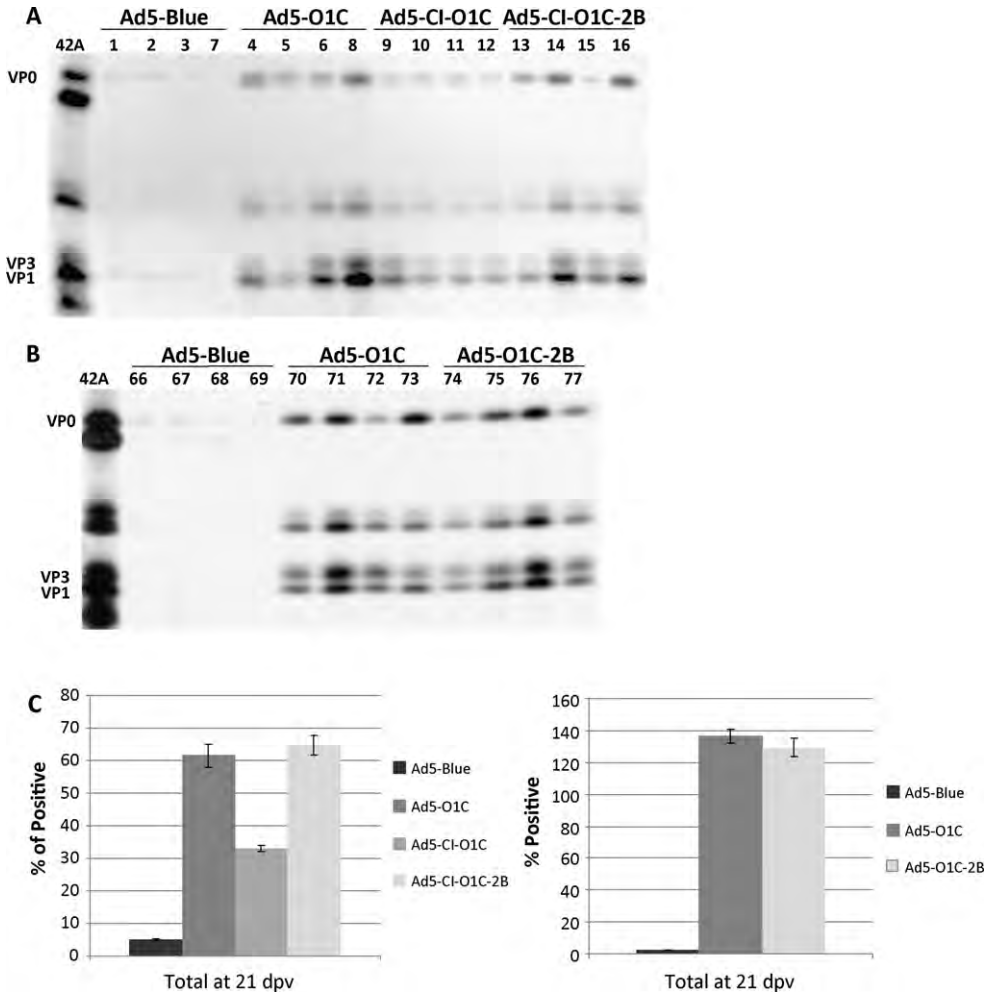

Fig. 3. Examination by RIP of 21 dpv serum from vaccinated animals. [^{35}S]-methionine labeled cell lysates from FMDV O1 Campos infected BHK cells were immunoprecipitated with sera from bovines vaccinated with Ad5-Blue, Ad5-O1C, Ad5-CI-O1C, Ad5-CI-O1C-2B or Ad5-O1C-2B as indicated. Panels A and B represent each group in experiments 1 and 2, respectively. Panel C represents the average relative quantitation of band intensity of Panels A and B normalized to immunoprecipitation using hyperimmune serum.

3.2. Antibody response after immunization with Ad5-vectored vaccines

We performed two vaccine studies in bovines in order to access the potency of the new vectors. In the first experiment, we compared the original vector with the vectors containing the pCI promoter and partial or full-length 2B. Four groups of bovines were vaccinated with Ad5-Blue, Ad5-O1C, Ad5-CI-O1C or Ad5-CI-O1C-2B. The antibody response was monitored at 0, 4, 7, 14 and 21 dpv and 7, 14 and 21 dpc by PRN_{70}. All animals vaccinated with Ad5 vectors encoding FMDV antigens developed FMDV-specific neutralizing antibodies by 4 dpv, except for the group receiving Ad5-CI-O1C (Fig. 2, Panel A), and neutralizing antibody titers peaked at 14 dpv in all Ad5-FMD vaccinated animals (Fig. 2, Panel A). The individual neutralizing antibody titers at the day of challenge (21 dpv) are shown in Table 1. In addition, all vaccinated animals, except for the Ad5-Blue control group, developed antibodies against the FMDV capsid proteins as detected by RIP (Fig. 3, Panel A).

In the second experiment, we performed a direct comparison between vectors with the pCMV promoter containing the empty capsid construct in the presence of the partial or complete 2B coding region. We detected neutralizing antibodies at 4 dpv in both groups inoculated with the vaccine candidates (Fig. 2, Panel B). The kinetics of the neutralizing antibody levels were very similar between the two vaccinated groups and with the vaccinated groups from the first experiment, with the highest levels detected at 14 dpv (Fig. 2, Panels A and B). Likewise, the level of antibodies against the capsid proteins detected by RIP (Fig. 3, Panels B and C) was similar in both vaccinated groups.

3.3. Clinical outcome after challenge

In both experiments the animals were challenged at 21 dpv by ID inoculation in the tongue with 10^4 BID_{50} of FMDV O1 Campos [9]. All animals inoculated with the control vector, Ad5-Blue, developed the maximum clinical score (5) as early as 3 dpc, except for animal #7 from the first trial that had a score of 5 only at 4 dpc. Viremia was detected in all the Ad5-Blue inoculated animals and was generally present from 1 to 3 dpc (Table 1). None of the Ad5-FMD vaccinated animals had detectable viremia. In the first experiment, we detected virus shedding in all animals after challenge, but the amount of virus shed was lower and the average duration of shedding in those vaccinated with the Ad5-FMD vectors was reduced by approximately 1.5–2 days as compared to the control group (Ad5-Blue) (Fig. 4, Panel A and Table 1).

In experiment 1 50% of the animals in the Ad5-FMD vaccinated groups did not develop clinical disease and in the other animals disease was less severe as compared to the control group (a clinical score of 1–2 vs. 5 for all control animals) (Table 1). In the Ad5-O1C

Table 1
Clinical performance of bovines immunized with Ad5 constructs and challenged with FMDV O1 Campos 21 days postvaccination.

Vaccine	Bovine#	Trial	PRN_{70} titer[a]	Clinical score[b]		Virus shedding[c] (day of onset, duration)	Viremia[d] (day of onset, duration)
				Assessment on 4	Assessment on 10		
Ad5-Blue	1	1	<8	5	5	2.7×10^5 (1, 6)	1.9×10^6 (1, 3)
Ad5-Blue	2	1	<8	5	5	1.5×10^4 (1, 6)	8.8×10^3 (2, 2)
Ad5-Blue	3	1	<8	5	5	5.1×10^6 (1, 6)	1.4×10^6 (1, 3)
Ad5-Blue	7	1	<8	5	5	1.3×10^5 (1, 5)	2.3×10^6 (1, 3)
Mean[e]			<8	5	5	1.5×10^5 (5.8)	
Ad5-O1C	4	1	256	0	1	1.3×10^4 (1, 4)	0 (0, 0)
Ad5-O1C	5	1	32	2	2	9.0×10^3 (1, 5)	0 (0, 0)
Ad5-O1C	6	1	128	0	0	2.7×10^4 (1, 4)	0 (0, 0)
Ad5-O1C	8	1	256	0	0	1.8×10^1 (4, 1)	0 (0, 0)
Mean[e]			128	0.5	0.75	7.2×10^3 (3.5)	
Ad5-CI-O1C	9	1	64	1	2	1.7×10^3 (3, 4)	0 (0, 0)
Ad5-CI-O1C	10	1	64	1	1	2.9×10^3 (1, 4)	0 (0, 0)
Ad5-CI-O1C	11	1	128	0	0	3.6×10^4 (2, 3)	0 (0, 0)
Ad5-CI-O1C	12	1	128	0	0	3.0×10^3 (1, 5)	0 (0, 0)
Mean[e]			90	0.5	0.75	1.1×10^4 (4.0)	
Ad5-CI-O1C-2B	13	1	32	1	1	3.3×10^3 (1, 4)	0 (0, 0)
Ad5-CI-O1C-2B	14	1	64	0	0	3.2×10^1 (1, 4)	0 (0, 0)
Ad5-CI-O1C-2B	15	1	64	1	2	1.9×10^2 (2, 4)	0 (0, 0)
Ad5-CI-O1C-2B	16	1	64	0	0	4.0×10^4 (1, 5)	0 (0, 0)
Mean[e]			54	0.5	0.75	1.1×10^4 (4.3)	
Ad5-Blue	66	2	<8	5	5	2.5×10^4 (1, 5)	2.0×10^6 (1, 3)
Ad5-Blue	67	2	<8	5	5	7.0×10^4 (1, 6)	2.0×10^6 (1, 3)
Ad5-Blue	68	2	<8	5	5	3.6×10^6 (1, 6)	2.0×10^6 (1, 3)
Ad5-Blue	69	2	<8	5	5	1.3×10^5 (1, 5)	2.0×10^6 (1, 3)
Mean[e]			<8	5	5	9.6×10^5 (5.5)	
Ad5-O1C	70	2	128	0	0	1.8×10^1 (2, 1)	0 (0, 0)
Ad5-O1C	71	2	64	0	0	5.5×10^0 (1, 1)	0 (0, 0)
Ad5-O1C	72	2	64	1	3	7.5×10^3 (1, 5)	0 (0, 0)
Ad5-O1C	73	2	64	1	1	2.3×10^1 (1, 2)	0 (0, 0)
Mean[e]			76	0.5	1	2.0×10^2 (2.3)	
Ad5-O1C-2B	74	2	64	0	1	2.2×10^2 (1, 2)	0 (0, 0)
Ad5-O1C-2B	75	2	128	0	0	7.5×10^1 (1, 3)	0 (0, 0)
Ad5-O1C-2B	76	2	128	0	0	5.2×10^2 (2, 1)	0 (0, 0)
Ad5-O1C-2B	77	2	64	0	0	1.2×10^2 (1, 3)	0 (0, 0)
Mean[e]			90	0	0.25	2.2×10^2 (2.3)	

[a] Neutralizing antibody response reported as the inverse of the serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) at the day of challenge (21 dpv).
[b] The clinical score was determined by the number of toes with a lesion and the presence of lesions on the snout and tongue combined. The maximum score is 5, and lesions restricted to the site of challenge were not counted.
[c] Maximum viral titer measured as plaque forming units per ml fluid (pfu/ml) present in nasal swabs collected after challenge. The numbers within parenthesis represents the first day that virus was detected in nasal secretions and the duration (in days) of shedding, respectively.
[d] Maximum viral titer, pfu/ml, detected from whole blood samples collected after challenge. The numbers within parenthesis have the same meaning as described in footnote c.
[e] Mean of the PRN_{70} titer, clinical score, and maximum virus shedding as described in footnotes a, b and c. The numbers within parenthesis, in the virus shedding column, represents average duration of virus shedding (in days).

vaccinated group animal #4 only developed a single lesion detected at 10 dpc.

When we directly compared the efficacy of the Ad5-O1C and Ad5-O1C-2B vectors in experiment 2, there was an increase of protection in the Ad5-O1C-2B group, with only one animal, #74, with a single lesion that was first detected at 6 dpc, a 2–3 day delay compared with animals, #72 and 73, in the Ad5-O1C group that developed lesions at 3 or 4 dpc (Table 1). All vaccinated animals had a significant reduction in the amount of virus shed, approximately 3–4 logs, as compared with the Ad5-Blue group. Furthermore, the Ad5-O1C-2B inoculated animals had almost a log reduction at the peak of shedding, 1 and 2 dpc, as compared to the Ad5-O1C group (Fig. 4, Panel B). In addition, the duration of shedding on average was reduced by more than 3 days in the Ad5-FMD vaccinated animals as compared to the control group (Table 1).

3.4. T cell responses

We also examined T cell responses by analysis of IFNγ producing $CD4^+$ and $CD8^+$ T cells. In the first experiment we examined samples weekly prior to challenge and one day post-challenge only from the Ad5-Blue, Ad5-CI-O1C and Ad5-CI-O1C-2B inoculated groups. No significant differences could be detected in the number of IFNγ producing $CD4^+$ or $CD8^+$ T cells prior to challenge, however, there was a statistically significant increase in the number of IFNγ producing $CD8^+$ T cells one day after challenge in both Ad5-FMD vaccinated groups as compared to the control group (data not shown). Furthermore, at 1 dpc the Ad5-CI-O1C-2B vaccinated group had a statistically significantly higher IFNγ response in comparison to the Ad5-CI-O1C group (data not shown).

Based on these results, in the second experiment, we expanded the analysis of T cell responses by testing all animals weekly prior to challenge and at 1, 3, 5, 7, 14 and 21 dpc. Again, there were no differences among the groups prior to challenge or at 14 or 21 dpc for either $CD4^+$ or $CD8^+$ T cells (data not shown). However, we detected a statistically significant increase in the number of IFNγ-producing $CD4^+$ T cells at 1 dpc until 7 dpc in both vaccinated groups as compared with the control group (Fig. 5, Panel A). Furthermore, at 5 dpc the Ad5-O1C-2B inoculated group had a statistically

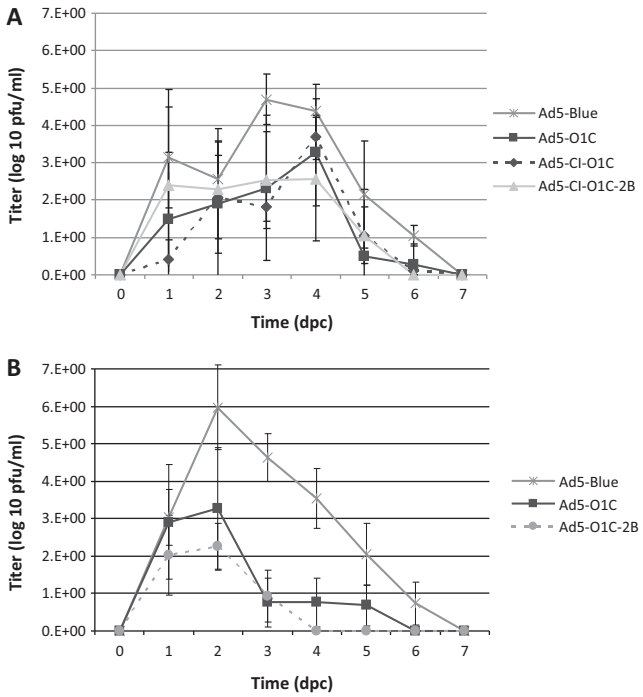

Fig. 4. Virus shedding in nasal swabs of bovines challenged with FMDV O1 Campos. Virus titers, pfu/ml, were determined by plaque assay on BHK-21 cells. Panels A and B represent the mean (±S.D.) of each group in experiments 1 and 2, respectively.

significant increase in the number of IFNγ-producing CD4$^+$ T cells as compared with Ad5-O1C group. We also observed a statistically significant increase in the CD8$^+$ T cell response in both the Ad5-O1C and Ad5-O1C-2B groups at 1, 3, 5 and 7 dpc compared with Ad5-Blue group (Fig. 5, Panel B). In addition, the animals receiving Ad5-O1C-2B had a statistically significant increase in the IFNγ CD8$^+$ T cell response at 3 and 5 dpc compared to the Ad5-O1C group.

4. Discussion

Adenovirus-vectored FMD vaccines are currently the most effective alternative to the commercially available inactivated vaccine [28–30,41–44]. These vaccines can protect both cattle and swine from clinical disease in 7 days, can be produced in FMD-free countries without the requirement of a biosecure facility, and have DIVA properties. One of the requirements for a commercially viable animal vaccine is the need to produce a low cost product. As a result we have been exploring approaches to enhance the potency of the Ad5-FMD vector. Recently, we demonstrated that a second-generation Ad5-A24 vaccine containing the full-length coding region of NS protein 2B, under control of a modified CMV promoter (pCI), has enhanced efficacy in swine as compared to our first generation vector that contained a truncated version of 2B [34].

FMDV serotype O is of particular concern to livestock producing countries since it is prevalent worldwide and has caused recent outbreaks in numerous countries of Asia including South Korea, Japan, China, and Taiwan as well as in Bulgaria in Eastern Europe [7,8,45]. Moreover, the inactivated serotype O vaccine requires 4–5 times greater antigenic payload than serotype A to be protective

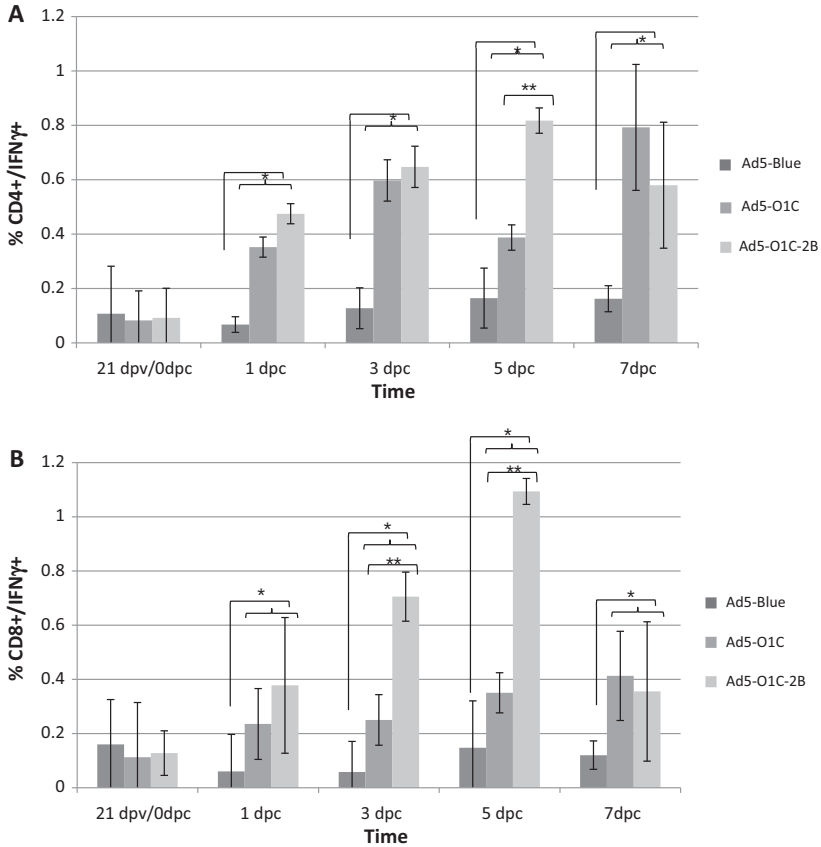

Fig. 5. Cell mediated immune response of bovines vaccinated with Ad5 vectors and challenged with FMDV O1 Campos. Panel A. Number of IFNγ-producing CD4$^+$ T cells in the vaccinated groups from experiment 2. Panel B. Number of IFNγ-producing CD8$^+$ T cells in the vaccinated groups from experiment 2. Error bars represent the standard deviation of the mean. *Statistically significant difference between Ad5-O1C and Ad5-O1C-2B vaccinated groups compared to the Ad5-Blue control group by Student's *t*-test ($p < 0.05$) and **statistically significant difference between the Ad5-O1C-2B and the Ad5-O1C vaccinated groups ($p < 0.05$).

[31,32]. Similarly an Ad5-O1C vaccine that we constructed only partially protected swine when used at a dose sufficient to confer full protection after vaccination with a comparable Ad5-A24 vaccine [29,30,33].

Here, we examined the ability of modified versions of this Ad5-O1C vaccine to induce protection in bovines, a natural host of FMDV. Based on our work with the Ad5-A24 vector we compared vaccines containing a partial or full-length 2B coding region under the control of either a CMV or a modified CMV promoter, pCI. The FMDV NS protein 2B is predicted to contain hydrophobic domains and we have previously found that the presence of full-length 2B in the Ad5-A24 vector resulted in significant proliferation of cytoplasmic vesicles in infected cells which become the sites of subsequent virus replication [34]. Bioinformatic analysis also suggests that the C-terminal region of 2B may be involved in membrane interaction. Furthermore, as mentioned above we have demonstrated the adjuvant effect of the addition of full-length 2B in an Ad5-A24 construct in swine [34].

In the first study in this report, we found that the groups inoculated with vaccine candidates containing a partial 2B with the CMV promoter or a full-length 2B with the pCI promoter had higher levels of FMDV-specific antibodies by RIP prior to challenge as compared to the Ad5-CI-O1C vaccinated group (Fig. 3, Panel C). However, levels of protection were the same among all the groups. As we and others have previously reported sometimes there is a lack of correlation between the levels of neutralizing antibodies and the clinical outcome [18,19]. Two of the animals vaccinated with Ad5-CI-O1C-2B (#14 and #16) had relatively low levels of neutralizing antibodies (1:64) at the day of challenge yet both were clinically protected, while #9 and #10 with the same neutralizing antibody titers developed disease and #4, vaccinated with Ad5-O1C, had the highest level of neutralizing antibodies (1:256) but developed disease although there was a considerable delay in its onset and reduced severity (10 dpc).

In a number of DNA vaccination studies it has been shown that the duration of antigen expression rather than initial exposure to antigen *in vivo* regulates the magnitude of the immune response [46–48]. It is possible that the high levels of expression of $3C^{pro}$, which is toxic to cells, in the animals vaccinated with the modified CMV promoter, limited the duration of exposure to antigen, overcoming the benefits of increased initial levels of expression of capsid proteins. Recently, we demonstrated that swine immunized with an Ad5-A24-vectored vaccine containing an inactive $3C^{pro}$ had increased FMDV-specific $CD8^+$ CTL killing of MHC-matched target cells as compared to swine immunized with vector containing wild-type $3C^{pro}$ [49].

The role of cell-mediated immunity in protection against FMDV infection is unclear. A number of groups have shown that FMDV infection suppresses various aspects of the cell-mediated immune response [50–57]. But demonstration of bovine T cell responses has been reported after FMDV infection or vaccination using assays detecting the secretion of IFNγ [23,58–60]. There are also reports of partial protection of animals vaccinated with Ad5-FMD vectors lacking detectable levels of neutralizing antibodies, suggesting that other mechanisms of protection are involved and might be responsible for the differences in protection observed in our study [25,61].

A number of studies have shown that Ad5 vectored vaccines induce higher levels of cell-mediated immune responses to the vaccine antigen as compared to vaccinia virus vectors or DNA immunization [62]. However, we could not detect a T cell response as measured by IFNγ producing $CD4^+$ or $CD8^+$ T cells prior to challenge in either experiment. But in a preliminary examination at 1 dpc in the first experiment there was a detectable $CD8^+$ T cell response in both Ad5-CI-O1C and Ad5-CI-O1C-2B vaccinated groups as compared to no response in the control group suggesting induction of memory by these vaccines. Furthermore, the IFNγ response in the Ad5-CI-O1C-2B group was statistically higher than the Ad5-CI-O1C group. In the second experiment, we expanded the analysis and also examined T cell responses after challenge. We demonstrated that either Ad5-O1C or Ad5-O1C-2B induced a statistically significant increase in IFNγ producing $CD4^+$ and $CD8^+$ T cells as early as 1 day after challenge which lasted until at least 7 dpc compared with control Ad5-Blue inoculated animals. Furthermore, animals receiving the vector with the full-length 2B protein again developed statistically significantly higher levels of both $CD4^+$ and $CD8^+$ T cells expressing IFNγ as compared to the Ad5-O1C vaccinated group at 5 dpc and at 3–5 dpc, respectively. These observations were associated with an improved clinical outcome of the animals from the Ad5-O1C-2B group. Three animals in this latter group never developed clinical disease and animal #74 presented a clinical score of only one at 6 dpc. The shedding of virus was reduced in both vaccinated groups with a further reduction in magnitude and length in the Ad5-O1C-2B group as compared to Ad5-O1C vaccinated animals (Fig. 4, Panel B and Table 1). The mechanisms of protection mediated by $CD8^+$ and $CD4^+$ T cells include direct cytotoxicity and induction and secretion of IFNγ. We and others have shown that IFNγ has antiviral activity against FMDV [38,63]. Thus, the increased production of IFNγ in animals vaccinated with Ad5-O1C-2B and challenged, as well as additional T cell effector mechanisms, may play a role in the enhanced protection induced in these animals.

Here we demonstrated that expression of the full-length FMDV 2B protein induced an increase in transgene expression independent of the version of the CMV promoter used, but the overall increase in efficacy of these vaccines is not dependent on the initial level of expression demonstrated *in vitro* or only on the level of neutralizing antibodies induced in response to vaccination. Our results demonstrate that the Ad5-O1C vaccines also induce a memory T cell response. Furthermore, the Ad5-O1C vector containing the full-length 2B induces an enhanced memory T cell response as compared to the vector containing only a partial 2B coding region [20–22,64]. These results suggest that a more comprehensive understanding of the mechanisms of induction of efficient humoral and cell-mediated immune responses should aid in the development of a more potent Ad5-FMD vaccine.

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ORIGINAL PAPER

Porcine major histocompatibility complex (MHC) class I molecules and analysis of their peptide-binding specificities

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Abstract In all vertebrate animals, CD8$^+$ cytotoxic T lymphocytes (CTLs) are controlled by major histocompatibility complex class I (MHC-I) molecules. These are highly polymorphic peptide receptors selecting and presenting endogenously derived epitopes to circulating CTLs. The polymorphism of the MHC effectively individualizes the immune response of each member of the species. We have recently developed efficient methods to generate recombinant human MHC-I (also known as human leukocyte antigen class I, HLA-I) molecules, accompanying peptide-binding assays and predictors, and HLA tetramers for specific CTL staining and manipulation. This has enabled a complete mapping of all HLA-I specificities ("the Human MHC Project"). Here, we demonstrate that these approaches can be applied to other species. We systematically transferred domains of the frequently expressed swine MHC-I molecule, SLA-1*0401, onto a HLA-I molecule (HLA-A*11:01), thereby generating recombinant human/swine chimeric MHC-I molecules as well as the intact SLA-1*0401 molecule. Biochemical peptide-binding assays and positional scanning combinatorial peptide libraries were used to analyze the peptide-binding motifs of these molecules. A pan-specific predictor of peptide–MHC-I binding, *NetMHCpan*, which was originally developed to cover the binding specificities of all known HLA-I molecules, was successfully used to predict the specificities of the SLA-1*0401 molecule as well as the porcine/human chimeric MHC-I molecules. These data indicate that it is possible to extend the biochemical and bioinformatics tools of the Human MHC Project to other vertebrate species.

Keywords Recombinant MHC · Peptide specificity · Binding predictions

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Introduction

Major histocompatibility complex class I (MHC-I) molecules are found in all vertebrate animals where they play a crucial role in generating specific cellular immune responses against viruses and other intracellular pathogens. They are highly polymorphic proteins that bind 8–11 amino acid long peptides derived from the intracellular protein metabolism. The resulting heterotrimeric complexes—consisting of the MHC-I heavy chain, the monomorphic light chain, beta-2 microglobulin (β_2m), and specifically bound peptides—are translocated to the cell surface where they displayed as target structures for peptide-specific, MHC-I-restricted CTLs. If a peptide of foreign origin is detected, the T cells may become activated and kill the infected target cell.

MHC-I is extremely polymorphic. In humans, more than 3,400 different human leukocyte antigen class I (HLA-I) molecules have been registered (as of January 2011), and this number is currently growing rapidly as more efficient HLA typing techniques are employed worldwide. The polymorphism of the MHC-I molecule is concentrated in and around the peptide-binding groove, where it determines the peptide-binding specificity. Due to this polymorphism, it is highly unlikely that any two individuals will share the same set of HLA-I molecules thereby presenting the same peptides and generating T cell responses of the same specificities—something, that otherwise would give microorganisms a strong evolutionary chance of escape. Rather, this polymorphism can be seen as diversifying peptide presentation thereby individualizing T cell responses and reducing the risk that escape variants of microorganisms might evolve.

In 1999, we proposed that all human MHC specificities should be mapped ("the Human MHC Project") as a preamble for the application of MHC information and technologies in humans (Buus 1999). Since then, we have developed large-scale tools that are generally applicable towards this goal: production, analysis, prediction and validation of peptide–MHC interactions (Ferre et al. 2003; Harndahl et al. 2009; Hoof et al. 2009; Larsen et al. 2005; Lundegaard et al. 2008; Nielsen et al. 2003, 2007; Ostergaard et al. 2001; Pedersen et al. 1995; Stranzl et al. 2010; Stryhn et al. 1996), and a "one-pot, read-and-mix" HLA-I tetramer technology for specific T cell analysis (Leisner et al. 2008). Here, we demonstrate that many of these tools can be transferred to other vertebrate animals as exemplified by an important livestock animal, the pig. We have successfully generated a recombinant swine leukocyte antigen I (SLA-I) protein, SLA-1*0401, one of the most common SLA molecules of swine (Smith et al. 2005). Using this protein, we have developed the accompanying biochemical peptide-binding assays and demonstrated that the immunoinformatics tools originally developed to cover all HLA-I molecules, despite the evolutionary distance, can be applied to SLA-I molecules. We suggest that the "human MHC project" can be extended to cover other species of interest.

Materials and methods

Peptides and peptide libraries

All peptides were purchased from Schafer-N, Denmark (www.schafer-n.com). Briefly, they were synthesized by standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, purified by reversed-phase high-performance liquid chromatography (to at least >80% purity, frequently 95–99% purity), validated by mass spectrometry, and quantitated by weight.

Positional scanning combinatorial peptide libraries (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 amino acids (excluding cysteine) was prepared for each synthesis and used for coupling in 8 positions, whereas a single type of Fmoc amino acid (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 amino acid pool was used in all nine positions. The library therefore consisted of $20\times 9+1=181$ individual peptide libraries:

- Twenty PSCPL sublibraries describing position 1: AX_8, CX_8, DX_8, …. YX_8
- Twenty PSCPL sublibraries describing position 2: XAX_7, XCX_7, XDX_7, …. XYX_7
- etc
- Twenty 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 amino acids from the mixture, whereas the single letter amino acid abbreviation is used to denote identity of the fixed amino acid.

The peptides in each synthesis were cleaved from the resin in trifluoroacetic acid/1,2-ethanedithiol/triisopropylsilane/water 95:2:1:3 *v*/*v*/*v*/*v*, precipitated in cold diethylether, and extracted with water before desalting on C18 columns, freeze drying, and weighting.

Recombinant constructs encoding chimeric and SLA-1*0401 molecules

A synthetic gene encoding a transmembrane-truncated fragment encompassing residues 1 to 275 of human HLA-A*11:01 alpha chain followed by a FXa–BSP–HAT tag (FXa = factor Xa cleavage site comprised of the amino acid sequence IEGR, BSP = biotinylation signal peptide, HAT = histidine affinity tag for purification purposes; see Online Resource 1) had previously been generated and inserted into the pET28 expression plasmid (Novagen) (Ferre et al. 2003). Synthetic genes encoding the corresponding fragments of the SLA-1*0401 alpha chain ($\alpha_1\alpha_2$) and α_3, respectively, (Sullivan et al. 1997) were purchased from GenScript. To exchange domains and generate chimeric human/swine MHC-I gene constructs, a type II restriction endonuclease-based cloning strategy (SeamLess® Strategene; Cat#214400, Revision#021003a), with modifications, was used. All primers were purchased HPLC-purified from Eurofins MWG Operon (Ebersberg, Germany), and all PCR amplifications were performed in a DNA Engine Dyad

PCR instrument (MJ Research, MN, USA). All constructs were validated by DNA sequencing. The following MHC-I heavy chain constructs were made HHH, HHP, HPP, PHP, and PPP, where the first, second, and third letter indicates domains α_1 (positions 1–90), α_2 (positions 91–181), and α_3 (positions 182–275), respectively, and H indicates that the domain is of HLA-A*11:01 origin, whereas P indicates that it is of SLA-1*0401 origin.

Constructs were transformed into DH5α cells, cloned, and sequenced (ABI Prism 3100Avant, Applied Biosystems) (Hansen et al. 2001). Validated constructs of interest were transformed into an *Escherichia coli* production cell line, BL21(DE3), containing the pACYC184 expression plasmid (Avidity, Denver, USA) containing an isopropyl-β-d-1-thiogalactopyranoside (IPTG)-inducible BirA gene to express biotin-ligase. This leads to almost complete in vivo biotinylation of the desired product (Leisner et al. 2008).

Expression of recombinant proteins

To maintain the pET28-derived plasmids, the media was supplemented with kanamycin (50 μg/ml) throughout the expression cultures. When appropriate, the media was further supplemented with chloroamphenicol (20 μg/ml) to maintain the BirA containing pACYC184 plasmid. *E. coli* BL21(DE3) cells transformed with appropriate plasmids were grown for 5 h at 30°C, and a 10-ml sample adjusted to $OD_{(600)}=1$ was then transferred to a 2-l fed-batch fermentor (LabFors®). To induce protein expression, IPTG (1 mM) was added at $OD_{(600)}\sim25$ and the culture was continued for an additional 3 h at 42°C (for in vivo biotinylation of the product, the induction media was further supplemented with biotin (Sigma #B4501, 125 μg/ml)). Samples were analyzed by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before and after IPTG induction. At the end of the induction culture, protease inhibitor (PMSF, 80 μg/l) was added, and cells were lysed in a cell disrupter (Constant Cell Disruptor Systems set at 2,300 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), and 0.1% deoxycholic acid (Sigma) and extracted into urea–Tris buffer (8 M urea, 25 mM Tris, pH 8.0), and any contaminating DNA was precipitated with streptomycin sulfate (1% *w*/*v*).

MHC class I heavy chain purification

The dissolved MHC-I proteins were purified by Ni^2+/IDA metal chelating affinity column chromatography followed by Q-Sepharose ion exchange column chromatography, hydrophobic interaction chromatography, and eventually by Superdex-200 size exclusion chromatography. Fractions containing MHC-I heavy chain molecules were identified by A280 absorbance and SDS-PAGE and pooled. Throughout purification and storage, the MHC-I heavy chain proteins were dissolved in 8 M urea to keep them denatured. Note that the MHC-I heavy chain proteins at no time were exposed to reducing conditions. This allowed purification of highly active pre-oxidized moieties as previously described (Ostergaard et al. 2001). Protein concentrations were determined by bicinchoninic acid assay. The degree of biotinylation (usually >95%) was determined by a gel-shift assay (Leisner et al. 2008). The pre-oxidized, denatured proteins were stored at −20°C in Tris-buffered 8 M urea.

Recombinant constructs encoding human and porcine beta-2 microglobulin

Recombinant human β_2m was expressed and purified as described elsewhere (Ostergaard et al. 2001), (Ferre et al. 2003). Using a previously reported *E. coli* codon-optimized gene encoding human β_2m as template (Pedersen et al. 1995), a gene encoding porcine β_2m was generated by multiple rounds of site-directed mutagenesis (QuikChange® Stratagene, according to the manufacturer's instructions) (Online Resource 2). Briefly, the genes encoding human or pig β_2m were N-terminally fused to a histidine affinity encoding tag (HAT) followed by a restriction enzyme encoding tag (FXa), inserted into the pET28 vector and expressed in inclusion bodies in *E. coli*. The fusion proteins were extracted into 8 M urea, purified by immobilized metal affinity chromatography (IMAC), and refolded by dilution. The fusion tags were then removed by FXa restriction protease digestion. The liberated intact and native human or pig β_2m were purified by IMAC and gel filtration chromatography, analyzed by SDS-PAGE analysis, concentrated, and stored at −20°C until use (Fig. 1).

Purification and refolding of recombinant porcine β_2m proteins

Porcine β_2m was purified in the same way as human β_2m (Ostergaard et al. 2001; Ferre et al. 2003). Briefly, the urea-dissolved β_2m protein was purified by Ni^2+/IDA metal chelating affinity column chromatography, refolded by drop-wise dilution into an excess refolding buffer under stirring (25 mM Tris, 300 mM urea, pH 8.00), and then concentrated (VivaFlow, 10 kDa). The refolded product was purified by Ni^2+/IDA metal chelating affinity column chromatography again (this time in aqueous buffer, i.e., without urea). Fractions containing HAT-pβ_2m were iden-

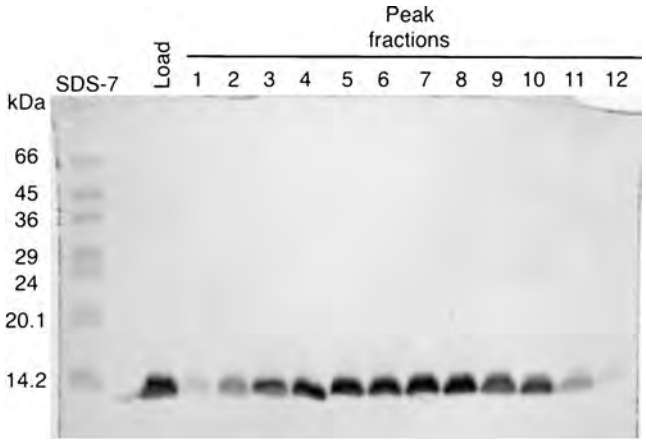

Fig. 1 SDS-PAGE analysis of peak fractions from size exclusion chromatography of porcine β_2m (11.4 kDa) after removal of histidine affinity tag (HAT) by FXa protease digestion. Samples were mixed 1:1 in non-reducing SDS sample buffer and loaded onto a 15% polyacrylamide gel

tified by SDS-PAGE and pooled. Removal of the HAT tag was performed by cleavage with factor Xa restriction protease (FXa) followed by renewed purified by Ni^{2}+/IDA metal chelating affinity and Superdex200 gel filtration column chromatography, concentrated by spin ultrafiltration (10 kDa), mixed 1:1 with glycerol, and stored at −20°C.

SDS-PAGE analysis

Protein samples were mixed 1:1 in SDS sample buffer (4% SDS, 17.4% glycerol, 0.003% bromophenol blue, 0.125 M Tris, 8 mM IAA (iodoacetamide)) with or without reducing agent (2-mercaptoethanol) as indicated, boiled for 3 min, spun at 20,000×*g* for 1 min, and loaded onto a 12% or 15% running gel with a 5% stacking gel. Gels were run at 180 V, 40 mA for 50 min.

Peptide–MHC class I interaction measured by radioassay and spun column chromatography

A HLA-A*11:01-binding peptide, KVFPYALINK (non-natural consensus sequence A3CON1 (Sette et al. 1994)), was radiolabeled with iodine (^{125}I) using a chloramine-T procedure (Hunter and Greenwood 1962). Dose titrations of MHC-I heavy chains (HHH or HHP) were diluted into refolding buffer (Tris–maleate–PBS) and mixed with β_2m (human or porcine) and radiolabeled peptide, and incubated at 18°C overnight. Then binding of radiolabeled peptide to MHC-I was determined in duplicate by Sephadex™ G50 spun column gel chromatography as previously described (Buus et al. 1995). MHC bound peptide eluted in the excluded volume, whereas free peptide was retained on the microcolumn. Both fractions were counted by gamma spectroscopy, and the fraction peptide bound was calculated as excluded radioactivity divided by total radioactivity.

To examine the affinity of the interaction, increasing concentrations of unlabeled competitor peptide were added. When conducted under limiting concentrations of MHC-I molecule, the concentration of competitor peptide needed to effect 50% inhibition of the interaction, the IC_{50}, is an approximation of the affinity of the interaction between MHC-I and the competitor peptide.

Peptide–MHC class I interaction measured by an enzyme-linked immunosorbent assay

Peptide–MHC-I interaction was also measured in a modified version of a previously described enzyme-linked immunosorbent assay (ELISA) (Sylvester-Hvid et al. 2002). Briefly, denatured biotinylated recombinant MHC-I heavy chains were diluted into a renaturation buffer containing β_2m and graded concentrations of the peptide to be tested and incubated at 18°C for 48 h allowing equilibrium to be reached. We have previously demonstrated that denatured MHC molecules can de novo fold efficiently, however, only in the presence of appropriate peptide. The concentration of peptide–MHC complexes generated was measured in a quantitative sandwich ELISA (using streptavidin as capture layer and the monoclonal anti-β_2m antibody, BBM1, as detection layer) and plotted against the concentration of peptide offered (Sylvester-Hvid et al. 2002). A prefolded, biotinylated FLPSDYFPSV/HLA-A*02:01 (Kast et al. 1994) complex was used as standard. Because the effective concentration of MHC (3–5 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.

Using peptide libraries to perform an unbiased analysis of MHC specificity

The experimental strategy of PSCPL has previously been described (Stryhn et al. 1996). The construction of the sublibraries and the ELISA-driven quantitative measurements of MHC interaction are as given above.

Briefly, the relative binding (RB) affinity of each PSCPL sublibrary was determined as RB (PSCPL)=ED_{50} (X_9)/ED_{50} (PSCPL) (where ED_{50} is the concentration needed to half-saturate a low concentration of MHC-I molecules) and normalized so that the sum of the RB values of the 20 naturally occurring amino acids equals 20 (since peptides with a given amino acid in a given position are 20 times more frequent in the corresponding PSCPL sublibrary than

in the completely random X_9 library). A RB value above 2 was considered as the corresponding position and amino acid being favored, whereas a RB value below 0.5 was considered as being unfavorable (these thresholds represent the 95% confidence intervals). An anchor position (AP) value was calculated by the equation $\sum(RB-1)^2$. A primary anchor position is characterized by one or few amino acids being strongly preferred and many amino acids being unacceptable. We have arbitrarily defined anchor residues as having an AP value above 15 (Lamberth et al. 2008). The peptide–SLA-I*0401 binding activity of each sublibrary was determined using previously published biochemical binding assay (ELISA) (Sylvester-Hvid et al. 2002) (with the modifications described above).

Sequence logos

Sequences logos describing the predicted binding motif for each MHC molecule were calculated as described by Rapin et al. (2010). In short, the binding affinity for a set of 1,000,000 random natural 9mer peptides was predicted using the *NetMHCpan* method, and the 1% strongest binding peptides were selected for construction of a position-specific scoring matrix (PSSM). The PSSM was constructed as previously described (Nielsen et al. 2004) including pseudo-count correction for low counts. Next, sequence logos were generated from the amino acid frequencies identified in the PSSM construction. For each position, the frequency of all 20 amino acids is displayed as a stack of letters. The total height of the stack represents the sequence conservation (the information content), while the individual height of the symbols relates to the relative frequency of that particular symbol at that position. Letter shown upside-down are underrepresented compared to the background (for details see Rapin et al. (2010)).

MHC distance trees

MHC distance trees were derived from correlations between predicted binding affinities. For each allelic MHC-I molecule, the binding affinity was predicted for a set of 200,000 random natural peptides using the *NetMHCpan* method. Next, the distance between any two alleles was defined, as $D=1-PCC$, where PCC is the Pearson correlation between the subset of peptides within the superset of top 10% best binding peptides for each allele. In this measure, two molecules that share a similar binding specificity will have a distance close to 0 whereas two molecules with non-overlapping binding specificities would have a distance close to 2. Using bootstrap, 100 such distance trees were generated, and branch bootstrap values and the consensus tree were calculated.

Results

Generation of chimeric MHC class I molecules

We have previously generated highly active, recombinant human MHC-I (HLA-I) molecules and accompanying high-throughput assays and bioinformatics prediction resources. Here, we transfer the underlying approaches to an important domesticated livestock animal, the pig, and its MHC system, the SLAs. MHC-I molecules are composed of a unique and highly variable distal peptide-binding platform consisting of the alpha 1 (α_1) and alpha 2 (α_2) domains of the MHC-I heavy chain (HC) and a much more conserved proximal immunoglobulin-like membrane attaching stalk consisting of the alpha 3 (α_3) domain of the HC non-covalently associated with the soluble MHC-I light chain (β_2m).

A priori, the establishment of recombinant SLA molecules is complicated by the lack of validated reagents. Any failure could therefore be caused either by real technical problems in generating SLA molecules, or merely by a lack of information about strong peptide binders to the SLA in question. To reduce this uncertainty, we decided to migrate from human to pig MHC-I in a step-wise manner and generate an intermediary chimeric MHC-I molecule composed of a well-known human peptide-binding platform attached to a SLA stalk, which might allow us to assess whether we could generate a functional SLA stalk consisting of SLA-1*0401 α_3 HC and pig β_2m. To this end, we used the $\alpha_1\alpha_2$ domains of the HLA-A*11:01 molecule, which we expected should be able to bind a known high-affinity HLA-A*11:01-binding peptide (KVFPYALINK). This peptide could be ^{125}I radiolabeled and used in a very robust peptide-binding assay testing whether the human stalk could be replaced with the corresponding SLA stalk. Once that had been successfully established, the entire SLA-1*0401 molecule would be constructed and tested.

We have previously expressed and purified the extracellular segment spanning positions 1–275 of the human HLA-A*11:01 in a denatured and pre-oxidized version that rapidly refold and bind appropriate target peptides (Ostergaard et al. 2001; Ferre et al. 2003). Codon-optimized genes encoding the corresponding segments of SLA-1*0401 ($\alpha_1\alpha_2$) and SLA-1*0401 (α_3) were constructed as described in the "Materials and methods" section and used to replace the HLA-A*11:01 gene segment in the above construct generating a new construct allowing for the expression of SLA-1*0401. For the generation of HLA-A*11:01/SLA-1*0401 chimeras, the genes encoding α_1 (spanning positions 1–90), α_2 (spanning positions 91–181), and/or α_3 (spanning positions 182–275) domains of HLA-A*11:01 and SLA-1*0401 were exchanged using Seam-Less and touch-down cloning strategies. Genes encoding

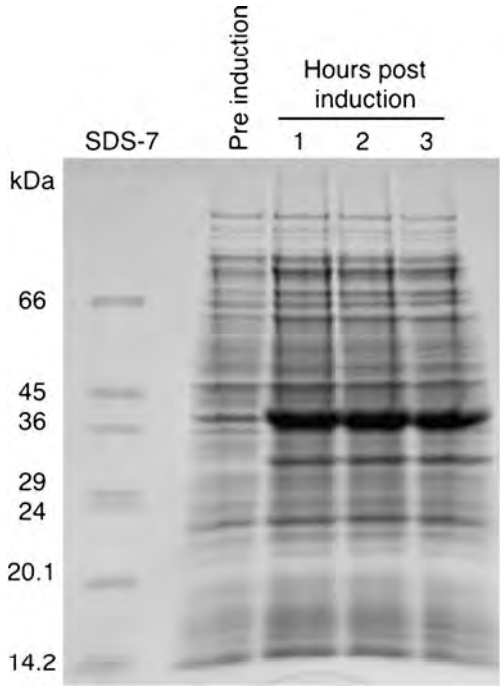

Fig. 2 SDS-PAGE analysis of cell lysates of SLA-1*0401 expression in *E. coli* before and after induction with IPTG. Samples were mixed 1:1 in a reducing SDS sample buffer and loaded onto a 12% polyacrylamide gel

the extracellular segments 1–275 of the above natural or chimeric MHC-I molecules were C-terminally fused to a biotinylation tag (as indicated for SLA-1*0401 in Online Resource 1), inserted into pET28, and expressed in inclusion bodies in *E. coli* (Fig. 2 shows SDS-PAGE of lysates of recombinant *E. coli* before and 3 h after IPTG induction). The fusion proteins were extracted into 8 M urea (without any reducing agents), purified by ion exchange, hydrophobic and gel filtration chromatography (all conducted in 8 M urea, without any reducing agents) (Fig. 3 shows SDS-PAGE of the purified SLA-1*0401 after gel filtration), concentrated, and stored in urea at −20°C.

Testing a chimeric molecule consisting of a SLA-1*0401 stalk and a HLA-A*11:01 peptide-binding platform—comparing human versus porcine β_2m

To test the proximal immunoglobulin-like membrane attaching SLA stalk, we generated recombinant porcine β_2m and a chimeric human/porcine MHC-I heavy chain molecule where the $\alpha_1\alpha_2$ were derived from the human HLA-A*11:01, and the α_3 was derived from the porcine SLA-1*0401. Since this construct contains the entire peptide-binding platform of HLA-A*11:01, we reasoned that the binding of the HLA-A*11:01 restricted peptide, KVFPYALINK, could be used as a functional readout of the refolding, activity, and assembly of the entire chimeric molecule including the porcine SLA stalk. For comparison, we tested the supportive capacity of human β_2m and folding ability of the entirely human HLA-A*11:01. A total of four combinations could therefore be tested: porcine or human β_2m in combination with either HHP or HHH (where the first letter indicates the origin of the α_1 domain (*H*uman HLA-A*11:01 or *P*orcine SLA-1*0401), the second letter the origin of the α_2 domain, and the third letter the origin of the α_3 domain). A concentration–titration of heavy chain was added to a fixed excess concentration (3 μM) of β_2m and a fixed trace concentration (23 nM) of radiolabeled peptide. As shown in Figs. 4 and 5, the four combinations gave almost the same heavy chain dose titration with a half-saturation occurring around 1–2 nM heavy chain. Porcine β_2m supported folding of the chimeric (HHP) α chain slightly better than it supported folding of the human (HHH) α chain. Human β_2m

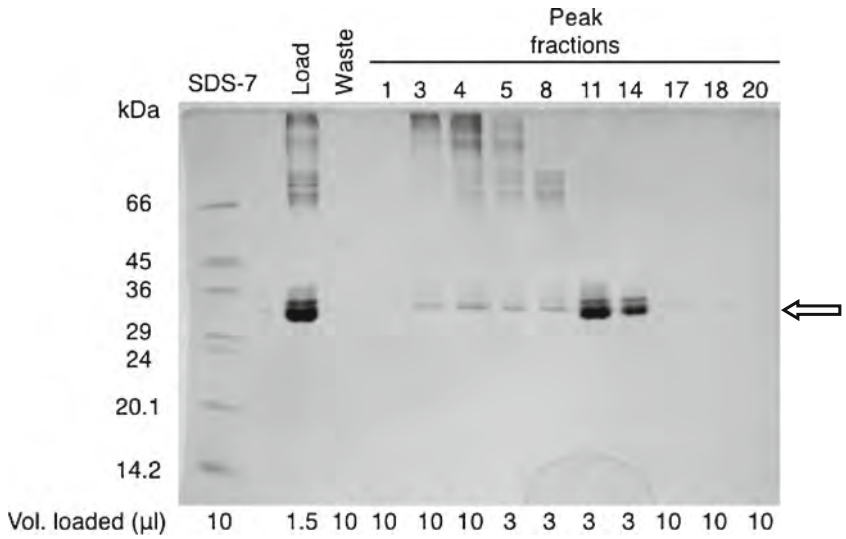

Fig. 3 SDS-PAGE analysis of size exclusion chromatography peak fractions from SLA-1*0401 purification. Fraction numbers are shown above each lane. Samples were mixed 1:1 in non-reducing SDS sample buffer and loaded onto a 12% polyacrylamide gel. Lanes were loaded with different volumes, indicated below each lane, to avoid overloading. An *arrow* indicates the band representing purified SLA-1*0401-BSP-HAT heavy chain (36,675 Da)

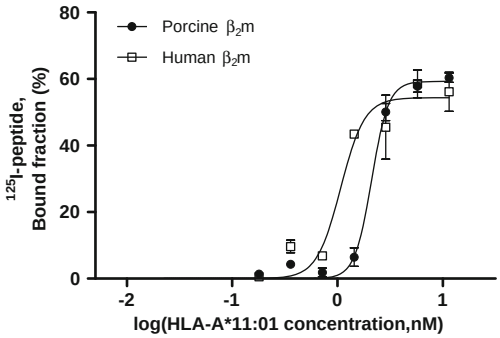

Fig. 4 HLA-A*11:01 complex formation with a known HLA-A*11:01-binding peptide (KVFPYALINK) using either human (*open squares*) or porcine (*filled circles*) β_2m. The affinity of HLA-A*11:01 to porcine and human β_2m was determined as 2.11 nM (95% confidence interval, 1.96 to 2.27) and 1.07 nM (95% confidence interval, 0.871 to 1.32), respectively

supported folding of HHP and HHH equally well. Thus, a recombinant SLA stalk can fold and support peptide binding of the peptide-binding platform. These results also suggest that human β_2m can support folding and peptide binding of porcine MHC-I heavy chain molecules.

Using a positional scanning combinatorial peptide library approach to perform an unbiased analysis of the specificity of SLA-1*0401 and human–pig chimeric MHC class I molecules

Using human β_2m to support folding, the recombinant SLA-1*0401 and human–pig chimeric MHC-I molecule were tested for peptide binding. We have previously described how PSCPL can be used to perform an unbiased analysis of MHC-I molecules (Stryhn et al. 1996). A PSCPL consists of 20 sublibraries for each position where one of each of the 20 natural amino acids have been locked and all other positions contain random amino acids. Analyzing how much of each PSCPL sublibrary is needed to support MHC-I folding (see examples in Fig. 6) and comparing each sublibrary with a completely random library, the effect of any amino acid in any position can be examined and expressed as a RB value. Further, an AP value calculated as the sum of squared deviations of RB values for each position can be used to identify the most prominent anchor position (see "Materials and methods" for calculations). Thus, the specificity of a nonamer binding MHC-I molecule can be analyzed comprehensively with 9×20+1 completely random library=181 sublibraries (Stryhn et al. 1996).

Here, this approach was used to perform a complete experimental analysis of SLA-1*0401 and a limited analysis of the chimeric HPP and PHP molecules. A nonamer PSCPL analysis of SLA-1*0401 can be seen in Table 1. AP values identified positions 9, 3, and 2 (in that order of importance) as the anchor positions of SLA-1*0401. In position 9, the amino acid preferences were dominated by the large and bulky aromatic tyrosine (Y), tryptophane (W), and phenylalanine (F), all having RB values above 4 (Table 1). In the almost equally important position 3, preferences for negatively charged amino acids glutamic acid (E) and aspartic acid (D) were observed. In the lesser important position 2, the most preferred amino acids were the hydrophobic amino acids valine (V), isoleucine (I), and leucine (L), followed by the polar amino acids threonine (T) and serine (S).

Finally, a very limited PSCPL analysis was performed for the two chimeric human HLA-A*11:01/porcine SLA-1*0401 MHC-I molecules, HPP and PHP (Table 2). For both chimeric molecules, it could be demonstrated that position 9 is a strong anchor position. The positively charged amino acids, arginine (R) and lysine (K), were preferred in position 9 of the chimeric HPP molecule, whereas the aromatic amino acid, tyrosine (Y), was exclusively preferred in position 9 of the chimeric PHP molecule.

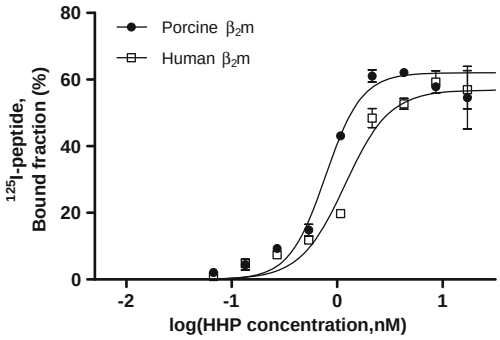

Fig. 5 MHC-I complex formation of the chimeric class I molecule HHP (HLA-A*11:01 ($\alpha_1\alpha_2$), SLA-1*0401 (α_3)) with a known HLA-A*11:01-binding peptide (KVFPYALINK), and human (*open squares*) versus porcine β_2m (*filled circles*). The affinity of HHP to porcine and human β_2m was determined as 0.774 nM (95% confidence interval, 0.630 to 0.951) and 1.19 nM (95% confidence interval, 0.958 to 1.47), respectively

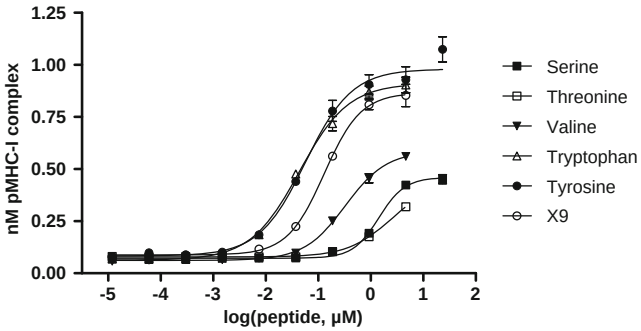

Fig. 6 PSCPL position 9 sublibrary analysis of the SLA-1*0401 peptide-binding motif. The amino acids valine, serine, and threonine are disfavored in position 9, evident by a decrease in affinity compared to the reference peptide (X9), whereas the large and bulky amino acids tryptophan and tyrosine are favored as seen by an increase in affinity compared to the reference peptide

Table 1 Binding motif of the SLA-1*0401 allele determined by PSCPL strategy

SLA-1*0401	Amino acid position in peptide								
	1	2	3	4	5	6	7	8	9
A	1.7	0.8	1.6	1.1	0.8	*0.4*	1.7	0.7	*0.0*
C	0.8	*0.2*	*0.4*	**2.7**	1.1	1.0	0.7	1.7	*0.0*
D	*0.4*	0.7	**7.7**	**2.1**	0.8	*0.1*	0.5	1.8	*0.0*
E	1.6	*0.0*	**2.8**	0.6	1.0	1.4	0.9	0.9	*0.2*
F	*0.4*	*0.2*	0.8	*0.0*	1.0	1.1	1.1	1.1	**5.8**
G	0.5	*0.3*	*0.1*	1.5	1.1	1.4	*0.0*	1.0	*0.0*
H	0.5	*0.2*	*0.1*	*0.1*	1.3	**2.4**	0.5	0.7	*0.4*
I	0.7	**3.4**	*0.3*	0.8	0.8	0.9	0.7	0.9	*0.3*
K	1.1	*0.2*	*0.1*	*0.4*	*0.4*	1.2	0.4	0.9	*0.0*
L	0.6	**2.1**	*0.2*	0.6	1.0	1.7	1.0	1.2	0.6
M	1.7	1.9	1.0	1.2	1.4	1.4	1.2	1.4	0.7
N	1.4	0.5	0.6	1.4	1.9	0.7	1.0	1.9	*0.0*
P	*0.0*	*0.0*	*0.1*	1.6	0.5	1.1	1.9	1.2	1.1
Q	1.0	0.9	*0.2*	1.0	0.5	*0.4*	0.8	0.7	*0.0*
R	**2.3**	*0.0*	*0.1*	0.6	**2.4**	0.9	1.0	0.5	*0.2*
S	1.8	**2.4**	1.8	1.1	0.8	1.5	1.6	0.7	*0.0*
T	1.4	**2.5**	1.6	0.7	0.9	0.5	2.0	0.7	*0.0*
V	1.1	**3.7**	*0.3*	0.5	0.7	0.8	0.9	0.6	0.8
W	*0.3*	*0.0*	*0.2*	0.6	1.0	0.8	0.6	1.4	**5.6**
Y	0.6	*0.0*	*0.5*	1.3	0.6	*0.2*	1.6	*0.3*	**4.3**
Sum	20	20	20	20	20	20	20	20	20
AP value	7	28	57	8	4	6	5	4	67

The normalized relative binding (RB) value indicates whether an amino acid is favored (RB>2, bold numbers) or disfavored (RB<0.5, italic numbers) in a given peptide position. The anchor position (AP) value is given by the equation $\sum(RB-1)^2$. The important anchor positions 2, 3, and 9 for SLA-1*0401 are underlined

The positively charged amino acids, arginine (R) and lysine (K), were preferred in position 9 of the chimeric HPP molecule similar to the position 9 specificity of the HLA-A*11:01 molecule. In contrast, the aromatic amino acid tyrosine (Y) was preferred in position 9 of the chimeric PHP molecule similar to the position 9 specificity of the SLA-1*0401 molecule.

Using *NetMHCpan* to predict peptides that bind to SLA-1*0401 or to human–pig chimeric MHC class I molecules

Our recently described neural network-driven bioinformatics predictor, *NetMHCpan* (version 2.0), has been trained on about 88,000 peptide-binding data points representing more than 80 different MHC-I molecules (primarily HLA-A and HLA-B molecules). We have previously shown that *NetMHCpan* is an efficient tool to identify peptides that bind to HLA molecules where no prior data exist (Nielsen et al. 2007) and demonstrated that *NetMHCpan* can be extended to MHC-I molecules of other species[1] (Hoof et al. 2009). We applied *NetMHCpan* to our peptide repository of about 10,000 peptides, which over the past decade have been selected to scan infectious agents (e.g., SARS and influenza, Sylvester-Hvid et al. 2004; Wang et al. 2010), improve coverage of MHC-I specificities (e.g., Buus et al. 2003; Christensen et al. 2003), etc. We extracted 29 peptides as predicted binders to either the SLA-1*0401, the HPP, or the PHP human/porcine chimeric class I molecules (some of the peptides were predicted to bind to two or even all three of these molecules). All these peptide–MHC-I combinations were tested for binding (Table 3); 13 of 14 peptides bound to the SLA-1*0401 molecule with an affinity (IC_{50} value) better than 500 nM (6 with an affinity less than 50 nM); all 13 peptides tested on the PHP molecule were strong binders with IC_{50} values below 50 nM; and 3 of 12 peptides tested on the HPP molecule bound with an affinity better than 500 nM. Of the 39 peptide–MHC-I combinations tested, 20 (51%) were found to be good binders, 9 (23%) were average binders, and 10 (26%) did not bind well (Table 3). This is in stark contrast to the 0.5% frequency of binders among randomly selected peptides (Yewdell and Bennink 1999).

Next, the *NetMHCpan* method was used to generate PSSMs and sequence logos from the corresponding amino acid frequencies as described by Nielsen et al. (2004). For each position, the frequencies of all 20 amino acids were displayed as a stack of letters showing the sequence conservation/information content (the height of the entire

[1] A preliminary report of SLA-1*0401 binding was given in Hoof et al. (2009).

Table 2 Comparison of PSCPL derived binding motifs for the three MHC-I heavy chains HPP, PHP, and PPP ($h\alpha_1 p\alpha_2 p\alpha_3$, $p\alpha_1 h\alpha_2 p\alpha_3$, and SLA-I*0401) regarding peptide positions 2 and 9

Molecule: HPP Position in peptide			Molecule: PPP Position in peptide			Molecule: PHP Position in peptide		
Amino acid	2	9	Amino acid	2	9	Amino acid	2	9
A	*0.3*	*0.4*	A	0.8	*0.0*	A	1.0	*0.0*
C	*0.2*	*0.4*	C	*0.2*	*0.0*	C	*0.0*	*0.0*
D	*0.2*	*0.4*	D	0.7	*0.0*	D	*0.0*	*0.0*
E	*0.2*	*0.4*	E	0.0	*0.2*	E	*0.0*	*0.0*
F	*0.0*	*0.4*	F	*0.2*	**5.8**	F	*0.1*	0.5
G	*0.2*	*0.4*	G	*0.3*	*0.0*	G	*0.4*	*0.0*
H	*0.2*	*0.4*	H	*0.2*	*0.4*	H	*0.2*	0.5
I	**2.4**	*0.4*	I	**3.4**	*0.3*	I	**2.7**	*0.1*
K	*0.2*	**2.3**	K	*0.2*	*0.0*	K	*0.1*	*0.0*
L	*0.0*	*0.4*	L	**2.1**	0.6	L	1.8	*0.4*
M	**5.3**	*0.4*	M	1.9	0.7	M	**2.1**	0.7
N	*0.0*	*0.4*	N	0.5	*0.0*	N	*0.2*	*0.0*
P	*0.2*	*0.4*	P	*0.0*	1.1	P	*0.0*	0.7
Q	*0.0*	*0.4*	Q	0.9	*0.0*	Q	1.1	*0.0*
R	*0.2*	**9.6**	R	*0.0*	*0.2*	R	*0.2*	*0.0*
S	**3.4**	*0.4*	S	**2.4**	*0.0*	S	**2.8**	*0.0*
T	**4.8**	*0.4*	T	**2.5**	*0.0*	T	**3.6**	*0.0*
V	1.6	*0.4*	V	**3.7**	0.8	V	**3.3**	*0.3*
W	*0.2*	*0.4*	W	*0.0*	**5.6**	W	*0.1*	*0.2*
Y	*0.2*	*0.4*	Y	*0.0*	**4.3**	Y	*0.2*	**16.5**
Sum	20	20	Sum	20	20	Sum	20	20
AP value	51	81	AP value	28	67	AP value	30	254

RB and AP values are defined as described in Table 1

stack) and the relative frequency of amino acids (the height of the individual amino acids). Figure 7 shows a specificity tree clustering of the SLA-1*0401 molecule compared to prevalent representatives of the 12 common HLA supertypes that *NetMHCpan* originally intended to cover (Lund et al. 2004). By this token, SLA-1*0401 most closely resembles that of HLA-A*01:01.

The limited PSCPL analysis of the chimeric MHC-I molecules revealed strong P9 signals with specificities that seemed to be determined by the origin of the α_1 domain: the HPP chimera showed an HLA-A*11:01-like P9 specificity, whereas the PHP chimera showed a SLA-1*0401/HLA-A*01:01-like specificity. Since the *NetMHCpan* predictor successfully captured these chimeric specificities (see above), we reasoned that the predictor might also be used to perform in silico dissection of these specificities and used the P9 specificity as an example of such an in silico analysis. The *NetMHCpan* predictor considers a pseudo-sequence consisting of 34 polymorphic positions, which contain residues that are within 4.0 Å of the atoms of bound nonamer peptides (Nielsen et al. 2007). Of the 34 positions of the pseudo-sequence, 10 delineates the P9 binding pocket; however, only 3 of these, positions 74, 77, and 97, differ between SLA-1*0401 and HLA-A*11:01. To explore the effect of these three residues, we performed in silico experiments where we examined single substitutions Y74D, G77D, and S97I (the letter before the position number indicates the SLA-A*0401 single letter residue, whereas the letter after indicates the HLA-A*11:01 residue) as well as the corresponding triple substitution (YGS-DDI). As described above, PSSMs were generated for each of the in silico molecules followed by a specificity tree clustering (including SLA-A*0401, HLA-A*01:01, and HLA-A*11:01). Figure 8 shows this tree along with the sequence logo plots showing the predicted binding specificity of each in silico MHC-I molecule. Albeit the Y74D and G77D single substitutions showing some of the positively charged P9 peptide residue preference of HLA-A*11:01, they still clustered with HLA-A*01:01. In contrast, the in silico (YGS-DDI) triple substitution clustered with the HLA-A*11:01. This suggests that the *NetMHCpan* method is capable of defining the residues of the F pocket that determine the specificity of position 9.

Discussion

We have previously suggested that the specificities of the entire human MHC-I system should be solved ("the human

Table 3 Peptide sequences and in vitro determined K_D values for the three different MHC molecules PPP (SLA-1*0401) (top), PHP ($p\alpha_1h\alpha_2p\alpha_3$) (middle), and HPP ($h\alpha_1p\alpha_2p\alpha_3$) (bottom), respectively

Peptide #	Sequence	Affinity (K_D, nM)
PPP		
1	HSNASTLLY	452
2	SSMNSFLLY	192
3	YSAEALLPY	202
4	MTFPVSLEY	15
5	MSSAAHLLY	103
6	STFATVLEY	32
7	MTAASYARY	16
8	HTSALSLGY	163
9	ASYQFQLPY	8
10	YANMWSLMY	386
11	STYQPLPLY	38
12	HTAAPWGSY	Non-binder
13	ITMVNSLTY	169
14	ATAAATEAY	5
PHP		
1	HSNASTLLY	5
2	SSMNSFLLY	2
4	MTFPVSLEY	1
5	MSSAAHLLY	1
6	STFATVLEY	3
9	ASYQFQLPY	1
13	ITMVNSLTY	2
15	STMPLSWMY	1
16	HMMAVTLFY	11
17	KTFEWGVFY	6
18	ATNNLGFMY	1
19	ATATWFQYY	3
20	ITMVNSLTY	2
HPP		
2	SSMNSFLLY	Non-binder
4	MTFPVSLEY	Non-binder
6	STFATVLEY	Non-binder
21	FSFGGFTFK	Non-binder
22	KVFDKSLLY	Non-binder
23	ATFSVPMEK	Non-binder
24	RVMPVFAFK	17
25	AVYSSSMVK	127
26	AVARPFFAK	231
27	GSYFSGFYK	Non-binder
28	LTFLHTLYK	Non-binder
29	MTMRRRLFK	Non-binder

The NetMHCpan percentage of success for predicting binders for the PPP, PHP, and HPP heavy chains were 93%, 100%, and 33%, respectively

MHC", Buus 1999; Lauemoller et al. 2000). However, due to the extreme polymorphism of the MHCs, any attempt to address the specificity of the entire MHC system is a significant experimental undertaking. During the past decade, we have established a series of technologies to support a general solution of human MHC class I and II specificities. For MHC-I, this includes (1) a highly efficient *E. coli* expression system for production of recombinant human and mouse MHC-I molecules (both heavy chain and light chain (β_2m) molecules) (Pedersen et al. 1995; Ostergaard et al. 2001), (2) a purification system for obtaining the highly active pre-oxidized MHC-I heavy chain species (Ferre et al. 2003), (3) a high-throughput homogenous peptide–MHC-I binding assay for obtaining large data sets on peptide–MHC-I interactions (Harndahl et al. 2009), (4) a positional scanning combinatorial peptide library approach for a robust and unbiased analysis of the specificity of any MHC-I molecule (Stryhn et al. 1996), (5) an immunobioinformatics approach to generate predictors of the peptide–MHC-I interaction, *NetMHCpan*, that allows predictions to be made for any human MHC-I molecules, HLA-I, even those that have not yet been covered by existing data set (Hoof et al. 2009; Nielsen et al. 2007), and finally (6) we have demonstrated that pre-oxidized MHC-I molecules can be used to generate MHC-I tetramers in a simple "one-pot, mix-and-read" manner (Leisner et al. 2008). Here, we propose that the next goal should be to extend the overall approach to MHC-I molecules of other species of interest. Mouse and rats have been extensively studied in the past, but much less reagents and information have accrued for the MHC-I molecules of other species. Here, we have used an important livestock animal, the pig, as a model system and demonstrated that it indeed is possible to transfer the original human approach to other species.

Before attempting to generate a recombinant version of the entire porcine SLA-1*0401 molecule, we grafted the more conserved membrane-proximal "stalk" (the immunoglobulin-like class I heavy chain α_3 and β_2m domains) of porcine SLA-1*0401 onto the peptide-binding domain of HLA-A*11:01 generating a chimeric human/porcine MHC-I molecule. This chimeric molecule retained the peptide-binding specificity of the HLA-A*11:01 molecule, and it clearly demonstrated that the recombinant porcine stalk was functional and, by inference, also properly folded. It also suggests that the peptide-binding specificity of the distal domains do not crucially depend upon the identity of the proximal stalk. Further, comparing the ability of human and porcine β_2m to support MHC-I complex formation using either a human or a porcine MHC-I stalk, we demonstrated that every combination (porcine β_2m/human-α_3, porcine β_2m/porcine-α_3, human β_2m/human-α_3, and human β_2m/porcine-α_3) showed al-

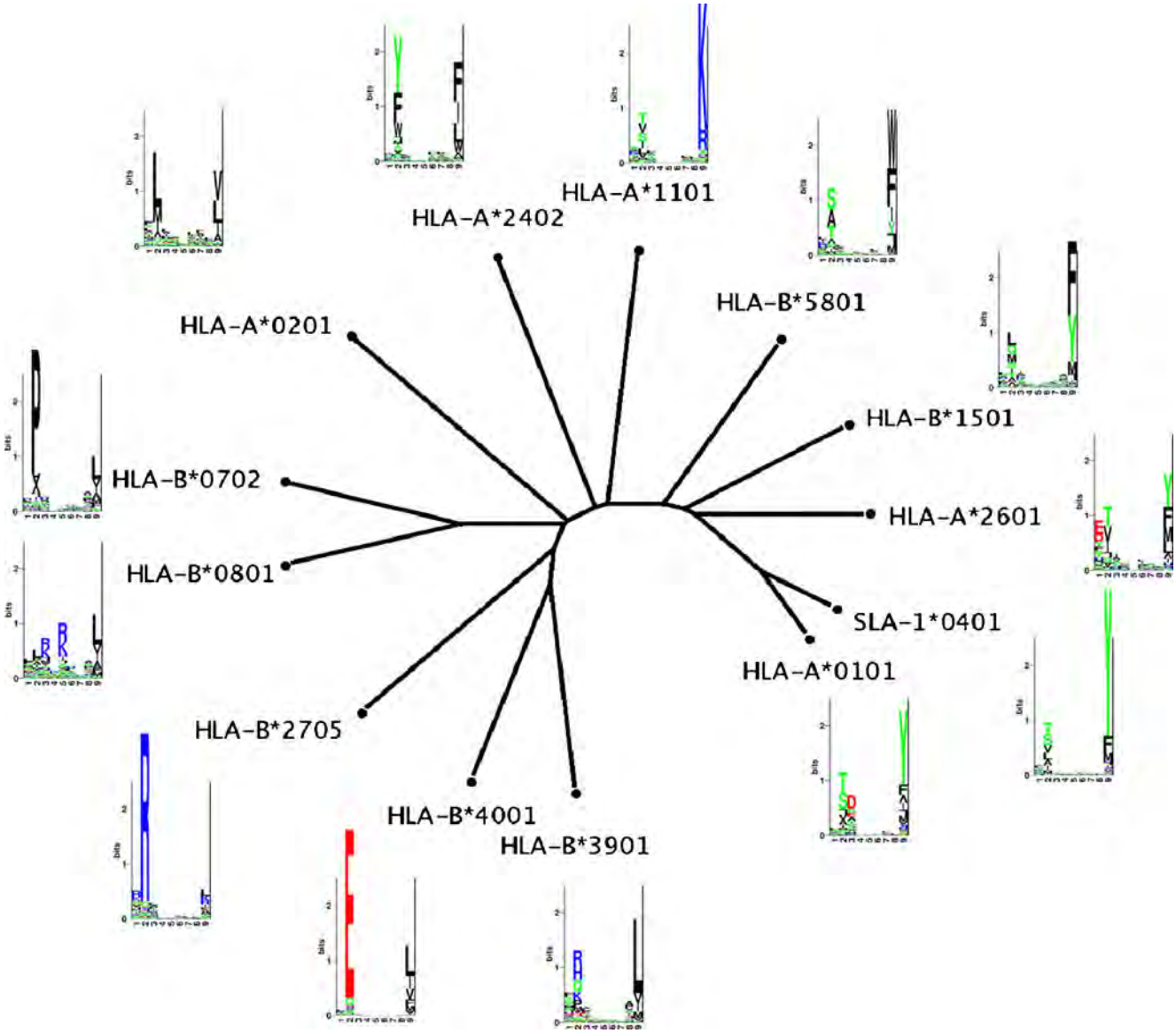

Fig. 7 Specificity tree clustering of the SLA-1*0401 molecule compared to prevalent representatives of the 12 common HLA supertypes (Lund et al. 2004). The distance between any two MHC molecules and the consensus tree is calculated as described in "Materials and methods". All branch points in the tree have bootstrap values of 100%. Sequence logos of the predicted binding specificity are shown for each molecule. In the logo, acidic amino acids [DE] are shown in *red*, basic amino acids [HKR] in *blue*, hydrophobic amino acids [ACFILMPVW] in *black*, and neutral amino acids [GNQSTY] in *green*. The axis of the LOGOs indicates in all case positions one through nine of the motif, and the y-axis the information content (see Materials and methods)

most the same heavy chain dose titration with identical half-saturations. These results illustrate the ability for porcine and human β_2m to support complex formation of SLA molecules and vice versa and suggest evolutionary that the stalk is quite conserved.

Next, we generated the entire SLA-1*0401 heavy chain and succeeded in generating complexes using human β_2m as the light chain and PCSPL as peptide donors. The latter solved the a priori problem of not knowing which peptides would be needed to support proper folding of SLA-1*0401, and it did so in an unbiased manner. Furthermore, this approach is highly efficient since it readily establishes a complete matrix representing the amino acid preference for each amino acid and each position of a nonamer peptide. The specificity of SLA-1*0401 shows two primary anchors: one in positions 9 with a preference for aromatic amino acids and another in position 3 with a preference for negatively charged amino acids. In addition, the SLA-1*0401 features a secondary anchor in position 2 with hydrophobic or polar amino acid preferences.

An alternative approach to solve the problem of identifying peptides that support folding of MHC-I molecules of so far unknown specificity is to use our recently developed pan-specific predictor, *NetMHCpan*. The successful use of this predictor to initiate peptide-binding studies was recently

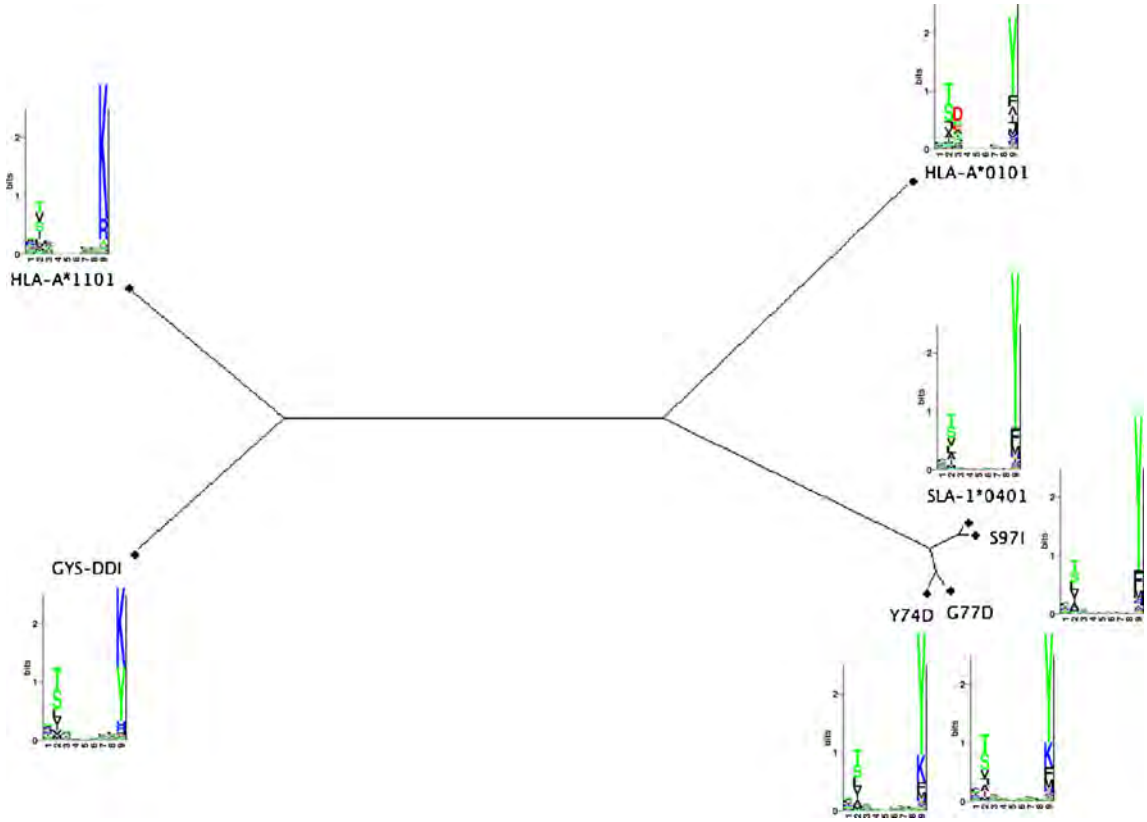

Fig. 8 Comparison of specific in silico mutations of the SLA-1*0401 molecule and comparison with the two HLA molecules: HLA-A*11:01 and HLA-A*01:01. The distance between any two MHC molecules and the consensus tree is calculated as described in "Materials and methods". All branch points in the tree have bootstrap values of 100%. The SLA-1*0401 mutations are indicated as Y74D, G77D, and S97I, where the letter before the position number indicates the SLA-1*0401 single letter residue and the letter after indicates the HLA-A*11:01 residue. YGS-DDI is the corresponding triple substitution. Sequence logos are calculated and visualized as described in Fig. 7. The axis of the LOGOs indicates in all case positions one through nine of the motif, and the y-axis the information content (see Materials and methods)

demonstrated for HLA-A*3001 (Lamberth et al. 2008). Although originally developed to cover all HLA-A and HLA-B molecules, it has also been shown to extend to MHC-I molecules of other species (Hoof et al. 2009). Here, we demonstrate that the *NetMHCpan* predictor is capable of extracting MHC-I sequence information across species and correctly relate this to peptide binding even in the absence of any available data for the specific query MHC-I molecule, i.e., the SLA-1*0401 as well as the chimeric HPP ($h\alpha_1 p\alpha_2 p\alpha_3$) and PHP ($p\alpha_1 h\alpha_2 p\alpha_3$) molecules. It is not clear why binding of the PHP chimera was more efficiently predicted than binding of the HPP chimera. One could speculate that *NetMHCpan* has not captured the effect of the different positions of the pseudo-sequence equally well and not all positions and pockets (and by inference—not all chimeric molecules) are therefore predicted equally well.

Using the *NetMHCpan* predictor to cluster SLA-1*0401 and representative molecules of 12 human HLA supertypes according to predicted peptide-binding specificities, the SLA-1*0401 specificity closely resembled that of HLA-A*01:01 (IEDB, http://www.immuneepitope.org/MHCalleleId/142, accessed March 9th 2011). This result was also obvious from an inspection of the PSCPL analysis of the SLA-1*0401. The PSCPL analysis of the P9 specificity of the SLA-1*0401 and the two chimeric molecules suggested that the P9 specificity primarily was determined by the α_1 domain. This contention was further strengthened by a *NetMHCpan*-driven in silico analysis of the residues delineating the F pocket, which interacts with P9. This suggests that *NetMHCpan* can be used to design and interpret detailed experiments addressing the structure–function relationship of peptide–MHC-I interaction. In the case of SLA-1*0401, *NetMHCpan* suggests that Y74, G77, and S97 play a prominent role in defining the P9 F pocket. Whereas the *NetMHCpan* readily captured the P9 anchor residue of SLA-1*0401, it did not capture the P3 anchor (at least not in the 2.4 version). We surmise that this shortcoming is due to insufficient examples of the use of P3 anchors within the currently available peptide–MHC-I binding data. Inspecting the pseudo-sequence of SLA-1*0401 and HLA-A*01:01 vs. HLA-A*11:01 suggests that the presence of an arginine in position 156 might

explain the preference for negatively charged amino acid residues in P3. Future *NetMHCpan*-guided experiments could pointedly address this question, and the resulting data could complement existing data and be used to update and improve the *NetMHCpan* predictor.

All in all the two complementary approaches, PSCPL and *NetMHCpan*, agreed on the specificity of the SLA-1*0401 molecule, as well as of the two chimeric MHC-I molecules. Thus, the specificity of SLA-1*0401 appear to be well established. This specificity has successfully been used to search for foot-and-mouth disease virus (FMDV)-specific CTL epitopes in FMDV-vaccinated, SLA-1*0401-positive pigs, and the recombinant SLA-1*0401 molecules have been used to generate corresponding tetramers and stain pig CTLs (Patch et al. 2011). In conclusion, we here present a set of methods that can be used to generate functional recombinant MHC-I molecules, map their specificities and identify MHC-I-restricted epitopes, and eventually generate peptide–MHC-I tetramers for validation of CTL responses. This suite of methods is not only applicable to humans, but potentially to any species of interest.

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Research, Education, and Economics
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Development of a Marker Foot-and-Mouth Disease Virus Vaccine Candidate that is Attenuated in the Natural Host

(Patent docket # 0193.07)

Inventors:
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Description

This invention provides a safe platform for production of Foot-and-Mouth Disease Virus (FMDV) inactivated vaccine that is (i) attenuated in the natural host (ii) lacks a conserved FMDV antigenic epitope and (iii) contains unique restriction endonuclease sites for easy exchange of capsid coding DNA sequences of FMD viruses. The vaccine production platform consists of a recombinant FMDV (LL-FMDV-YR) that lacks the coding region for the Leader protein and contains 2-amino acid substitutions(Y-R at amino acid residues 26 and 30) in a specific antigenic region of non-structural protein 3Dpol. The introduced YR mutations in FMDV result in severe attenuation of this virus in cattle. Further, YR mutations introduced in LL-FMDV results in complete attenuation in cattle. The YR mutations, based on the corresponding 3Dpol sequences of the closely related bovine rhinovirus type 2, also erase an epitope recognized by an FMDV-specific monoclonal antibody (mAb44.2) that recognizes all FMDV serotypes. The absence of this antigenic site provides the molecular basis for a differential recognition between LL-FMDV-YR vaccinated from FMDV infected animals using serological tests such as competitive ELISA with mAb44.2.

This vaccine platform builds upon a previous ARS patent (LL-FMDV) and provides three major improvements over the previous invention including having an additional attenuation factor, new restriction enzyme sites for easy swapping of capsid coding sequences and the absence of an antigenic epitope in 3D that allows serological differentiation of vaccinated from infected animals.

Commercial Potential/Advantages:

FMD vaccines are one of the largest sources of income for vaccine companies around the world, with annual sales surpassing 1 billion doses. Currently commercially available FMDV vaccines consist of inactivated virus antigens produced by growing large volumes of field strains in cell culture. These vaccines have several shortcomings including the need for biosafety level 3 containment facilities in order to prevent escape of the virus; problems during adaptation to cell culture of field strains; and difficulty in differentiating vaccinated from infected animals. There have been escapes of FMDV

form vaccine production plants, the most recent example in August 2007 in the UK where an ongoing outbreak of FMD was linked to the accidental release of a FMDV vaccine strain. The two largest FMDV vaccine production companies have manifested their interest in safer vaccine production platforms, particularly after the latest outbreak. Also there is great interest for vaccine seeds that would not require extensive cell culture adaptation and potential antigenic change, and that would allow to easily differentiate vaccinated from infected animals (DIVA). Safe DIVA vaccines would also have the potential to be produced in the United States, where work with live FMDV is currently only allowed at PIADC.

Currently the US maintains the North American FMD Vaccine Bank at the Plum Island Animal Disease Center (PIADC). Various inactivated FMDV antigens are purchased overseas. Vaccine would be made available in case of outbreak in the US, Canada, or Mexico, but must be formulated by the manufacturer (currently in the UK) in the event of an emergency. A safe FMDV marker vaccine production platform has the potential for production in the US and could become part of the National Veterinary Stockpile. The platform presented here has the added advantage that it uses the same industrial technology currently used for FMDV vaccine production.

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Recombinant Live Attenuated Foot-and-Mouth Disease (FMD) Vaccine Containing Mutations in the L Protein Coding Region

(Patent docket # 0026.10)

Inventors:
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[1]Plum Island Animal Disease Center, Orient NY

Description

This invention relates to the discovery of a functional domain and introduction of mutations within this coding region of the leader (L) protein of foot-and-mouth disease virus (FMDV) that are stable and confer mild attenuation to the mutant virus in vitro but significant attenuation in vivo in swine and cattle. Identification of these mutations should serve as a basis to construct novel live-attenuated vaccine candidates to control FMD.

The identified mutations lie in a SAP (for SAF-A/B, Acinus, and PIAS) domain, a conserved protein motif associated with the nuclear retention of molecules involved in transcriptional control. The mutations include isoleucine 55 to alanine (I55A) and leucine 58 to alanine (L58A) based on the amino acid sequence of the FMDV Lb protein. In vitro, the double mutant displays a moderate attenuated phenotype, resulting from inadequate sub-cellular distribution of the Lb protein and its inability to induce degradation of the transcription factor nuclear factor kappa-B. Remarkably, inoculation of swine and cattle with this mutant virus results in the absence of clinical disease, the induction of a significant FMDV-specific neutralizing antibody response, and protection against subsequent homologous virus challenge.

This FMDV variant should serve as the basis for construction of other targeted mutations that could further attenuate the virus thus decreasing the probability of its reversion to virulence. Multiple mutations could be included in a live-attenuated FMD vaccine candidate. Furthermore, both negative and positive markers can be included to allow differentiation of animals vaccinated with this live-attenuated vaccine from animals infected with wild-type virulent FMDV.

A live-attenuated FMD vaccine can potentially induce longer protection than the current vaccine thus reducing the need for bi- or tri-annual vaccination.

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Commercial Potential/Advantages:

MDV, a member of the Picornaviridae family, is the most contagious pathogen of cloven-hoofed animals including bovines, swine, sheep, and goats, and causes a rapidly-spreading, acute infection characterized by fever, lameness and vesicular lesions on the feet, tongue, snout and teats (Grubman and Baxt, 2004). In areas where FMD is enzootic, disease control is achieved by slaughter of infected animals, movement control of susceptible animals, and vaccination. The current inactivated whole virus antigen is not ideal to eliminate FMD since outbreaks are currently reported in several parts of the world even in areas where vaccination programs are in place. Additionally, protection conferred by the inactivated vaccine is not as long-lasting as protection induced by natural virus infection suggesting that different specific immune responses are induced in animals infected with FMDV as compared to animals vaccinated with an inactive whole virus antigen even in the presence of adjuvant.

FMD vaccines are one of the largest sources of income for vaccine companies around the world, with annual sales surpassing 1 billion doses. Although recombinant FMD vaccines are currently in a pipeline for inclusion in the U.S. Veterinary Vaccine Stockpile and potential commercialization, economic constraints of the potential users, which mostly include underdeveloped nations, may limit the immediate success of such an approach. Furthermore, a live-attenuated FMD vaccine may also be less expensive than the current vaccine, since fewer vaccinations may be required and purification may not be needed.

Production of a live-attenuated FMD vaccine has the added advantage of using the same industrial technology currently used for manufacturing inactivated FMD vaccines and it is expected that accidental release of such strains from production facilities (e.g.: United Kingdom, 2007 outbreak presumably from the Merial vaccine facility in Pirbright) should have lesser consequences due to the decreased virulence of attenuated viral strains.

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