RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of the epitope driven pDNA vaccine PigMatrix EDV in the pig model as a candidate vaccine for universal flu protection – **NPB #17-185**

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Date Submitted: December 19, 2018

Industry Summary

Most commercial Influenza A virus (IAV) vaccines provide homologous protection, but fail to prevent heterologous infections. In this study, the efficacy of an intradermal administered pDNA vaccine (EPITOPE) was evaluated and compared to an intramuscular commercial inactivated whole virus vaccine (INACT), and a combined vaccine regimen against virulent IAV challenge. Thirty-nine IAV-free, 3-week-old pigs were randomly assigned to one of five groups including a NEG-CONTROL group (unvaccinated, sham-challenged), an INACT-INACT-IAV group (vaccinated with FluSure XP® at 4 and 7 weeks, pH1N1 challenged), an EPITOPE-INACT-IAV group (vaccinated with PigMatrix EDV at 4 and FluSure XP® at 7 weeks, pH1N1 challenged), an EPITOPE-EPITOPE-IAV group (vaccinated with PigMatrix EDV at 4 and 7 weeks, pH1N1 challenged), and a POS-CONTROL group (unvaccinated, pH1N1 challenged). The challenge and sham-challenge were done at 9 weeks of age, and all pigs were necropsied at day post challenge (dpc) 5. At the time of challenge, all INACT-INACT-IAV pigs, and by dpc 5 all INACT-INACT-IAV and EPITOPE-INACT-IAV pigs, were seropositive for IAV. IFNγ secreting cells, recognizing T cell epitope specific vaccine peptides as well as pH1N1 challenge virus, were increased for the EPITOPE-INACT-IAV over the EPITOPE-EPITOPE-IAV and INACT-INACT-IAV groups at challenge. On dpc 1, EPITOPE-INACT-IAV pigs and INACT-INACT-IAV pigs had significantly lower body temperatures compared to the POS-CONTROL and EPITOPE-EPITOPE-IAV. Macroscopic lung lesion scores were reduced in all EPITOPE-INACT-IAV pigs while INACT-INACT-IAV pigs exhibited a bimodal distribution of low and high scores akin to naïve challenged animals. No detectable IAV antigen in lung tissues at dpc 5 was observed in the EPITOPE-INACT-IAV group similar to naïve unchallenged pigs and different from all other challenged groups. These results suggest that the prime boost approach using an epitope driven DNA vaccine with inactivated vaccines merits further exploration as a practical control measure against IAV.

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Keywords: IAV; vaccination; pigs; prime boosting; DNA vaccine; inactivated vaccine.

Scientific Abstract

Most commercial influenza A virus (IAV) vaccines provide homologous protection, but fail to prevent heterologous infections. In this study, the efficacy of an intradermal administered pDNA vaccine (EPITOPE) was evaluated and compared to an intramuscular commercial inactivated whole virus vaccine (INACT), and a combined vaccine regimen against virulent IAV challenge. Thirty-nine IAV-free, 3-week-old pigs were randomly assigned to one of five groups including a NEG-CONTROL group (unvaccinated, sham-challenged), an INACT-INACT-IAV group (vaccinated with FluSure XP® at 4 and 7 weeks, pH1N1 challenged), an EPITOPE-INACT-IAV group (vaccinated with PigMatrix EDV at 4 and FluSure XP® at 7 weeks, pH1N1 challenged), an EPITOPE-EPITOPE-IAV group (vaccinated with PigMatrix EDV at 4 and 7 weeks, pH1N1 challenged), and a POS-CONTROL group (unvaccinated, pH1N1 challenged). The challenge and sham-challenge were done at 9 weeks of age, and all pigs were necropsied at day post challenge (dpc) 5. At the time of challenge, all INACT-INACT-IAV pigs, and by dpc 5 all INACT-INACT-IAV and EPITOPE-INACT-IAV pigs, were seropositive for IAV. IFNγ secreting cells, recognizing T cell epitope specific vaccine peptides as well as pH1N1 challenge virus, were increased for the EPITOPE-INACT-IAV over the EPITOPE-EPITOPE-IAV and INACT-INACT-IAV groups at challenge. On dpc 1, EPITOPE-INACT-IAV pigs and INACT-INACT-IAV pigs had significantly lower body temperatures compared to the POS-CONTROL and EPITOPE-EPITOPE-IAV. Macroscopic lung lesion scores were reduced in all EPITOPE-INACT-IAV pigs while INACT-INACT-IAV pigs exhibited a bimodal distribution of low and high scores akin to naïve challenged animals. No detectable IAV antigen in lung tissues at dpc 5 was observed in the EPITOPE-INACT-IAV group similar to naïve unchallenged pigs and different from all other challenged groups. These results suggest that the prime boost approach using an epitope driven DNA vaccine with inactivated vaccines merits further exploration as a practical control measure against IAV.

Introduction

Viruses are a major cause of respiratory disease in pigs, decreasing both the welfare of pigs and economic gains of pig farmers. Clinical signs of IAV infection in pigs includes loss of appetite, fever, lethargy, paroxysmal coughing, conjunctivitis, and nasal discharge (1). The incubation period is short, with the disease spreading across a herd and causing clinical disease within 2 or 3 days (2). Stress, high population density, and environmental factors increase the chance of the virus spreading among a population (3). The prevalence of IAV as a cause of acute respiratory disease or endemic infections in pig herds is likely underestimated (4). Diseased individuals can become economic burdens due to weight loss in growing pigs or reproductive failures secondary to fever in breeding herds (5). North American subtypes of IAV which commonly circulate in the pig population include H1N1, H1N2, and H3N2 (6). Within a subtype, strains vary due to minor amino acid (7) or glycosylation (8) differences in the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (9). The ease and frequency of antigenic drift indicates a need for a cross-protective vaccine to offer heterosubtypic (often called “universal”) immunity. Cell-mediated immunity has demonstrated protection across heterosubtypic IAV strains in mice (10) and pigs (11).

DNA vaccines have demonstrated the ability to induce both humoral and cell mediated immune responses (12). Humoral immunity neutralizes viruses before host cells are infected, while cell mediated immunity (CMI) prevents infected individuals from prolonged infections leading to

chronic symptoms or death. The combination of both humoral immunity and CMI induced by DNA vaccines offers broad protection to the vaccinated animal (13). Potential causes of DNA vaccine failure include the use of low numbers of epitopes, poor epitope sequence conservation among strains, poor matching with leukocyte antigen populations, inefficient delivery, and epitopes activating regulatory T cell responses (14). To combat these problems and to determine which epitopes will best provide protection from infection, an in silico epitope prediction tool for swine has been developed. PigMatrix, in conjunction with the iVAX toolkit, is an algorithm which calculates swine leukocyte antigen peptide binding preferences (15). PigMatrix can be used to predict immunogenic T cell epitopes, which has allowed for the development of an epitope driven vaccine (EDV) (16). A previous study utilizing intramuscular injections of PigMatrix EDV in young growing pigs showed that a DNA vaccine composed of cross-conserved T cell epitopes identified using immunoinformatics tools could stimulate T cell responses reactive to a whole influenza virus in vitro (16). The cross-conserved T cell epitope-based PigMatrix EDV was further evaluated for efficacy in this study by investigating the vaccine regimen (prime boost) and route of administration (intra-dermal).

Objective

The objective of this study is to determine if vaccination with the novel epitope-based IAV vaccine antigen PigMatrix-EDV is successful in protecting pigs from the effects of pH1N1 challenge in comparison to a commercial inactivated vaccine and a prime boosting approach using both vaccines.

Materials and Methods

Pigs and experimental design. Thirty-nine 3-week-old IAV free pigs were randomly assigned to five groups and rooms. At 4 weeks of age and again at 7 weeks of age, pigs were vaccinated intradermally with an experimental epitope-driven pDNA vaccine (PigMatrix EDV; Nature Technology) i.e. EPITOPE, or intramuscularly with a commercial inactivated whole virus vaccine (FluSure XP®; Zoetis) i.e. INACT (Table I). At 9 weeks of age, the pigs were challenged with pH1N1 virus or sham-inoculated. Euthanasia and necropsy were conducted at day post challenge (dpc) 5. The study protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (Approval number: 8-17-8586-S) and included environmental enrichment of pens and independent veterinary supervision.

Sample collection. Blood samples were collected from the pigs at arrival and weekly thereafter, at dpc -1, and at dpc 5, centrifuged, aliquoted and stored at -80 ℃ until testing. Cotton-tipped swabs were used to collect nasal secretions by swabbing both nostrils of each pig at dpc -1, 1, 2, 3, 4 and 5. After collection, the swabs were immediately placed in 1 mL of phosphate buffered saline (PBS) in 5 mL plastic tubes and stored at -80 ℃ until testing.

Clinical assessment. All pigs were weighed upon arrival, challenge, and at necropsy and the average daily gain (ADG) was calculated. Rectal temperatures, nasal discharge, coughing, sneezing, and respiratory scores were assessed daily, beginning at the day of challenge. Nasal discharge was scored ranging from 0 = none to 2 = severe, and further characterized for location (left, right, or both nostrils), color (clear, yellow, or white), and consistency (watery or mucoid) (23). Clinical signs including presence and duration of cough (0 = none, 1 = single cough, and 2 = persistent coughing) and respiratory scores (0 = normal to 6 = severe dyspnea and/or tachypnea at rest) were assessed as described (23).

Vaccination. An experimental pDNA vaccine (16) was used to vaccinate the pigs in the EPITOPE-EPITOPE-IAV and EPITOPE-INACT-IAV groups. The vaccine is composed of a 1:1

mixture of two plasmids: one carries a synthetic gene encoding 28 SLA class I epitopes targeted to the proteasome by an N-terminal ubiquitin fusion for endogenous antigen processing; the other, 20 SLA class II epitopes targeted for secretion by a tissue plasminogen activator signal sequence for processing via the exogenous pathway. The epitope selection process is described in Gutierrez AH et al, 2016 (16). High-purity plasmids for immunizations were prepared at research grade (Nature Technology; Lincoln, Nebraska, USA). All pigs in the EPITOPE-EPITOPE-IAV group were vaccinated with the EPITOPE at 4 and 7 weeks of age and all EPITOPE-INACT-IAV pigs were vaccinated at 4 weeks of age. The vaccine was prepared using 2 mg/mL DNA plasmid in Tris-EDTA buffer (10 mM Tris pH 8.0, 1 mM EDTA) diluted to 266 µg/mL with phosphate buffered saline (PBS). The pDNA vaccine (0.5 mL dose containing 133 µg of plasmid) was administered intradermally in the neck using a commercial needle-free high-pressure device (Pulse 50TM Micro Dose Injection System, Pulse NeedleFree Systems; Lenexa, KS, USA) set at 65 pounds per square inch (PSI). A commercially available, inactivated whole IAV-S vaccine (FluSure XP®, Zoetis; Lot 275030; Parsippany, New Jersey, USA) was administered at 4 and 7 weeks of age to INACT-IAV pigs and at 7 weeks to the EPITOPE-INACT-IAV pigs. Manufacturer instructions were followed, and 2 mL of the INACT vaccine was administered intramuscularly into the neck area of each pig. The FluSure XP® vaccine contained H3N2 Cluster IV-A&B, H1N2 Delta-1, and H1N1 Gamma IAV strains.

IAV challenge. For the challenge, the pigs were anaesthetized as described (17). The pH1N1 challenge strain A/swine/Iowa/A01104104/2017 was purchased through the National Veterinary Services Laboratories and the USDA swine surveillance system. The challenge strain was selected using immunoinformatic methods (25, 26). Each pig was inoculated with the pH1N1 by administering 2 mL intratracheally and 1 mL intranasally for a total of $3\times10^{5.1}$ 50% tissue culture infectious dose ($TCID_{50}$) per pig. NEG-CONTROL pigs were similarly inoculated with saline.

Serology. Serum antibody levels against IAV were measured using a commercial blocking ELISA kit (IDEXX Laboratories) based on detecting antibodies against the IAV nucleoprotein, as per manufacturer's instructions. A sample to negative (S/N) ratio ≥0.60 was considered antibody negative.

Enzyme-linked immunospot (ELISPOT) assay. Peripheral blood mononuclear cells (PBMCs) collected on dpc -1 were tested for the presence of a CMI response using a commercial IFNγ ELISpot kit (Porcine IFN-gamma ELISpot kit, R&D Systems Inc, Minneapolis, MN, USA) as per the manufacturer's directions. To each well, 50 µL of complete RPMI was added to pre-wet the membranes, as suggested (18). Blue-black colored precipitate spots corresponding to activated IFNγ secreting cells were counted with an ELISPOT reader (ImmunoSpot ELISPOT analyzer, Cellular Technology Limited, Cleveland, OH, USA).

Detection and quantification of IAV-S specific nucleic acids. Nucleic acids were extracted from nasal swabs using the MagMAXTM Pathogen RNA/DNA 96-well kit (Applied Biosystems, Life Technologies, Foster City, CA, USA) on a KingFisher™ Flex platform (Thermo-Fisher Scientific, Waltham, MA, USA). A quantitative real-time reverse transcriptase (RT) PCR assay was performed using a VetMAXTM-Gold SIV Detection Kit (Applied Biosystems, Life Technologies, Foster City, CA, USA) as per the manufacturer's instructions and based on a standard curve using 50% tissue culture infectious dose per ml of an IAV isolate. A sample with a threshold (TH) value below 38 cycles was considered positive. Suspect samples with a TH between 38 and 40 cycles were considered negative for this study. Appropriate negative and positive controls were included in each run.

Necropsy and gross lung lesions. On dpc 5, all pigs were euthanized by intravenous administration of pentobarbital overdose. A pathologist blinded to the pig treatment status assessed

the lung lesions based on the percentage of lung surface affected (28). Sections of fresh lung and distal trachea were collected in 10% neutral-buffered formalin and processed for histopathology.

Histopathology and immunohistochemistry. Microscopic lung lesions were assessed by a veterinary pathologist blinded to the pig treatment status (28). Specifically, percentage of intrapulmonary airway epithelial necrosis and magnitude of peribronchiolar lymphocytic cuffing were scored. Immunohistochemistry (IHC) was used to assess the intralesional amount of IAV antigen as described (19), with scores ranging from 0 = IAV antigen negative to 3 = presence of abundant diffusely distributed IAV antigen.

Statistics. Quantitative RT-PCR data was log transformed prior to analysis. Repeated measures (nasal shedding and rectal temperature) were analyzed by using a REML model fitting pig nested within treatment as random effect and treatment and days post-challenge and their interactions as fixed effects. Significance of differences between more than two means was tested using Tukey's honest significant difference. The null hypothesis rejection level was $P < 0.05$. Non-repeated measures were assessed using nonparametric Kruskal-Wallis ANOVA. When group variances were different, pairwise comparisons were performed using the Wilcoxon rank sum test. Differences in incidence were evaluated by using Fisher's exact test. All analyses were performed with JMP® Pro Version 13.0.0 statistical software.

Results

Humoral and CMI responses. At dpc -1, all INACT-INACT-IAV pigs had detectable IAV antibodies and by dpc 5, all pigs in the INACT-INACT-IAV and the EPITOPE-INACT-IAV groups were seropositive. None of the other pigs seroconverted over the duration of the study. Pigs vaccinated with the EPITOPE vaccine once (EPITOPE-INACT-IAV) or twice (EPITOPE-EPITOPE-IAV) had significantly higher IFNγ producing cells in response to the peptides encoded by the EPITOPE vaccine. All vaccinated pigs had a significantly higher IFNγ producing cell response against the pH1N1 challenge strain compared to non-vaccinated control groups.

Clinical disease. Clinical signs of respiratory disease were not observed in any of the pigs before the IAV challenge and were never observed in any of the NEG-CONTROL pigs. The ADG (in g ± SEM) of the pigs between the time of pH1N1 challenge and the necropsy was 631.8±20.3 for the NEG-CONTROL group, 558.9±64.7 for the INACT-INACT-IAV group, 526.5±37.6 for the EPITOPE-INACT-IAV group, 601.7±27.3 for the EPITOPE-EPITOPE-IAV group, and 502.2±26.7 for the POS-CONTROL group. Rectal temperatures spiked 24 hours after challenge in all IAV challenged pigs and 1/8 INACT-INACT-IAV, 1/8 EPITOPE-INACT-IAV pigs, 5/7 EPITOPE-EPITOPE-IAV pigs, and 8/8 POS-CONTROL-IAV pigs had temperatures above 40.5ºC at dpc 1. A mild increase in respiratory scores (score of 1 or 2) was observed in 1-4 IAV infected pigs per group regardless of vaccination status. A sporadic cough was first recognized between dpc 1-3 in the different treatment groups and became persistent by dpc 3-5 in individual IAV infected pigs across all treatments.

IAV RNA shedding. All nasal swabs obtained from the NEG-CONTROL pigs were negative for the presence of IAV RNA. In contrast, apart from one INACT-INACT-IAV pig that was IAV RNA negative on dpc 1 and another INACT-INACT-IAV pig that was negative on dpc 1, 2 and 3, all nasal swabs from IAV infected pigs were IAV RNA positive regardless of treatment status. The INACT vaccine was more effective than the EPITOPE vaccine in reducing viral shedding during the first four days post challenge, while the combination regimen (EPITOPE-INACT-IAV) was not different from either group. By dpc 5, all IAV infected pigs regardless of treatment had similar IAV RNA shedding.

Lesions and IAV antigen in tissue sections. Lesions ranged from moderate to severe, and were characterized as cranioventral, red to purple consolidation that ranged from a checkerboard or lobular pattern to involving the entire cranioventral lobe. Consolidation extended into the cranial portion of the caudodorsal lung lobe in some pigs. There were no significant differences among treatment groups; however, INACT-INACT-IAV pigs (mean score±SEM, 22.9±6.5) had significantly higher lesion scores compared to the NEG-CONTROL pigs (0.2±0.1), while the EPITOPE-INACT-IAV (12.4±1.8) and the EPITOPE-EPITOPE-IAV (14.2±2.1) groups had fewer lesions than the INACT-INACT-IAV group. Microscopically, most lungs had focal to diffuse mild to severe necrotizing bronchiolitis and mild to severe peribronchiolar accumulation of inflammatory cells. IAV antigen was demonstrated by IHC stainsin all treatment groups except NEG-CONTROL pigs and EPITOPE-INACT-IAV pigs.

Discussion

A contemporary Iowa pandemic strain, A/swine/Iowa/A01104104/2017 recovered in 2017, was selected for challenge. This strain is not present in the FluSure XP® vaccine, which was chosen based on its wide usage and assumption that producers without access to sequencing tools would choose it by default. An epitope driven DNA vaccine, which was developed to target highly conserved epitopes across many influenza subtypes was also chosen. The potentially poor immunogenicity of DNA vaccines due to low immunogen expression was addressed with the boosting regimen and the intradermal vaccination route. DNA vaccines are an advantageous vaccination platform due to their ease of production, long shelf stability, and potential for rapidly incorporating precise vaccination targets on demand (20). DNA vaccines can be designed to code for entire antigenic proteins or for defined epitopes, in an effort to increase the specificity of the immune response. Incorporating whole antigens into DNA vaccines has shown protection against both homologous and heterologous IAV challenges in mice (21, 22). T cell responses induced by DNA vaccines have been shown to be effective against viral challenge in non-human primates, while antibody responses were found to be poorly neutralizing (23). In our study, the DNA vaccine specifically incorporated conserved T cell epitopes of structural and non-structural proteins predicted to have good binding profiles to the SLA class I and class II alleles common in US pig populations (15, 16). In a previously published study, the EPITOPE vaccine was delivered intramuscularly and stimulated T cells that recognize class II epitopes; recall responses to class I epitopes were not observed (16). ELISpot assays performed here did not determine sub-specificity of T cell responses.

Our results indicate that the EPITOPE-EPITOPE-IAV vaccinated pigs, while having no detectable seroconversion to IAV, had detectable T cell responses. Specifically, these pigs were able to produce a recall response to pH1N1 virus stimulation. Importantly, the prime-boosting approach in the EPITOPE-INACT-IAV group improved recall responses to EPITOPE vaccine peptides and pH1N1 challenge virus over homologous prime-boost with EPITOPE and INACT vaccines. A previous IAV vaccination study, using a similar prime-boosting strategy with DNA vaccination followed by an inactivated vaccine, demonstrated similar improvements in immunity. However, unlike the DNA vaccine in that study that encodes a strain-specific HA, our vaccine carries T cell epitopes sourced from multiple antigens conserved across multiple IAV strains and subtypes.

Under the conditions of this study, the EPITOPE vaccine induced a detectable CMI response against IAV, which had no impact on lung lesions scores, shedding or IAV antigen in lung lesions. On the other hand, pigs vaccinated with the INACT vaccine had a stronger humoral response, which could be correlated with a reduction in IAV shedding. When the two vaccines were

combined in a prime-boost regimen, CMI was enhanced. In these pigs, IAV antigen was not detectable in lung tissues and significantly lower compared to all other groups. As the IAV lesions and IAV RNA shedding levels were not different between the EPITOPE-INACT-IAV and INACT-INACT-IAV groups but the lung IAV antigen levels were reduced for the prime boost regimen, it may be an ideal choice for vaccination because it improves overall humoral and cell-mediated immunity. While the results are encouraging, future studies are needed to evaluate whether the number and breadth of T cell epitopes in the vaccine design, changes to the prime-boosting regimen, and additional modifications to the vaccination regimen of inactivated and epitope-based DNA IAV vaccines will improve swine influenza outcomes.

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