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A prime-boost concept using a T-cell epitope-driven DNA vaccine followed by a whole virus vaccine effectively protected pigs in the pandemic H1N1 pig challenge model

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ABSTRACT

Influenza A virus (IAV) vaccines in pigs generally provide homosubtypic protection but fail to prevent heterologous infections. In this pilot study, the efficacy of an intradermal pDNA vaccine composed of conserved SLA class I and class II T cell epitopes (EPITOPE) against a homosubtypic challenge was compared to an intramuscular commercial inactivated whole virus vaccine (INACT) and a heterologous prime boost approach using both vaccines. Thirty-nine IAV-free, 3-week-old pigs were randomly assigned to one of five groups including NEG-CONTROL (unvaccinated, sham-challenged), INACT-INACT-IAV (vaccinated with FluSure XP® at 4 and 7 weeks, pH1N1 challenged), EPITOPE-INACT-IAV (vaccinated with PigMatrix EDV at 4 and FluSure XP® at 7 weeks, pH1N1 challenged), EPITOPE-EPITOPE-IAV (vaccinated with PigMatrix EDV at 4 and 7 weeks, pH1N1 challenged), and a POS-CONTROL group (unvaccinated, pH1N1 challenged). The challenge was done at 9 weeks of age and pigs were necropsied at day post challenge (dpc) 5. At the time of challenge, all INACT-INACT-IAV pigs, and by dpc 5 all EPITOPE-INACT-IAV pigs were IAV seropositive. IFNγ secreting cells, recognizing vaccine epitope-specific peptides and pH1N1 challenge virus were highest in the EPITOPE-INACT-IAV pigs at challenge. 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 IAV antigen in lung tissues was detected at necropsy in the EPITOPE-INACT-IAV group, which was similar to naïve unchallenged pigs and different from all other challenged groups. Results suggest that the heterologous prime boost approach using an epitope-driven DNA vaccine followed by an inactivated vaccine was effective against a homosubtypic challenge, and further exploration of this vaccine approach as a practical control measure against heterosubtypic IAV infections is warranted.

1. Introduction

Viruses are a major cause of respiratory disease in pigs, decreasing both the welfare of pigs and economic gains of pig farmers. Influenza A virus is an enveloped virus of the *Orthomyxoviridae* family, composed of segmented negative-sense single-stranded RNA [1]. The virus in pigs, often referred to as swine IAV or IAV-S, is transmitted quickly and efficiently from pig to pig by nasal mucus aerosols or droplets [2]. Clinical signs of IAV infection in pigs includes loss of appetite, fever, lethargy, paroxysmal coughing, conjunctivitis, and nasal discharge [3]. The incubation period is short, with the disease spreading across a herd and causing clinical disease within 2 or 3 days [3]. Stress, high population density, and environmental factors increase the chance of the virus spreading among a population [4]. The prevalence of IAV as a cause of acute

Abbreviations: CMI, cell mediated immunity; EDV, epitope driven vaccine; HA, hemagglutinin; IAV, influenza A virus; NA, neuraminidase; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; pH1N1, pandemic H1N1; PSI, pounds per square inch; SLA, swine leukocyte antigen.

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respiratory disease or endemic infections in pig herds is likely underestimated [5]. Diseased individuals can become economic burdens due to weight loss in growing pigs or reproductive failures secondary to fever in breeding herds [6].

"Swine influenza" was first clinically recognized in 1918, however the virus was not isolated and identified from pigs until 1930 [7]. IAV in pigs has recently increased in importance in connection with the pandemic IAV outbreak in 2009, which caused concern over the ability of avian, porcine, and human influenza viruses to re-assort and create strains with enhanced pathogenic properties [8]. North American subtypes of IAV which commonly circulate in the pig population include H1N1, H1N2, and H3N2 [9]. Within a subtype, strains vary due to minor amino acid [10] or glycosylation [11] differences in the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). These small changes are positively selected for by immune pressure [12], especially in pandemic strains [13]. These slight alterations in important viral epitopes lead to antigenic changes in HA or NA known as antigenic drift [14]. 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 [15] and pigs [16]. While this experiment only utilized a homosubtypic IAV challenge for the experimental vaccine, a strong CMI response would support further evaluations for heterosubtypic protection.

DNA vaccines have demonstrated the ability to induce both humoral and cell mediated immune responses [17]. Humoral immunity neutralizes viruses before host cells are infected or, in the case of non-neutralizing antibodies, may facilitate resistance [18] or contribute to protection [19]. Meanwhile, 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 [20]. 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 [21]. 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 [22]. PigMatrix can be used to predict immunogenic T cell epitopes, which has allowed for the development of an epitope driven vaccine (EDV) [23]. 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* [23]. 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).

The objective of this study was to compare the efficacies of an epitope driven pDNA vaccine administered intradermally, a commercial inactivated whole virus vaccine administered intramuscularly, and a combination of these vaccines in protecting growing pigs from the effects of a pandemic H1N1 IAV challenge.

2. Materials and methods

2.1. Ethical statement

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.

2.2. Pigs and experimental design

Forty 3-week-old pigs from an IAV free source farm were randomly assigned to five groups and rooms. Pig groups were kept in pens approximately 3.8 m × 3.8 m in size. Feed from wall-mounted conventional self-feeders and water from nipple waterers were offered *ad libitum*. 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 1). One EPITOPE-EPITOPE pig was found dead 8 days after initial vaccination, and a *Streptococcus suis* associated meningitis unrelated to the project was diagnosed. This pig was removed from the study. 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.

2.3. Sample collection

To obtain serum, blood samples were collected from the pigs at arrival and weekly thereafter, at dpc −1, and at dpc 5 using BD Vacutainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The vacutainers were centrifuged at 1500*g* for 8 min at 4 °C, and then the serum was aliquoted and stored at −80 °C until testing.

To obtain peripheral blood mononuclear cells (PBMCs) 8–10 mL blood was collected from each pig on the day of IAV challenge using BD Vacutainer® CPT™ cell preparation tubes with sodium citrate (Becton, Dickinson and Company). Within 2 h of blood collection, the tubes were centrifuged at 1800*g* for 20 min at room

Table 1
Experimental design and treatment groups.

Group designation	Number of pigs	Vaccination 1 *4 weeks of age*	Vaccination 2 *7 weeks of age*	IAV challenge *9 weeks of age*
INACT-INACT-IAV	8	Inactivated vaccine**	Inactivated vaccine	pH1N1****
EPITOPE-INACT-IAV	8	Epitope DNA vaccine***	Inactivated vaccine	pH1N1
EPITOPE-EPITOPE-IAV	7*	Epitope DNA vaccine	Epitope DNA vaccine	pH1N1
NEG-CONTROL	8	Saline	Saline	Saline
POS-CONTROL	8	Saline	Saline	pH1N1

* One pig in this group was found dead 8 days after initial vaccination, a *Streptotococcus suis* associated meningitis unrelated to this project was diagnosed, and the pig was therefore removed from the study.
** Commercial inactivated whole IAV-S vaccine (FluSure XP®, Zoetis; Lot 275030; Parsippany, New Jersey, USA). Each pig received 2 mL by the intramuscular route.
*** Experimental pDNA vaccine (PigMatrix EDV, Nature Technology; Lincoln, Nebraska, USA). Each pig received 0.5 mL by the intradermal route.
**** pH1N1 challenge strain A/swine/Iowa/A01104104/2017 at a dose of $3 \times 10^{5.1}$ 50% tissue culture infectious dose ($TCID_{50}$) per pig. Each pig received 2 mL intratracheally and 1 mL intranasally.

temperature. The buffy coat was collected and resuspended in PBS. Cells were washed and centrifuged at 500g for 5 min at 4 °C, the supernatant was discarded, and the pellet was used immediately for the ELISpot assay.

Cotton-tipped swabs (Fisher Scientific, Pittsburgh, PA, USA) 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 °C until testing.

2.4. Clinical assessment

All pigs were weighed upon arrival, just prior to challenge, and at necropsy and the average daily gain was calculated. Rectal temperatures, nasal discharge, coughing, sneezing, and respiratory scores were assessed daily, beginning at the day of challenge. Pigs with rectal temperatures equal to or greater than 40.5 °C were considered febrile. 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) [24]. 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 [24].

2.5. Vaccination

An experimental pDNA vaccine [23] (PigMatrix EDV, Nature Technology; Lincoln, Nebraska, USA) was used to vaccinate the pigs in the EPITOPE-EPITOPE-IAV and EPITOPE-INACT-IAV groups (Table 1). The vaccine was 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 plasmid contained 20 SLA class II epitopes targeted for secretion by a tissue plasminogen activator signal sequence for processing via the exogenous pathway. IAV epitopes were included in the EPITOPE vaccine for representation by prevalent SLA alleles, and because the epitopes were highly conserved in circulating strains of swine IAV. The epitope selection process is described in Gutierrez et al, 2016 [23].

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 vaccine 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 50™ Micro Dose Injection System, Pulse NeedleFree Systems; Lenexa, KS, USA) set at 65 lb 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 but did not contain pH1N1 antigens.

2.6. IAV challenge

The IAV challenge strain used in this study, pH1N1 strain A/swine/Iowa/A01104104/2017, was selected using immunoinformatic methods [25,26] from among 72 swine IAV pH1N1 2017 isolates for which whole genomes were available (Table S1) for closest T cell epitope relatedness to the pDNA vaccine. Two validated immunoinformatic algorithms were applied: T cell epitope content comparison (EpiCC) [26], and JanusMatrix (JMX) [25]. Pairwise comparisons between the EPITOPE-IAV vaccine and circulating strains were conducted using EpiCC to analyze overall vaccine epitope cross-conservation on an antigen-by-antigen basis (Fig. S1). Higher EpiCC scores are associated with greater T cell epitope relatedness between the EPITOPE-IAV vaccine and circulating strains. EpiCC scores were summed over all IAV antigens per SLA class and ranked for challenge strain selection (data not shown). As a complementary approach, JanusMatrix (JMX) was used to analyze sequences on an epitope-by-epitope basis to identify identical T cell epitopes in the EPITOPE-IAV vaccine among the set of 72 H1N1 strains circulating in 2017 (Table S2, Fig. S2). JMX calculates the Janus Homology Score, which represents the average depth of coverage in the search database of circulating strains for each EpiMatrix hit in the input vaccine sequence. It considers all constituent 9-mers in any given peptide, including flanks. Strains showing the highest JMX matched SLA class I and II epitopes were ranked for challenge strain selection (data not shown).

The pH1N1 challenge strain A/swine/Iowa/A01104104/2017 was purchased through the National Veterinary Services Laboratories and the USDA swine surveillance system. For the challenge, the pigs were anaesthetized using a ketamine (8 mg/kg), xylazine (4 mg/kg), and telazol (6 mg/kg) combination as described [27]. Each pig was inoculated with the pH1N1 by administering 2 mL intratracheally and 1 mL intranasally for a total of $3 \times 10^{5.1}$ 50% tissue culture infectious dose ($TCID_{50}$) per pig. NEG-CONTROL pigs were similarly inoculated with saline.

2.7. Serology

Serum antibody levels against IAV were measured using a commercial blocking ELISA kit (Swine Influenza Virus Antibody Test Kit, IDEXX Laboratories, Inc.; Westbrook, Maine, USA) 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.

2.8. Enzyme-linked immunospot (ELISPOT) assay

PBMCs collected on dpc 0 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 [28]. A total of 2.5×10^5 viable PBMC in 100 μL of complete RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum were seeded into pretreated microplates (provided in the kit). The seeded cells were stimulated with either the challenge pH1N1 at a concentration of 2.5×10^5 $TCID_{50}$ to produce a multiplicity of infection of 1 or pooled peptides at a concentration of 2 μg in 100 μL per well. The peptides were selected to match the epitopes presented in the pDNA vaccine which included sequences derived from influenza structural and nonstructural proteins. For control purposes, PBMCs were stimulated with 0.25 μg pokeweed mitogen (MP Biomedicals™, Santa Ana, CA, USA) in 100 μL of complete RPMI. The cells were then incubated for 36 h at 37 °C in a 5% CO_2 incubator. Subsequently, the ELISPOT assay was performed according to the manufacturer's instructions. 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).

2.9. Detection and quantification of IAV-S specific nucleic acids

Nucleic acids were extracted from nasal swabs using the MagMAX™ 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 VetMAX™-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 (T_H) value below 38 cycles was considered positive. Suspect samples with a T_H between 38 and 40 cycles were considered negative for this study. Appropriate negative and positive controls were included in each run.

2.10. Necropsy and gross lung lesions

On dpc 5, all pigs were euthanized by intravenous administration of pentobarbital overdose (FATAL-PLUS®, Vortech Pharmaceuticals LTD, Dearborn, MI, USA). A pathologist (PCG) blinded to the pig treatment status assessed the lung lesions based on the percentage of lung surface affected [29]. Sections of fresh lung and distal trachea were collected in 10% neutral-buffered formalin and processed for histopathology.

2.11. Histopathology and immunohistochemistry

Microscopic lung lesions were assessed by a veterinary pathologist (TO) blinded to the pig treatment status [29]. Specifically, percentage of intrapulmonary airway epithelial necrosis and magnitude of peribronchiolar lymphohistiocytic cuffing were scored. Immunohistochemistry (IHC) was used to assess the intralesional amount of IAV antigen as described [30], with scores ranging from 0 = IAV antigen negative to 3 = presence of abundant diffusely distributed IAV antigen.

2.12. Statistics

Summary statistics were calculated for groups to assess the distributional property. 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, pair-wise comparisons were performed using the Wilcoxon rank sum test. Differences in incidence were evaluated by using Fisher's exact test. Correlations were estimated by Pearson's method. All analyses were performed with JMP® Pro Version 13.0.0 statistical software.

3. Results

3.1. Humoral and CMI responses

In this study, the efficacy of three different vaccination strategies including (1) two vaccinations of an inactivated commercial vaccine (INACT-INACT-IAV), (2) two vaccination of an epitope DNA vaccine (EPITOPE-EPITOPE-IAV), or (3) a single dose of the epitope DNA vaccine followed by a single dose of the inactivated vaccine (EPITOPE-INACT-IAV). All of the vaccine regimens were followed by challenge with a pH1N1 strain and were compared to each other and to non-vaccinated pH1N1 challenged pigs (POS-CONTROL). The mean group IAV NP ELISA S/N ratios are summarized in Table 2. 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. ELISpot results from assays using PBMCs sampled before challenge are summarized in Fig. 1. Pigs vaccinated with the EPITOPE vaccine once (EPITOPE-INACT-IAV) were not different from the group vaccinated with the EPITOPE twice (EPITOPE-EPITOPE-IAV) but had significantly higher IFNγ producing cells in response to the peptides compared to all other groups. Notably, recall responses to the EPITOPE vaccine peptides were boosted in pigs vaccinated with EPITOPE-INACT-IAV over pigs that received two doses of the EPITOPE vaccine.

3.2. 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

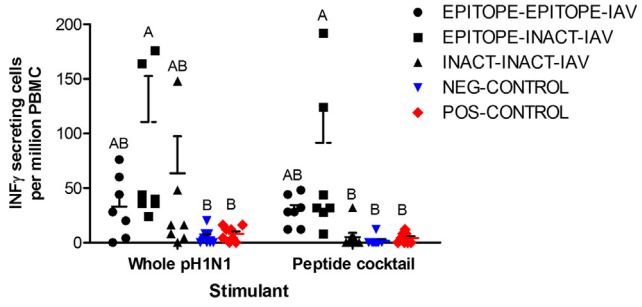

Fig. 1. Group mean and individual pig numbers of IFNγ producing cells per million PBMC ± SEM in the treatment stimulated by using the challenge virus or peptides at day post challenge (dpc) −1. Different superscripts ($^{A, B}$) for a stimulant indicated significant differences among groups. A nonparametric Kruskal-Wallis ANOVA was used for analysis. When group variances were different, pair-wise comparisons were performed between all groups using the Wilcoxon rank sum test. The null hypothesis rejection level was $P < 0.05$.

Table 2
Prevalence of IAV antibody positive pigs (mean group S/N ratio ± SEM) in the different treatment groups as determined by a commercial blocking IAV NP ELISA test before vaccination and at days post challenge −1 and 5.

Group	Pre-vaccination	Day post challenge	
		1	5
INACT-INACT-IAV	0/8 (0.98 ± 0.02)^{A,*}	8/8 (0.35 ± 0.03)^{A}	8/8 (0.25 ± 0.03)^{A}
EPITOPE-INACT-IAV	0/8 (0.93 ± 0.02)^{A}	0/8 (0.91 ± 0.03)^{B}	8/8 (0.26 ± 0.04)^{A}
EPITOPE-EPITOPE- IAV	0/7 (0.97 ± 0.03)^{A}	0/7 (0.97 ± 0.08)^{B}	0/7 (0.86 ± 0.12)^{B}
NEG-CONTROL	0/8 (0.92 ± 0.01)^{A}	0/8 (0.91 ± 0.03)^{B}	0/8 (0.92 ± 0.02)^{B}
POS-CONTROL	0/8 (0.94 ± 0.02)^{A}	0/8 (1.04 ± 0.12)^{B}	0/8 (0.88 ± 0.07)^{B}

* Different superscripts ($^{A, B}$) indicate significant ($P < 0.05$) differences in mean group S/N ratios on a certain treatment day.

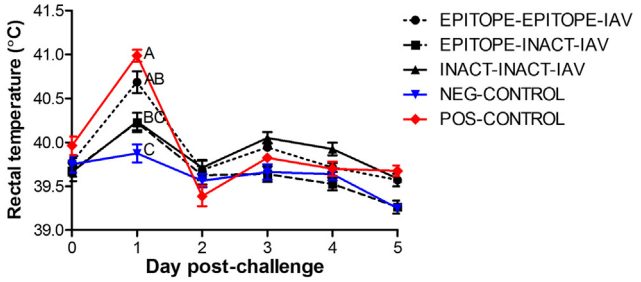

Fig. 2. Mean rectal temperatures ± SEM in the different treatment groups at certain days post challenge (dpc) with pH1N1. Different superscripts (^{A,B}) indicate significantly ($P < 0.05$) different group means at a certain dpc. A REML model was used for analysis. When group variances were different, pair-wise comparisons were performed between all groups using Tukey's honest significant difference. The null hypothesis rejection level was $P < 0.05$.

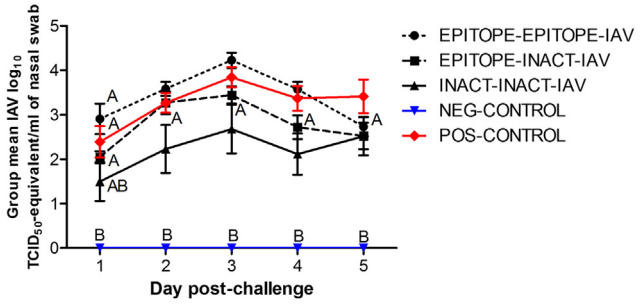

Fig. 3. Group mean $\log_{10}$ IAV $TCID_{50}$-equivalent/ml ± SEM in nasal swabs at different days post IAV challenge. Different superscripts (^{A,B}) indicate significantly ($P < 0.05$) different group means at a certain dpc. A REML model was used for analysis. When group variances were different, pair-wise comparisons were performed between all groups using Tukey's honest significant difference. The null hypothesis rejection level was $P < 0.05$.

of the NEG-CONTROL pigs. The average daily gain (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. The groups were not significantly different from each other ($P = 0.14$). The rectal temperatures after IAV challenge are summarized in Fig. 2. Rectal temperatures spiked 24 h after challenge in all IAV challenged pig groups; 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 40.5 °C at dpc 1. 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. The average length of coughing in a pig was 1.6 ± 0.7 days in INACT-INACT-IAV pigs, 0.6 ± 0.3 days in EPITOPE-INACT-IAV pig, 1.4 ± 0.7 days in EPITOPE-EPITOPE-IAV pigs, and 0.4 ± 0.2 days in the POS-CONTROL-IAV pigs. Nasal discharge was noted as watery and present in both nostrils at least once in 5/8 INACT-INACT-IAV pigs, 2/8 EPITOPE-INACT-IAV pigs, 1/7 EPITOPE-EPITOPE-IAV pigs, and 4/8 POS-CONTROL pigs.

3.3. IAV RNA shedding

The overall shedding results were significant ($P < 0.001$) for treatment, dpc, and treatment within dpc. All nasal swabs obtained from the NEG-CONTROL pigs were negative for the presence of IAV RNA (data not shown). 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 (Fig. 3). By dpc 5, all IAV infected pigs regardless of treatment had similar IAV RNA shedding.

3.4. Lesions and IAV antigen in tissue sections

The macroscopic lung lesions are summarized in Fig. 4. 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. Of note, the distribution of pigs with more severe lung lesions was wider in the INACT-INACT-IAV and the POS-CONTROL group compared to all other groups and appeared to be bimodal in 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 stains (Fig. 4) in all treatment groups except NEG-CONTROL pigs and EPITOPE-INACT-IAV pigs.

4. Discussion

Vaccination to control IAV and prevent the economic losses and welfare problems associated with sick pigs has been difficult. The variable genetic nature of IAV necessitates "universal" vaccine strategies to offer more broad protection against a wide variety of IAV strains. In this study, the efficacies of a T cell epitope-encoding DNA vaccine administered intradermally, a commercial whole virus inactivated vaccine given intramuscularly, and a mixed prime boost concept using both vaccines were assessed for protecting growing pigs from the effects of a pandemic H1N1 IAV challenge.

A previous proof of concept study used SLA predictive epitope mapping matrices to identify immunogenic T cell epitopes, and then pigs were immunized with the epitope-based pDNA IAV vaccine alone [23]. In the present study, both immunogenicity and efficacy in the context of homologous (EPITOPE-EPITOPE) and heterologous (EPITOPE-INACT) prime boost regimens were investigated.

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 typically administered intramuscularly via gene gun or electroporation which have shown promising results in laboratory animals [31,32], but are impractical for mass vaccination of pigs under field conditions. In this study, the intradermal route was chosen to administer the DNA vaccine primarily due to the

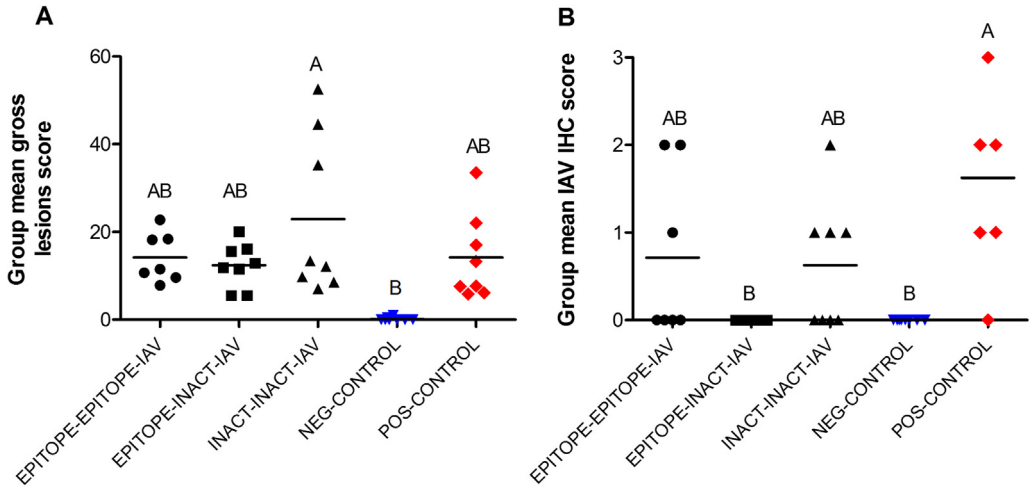

Fig. 4. A. Group mean gross lung lesion scores ranging from 0 to 100% of the lung surface affected by consolidation with individual pig scores. **B.** Group mean IAV antigen in lungs as determined by immunohistochemistry with individual scores for each pig (score range from 0 = negative to 3 = abundant, multifocal IAV antigen present). Different superscripts (^{A,B}) indicate significantly ($P < 0.05$) different group means. A nonparametric Kruskal-Wallis ANOVA was used for analysis. When group variances were different, pair-wise comparisons were performed using the Wilcoxon rank sum test. The null hypothesis rejection level was $P < 0.05$.

effectiveness of dermal dendritic cells in antigen capture and T lymphocyte presentation, whereas muscle tissue has many fewer antigen presenting cells [33]. In addition, intradermal vaccination of pigs is now becoming a more common practice on many farms due to the ease of using the newer needle-free devices and the avoidance of problems with broken needles.

Currently, the most commonly utilized vaccines to protect pigs against IAV are inactivated whole virus preparations. These vaccines are advantageous due to their relatively low production cost and the lack of live virus which eliminates reversion to virulence or shedding of the vaccine strain [34]. On the other hand, inactivated whole virus vaccines can be difficult to properly employ due to low shelf life and heat stability requirements, lack of cell mediated immune stimulation, and the inability to tailor the vaccine to specific target strains [34]. Inactivated vaccines, usually containing one to four viral strains, in rare cases may elicit weakly cross-reactive HA antibodies which lead to vaccine-associated enhanced respiratory disease [35]. In this study, enhanced disease was not observed in EPITOPE-EPITOPE-IAV and EPITOPE-INACT-IAV groups. However, three pigs in the INACT-INACT-IAV group had higher percentages of pneumonia compared to the non-vaccinated, challenged group suggesting enhanced pneumonia in specific pigs in the group.

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 [34]. 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 homosubtypic and heterosubtypic IAV challenges in mice [36,37]. 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 [38]. 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 [22,23]. The SLA alleles of pigs in this study were not typed; hence there is no information about individual SLA types and compatibility with the epitopes in the vaccine. Unlike in the previous study where class II and class I epitopes were tested separately [23], the ELISpot assays performed here did not determine sub-specificity of T cell responses. Therefore, it is not possible to draw conclusions about the immunogenicity of particular peptides, nor how accurate the predictive algorithm is. Mismatches between SLA alleles used for epitope predictions and the SLA types of pigs in the study may reduce the potential of the vaccine to stimulate protective immune responses. In addition, SLA allele frequency data are limited and SLA diversity is high; thus, there is insufficient information to describe the proportion of pigs covered by the predictive tools. The overall outcomes obtained from the present study suggest that the cohort was well matched to the alleles used to make predictions. To determine vaccine coverage for the North American or global pig population, SLA typing would need to be conducted on larger numbers of pigs sourced from farms in multiple locations. Also, despite a high MHC diversity, alleles can be clustered by sequence relationship into families or supertypes with common epitope binding preferences [39]. Thus, the possibility exists that despite a mismatch, a study pig's alleles may bind a predicted epitope and in reality, pigs are unlikely to be individually typed prior to vaccination on a regular basis.

Our results indicate that the EPITOPE-EPITOPE-IAV vaccinated pigs, while having no detectable seroconversion to IAV, had detectable T cell responses (Fig. 1). 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 [40]. However, unlike the DNA vaccine in that study that encodes a strain-specific HA, our vaccine carried T cell epitopes sourced from multiple antigens conserved across multiple IAV strains and subtypes.

The nucleoprotein (NP) blocking ELISA that was used to determine the presence of IAV antibodies showed the highest antibody production in the INACT and EPITOPE-INACT groups. This assay is a good indicator of humoral immunity for IAV, as it has been shown that anti-NP IgG antibodies promoted viral clearance in both IAV active immunized mice and naïve mice receiving donor serum from vaccinated mice by the intraperitoneal route [41]. Our results show that humoral immunity stimulated in the INACT-INACT-IAV group was effective in reducing IAV shedding, but it did not result

in a significant decrease in lung lesions or detectable IAV antigen in lungs. This may be due to the lack of cytotoxic T cell response by CD8+ T cells stimulated by inactivated virus, which are responsible for clearing the viral infection by killing infected cells. Moreover, three pigs of the INACT-INACT-IAV group had more severe lung lesion scores than any of the pigs in the POS-CONTROL group (Fig. 4). This could perhaps indicate antibody-enhancement of IAV infection in this group [27,42]. In both this and previous studies [43], some but not all pigs had evidence of enhanced lesions, which may be due to variations in the biology of pigs, responses to vaccination, and development of aberrant immune responses.

Conversely, the EPITOPE-EPITOPE-IAV group showed no evidence of seroconversion and the rectal temperatures at dpc 1 in these pigs after IAV challenge were similar to the POS-CONTROLs and significantly higher compared to all other groups. The ELISpot response against the peptide cocktail did show evidence of good epitope prediction and an effective DNA vaccine design. In contrast, the heterologous prime-boost regimen with the DNA vaccine followed by the inactivated vaccine showed an additive increase in CMI and a rapid increase in nucleoprotein specific antibody levels upon challenge, as well as good clinical protection. Further prime-boosting using this approach with more animals over a longer challenge period is needed to more effectively assess the effect on protection against various IAV strains.

5. Conclusions

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. Pigs vaccinated with the INACT vaccine had a strong humoral immune response which could be correlated with a reduction in IAV shedding. This group also had the second highest CMI response among all groups. In this study, class I and II SLA responses were not separated and some of the measured responses may be class II; nevertheless, the data are indicative of some degree of a cytotoxic T-cell response. When the two vaccines were combined in a prime-boost regimen, CMI was enhanced and the humoral response on day 5 after IAV challenge was similar to the INACT vaccine group. In the same pigs, IAV antigen was not detectable in lung tissues. 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 heterologous prime-boost regimen, it may be an ideal choice for vaccination because of the improved outcome resulting from enhanced 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. Specifically, the relative importance of the class I and II epitopes, the significance of the DNA prime (a challenge group that only received a single dose of the inactivated virus vaccine), and challenges with heterosubtypic viral strains need to be assessed.

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Declaration of Competing Interest

ADG and LM are employees of EpiVax. ADG is a majority stockholder and LM holds stock options. These authors recognize the presence of a potential conflict of interest and affirm that the information represented in this paper is original and based on unbiased observations. All other authors declare no financial and personal relationships with other people or organizations that could inappropriately influence this work.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.044.

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