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Delivery of a thermo-enzymatically treated influenza vaccine using pulmonary surfactant in pigs

Heather Vinson[a], Gagandeep Singh[a], Angela Pillatzki[b], Brett Webb[c], Eric Nelson[b], Sheela Ramamoorthy[a,*]

[a] Department of Microbiological Sciences, N. Dakota State University, Fargo, ND, United States
[b] Animal Disease Research and Diagnostic Laboratory, S. Dakota State University, Brookings, SD, United States
[c] Veterinary Diagnostic Laboratory, N. Dakota State University, Fargo, ND, United States

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ABSTRACT

Swine influenza A virus (IAV-S) infections are a major cause of economic losses for the swine industry. The vast genetic and antigenic diversity often results in mismatch between the vaccine and field strains, necessitating frequent updates of vaccines. Inactivated IAV-S vaccines are of questionable efficacy. Intra-nasally administered live vaccines are more effective but are associated with safety concerns. The objective of this study was to develop a first-generation vaccine which combines the safety and efficacy advantages of inactivated and attenuated vaccines respectively. The approach targeted fragmentation of viral nucleic acids while preserving structure. Hence, cultures of influenza A/CA/04/09 H1N1 were exposed to 44 °C for 10 min. to reversibly denature the capsid, followed by RNase treatment to digest the genomic RNA and then refolded at lower temperatures. As targeted, treated virions retained an intact structure and were not detected in the first passage in infected cells. To improve intra-nasal delivery of the vaccine antigen, the vaccine antigen was delivered in porcine lung surfactant. Both the treated vaccine alone or vaccine in combination with the surfactant elicited strong anti-HA and virus neutralizing antibodies, protection against viral shedding and lung lesions in 3-week-old piglets. There were no significant differences between the groups. Vaccine viral replication was not detected in the vaccinated pigs. The described approach can advance current immunization practices against swine influenza viruses due to the relative simplicity, high efficacy and safety and ease of adaptation to newly emerging field strains.

1. Introduction

Among the economically important swine infectious diseases, swine influenza A viruses (IAV-S) are a leading cause of respiratory illness, morbidity and poor weight gain in production swine of all ages. Further, as coinfections are common in pigs affected by the porcine respiratory disease complex, the presence of one agent is often exacerbated by another. Influenza viruses are well known for their genetic and antigenic diversity, attributed to the high frequency of genetic drift and shift associated with their segmented RNA genomes. As pigs harbor sialic acid receptors for both human and avian IAVs they play an important role in the evolution of IAVs. As a result of the complex evolution of IAV-S over the last few decades, three different subtypes, H1N1, H1N2 and H3N2, currently co-circulate in U.S swine herds and are represented in commercial IAV-S vaccines (Lorusso et al., 2013).

The majority of commercial IAV-S vaccines are inactivated preparations administered at 3 or more weeks of age, as 2 doses administered 3 weeks apart. While these vaccines are effective against homologous strains, they show variable protection against heterologous or newly emerging strains due to a mismatch between vaccine and field strains. Further, when combined with adjuvants, inactivated IAV-S vaccines have been associated with vaccine associated enhanced respiratory disease (VAERD) (Vincent et al., 2008a). Therefore, improving the efficacy of current IAV-S vaccines entails extensive surveillance, serological profiling and regular updating of vaccines strains (Corzo et al., 2013). Experimental, live attenuated IAV-S vaccines (LAIV-S) are developed by introducing attenuating mutations in the genome. When administered intra-nasally, the vaccine virus undergoes moderate replication in the upper respiratory tract to stimulate mucosal immunity and is not associated with VAERD (Masic et al., 2009). As with other attenuated vaccines, the time for development time can be quite long. Until very recently, LAIV-S were not licensed for field use

* Corresponding author at: Microbiological Sciences, 1523 Centennial Blvd, Fargo, ND, 58102, United States.
E-mail address: sheela.ramamoorthy@ndsu.edu (S. Ramamoorthy).

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due to safety and regulatory concerns. The first LAIV-S vaccine in the U.S (Inglevac Provenza ™, Boehringer Ingelheim Vetmedica, Inc), containing H1N1 and H3N2 strains, was launched in 2018. As a result, currently, more than 50% of the IAV-S vaccine market is captured by the autogenous vaccine industry (Sandbulte et al., 2015). Autogenous vaccines consist of field isolates which are cultured and inactivated by the manufacturer for herd-specific use and are often associated with inconsistencies in performance.

While intranasal delivery is accepted as the best way to stimulate mucosal responses against IAV-S, rapid muco-ciliary clearance and elimination of the vaccine antigen pose a risk to achieving adequate vaccine efficacy. Pulmonary surfactant is a naturally occurring substance which bathes the lung surface to reduce surface tension. It contains phospholipids and proteins, which can form multi-lamellar vesicles to deliver drugs and vaccines via the intranasal or thoracic routes (Baer et al., 2019; Mizuno et al., 2006). Surfactant proteins are known to modulate host immunity and have an adjuvant effect in stimulating mucosal and cell mediated immune responses (Han and Mallampalli, 2015; Mizuno et al., 2006). The use of porcine surfactant as a vaccine delivery vehicle for swine has not been tested thus far. Considering the above-described limitations of IAV-S vaccines, the objective of this study was to develop an approach which combined the safety and efficacy advantages of inactivated and attenuated IAV vaccines, while being relatively simple to develop and easily adaptable to new strains. The goal was to diminish viral replication but retain structural integrity. To achieve this end, influenza A/CA/04/09 H1N1 virus cultures were subjected to low heat to reversibly denature the capsid, followed by treatment with RNase to digest genomic RNA and subsequent refolding at lower temperatures. The study objectives were to test the heat and RNase treated vaccine in a swine challenge model and determine whether delivery of the vaccine using porcine pulmonary surfactant would further enhance vaccine efficacy. The data described can improve current immunization practices against IAV-S and could also benefit the autogenous vaccine industry.

2. Methods

2.1. Cell and virus culture

A stock of Influenza A/CA/04/09 H1N1 virus was propagated in Madin-Darby kidney (MDCK) cells as previously described (Abdoli et al., 2016; Singh et al., 2019). Briefly, prepared MDCK cell monolayers were washed with Dulbecco's Modified Eagles Media (DMEM, Thermo Scientific, Waltham, MA). The viral inoculum was incubated on the cells for 30 min. at 37 °C in a CO_2 incubator. Viral growth medium containing 2% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 100 U/ml penicillin and100 µg/ml streptomycin (Thermo Scientific, Waltham, MA) and 2 µg/ml of TPCK-trypsin solution (Thermo Scientific, Waltham, MA) was added to the culture. The flasks were incubated at 37 °C in a CO_2 incubator for 48 h. After 48 h of incubation the virus culture was subjected to three freeze-thaw cycles, followed by centrifugation at 5000xG for 5 min.. The titer of the culture was quantified by the tissue culture infective dose 50% ($TCID_{50}$) method using the Reed-Muench formula (Reed and Muench, 1938). Virus cultures were resuspended to a concentration of 1×10^5 $TCID_{50}$/ml and stored as aliquots at −80 °C.

2.2. Vaccine preparation

To achieve the targeted outcome of maintaining structure while diminishing replication, virus cultures were subjected to low heat to reversibly denature the capsid protein followed by digestion of the RNA genome with Ribonuclease (RNase) and subsequent refolding of proteins. Influenza A/CA/04/09 H1N1 virus cultures resuspended to 1×10^5 $TCID_{50}$/ml were exposed to 44 °C for 10 min. to reversibly denature of the capsid. A combination of RNase concentrations and times of incubation were tested and evaluated by electron microscopy to ensure the structure remained intact and treated virus was not detected until the 3rd passage in MDCK cells. The final optimized process consisted of incubation at 44 °C for 10 min, followed by the addition of 20 units of RNase A (VWR, Radnor, PA) and 2000 units of RNase T (Thermo Scientific, Waltham, MA) and incubation at 44 °C for an additional 8 h 7 min, after which the culture was immediately placed at 25 °C for 2 h, moved to 4 °C and then directly used to inoculate pigs. To integrate the treated vaccine virus with the surfactant, a commercial porcine pulmonary surfactant preparation (Poractant alfa, Curosurf®, Chiesi Farmaceutici, Cary, NC), was mixed with chloroform to achieve a final concentration of 1 mg/ml. The mixture was heated to 60 °C to evaporate the chloroform and prepare a thin film. The thin film was then resuspended with an equal volume of the final vaccine product prepared as described as above, and mixed for 1 h at room temperature for resuspension.

2.3. Electron microscopy

Standard negative staining methods were used to examine the ultrastructure of samples (Booth et al., 2011). Treated virus, lung surfactant and an untreated virus control were examined. An irradiated Formvar/Carbon 300 electron microscopy film (Ted Pella Inc., Redding, CA) was placed on a 50 µl droplet of the sample for 10 min. followed by transfer to a 50 µl drop of 2% phosphotungstic acid (PTA) (Millipore Sigma, St. Louis, MO) for 30 s. The thin film was analyzed at the North Dakota State University Electron Microscopy Center using a transmission electron microscope (JEM-100CS II, JEOL, Peabody, MA). Images were acquired using a digital micrograph software (Gatan, Pleasanton, CA) (Fig. 1).

2.4. Immunofluorescence assay (IFA)

To assess the effect of the heat and RNase treatment on viral replication, MDCK cell monolayers in 25 cm^2 flasks and 8-well cell culture chamber slides (Corning Life Sciences, Tewksbury, MA) were infected with 2mls and 100 µl of the treated virus respectively and cultured as described above (Abdoli et al., 2016). Untreated virus was used as a control. After 48 h of incubation, cells in the chamber slide were fixed with a 1:1 methanol: acetone solution. Fixed slides were stained with 100 µl of 1:500 polyclonal anti- Influenza A/CA/04/09 H1N1 swine serum collected from infected animals at 33 days post infection (provided by Dr. Amy Vincent, National Animal Disease Center, Ames, Iowa) and anti-swine IgG fluorescein-conjugated secondary antibody (Ab) (Seracare Life Sciences, Milford, MA). Slides were examined with a fluorescent microscope for the presence of green fluorescence characteristic of viral replication. Infected cell cultures in the 25cm^2 flasks were serially passaged 4 times, with each passage being visualized by staining 8-well cell culture chamber slides infected with the passaged virus by IFA (Fig. 1A, B, C). Virus culture from the 3rd and 4th passage was quantified by the $TCID_{50}$ method.

2.5. Antigen delivery by the lung surfactant

To determine if the pulmonary surfactant enhanced delivery of the vaccine antigen in-vitro, treated vaccine in combination with the surfactant was also layered on MDCK cells and passaged three times. Enhanced delivery of vaccine antigen by the surfactant was assessed as the difference in the time of detection of viral antigen between the vaccine alone and vaccine combined with the surfactant by IFA, with earlier detection indicating improved delivery. The IFA was carried out as described in the above section (Fig. 1C, D, E).

2.6. Swine vaccination and challenge

Twenty-four 3-week old pigs, which were IAV-S seronegative and

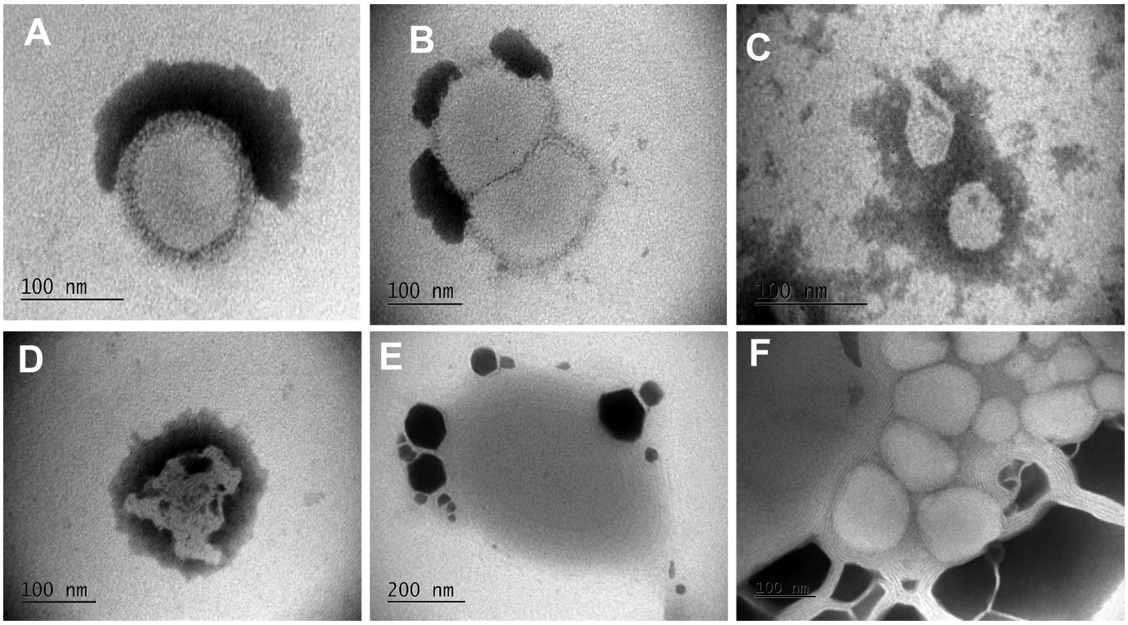

Fig. 1. Ultrastructure of IAV-S virions and pulmonary surfactant: Pictures depict the pleomorphic, spherical structure of the enveloped influenza A virus with the HA and NA protein spikes on the surface of the virions. A. Untreated virus; B. Treated vaccine virus; C. Virus incubated to 55 °C; D. Virus incubated to 60 °C; E. Thin film of the surfactant; F. Virus packaged in the pulmonary surfactant.

virus negative by ELISA (IDEXX Influenza A antibody kit), and PCR (Path-ID RT-PCR Kit, Thermo Scientific, Waltham, MA) respectively, were supplied by the North Dakota State University swine facility and transferred to South Dakota State University animal experimentation facilities for the duration of this study. All animal experimentation was carried out in compliance with the Institutional Biosafety Committee and Institutional Animal Care and Use (IACUC) committees of North Dakota and South Dakota State Universities. Piglets were assigned to one of three groups. Group I (N = 8) remained unvaccinated, Group II (N = 8) received the heat and RNase treated vaccine and Group III (N = 8) received the heat and RNase treated vaccine in combination with lung surfactant. Immunization involved 4 ml of 1×10^5 $TCID_{50}$/ml of treated virus with 2 ml delivered intranasally and 2 ml subcutaneously. Unvaccinated pigs received DMEM media, at the same volume and route. Pigs were observed post-vaccination for any side reactions or clinical signs of influenza. The first dose was administered at day 0 post vaccination (DPV 0), followed by two boosters at DPV 12 and DPV 20 days post-vaccination at the same dosage. Serum was collected at DPV 0, DPV 14, DPV 20 and DPV 31. Nasal swabs were collected at DPV 2, DPV 14, DPV 31 (or DPC 0), DPC 4 and 8. At DPV 31 (or 0 days post-challenge – DPC 0), all pigs were challenged with a total of 4 ml of 2.5×10^6 $TCID_{50}$/ml of Influenza A/CA/04/09 H1N1 virus culture administered equally into each nostril. Prior to challenge at DPV 31, two pigs from each group were euthanized to evaluate safety of the vaccine. Daily observations for clinical signs of influenza infection including pyrexia, nasal or ocular discharge, coughing and temperatures were recorded during the eight days following challenge. All pigs were humanely euthanized at DPC 8 and necropsies conducted as described below.

2.7. Pathological examination

Pathological evaluation and scoring was carried out in a blinded fashion by a board-certified veterinary pathologist. For the pigs (N = 2/group) euthanized prior to challenge to assess vaccine safety, the presence of any gross lesions due to vaccination was assessed. Heart, liver, spleen, kidney, lung and lymph node tissues were collected for microscopic examination. In addition, lung sections were assessed by immuno-histochemistry (IHC).

The remaining pigs (N = 6/group) were euthanized on DPC 8. The presence of gross lesions was assessed as the percentage of lung affected in the right and left cranial, medial and caudal lobes and accessory lobes of the lungs, normalized to the size of the lung. Microscopic lesions were assessed using hematoxylin and eosin stained sections as described before with some modification (Gauger et al., 2012; Halbur et al., 1996). Lung airways were examined for lymphocytic cuffing and interstitial pneumonia in the six selected lung sections and assigned scores from 1 to 4 as follows: 25% airways affected = 1, 26–50% airways affected = 2, 51–75% airways affected = 3, 76–100% airways affected = 4. The severity of the lesions was assessed as follows; lymphocytic cuffing of the peribronchiolar areas were scored as 0 = none, 1 = minimal, loosely formed, 2 = mild, loosely formed, 3 = moderate, well formed, 4 = severe, thick, well-formed cuffs. Interstitial pneumonia was scored as 0 = none, 1 = mild, focal to multifocal IP, 2 = moderate, locally extensive to multifocal IP, 3 = moderate, multifocal to coalescing IP, 4 = severe, coalescing to diffuse. The total average scores per experimental group are presented in Table 1.

A swine influenza-specific monoclonal antibody (ATCC HB65) was used to detect viral antigen in the lung sections by IHC at the South Dakota State University Veterinary Diagnostic laboratory, following their standard operating procedures. The number of sections with detectable antigen were enumerated to assign the IHC score. The gross, microscopic and IHC scores were summed to obtain the total lesion scores (Table 1). Statistically significant differences were assessed by the Mann-Whitney U test at $p \leq 0.05$.

2.8. Quantification of virus loads in nasal swabs

Automated extraction of total nucleic acids was carried out with a commercial kit (MagMAX total nucleic acid isolation kit, Thermo Scientific, Waltham, MA) and extractor (Biosprint 96, Qiagen, Valencia, CA) following the manufacturer's instructions. A standard curve was generated using logarithmic dilutions of the virus stock prepared as described above. A commercial one-step quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay, the Path-ID RT-PCR Kit (Thermo Scientific, Waltham, MA), which targets the matrix gene, was used to assess the extracted samples, following the manufacturer's instructions and previously validated standard operating

Table 1
Lung lesion scores.

Group	Gross Lesion Score/ No of positive animals	Microscopic Lesions Score/ No of positive animals	Immunohisto- chemistry Score/ No of positive animals	Total Lesion Score
PBS	17.00 ± 1.83 (6/6)	6.83 ± 0.19 (6/6)	10.00 ± 1.37 (4/6)	33.83 ± 2.05
Vaccine	4.50 ± 1.60 [a] (2/6)	2.66 ± 0.27 [a] (2/6)	1.0 ± 0.41 [a] (1/6)	8.16 ± 2.06 [a]
Vaccine + Surfactant	2.00 ± 0.82 [a] (1/6)	2.66 ± 0.14 [a] (5/6)	0.00 ± 0.00 [a] (0/6)	4.66 ± 0.93 [a]

Gross lesion scores – Total percentage of lungs affected (N = 6 pigs/group).
Microscopic lesion scores – Sum of the percentage of each lung section affected (N = 6pigs/group, 6 lung sections per pig), scored as follows:
Bronchial/bronchiolar epithelial changes, and/or bronchitis and bronchiolitis - Scoring -25% airways affected = 1, 26–50% airways affected = 2 =, 51–75% airways affected = 3, 76–100% airways affected = 4.
Interstitial pneumonia (IP) - Scoring - 0 = none, 1 = mild, focal to multifocal IP, 2 = moderate, locally extensive to multifocal IP, 3 = moderate, multifocal to coalescing IP, 4 = severe, coalescing to diffuse.
Peribronchiolar lymphocytic cuffing - 0 = none, 1 = minimal, loosely formed, 2 = mild, loosely formed, 3 = moderate, well formed, 4 = severe, thick, well-formed cuffs.
Immunohistochemistry (IHC) scores – Sum of the number of sections positive for antigen as detected by a SIV specific monoclonal antibody and IHC score (N = 5 pigs/group, 6 lung sections per pig), Scoring - weak = 1, moderate = 2, strong = 3.
Total lesion scores – Sum of the gross, microscopic and IHC scores.
a- significantly different from the unvaccinated group, b- significantly different from the other vaccine group, Mann-Whitney U test at $p \leq 0.05$.

procedures of the North Dakota State University Veterinary Diagnostic Laboratory.

2.9. Antibody responses to vaccination

Sera collected from the experimental pigs were assessed in duplicate by an enzyme linked immunosorbent assay (ELISA) for antibody (Ab) responses. Responses against recombinant hemagglutinin (HA) protein and nucleoprotein (NP) were measured. For the HA ELISA, 96-microwell ELISA plates (Corning, USA) were coated with 100 µl/well of a 1:1000 dilution of influenza A/CA/04/09 HA protein (NR-15749, BEI resources) in carbonate coating buffer (pH 9.6), and incubated overnight at room temperature, followed by 5 washes with phosphate buffered saline with tween 20 (PBST). Plates were blocked with 200 µl/well of blocking buffer (2% BSA in 1X PBST) for 2 h at 37 °C. After blocking, plates were washed 5 times using 1X PBST. To each well, 50 µl of test serum diluted 1:50 in PBST was added in duplicate and incubated for 2 h at 37 °C. After 4 washes with PBST, 50 µl/well of a 1:10,000 diluted anti-swine IgG peroxidase-conjugated secondary Ab (KPL, USA) was added. The plates were incubated at 37 °C for 1 h. After washing 5 times, 50 µl/well of TMB substrate (KPL, USA) was added to plates and incubated in the dark for 5 min. at room temperature to catalyze the reaction. Finally, 50 µl/well of 1 M HCl was added to stop the reaction. The OD readings were obtained at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT) (Fig. 4).

Antibody responses to the conserved nucleoprotein (NP) were measured using a commercial ELISA kit (IDEXX Influenza A antibody kit), following the manufacturer's instructions and standard operating procedures of the S. Dakota State University Veterinary Diagnostic laboratory. The manufacturer recommended signal/ negative cutoff ratio to distinguish between positive and negative samples was ≤ 0.6, and so a lower value indicates a stronger response and vice versa.

2.10. Hemagglutination inhibition assay (HAI)

A standard protocol was used to test sera for HAI. Briefly, test sera were treated with 15 µl of receptor destroying enzyme (Jing et al.) (Denka Seiken, USA) in 45 µl of serum and incubating overnight at 37 °C, followed by RDE inactivation at 56 °C for 30 min. Serial, doubling dilutions of the sera were performed starting at a 1:10 dilution. Diluted samples were then mixed with 8 HA units of Iinfluenza A/CA/04/09 H1N1 virus and incubated at 37 °C for 1 h. Following the incubation, 50 µl of 1% turkey RBCs (Lampire Biological Laboratories, USA) was added to each well, mixed gently and incubated for 15 min. Wells containing button of RBCs were recorded as positive.

2.10.1. Statistical analysis

Antibody responses, qRT-PCR data and HAI data was analyzed by a Student T test using Microsoft Excel 2016. The lesions scores were analyzed by the Mann Whitney U test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Heat and RNase treated virus is ultra-structurally stable

At the selected temperature of 44 °C for 10 min. for reversible denaturation, followed by RNase treatment and refolding, treated virions were found to retain the characteristic pleomorphic, spherical structure of influenza viruses with the HA and NA protein spikes embedded in the envelope (Fig. 1A & B). As expected, examination of the thin films formed by the lung surfactant alone showed the presence of vesicular structures as described before (Braun et al., 2007) (Fig. 1C). No toxicity was detected when the surfactant preparation was incubated on MDCK cells for 48 h. Examination of the vaccine preparation containing a combination of treated virions and the surfactant showed the presence of aggregates of virions enclosed in the multi-lamellar vesicles formed by the surfactant (Fig. 1D)

3.2. The treated virus is not detected until the 3rd passage in cells

Incubation of the reversibly denatured virus culture to various time points ranging from 2 h to 24 h in the presence of RNase, helped to narrow down the optimal time of exposure to 8 h and 7 min.. Replication of the treated virus was not detected in the 1st passage when MDCK cell monolayers were infected with the treated virus and stained with a H1N1-specific Ab after 48 h (Fig. 2A). A low level of fluorescence was detected by IFA in the 3rd passage (Fig. 2C). Quantification of the $TCID_{50}$ values of the virus culture from the 3rd and 4th passage of treated virus did not produce a detectable signal in the lowest dilution of 10^{-1}, while the untreated control virus has a titer of $10^{6.1}$ $TCID_{50}$.

3.3. Lung surfactant improves delivery of vaccine antigen to cells

When the vaccine virus packaged in the surfactant was similarly tested to determine if the surfactant improved antigen delivery to cells, a green signal indicative of viral antigen was not detected by IFA in the first passage (Fig. 2D). However, while the treated virus alone was not detected until the 3rd passage, a low-level signal was detected earlier, in the 2nd passage (Fig. 2E), in cells exposed to the treated virus and surfactant, indicating that the surfactant promoted a more efficient

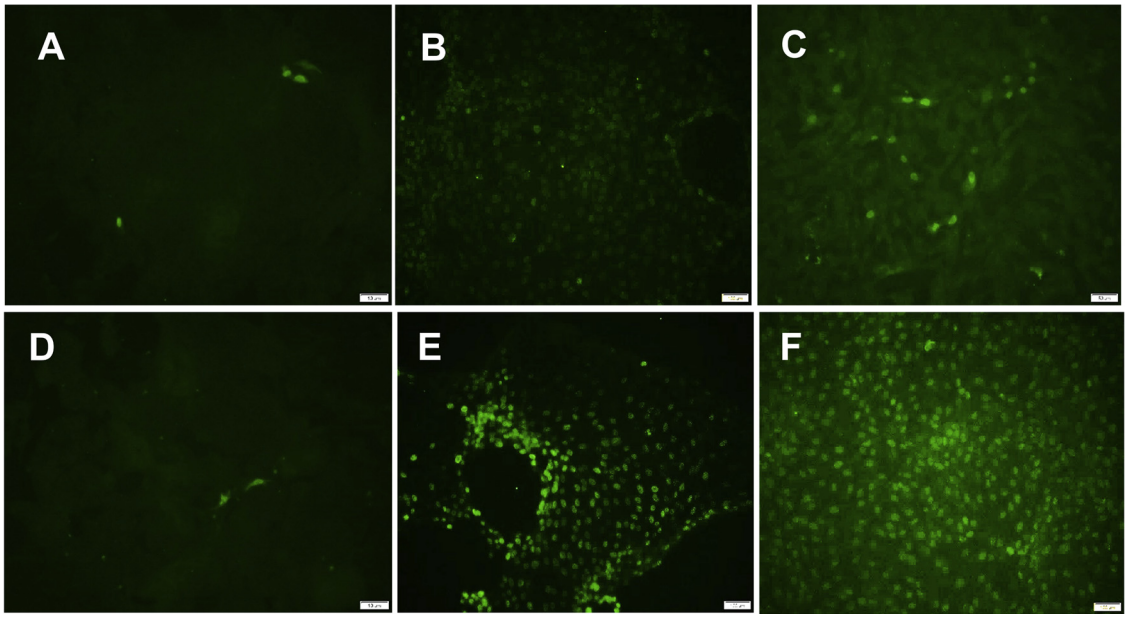

Fig. 2. In vitro antigen delivery by the pulmonary surfactant. Vaccine antigen delivery in MDCK cells infected with treated virus alone (A, B, C); or treated virus combined with pulmonary surfactant (D, E, F) at passage 1 (A, D); passage 2 (B, E) and passage 3 (C, F). Cells were stained with an influenza A/ H1N1-specific swine polyclonal antibody. The strength of the apple green fluorescent signal is indicative of the amount of viral antigen detected (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

intracellular delivery of the vaccine virus (Fig. 2F). The $TCID_{50}$ value for the 3rd and 4th passages were below the limit of detection.

3.4. Vaccination protects against development of lung lesions

Following challenge with the homologous virus, all six unvaccinated control pigs developed gross and microscopic scores with a total score of 33.83 (Table 1). However, clinical signs of influenza such as an elevated temperature, coughing or sneezing were not observed in the unvaccinated group. Lesions scores at the gross, microscopic and IHC levels were significantly lower in both the vaccine groups when compared to the unvaccinated controls. While the overall scores of the pigs immunized with vaccine and surfactant were slightly lower than that of the pigs administered the vaccine alone, the differences were not statistically significant (Table 1).

3.5. Vaccinated pigs are protected against challenge viral replication

The pathological lesion scores followed a similar trend as the qRT-PCR results. One pig in the group administered the vaccine alone had a low value on DPC 8 and challenge virus was not detected by qRT-PCR in the nasal swabs of pigs in both vaccinated groups at DPC 4. The unvaccinated control pigs shed high titers of challenge virus in their nasal secretions on DPC 4, with the infection resolving by DPC 8 as only low viral loads were detected at this time point. As expected, all pigs were negative on DPC 0, prior to challenge (Fig. 3).

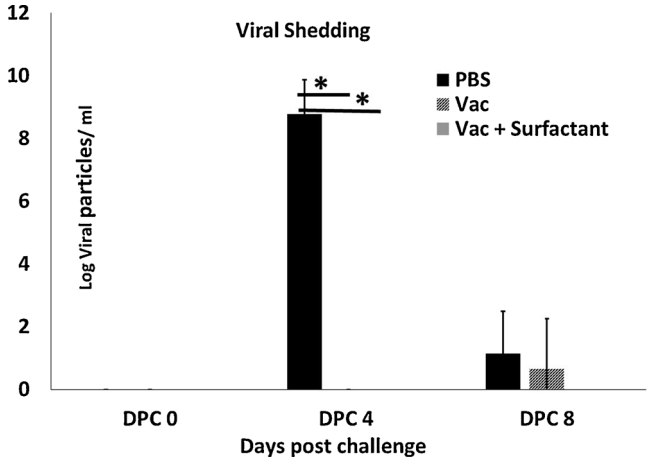

Fig. 3. Protection against viral shedding. Viral loads in nasal swab samples measured by qRT-PCR. N = 8 pigs for DPC 0 and N = 6 pigs for DPC 4 & 8. X axis- days post challenge (DPC), Y axis – log viral particles/ml. Error bars represent the standard deviation. An asterisk (*) symbol represents groups that are statistically different ($p < 0.05$, Student's T test) from the unvaccinated group.

3.6. Vaccination induces strong antibody responses against the HA protein

Measurement of serum Ab responses against the HA and NP proteins by ELISA showed that both vaccine groups mounted strong HA specific Ab responses which were significantly different from the unvaccinated group at 2 weeks after the primary vaccination and continued to increase in magnitude after the boosters (Fig. 4A). However, Ab responses to the NP were very low throughout the study, although they were significantly different from the unvaccinated controls at DPV 14, 20 and 31. The mean signal / negative ratio values remained above the manufacturer prescribed cut-off value of 0.6 to distinguish between positive and negative samples for all test groups

(Fig. 4B). Therefore, vaccination induced strong Ab responses to the surface HA protein but not the NP protein.

3.7. Vaccination induces HAI responses

Similar to the patterns detected with the binding antibody assays, robust HAI titers were noted at DPV 14 in both vaccine groups, after the primary vaccination. The titers were significantly different from the unvaccinated controls. Although there were no statistically significant differences between the two vaccine groups, the pigs administered the vaccine in combination with the surfactant had slightly lower values. Administration of the booster vaccines did not appear to have a significant incremental effect on induction of virus neutralizing Abs (Fig. 5).

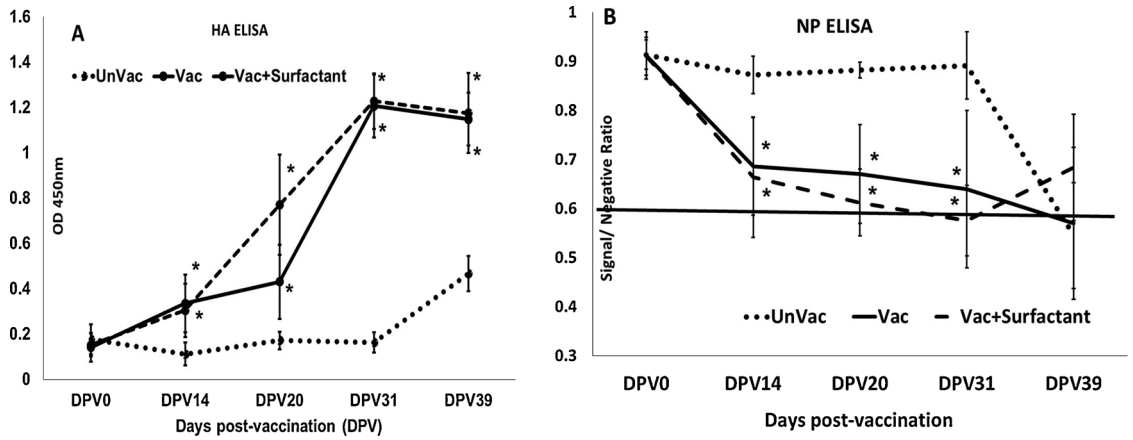

Fig. 4. Antibody responses in vaccinated pigs. Antibody responses against the influenza hemagglutinin (HA) and nucleoprotein (NP) proteins as measured by ELISA. N = 8 pigs for DPV 0,14, 20 and 31 and N = 6 pigs for DPV 39. Dotted line – Unvaccinated control, Solid line – pigs vaccinated with the heat and RNase treated vaccine, dashed line - pigs vaccinated with the heat and RNase treated vaccine and surfactant. 4A. Antibody responses to the HA protein. X axis- days post vaccination (DPV), Y axis – Mean OD reading (450 nm). 4B. Antibody responses to the NP. X axis - days post vaccination (DPV), Y axis – Mean signal: negative (S/N) ratio (450 nm). Solid horizontal bar – manufacturer prescribed S/N cut off value of ≤ 0.6 to distinguish between positive and negative samples. Error bars represent the standard deviation. An asterisk (*) symbol represents groups that are statistically different ($p < 0.05$, Student's T test) from the unvaccinated group at the respective days post vaccination.

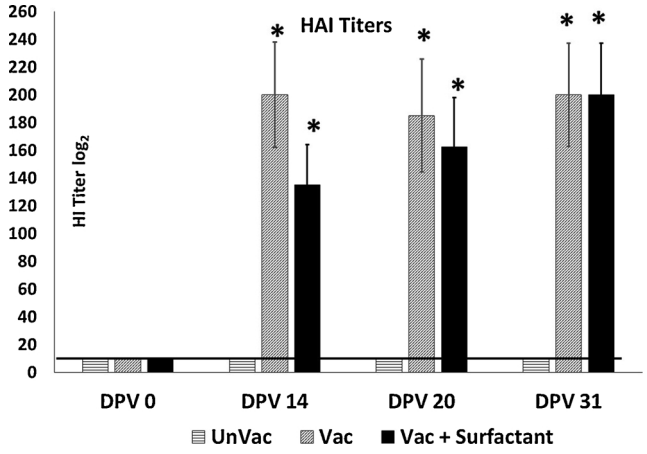

Fig. 5. Virus neutralizing antibody responses: Hemagglutination inhibition (HAI) titers measured against the homologous influenza A/CA/04/09 strain. N = 8 pigs/ group. X axis – Days post vaccination (DPV), Y axis – Mean $\log_2$ HAI titer, Bars with horizontal lines – Unvaccinated controls, Bars with slanted lines – Heat and RNase treated vaccine, Solid black bars - Heat and RNase treated vaccine + surfactant. Solid horizontal line – lowest detection limit of the test. Error bars represent standard deviation. * Significantly different from the unvaccinated group at the respective days post vaccination (DPV) at $p < 0.05$. No significant differences were noted between the two vaccine groups.

3.8. The heat and RNase treated vaccine and surfactant are safe

No side-effects or untoward reactions due to vaccination were observed in any of the vaccinated pigs prior to challenge. Shedding of vaccine virus in nasal secretions by qRT-PCR was also not detected after the primary or booster vaccinations respectively. Similarly, there were no significant differences in the pathological lesion scores between the three experimental groups in the pigs sacrificed prior to challenge. No gross lesions were detected in any of the pigs. Low microscopic scores of 1.0 for the unvaccinated group, 1.3 for the pigs administered the treated vaccine alone and 0.4 for the pigs administered the vaccine with the surfactant were noted. These low scores were primarily related to mild, non-specific peribronchiolar and perivascular lymphocytic cuffing. The presence of viral antigen in lungs as detected by IHC showed that the unvaccinated pigs had a baseline score of 1.5, while the pigs administered the vaccine alone had a score of 2.0 and the pigs administered the vaccine with the surfactant had a score of 3.5.

4. Discussion

Commonly used inactivation methods for influenza vaccines, such as beta propiolactone and formaldehyde, are safe but may interfere with the integrity of the HA protein which is critical for binding, fusion and effective antigen presentation in vivo (Herrera-Rodriguez et al., 2019). Similar drawbacks are reported for non-chemical methods of vaccine development, such as ultraviolet ray exposure, gamma irradiation or high heat, where aggregation and change to surface structures due to oxidation or generation of carbonyl groups may compromise the integrity of B cell epitopes, lead to suboptimal vaccine efficacy and contribute (De Flora and Badolati, 1973; Scheidegger et al., 2010). Exposure to temperatures above 56 °C for 10 min. or more (De Flora and Badolati, 1973; Isbarn et al., 2007; Wanaratana et al., 2010) can inactivate influenza viruses in media or various matrices such as feed or poultry waste. Depending on the amino acid composition, complete denaturation of the HA protein can occur at temperatures above 49 °C. For the influenza A/CA/04/09 strain, the HA protein was completely denatured by exposure to 52 °C for 40 min. (Epand and Epand, 2002; Rudneva et al., 2018). Hence in this study, 44 °C was used as the optimal temperature to reversibly denature the viral capsid. At this temperature, no damage to the surface structures was noted in electron micrographs (Fig. 1B).

Similarly, the RNase protein has a diameter of 3–4 nm and is metastably active over a range of temperatures (Ramm et al., 1985). The structural influenza M2 protein is considered a viroporin and a transmembrane ion channel (Torres, 2019). Viroporins are known to permit the entry of small molecules and drugs (Jing et al., 2008) and interact with other glycoproteins (Nieva et al., 2012). While characterizing the exact physical mechanisms involved is not within the scope of this study, it is possible that RNase is able to access the interior of the capsid to digest viral RNA when the capsid is in the reversibly denatured state at 44 °C.

Serial passage of the foot and mouth disease virus (FMDV) in cell culture for attenuated vaccine development results in the production of replication deficient genomes. When two or more replication deficient genomes infect the same cell, they were found to complement each other to produce plaques in vitro (Rodriguez-Calvo et al., 2010). In this study, replication of the heat and RNase treated IAV-S was not detected

in the first passage in MDCK cells. However, it is possible that repair, mutation and complementation mechanisms similar to those reported in the cited study on FMDV, were responsible for detection of the treated virus in the 3rd passage (Fig. 1C). Similar to this study, strong protection was elicited, and vaccine viral replication was not detected in pigs vaccinated with the FMDV defective-replication-complementation system (Rodriguez-Calvo et al., 2010). In vivo, the impediment posed by an active immune system to viral replication and the requirement for coinfection and complementation of the same cell by 2 or more defective genomes (or partially digested genomes in the case of the heat and RNase treated vaccine) provides for an additional vaccine safety barrier. Further, strong Ab responses to the NP are detected as early as 10 days post infection in pigs with active influenza viral replication (Panyasing et al., 2014). The absence of Ab responses to the NP despite booster vaccinations, and the lack of detection of vaccine virus by qRT-PCR in vaccinated pigs, indicates that vaccine viral replication was absent or minimal enough to be curtailed by host immunity, as originally targeted.

The field performance of the newly introduced LAIV-S vaccine remains to be evaluated in comparison to its experimental efficacy and safety (Masic et al., 2009; Solorzano et al., 2005). Current inactivated vaccines, which still constitute the majority of available commercial vaccines, were only recently updated to multi-valent preparations containing contemporary strains. However, given the vast diversity of IAV-S in the U.S (Anderson et al., 2013), there is a strong possibility of serological mismatch between the field and vaccine strains (Lee et al., 2007), and suboptimal vaccine performance in the field. Hence the availability of safe and effective IAV-S vaccines that can be rapidly updated to accommodate new strains is a critical industry need, which is being imprecisely addressed by the autogenous vaccine industry. Both commercial and autogenous vaccines which are killed, adjuvanted preparations, administered intra-muscularly, can be associated with VAERD. Strong binding, but non-neutralizing Ab responses elicited by vaccination are believed to bind non-specifically to new viral strains encountered on challenge to promote internalization, resulting in VAERD (Gauger et al., 2011; Vincent et al., 2008b). In this study, VAERD was not detected in either vaccine group as lesion scores in vaccinated pigs were significantly lower than the controls and no clinical signs were detected but warrants further testing with a heterologous challenge, which is not within the scope of this study. As viral loads were declining at DPC 8 (Fig. 4), the lung lesions could also have been resolving at this time point (Table 1).

While it is clear that intranasal delivery of attenuated vaccines is the most successful approach for immunization against IAVs, intranasal delivery of vaccines is often inefficient due to the rapid muco-ciliary clearance of vaccine antigen and the complex branched structure of the respiratory tract requiring high antigen payloads (Calzas and Chevalier, 2019). Further as the A/CA/04/09 strain can cause systemic signs such as vomiting (Lange et al., 2009), the vaccine was administered by both the intranasal and subcutaneous routes in this study, to target both local and systeminc protection. Pulmonary surfactant is a natural substance which reduces surface tension in the lung and is used in the treatment of airway distress in infants. It has also been effectively used as a drug and vaccine delivery system as it can spread throughout the lung surface (Baer et al., 2019; Mizuno et al., 2006). Lung surfactant is composed of phospholipids, and proteins such as SP-A, B, C and D. Surfactant proteins play a role in host defenses and clearance of bacterial and viral pathogens (Han and Mallampalli, 2015). As seen in this study (Fig. 1D), lung surfactant is known to spontaneously form uni or multilamellar, bi-layered vesicles (Braun et al., 2007) which can aggregate to deliver cargo with diverse physical properties to cells. When bovine lung surfactant was used as a vaccine delivery system for a subunit vaccine containing the influenza HA protein, HAI responses were elevated in the mini-pigs administered the antigen with the surfactant when compared to mini-pigs administered the antigen alone. The vaccinated pigs were not challenged in the study where bovine lung surfactant was used for vaccine delivery (Nishino et al., 2009). In this study, there was no statistically significant improvement in Ab responses or vaccine efficacy in the pigs administered the vaccine and surfactant, although the lesion scores were slightly lower in pigs vaccinated with the surfactant and antigen (Table 1). Bovine lung surfactant contains proteins SP-A and D while porcine surfactant contains SP-B and C. It is possible that the use of conventional pigs and porcine surfactant could have influenced the outcomes in this study. Niosomes are vesicular, non-ionic surfactant-based vaccine delivery systems which contain lipids and cholesterol. Phospholipids, similar to those present in lung surfactant, are common components of niosomes (Mahale et al., 2012). As shown in Fig. 1, the lung surfactant was able to form vesicles enclosing the cargo, similar to niosomes. While lung surfactant has not been evaluated for systemic vaccine delivery before, in this study, the presence of strong serum Ab responses to the HA protein indicates that antigen delivery by the surfactant via the subcutaneous route was also effective.

The pathogenicity of IAV-S varies between strains and challenge with other heterologous and homologous strains could affect the outcomes for the vaccine tested. In recent swine studies using A/CA/04/09 as the challenge strain, the patterns of viral replication and clinical disease reported are similar to this study (Everett et al., 2019; Mamerow et al., 2019). Vaccination of swine with the A/CA/04/09 strain has been shown to induce strong immunity against homologous, but not antigenically distant strains (Everett et al., 2019). Since the primary objective of this study was not related to the induction of cross protection, HAI responses against other strains were not tested.

While a limitation of the described method is that culturable virus should be readily available, it has several advantages in addressing current gaps in veterinary influenza vaccines. The heat and RNase treatment process can be rapidly adapted to newly emerging strains, can be delivered intra-nasally either alone, or in combination with adjuvants which are compatible with the respiratory tract. As vaccine viral replication is not detected in vaccinated animals, concerns about reversion to virulence or recombination to produce new strains would be negligible. While the ability of the vaccine to induce heterologous protection and cell mediated immunity remains to be evaluated, the high efficacy margin obtained in this experimental model supports further exploration of the described method in preserving critical surface structures without compromising safety and efficacy, presenting new options for influenza vaccine production.

Author contributions

H.V - data acquisition, analysis, manuscript preparation, G.S – data acquisition, analysis, manuscript preparation, A.P – pathological evaluation, manuscript review, B.W – q RT-PCR, manuscript review, E.N – NP ELISA, manuscript review, S.R – conception, funding, manuscript preparation.

Ethical approval

All animal experimentation was carried out in compliance with the Institutional Biosafety Committee and Institutional Animal Care and Use (IACUC) committees of North Dakota and South Dakota State Universities.

The authors declare no financial or commercial conflicts of interest. The described method and vaccine are protected by a provisional U.S. Patent Application (Serial No. 15/906,685).

Declaration of Competing Interest

The authors declare no financial or commercial conflicts of interest. The described method and vaccine are protected by a provisional U.S. Patent Application (Serial No. 15/906,685).

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