

Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine

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Abstract

Two US swine influenza virus (SIV) isolates, A/Swine/Iowa/15/1930 H1N1 (IA30) and A/Swine/Minnesota/00194/2003 H1N2 (MN03), were evaluated in an *in vivo* vaccination and challenge model. Inactivated vaccines were prepared from each isolate and used to immunize conventional pigs, followed by challenge with homologous or heterologous virus. Both inactivated vaccines provided complete protection against homologous challenge. However, the IA30 vaccine failed to protect against the heterologous MN03 challenge. Three of the nine pigs in this group had substantially greater percentages of lung lesions, suggesting the vaccine potentiated the pneumonia. In contrast, priming with live IA30 virus provided protection from nasal shedding and virus replication in the lung in MN03 challenged pigs. These data indicate that divergent viruses that did not cross-react serologically did not provide complete cross-protection when used in inactivated vaccines against heterologous challenge and may have enhanced disease. In addition, live virus infection conferred protection against heterologous challenge.

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1. Introduction

Three major subtypes of swine influenza virus (SIV) currently circulate in US swine populations,

H1N1, H1N2, and H3N2, with multiple genetic and antigenic variants within each subtype (Choi et al., 2002b; Vincent et al., 2006; Webby et al., 2004). Until 1998, SIV in North America was relatively stable with only one predominant circulating subtype, known as the classical swine H1N1 (cH1N1) (Easterday and Reeth, 1999). However in 1998, H3N2 isolates with human, avian, and swine genes were identified in

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multiple swine populations across the US (Zhou et al., 1999, 2000), and reassortants between the classical H1N1 and the newly introduced H3N2 viruses rapidly appeared. The reassortments produced H1 swine viruses with the hemagglutinin (HA) and neuraminidase (NA) from the cH1N1 swine virus and the internal genes from the H3N2 viruses (rH1N1) or the HA from the cH1N1 swine virus and the NA and internal genes from the H3N2 viruses (H1N2) (Gramer, 2006; Janke et al., 2004; Karasin et al., 2002; Webby et al., 2004). With the acquisition of the avian polymerase genes, an increase in the rate of genetic change in North American swine influenza isolates appears to have occurred in both H3 and H1 virus subtypes. Reassortant H1 viruses are reported to be infecting and causing disease in herds that have been routinely vaccinated with commercial vaccines containing cH1N1, despite the observation that the HA gene in the circulating H1N2 and rH1N1 viruses originated from the cH1N1 virus.

Antibodies that block binding of the HA protein to host receptors are thought to be responsible for much of the protection conferred by natural or vaccine induced immunity to homologous or antigenically related heterologous viruses, and this is most commonly measured using the hemagglutinin inhibition (HI) assay. However, antibodies raised against non-HI epitopes or other viral proteins (Epstein et al., 1997; Mozdzanowska et al., 1997, 1999; Tumpey et al., 2001), as well as cell mediated immunity (CMI) (Flynn et al., 1998, 1999; Liang et al., 1994; Nguyen et al., 1999; Taylor and Askonas, 1986; Wraith et al., 1987), clearly play a role in the heterologous cross-reactive immune responses (Het-I) against influenza virus infection. However, these studies have been predominantly conducted in mice, and reports on the *in vivo* evaluation of cross-protection between antigenically distinct viruses in a natural host are limited. In the swine host, cross-protection studies have been reported, but primarily with European isolates that either had some degree of serologic cross-reactivity or in models that did not directly compare live versus inactivated vaccine priming (Heinen et al., 2001a,b; Van Reeth et al., 2003, 2001).

We have reported previously that US H1 viruses isolated since 1998 have variable cross-reactivity with immune-serum raised against genetic variant H1 viruses, especially those isolated prior to 1998

(Vincent et al., 2006). In the study reported here, two isolates, IA30 and MN03, were chosen to evaluate the efficacy of killed vaccines against live virus challenge with homologous and heterologous challenge. IA30 and MN03 were shown to have no cross-reactivity either as anti-sera or antigen in the HI assay, therefore providing a model for viruses within an HA subtype that are genetically related but fail to cross-react in the HI assay. In addition, priming by live virus was compared to protection elicited by the inactivated vaccines. Vaccine efficacy was measured by nasal virus shedding and by replication of the virus in the lower respiratory tract, evidence of pneumonia, and the humoral immune response in serum and at the respiratory mucosa. We report that one of the inactivated vaccines failed to protect against heterologous challenge and led to enhancement of pneumonic lesions in one-third of the pigs. Serum HI titers were not predictive of heterologous protection, nor were they correlated with antibody levels in the lung, especially in animals with live exposure.

2. Materials and methods

2.1. Experimental design

Eighty-three 2-week-old conventional pigs obtained from a herd free of SIV and PRRSV were randomly divided into treatment groups. The experimental design is described in Table 1. All pigs were treated with ceftiofur hydrochloride (Pharmacia & Upjohn, Kalamazoo, MI) for three consecutive days to reduce bacterial contaminants prior to the start of the study. The appropriate groups of pigs were vaccinated with experimentally prepared inactivated vaccines at 3 and 6 weeks of age. At 8 weeks of age (2 weeks following the second vaccination in vaccinated groups), pigs were challenged with live virus or sham inoculum intratracheally while anesthetized with an intramuscular injection of ketamine, xylazine, tiletamine and zolazepam. In a subsequent experiment to evaluate the effect of live challenge and recovery as compared to immunization with killed vaccine, two groups of eight pigs were immunized with live virus at 3 weeks of age and challenged via the intratracheal route 5 weeks after the vaccination with live virus at approximately 8 weeks of age. No boost was given to

Table 1
Experimental design

Group	Vaccine virus	Challenge virus	<i>N</i>	Weeks of age at priming	Weeks of age at boost	Weeks of age at challenge
1	None	Sham	5	n.d.	n.d.	8
2	None	IA30	8	n.d.	n.d.	8
3	None	MN03	9	n.d.	n.d.	8
4	IA30	Sham	5	3	6	8
5	MN03	Sham	5	3	6	8
6	IA30	IA30	8	3	6	8
7	IA30	MN03	9	3	6	8
8	MN03	MN03	9	3	6	8
9	MN03	IA30	9	3	6	8
10	Live IA30 ^a	MN03	8	3	n.d.	8
11	Live MN03 ^a	IA30	8	3	n.d.	8

n.d.: not done.

^a Live infection and recovery conducted in a separate experiment.

the live-immunized group. Challenge groups were housed in individual isolation rooms and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. All animals were humanely euthanized 5 days post-infection (dpi) with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) and exsanguination.

2.2. Viruses and vaccine preparation

The viruses used in this study were A/Swine/Iowa/15/1930 H1N1 (IA30) and A/Swine/Minnesota/00194/2003 H1N2 (MN03). These two H1 isolates were shown to have no cross-reactivity in the HI assay with hyper-immunized swine sera in a previous study (Vincent et al., 2006). Challenge isolates were grown in specific pathogen free embryonated chicken eggs (Charles River Laboratories), and allantoic fluids were harvested to collect the viruses. To make pig-adapted challenge inoculum, allantoic fluids were given intratracheally at a dose of 2 ml of 1×10^5 50% tissue culture infective dose (TCID₅₀)/ml to anesthetized 2-week-old caesarian derived-colostrum deprived pigs. Sham inoculated pigs were given negative allantoic fluid from age-matched eggs. The lungs from infected and sham inoculated pigs were lavaged with Minimal Essential Medium (MEM) to collect bronchioalveolar lavage fluid (BALF) at necropsy on 3 dpi for use as challenge material in the study. To prepare the vaccines, MDCK-grown viruses at approximately 512 HA units and

$10^{7.5}$ TCID₅₀/ml were diluted to 10^6 TCID₅₀/ml. The viruses were then inactivated in media using the sterilize setting in a ultraviolet cross-linking chamber (GS Gene Linker, Bio-Rad, Hercules, CA), and a commercial adjuvant was added at a 1:1 ratio (Emulsigen, MVP Laboratories, Inc., Ralston, NE). Inactivation of the virus was confirmed by failure to replicate in two serial passages on MDCK cells. The appropriate vaccine was given intramuscularly to groups 4–9 at a dose of 2 ml per pig at 3 and 6 weeks of age. Each dose contained approximately 10^6 TCID₅₀ units of virus before inactivation. Groups 10 and 11 received one live exposure at 3 weeks of age with 2 ml of 1×10^5 TCID₅₀/ml of the appropriate pig-passed virus given intratracheally while anesthetized. At approximately 8 weeks of age, pigs in groups 2, 3, 6, 7, 8, 9, 10, and 11 were challenged with 2 ml of 1×10^5 TCID₅₀/ml of the appropriate pig-passed virus given via the intratracheal route while anesthetized. Groups 1, 4, and 5 were challenged with sham BALF at a similar dilution.

2.3. Clinical observation and sampling

Pigs were observed daily for clinical signs, and rectal temperatures were taken daily from –2 to 5 dpi. Nasal swabs were taken on 0, 3, and 5 dpi, placed in 2 ml MEM and frozen at –80 °C until study completion. After euthanasia, each lung was lavaged with 50 ml MEM to obtain BALF. Each nasal swab sample was subsequently thawed and vortexed for 15 s, centrifuged for 10 min at $640 \times g$ and the

supernatant passed through 0.45 μm filters to reduce bacterial contaminants. An aliquot of 200 μl of the filtrate was plated onto confluent phosphate buffered saline (PBS)-washed MDCK cells in 24-well plates. After 1 h incubation at 37 °C, 200 μl serum-free MEM supplemented with 1 $\mu\text{g/ml}$ TPCK trypsin and antibiotics was added. All wells were evaluated for cytopathic effect (CPE) between 24 and 48 h and subsequently frozen. Aliquots of 200 μl from the 24-well frozen–thawed plates were transferred onto confluent MDCK cells in 48-well plates and again evaluated for CPE at between 24 and 48 h post-infection. Ten-fold serial dilutions in serum-free MEM supplemented with TPCK trypsin and antibiotics were made with each BALF sample and virus isolation positive nasal swab filtrate sample. Each dilution was plated in triplicate in 100 μl volumes onto PBS-washed confluent MDCK cells in 96-well plates. Plates were evaluated for CPE between 48 and 72 h post-infection. A TCID₅₀ was calculated for each sample using the method of [Reed and Muench \(1938\)](#).

2.4. Pathologic examination of lungs

At necropsy, lungs were removed and evaluated for the percentage of the lung affected with purple-red consolidation typical of SIV infection. The percentage of the surface affected with pneumonia was visually estimated for each lung lobe, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume ([Halbur et al., 1995](#)). Tissue samples from the trachea and right cardiac lung lobe and other affected lobes were taken and fixed in 10% buffered formalin for histopathologic examination. Tissues were routinely processed and stained with hematoxylin and eosin. Lung sections were given a score from 0–3 to reflect the severity of bronchial epithelial injury based on previously described methods ([Richt et al., 2003](#)). The lung sections were scored according to the following criteria: (0.0) no significant lesions; (1.0) a few airways affected with bronchiolar epithelial damage and light peribronchiolar lymphocytic cuffing often accompanied by mild focal interstitial pneumonia; (1.5) more than a few airways affected (up to 25%); (2.0) 50% airways affected often with interstitial pneumonia; (2.5) approximately 75% airways affected, usually with significant interstitial pneumonia; (3.0)

greater than 75% airways affected, usually with interstitial pneumonia. A single pathologist scored all slides and was blinded to the treatment groups.

SIV-specific antigen was detected in lung tissues using a previously described immunohistochemical (IHC) method with minor alterations ([Vincent et al., 1997](#)). Briefly, tissue sections were deparaffinized and hydrated in distilled water. Slides were quenched in 3% hydrogen peroxide for 10 min, rinsed three times in de-ionized water and treated in 0.1% proteinase K for 2 min. Slides were then rinsed twice in de-ionized water and once in Tris-buffered saline (TBS). SIV-specific monoclonal antibody HB65 (ATCC, Manassas, VA), specific for the nucleoprotein of influenza A viruses, was applied at 1:1000 dilution and slides were incubated at room temperature for 1 h. To detect positive signals, bound MAbs were stained with peroxidase-labeled anti-mouse IgG followed by chromogen using the DAKO Envision IHC System (DAKO, Carpinteria, CA) according to the manufacturer's instruction. The slides then were rinsed in de-ionized water and counterstained with Gill's hematoxylin. Positive IHC signals were scored according to the following criteria: 0, no signal present; 1, only a few cells were positive in an occasional airway; 2, only a few cells were positive in scattered airways; 3, moderate numbers of cells were positive in an occasional airway. 4, moderate numbers of cells were positive in scattered airways and alveoli.

2.5. Serologic and mucosal antibody assays

Serum samples were collected by jugular venipuncture at the following time-points: pre-vaccination, pre-boost, pre-challenge, and at necropsy. For use in the HI assay, sera were heat inactivated at 56 °C and treated to remove non-specific agglutinators with a 20% suspension of Kaolin (Sigma Aldrich, St. Louis, MO) followed by adsorption with 0.5% turkey red blood cells (RBC). The HI assays were done with the IA30 H1N1 and MN03 H1N2 viruses as antigens and turkey RBC using standard techniques ([Palmer et al., 1975](#)).

An ELISA used to detect SIV-specific antibodies present in the respiratory tract or in the serum was performed as previously described ([Larsen et al., 2000](#)) with slight modifications. The BALF samples from 5 dpi were incubated at 37 °C for 1 h with an equal volume of 10 mmol dithiothreitol (DTT) to

disrupt mucus present in the fluids. Serum samples were diluted 1:1000 in PBS. Independent assays were run using the IA30 H1N1 and MN03 H1N2 as ELISA antigen. Concentrated wild type virus was resuspended in Tris–EDTA basic buffer, pH 7.8, and diluted to a hemagglutination (HA) concentration of 100 HA units/50 μ l. Immulon-2HB 96-well plates (Dynex, Chantilly, VA) were coated with 100 μ l of SIV antigen and incubated at room temperature overnight. Plates were blocked for 1 h with 100 μ l of 10% BSA in PBS and washed three times with 0.05% Tween 20 in PBS (PBS-T). The assays were performed on each BALF or serum sample in triplicate. Plates were incubated at room temperature for 1 h, washed three times with PBS-T, then incubated with peroxidase-labeled goat anti-swine IgA (Bethyl, Montgomery, TX), IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), IgG1 (Serotec, Raleigh, NC), or IgG2 (Serotec) at 37 °C for 1 h. 2,2'-Azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS)-peroxide was added as the substrate (Kirkegaard & Perry Laboratories) and optical density (OD) was measured at 405 nm wavelength with an automated ELISA reader. Antibody levels were reported as the mean OD and the mean OD of each treatment group was compared.

2.6. Statistical analysis

Macroscopic pneumonia scores, microscopic pneumonia scores, \log_{10} transformed BALF and nasal swab virus titers, ELISA OD readings, and

\log_2 transformations of HI reciprocal titers were analyzed using analysis of variance (ANOVA) with a p -value ≤ 0.05 considered significant (JMP, SAS Institute, Cary, NC). Response variables shown to have a significant effect by treatment group were subjected to pair-wise comparisons using the Tukey–Kramer test.

3. Results

3.1. Clinical evaluation and pneumonia scores

Clinical signs were mild in the 8–9-week-old pigs for all of the IA30 H1N1 and the MN03 H1N2 challenge groups regardless of vaccination status. Although there were slight differences for rectal temperatures between treatment groups and in individual animals, no groups had mean febrile temperatures (≥ 40.2 °C) for any day post challenge as defined by two standard deviations above the mean rectal temperatures before challenge (data not shown). In addition, the macroscopic and microscopic pneumonia scores were quite mild, even in the non-vaccinated challenge control groups (groups 2 and 3) (Table 2). The group vaccinated against IA30 H1N1 and challenged with the heterologous MN03 H1N2 (group 7) had significantly greater macroscopic and microscopic lung lesions over all other treatment groups, including the non-vaccinated MN03 challenge control group (group 3). Within group 7, six of the pigs

Table 2
Lung lesions and \log_{10} virus titers in the BALF at 5 dpi and in nasal swabs (NS) at 3 and 5 dpi^a

Group	Macroscopic pneumonia (%)	Microscopic pneumonia (0–3)	BALF virus titers	NS virus titers 3 dpi	NS virus titers 5 dpi
1 NV/NC	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
2 NV/IA30	1.9 \pm 0.6 a	1.3 \pm 0.4 b	4.8 \pm 0.4 b	0.0 \pm 0.0 a	0.0 \pm 0.0 a
3 NV/MN03	3.4 \pm 0.5 a	0.5 \pm 0.2 ab	6.4 \pm 0.1 c	4.2 \pm 0.2 b	5.0 \pm 0.3 b
4 IA30/NC	0.1 \pm 0.1 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
5 MN03/NC	0.1 \pm 0.1 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
6 IA30/IA30	0.6 \pm 0.4 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
7 IA30/MN03	13.6 \pm 4.7 b	2.9 \pm 0.1 c	5.8 \pm 0.2 bc	1.7 \pm 0.6 c	4.1 \pm 0.3 c
8 MN03/MN03	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
9 MN03/IA30	1.1 \pm 0.5 a	0.2 \pm 0.2 a	1.2 \pm 0.6 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a
10 IA30/MN03 ^b	3.5 \pm 0.9 a	0.3 \pm 0.2 a	0.0 \pm 0.0 a	n.d.	0.0 \pm 0.0 a
11 MN03/IA30 ^b	0.6 \pm 0.2 a	0.0 \pm 0.0 a	0.0 \pm 0.0 a	n.d.	0.0 \pm 0.0 a

n.d. = not done.

^a Mean \pm standard error. Group mean values with different letters within a column are significantly different at $p < 0.05$. Treatment for each group is indicated by vaccine/challenge strain and (NV) non-vaccinated; (NC) sham-challenged.

^b Live infection and recovery conducted in a separate experiment.

had macroscopic lesions similar to the non-vaccinated control group (group 3). However, the remaining three pigs had markedly increased severity of lesions with greater lung involvement. The percentage of lung involvement was 36.5, 26.9, and 32.0% in the three affected pigs compared to an average of 4.5% for the other six pigs in group 7 (Fig. S1). In addition, the distribution of viral antigen in diseased lung sections as determined by IHC differed between groups 3 and 7 pigs (Fig. S2). The location of influenza antigen in the MN03 challenge control group tended to be in tracheal or large airway epithelial cells only. However, in pigs immunized with IA30 and challenged with MN03 (group 7), the lung sections consistently demonstrated a broader distribution of antigen, with viral antigen in tracheal epithelium, large and small airway epithelium, as well as within alveoli. There were no lung lesions in the homologous vaccination and challenge groups (groups 6 and 8) or in the non-challenged control groups (groups 1, 4, and 5). The groups primed with live virus and challenged with heterologous virus (groups 10 and 11) demonstrated mild macroscopic pneumonia, although not different than the challenge controls, and very mild to no microscopic lesions characteristic of influenza pneumonia.

3.2. Virus levels in the lung and nasal secretions

Despite the mild clinical signs and lung lesions in the challenge control groups (groups 2 and 3), viral replication was detected in the lungs of all pigs in these groups with titers similar to that in a previous study using a 4-week-old pig model (Vincent et al., 2006), demonstrating a successful virus challenge with both isolates (Table 2). The homologous vaccination and challenge groups (groups 6 and 8) showed complete protection against virus replication in the lung. However, all pigs in the IA30 vaccinated and MN03 challenge group (group 7) had similar virus titers in the lung as the challenge control group (group 3). In the reciprocal heterologous challenge group (group 9), three pigs in the group receiving MN03 vaccine followed by challenge with IA30 had virus replicating in the lung at 5 dpi.

The IA30 H1N1 virus did not efficiently replicate in the nasal cavity in the 8–9-week-old pig model (Table 2), consistent with results previously demonstrated in the 4-week-old pig model. In contrast, the

MN03 H1N2 virus was detected at moderately high titers in the nasal cavity of all pigs on 3 and 5 dpi in the challenge control group (group 3). In addition to a failure to reduce lung lesions and virus titers in the lung, six of the nine pigs in the IA30 vaccinated and MN03 challenged group (group 7) were positive for nasal shedding at 3 dpi and all of the pigs had substantial titers at 5 dpi. It should be noted that the IA30 vaccine was effective in preventing virus replication in the lung against homologous challenge. None of the pigs in groups 10 and 11 that were primed with live virus and challenged with heterologous virus had virus detected in nasal swabs or in the lungs.

3.3. Serologic and local antibody responses

There were no positive HI titers detected in any pig prior to vaccination or in any non-vaccinated pig throughout the study, indicating the pigs were negative for SIV viruses and antibodies prior to the start of the study (Fig. 1). As expected, isolate specific HI antibody titers developed only in pigs primed with killed vaccine or live virus, with no cross-reactivity between sera from vaccinated pigs and heterologous antigens prior to challenge. However, a rise in IA30-specific HI titers could be seen in the group primed with live MN03 and challenged with IA30 (group 11) by 5 dpi. This is in contrast to group 10 pigs that were primed with live IA30 and both groups primed with killed vaccine and challenged with heterologous virus, as no pigs in any of these groups had a rise in heterologous antigen-specific HI titers at any time point. Results from the IA30 HI assays are summarized in Fig. 1A and the MN03 HI assays are summarized in Fig. 1B. In contrast to the lack of cross-reacting HI antibodies induced by the inactivated vaccines, cross-reacting IgG antibodies were detected in the sera of vaccinated and challenged pigs as measured by the whole virus ELISA (Table 3). The cross-reacting antibodies were seen in the vaccinated only groups (groups 4 and 5), as well as the homologous challenge groups (groups 6 and 8) and in the heterologous challenge groups (groups 7, 9, 10, and 11). The cross-reacting IgG antibodies in the serum seemed primarily induced by vaccination, since challenge by homologous or heterologous virus did not significantly alter the OD levels between the

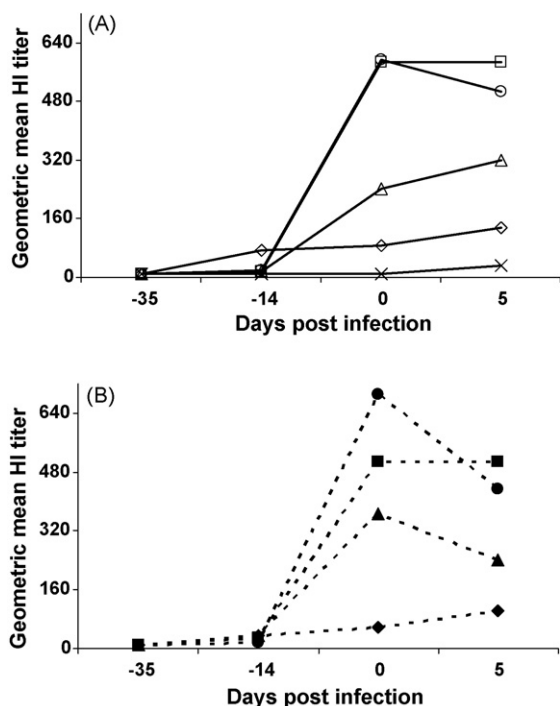


Fig. 1. (A) Reciprocal geometric mean hemagglutination inhibition (HI) titers against the IA30 H1N1 antigen. The groups are represented by (△) IA30 vaccine only; (□) IA30 homologous vaccine and challenge virus; (○) IA30 vaccine virus and MN03 challenge virus; (◇) IA30 live vaccine and MN03 challenge virus; (×) MN03 live vaccine and IA30 challenge virus. (B) Reciprocal geometric mean HI titers against the MN03 H1N2 antigen. The groups are represented by (▲) MN03 vaccine only; (■) MN03 vaccine and homologous challenge virus; (●) MN03 vaccine virus and IA30 challenge virus; (◆) MN03 live vaccine and IA30 challenge virus. There were no positive HI titers against either virus in any non-vaccinated pig and there was no cross-reactivity between the sera from killed virus vaccinated pigs and heterologous antigens. In contrast, the group primed with live MNO3 had begun to have a rise in IA30-specific HI titers by 5 dpi. The group primed with live IA30 did not have a rise in MN03-specific HI titers at 5 dpi.

vaccinated and challenged groups and the vaccinated only groups.

In the lung, vaccination by the inactivated vaccines induced a rise in IgG against homologous virus, although not always statistically different from the non-vaccinated, non-challenged control group (group 1) (Table 3). The inactivated vaccine induced rise in BALF IgG against heterologous virus was less apparent compared to serum. In addition, groups 7 and 9 that received inactivated vaccines and heterologous challenge demonstrated a boost in IgG levels

against the priming antigen without a subsequent rise in antibodies against the challenge virus. This is in contrast to the respective homologous vaccinated and challenged groups. Groups 10 and 11 primed with live virus had significantly higher local IgG levels against the homologous virus as well as cross-reacting antibodies against heterologous virus.

The IgA levels in BALF are summarized in Table 4. The inactivated vaccines alone did not induce significant rises in IgA levels in the lung, however vaccination and challenge tended to increase IgA levels against the ELISA antigen homologous to the vaccine by 5 dpi (groups 6 and 7 with IA30 and groups 8 and 9 with MN03). This was most pronounced in the groups vaccinated and challenged with heterologous virus (groups 7 and 9). Similar to the local IgG levels, there was a substantial rise in IgA antibodies in the live infection and recovery groups, and the IgA appeared to be more cross-reactive compared to the IgA induced by inactivated vaccine.

Of interest, the boost in local IgG antibody levels in heterologous challenged animals was most pronounced in the three pigs in group 7 with the marked increase in macroscopic lung lesions, with an average OD value of 0.97 against the heterologous MN03 and 1.26 against the homologous IA30 compared to 0.50 and 0.56 in the remaining six pigs, respectively (Fig. 2). Similarly, IgG1 mean OD level against IA30 was 0.41 in the three affected pigs compared to 0.20 in the remaining pigs, whereas the IgG1 levels against MN03 were also equivocal between the high and low lesion pigs of group 7. Similarly, the IgG2 mean OD level against IA30 was 0.60 in the three affected pigs compared to 0.33 in the remaining pigs, and levels against MN03 were equivocal between the high and low lesion pigs of group 7. In stark contrast to the elevated IgG levels in the lungs of the three high lesion pigs, the average OD for IgA in the affected pigs was dramatically lower, with 0.39 against the homologous IA30 and 0.25 against the heterologous MN03 compared to 1.19 and 0.81 in the remaining pigs, respectively.

4. Discussion

This study demonstrates a lack of cross-protection between two US H1 isolates of swine influenza virus used in inactivated vaccines. The two isolates were

Table 3
Isolate specific IgG levels against whole virus in serum and BALF at 5 dpi^a

Group		Serum IA30	Serum MN03	BALF IA30	BALF MN03
1	NV/NC	0.34 ± 0.07 a	0.28 ± 0.06 a	0.15 ± 0.05 abc	0.47 ± 0.04 a
2	NV/IA30	0.51 ± 0.06 a	0.39 ± 0.04 a	0.05 ± 0.02 ab	0.45 ± 0.05 a
3	NV/MN03	0.43 ± 0.03 a	0.35 ± 0.02 a	0.03 ± 0.02 a	0.38 ± 0.05 a
4	IA30/NC	1.82 ± 0.15 cde	1.08 ± 0.26 bc	0.43 ± 0.14 bcd	0.67 ± 0.14 abc
5	MN03/NC	1.26 ± 0.26 bc	1.65 ± 0.13 cd	0.23 ± 0.03 abc	1.00 ± 0.15 bc
6	IA30/IA30	2.06 ± 0.04 e	1.53 ± 0.19 cd	0.49 ± 0.07 cd	0.56 ± 0.02 ab
7	IA30/MN03	1.91 ± 0.04 de	1.28 ± 0.10 bcd	0.79 ± 0.16 de	0.65 ± 0.10 ab
8	MN03/MN03	1.12 ± 0.17 b	1.65 ± 0.11 d	0.18 ± 0.04 abc	0.72 ± 0.10 ab
9	MN03/IA30	1.07 ± 0.14 b	1.66 ± 0.07 d	0.42 ± 0.06 c	1.09 ± 0.12 c
10	IA30/MN03 ^b	1.50 ± 0.06 bcd	0.95 ± 0.05 b	1.29 ± 0.04 f	1.66 ± 0.04 d
11	MN03/IA30 ^b	1.07 ± 0.12 b	1.35 ± 0.11 bcd	1.00 ± 0.07 ef	1.71 ± 0.05 d

^a Mean OD ± standard error in the whole virus ELISA. Sample type and virus used as antigen indicated at the top of each column. Group mean values with different letters within a column are significantly different at $p < 0.05$. Treatment for each group is indicated by vaccine/challenge strain and (NV) non-vaccinated; (NC) sham-challenged.

^b Live infection and recovery conducted in a separate experiment.

chosen for evaluation in a vaccination and challenge study due to their lack of cross-reactivity with sera from heterologous primed animals in the HI assay as a model for protection against antigenically drifted influenza viruses. The lack of serum HI cross-reactivity was confirmed in this study, both in pigs primed with inactivated vaccine and in pigs primed by infection and recovery. The lack of cross-protection, however, was primarily limited to parenteral priming with the IA30 inactivated vaccine and challenge with MN03. In contrast, infection and recovery conferred

complete heterologous protection from shedding of virus from the nose and virus replication in the lungs at 5 dpi. In addition, an enhancement of pneumonia was demonstrated in one-third of the inactivated IA30-vaccinated pigs challenged with MN03. This enhancement has been reproduced in subsequent studies (data not shown). The failure was not complete with both vaccines, as priming with the inactivated MN03 vaccine conferred a greater percent reduction in IA30 virus titers in the lungs as compared to the reciprocal heterologous challenge group.

Table 4
Isolate specific IgA levels against whole virus in BALF at 5 dpi^a

Group		BALF IA30	BALF MN03
1	NV/NC	0.41 ± 0.11 abcd	0.63 ± 0.17 ab
2	NV/IA30	0.11 ± 0.04 a	0.28 ± 0.07 a
3	NV/MN03	0.06 ± 0.02 a	0.17 ± 0.03 a
4	IA30/NC	0.29 ± 0.10 abc	0.35 ± 0.14 ab
5	MN03/NC	0.26 ± 0.09 ab	0.56 ± 0.11 ab
6	IA30/IA30	0.83 ± 0.09 de	0.87 ± 0.12 bc
7	IA30/MN03	0.92 ± 0.15 e	0.62 ± 0.12 ab
8	MN03/MN03	0.53 ± 0.05 bcd	0.84 ± 0.07 b
9	MN03/IA30	0.71 ± 0.12 cde	1.33 ± 0.20 c
10	IA30/MN03 ^b	1.88 ± 0.03 f	2.05 ± 0.07 d
11	MN03/IA30 ^b	1.77 ± 0.04 f	2.16 ± 0.03 d

^a Mean OD ± standard error in the whole virus ELISA. Sample type and virus used as antigen indicated at the top of each column. Group mean values with different letters within a column are significantly different at $p < 0.05$. Treatment for each group is indicated by vaccine/challenge strain and NV = non-vaccinated and NC = sham-challenged.

^b Live infection and recovery conducted in a separate experiment.

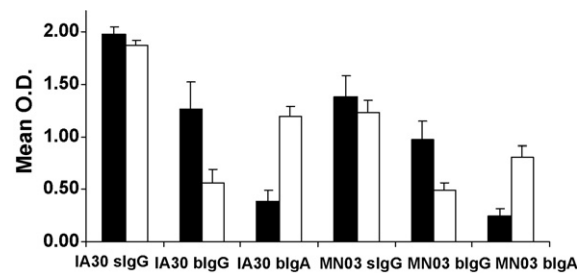


Fig. 2. Mean OD readings for the three pigs in group 7 with enhanced macroscopic lung lesions (black bar) compared to mean OD readings for the remaining six pigs in group 7 (white bar). The virus antigen coated on the ELISA plate is indicated on the x-axis with sIgG: serum IgG; bIgG: BALF IgG; bIgA: BALF IgA. The three affected pigs had higher total IgG levels against IA30 and MN03 in the BALF compared to their group-mates, whereas the serum levels were nearly equivalent. However, in distinct contrast to IgG levels, the three high lesion pigs had lower IgA levels against both the priming and challenge viruses in the lower respiratory tract. Error bar represents standard error of the mean.

In previous studies conducted in Europe using the swine model, immunity induced by vaccination with an H1N1 or an avian-like swine H1N1 was shown to elicit partial to total protection against a drifted avian-like swine H1N1 challenge when the pre-challenge HI titers were sufficiently high, although more antigenically similar isolates provided more effective protection (Van Reeth et al., 2001). The European study suggested that vaccines that induce high HI titers might be more effective against drifted or antigenically divergent viruses. However, the challenge isolate was cross-reactive in HI assays with anti-sera induced by the human-like H1N1 in the commercial vaccine and with a drifted H1N1 isolate present in one experimental vaccine. Thus, it was not clear if high HI titers would have been effective in providing protection against viruses that fail to cross-react in the HI assay. The inactivated vaccines used in the study reported here induced HI titers with averages greater than 1:160 against homologous antigen, yet there appeared to be no correlation between HI titer and cross-protection against the heterologous antigen.

Other European swine studies have demonstrated that immunity induced by a live exposure can provide Het-I. Coinfection of H1N1 and H3N2 SIV conferred complete protection against an H1N2 with an unrelated HA gene and no serologic cross-reactivity with the priming H1N1 (Van Reeth et al., 2003). The immunity induced by a live exposure with an H1N1 followed by challenge with a heterosubtypic H3N2 was shown to reduce viral shedding and transmission in pigs (Heinen et al., 2001a). In addition, an anamnestic humoral response was shown against the extracellular domain of the M2 viral protein in the H1N1 primed and H3N2 challenged group. This suggests that challenge with live virus can provide Het-I that is effective in reducing viral load and/or duration of shedding and could be in part due to the humoral response to conserved viral antigens such as M2. The Het-I conferred by live exposure seemed to be independent of HI-antibody levels to the priming virus in these previous studies as well as the data we report here. As the ELISA used in our study utilized whole virus antigen, it is likely that conserved epitopes on the surface proteins such as HA and NA, and to a lesser degree M2, were involved in the cross-reactivity seen in the mucosal antibodies primarily from live-primed animals. It is unknown the role antibody to

internal viral proteins such as NP played in the mucosal response measured by the whole virus ELISA described in this report.

To address the role that mucosal antibodies play in immunity against influenza infections, the European researchers conducted an additional study in pigs to compare the efficacy of an inactivated vaccine to the efficacy of live challenge and recovery in protection against a drifted H3N2 (Heinen et al., 2001b). Indeed, it was demonstrated that mucosal exposure of live virus was superior in reducing virus excretion and in inducing mucosal IgA directed against the conserved nucleoprotein (NP) compared to the inactivated vaccine. The killed vaccine induced high serum HI and neutralizing antibodies that cross-reacted to the drifted H3N2 despite only conferring partial protection, again suggesting that serum antibody levels may not be an accurate measure of immunity at the respiratory mucosa. In each of these European studies in the swine host, assessment of macroscopic or microscopic lung lesions was not reported nor was inactivated vaccine enhancement of macroscopic lung lesions described.

Most recently, an NS-1 truncated mutant H3N2 SIV has been demonstrated to provide partial Het-I against a heterosubtypic H1N1 when used as a MLV given via the respiratory mucosal route (Richt et al., 2006). Cross-reacting antibodies against the heterosubtypic virus were seen in the lung by 5 days post-infection while there was no serum HI cross-reactivity. However, the MLV was not compared with inactivated vaccines.

The IA30 H1N1 used in this study is the earliest isolated influenza virus from swine and has become the prototypic classical swine H1N1 virus. The MN03 H1N2 isolate was demonstrated to have considerable drift in the HA gene compared with IA30, although the HA from MN03 is swine in origin, with 83% identity at the nucleotide level (data not shown). The genetic composition of the MN03 H1N2 is the result of reassortant events between classical swine H1N1 viruses and human-, swine-, and avian-reassortant H3N2 viruses. In US H1N2 swine viruses studied to date, the HA, M, NP, and NS genes are of classical swine H1N1 origin, and the NA, PA, PB1 and PB2 genes are of human or avian origin (Choi et al., 2002a; Karasin et al., 2002). The M and NP genes have been demonstrated to be conserved between influenza

isolates (Gorman et al., 1991; Ito et al., 1991), and their proteins are reported to be involved in heterologous immunity (Flynn et al., 1998; Neiryneck et al., 1999; Scherle and Gerhard, 1986; Slepishkin et al., 1995; Ulmer et al., 1993; Wesley et al., 2004). A serologic profile of pigs vaccinated with an inactivated vaccine demonstrated IgG primarily against the NP protein in addition to the HA protein, whereas natural infection induced antibodies against HA, NA, NP, M1, NS1, and NS2 (Kim et al., 2006). This, however, may not reflect the antibody profile at the respiratory mucosa. The role of antibodies generated from sub-unit or inactivated vaccines against minor immunogenic proteins such as NP or M in Het-I is not clear (Mozdzanowska et al., 1999; Slepishkin et al., 1995; Wraith et al., 1987), especially in swine where a DNA vaccine expressing a M2-NP fusion protein led to an enhancement of disease (Heinen et al., 2002). The outcome of the DNA vaccine study, however, suggests the exacerbation was likely due to immunopathology induced by vaccination, as is suggested in the study we report.

Infection with live virus, MLV or DNA vaccines prime the immune system through intracellular mechanisms that are more likely to induce a response against viral internal or minor surface proteins that activate cross-reactive T- and B-lymphocytes (Flynn et al., 1999; Scherle and Gerhard, 1986; Taylor and Askonas, 1986; Ulmer et al., 1993), promoting a more balanced and effective immune response. Here, live priming was demonstrated to be superior to priming with inactivated vaccine, as protection was complete for both heterologous challenges. The enhanced protection is likely to be due to the combined activation of CMI and humoral systems, however only antibody responses at the systemic and local mucosal levels were evaluated in this study. No cross-reacting serum HI antibodies were detected in these pigs. Future work is planned to assess the role of CMI in heterologous immunity using the IA30 vaccinated and MN03 challenged model.

Despite lower serum HI titers in live compared to inactivated vaccine groups, a dramatically enhanced IgG and IgA response was shown at the mucosal level. These antibodies appeared to be much more cross-reactive as there was no statistical difference between the two live primed groups in IgG or IgA in the lung or total IgG in the serum against the priming virus compared to challenge virus antigen. In addition, pigs

in the MN03 live-primed and IA30-challenged group (group 11) had begun to sero-convert against IA30 in the HI assay by 5 dpi. No pigs were evaluated beyond 5 dpi, so it is not clear how rapidly HI antibodies against the heterologous challenge virus would appear in the other vaccinated groups. Five days post infection may have been too early to appreciate any boost in serum immunity against the priming antigen, known as original antigenic sin (Fazekas de St and Webster, 1966a,b). Alternatively, the timing between vaccination and challenge has been shown to be important in the development of antigenic sin (Masurel and Drescher, 1976). The 5-week interval between the first dose of vaccine and virus challenge used in this study may have been too short in duration for the development of clonal-restricted memory cells involved in production of serum antibodies.

A boost in mucosal IgA antibodies, however, was apparent by 5 dpi in groups challenged with either homologous or heterologous virus. This was especially evident in the inactivated MN03-primed and IA30-challenged group, with a rise in IgA against the priming MN03 antigen compared to the inactivated MN03-primed homologous challenged group. This boost against the priming antigen may exemplify what is reported as antigenic sin; however there was a corresponding rise in local IgG and IgA antibodies against the challenge IA30 virus as well. In contrast, although the inactivated IA30-vaccinated and MN03-challenged group demonstrated a rise in local IgG levels against the IA30 antigen, there did not appear to be any increase in cross-reacting antibodies against the heterologous MN03 antigen. At first glance, it would appear that this lack of cross-reacting antibodies during the acute stage of disease played a role in the vaccine failure of the inactivated IA30-primed and MN03-challenged group. However, the group 7 mean IgG and IgA levels are skewed by the antibody levels in the three pigs with enhanced lung lesions. These three pigs had increased levels of IgG against both the priming and the challenge antigens in concert with decreased levels of IgA against both antigens. An important role for cross-reacting IgA has been demonstrated in mice (Liew et al., 1984; Renegar and Small, 1991; Tamura et al., 1991b), and might also be the basis for the different outcomes of the vaccination in the three pigs with enhanced lesions.

The distribution of the influenza virus antigen in the microscopic sections of lung lesions from the IA30 vaccinated and MN03 challenged pigs was remarkably different compared to the unvaccinated MN03 challenge controls. This was demonstrated in the diseased lung tissue in all group 7 pigs, including the three with and the six without enhanced macroscopic pneumonia. It could be speculated that the vaccine-induced antibodies in the lung are related to the presence and distribution of virus antigen as well as the enhanced macroscopic lesions. Pigs vaccinated against IA30 (group 4) tended to have more IgG than IgA in the lung, so it can be assumed that at the time of challenge, the major isotype of SIV-specific antibodies in the lungs of vaccinated pigs belong to the IgG class. A possible role for the vaccine-induced IgG in the virus distribution is through antibody dependent enhancement (ADE) through receptor mediated uptake of virus by the resident antigen presenting cells in the lung, pulmonary alveolar macrophages (PAM). The cells staining positive for influenza virus in the alveoli in Fig. 2 are likely to include PAM. Uptake of influenza virus into murine macrophages was enhanced in the presence of anti-HA antibody (Ochiai et al., 1992), and enhanced uptake via antibodies against HA and NA were shown to be mediated through the Fc receptor (Tamura et al., 1991a). Influenza virus has been shown to infect and replicate in swine PAM (Seo et al., 2004), further supporting the role of macrophages and ADE in the virus antigen distribution reported here. In addition, it is possible that vaccine-induced IgG contributed to lung pathology through antibody dependent cell-mediated cytotoxicity (ADCC). Inactivated influenza vaccine has been shown to induce antibodies reactive in ADCC (Vella et al., 1980), and ADCC antibodies appear rapidly in primed individuals, even in the absence of HI antibodies (Hashimoto et al., 1983; Reichman et al., 1979). In addition to ADCC, IgG has been shown to be effective in promoting complement fixation in response to influenza infection (Kopf et al., 2002), and may also promote cell-mediated cytotoxicity, although to a lesser degree than natural killer cell ADCC (Jegerlehner et al., 2004).

The IA30 vaccinated and MN03 challenged pigs that had less macroscopic pneumonia at 5 dpi (4.5% compared to 31.8%) demonstrated higher IgA against both the vaccine virus and the challenge virus.

Although the virus distribution demonstrated by IHC was the same in the diseased lung tissue of all the pigs in group 7, it is assumed that the areas of lung that were macroscopically healthy in appearance would have limited SIV antigen distribution compared to the diseased sections in the 6 pigs with minimal lesions. Antibodies of the IgA isotype have been shown to be highly effective in neutralizing influenza virus infection (Taylor and Dimmock, 1985), more cross-reactive than IgG (Tamura et al., 1991b), and correlated to protection from infection by heterologous virus (Liew et al., 1984). The appearance of cross-reacting IgA in six of the nine pigs in group 7 could be responsible for improved virus neutralization and limiting the spread of the virus from sites of initial infection to further areas of the lung, while a subsequent decrease in IgG might reduce ADE- and ADCC-associated pathology. This combined effect of increased IgA and decreased IgG might thus limit the lung pathology induced by cell-mediated cytotoxicity to areas near the primary infection.

Pigs are a natural host for influenza virus and serve as an excellent model for the study of influenza disease and vaccination strategies. The vaccination and challenge model we have described provides an opportunity to further explore the molecular and cellular control mechanisms for heterologous immunity induced by inactivated vaccines as compared with live exposure in a natural host. The role of early innate and inflammatory signals as well as the involvement of the humoral and CMI systems can be more carefully examined in future studies utilizing this model. With the continual emergence of swine influenza virus genetic and antigenic variants, the swine industry around the world is faced with the challenge of controlling this disease with inactivated vaccines prepared from parent strains with limited representation of the current milieu of viruses circulating among swine. The development of vaccination strategies that induce greater Het-I is necessary to reduce the costly ramifications of this disease and may play a significant role in public health due to the zoonotic potential of swine influenza virus. Furthermore, caution is needed when relying on HI cross-reactivity to estimate cross-protection from live or MLV priming, even within the same HA subtypes, as demonstrated by the swine H1N1 and H1N2 viruses used in this model.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vetmic.2007.07.011](https://doi.org/10.1016/j.vetmic.2007.07.011).

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