RESEARCH REPORT
SWINE HEALTH

Title: A candidate swine influenza virus vaccine: *in vivo* evaluation of novel chimeric hemagglutinins expressed by parainfluenza virus 5 (PIV5) vector; **NPB#15-020**

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Industry Summary:

The goal of this project was to evaluate candidate influenza vaccines that induce broad immunity against multiple influenza viruses in pigs. These vaccines have been designed to induce immune responses against viruses within the influenza A virus H1N1 subtype, which includes multiple clusters that co-circulate in pigs. Using parainfluenza virus 5 (PIV5) as a vector, we now show that each of our two influenza virus hemagglutinins (HAs) can induce antibodies against multiple influenza viruses within the H1N1 subtype, and that both can protect pigs against infection with the A/swine/Alberta/25/2009-H1N1 influenza virus. These results show that both vaccines have the ability to induce broad immunity within the H1N1 subtype, with evidence of protection against one of these viruses in a direct vaccine:challenge. Vaccines designed in this manner can be used to minimize the number of HAs included in current influenza vaccines, while simultaneously increasing the breadth of immunity induced.

Keywords:

Vaccination, influenza, immunity, heterologous, protection

Scientific Abstract:

Vaccination remains our best method of protection against influenza virus infection, and vaccines are used to limit spread of these viruses in both pigs and humans. Unlike the human situation, multiple clusters of influenza A viruses co-circulate within pigs, with as many as four clusters identified in pigs. This makes the vaccine selection process more difficult for pigs than it is for humans, and current vaccines include multiple hemagglutinins (HAs) representing these distinct clusters in an effort to induce broad immunity. We recently demonstrated that chimeric HA constructs created using DNA shuffling could induce broad immunity against HAs within the H1N1 subtype. Specifically, two of these HAs, HA-111 and HA-113, induced broad immunity within the H1N1 subtype. In this study, we used a parainfluenza virus 5 (PIV5) vector to express HA-111 and HA-113 as candidate vaccines. Immunogenicity of the PIV5-111 and PIV5-113 vaccines was tested in mice as well as in nursery pigs. Serum ELISA results showed that mice immunized with the PIV5-111 and PIV5-113

candidate vaccines produced antibody responses against genetically diversified influenza virus H1N1 strains, including parental viruses and heterologous viruses. In PIV5-111 and PIV5-113 vaccinated pigs, both vaccines were able to induce antibodies against parental HAs, as detected using hemagglutination inhibition. More importantly, PIV5-111 and PIV5-113 vaccinated pigs were protected from virulent virus challenge, as demonstrated by reductions in virus load in both nasal swab and bronchoalveolar lavage fluid samples. Our data suggest that PIV5-vectored chimeric HA vaccine candidates can induce broad, protective immunity in vaccinated animals. This study provides a new vaccine platform for development of broadly protective vaccines for other important swine pathogens.

Introduction

Influenza viruses have the ability to infect a large number of animal species, including birds, pigs, and humans. These viruses are characterized by the hemagglutinin (HA) protein expressed at their surface, which is also the target of host adaptive immunity against these viruses [1]. Distinct clusters of HA proteins currently circulate in pigs [2,3] and humans [4,5], and this can be problematic when these viruses transmit between the two species, as they did in 2009 [4,6–8]. These interspecies transmission events resulted in a pandemic in humans [9], which was accompanied by illnesses in pigs that had an economic impact on pork production [10].

A vaccine that provides immunity against multiple swine HAs at the same time would be beneficial, and the commercially available FluSure XP® vaccine uses viruses expressing individual H1 HAs (A/Swine/North Carolina/031/05-H1N1, A/Swine/Iowa/110600/00-H1N1, and A/Swine/Oklahoma/0726H/2008-H1N2), as well as a single H3N2 variant (A/Swine/Missouri/069/05-H3N2) to induce this broad immunity. We are now using a technique known as “molecular breeding (DNA shuffling)” to create HA genes in the lab that express multiple epitopes within a specific HA subtype, in an unbiased manner. The DNA shuffling process allows for the random recombination of parental HA genes into novel gene constructs that can be evaluated for immunogenicity [11–15]. The chimeric HAs we created have demonstrated an ability to induce immunity against many, if not all, of the parental HAs used for their creation. Having demonstrated the ability of our vaccines to induce broad immunity, we decided to evaluate two of our candidate vaccines using the parainfluenza 5 (PIV5) virus vector, which would allow these HA proteins to be expressed as a vaccine that can be delivered intranasally (IN) in a manner that induces robust mucosal immune responses [16]. In this proposal, we used PIV5 as a vaccine vector that expresses laboratory-derived H1 chimeric HA proteins that do not exist in nature, but have demonstrated the potential to induce antibodies that recognize HA proteins expressed by both swine and human influenza viruses.

Objectives:

Objective 1: To construct PIV5-vectored candidate vaccines that express influenza virus HA-111 and HA-113 chimeras (Biao He, University of Georgia).

The first objective of this project is to create PIV5 viruses that individually express either the HA-111 or the HA-113 DNA constructs as recombinant proteins. Dr. Biao He’s laboratory has previously expressed native influenza HA proteins within the PIV5 vector system [17], indicating a high likelihood for success in creating these recombinant viruses. To show that the recombinant PIV5 viruses express the desired HA-111 and HA-113 proteins, immunoblotting and immunofluorescence assays will be performed as described previously [17], using antibodies from our DNA vaccine study, which were induced by the HA-111 and HA-113 DNA constructs. The metric for success in this objective will be the creation of PIV5 vectors that express HA-111 (PIV5-111) and HA-113 (PIV5-113).

Objective 2: To evaluate the safety and efficacy of PIV5-111 and PIV5-113 candidate vaccines in a nursery pig model (Ying Fang, Kansas State University).

The safety and efficacy of PIV5-111 and PIV5-113 candidate vaccines will be evaluated in a nursery pig model. Since we have already demonstrated that our HA-111 and HA-113 proteins can induce antibodies against 4 parental viruses, we anticipate that the PIV5-111 and PIV5-113 vaccines will also induce broad

immunity against these viruses. As mentioned above, PIV5 was tested in pigs, with both clinical signs, virus titers, and immunogenicity evaluated. The metric for success in this objective will be the safe delivery of our vaccine to pigs using the PIV5 vector system, with the desired outcome being the induction of antibodies against influenza H1 HA after vaccination.

Objective 3: To evaluate pig serum antibodies for the breadth of immunity against genetically diversified H1N1 viruses (Victor Huber, University of South Dakota).

After vaccination with either PIV5-111 or PIV5-113, serum samples from individual pigs will be evaluated for the breadth of immunity induced, using a standard hemagglutination inhibibition (HAI) assay, as described [18]. Evaluation of the breadth of the antibody response induced by HA-111 and HA-113 will include testing serum reactivity induced using a panel of genetically diversified viruses, including both human and pig H1 HA-expressing viruses that represent distinct H1 HA clusters. The metric for success in this objective will be induction of antibodies against genetically diversified influenza viruses.

Materials & Methods:

Creation of PIV5-111 and PIV5-113: DNA encoding either HA-111 or HA-113 was cloned into the PIV5 vector, between the gene encoding the SH protein and the gene encoding the HN protein, as described [17]. This included the whole HA gene, representing both the HA1 and HA2 regions, and previous creation of PIV5 viruses that contain this genetic makeup (PIV5-H5) have demonstrated optimal success within Dr. He's recently published murine vaccine:challenge model [17].

Confirmation of PIV5-111 and PIV5-113 protein expression: Both immunoblotting and immunofluorescence were performed using anti-H1N1 HA antibodies to demonstrate expression of HA-111 and HA-113 by MDBK cells infected with PIV5-111 or PIV5-113, respectively. Included in the anti-H1 HA antibodies were those directed against the HA-111 and HA-113 constructs, produced previously using DNA vaccination. These sera were included to demonstrate that the HA proteins expressed by PIV5 are recognized by antibodies that specifically react with the proteins expressed by our HA-DNA constructs.

Mice: Adult (6–8-week-old) female BALB/cJ mice were obtained from Harlan Laboratories (Indianapolis, IN) and housed in groups of five, with 24-hour access to food and water. All animal experiments were performed following the guidelines established and approved by the Animal Care and Use committee at the University of Georgia (Athens, GA).

Mouse PIV5-111 and PIV5-113 Inoculation: Mice in a group of 5 were inoculated with PBS, PIV5, PIV5-111 or PIV5-113 at a dose of 10^7 plaque forming units (PFU) intranasally. At 21 days after the immunization, blood samples were collected from mice. After 28 days after initial immunization, the mice were boosted with the same virus at the same dose. At 14 days after the boost, blood samples were collected.

Hemagglutination Inhibition (HAI) Assay and ELISA: RDE-treated sera were analyzed for influenza-reactive antibody using standard HAI titer and ELISA assays. For HAI assays, chicken red blood cells were used, and titers are reported as the reciprocal of the final serum dilution that inhibited hemagglutination. A titer of less than 10 (starting serum dilution) was assigned for serum samples that did not inhibit hemagglutination. HAI assays were performed using standard techniques. Briefly, sera were diluted, and 4 HA units of virus of interest was added in each well. The virus:sera mixtures were incubated for one hour at 4°C, and 50µL of 0.5% chicken red blood cells (RBC) was subsequently added to each well. For ELISA, plates were coated with 1 µg/mL influenza virus HA content, and diluted sera were added to the plates after blocking. Antibodies of the IgG isotype were detected using goat anti-mouse IgG (H+L) conjugated to alkaline phosphatase. After washing, substrate was added, and titers are reported using 50% maximal binding on the titration curve.

Pig PIV5-111 and PIV5-113 Inoculation: Groups of pigs were inoculated with PBS (n = 12), PIV5 (n = 6), PIV5-111 (n = 6), or PIV5-113 (n = 6) at a dose of 10^7 plaque forming units (PFU) intranasally. Serum samples were collected on days 7, 14, 21 and 28 post-vaccination for evaluation of influenza-reactive antibodies.

Pig PIV5-111 and PIV5-113 Challenge: Groups of pigs that were inoculated with PBS were either not infected with influenza virus (n = 6) or infected with the A/swine/Alberta/25/2009-H1N1 influenza virus (n = 6) at a dose of 10^6 $TCID_{50}$ intranasally. Similarly, pigs inoculated with PIV5 (n = 6), PIV5-111 (n = 6), or PIV5-113 (n = 6) were infected with the A/swine/Alberta/25/2009-H1N1 influenza virus (n = 6) at a dose of 10^6 $TCID_{50}$ intranasally. Nasal swab samples were collected on days 0, 1, 3, and 5 post-challenge, and bronchoalveolar lavage fluid was collected on day 5 post-challenge. Serum samples were collected on day 5 post-challenge for evaluation of influenza-reactive antibodies.

Results:

Objective 1: To construct PIV5-vectored candidate vaccines that express influenza virus HA-111 and HA-113 chimeras

Metric of success: The metric for success in this objective will be the creation of PIV5 vectors that express HA-111 (PIV5-111) and HA-113 (PIV5-113).

The PIV5-111 and PIV5-113 candidate vaccines were created, with sequence confirmation of the HA-111 and HA-113 genes being contained within the vectors. Despite our best efforts, we were unable to confirm HA-111 and HA-113 protein expression by our PIV5 vectors using anti-HA antibodies. Thus, to confirm expression of these HA constructs, and to demonstrate immunogenicity before moving into the pig model, we performed a mouse pilot vaccine study (**Figure 1**). Antibodies generated against PIV5-111 and PIV5-113 were tested against the parental influenza viruses H1 HA clusters (A/New Jersey/8/76-H1N1, **NJ76**, α cluster; A/Iowa/1/06-H1N1, **IA06**, β cluster; and A/Ohio/1/07-H1N1, **OH07**, γ cluster), as well as an H1 HA cluster that established stable transmission in humans (A/Tennessee/1-560/09-H1N1, **CA09**, 2009 pandemic-like cluster). These antibodies were also tested against the HA expressed by the human virus A/Memphis/3/08 (**ME08,** δ cluster). The metric of this objective was achieved as our data show that we created PIV5 vaccines that express HA-111 and HA-113, and that these vaccines can induce broad immunity against genetically diversified HA proteins.

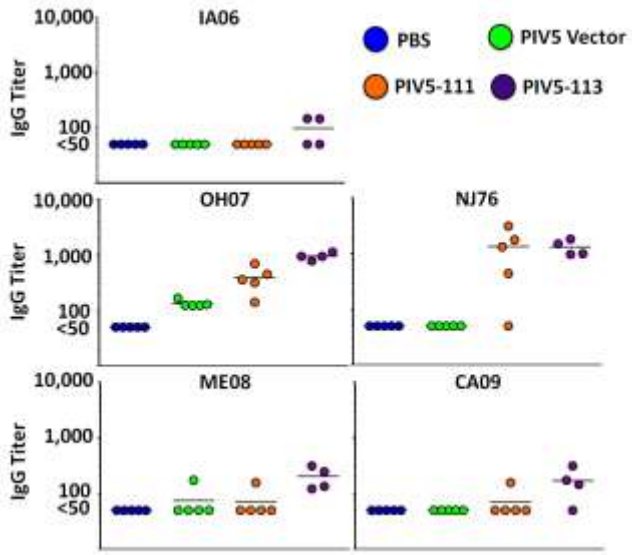

Figure 1: Detection of antibodies after vaccination with HA-111 and HA-113 expressed by PIV5. Mice were vaccinated with PBS (n = 5), PIV5 Vector (n = 5), PIV5 expressing HA-111 (PIV5-111, n = 5), or PIV5 expressing HA-113 (PIV5-113, n = 4). Mice were vaccinated twice, with 21 days separating vaccine doses. Sera were collected on Day 42 after initial vaccination and analyzed by ELISA (IgG(H+L)). Results are shown as titers based on 50% maximal binding on the titration curve. Vaccine-induced antibodies were detected against parental HA-expressing viruses, with the PIV5-113 vaccine inducing antibodies against all five parental HAs: IA06, OH07, NJ76, ME08, and CA09.

Objective 2: To evaluate the safety and efficacy of PIV5-111 and PIV5-113 candidate vaccines in a nursery pig model.

Metric of success: The metric for success in this objective will be the safe delivery of our vaccine to pigs using the PIV5 vector system, with the desired outcome being the induction of antibodies against influenza H1 HA after vaccination.

Having created the PIV5-111and PIV5-113 vaccines, and demonstrating their ability to induce antibodies in mice, we proceeded with our vaccination of pigs. Pigs vaccinated with either PIV5-111 or PIV5-113 were challenged with the A/swine/Alberta/25/2009-H1N1 influenza virus 28 days post-vaccination, and nasal swab samples were collected on days 1, 3, and 5 post-challenge (**Figure 2**). On day 5 post-challenge, bronchoalveolar lavage fluids were also collected on day 5 post-challenge, which was the end of the experiment. The A/swine/Alberta/25/2009-H1N1 is similar to the CA09 influenza virus that was responsible for the human pandemic in 2009. The metric of success for this objective was achieved as our data show that we vaccination with PIV5-111 and PIV5-113 can protect pigs against challenge with the A/swine/Alberta/25/2009-H1N1 influenza virus. As indicated below (**Figure 3**), this protection correlates with antibodies against H1N1 influenza viruses, including CA09.

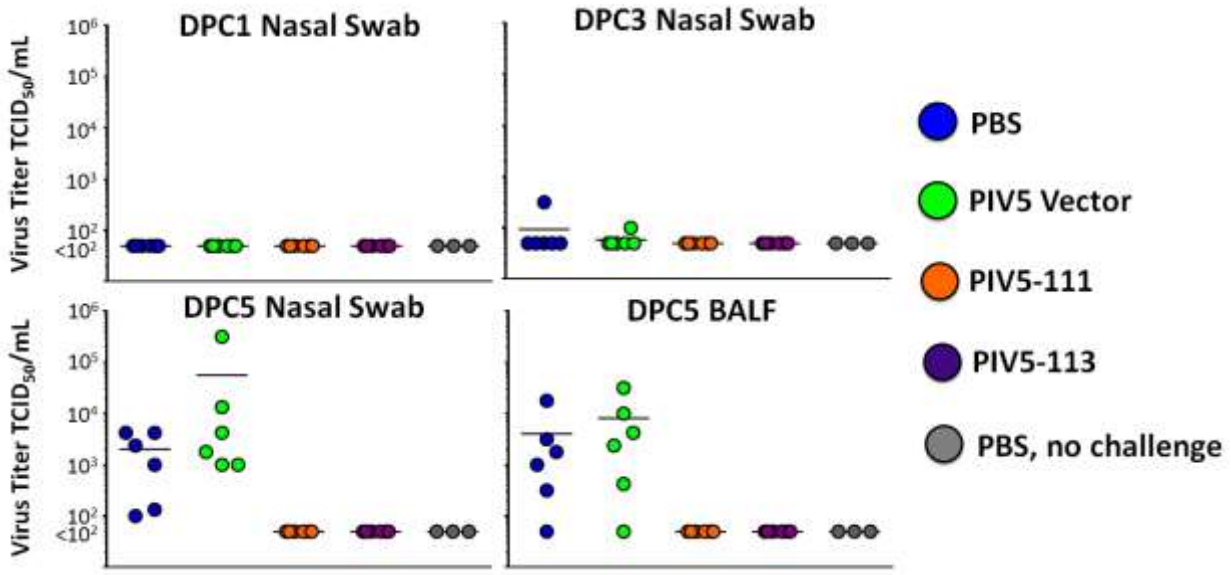

Figure 2: Virus titer
PIV5-113 twice with
A/swine/Alberta/25/
post-challenge. Vir
pigs, but not vaccin

Objective 3: To evaluate pig serum antibodies for the breadth of immunity against genetically diversified H1N1 viruses

Metric of success: The metric for success in this objective will be induction of antibodies against ge
diversified influenza viruses.

As presented above, we have created two candidate influenza vaccines, PIV5-111 and PIV5
induce broad immunity against viruses expressing parental HA proteins (**Figure 1**), and that can ind
protective immunity against a virus expressing an HA that is similar to one of the parental proteins (**Figure 2**). Using sera that were collected 5 days post-challenge, here we show that antibodies against all 5 parental viruses are detected in pigs that were vaccinated with either PIV5-111 or PIV5-113 (**Figure 3**). The metric of success for this objective was achieved, as we demonstrated that vaccine-induced antibodies recognize genetically diversified HA proteins expressed by individual influenza viruses.

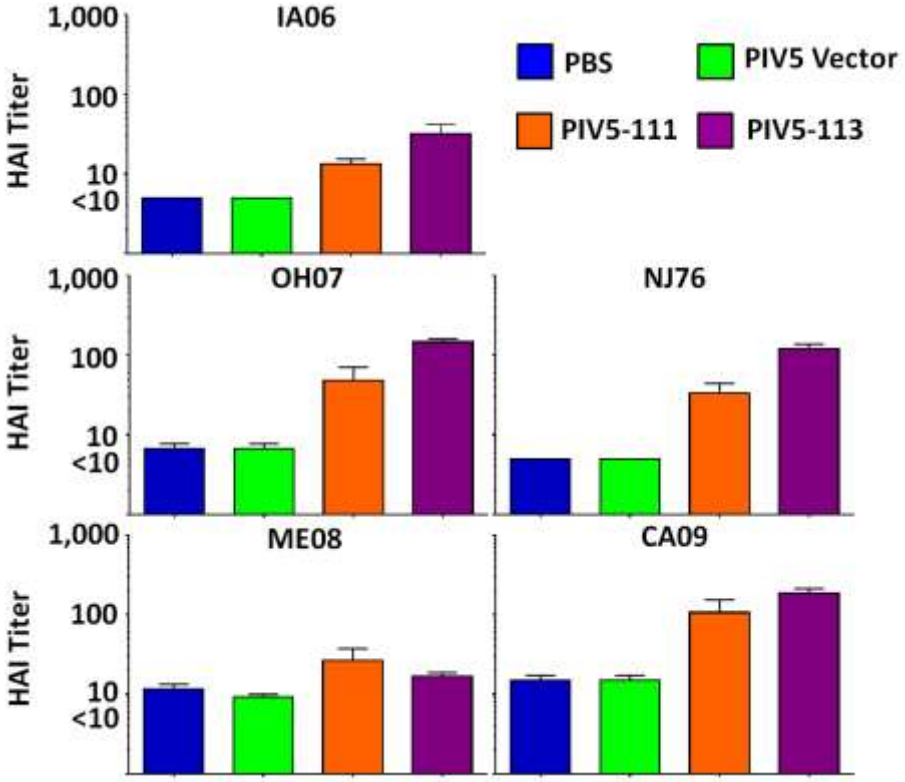

Figure 3: Hemagglutination Inhibition Assay. Pigs (n = 6 per group) were vaccinated with PBS, PIV5 Vector, PIV5-111, or PIV5-113 twice with 14 days separating vaccine doses. Sera were collected 5 days after challenge, and were analyzed using a standard hemagglutination inhibition (HAI) assay. Vaccine-induced antibodies were detected against the five parental HAs IA06, OH07, NJ76, ME08, and CA09.

Discussion:

Our data show that the two candidate vaccines we created, PIV5-111 and PIV5-113, can induce broad immunity against genetically diversified influenza viruses within the H1N1 subtype, and that these vaccines can prevent infection of pigs with at least one of these influenza viruses. These vaccines show significant promise for both immediate and future use in pigs. However, in order to more adequately demonstrate the breadth of protective immunity, we will need to perform at least one additional challenge study using another parental influenza virus, possibly the OH07-like virus. The breadth of antibodies induced is very promising, and additional experiments would include further evaluation of the antibody response that correlates with broad, protective immunity.

It is worth noting that our vaccine was delivered via the intranasal route, and that our attempts to measure vaccine-induced immunity using post-vaccination serum from pigs did not show strong antibody titers in circulation. However, when post-challenge sera were evaluated (**Figure 3**), and vaccine-induced protection is considered (**Figure 2**), there is not only a protective response observed, but the breadth of vaccine-induced immunity is fully realized. This has led us to ponder whether the vaccine-induced antibody responses may be higher in the mucosal compartment, and experiments designed to measure antibodies in nasal swab and bronchoalveolar lavage samples are planned. Results from these experiments may indicate that a better correlate of immunity for these vaccines can be detected in this compartment. Future work will focus on identifying the pre-challenge immune response that correlates with the protection observed. Despite this

limitation in antibody detection, our findings from the vaccine:challenge study in pigs clearly show that protection can be induced by these vaccines.

The data acquired from this study will be critical for advancing this vaccine into use in the near future. Further validation of the breadth of immunity induced, using either additional pig or mouse vaccine:challenge studies, will help strengthen our position that this vaccine could improve the health of pigs. Further, our future efforts will focus on identifying the vaccine-induced correlate of protective immunity that is associated with the protection that we observed in the pig vaccine:challenge study. Determining this correlate of protection will help us determine the immune response that we should target using this vaccine, or any other intranasal vaccine. These findings indicate that our vaccines have both immediate and future benefit to pork producers in their efforts to minimize the burden of influenza virus infection.

List of Publications and Presentations

The data generated were presented in poster form at the Options for the Control of Influenza Conference in Chicago on August 24-28 of 2016, and an abstract for this work was selected for an oral presentation at the CRWAD meeting that will be held on December 6-8 in Chicago. All publications, presentations, and abstracts of the project results will include an acknowledgment of funding, as described in the "NPB Interim Research Grant Report Form."

Invention statement

An invention has been disclosed to the Technology Transfer Office at the University of South Dakota, and a patent application was filed on April 17, 2016 to protect the use of DNA shuffling to create chimeric HA genes (attorney Docket Number 8967292/109135)

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