RESEARCH REPORT
SWINE HEALTH

Title: Attempts to broaden cross-protective immunity against swine influenza viruses – **(#19-215 IPPA)**

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Industry Summary:

Swine influenza is an economic burden to pig producers due to decreased feed consumption, weight loss, and increased death loss that often occurs from secondary bacterial infections. In addition, influenza A virus - swine (IAV-S), also known as swine influenza virus (SIV), is a zoonotic pathogen, which means it can be transmitted from pigs to people and causes influenza illness. Susceptible individuals include swine farm employees and their families, as well as youth attending agriculture fairs and in close proximity to infected swine. Therefore, the disease is a health and economic threat to both humans and swine worldwide.

Strict biosecurity, proper pig flow in a production system, and preventing transmission of human IAV to pigs through personal protective equipment have been essential control measures for IAV on swine farms. At the same time, swine producers need effective vaccines and vaccination strategies to help control virus infection, disease, and transmission among pigs and potentially between pigs and people. Unfortunately, influenza viruses in swine continue to evolve rapidly, complicating the ability to effectively control infection and transmission through the use of inactivated vaccines. Current inactivated virus vaccines, however, fail to cross-protect against the massive number of antigenically diverse strains circulating in swine and against the threat of human spillover IAV. Since vaccination still remains one of the most important methods to control IAV in swine, it is imperative the swine industry supports research and development of new vaccine platforms, immunogens, or alternative vaccination protocols.

Recently, our team developed a HA stem-based immunogen designated "HIV6HB-HA_{STEM}" based on the hemagglutinin (HA) of a human H3 virus. It had shown its binding with the monoclonal antibody specific for the most conserved epitope (CR9114) among influenza A and B viruses to date. More importantly, mice immunized with this antigen (10 µg, three times) not only developed a high level of ELISA antibody against the immunogen but also were protected from a lethal challenge of H1 and H3 IAV strains.

Based on these previous observations, an HA_{STEM}-based immunogen was constructed and produced based on the consensus sequence of H3 strains of IAV-S as H1-based immunogen was unstable to use for vaccination. Then we conducted a pigs study to evaluate the efficacy of the H3 HA_{STEM} immunogen in pigs against both H1 and H3 IAV-S simultaneously as a proof-of-concept for the universal vaccine platform. The immunogen was given intramuscularly to pigs with one of the three different adjuvants (Alum, Zn-chitosan, Emulsigen), 3 times at 2-week intervals. A group of pigs was kept unvaccinated as a control (NV group). Two weeks after the last immunization, all pigs were challenged with a mix of H1 and H3 IAV-S at the same titer. Some of the immunized and NV pigs were left unchallenged. All pigs were bled at 0, 14, 28, and 35 days after the first immunization and at 5 days post inoculation (dpi) for antibody tests (ELISAs, VN, HI). Pigs were weighed on days of challenge and at 5 dpi to calculate the average daily gain (ADG). Oral fluids and nasal swabs were collected to assess viral shedding by qPCR at 1, 3, and 5 dpi. All pigs were necropsied at 5 dpi, and lungs were collected for gross and microscopic evaluation of lesions and tested by IHC and qPCR.

No injection site reaction was observed in any of the immunized pigs. Pigs developed antibodies specific for the immunogen but no VN or HI antibodies. In general, no febrile response (>104 ºF) was observed in the vaccinated pigs after challenge except for some of the pigs immunized with Zn-chitosan adjuvant (for one day). Yet, the immunized pigs had a lower ADG than the NV group after the challenge. The immunization could not establish sterile immunity as all pigs had both H1 and H3 IAV-S at a similar level in lung and lung lavage fluid samples collected at 5 dpi. Viral load in the lung was lower in pigs who received the immunogen with Zn-chitosan adjuvant than the NV group, while the other immunized groups had a higher viral load. Likewise, pigs who received the immunogen with Zn-chitosan adjuvant developed gross lung lesion scores similar to those of the NV group but lower than those of other vaccinated pigs. The same group shed significantly less virus in nasal secretion as compared to the NV group.

Overall, the study demonstrated that the H3 HA_{STEM}-based antigen was immunogenic to pigs, even though the vaccine-induced antibodies did not neutralize the virus. The vaccine-induced immunity had the same antiviral impact on both H1 and H3 viruses, i.e. no subtype bias. While no sterile immunity could be conferred by this immunogen, the immunization, particularly when given with H3 HA_{STEM} + Zn-chitosan adjuvant, appears to induce some degree of protective immunity based on lack of fever, lower lung scores, lower lung viral load, and lower viral shedding in nasal secretions after challenge. Therefore, the immunogen used in the study may provide a design concept/platform toward a universal IAV vaccine with further optimization, including vaccination strategies.

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Key Findings:

- The HA_{STEM} antigen based on the consensus of H3 IAV is immunogenic to pigs.
- Antibodies induced by the immunogen do not neutralize IAV-S.
- The immunity induced by the designed immunogen may have the same antiviral effect on both H1 and H3 IAV-S.
- The immunogen may provide some degree of protective immunity (lower lung lesion score, lower lung viral load, lower viral shedding), particularly when it is given with Zn-chitosan adjuvant.
- The immunogen used in the study could be a new vaccine design platform toward a universal IAV vaccine.

Keywords:
Influenza A virus, swine, universal vaccine, HA stem-based immunogen, protection

Scientific Abstract:

Swine influenza is an economic burden to pig producers due to decreased feed consumption, weight loss and increased death loss that often occurs from secondary bacterial infections. In addition, influenza A virus - swine (IAV-S), also known as swine influenza virus (SIV), is a zoonotic pathogen. Therefore, the disease is a health and economic threat to both humans and swine worldwide. Although strict biosecurity, proper pig flow in a production system, and preventing transmission of human IAV to pigs have been important control measures for IAV on swine farms, vaccinations for the virus are the most common and necessary methods to control swine influenza. Current inactivated virus vaccines, however, fail to cross-protect against the massive number of antigenically diverse strains circulating in swine. Since vaccination still remains one of the most important methods to control IAV in swine, it is imperative the swine industry supports research and development of new vaccine platforms, immunogens, or alternative vaccination protocols.

Based on recent observations in our laboratories with a HA stem-based immunogen concerning universal 'flu' vaccines, an HA_{STEM}-based immunogen was constructed and produced based on consensus sequence of H3 strains of IAV ($H3HA_{STEM}$). Then a pig study was conducted to evaluate the efficacy of the immunogen against H1 and H3 IAV-S as a proof-of-concept for universal IAV vaccine. The immunogen was given intramuscularly to pigs with one of the 3 different adjuvants (Alum, Zn-chitosan, Emulsigen), 3 times at 2-week intervals. A group of pigs were kept unvaccinated as control (NV group). Two weeks after the last immunization, all pigs were challenged with a mix of H1 and H3 IAV-S. All pigs were bled at 0 and 35 days after the first immunization and at 5 days post inoculation (dpi) for antibody tests (ELISA, VN, HI). Pigs were weighed on days of challenge and at 5 dpi to calculate ADG. Oral fluids and nasal swabs were collected to assess viral shedding by qPCR at 1, 3 and 5 dpi. All pigs were necropsied at 5 dpi and lungs and trachea were collected for gross and microscopic evaluation of lesions and also tested by IHC and qPCR.

Pigs developed antibodies specific for the immunogen but no VN or HI antibodies. After challenge, no febrile response (>104 ºF) was observed in vaccinated pigs except some of the pigs received the immunogen with Zn-chitosan adjuvant (for one day). Yet, the immunized pigs had a lower ADG than the NV pigs. All pigs had both H1 and H3 IAV-S in lung and BALF samples collected at 5 dpi. Viral load in the lung was lower in pigs received the immunogen with Zn-chitosan than the NV group, while the other immunized group had a higher viral load. Likewise, pigs received the immunogen with Zn-chitosan developed gross lung lesion scores similar to the NV group but lower than any other vaccinated pigs. The same group shed significantly less virus in nasal secretion when compared to the NV group.

Overall, the study demonstrated that the H3 HA_{STEM}-based antigen was immunogenic to pigs, even though the vaccine-induced antibodies did not neutralize the virus. No subtype bias was observed in antiviral impact against H1 versus H3. While no sterile immunity could be conferred by this immunogen, the immunization appears to induce some degree of protective immunity based on lack of fever, lower lung scores, lower lung viral load, and lower viral shedding in nasal secretions after challenge particularly when the immunogen was given with Zn-chitosan adjuvant,. Therefore, the immunogen used in the study may provide a design concept toward a universal IAV vaccine even though further optimization and evaluation is necessary.

Introduction:

Influenza A virus in swine (IAV-S), also known as swine influenza virus (SIV), causes acute, severe respiratory disease in pigs, and is considered one of the top three health challenges facing the swine industry in the United States.[1,2] In addition, IAV-S is a zoonotic pathogen readily shared between pigs and people representing a health and economic threat to humans and swine worldwide.[3-10] Clinically, IAV-S decreases growth and production as well as increasing treatment costs leading to economic losses.[11] Estimates have shown a reduction of $10.31 per market hog per year.[11]

Influenza A virus is a member of the *Orthomyxoviridae* family and contains 8 negative-sense, single-stranded RNA segments.[12] Due to the segmented RNA genome, IAV genetic and antigenic diversity rapidly increases via reassortment of gene segments from different IAV strains and accumulation of point mutations[8,9,13] impeding the ability to effectively prevent or control IAV through vaccination. Currently, there are 8 H1 and 8 H3 genetically distinct IAV lineages circulating in North American swine.[14,15] Importantly, each H1 and H3 cluster is antigenically distinct from each other,[16,17] which has complicated the ability to develop broadly cross-protective IAV vaccines in swine. Therefore, the swine industry needs new, improved and broadly efficacious IAV vaccines.

Commercially available swine IAV vaccines for use in the U.S. are multivalent, whole inactivated virus (WIV) products administered by the intramuscular (IM) route or as non-replicating RNA particle subunit vaccines.[18-20] Due to the marked genetic and antigenic diversity observed in IAV-S, producing a broadly cross-protective vaccine based on the current WIV platform that prevents clinical disease and reduces transmission has become increasingly difficult.[2,18,21,22] In addition, live attenuated influenza virus (LAIV) vaccines administered by the mucosal route have shown to induce broader cross-protective immunity.[23-25] However, unintended transmission and reassortment with wild type IAV and persistence of vaccine virus have been observed with use of LAIV.

Recently, the concept of a universal influenza vaccine in humans has increased in popularity and may be applicable to IAV in swine. Universal vaccine antigens are designed to target highly conserved epitopes in the HA stalk region (HA2) or the matrix 2. These antigens are expected to induce broadly cross-reactive immune responses if appropriately presented to the immune system.[26-30] In particular, the HA2 is highly conserved among IAV in the same phylogenetic group and is a promising target for universal vaccine design.[31,32] Differing strategies for immunogen, dose, delivery, and schedule have been investigated for the HA2 as it may be shielded from the immune system by the immune-dominant HA1 globular head during infection or vaccination with WIV[33] increasing the need for novel immunogen formulations and vaccine strategies.

Objectives:

Our long-term goal is to develop a universal IAV vaccine for swine. The primary objective of the project was to generate novel immunogens and establish innovative vaccine strategies to elicit broadly cross-protective antibodies in swine that can provide protective immunity against IAV-S. The specific aim of the study was to determine the efficacy of the HA stem-based immunogens formulated based on consensus sequence of influenza A viruses against heterologous IAV-S.

Materials & Methods:

Study design. Initially, we proposed to study the protective efficacy of two HA stem (HA_{STEM})-based immunogens (H1 and H3), in comparison to selected commercial vaccines (WIV and LAIV), against selected heterologous H1 and H3 strains in the presence and absence of maternal antibodies. Due to COVID-19 and technical difficulties with HA_{STEM}-based immunogens (e.g., instability of H1 HA_{STEM} immunogen and a low concentration of immunogen), we modified the study design as shown below based on observations made from another NPB-funded project:

Immunogen	Adjuvant	H1 & H3 challenge	No virus challenge (NC)
HA_{STEM}-H3[a]	Alum	6[b]	2
HA_{STEM}-H3	Zn-Chitosan	6	2
HA_{STEM}-H3	Emulsigen	6	2
PBS (NV[c])	NA[d]	6	6

[a]H3 HA stem-based immunogen

[b]Number of pigs assigned per group

cNV: non-vaccinated

[d]NA: Not applicable

The study was intended to determine if the HA_{STEM} immunogen can confer cross-protective immunity against a wide range of IAV-S while optimizing vaccination strategies. The immunogen was designed based on the HA consensus sequence of H3 strains of IAV-S. The immunogen based on the consensus sequence of H1 strains of IAV-S was not used due to the instability of the protein after production.

Animals and immunization. Crossbred weaned pigs (n=36), 3-to-4 weeks of age and seropositive or seronegative for IAV, were purchased from commercial vendors historically negative for PRRSV, PCV2, and *M. hyopneumoniae* and housed in Livestock Infectious Disease Isolation Facility (LIDIF) at the Iowa State University. Upon receiving, pigs were ear-tagged, bled, weighed and randomly assigned to one of nine treatment groups shown in the table above. While pigs in "no-virus challenge" (NC) groups were housed in the same room, pigs in different vaccine/challenge groups were housed separately. After 3-day acclimation, pigs were administered intramuscularly with sham (i.e., no immunogen, NV group) or one of the immunogens (approximately 200-250 µg/dose) three times at 2-week intervals. During the vaccination phase, pigs were monitored for injection site reactions and clinical abnormalities. Blood samples (PBMC and serum) and nasal washes were collected on day 0 (before prime immunization) and weekly thereafter until the virus challenge. Blood for serum collection was collected by standard venipuncture of the anterior vena cava using SST Vacutainer™ tubes. Nasal wash was collected by flushing 5 ml sterile PBS into one nostril and immediately collecting the solution from the alternate nostril into a sterile cup as previously described.[34] Blood for collecting peripheral blood mononuclear cells (PBMC) were collected in BD Vacutainer® blood collection tubes with CPT and processed to isolate PBMC as previously reported.[35] All samples were stored frozen until tested.

Virus challenge, evaluation of response and sample collection. Two weeks after the final immunization or sham inoculation, pigs (at 11-to-12 weeks of age) were inoculated with a 2-ml intratracheal and 2-ml IN dose of H1 and H3 IAV-S at 1 x 10^5 $TCID_{50}$/ml, except the NC groups. Contemporary H1 γ1 [A/Sw/MN/A01567490/2014(H1N1)] and 2010 Human-like H3 [A/Sw/MO/23637/2014(H3N2)] isolates, commonly implicated in swine influenza outbreaks, were used as challenge viruses. Inoculation was performed under injectable anesthesia as previously described.[14] After challenge, body temperature and clinical signs (appetite, lethargy, nasal discharge, sneezing, coughing) were monitored daily. Body weight was measured at 0 and 5 days post inoculation (dpi). Oral fluids[36] and nasal swabs were collected on 0, 1, 3, and 5 dpi, processed and stored frozen until tested. Serum samples were collected at 0 and 5 dpi and stored frozen until tested for antibodies.

At 5 dpi, all pigs were humanely euthanized with a lethal dose of pentobarbital (Fatal Plus®, Vortech Pharmaceuticals) and necropsied. Postmortem samples included fresh bronchoalveolar lavage fluid (BALF), trachea, and right cardiac or the affected lung lobe. BALF samples were collected using 50ml of MEM as previously described[37], then stored frozen until evaluated for both virus and antibodies. Lungs were grossly and microscopically evaluated for the percent of the surface affected with pneumonia as previously described.[38] Trachea and lung tissue samples were fixed in 10% buffered formalin, routinely processed, and stained with H&E per VDL protocols.

Laboratory analyses. Sera collected at day 0, 14, 28, and 35 after prime immunization and ones collected at 5 dpi were evaluated for antibody responses using various assays, including 1) ELISA to monitor IgG against HA stem antigens, 2) VN assay against a large panel of IAV-S of different subtypes and lineages, and 3) HI test against IAV-S of different subtypes and lineages. ELISA, VN, and HI tests will be conducted using standard protocols established at ISU VDL and research labs.[39,40]

Oral fluids and nasal swabs collected on 1, 3, and 5 dpi were tested by a commercially available IAV RT-qPCR (ThermoFisher). Microscopic lesions were evaluated by a veterinary pathologist blinded to treatment groups and scored according to previously described parameters.[43] The level of IAV-specific antigen in lung tissues (i.e., IHC scores) was assessed using a previously described immunohistochemical (IHC) method with minor modification.[43,44]

Data analysis. Summary statistics were calculated for continuous variables from all groups to assess the overall quality of the data. Analysis of variance, with a p-value ≤ 0.05 considered significant, was used to analyze log-transformed virus titers and macroscopic pneumonia scores. Kruskal-Wallis test was employed to analyze microscopic pneumonia and IHC scores. Response variables shown to have a significant effect by treatment group were then subjected to pair-wise comparisons using the Tukey-Kramer test or the Dunn's test with Bonferroni correction.

Results:

All immunized pigs did not show any injection site reactions or were negatively impacted by vaccination clinically. After immunizations, all pigs developed antibodies against the immunogen regardless of immunogen formula as determined by antigen-specific ELISA. Two weeks after the 3rd shot (i.e., before challenge), the antibody titers ranged between 1:10000 and 1:100000, as shown in Figure 1. Numerically, pigs received the immunogen with oil-emulsion adjuvant had more consistent and higher antibody titers as compared to the other two vaccination groups. Antibody levels in all immunized pigs did not change after exposure to a mix of H1 and H3 IAV-S.

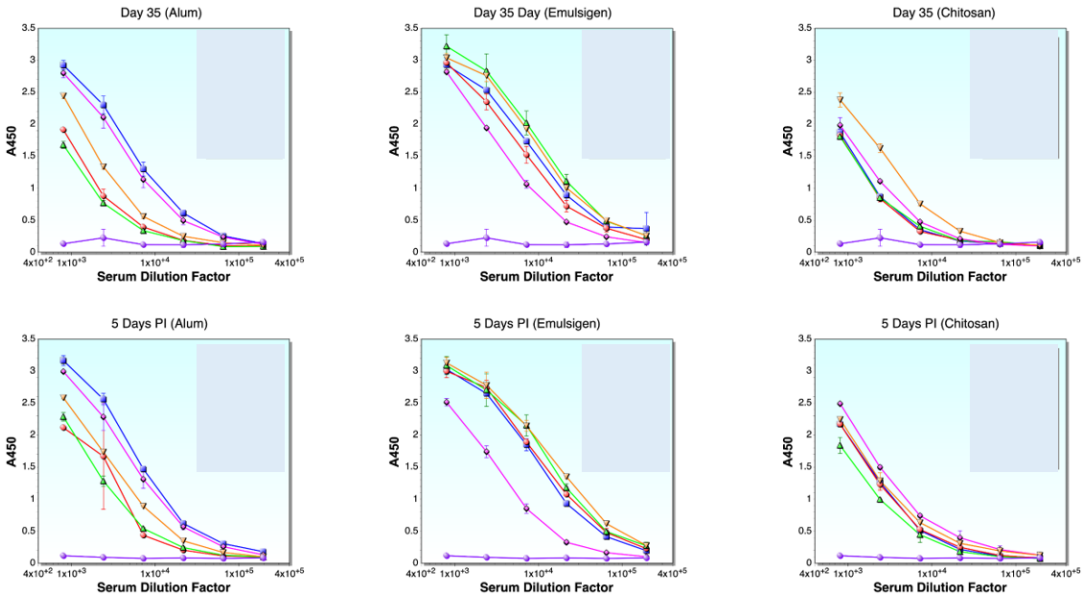

Figure 1. Antibody response in immunized and non-immunized pigs before and after challenge

None of the ELISA-positive serum samples were positive by HI and VN assays. None of the pigs were seropositive when tested by a commercial multispecies IAV ELISA which is based on NP antigen.

After challenge, NV pigs and pigs received the immunogen with Zn-chitosan were febrile (i.e., >104ºF) at 1 dpi based on anal temperature. Their body temperature became normal after that. No increased anal temperature was observed in the rest of the groups. Clinical signs, such as coughing, respiratory distress, anorexia, lethargy which are commonly manifested in young pigs affected swine influenza, were not apparent in any of the pigs, including NV pigs. Interestingly, while the NV pigs maintained an average daily gain (ADG) of approximately 1.7 LB, the immunized pigs had a lower ADG (around 1.2 LB) after challenge.

At necropsy, lung consolidation due to viral infection was significantly more severe in pigs received the immunogen with Alum or Emulsigen adjuvant (gross lung lesion score = 17-18) than pigs received the immunogen with Zn-chitosan adjuvant. The gross lung lesion score of this group was similar to that of NV pigs (Fig. 2). Microscopically, however, all vaccinated pigs had a higher lung score than NV pigs (data not shown).

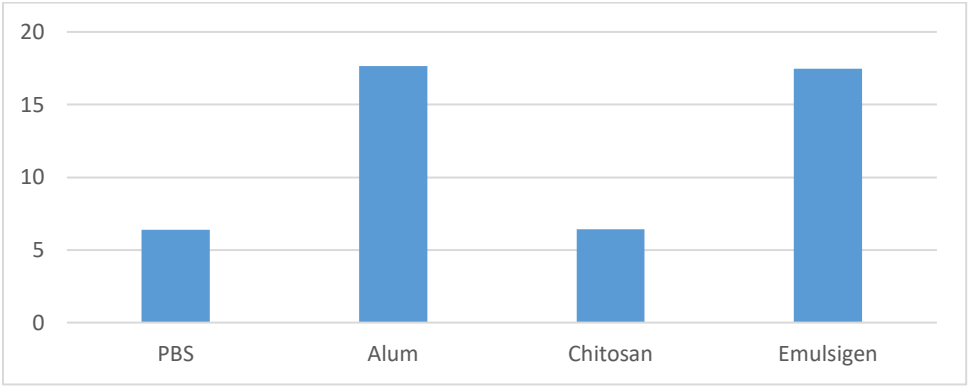

Figure 2. Gross lung lesions scores at 5 days post inoculation

At 5 dpi, all pigs had IAV-S in their lungs except NV/NC pigs. Pigs received the immunogen with Zn-chitosan had the lowest amount of IAV-S as determined by IHC. Pigs immunized with Alum or Emulsigen-adjuvanted immunogen had a higher IHC scores for IAV-S in their lungs than did NV pigs (Fig. 3). When the lungs were tested by subtype-specific qPCRs, all IAV positive lungs were determined to contain both H1 and H3 challenge viruses at similar titers. qPCR results on BALF samples were similar to those of the lung samples.

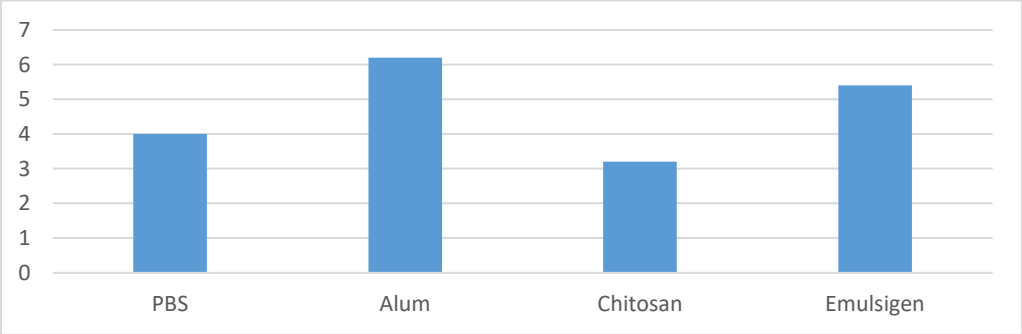

Figure 3. Lung viral load determined by IHC at 5 dpi

A significantly lower level (almost 100-fold) of IAV-S was detected by qPCR in nasal swabs from pigs received the immunogen with Zn-chitosan than any other treatment groups. About 40% of the pigs in this group were determined to shed the virus in their nasal swabs, while 80-100% of the pigs in the other treatment groups shed the virus in their nasal secretions. qPCR testing on oral fluids showed results similar to what was observed with nasal swabs among the treatment groups.

Discussion:

The study demonstrated that a HA_{STEM}-based antigen based on the consensus of H3 IAV (H3 HA_{STEM}) was immunogenic to pigs, even though vaccine-induced antibodies did not neutralize the virus or block the virus' ability to agglutinates RBCs in-vitro. The immunogen-specific antibody was detectable even after the 1st shot, implying that less than 200 µg of the immunogen could be used to dose a pig. The influence of adjuvant on the antibody response was apparent as the immunogen with an oil emulsion adjuvant induced a higher antibody level than the immunogen with the other adjuvants. However, it must be pointed out that the antibody level induced by this immunogen in pigs was not as high as what was observed in mice immunized with HA_{STEM}-based antigen made of a human IAV.

As no booster effect or reduction of antibody titer was observed after challenge, it can be speculated that the host immune system may not readily recognize the HA stem area within the IAV, at least in the early stage of infection. For the same reason, one can speculate that antibodies induced by a HA stem immunogen may not be consumed quickly by the immune system or outcompete by immunodominant epitopes of the virus. This property may be an advantage when pigs with maternal antibodies need to be immunized.

Under study conditions, immunization does not seem to confer sterile immunity against IAV-S as breakthrough infection occurred among immunized pigs regardless of adjuvant types. Yet, immunization with H3 HA_{STEM}+Zn-chitosan adjuvant had more positive impacts in terms of gross lung lesions and viral load in the lung compared to the other vaccinated groups and even the NV group. More importantly, immunization with H3 HA_{STEM}+Zn-chitosan adjuvant was able to induce immunity in pigs which contributed to a substantial reduction of virus shedding in nasal secretion.

One variable that needs to be taken into consideration is the age of pigs (12 weeks of age) by the time of challenge in this study. Knowing swine influenza is clinically more severe in young naïve pigs (2-6 weeks of age), 12-week old pigs may not have developed a severe clinical response to IAV challenge even if unvaccinated. If the study were done in younger pigs, the vaccination effect on clinical responses such as body temperature or ADG might have been more apparent.

Even though this immunogen could not induce sterile immunity and did not provide immunity for better clinical protection in this study, the immunogen was able to generate the immunity against both H1 and H3 IAV since any protective outcome was not skewed to the homologous serotype (i.e., H3) based on subtype-specific qPCR results on all IAV-positive samples, suggesting that this immunogen design may provide a concept or platform toward a universal IAV vaccine design. However, further optimization, including vaccination strategies, may be necessary.

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