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Short communication

Pre-exposure with influenza A virus A/WSN/1933(H1N1) resulted in viral shedding reduction from pigs challenged with either swine H1N1 or H3N2 virus

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ABSTRACT

There is an urgent need to develop a broad-spectrum vaccine that can effectively prevent or eliminate the spread of co-circulating swine influenza virus strains in multiple lineages or subtypes. We describe here that pre-exposure with a live virus generated via a A/WSN/1933(H1N1) reverse genetics system resulted in a significant reduction of viral shedding from pigs exposed to either a swine H1N1 virus or a swine H3N2 virus. At 3-day post challenge (DPC), approximately 1 log and 1.5 logs reductions of viral shedding were observed in the swine H1N1- and H3N2-challenged vaccinated pigs when compared to unvaccinated animals. A further decline in viral load was observed at 5 DPC where viral shedding was decreased by greater than 3 logs in vaccinated pigs receiving either the H1N1 or H3N2 virus challenge. Although the sera of the vaccinated pigs contained high titers of neutralizing antibodies against the vaccine strain, measured by Hemagglutination Inhibition (HI) assay, only suboptimal HI titers of neutralizing antibody were detected in the post-challenge serum of the vaccinated animals using the challenge swine H1N1 virus. The substantial genetic and antigenic differences between the vaccine virus and the challenge viruses imply that the observed protection may be mediated by mechanisms other than neutralization by IgG, such as non-neutralizing antibody activities, mucosal immunity, or conserved T cell immunity, which warrants further investigation.

1. Introduction

Swine Influenza (SI) is an acute respiratory disease caused by Influenza A Virus (IAV). Swine influenza virus (SIV) is a pathogen of economic significance to the swine industry, and a zoonotic organism (Easterday, 1980; Rajao et al., 2018). H1N1, H1N2, and H3N2 subtypes are largely responsible for annual outbreaks of SI, although three rare subtypes (H3N1, H2N3, and H4N6) have been isolated from the diseased pigs (Vincent et al., 2008). Each subtype also consists of several genetic and antigenic clusters (Hause et al., 2012b).

Currently available SI vaccines are based on killed viruses containing both H1N1 and H3N2 subtypes (Loving et al., 2013, 2012; Van Reeth and Ma, 2013). Due to antigenic drift and shift, the killed vaccines are very limited in offering protection against SIV strains that are genetically and antigenically different from the vaccine strains. Also, these killed vaccines appear to be associated with disease enhancement in vaccinated pigs after heterologous virus infections (Khurana et al., 2013; Rajao et al., 2014). The currently licensed vaccines lack a delivery strategy that can induce sufficient mucosal immunity to effectively prevent SIV infection, which occurs primarily through the mucosal route. Recently, a bivalent swine H1N1 and H3N2 NS1-truncated live attenuated vaccine has become available to swine industry in the United States (Genzow et al., 2018). Currently, experimental challenge data supported that this bivalent vaccine was effective in protecting piglets from heterologous challenges. Despite the encouraging result, there is ample evidence, derived from human influenza vaccine studies; that the influenza vaccines of any approved form are largely strain-specific and those provide limited protection against circulating strains that are not well-matched to vaccine strains (Erbelding et al., 2018). To limit the public health concern of SI, developing a vaccine that elicits

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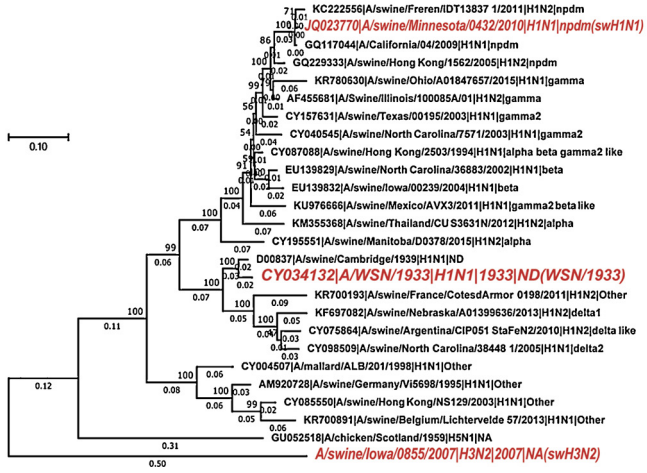
A



B

US swine H1 clade	Stain	Distance to WSN/1933
ND	A/swine/Cambridge/1939|H1N1	0.035
delta2	A/swine/North Carolina/38448 1/2005|H1N1	0.145
delta like	A/swine/Argentina/CIP051 StaFeN2/2010|H1N2	0.152
other	A/swine/France/CotesdArmor 0198/2011|H1N2	0.171
delta1	A/swine/Nebraska/A01399636/2013|H1N2	0.172
alpha beta gamma2 like	A/swine/Hong Kong/2503/1994|H1N1	0.225
gamma2 beta like	A/swine/Mexico/AVX3/2011|H1N1	0.228
gamma2	A/swine/Texas/00195/2003|H1N1	0.231
beta	A/swine/North Carolina/36883/2002|H1N1	0.233
alpha	A/swine/Manitoba/D0378/2015|H1N2	0.235
beta	A/swine/Iowa/00239/2004|H1N1	0.238
npdm	A/swine/Hong Kong/1562/2005|H1N2	0.241
gamma	A/Swine/Illinois/100085A/01|H1N2	0.243
gamma2	A/swine/North Carolina/7571/2003|H1N1	0.243
npdm	A/California/04/2009|H1N1	0.247
npdm	A/swine/Minnesota/0432/2010|H1N1(swH1N1)	0.249
npdm	A/swine/Freren/IDT13837 1/2011|H1N2	0.249
alpha	A/swine/Thailand/CU S3631N/2012|H1N2	0.253
gamma	A/swine/Ohio/A01847657/2015|H1N1	0.267
other	A/mallard/ALB/201/1998|H1N1	0.284
other	A/swine/Germany/Vi5698/1995|H1N1	0.305
other	A/swine/Hong Kong/NS129/2003|H1N1	0.316
other	A/swine/Belgium/Lichtervelde 57/2013|H1N1	0.343
NA (H5N1)	A/chicken/Scotland/1959|H5N1	0.512
NA (H3N2)	A/swine/Iowa/0855/2007|H3N2|2007|NA(swH3N2)	0.820

Fig. 1. Phylogenetic analysis of the 26 HA nucleotide sequences by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model with a bootstrapping of 1000 replicates. **A**. Phylogenetic tree with the highest log likelihood (-17706.31). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). There were a total of 1853 positions in the final dataset. **B**. Pairwise distances to WSN/1933 HA. Pairwise nucleotide sequence divergence to WSN/33 HA was computed using the Maximum Composite Likelihood (MCL). The number of base substitutions per site from between sequences are shown. The analysis involved 26 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1612 positions in the final dataset. The evolutionary analyses were conducted in MEGA7. US swine H1 clade classification of each strain was indicated as "ND" (not determined); alpha; beta; delta1; delta2; delta like; npdm (new pandemic); gamma; gamma2; alpha beta gamma2 like; gamma2 beta like; other; or NA (not applicable) (www.fludb.org).

broad and durable protection against multiple influenza strains of the virus in pigs, including those that may cause human infections with a pandemic potential, is critically needed. The aim of this study was to investigate whether intranasal vaccination with live A/WSN/1933(H1N1), a laboratory mouse-adapted human IAV strain, would protect pigs from infections by a pandemic swine H1N1 strain A/swine/Minnesota/0432/2010 (swH1N1) and a recent H3N2 strain A/swine/Iowa/0855/2007 (swH3N2).

2. Materials and methods

2.1. Cells, viruses, and virus titration

Swine testicle (ST), Madin-Darby Canine Kidney (MDCK), and HEK293 T human embryonic kidney cells were purchased from ATCC. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37 °C with 5% CO_2. For viral propagation, fetal bovine serum was omitted from the DMEM. A/swine/Minnesota/0432/2010 (swH1N1) is a pandemic H1N1 2009 virus (Hause et al., 2012a). A/swine/Iowa/0855/2007 (swH3N2) was isolated from pig specimen in 2007 and was used in a previously study (Thomas et al., 2018). The viruses were propagated in ST cells. A/WSN/1933 (H1N1) (WSN/1933) was generated by using the reverse genetics system that contains eight dual-promoter plasmids (a gift of Erich Hoffmann at St. Jude Children's Research Hospital) through transfection of co-cultured HEK293 T and MDCK cells as described previously (Hoffmann et al., 2000). At 72 h post-transfection, viral supernatants were collected, centrifuged at 2000 r.p.m. (Sorvall Legend Mach 1.6R, rotor 75,003,348) for 10 min, aliquoted and stored at −80 °C for further analysis. Viral stocks were determined for viral infectivity (log_{10} 50% Tissue Culture Infectious Dose per ml, $log_{10}TCID_{50}$/ml) in MDCK cells, as described previously (Ran et al., 2015).

2.2. Sequencing, virus annotation, and phylogenetic analyses

The full sequences were obtained from RNA extracted from the virus culture from ST cells using MiSeq Illumina. All the genome segments were assembled using the CAP3 assembly tool (Huang and Madan, 1999). The HA genes of the H1 viruses were classified according to the swine H1 classifier tool implemented on the web through IRD (http://www.fludb.org) (Anderson et al., 2016). The molecular evolutionary distances were computed using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Molecular Phylogenetic analysis by the Maximum Likelihood method was conducted in MEGA7 (Kumar et al., 2016). All nucleotide positions containing gaps and missing data were partially deleted and very strong branch filters were applied to run the analysis. For each taxon, the bootstrap value was determined from 1000 replicates to verify the tree topology.

2.3. Hemagglutination inhibition (HI) assay

HI assay was performed following the standard procedure (Hause et al., 2012b). In brief, sera were treated with a receptor-destroying enzyme for 24 h at 37 °C and then adsorbed with a 20% suspension of turkey erythrocytes in phosphate-buffered saline (PBS) for 30 min at room temperature. 25 µl of virus suspensions containing 4 HA units of virus were incubated for 1 h with serial 2-fold dilutions of antisera (1:10, 1:20, 1:40 and 1:80 v/v), and the HI titers were determined as the reciprocal of the highest dilution that showed complete inhibition of hemagglutination using 0.5% washed turkey erythrocytes.

2.4. Experimental immunization and virus challenge procedures

Six-week-old commercial influenza antibody-negative pigs were obtained from a commercial vendor in Minnesota. Vaccination and challenge studies were conducted in BSL-2 animal facility at South Dakota State University as required by CDC and USDA on swine influenza work (http://www.cdc.gov/). The project was reviewed and approved by the institution's Animal Care and Use Committee (IACUC) prior to its being conducted. Euthanasia procedures were compliant with the recommendations of the most recent American Veterinary Medical Association (AVMA) Panel on Euthanasia.

Pigs were divided into five experimental groups with four animals per group. Each group of pigs was placed in a separate room, and baseline samples including serum and temperature were collected prior to vaccination. As summarized in Fig. 1A, after 1-week acclimation, two

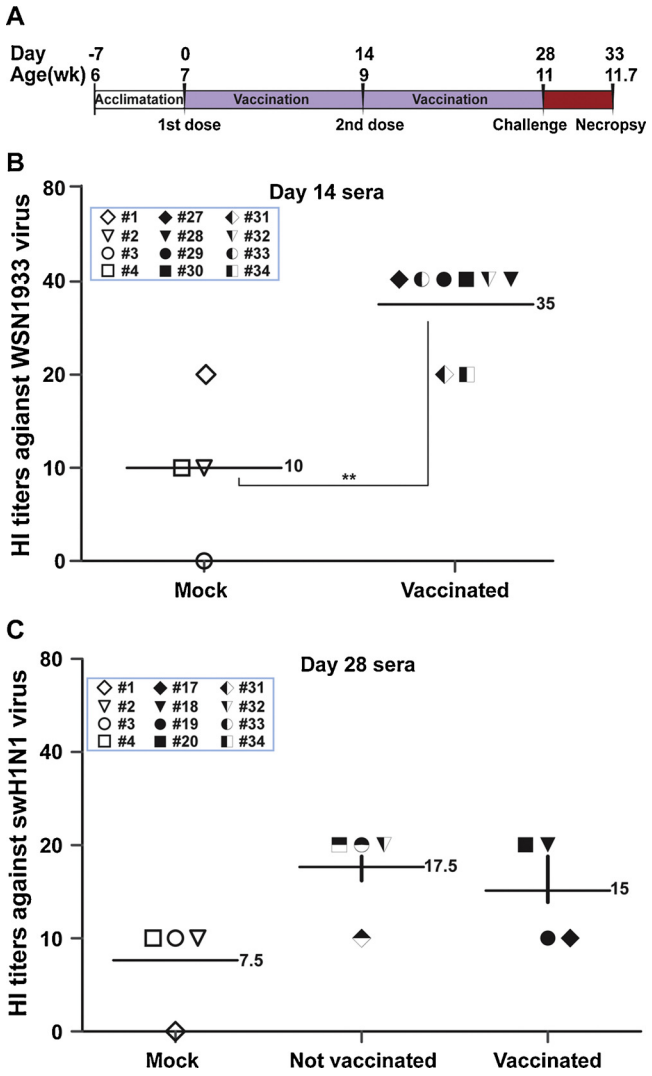


Fig. 2. Experimental immunization and virus challenge procedure and seroconversion after immunization. **A**. A schematic description of intranasal immunization and challenge procedure used in this pig experiment. **B**. Pig serum samples from Day 0 (pre-vaccination) and Day 14 were tested for the presence of virus-specific antibodies against WSN/1933 by HI assay. Horizontal lines represent the mean antibody titers for each group. Statistically significant difference (**P < 0.01) for WSN/1933-specific HI antibody titers was detected between non-vaccinated and vaccinated groups. **C**. HI antibody levels against swH1N1 in pigs vaccinated intranasally with WSN/1933 virus on Day 28 (prior to challenge). The HI antibody titer is expressed as the reciprocal of the highest serum dilution showing complete inhibition of agglutination using 4 hemagglutinating units/25 µl. Horizontal lines represent the mean antibody titers for each group.

groups of pigs were inoculated two times at a two-week interval by intranasal administration of 10^6 $TCID_{50}$ of WSN/1933. Remaining three groups of pigs received PBS control intranasally.

Two weeks following the second inoculation (11 weeks of age), two vaccinated groups and two PBS control groups were challenged intranasally either with 1 ml of 10^6 $TCID_{50}$/ml swH1N1 or swH3N2 viruses (Fig. 2A). Remaining PBS control group was mock challenged with regular culture medium by the same route. Pigs were monitored 0–5 DPC on a daily basis for both clinical respiratory disease and fever based on rectal temperature. All of the animals were euthanized at 5 DPC. Serum samples were collected prior to 1 st and 2nd doses of vaccination, virus challenge, and at euthanasia, to assess for humoral immune responses. Nasal swabs were collected immediately before virus challenge, 3 DPC, and at euthanasia, and transferred to tubes containing 1 ml of DMEM, supplemented with 2% pen-strep. After brief vortexing for 30 s, the swabs were discarded, and the tubes were centrifuged at 1500 g for 10 min to remove debris. The supernatant was collected, stored at −80 °C, and used for viral titration. In addition, lung homogenates were collected at euthanasia and prepared for virus isolation and titration. Lung tissues of 2 x 2 cm was collected from the right middle lobe for virus isolation and histopathology from all the animals. For virus isolation, 100% w/v lung homogenate was prepared in DMEM supplemented with 2% pen-strep. The homogenate was then centrifuged at 1500 g for 10 min to remove debris and the supernatant was collected and stored at −80 °C until being cultivated in MDCK cells.

2.5. Statistical analysis

All assays were performed at least three times independently in duplicate. To determine the differences between groups, One-way ANOVA statistical analysis followed by Tukey's multiple comparison test was performed using the GraphPad Prism software version 5.0. $p < 0.05$ indicated a statistically significant difference.

3. Results and discussion

The HA segment of the swH1N1 was classified as clade "npdm" (US SOP) or "1 A.3.3.2″ (Global SOP), and WSN/1933 was classified as clade "ND" (US SOP) or "Other-Human" (Global SOP) according to the swine H1 classifier tool implemented on the web through IRD (http://www.fludb.org) (Anderson et al., 2016). The HA of swH3N2 was classified as H3 clade IV according to the phylogenetic analysis (data not shown). To further determine the genetic distances between WSN/1933 and the challenge viruses, phylogenetic analysis by Maximum Likelihood method were conducted in MEGA7 (Kumar et al., 2016). The phylogenetic tree and the pairwise distances (Fig. 1) shows the evolutionary distances (the number of base substitutions per site) of the swH1N1 HA, WSN/1933 HA, and the HA reference sequences representing all US SOP H1 clades. A substantial distance was observed between WSN/1933 and swH1N1 (Fig. 1). A dramatic distance was observed between the HAs in H1 subtype and the HA of swH3N2 (Fig. 1).

To determine whether WSN/1933 vaccination elicited humoral immune responses in vaccinated pigs, we performed HI assay to analyze HI antibody levels in vaccinated animals (Fig. 2B). Six of the eight vaccinated pigs seroconverted as detected at two weeks post vaccination with an HI titer of 40 or above, and two pigs (#31 and 34) exhibited an HI titer of 20. In contrast, all four pigs that received PBS inoculation did not show measurable HI antibody titers (Fig. 2B). The seroconversion result indicates that live virus vaccination via the nasal route was able to induce significant humoral immune responses in pigs. Previous studies reported that human H1N1 strains could replicate in pigs, although with a limited efficiency (Meng et al., 2013; Van Poucke et al., 2010). In this pilot study, the replication of WSN/1933 in pigs was not monitored during the 4-week vaccination period. Due to this limitation, it is not clear if the virus had replicated effectively in pigs, which should be investigated in a future study.

We next used the standard serological assay to examine virus-specific antibody responses in vaccinated animals on the day of the challenge. The swH3N2 challenge virus was not included in this analysis as our previous work revealed no detectable cross-reaction between WSN/1933 and swH3N2 viruses (data not shown). With 40 as a cut-off value of positive HI, neither non-vaccinated nor vaccinated pigs had positive HI titers against the swH1N1 challenge virus (Fig. 2C). We did not observe a correlation between the presence of HI antibody titer and protection from swH1N1 infection, as some WSN/1933-vaccinated pigs that were protected from detectable infections following challenge were negative in the HI assays (i.e., titers below 40).

Pigs challenged with either the swH1N1 strain or the swH3N2 strain did not exhibit obvious clinical signs including respiratory distress and

nasal or ocular discharge. No significant differences between the vaccinated and non-vaccinated controls were observed with regard to rectal temperature and body weight (data not shown). At 5 DPC, pigs were euthanized and necropsies were performed. Neither the vaccinated nor the control pigs displayed gross clinical lung lesions. The failure of the swH3N2 in producing macroscopic lung lesions is in contrast to the pathogenic properties originally described for these viruses when young piglets were infected (Thomas et al., 2015). It was not clear why there were no detectable clinical signs and lung lesions developed in these non-vaccinated control pigs, which should be investigated in a future experiment with consideration of factors including age, breed, and gender.

Influenza vaccine effectiveness has been correlated closely with the levels of virus replication in experimentally infected animals, as observed with other influenza animal models (Wang et al., 2018, 2017). Therefore, in the absence of defined influenza disease symptoms, we closely monitored the levels of virus replication in the experimental infections to assess the protection efficacy of WSN/1933 against swH1N1 and swH3N2. To quantify the level of virus replication, we used a traditional MDCK cell-based assay to monitor infectious influenza particle levels in nasal swabs taken at 3 and 5 DPC and lung samples taken at 5 DPC. The individual nasal sample viral load in $\log_{10}TCID_{50}$/ml was measured and was presented, and a calculated mean of the viral load levels for the groups of pigs was shown with horizontal bars in Fig. 3.

As detailed in Fig. 3A, all four of the swH1N1-challenged non-vaccinated animals, at 3 DPC, displayed similar levels of viral load at nasal washes. Among the vaccinated animals, two pigs (#31 and 34) had viral load levels approximately 2 logs lower than those seen in the non-vaccinated animals. In marked contrast, the other two vaccinated animals (#32 and 33) displayed viral load level that was higher or similar to those in the non-vaccinated animals. In the swH3N2 challenge group (Fig. 3B), three swH3N2-challenged non-vaccinated pigs (#5, 7, and 8) exhibited similar levels of viral replication with one animal (#6) displaying 1 log lower viral load level at 3 DPC. Interestingly, among the vaccinated pigs, one animal (#30) showed undetectable viral load and the other three (#27, 28, and 29) had the levels of viral replication ranged between 2–3 $\log_{10}TCID_{50}$/ml. To account for the natural variation observed in SIV infection in outbred animals, we also calculated mean viral load levels for each group of four pigs. In this context, the general pattern of the vaccination-mediated reduction of the swH1N1 and the swH3N2 titers becomes more obvious. These data revealed that at 3 DPC, approximately 1 log and 1.5 logs reductions of viral shedding were observed in the swH1N1- and the swH3N2-challenged vaccinated pigs when compared to unvaccinated animals (Fig. 3A–B).

As shown in Fig. 3C–D, a further decline in viral load was observed at 5 DPC where viral shedding was decreased by greater than 3 logs in both the swH1N1- and the swH3N2-challenged pigs receiving the vaccination. For example, all four of the swH1N1-infected non-vaccinated pigs had viral loads ranging from 1.5 to 4.0 logs with an overall mean of 3.5 $\log_{10}TCID_{50}$/ml nasal washes (Fig. 3C). In marked contrast, three of vaccinated animals (#31, 32, and 34) were completely protected from the swH1N1 virus challenge. One vaccinated pig (#33) showed a relatively consistent low level of challenge virus titers, averaging less than 0.5 $\log_{10}TCID_{50}$/ml. A similar trend of the vaccine effectiveness was observed in swine H3N2 challenge group at 5 DPC (Fig. 3D). All four of the swH3N2-challenged non-vaccinated animals displayed similar levels of viral load, with an overall mean of 3.8 $\log_{10}TCID_{50}$/ml.

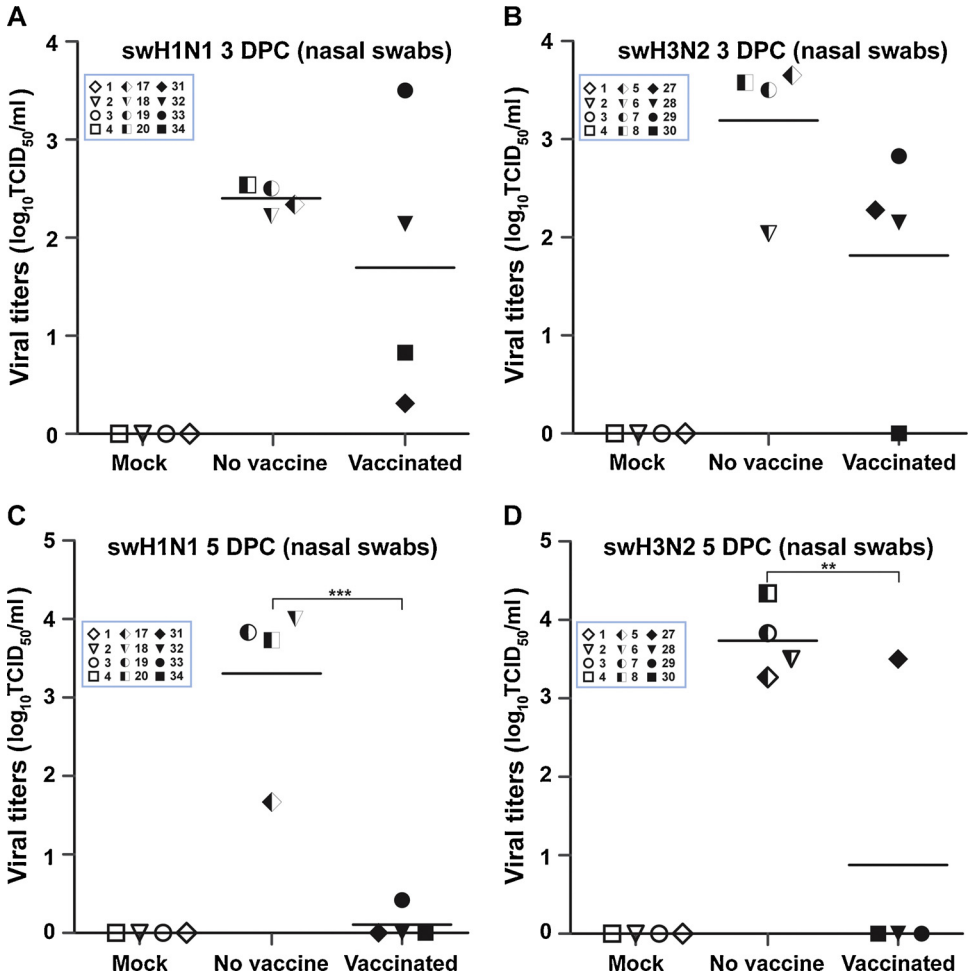


Fig. 3. Nasal swab virus titration post challenge. Serial 10-fold dilutions of the nasal swab sample from pigs were inoculated on MDCK cells followed by 5-day cultivation at 37 °C. The infected cell culture plates were then washed with PBS and fixed in 80% acetone. The plates were stained for determinations of virus titers ($\log_{10}TCID_{50}$/ml) in nasal washes by indirect immunofluorescence assay. **A**. Viral loads for swH1N1-challenged pigs at 3 DPC. **B**. Viral loads for swH3N2-challenged groups at 3 DPC. **C**. Viral loads for swH1N1-challenged pigs at 5 DPC. **D**. Viral loads for swH3N2-challenged groups at 5 DPC. Horizontal lines represent the mean viral titers for each group. Statistically significant differences were indicated as ** ($P < 0.01$) and *** ($P < 0.001$), respectively.

Three of these four vaccinated animals (#28, 29, and 30) had no detected virus in nasal swab samples after challenge with the swH3N2. The fourth vaccinate (#27) had a virus titer similar to those observed in non-vaccinated pigs after challenge with swH3N2. To determine the differences between groups, One-way ANOVA statistical analysis followed by Tukey's multiple comparison test was performed using the GraphPad Prism5 software. There was no significant difference in nasal virus titers between non-vaccinated and vaccinated groups at 3 DPC (Fig. 3). However, the nasal virus titers of the vaccinated groups were significantly lower ($P < 0.001$ for the swH1N1 challenge group and $P < 0.01$ for the swH3N2 challenge group) than that of the non-vaccinated groups at 5 DPC (Fig. 3). It should be noted that neither non-vaccinated nor vaccinated animals showed detectable viral replication in the lungs of challenged pigs at 5 DPC (data not shown). In summary, by viral load analysis, intranasal vaccination by WSN/1933 substantially protected pigs from infections of both the swH1N1 (same subtype, different clade) and the swH3N2 (different subtype) viruses.

The antigenic diversity of existing and emerging SIV strains is a big challenge to the development of cross-clade/subtype effective vaccines for swine. The results presented in this study suggest that pigs responded to intranasal immunization with the live WSN/1933 virus. The derived immune response specific to WSN/1933 via the intranasal route limited virus shedding upon challenged by either the swH1N1 or the swH3N2 strain. These results suggest that the vaccination with WSN/1933 may have the potential to limit heterogeneous or even heterosubtypic influenza virus infection and transmission in pigs.

Our work described here suggests that the live vaccine WSN/1933 had resulted in the reduction of virus shedding when challenged by a distantly related homosubtypic virus swH1N1, or by a heterosubtypic virus swH3N2. Traditional multivalent, inactivated SIV vaccines are able to effectively protect animals against strains that are antigenically similar to the vaccine strains. The protection usually correlates with the induction of anti-HA neutralizing antibodies (Guo et al., 2014; Van Reeth and Ma, 2013). We have demonstrated that after 2 doses of WSN/1933 immunization, the level of serum neutralizing antibodies against swH1N1 virus was suboptimal (Fig. 2C). Recent studies have suggested that live-attenuated vaccines are better at inducing mucosal IgA and CD8 + T cell responses, which are possible mechanisms of the broad-spectrum protection observed (Genzow et al., 2018; Guo et al., 2014; Vincent et al., 2012). Our work had the limitations because the IgA and CD8 + T cell responses were not evaluated. Since the immunization failed to induce a significant level of neutralizing antibodies against the challenge viruses, we speculate the cross-subtype protection mechanism is associated with mucosal IgA and CD8 + T cell-mediated immune responses. In addition, influenza vaccine protection has been correlated to antibody-dependent cellular cytotoxicity (ADCC) (Jegaskanda et al., 2013), which provide us with another direction for follow-up studies. A universal SIV vaccine needs to incorporate multiple viral components that stimulate immune responses involving both B cells and T cells, which may provide durable and broad protection for swine against multiple influenza strains of the virus (Erbelding et al., 2018). Further investigation into the cross-subtype protection mechanism, especially mucosal immunity, T cell-mediated immunity, and ADCC, provided by WSN/1933, may offer novel insights into the development of a universal SIV vaccine.

Declaration of interest

The authors have read the journal's policy and have the following conflicts: BMH is employed by Cambridge Technologies, a company that produces swine influenza virus vaccines. This does not alter the authors' adherence to all the Journal policies on sharing data and materials.

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