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From: Amy L. Vincent, Wenjun Ma, Kelly M. Lager, Bruce H. Janke, and Jürgen A. Richt, Swine Influenza Viruses: A North American Perspective.

In Karl Maramorosch, Aaron J. Shatkin, and Frederick A. Murphy, editors:
Advances in Virus Research, Vol. 72, Burlington: Academic Press, 2008, pp.127-154.
ISBN: 978-0-12-374322-0

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CHAPTER 3

Swine Influenza Viruses: A North American Perspective

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Contents		
	I. Introduction to Influenza A Viruses	128
	A. The Virus	128
	B. Influenza A Virus Infection of Pigs	130
	II. Evolution of North American SI Viruses of the H1 and H3 Subtype	132
	III. Cross-Species Transmission of Influenza A Viruses and Novel Subtypes in North American Swine	136
	A. SI Infections of Humans	136
	B. Novel SI Isolates in North America	137
	IV. Vaccination of Pigs Against SI	139
	V. Conclusions and Outlook	147
	Acknowledgments	148
	References	148

Abstract

Influenza is a zoonotic viral disease that represents a health and economic threat to both humans and animals worldwide. Swine influenza (SI) was first recognized clinically in pigs in the Midwestern U.S., in 1918, coinciding with the human influenza pandemic known as the Spanish flu. Since that time SI has remained of importance to the swine industry throughout the world. In this

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review, the epidemiology of swine influenza virus (SIV) infection in North American pigs is described in detail. The first 80 years of SI remained relatively static, whereas the last decade has become dynamic with the establishment of many emerging subtypes. With the increasing number of novel subtypes and genetic variants, the control of SI has become increasingly difficult and innovative strategies to combat this economically important zoonotic disease are critical. Therefore, protective immune responses against influenza virus infections as well as new paradigms of vaccine development in pigs are discussed in the review. It is expected that the dynamic evolutionary changes of SIVs in North American pigs will continue, making currently available prophylactic approaches of limited use to control the spread and economic losses associated with this important swine pathogen.

I. INTRODUCTION TO INFLUENZA A VIRUSES

Influenza is a zoonotic viral disease that represents a health and economic threat to both humans and animals worldwide. Influenza A viruses infect a wide variety of species and exhibit only a partial restriction of their host range, that is, there is occasional transmission from one species to another. Annual epidemics/epizootics in humans and animals and occasional influenza pandemics in humans depend on the continued molecular evolution of influenza viruses giving rise to new antigenic variants. The surface hemagglutinin (HA) and neuraminidase (NA) antigens undergo two types of variation called antigenic drift and antigenic shift. Antigenic drift involves minor changes in the HA and NA, whereas antigenic shift involves major changes in these molecules resulting from replacement of the entire gene segment. The segmented nature of the influenza virus genome is a key feature of influenza viruses and supports antigenic shift or reassortment. In the event that cells are infected with two (or more) different influenza viruses, exchange of RNA segments between the viruses allows the generation of progeny viruses containing a novel combination of genes. In mammals, influenza viruses replicate mainly in the respiratory tract, usually accompanied with clinical signs, whereas in avian species, the major replication site is the intestinal tract without clinical signs (Webster, 2002). In aquatic birds, influenza viruses are generally highly host-adapted and show low evolutionary rates (Webby and Webster, 2001), whereas in mammalian species the evolutionary rate is much greater (Buonagurio *et al.*, 1986).

A. The Virus

Influenza viruses are members of the family *Orthomyxoviridae* comprising five genera: Influenza A, B and C viruses, Thogotovirus, and Isavirus (Knipe *et al.*, 2007). Of these, only influenza A viruses are true zoonotic

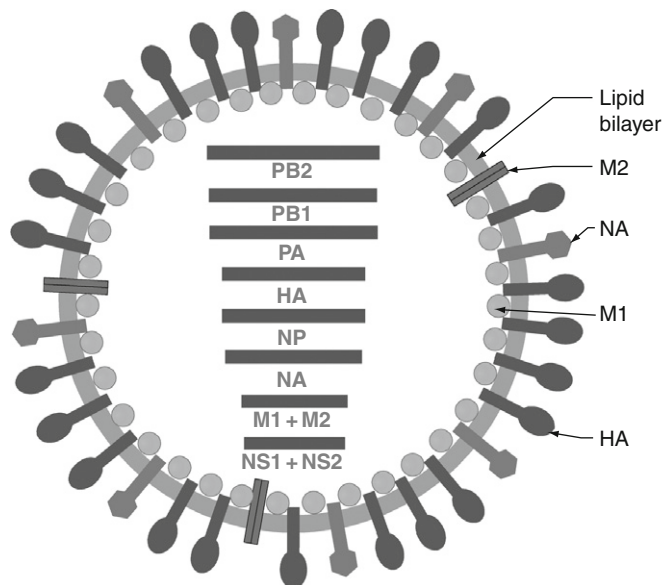


FIGURE 1 Diagram of an influenza A virion with the viral envelope and the eight RNA gene segments.

agents. Influenza B and C viruses are primarily human pathogens; influenza C can occasionally infect pigs and dogs (Ohwada *et al.*, 1987). Influenza A viruses are 80–120 nm enveloped viruses with segmented, single-stranded, negative-sense RNA genomes (Fig. 1). The eight RNA segments within the viral genome, varying in length between 890 and 2341 nucleotides, encode 10 and in some cases 11 proteins. Segment 7 (Matrix, M) and segment 8 (Nonstructural, NS) encode two proteins (M1/M2 and NS1/NS2; Knipe *et al.*, 2007) due to differentially spliced transcripts, and in some virus strains segment 2 (polymerase basic 1, PB1) encodes a second short protein, called PB1-F2, from an additional open-reading frame (Conenello and Palese, 2007). The RNA fragments are bound and protected by the viral nucleoprotein (NP; Compans *et al.*, 1972). The trimeric RNA polymerase complex (PB1, polymerase basic 2, PB2 and polymerase acidic, PA) binds to the 5' and 3' ends of the viral RNA forming a noncovalent circular complex (Klump *et al.*, 1997). The complex consisting of viral RNA, the polymerase complex, and the NP is called the ribonucleoprotein (RNP) complex. Influenza A viruses are typed according to their surface glycoproteins, HA and NA. The HA and NA are also the main targets of the host humoral immune response. Host immune pressure is the driving force in selecting mutant viruses with amino acid substitutions, a process called antigenic drift. The HA serves as the viral receptor-binding protein and mediates fusion of the

virus envelope with the host cell membrane (Skehel and Wiley, 2000). The HA binds to *N*-acetylneuraminic acid-2,3-galactose linkage or *N*-acetylneuraminic acid-2,6-galactose linkage on sialyloligosaccharides for avian and mammalian viruses, respectively (Rogers and Paulson, 1983). The NA is responsible for cleaving terminal sialic acid residues from carbohydrate moieties on the surfaces of the host cell and virus (Gottschalk, 1957), thus assisting in virus cell entry by mucus degradation (Matrosovich *et al.*, 2004) and the release and spread of progeny virions (Palese *et al.*, 1974). Like the HA, the NA undergoes substantial antigenic variation in response to immune pressure. The M2 protein, the third envelope glycoprotein present in the influenza virion, serves as an ion channel (Wang *et al.*, 1993). The M1 protein is the most abundant protein present in the influenza virion and lies beneath the lipid envelope (Fig. 1). Influenza viruses encode two nonstructural (NS) proteins, NS1 and NS2. While the NS2 or nuclear export protein (NEP) was originally thought to be a nonstructural protein; it has since been found to be a part of the influenza virion (Richardson and Akkina, 1991). In contrast, although NS1 is abundantly present in infected cells during virus replication, the protein is not incorporated into the progeny virions (Palese *et al.*, 1999).

B. Influenza A Virus Infection of Pigs

Swine influenza (SI) was first recognized clinically in pigs in the Midwestern U.S. in summer/fall of 1918 (Koen, 1919), coinciding with the human influenza pandemic known as the Spanish flu (Webster, 2002). Since then SI has been of importance to the swine industry throughout the world (Olsen, 2002). The first SI virus (SIV) isolated from pigs in 1930 (Shope, 1931) belonged to the H1N1 lineage of SIVs. Clinical signs of influenza in pigs are similar to those observed in humans, making it an important model to study influenza pathogenesis in a natural host. Specifically, SIV infections are manifested as acute respiratory disease characterized by fever, inactivity, decreased food intake, respiratory distress, coughing, sneezing, conjunctivitis, and nasal discharge (Alexander and Brown, 2000; McQueen *et al.*, 1968; Richt *et al.*, 2003). Although the severity is affected by many factors, including viral strain, the onset of disease is typically sudden. The disease incubation period is between 1 and 3 days with rapid recovery beginning 4–7 days after onset. SI is a herd disease characterized by high morbidity (approaching 100%) and generally low mortality (<1%) rates. Macroscopically, SIV-infected lungs display a purple-red, multifocal to coalescing consolidation of predominantly cranio-ventral portions of the lung (Fig. 2A). Microscopic changes in the lung consist of necrosis of bronchiolar epithelial cells and sloughing of these cells into airway lumen, which often contains cellular debris, proteinaceous fluid and a few leukocytes (Fig. 2B). This necrosis

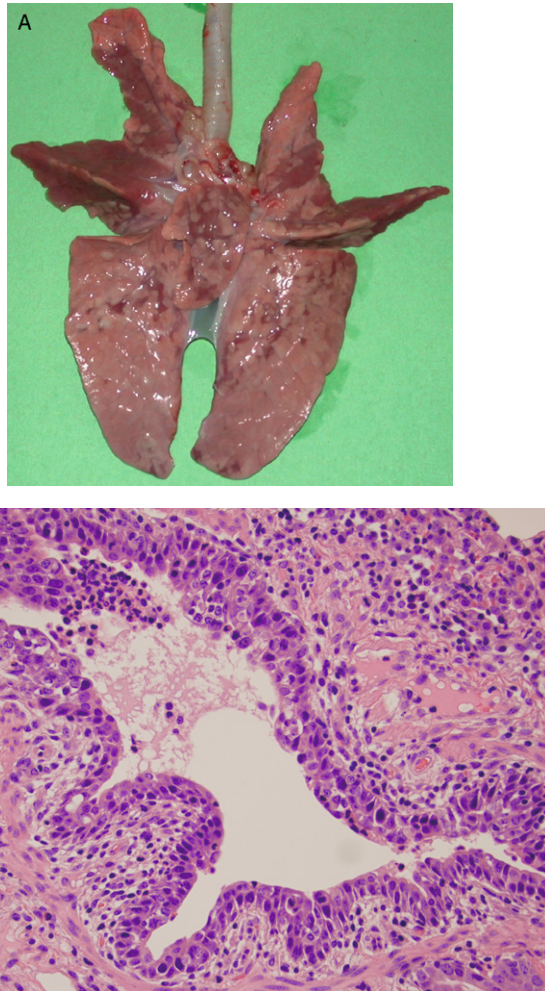


FIGURE 2 Macroscopic and microscopic pneumonia associated with swine H1N1 influenza virus infection of pigs. (A) Macroscopic pneumonia characterized as purple-red consolidation located primarily in cranial and middle lung lobes. (B) Necrotizing bronchiolitis in a pig 3 days postinoculation with SIV. Necrosis and reactive proliferation of epithelial cells is occurring with sloughing of these cells into the airway lumen which contains cellular debris, proteinaceous fluid and a few leukocytes.

is accompanied by peribronchiolar lymphocytic infiltration and interstitial pneumonia of variable severity. In recovery, bronchiolar epithelium becomes proliferative and lymphocytic cuffing becomes more prominent. Influenza viruses are part of the porcine respiratory disease complex (PRDC), acting in concert with other pathogens such as *Mycoplasma*

hyopneumoniae, *Actinobacillus pleuropneumonia*, *Pasteurella multocida*, porcine reproductive and respiratory syndrome virus (PRRSV), and porcine circovirus type 2 (PCV-2; Ellis *et al.*, 2004; Thacker *et al.*, 2001).

Current human influenza viruses are believed to have arisen by genetic reassortment between pre-existing human influenza viruses and nonhuman primarily avian influenza viruses. Swine have been considered a potential "mixing vessel" (Scholtissek, 1995), because they have receptors for both avian and human influenza viruses (Ito, 2000; Ito *et al.*, 1998). Therefore, they can serve as hosts for viruses from either birds or humans.

II. EVOLUTION OF NORTH AMERICAN SI VIRUSES OF THE H1 AND H3 SUBTYPE

Historically, SI in the United States had a predictable pattern with an epizootic in the late fall and early winter months similar to that in humans. Prior to 1998, this acute respiratory disease was almost exclusively caused by viruses of the classical-swine H1N1 lineage (cH1N1; Easterday and van Reeth, 1999). The cH1N1 virus, first isolated and identified in North America in 1930 (Shope, 1931), is believed to have been introduced into the U.S. pig population during the 1918 Spanish influenza pandemic since a concurrent disease similar to that of people was described in the pig population (Fig. 3A). For nearly 70 years, SIV in North America was relatively stable with the cH1N1 as the only predominant subtype. However, serological evidence indicated that human subtype H3 influenza viruses were circulating at a low frequency in U.S. pigs (Chambers *et al.*, 1991), but failed to establish a stable lineage (Fig. 3A). In 1998, a severe influenza-like illness was observed in pigs on a farm in North Carolina with additional outbreaks in swine herds in Minnesota, Iowa, and Texas. The causative agents for these outbreaks were identified as influenza viruses of the subtype H3N2. Genetic analysis of these H3N2 viruses showed that at least two different genotypes were present (Fig. 3A). The initial North Carolina isolate contained gene segments similar to those of the human (HA, NA, PB1) and classical-swine (NS, NP, M, PB2, PA) lineages (double reassortant), whereas the isolates from Minnesota, Iowa, and Texas contained genes from the human (HA, NA, PB1), swine (NS, NP, M), and avian (PB2, PA) lineages (triple reassortant; Zhou *et al.*, 1999). By the end of 1999, viruses antigenically and genetically related to the triple reassortant lineage were widespread in the U.S. swine population (Webby *et al.*, 2000), whereas the double reassortant virus did not spread efficiently among swine. The double and triple reassortant H3N2 viruses contained similar HA genes with identical residues in critical receptor binding regions, suggesting that their different successes were due to factors not associated with the HA and receptor binding. The

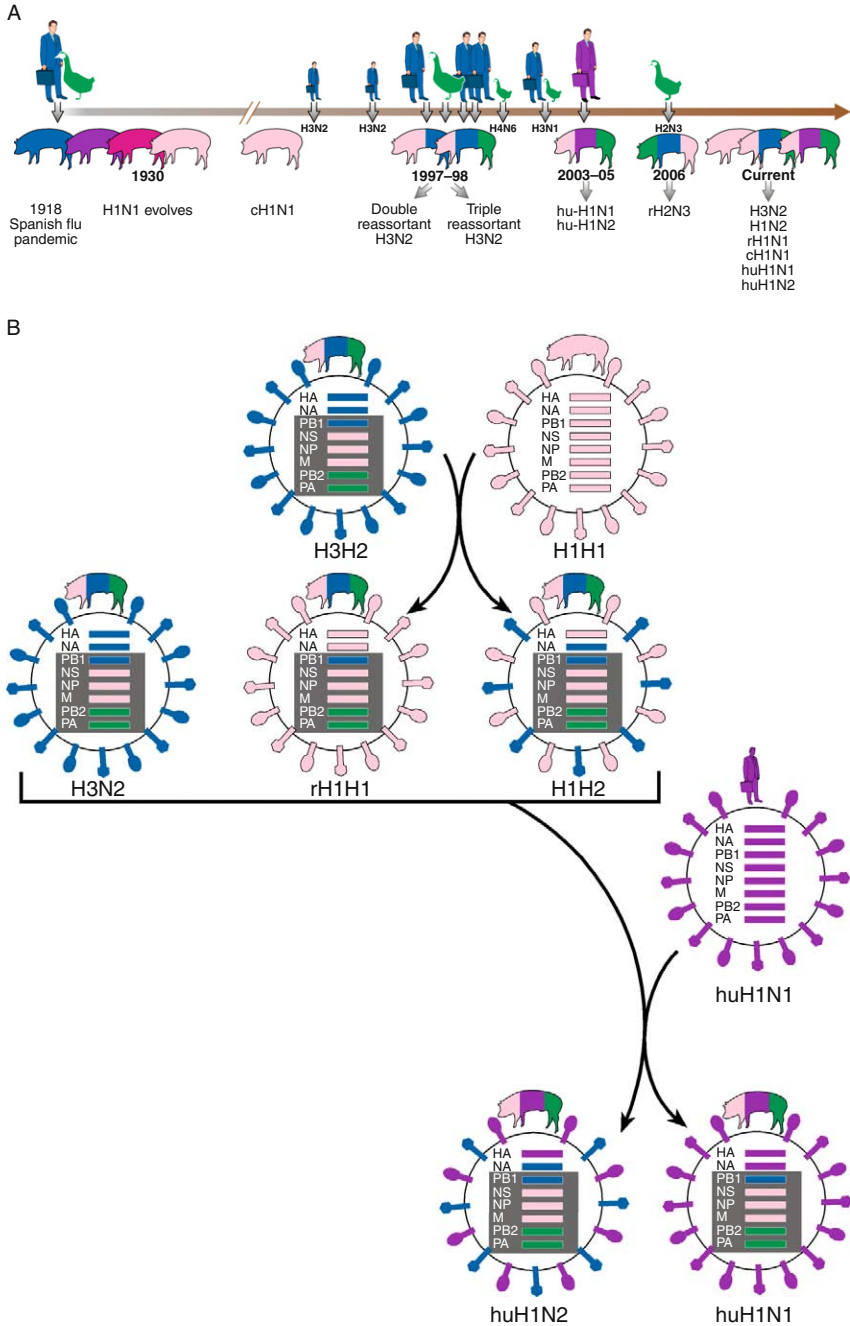


FIGURE 3 Epidemiology of SIVs in North America since 1918. Swine virus lineage is color coded pink, avian lineage is coded green, human lineage is coded blue or purple. (A) Chronology of transmission events leading to reassortant viruses with genes from

major difference between the two viruses was the acquisition of two avian polymerase genes (PA, PB2) in the triple reassortant H3N2 (Fig. 3B).

Once established in the swine population, the H3N2 viruses evolved through genetic mutation and reassortment with cH1N1 swine viruses. Currently, there are a number of reassortant viruses that have been identified, including further H3N2 genotypes (Richt *et al.*, 2003; Webby *et al.*, 2000, 2004), H1N2 (Choi *et al.*, 2002; Karasin *et al.*, 2002), reassortant H1N1 (rH1N1; Webby *et al.*, 2004), and H3N1 viruses (Lekcharoensuk *et al.*, 2006; Ma *et al.*, 2006; Fig. 3B). The H3N2, rH1N1, and H1N2 viruses have become endemic and co-circulate in most major swine producing regions of both the U.S. and Canada. More recently, introductions of human-like H1 viruses that are genetically and antigenically distinct from the classical swine H1 lineage were identified in pigs in Canada (Karasin *et al.*, 2006).

All of the successful SIV reassortants that have become endemic in the U.S. pig population that have been characterized to date contain a similar triple reassortant internal gene (TRIG) cassette including the PA and PB2 genes of avian lineage, NS, NP, and M genes of classical swine lineage, and the PB1 gene of human lineage (Fig. 3B). This would suggest that the TRIG cassette can accept multiple HA and NA types and may endow a selective advantage to swine viruses possessing this gene constellation. With the acquisition of the avian PA and PB2 genes and the human PB1 gene, the current swine viruses appear to have increased the rate of antigenic drift and reassortment, and thereby, the ability to evade established herd immunity. This was not seen with the classical swine H1N1, which remained relatively stable antigenically for nearly 70 years (Luoh *et al.*, 1992; Noble *et al.*, 1993; Olsen *et al.*, 1993; Sheerar *et al.*, 1989). Classical swine H1N1 isolated as recently as 1999 maintained moderate to good cross-reactivity with viruses isolated decades earlier (Vincent *et al.*, 2006).

swine, human and avian influenza virus (AIV) lineages. The “Spanish flu” virus was transmitted from avian/human origin to pigs and evolved into the cH1N1, as indicated by the transition in color of pigs from blue to purple to red to pink. The human and avian images above the horizontal timeline represent the species origin of viral gene segments donated to give rise to the swine influenza virus (SIV) reassortants listed below the horizontal timeline. Note, timeline not drawn to scale. (B) Diagram of viruses with their eight gene segment constellations in currently circulating SIVs. The triple reassortant H3N2 reassorted with the cH1N1 to produce rH1N1 and H1N2 subtype viruses with the triple reassortant internal gene (TRIG) cassette. Subsequent reassortment events with human H1 subtype viruses led to the human-like H1N2 and human-like H1N1 SIVs. The TRIG cassette is highlighted by the gray box.

H3N2 SIV isolated since 1998 have been evaluated at the genetic and antigenic level (Richt *et al.*, 2003; Webby *et al.*, 2004) and were demonstrated to have arisen from at least three introductions of human H3-subtype viruses, leading to phylogenetic clusters I, II, and III. There was variable antigenic cross-reactivity between the clusters. The cluster III viruses have become dominant in North America (Gramer, 2007) and have continued to evolve into cluster III variants, also known as cluster IV (Olsen *et al.*, 2006). We have evaluated and compared the pathogenesis of 10 H1 SIV isolates dating from 1930 to more recent isolates (Vincent *et al.*, 2006). In addition, the HA and NA genes of each isolate were sequenced for genetic comparison, and serological cross-reactivity was evaluated using sera and virus combinations in HI assays. Differences in pathogenicity were detected between H1 isolates, with recent isolates tending to produce more severe disease, increased nasal shedding, and higher virus titers in the lung. Serologically, the historical classical viruses tended to have better cross-reaction between historical sera and antigens, with moderate to good cross-reactivity with modern viral antigens. However, the modern sera were less reactive to historical viruses and tended to be less consistent in cross-reactivity within the modern group. There appeared to be an increase in genetic and antigenic diversity coincident with the emergence of the swine triple reassortant H3N2 in 1998 and the acquisition of the TRIG cassette. Many of the recent isolates had accumulated amino acid changes in the predicted antigenic and receptor binding sites on the HA protein. Existence of antigenic diversity in H1N1 and H1N2 SIVs is similar to the observations made in the diversity of the triple reassortant H3N2 SIV (Richt *et al.*, 2003; Vincent *et al.*, 2006).

Since 2005, H1N1 and H1N2 viruses with the HA gene derived from human viruses have spread across the U.S. in swine herds (Gramer, 2007). The HA from the human-like swine H1 (hu-H1) viruses are genetically and antigenically distinct from swine H1 viruses. However, the six internal genes appear to be similar to those found in the TRIG cassette of contemporary swine triple reassortant viruses (Vincent, unpublished results). The NAs from these newly emerged viruses also are primarily human lineage N1 or N2. The hu-H1 SIVs have become one of the major types of SIV isolated and characterized from swine respiratory disease outbreaks (Gramer, personal communication). We evaluated one hu-H1N1 isolate in our experimental infection and transmission model and demonstrated that it was pathogenic and transmissible in 4-week-old pigs (Vincent, unpublished results). The hu-H1N1 isolate evaluated in our studies demonstrated differences in kinetics of lung lesion development, viral load in the lung, and nasal shedding when compared to a virulent rH1N1 SIV. These studies suggest this emerging virus genotype may not be fully adapted to the swine host since virus replication in the lung and virus shedding from the nose were reduced compared to the contemporary rH1N1 SIV. Nonetheless, the hu-H1 viruses have become established in the U.S. pig population.

III. CROSS-SPECIES TRANSMISSION OF INFLUENZA A VIRUSES AND NOVEL SUBTYPES IN NORTH AMERICAN SWINE

Influenza A viruses of all 16 HA and 9 NA subtypes have been recovered from wild waterfowl and seabirds (Fouchier *et al.*, 2005; Webster *et al.*, 1992). From these studies it was concluded that waterfowl provide a vast global reservoir of influenza viruses in nature from which novel viruses can emerge and infect mammalian species (Webby and Webster, 2001). Prominent examples of cross-species transmission of influenza viruses from avian to mammalian species or vice versa are the recent infections of humans, cats, and martens with the highly pathogenic avian H5N1 viruses (Klopfleisch *et al.*, 2007; Tiensin *et al.*, 2005; Webster *et al.*, 2006) and the transmission of triple reassortant H3N2 SIVs to turkeys (Yassine *et al.*, 2007). The outbreak of severe respiratory disease in racing greyhounds due to infection with an H3N8 influenza virus closely related to an equine influenza virus (Crawford *et al.*, 2005) represents an intra-mammalian cross-species transmission of influenza viruses. Cross-species spill-over of influenza viruses occur rather frequently; however, they tend to be self-limiting and the viruses are rarely maintained in the new host species (Webster *et al.*, 1992; Webster, 2002). As discussed previously, the segmented nature of the influenza virus genome is a key feature for influenza virus evolution and cross-species transmissibility. However, specific subtypes differ in their ability to cross species barriers (Brown, 2000). Viral and host factors obviously play a role in cross-species transmissions and experimental evidence suggest that all eight gene segments, not only the surface proteins HA and NA, as well as specific gene combinations are involved in influenza virus species specificity (Horimoto and Kawaoka, 2001; Neumann and Kawaoka, 2006; Scholtissek *et al.*, 1985). Given the plasticity of the virus genome, influenza fulfills the prerequisites of a virus with emerging disease potential (Webster *et al.*, 1993). It is highly likely that sometime in the near future a “new” influenza A virus, for example, one of the H5N1 viruses currently circulating in the wild bird population in large parts of Asia or a different virus, will be able to emerge from its animal reservoir to cause widespread disease in mammalian species.

A. SI Infections of Humans

In a recent review by Myers and colleagues (Myers *et al.*, 2007) the entire literature on cases of SI in humans was reviewed. These authors reported that 50 cases of zoonotic SIV infections, 37 civilian cases and 13 military personnel cases, are described in the literature. The majority belonged to the H1N1 subtype, a few to the H3N2 subtype. The case-fatality rate of all reported cases was 14% (7/50). Civilian cases were described in the U.S.

(19 cases), Czechoslovakia (6 cases), The Netherlands (4 cases), Russia and Switzerland (3 cases each), and Canada and Hong Kong (1 case each). The median age of the patients was 24.5 years and the majority of the patients (61%) reported a recent exposure to pigs. A well publicized outbreak of SI due to an H1N1 virus resulted in 1 death and respiratory illness in 12 soldiers at Fort Dix, NJ, in early 1976 (Gaydos *et al.*, 1977). Interestingly, no evidence of exposure to pigs was ever found. It has since been shown, however, that persons who work with swine are at increased risk of zoonotic influenza virus infection (Myers *et al.*, 2006). Farmers, meat processing workers, and veterinarians were studied and all three exposed study groups demonstrated elevated serologic titers and higher odds for exposure to H1N1 and/or H1N2 SIV isolates, compared with control subjects. This indicates that occupational exposure to pigs greatly increases workers' risk of SIV infection. Recently, an H1N1 SIV infection of pigs and people at an Ohio county fair was reported (Swenson, 2008). Pigs and people in close contact with them became clinically affected with an acute influenza-like illness, and virus was isolated from several pigs and at least two people (parent and child). The viruses isolated from the humans were 100% identical to the viruses isolated from the pigs, indicating that the virus was shared between pigs and people at the fair, again emphasizing the zoonotic risk for SIV.

B. Novel SI Isolates in North America

A number of novel subtypes were isolated from swine in the past decade. Most of these novel SI subtypes were not able to establish themselves in the swine population. However, the following examples indicate that there is an ever-present chance of a new influenza subtype being established within the swine population which could have dire consequences for human health. The species barrier for the transmission of avian influenza viruses to pigs may be less stringent, since pigs contain receptors for both avian and mammalian influenza viruses in their respiratory tract (Ito *et al.*, 1998). It is therefore, not surprising that pigs can be experimentally infected at least transiently with a wide variety of subtypes of avian influenza viruses (Kida *et al.*, 1994). In addition, co-infection of pigs with a swine virus and with an avian virus unable to replicate in pigs generated reassortant viruses that could be passaged in pigs, indicating that even avian viruses that do not replicate in pigs can contribute genes to generate reassortant viruses (Kida *et al.*, 1994).

1. The H4 Experience

An example of infection of pigs with an avian influenza virus (AIV) occurred on a swine farm in Canada in October 1999. Genetic and antigenic analyses demonstrated that viruses isolated from pigs during an

outbreak of respiratory disease were wholly avian H4N6 viruses of the North American lineage (Karasin *et al.*, 2000). It was found that the farm of origin is located near a lake on which large numbers of waterfowl congregate each fall and from which the farm drew water. Therefore, the source of this virus was most likely ducks on the adjacent lake. It is well known that ducks shed high level of virus which can be isolated from unconcentrated lake water (Laver *et al.*, 2000). The H4N6 virus spread to additional units of the original farm, suggesting that it has the ability to spread from pig-to-pig (Olsen, 2002). Fortunately, it has not been detected outside the original farm system. Interestingly, the HA of this virus contained amino acids in the receptor binding pocket that have been associated with mammalian receptor binding (Karasin *et al.*, 2000).

2. The H2 Experience

Unique H2N3 influenza viruses were recently isolated from clinically affected pigs from two farms in the central U.S. (Ma *et al.*, 2007). Sequencing demonstrated they were H2N3 influenza A viruses with 99.3–99.9% homology between the isolates. The HA segment was similar to an AIV H2N3 isolated from mallards and the NA sequence was similar to an AIV H4N3 isolated from blue-winged teal. The PA segment had high homology to an AIV H6N5 isolated from mallards and the remaining genes were similar to influenza virus gene segments found in the contemporary TRIG cassette (human-like PB1, swine M, NP and NS, avian-like PB2) in U.S. SIVs.

In addition to half of the gene segments being avian-like, the avian-like H2 HA has an amino acid sequence constellation in the receptor binding area indicating a preferential binding to the mammalian influenza receptor. This HA mutation is identical to the initial reassortant human influenza isolates found at the beginning of the 1957 H2N2 pandemic. *In vivo* studies in mice, swine and in ferrets, surrogate model for human influenza infection, were conducted. Experimentally-infected pigs developed lung lesions following challenge and virus was shed to contact control pigs that became infected and seroconverted. Similarly, in ferrets, virus was transmitted to contact ferrets. In addition, mortality was induced in young mice. The only recognized common thread between the two pig farms were geographic location and the use of pond water for both drinking and cleaning in the pig barns. The ability of the H2N3 viruses with avian origin surface glycoproteins to infect and replicate in three mammalian hosts without serial passage for adaptation in each species suggests this virus is already adapted to the mammalian host and may have potential risk to the human population. Although viruses of each of the 16 influenza A HA subtypes are potential human pathogens, only viruses of the H1, H2, and H3 subtype are known to have been successfully established in humans (Hilleman, 2002). H2 influenza viruses have been absent from human circulation since 1968. As such they pose a substantial human pandemic risk because of lack of population immunity.

IV. VACCINATION OF PIGS AGAINST SI

Vaccinating pigs against influenza A virus has become a common practice in the U.S. swine industry over the last 10 years. Inactivated influenza vaccines became commercially available in 1994. In 1995, influenza vaccine usage was not reported in the National Animal Health Monitoring System survey of the U.S. swine operations (USDA, 1995). However, by 2000, over 40% of large producers reported that they vaccinated breeding females and approximately 20% vaccinated weaned pigs (USDA, 2003). In the survey conducted in 2006, the number of large producers vaccinating breeding females increased to 70%, whereas vaccinating weaned pigs remained relatively unchanged (USDA, 2007). Importantly, of those farms that vaccinated breeding females in 2006, approximately 20% reported using autogenous SIV vaccines rather than commercial vaccines. Autogenous vaccines prepared from virus cultures that have been inactivated may be used only in the herd of origin under the direction of a veterinarian. Autogenous vaccine usage against influenza virus has increased due to the diversity of viruses circulating in the North American pig population and the inability of the animal biologics industry to change the vaccine composition as rapidly as the viruses are changing. In contrast to human influenza virus epidemiology, SIV is no longer seasonal and there are too many circulating variants in North America to include a representative few in a bivalent or trivalent killed vaccine. There are three major problems with the control and prevention of SI in the U.S.: (a) SIV is changing faster than traditional vaccines can be developed, (b) There is a need for vaccines that can induce better cross-protection among SIV isolates, and (c) Passively acquired immunity is believed to block vaccine efficacy in pigs.

The first line of defense against influenza virus infection is the innate immune system. Host cells have molecular sensors that recognize specific motifs from prokaryotic, protozoan, and viral pathogens. Some of the known sensors for single stranded RNA viruses like influenza viruses include the RNA helicases RIG-I and MDA-5 and the RNA binding and signaling proteins TLR3 and TLR 7 (reviewed in Garcia-Sastre, 2006). Many of these sensors have pathways that converge to upregulate the type 1 interferons (IFN α/β). IFN α/β , in turn, sound the alarm to other nearby cells and activate them through the production of cytokines and chemokines. In addition, the presence of type 1 interferons upregulates the production of host antiviral proteins such as Mx, PKR, and OAS, impairing or destroying the invading virus. Many pathogens, including influenza virus, have evolved to interfere with the IFN α/β signaling cascade as part of their survival mechanisms. The NS1 protein of influenza virus contributes to virulence by interacting with the IFN α/β

antiviral response. The carboxy-terminus of NS1 is reported to contain the effector domain responsible for antagonizing the type 1 IFN pathway (Wang *et al.*, 2002). The amino terminus is reported to contain the RNA binding domain (Wang *et al.*, 2000), which may allow the NS1 protein to sequester viral RNA and therefore avoid detection by the host cell's virus sensor. Using reverse genetics approaches, we have produced H3N2 SIVs with deletions in the 3' end of the NS1 gene; The NS1-truncated mutants are highly attenuated *in vitro* and in pigs (Solorzano *et al.*, 2005), demonstrating that the NS1 is a virulence factor of SIV in pigs. The attenuation is due, at least in a major part, to a loss in the ability of the mutated virus to block antiviral defense mechanisms, with subsequent host cell upregulation of type 1 interferons (Solorzano *et al.*, 2005) and downstream effector molecules, such as Mx and PKR (Wang *et al.*, 2002).

Protective immunity against infection with influenza involves both the humoral and cell mediated (CMI) arms of the adaptive immune system. The responses of the humoral and CMI systems are interwoven and both are necessary for protective immunity. Antibodies play a significant role in attenuating and preventing swine influenza as shown by the protective capacity of colostrum (Renshaw, 1975) and inactivated vaccines (Bikour *et al.*, 1996). Clinical protection against challenge virus appears to be directly correlated with the hemagglutination inhibition (HI) titer in the serum of an individual animal, that is, a high HI titer provides better protection against challenge than a low HI titer. This information has led to the suggestion that the presence and magnitude of an HI titer could be a predictor of protection. Unfortunately, this seems only true when the priming HA antigen inducing the HI titer is antigenically closely related to the HA of the challenge virus. Other studies have demonstrated the protective qualities of antibodies at the mucosal level. Pigs immunized with virulent, live SIV, and then challenged with the same virus 42 days later did have a detectable anamnestic antibody response at the mucosal level but not in the serum (Larsen *et al.*, 2000). Specifically, a rise in IgA and IgG was detected in the nasal cavity, the site of challenge. This data supports the hypothesis that antibody mediated immune reactions at the mucosal level and not the systemic level are important for protecting the respiratory tract from SIV.

Extensive studies investigating the immune response of mice to influenza virus infection indicate they can develop homosubtypic (same subtype) and heterosubtypic (different subtype) immunity (Het-I). Homotypic immunity tends to exert a more complete protection, whereas Het-I may fail to prevent an initial infection, but is successful in reduction of virus shedding and a more rapid recovery from infection, (reviewed in Tamura *et al.*, 2005). Collectively, studies on hetero- and homosubtypic immunity in mice demonstrate that virus elimination and protection from disease are dependent on virus-specific neutralizing antibodies and T

cells, as well as the virus-specific mucosal immune response. In mice, cross-reactive IgA induced by natural infection was shown to be strongly correlated to protection from challenge with a homosubtypic virus belonging to a different, heterologous genotype (Liew *et al.*, 1984). Serum HI antibody titer and the presence of cross-reactive cytotoxic T-lymphocytes did not correlate with protection, but might be crucial for recovery. IgA was shown to be more cross-reactive than IgG against heterologous influenza viruses and passive transfer of IgA to non-immune mice conferred protection (Tamura *et al.*, 1991). Although cytotoxic T-lymphocyte activity has been shown to be stimulated in heterosubtypic primed mice (Nguyen *et al.*, 1999), protection against heterosubtypic challenge in mice was largely dependent on the presence of B-cells and CD4+ T-helper cells, specifically those with a Th1 phenotype (Moran *et al.*, 1999; Nguyen *et al.*, 1999, 2001). CD4+ T cells primed against conserved internal influenza proteins may be responsible for the rapid development of cross-reacting antibodies following a heterosubtypic challenge (Scherle and Gerhard, 1986). These cross-reacting antibodies appeared to provide at least partial protection and a more rapid recovery after heterosubtypic challenge. In contrast, an inactivated virus challenge in mice stimulated a Th2 response and no heterosubtypic immunity (Moran *et al.*, 1999). However, heterosubtypic immunity could be induced when mice were immunized with inactivated virus and, in addition, received an injection of interleukin (IL) 12 and antibodies against IL 4 (Moran *et al.*, 1999). Although impractical for swine vaccination, these results suggest that improved adjuvants may enhance the protective immunity of killed vaccines.

The continual consolidation of the swine industry into larger swine herds housed in swine-dense regions and the emergence of novel SIV subtypes ensures that future SIV control will be heavily dependent upon vaccination protocols. When swine are infected with a virulent influenza virus, complete protective immunity typically develops against rechallenge with the homologous virus, that is, there is little or no detectable virus replication following secondary challenge and there are no lung lesions associated with challenge (Larsen *et al.*, 2000). Exposure to live H1N1 and H3N2 viruses also conferred complete protection against an H1N2 with an unrelated HA protein (Van Reeth *et al.*, 2003), however vaccination with commercial killed vaccines containing H1N1 and H3N2 did not protect against H1N2 challenge (Reeth *et al.*, 2004). In studies using inactivated whole virus vaccines only partial protection was found following homologous challenge (Bikour *et al.*, 1996; Macklin *et al.*, 1998). These studies indicate that inactivated vaccines have limited ability to cross-protect against heterologous homosubtypic, or heterosubtypic viruses. Good protection can only be achieved when challenge and vaccine strain show cross-reactivity. The development of attenuated modified live-virus vaccine (MLV) or vector-based subunit vaccines for swine

that induce an immune response based on both humoral and cell mediated mechanisms are likely to improve homosubtypic and hetero-subtypic protection.

To evaluate the clinical relevance of *in vitro* serum cross-reactivity, we studied two H1 isolates, IA30 (H1N1) and MN03 (H1N2), with substantial genetic divergence in the HA gene and failure to cross-react in the HI assay (Vincent *et al.*, 2008) in more detail. Inactivated vaccines were prepared from both isolates and used to immunize two groups of conventional pigs. In addition, two groups of pigs were primed with live, virulent virus. The vaccinated pigs (either live or inactivated vaccine) were then challenged with the homologous and heterologous viruses. Both inactivated vaccines provided excellent protection against homologous challenge. However, the inactivated IA30 vaccine failed to protect against the heterologous MN03 challenge, whereas the MN03 vaccine was partially protective against the heterologous IA30 challenge. Surprisingly, 3 of the 9 pigs in the MN03-challenged, IA30-immunized group had substantially greater percentages of lung lesions compared to non-vaccinated MN03 challenge controls. This suggests that the IA30 inactivated vaccine may have potentiated the level of pneumonia when challenged with the heterologous MN03 virus. This was not true when MN03 vaccinated pigs were challenged with the IA30 virus. The potentiation of lung lesions may have been immune-mediated due to the induction of lower levels of IgA in conjunction with higher levels of IgG antibodies in the lungs of the three IA30-immunized pigs. The inactivated and live vaccines induced an isolate-specific serum HI response against homologous virus, but there was no cross-reactivity with heterologous viruses. We concluded from this study that divergent H1 viruses that do not cross-react serologically may not provide complete cross-protection when used as an inactivated vaccine. Although mild lung lesions consistent with SIV were seen in pigs primed with live IA30 or MN03 and challenged with MN03 or IA30, respectively, the live vaccination prevented virus shedding from the nose and no virus was isolated from the lungs in our experimental pig model (Vincent *et al.*, 2008). In summary, these results suggest that the use of live virus or a mucosal route for immunization may enhance the efficacy of vaccines and prevent virus shedding when used in the face of antigenically heterologous viruses of the same subtype.

Reverse genetics or the *de novo* synthesis of negative sense RNA viruses from cloned cDNA, has become a reliable laboratory method that provides a powerful tool for studying various aspects of the viral life cycle, the role of viral proteins in pathogenicity and the interplay of viral proteins with components of the host's immune system. It also opens the way to develop live attenuated virus vaccines and vaccine vectors. A reverse genetics system that allows the generation of influenza A viruses entirely from cloned cDNAs has been established (Fodor *et al.*,

1999; Hoffmann *et al.*, 2000; Neumann *et al.*, 1999). This technology allowed the generation of mouse-adapted viruses with mutations in the NS1 gene which exhibited an attenuated phenotype in cell culture, mice, and embryonated eggs (Talon *et al.*, 2000). The attenuation in these models was believed to be due to a loss of function of the viral NS1 protein, a type 1 interferon antagonist. We have investigated the role of the NS1 protein in the virulence of a SIV isolate in the natural host, the pig, producing various mutants encoding carboxy-truncated NS1 proteins. Similar to the other model systems, we found that these NS1 truncations decreased the ability of SIVs to prevent IFN- α/β synthesis in pig cells and conveyed attenuation in pigs (Solorzano *et al.*, 2005). We proposed NS1-mutated SIVs might have a great potential as live attenuated vaccine candidates against SIV infections of pigs (Solorzano *et al.*, 2005).

The development of attenuated MLV or vectored subunit vaccines for swine that induce a balanced immune response including humoral and cell mediated mechanisms are likely to improve homosubtypic and heterosubtypic protection. A cold-adapted live attenuated intranasal (IN) influenza vaccine has been approved in the U.S. for use in humans with results from clinical and field trials showing good efficacy (Belshe, 2004). A similar vaccine is available for horses as well (Townsend *et al.*, 2001). A prototype H3N2 SIV (Sw/A/TX/98) virus with a carboxy-terminal truncation of the NS1 gene starting at amino acid 126 (Δ 126) generated by reverse genetics has been shown to be highly attenuated in pigs, was not shed from the nose but was capable of stimulating an immune response (Solorzano *et al.*, 2005). The potential of this NS1 mutant, called TX98 NS1 Δ 126, for use as a MLV vaccine in pigs has been recently evaluated.

To evaluate the TX98 NS1 Δ 126 as an MLV vaccine, 4-week-old pigs were vaccinated and boosted with the TX98 NS1 Δ 126 MLV via the intratracheal route (Richt *et al.*, 2006). Pigs were challenged with wild type homologous H3N2 or heterosubtypic classical H1N1 SIVs and necropsied 5 days later. The MLV was highly attenuated and completely protected against challenge with the homologous virus. Vaccinated pigs challenged with the heterosubtypic cH1N1 virus demonstrated pathologic lung changes similar to the nonvaccinated H1N1 control pigs. However, vaccinated pigs challenged with cH1N1 had significantly reduced virus shedding from the respiratory tract when compared to nonvaccinated, cH1N1 challenged pigs. All vaccinated pigs developed a significant level of HI titer, serum IgG, and mucosal IgG and IgA antibodies against parental H3N2 SIV antigens (Richt *et al.*, 2006).

A separate study evaluated the efficacy of the TX98 NS1 Δ 126 MLV when used via the IN or intramuscular (IM) route and challenged with homologous virus (Fig. 4). Furthermore, pigs vaccinated via the IN route were also challenged with a homosubtypic, but genetically and

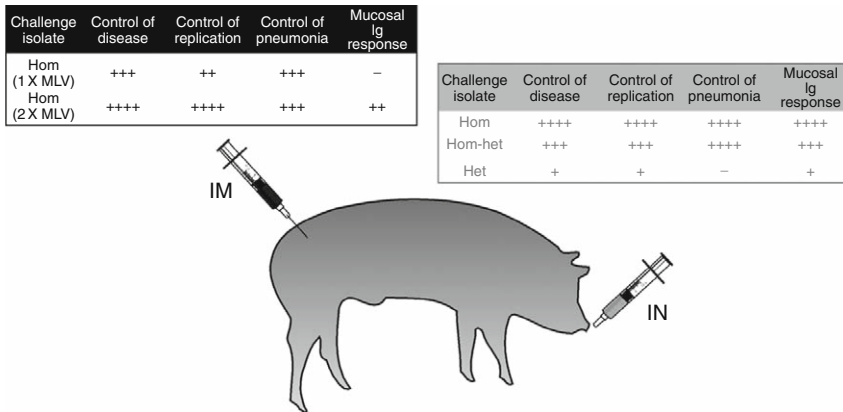


FIGURE 4 Influence of route and dose on the efficacy of the TX98 NS1 Δ 126 MLV against SIV. Challenge isolate: Hom = homologous wild type H3N2; Hom-het = homo-subtypic heterologous H3N2; and Het = heterosubtypic H1N1. Scale of 0–4+, with the greater number of + being most protective. Control of disease is based on clinical signs and rectal temperatures. Control of replication is based on virus titers from nasal swabs and bronchioalveolar lavage fluid. Control of pneumonia is based on percentage of macroscopic pneumonia and microscopic evaluation. Mucosal immunoglobulin (Ig) response is based on levels of IgG and IgA in the lung. IM = intramuscular route; vaccination results are summarized in the black box with one dose (1 \times) or two doses (2 \times). IN = intranasal route; vaccination results are summarized in the gray box.

antigenically heterologous H3N2 (CO99) and a rH1N1 (IA04) SIV that contained the TRIG cassette similar to the triple reassortant H3N2 viruses (Vincent *et al.*, 2007). A single dose of MLV administered intranasally conferred complete protection against homologous virus and nearly complete protection against the heterologous H3N2 CO99 virus challenge (Fig. 4). When challenged with the rH1N1 IA04 virus, MLV vaccinated animals displayed reduced fever and virus titers despite minimal reduction in lung lesions (Fig. 4). In vaccinated pigs, there was no serologic cross-reactivity by HI assays with the heterologous or heterosubtypic viruses. However, there appeared to be substantial cross-reactivity in antibodies at the mucosal level with the CO99 virus in MLV vaccinated pigs (Vincent *et al.*, 2007). It is apparent that a complex host response involving CMI and humoral mechanisms contribute to the immunity established via the TX98 NS1 Δ 126 MLV SIV vaccine and the immune response to MLV seems to be superior to that induced by inactivated influenza vaccines.

One of the primary reasons for vaccinating breeding sows with inactivated vaccines is to stimulate passive antibody transfer to the suckling pig. The level of protection is dependent on the level of maternal derived antibody (MDA). However, several studies have demonstrated that

MDA rarely prevents infection with influenza virus and only provides partial protection (Loeffen *et al.*, 2003; Renshaw, 1975). In addition, the presence of MDA interferes with a primary immune response to SIV, either by infection or vaccination (Kitikoon *et al.*, 2006; Loeffen *et al.*, 2003; Renshaw, 1975). We recently evaluated the ability of IN applied TX98 NS1 Δ 126 MLV to overcome maternal antibody interference when challenged with homologous (TX98 wild type) or heterologous (CO99) H3N2 virus. The MDA present in the vaccinated pigs was shown to interfere with the serologic SIV-antibody response to either an inactivated TX98 vaccine or the TX98 NS1 Δ 126 MLV; however, protection from challenge with homologous virus was demonstrated for both vaccines (Vincent, unpublished results). MDA reduced the efficacy of one-dose IN application of the MLV when compared to pigs vaccinated in the absence of MDA, although the virus levels in the respiratory tract were significantly reduced compared to nonvaccinated controls. The most remarkable finding from this study was the observation of a dramatic enhancement of disease and pneumonia in pigs which were vaccinated with an inactivated TX98-based vaccine in the presence of MDA, followed by challenge with heterologous CO99 virus. This was not seen in MDA positive pigs vaccinated with the MLV nor in MDA negative pigs given inactivated or MLV vaccine and challenged with heterologous H3N2 CO99 virus. Enhancement of pneumonia by inactivated vaccine used in the face of MDA with an H1N1 challenge was reported previously by Kitikoon *et al.* (2006), which supports our findings with H3N2 viruses. These results indicate a much more insidious role for MDA rather than simple interference with primary immune responses when using inactivated vaccines in young pigs.

Recombinant human adenoviruses have been demonstrated to be effective vectors for insertion of antigens from infectious agents for use as vaccine candidates in many species, including those of veterinary importance (Casimiro *et al.*, 2003; Elahi *et al.*, 1999; Eloit *et al.*, 1990; Mayr *et al.*, 2001; Pacheco *et al.*, 2005). Several of these vaccine candidates, specifically those created from human adenovirus serotype 5 (HAd5), have been shown to provide excellent protection from challenge with foot-and-mouth disease virus and SIV (Mayr *et al.*, 2001; Wesley *et al.*, 2004). Vaccination with HAd vectors has been shown to induce both humoral and cell-mediated immunity, making them potentially more effective than inactivated vaccines and more similar to the response elicited from MLV, reviewed in Gamvrellis *et al.* (2004). In addition, HAd vectored vaccines given by a mucosal route have been shown to provide superior, long lasting mucosal immunity (Baca-Estrada *et al.*, 1995). Replication-defective adenovirus recombinants were developed as potential vaccines against H3N2 influenza viruses (Wesley *et al.*, 2004). Pigs in the groups given the recombinant adenovirus expressing

HA protein developed high levels of virus-specific HI antibody by 4 weeks postvaccination. Pigs in the group vaccinated with recombinant viruses expressing both the HA and NP in a mixture were completely protected against homologous challenge, shown by the lack of nasal shedding of virus following challenge and by the lack of lung lesions at 1 week following the challenge infection. In addition, the efficacy of the HAd5 vaccine for protecting weaned pigs against SIV subtype H3N2 infection were evaluated when administered via two injection methods, either with a needle-free injection device or by traditional IM injection (Wesley and Lager, 2005). Traditional IM-administered vaccination induced consistently higher HI responses than vaccination via needle-free injection, but the differences were not significant. Likewise, traditional IM administration was superior at reducing nasal virus shedding except at the highest dose, at which both methods blocked virus replication. The severity of lung lesions was reduced in a dose-dependent manner by both vaccination methods. The replication-defective vaccine HAd5 virus was not transmitted to sentinel pigs (Wesley and Lager, 2005).

In addition to the success in naïve pigs, recombinant HAd5 vectored SIV vaccines were demonstrated to prime the immune system in the presence of MDA. Piglets with H3N2-specific MDA were sham-inoculated with a nonexpressing HAd5 vector or given a primary vaccination with replication-defective HAd5 expressing the HA and the NP of an H3N2 SIV subtype virus (Wesley and Lager, 2006). The HI titer of the sham-inoculated group showed continued antibody decay whereas piglets vaccinated with HAd5-SIV developed an active immune response by the second week postvaccination. At 4-weeks of age, when the HI titer of the sham-inoculated group had decayed, the sham-inoculated group and half of the HAd5 SIV vaccinated pigs were boosted with a commercial inactivated SIV vaccine. The boosted pigs that had been primed with the HAd5 expressing SIV genes in the presence of MDA had a strong anamnestic response while sham-inoculated pigs did not respond to the commercial vaccine. Two weeks after the booster vaccination the pigs were challenged with a heterologous virulent H3N2 SIV. The pigs primed with the HAd5-SIV vaccine and boosted with inactivated vaccine showed a reduction of clinical signs, reduced virus levels in the respiratory tract, and the absence lung lesions (Wesley and Lager, 2006). In contrast, MDA positive pigs not primed with the HAd5-SIV vaccine and only vaccinated with the inactivated vaccine demonstrated a vaccine failure (Wesley and Lager, 2006).

It is evident from the increasing number of novel subtypes and genetic variants isolated from pigs that controlling swine flu will only continue to be difficult. New strategies of vaccine development must be considered to keep up with the ever-evolving influenza viruses and to overcome the problem of maternal antibody interference with inactivated vaccines.

The demonstrated safety and efficacy of cold-adapted modified live virus vaccines in human and equine medicine has paved the way for investigating modified live vaccines in swine medicine. The few studies using different SIV vaccination concepts in pigs have shown that strain, route of administration, and use of vaccine additives can play a role in enhancing heterologous protection. Future studies are needed to address each of these areas. The use of reverse genetics to genetically engineer viruses with vaccine potential (live or inactivated) and to identify virulence genes will certainly help in this pursuit as will vectored vaccines. In order to gain a better understanding of homosubtypic and heterosubtypic vaccine efficacy, the CMI and humoral immune responses at the systemic and mucosal levels need to be included in future pig studies.

V. CONCLUSIONS AND OUTLOOK

The impact of influenza A in humans and animals, whether measured by morbidity, mortality, or economic losses, is significant. It is, therefore, essential to understand the mechanisms that allow these viruses to jump species barriers and establish themselves in new animal populations. The emergence of new subtypes of SIVs (hu-H1, H3N2, H4N6, and H2N3) in North American pigs has implications not only for pigs but also for the people who care for them. These newly emerging viruses are capable of epidemics at the herd or U.S. swine industry level since they are antigenically distinct from previously circulating and/or currently used commercial vaccine strains, are virulent in the pig and can infect and transmit from pig to pig. The potential for human infection as North American SIVs continue to drift, shift, and adapt to a mammalian host is unclear, but definitely remains a risk. It is increasingly evident that improved vaccination strategies with novel vaccine platforms are needed. Therefore, novel vaccine approaches using genetically engineered MLV and vectored vaccines are discussed in this review. The influenza epidemiology in North American pigs clearly indicates that the potential for pandemic influenza virus emergence exists not only in the traditionally considered "influenza hotbeds" of Southeast Asia ([Shortridge and Stuart-Harris, 1982](#)) but also in North America. This review underscores the need for vigilance in examining influenza A viruses from swine (and other species) for human pathogenic potential in addition to the major focus currently placed on AIVs. Although there does not appear to be a simple solution to the SIV problem in North America, some lessons can be learned from our and others experiences over the last two decades. People to pig, pig to people, pig to avian, avian to human, and avian to pig transmissions occur. Strategies that may reduce the risk for these transmission events should be employed in the swine industry, such as vaccination of workers

with occupational exposure to swine (Gray *et al.*, 2007); sick policies for workers, and the use of disposable respirators; bird-proofing swine facilities; and using only treated water for barn cleaning and consumption. The swine industry should be aware that the use of untreated pond or lake water can be a threat to animal health. Pig to people transmission must be emphasized as well. Education and caution for workers with occupational exposure as well as for those in the human health care system is critical for reducing and/or monitoring these transmission events. Our strength in monitoring and reacting to newly emerging or re-emerging subtypes in the swine or human population will be much greater as the veterinary and public health communities collaborate and engage together in these efforts.

ACKNOWLEDGMENTS

The authors thank the team of technical and animal care staff that has significantly contributed to the many studies described in this review. We thank Mike Marti for illustrations and Drs A. Garcia-Sastre, M. R. Gramer, and R.J. Webby for their continued support. Projects described in this review were funded by the USDA-Agricultural Research Service and in part with Federal funds from the National Institute Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN266200700005C and from the Center for Disease Control and Prevention, grant No. U01 CI000357-01.

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