RESEARCH REPORT
SWINE HEALTH

Title: Broadening cross-protective immunity against swine influenza viruses: A path forward a universal influenza vaccine **(NPB #19-106)**

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Industry Summary:

Influenza A virus - swine (IAV-S), commonly also known as swine influenza virus (SIV), is the cause of acute, severe respiratory disease in pigs and is considered one of the top three health challenges facing the swine industry in the United States (U.S.). 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. Morbidity from swine influenza in a pig population may achieve 100% with low mortality, although secondary bacterial infections may increase the death loss. However, IAV-S decreases growth and production as well as increasing treatment costs leading to further economic losses. Estimates have shown a reduction of $10.31 per market hog per year due to influenza virus infections. To put this into perspective, a large production system in the U.S. may sustain losses of over $65 million per year due to IAV and its associated costs. In addition, the increased use of antibiotics to treat secondary infections due to IAV is counter-intuitive to current initiatives to reduce bacterial antibiotic resistance.

Strict biosecurity, proper pig flow in a production system, and preventing transmission of human IAV to pigs through personal protective equipment are important control measures for IAV on swine farms. However, vaccinations for IAV-S are the most common and necessary methods to control swine influenza. Current inactivated virus vaccines fail to cross-protect against the massive number of antigenically diverse strains circulating in swine and against the threat of human spillover IAV that had significantly impacted the diverse IAV ecology since 1998 when the first H3N2 human transmission occurred that profoundly affected the industry. As traditional vaccines fail, the industry must explore novel immunogens/antigens and vaccination strategies that will demonstrate broad, cross-protective immune responses in the pig.

Our team recently developed a HA stem-based immunogen designated "HIV6HB-HA_{STEM}" based on the hemagglutinin (HA) of a human H3 virus (A/Hong Kong/8/68), which 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) developed a high level of ELISA antibody against the immunogen and were protected from a lethal challenge of H1 and H3 IAV strains. As a proof-of-concept for the universal vaccine platform, we conducted a pig study using a random block design to assess if the immunogenicity of HIV6HB-HA_{STEM} for swine and the degree of cross-reactivity of the antibodies with various H1 and H3 strains of IAV-S. Pigs were immunized three times with two different doses of the immunogen via two different routes. Two different adjuvants were used with the immunogen. All vaccinated pigs developed antibodies against the immunogen regardless of doses and routes, although IM injection and an oil-emulsion adjuvant induced a higher level of the antibodies, indicating the immunogenicity of the immungen for swine. However, neither virus-neutralizing antibodies nor HI antibodies were detected in these pigs, implying that this particular immunogen may elicit protective immunity in swine in a way different from what traditional IAV-S vaccines induce. Nonetheless, it remains to further investigate if the immune response induced by this immunogen can provide pigs clinical protection against IAV-S challenge.

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Key Findings:

- HA stem-based antigen is immunogenic to swine, even though the immunogen was designed based on the HA protein of a human H3 strain.
- The immunogen did not induce either virus-neutralizing or HI antibodies, suggesting that the protective immunity, if conferred, may not depend upon antibodies against HA protein.
- Immunogen design and immunization strategies would be essential for a universal IAV vaccine.

Keywords: Influenza A viruses, swine, universal vaccine, hemagglutin, HA-stem based immunogen

Scientific Abstract:

Influenza A virus - swine (IAV-S), commonly also known as swine influenza virus (SIV), is the cause of acute, severe respiratory disease in pigs and is considered one of the top three health challenges facing the swine industry in the United States (U.S.). 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. Although Strict biosecurity, proper pig flow in a production system, and preventing transmission of human IAV to pigs through personal protective equipment are 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. Hence, the industry must explore novel immunogens/antigens and vaccination strategies that will demonstrate broad, cross-protective immune responses in the pig.

Recently our team developed a HA stem-based immunogen designated "HIV6HB-HA$_{STEM}$" based on the hemagglutinin (HA) of a human H3 virus (A/Hong Kong/8/68). 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, 3 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, even though no virus neutralizing antibodies were detected in these mice. Therefore, the following study was conducted to evaluate this immunogen in pigs against IAV-S as a proof-of-concept for the universal vaccine platform.

A pig study using a random block design was conducted to assess if the immunogenicity of HIV6HB-HA$_{STEM}$ for swine and the degree of cross-reactivity of the antibodies with various H1 and H3 strains of IAV-S. Pigs were immunized 3 times with two different doses (200 and 400 µg/pig) of the immunogen via two different routes (IM and IN). Two different adjuvants (Zn-chitosan and Emulsigen) were used with the immunogen. All vaccinated pigs developed antibodies against the immunogen regardless of doses and routes, although IM injection and an oil-emulsion adjuvant induced a higher level of the antibodies, indicating the immunogenicity of the immungen for swine. However, the immunized pigs were not seropositive by HI assays against various H1 and H3 strains of IAV-S and IFA assay using cells infected with those IAV-S strains, implying that particular immunogen may elicit protective immunity in swine in a way different from what traditional vaccines induce. It remains, however, to further investigate if the immune response induced by this immunogen can provide pigs clinical protection against IAV-S challenge.

Introduction:

Influenza A virus in swine (IAV-S) is the cause of acute, severe respiratory disease in pigs and is considered one of the top three health challenges facing the swine industry in the United States (U.S.).[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]

Clinically, swine influenza is manifested as an abrupt onset of respiratory disease characterized by fever, inactivity, inappetence, respiratory distress, coughing, sneezing, and nasal discharge.[4,5] The viral incubation period is brief, approximately one to three days, with rapid recovery occurring from seven to ten days after onset.[1,6] Morbidity in a swine population may achieve 100% with low mortality, although secondary bacterial infections may increase the death loss.[1,4] However, IAV-S decreases growth and production as well as increasing treatment costs

leading to further economic losses.[16] Estimates have shown a reduction of $10.31 per market hog per year due to IAV-S.[7] More importantly, following the emergence and detection of the H1N1 2009 pandemic virus in US swine, 27 countries banned or threatened to ban U.S. pork and pork products, which cost the U.S. pork industry over $5 million per day due to the perceived uncertainty of the safety of U.S. pork.[8]

Influenza A virus is a member of the *Orthomyxoviridae* family and contains a negative-sense, single-stranded, 8-segmented RNA genome[18] encoding for at least 12-13 proteins. Two surface proteins, hemagglutinin (HA) and neuraminidase (NA), are the antigenic and/or genetic basis for subtyping.[10] The H1, H3, N1, and N2 are the common subtypes implicated in swine influenza even though pigs have demonstrated susceptibility to all known 17 HA subtypes of IAV as both human α-2,6 and avian α-2,3 influenza receptors are present in the respiratory tract of swine.[11] Due to the segmented RNA genome, IAV genetic and antigenic diversity rapidly increases via reassortment of gene segments from different influenza virus strains (genetic shift) and accumulation of point mutations (genetic drift)[12] impeding the ability to effectively prevent or control IAV through vaccination. Furthermore, spillovers of human-seasonal IAV to swine continues to complicate the ability to develop broadly cross-protective vaccines.[2] Currently, there are eight genetically distinct H1 IAV lineages circulating in North American swine designated the H1 α, β, γ1, γ2, δ1a, δ1b, δ2, pdm09 clades and eight different H3 cluster IV, IVA, IVB, IVC, IVD, IVE, IVF and 2010 human-like clades.[13,14] Importantly, each H1 and H3 cluster is antigenically distinct from each other with minimal serologic cross-reactivity.[15,16] Unfortunately, this has complicated the ability to develop broadly cross-protective IAV vaccines with the ability to control the spread and continued evolution of IAV in swine populations. Therefore, the swine industry is in great need of new, improved and broadly efficacious IAV vaccines incorporating novel antigens, platforms and delivery methods that protect against the broad genetic and antigenic diversity circulating in swine.

Commercially available IAV vaccines for use in the U.S. for swine are whole inactivated virus (WIV), multivalent, adjuvanted products administered by the intramuscular route or as non-replicating RNA particle subunit vaccines.[17-19] Due to the marked genetic and antigenic diversity observed in H1 and H3 IAV in swine, producing a broadly cross-protective vaccine based on the current WIV platform that prevents clinical disease and reduces transmission has become increasingly difficult.[2,17,20,21] Alternative IAV vaccine platforms and methods of delivery are needed to improve heterologous protection. Live attenuated influenza virus (LAIV) vaccines administered by the mucosal route mimic natural infection and has shown to induce broader cross-protective immunity.[22-24] However, an LAIV product recently licensed for use in US swine for the first time has shown evidence of reassortment with wild type IAV, unintended transmission, and persistence of the HA gene in some swine populations. This unexpected problem with the LAIV platform has caused a major issue for the swine industry with unknown consequences at this time.

More 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 protein 2 (M2). These antigens are expected to induce broadly cross-reactive immune responses if appropriately presented to the immune system.[25-27] In particular, the HA2 stalk domain is highly conserved among influenza viruses in the same phylogenetic group and is a promising target for universal vaccine design.[28,29] Differing strategies for type of immunogen, dose, delivery, and schedule have also been investigated for the HA2 stalk domain as it may be shielded from the immune system by the immune-dominant HA1 globular head during infection or vaccination with WIV.[30] To date, it has been difficult to induce broadly neutralizing antibody against the HA2 stalk domain reliably, raising the need for novel immunogen formulations and vaccine strategies.

Objectives:

The primary objective of the proposed work was to generate novel immunogens and to establish innovative vaccine strategies to elicit broadly cross-protective antibodies against IAV-S. The specific aim is to characterize and optimize the immunogenicity of HA_{STEM}-based immunogen (HIV6HB-HA_{STEM}) for swine.

Materials & Methods:

Pigs and study design. Large White crossbred weaned pigs (n=60) at three weeks of age that are seronegative for IAV and PRRS virus were purchased from commercial vendors and housed in the Livestock Infectious Disease Isolation facility (LIDIF) at Iowa State University (ISU). Upon receiving, pigs were ear-tagged, bled, and randomly assigned to 3 or 6 pigs per treatment group (total 12 groups). Next, one pig from each of 4 unvaccinated groups and

two pigs from each of 8 vaccinated groups were randomly selected and placed into a room, resulting in 3 rooms with 20 pigs each representing all treatment groups.

Immunization. Pre-trial serum samples were tested for antibodies against IAV, PRRS virus, PCV-2, porcine parainfluenza virus 1, and *Mycoplasma hyopneumoniae* to establish a baseline status. After 2- to 3-day acclimation, pigs were administered with sham (i.e., no immunogen) or H3 HIV6HB-HA_{STEM} at dosages of 200 µg or 400 µg with a Zn-chitosan or Emulsigen®-D (oil-in-water emulsion) adjuvant, delivered by the intramuscular (IM) or intranasal (IN) route, three times at 2-week intervals. For IM injection, 2ml of the immunogen were given in the neck area. For IN inoculation, 1ml of the antigen was given to each nostril of the pig using a Prima® Mist intranasal device (Neogen, Lexington, KY) attached to the syringe. During the vaccine phase of the study, pigs were monitored for injection site reactions and any clinical abnormalities.

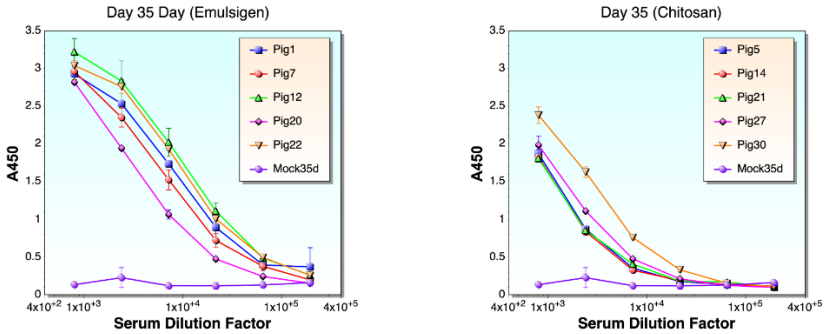
Sample collection. Blood samples and nasal washes were collected on day 0 before prime immunization and weekly thereafter until the end of the study (3 weeks after boost immunization). Blood samples for serum were collected by standard venipuncture of the anterior vena cava using SST Vacutainer™ tubes. Nasal wash was collected by flushing 5 ml sterile PBS into one nostril while the pig was lying on its back and immediately collecting the solution from the alternate nostril into a sterile cup as previously described.[31] All processed samples were stored at -80°C until tested.

Antibody analyses. Antibody responses were characterized using a multitude of assays, including 1) ELISA to monitor HA stem (i.e., immunogen)-specific antibody levels and to monitor antibody response to nucleocapsid protein (NP), 2) indirect fluorescent antibody (IFA) assay to assess antibody response against the native HA protein, 3) hemagglutinin-inhibition (HI) assay against a panel of IAV-S of different subtypes and lineages (H1 α, β, γ1, γ2, δ1a, δ1b, δ2, pdm09 clades and eight different H3 cluster IV, IVA, IVB, IVC, IVD, IVE, IVF, and 2010 human-like clades), and 3) VN assay against the panel of IAV-S of different subtypes and lineages. ELISAs, HI, and VN assay were conducted using standard protocols established at ISU VDL and research lab.[32,33]

Data analysis. Log-transformed, mean antibody titers detected in all assays were compared using an ANOVA with significant differences among groups measured with the Tukey-Kramer test.

Results:

Immunization with HIV6HB-HA_{STEM} induced antibodies specific for the immunogen in pigs as determined by the immunogen-specific ELISA. Antibody response was detectable after 1st short, although the antibody level was low, regardless of immunogen dose or route of immunization. The level of antibody against the immunogen on the 35 days (7 days after 3rd shot) was between 10^4 and 10^5 based on the serial dilution of samples as shown in the figure below. The figure shows representative antibody response from each group. The oil-emulsion adjuvanted immunogen induced a higher level of antibody numerically than did the Zn-chitosan adjuvanted immunogen. Immunization of pigs by IM injection tended to induce a higher antibody level than IN route.



None of the pigs developed an antibody against NP after immunizations as determined by a commercial ELISA specific for IAV of multispecies. No virus neutralization antibody to H1 or H3 IAV-S was detected in any of the immunized pigs either. Unexpectedly, none of the immunized pigs were seropositive against IAV-S when tested by IFA assay using MDCK cells infected with various H1 and H3 IAV-S.

Discussion:

The study demonstrated the HIV6HB-HA_{STEM} was immunogenic to pigs even though the immunogen was constructed based on the HA sequence of a human H3 virus, suggesting that the same approach of immunogen design is applicable to IAV-S specific vaccines. Furthermore, a high level of immunogen may not be required for immunization as pigs immunized with 200 μg/pig had detectable antibody response even after the first shot. Under the study condition, the route of immunization (IM vs. IN) did not matter, although adjuvant may influence the level of immune responses in pigs.

The immunogen used in the study was designed based on the HA2 unit of the HA protein of IAV. Therefore, the immunized pigs were not expected to develop antibodies against any other viral proteins such as NA, NP, or M. Furthermore, it was also anticipated that pigs immunized with this specific immunogen would not develop HI antibodies because the immunogen was based on the internal portion of the HA protein. Hence, all negative results on the NP ELISA or HI assay are not a surprise. In contrast, it was an unexpected finding that all immunized pigs were seronegative by IFA assay using MDCK cells infected with IAV-S, assuming that the infected cells would possess various portions of the HA protein besides many other viral proteins. Further work remains to investigate reasons.

It was disappointing that none of the immunized pigs developed antibodies which can neutralize IAV-S. It is, however, in agreement with observations from a previous study done in Dr. Cho's laboratory. His group was not able to demonstrate the presence of neutralizing antibody in mice immunized with the same antigen used in this study. Since the immunized mice were protected from a lethal challenge, we speculate that there must be another immune mechanim contributing to such protection. It remains to further study if the same protection can be seen in pigs.

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