RESEARCHREPORT
SWINE HEALTH

Title: Advances in Vaccine Design: Developing A Cross-conserved Influenza Vaccine for Swine **- NPB #13-121**

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Industry Summary:
The overall goal of this project was to implement the innovative iVAX suite of immunoinformatics vaccine design tools that have been extensively validated in pre-clinical studies of human vaccines, modify their core elements (matrices) for swine, and apply them to the design of a swine vaccine for Influenza A Virus (IAV). Vaccine design using the new PigMatrices and iVAX toolkit, offers significant advantages over other approaches to developing vaccines for pathogens affecting the pork industry because (i) the tools can be used to accelerate vaccine design for new pathogens; (ii) the tools can be used to improve existing vaccines; and (iii) the tools can be used to predict the efficacy of existing vaccines against new circulating strains. For example, validation of these tools will make it possible to accelerate the development of vaccines and diagnostics for new and emerging infections such as PEDv and ASF.

In the context of the current grant, we used the PigMatrices and iVAX toolkit to identify highly conserved genome components (T cell epitopes) of circulating IAV strains that should drive cross-protective immune responses to IAV. The concept driving this vaccine is that it would reduce the need to produce a new vaccine for each emerging IAV strain. **First**, the iVAX toolkit was adapted for swine influenza vaccine design. We developed six new swine SLA prediction matrices, four more the number of matrices originally proposed in the grant. **Second**, we selected 48 highly cross-conserved ("universal") IAV T cell epitopes, and integrated them into a vaccine that was used to validate the predictions and vaccinate pigs in a head-to-head comparison with an existing influenza vaccine. **Third**, we performed a challenge study that was carried out in collaboration with Crystal Loving of the USDA. All of the pigs received a standard intranasal flu challenge. The results were surprisingly good. *The first test of this prototype showed immunogenicity, but lack of protection in terms of viral load*. A repeat study will need to be performed, with epitopes representing additional SLA allele predictions, which can be expected to will improve the efficacy of the vaccine. **Overal**l, the program was remarkably successful, considering we went from "zero to sixty"; we validated our predictions and developed our first prototype PigMatrix-epitope based vaccine and performed a challenge study within the timeframe of the grant. Further development of the PigMatrix tool for iVAX is likely to yield a means of accelerating and improving vaccines for the pork industry.

These results provide a very **exciting proof-of-concept** that using the new PigMatrices with the iVAX toolkit, we can design vaccines that stimulate responses specific to the predicted

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epitopes in swine. In addition to designing new, and possibly more effective vaccines for PEDv, PRRSv, ASF and IAV, the tools could be used to identify components that will boost immune responses to any whole antigen vaccine. In addition, predicted immunogenic epitopes can be integrated into low immunogenic antigens to improve their protective response. We are eager to pursue additional tests of the potential of these tools for accelerating vaccine design.

We also developed a new tool for predicting vaccine efficacy. Using these tools we have also developed a new method for classifying vaccines that we hope to validate and then commercialize. We developed a "vaccine classification tool" called Epitope Content Comparison (EpiCC) tool. EpiCC compares the epitope content across strains and would allow pork producers to determine whether a given vaccine would work for emerging strains of influenza or PRRSv. We plan to test this tool, beginning by comparing existing viral strains to vaccines, so as to determine if we can accurately predict vaccine efficacy.

Keywords: Immunoinformatics, epitope prediction, T cell epitopes, epitope-driven vaccine (EDV), influenza A virus

Scientific Abstract:
Computer-driven algorithms enable accelerated vaccine design for emerging pathogens. iVAX is an integrated set of epitope-driven vaccine (EDV) design tools that is based on the EpiMatrix algorithm and has been extensively validated for human vaccine design. Comparable tools for the development of vaccines for food animals are not available. We used the pocket profile method to create T cell epitope-prediction matrices (PigMatrix) for porcine MHC (Swine Leukocyte Antigens, SLA). These new matrices were integrated into iVAX so that this comprehensive suite can be used to design EDV for pathogens affecting swine. For this study, we developed matrices for common class I and II SLA alleles and predicted 28 cross-conserved class I and 20 class II epitopes from seven circulating IAV.

Multi-epitope DNA vaccines encoding strings of class I and II epitopes separately were produced and pooled for intramuscular injection into pigs. Peptides induced specific recall responses as evidenced by IFN-γ production upon exposure to pooled peptides, demonstrating their immunogenicity and validating the SLA matrices. Pooled peptides induced similar recall responses as whole inactivated virus (WIV) used as antigen. Moreover, epitope-specific recall responses in DNA vaccinated pigs were equivalent in magnitude to WIV-induced responses in pigs immunized with quadrivalent inactivated vaccine (FluSureXP®). In other words, a reduced set of cross-conserved peptides (48), predicted using immunoinformatics tools, induced a similar IFN-γ response as four inactivated viral strains. In terms of viral loads, neither the DNA vaccine nor the commercial vaccine was protective against intranasal challenge with A/California/04/2009 (H1N1). From the T cell epitope perspective, the results are advantageous and demonstrate the potential of immunoinformatics tools for prediction of porcine T cell epitopes. We are now in a position to dissect the responses induced by individual epitopes to better understand the mechanism of action and potentially improve current available vaccines.

Introduction:
The majority of currently licensed swine IAV vaccines consist of whole inactivated viruses administered with adjuvant by intramuscular injection. This platform induces primarily systemic IgG antibody responses to the surface glycoproteins, mainly HA. However, antibody does not provide protection against antigenically drifted IAV strains; in addition, there is huge antigenic diversity in swine IAV HA. Frequent changes through antigenic drift allow IAV to escape antibody-mediated protection; however, cell-mediated immune responses to conserved internal genes can be broadly cross-reactive to a variety of IAV subtypes[1]. IAV vaccine efficacy is increased when cross-reactive T helper (Th) cells are elicited following vaccination and cytotoxic T lymphocytes (CTL) are required for clearing virus after infection[2]..

The triple-reassortant internal gene (TRIG) cassette in swine IAV is an obvious target for T cell epitope mapping, since identification of cross-conserved Th and CTL epitopes within TRIG, along with other highly conserved regions of the genome, will enable the development of genome-derived (GD) T cell ED IAV vaccines that may provide a broad level of cross-protection to a variety of strains. The approach is currently under extensive study for human influenza vaccines[3–7].

For this project we swapped out human EpiMatrix for swine leukocyte antigen (SLA) matrices in the iVAX toolkit, so as to take advantage of validated algorithms that have already proven successful in influenza challenge models[8]. Using these tools, we developed and tested an ED IAV vaccine.

Objectives:
The objective of this project is to identify highly antigenic T cell epitopes conserved across divergent swine IAV isolates to include them in a DNA-vaccine for heterologous protection. Preliminary data has demonstrated that it is feasible to extrapolate computer-driven T cell epitope identification, which has been successfully applied in human vaccine design, to swine. To carry this one step further, we propose the following specific objectives:

- Identify highly antigenic T cell epitopes conserved in diverse influenza subtypes. Regions that are highly conserved across influenza strains and also predicted to bind to SLA are to be identified using Conservatrix and PigMatrix. Previously, we developed one prediction matrix for SLA class I (SLA-1*0401) and one for class II (SLA-DRB1-0201). Additional matrices, once developed are to be integrated into the iVAX toolkit.
- Validation of predicted epitopes. Peptides are validated using SLA binding assays measuring their ability to compete with a biotinylated known-binder peptide for binding to purified SLA molecule.
- Design of vaccine construct. Epitope sequences are then concatenated to form a multi-epitope gene, containing SLA class II and I validated epitopes.
- Vaccination and challenge. Pigs are to be vaccinated using a DNA-vaccine and challenged three weeks after the final boost with A/California/04/2009 (H1N1) and A/turkey/Ohio/313053/2004 (H3N2) virus.
- Immunogenicity measure. Peripheral blood mononuclear cells (PBMC) will be collected at various times pre and post-vaccination and evaluated in an IFN-γ and IL-13 ELISpot assays. Antibody titers are then evaluated using ELISA.

Materials & Methods:

a) Sequences. Since the number the number of antigenically different IAV strains circulating in pigs is large[9], we focused on swine IAV genomes of frequent IAV subtypes in North American pig populations (H1N1, H1N2 and H3N2, Table 1). Complete genomes of IAV were downloaded from GenBank.

Table 1. IAV sequences used to predict Class I and II T cell epitopes

Subtype	Virus name	Short name
H1N1	A/California/04/2009	CA/04
	A/Swine/Ohio/511445/2007	OH/07
	A/Swine/Illinois/5265/2010	IL/10
H1N2	A/Swine/Minnesota/02011/2008	MN/08
	A/swine/Minnesota/A01301731/2012	MN/2012
H3N2	A/Swine/Texas/4199-2/1998	TX/98
	A/Turkey/Ohio/313053/2004	OH/04

b) Matrix development. Matrix development was based on the Pocket Profile Method10 and SLA crystal structures[11]. Matrices were developed for commonly expressed class I and II SLA alleles[12,13]. Matrices were integrated into the iVAX toolkit.

c) Identification and synthesis of highly antigenic T cell epitopes. Using PigMatrix and other tools available in the iVAX toolkit (e.g. Conservatrix, EpiAssembler, ClustiMer), we screened IAV proteins and identified potentially immunogenic class I and II peptides. Cross-conserved peptides were synthesized by 9-fluoronylmethoxy-carbonyl (Fmoc) synthesis at 21st Century Biochemicals (Marlboro, MA).

d) Epitope-based DNA vaccine design. Predicted epitope sequences were concatenated to form two multi-epitope genes (one for SLA class I and one for class II epitopes). In order to avoid creation of novel epitopes at epitope junctions and transmembrane (TM) domains, VaccineCAD and a concatamer optimization algorithm were used to re-order the epitopes to reduce junctional immunogenicity and potential TM helices. Two of the optimized constructs (one for class I and other for class II epitopes) were selected to move forward for gene synthesis. GeneArt (Life Technologies, NY, USA) synthesized the genes, which were subcloned in two different vaccine vectors. The class I gene was subcloned into pNTC8684 (Nature Technology Corporation, NE, USA), which targets proteins to the proteosome by fusion C-terminal to a destabilizing UbiquitinA76 tag. The class II gene was subcloned into pNTC8682 (Nature Technology Corporation, NE, USA) that targets the encoded protein for secretion using an optimized tissue plasminogen activator (TPA) signal peptide so the immunogen is processed via the class II SLA pathway of antigen presentation.

e) Vaccination and challenge. The concatamer genes were evaluated in a vaccination and challenge study using IAV-free SLA-typed pigs. Thirty-two, 3-week old pigs were delivered to the USDA-National Animal Disease Center. Pigs were randomly distributed into four groups of 8 and housed in separate isolation rooms. Eight pigs were vaccinated according to label directions with FluSureXP® (Pfizer Animal Health); another group was primed with the DNA-vaccine (plasmids carrying class I and class II concatemers were combined) and homologous boosted at 3 and 6 weeks post-priming; the third (sham vaccinated) group was vaccinated and boosted with empty plasmids. Eight non-vaccinated pigs served as controls. DNA vaccine was administered intramuscularly (i.m.) in the postauricular region of the neck by needle stick injection with 4 mg of DNA in 1 ml PBS as previously described[14]. Three weeks after the final boost, pigs were challenged intranasally with A/California/04/2009 (H1N1) as previously performed in Dr. Loving's lab[14]. Pigs were observed daily for clinical signs of disease; they were euthanized a week post-challenge. Protection was evaluated as reduction in lung lesions, viral titers in nasal swabs, and bronchoalveolar lavage fluid (BALF) using a plaque reduction assay.

f) Immunogenicity measure. To assess vaccine immunogenicity, both humoral and cell-mediated immune assays were performed. Cell-mediated immunity was measured by evaluating antigen-specific production of IFN-γ by PBMC collected (1) a day before boost2 (week 6), (2) a week post boost2 (week 7), and (3) a day before challenge (week 9) and restimulated with one of the following antigens: Culture medium, Pool 1 (all class I and II peptides), Pool 2 (class I and II peptides predicted from internal proteins), or whole inactivated CA/09 influenza virus (WIV), overnight. Results were recorded as the average number of spots over background and adjusted to spots per one million cells seeded. Responses were considered positive if the number of spots is greater than 20 spots forming cells per one million cells over background and at least twice the background. Humoral immunity was measured by evaluating serum hemagglutination inhibition (HI) titers and serum levels of IgG specific to virus and peptides.

Results:

a) **Matrix development.** We developed more matrices than we proposed. We initially proposed to use two prediction matrices, however, we have developed six additional matrices for common SLA alleles using the same approach. Using these matrices, we were able to predict potentially immunogenic epitopes that are not only cross-conserved across multiple IAV viruses, but also predicted to bind to multiple SLA alleles. Thus, vaccines containing these predicted epitopes may induce immune responses in a broader swine population. We developed and integrated into iVAX prediction matrices for commonly expressed SLA alleles. For class I: SLA-1*0101, 0401, SLA-2*0101, 0401; for class II: SLA-DRB1*0101, 0201, 0401, 0601.

b) **Epitope prediction**

- **Class I epitopes.** We identified 28 class I epitopes cross-conserved across multiple IAV subtypes using Conservatrix and class I PigMatrix (Table 2). We balanced between predicted binding potential to multiple alleles and conservation across IAV subtypes. Some epitopes were identified in one IAV subtype only, but those epitopes were predicted to bind to four alleles. We selected epitopes from NA to cover all the IAV subtypes analyzed and for HA we focused on the H1 subtype. We identified highly immunogenic and cross-conserved epitopes in internal proteins (NP, PA, PB1, and PB2).
- **Class II epitopes.** Similar to class I epitope predictions, conserved 9-mer sequences identified by Conservatrix were analyzed using class II PigMatrix to identify class II epitopes. Then we used EpiAssembler to construct highly immunogenic consensus sequences (ICS)[15]. We also screened individual proteins from the IAV viruses using class II PigMatrix and ClustiMer to identify protein's regions where potential T cell epitopes were "clustered". T cell epitope "clusters" range from 9 to roughly 25 amino acids in length and, considering their affinity to multiple alleles and across multiple frames, can contain anywhere from 4 to 40 binding motifs. Thus, we identified "promiscuous epitopes" (i.e. epitopes that have the potential to be recognized in the context of more than one MHC)[16] from IAV protein sequences. We identified 20 cross-conserved and potentially immunogenic class II epitopes (Table 3). We focused on the H1 subtype for prediction of HA epitopes. Epitopes predicted from M protein as well as internal proteins were highly conserved across all the IAV viruses. We selected no hydrophobic peptides to minimize technical difficulties with peptide synthesis and peptide solubility in aqueous solutions.

SLA binding studies could not be completed. A. Gutierrez traveled twice to Plum Island to stay for extended periods of time to learn how to perform SLA binding assays. These assays involve expression of SLA allele molecules in *E. coli*, isolating and purifying the proteins and analyzing the capacity of these proteins to bind antigenic peptides derived from pathogenic viruses in a sandwich ELISA assay. The assays failed (even though they were performed with the supervision of experts), but the technology is available at the Technical University of Denmark National Veterinary Institute (DTU). We tried to establish a collaboration agreement with them, but it was not possible. Nonetheless, the predicted epitopes were validated in the vaccinated animals (see immunogenicity studies). SLA-typing will soon be available on the study animals, enabling more accurate assessments of the predicted vs. observed immune response.

c) **Multi-epitope DNA vaccines.** We designed two constructs (one for class I and one for class II) with reduced predicted junctional immunogenicity and no transmembrane domains. Constructs were subcloned into DNA vaccines vectors including signals to target epitopes to the appropriate (class I or class II) processing pathway.

Table 2. Cross-conserved and potentially immunogenic class I epitopes

Peptide ID	Count	Percent	Z score* SLA1*0101	Z score SLA2*0101	Z score SLA1*0401	Z score SLA2*0401	hits	H1N1 CA/04	H1N1 IL/10	H1N1 OH/07	H1N2 MN/12	H1N2 MN/08	H3N2 TX/98	H3N2 OH/04
HA_4	3	43%	3.71	1.68	4.34	3.19	4							
HA_5	2	29%	1.71	2.95	4.15	2.1	4							
HA_8	3	43%	1.24	2.97	4.12	1.24	2							
HA_6	3	43%	2.37	2.19	3.95	1.99	4							
HA_2	1	14%	3.44	3.51	3.15	2.08	4							
HA_7	3	43%	3.32	3.84	3.07	2.25	4							
HA_1	4	57%	3.21	3.3	2.85	1.86	4							
HA_3	4	57%	2.94	3.38	2.71	1.91	4							
M_19	4	57%	4.43	3.44	4.18	2.85	4							
M_15	7	100%	2.35	2.06	3.96	1.74	4							
M_20	7	100%	0.78	-0.37	2.62	1.58	1							
M_16	6	86%	3.38	3.93	2.38	1.72	4							
M_18	4	57%	1.97	2.6	2.35	1.41	3							
M_21	7	100%	1.47	0.6	2.14	1.54	1							
M_17	7	100%	3.32	3.88	2.05	1.65	4							
NA_13	1	14%	5.14	3.34	4.59	3.22	4							
NA_9	2	29%	2.72	3.43	3.92	1.74	4							
NA_10	2	29%	4.07	3.58	3.72	2.38	4							
NA_11	4	57%	2.06	3.68	3.71	1.84	4							
NA_14	3	43%	2.64	2.56	3.61	1.66	4							
NA_12	4	57%	3.52	3.9	3.25	2.44	4							
NP_24	6	100%	2.33	1.69	5.13	1.81	4		ns					
NP_25	6	100%	3.37	3.6	4.18	2.96	4		ns					
NP_28	6	100%	1.93	3.17	3.44	1.92	4		ns					
PA_26	6	100%	3.53	3.07	3.78	2.59	4		ns					
PB1_22	6	100%	2.56	2.73	5.51	2.43	4		ns					
PB1_27	6	100%	2.96	3.52	4.01	2.13	4		ns					
PB2_23	6	100%	3.71	1.98	5.16	2.84	4		ns					

*Z score indicates the potential of a 9mer frame to bind to a given SLA allele; the strength of the score is indicated by the blue shading. For example:

Table 3. Cross-conserved and potentially immunogenic class II epitopes

Peptide ID	Length	Count	Percent	Hydro-phobicity	H1N1 CA/04	H1N1 IL/10	H1N1 OH/07	H1N2 MN/12	H1N2 MN/08	H3N2 TX/98	H3N2 OH/04
HA_17	17	3	43%	-0.16							
HA_18	16	5	71%	-1.03							
HA_19	19	2	29%	0.34							
HA_20	21	3	43%	1.23							
M_10	19	3	43%	-0.49							
M_11	23	7	100%	0.82							
M_12	23	7	100%	0.43							
NA_13	16	3	43%	-0.16							
NA_14	23	2	29%	0.05							
NA_15	16	2	29%	1.01							
NA_16	19	2	29%	1.1							
NP_1	25	6	100%	0.8		ns					
NP_2	18	6	100%	0.1		ns					
NP_3	23	6	100%	-0.73		ns					
NS_4	18	6	100%	-0.3		ns					
NS_5	17	6	100%	0.68		ns					
PA_6	23	6	100%	-0.55		ns					
PA_7	17	5	83%	0.01		ns					
PB_8	16	6	100%	1.52		ns					
PB_9	20	6	100%	0.25		ns					

100% id
>90 id

d) Immunogenicity studies. Peptides induced specific recall responses as evidenced by IFN-γ production upon exposure to pooled peptides (Pool 1 and 2). Statistically significant differences ($p<0.0001$) were observed between groups vaccinated with DNA vaccine and empty plasmid (Fig. 1). These differences were evidenced at all measured time points (weeks 6, 7 and 9). In addition, pooled peptides (Pool 1 and 2) induced similar recall responses as WIV. Moreover, epitope-specific recall responses in DNA vaccinated pigs were equivalent in magnitude to WIV-induced responses in pigs immunized with quadrivalent inactivated vaccine (FluSureXP).

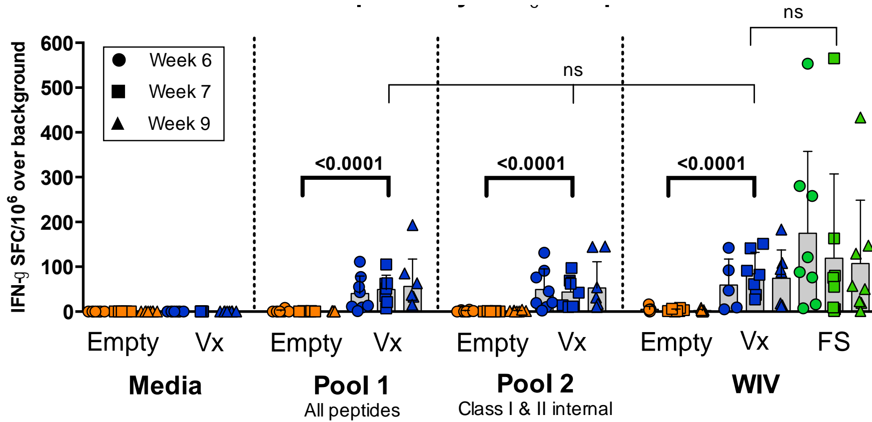

Figure 1. Peptides induced specific recall responses as evidenced by IFN-γ ELISpot. PBMC from eight pigs vaccinated with DNA vaccine (Vx) and eight pigs vaccinated with empty plasmid (Empty) were collected (1) a day before boost2 (week 6), (2) a week post boost2 (week 7), and (3) a day before challenge (week 9) and restimulated with one of the following antigens: Culture medium, Pool 1 (all class I and II peptides), Pool 2 (class I and II peptides predicted from internal proteins), or whole inactivated CA/09 influenza virus (WV), overnight. PBMC from the group vaccinated with FluSureXP (FS) was also evaluated. Control group (non-vaccinated/non-challenged) responses were minimal (not shown).

e) Vaccine efficacy. No significant differences were observed in the percentage of pneumonia between vaccinated groups (empty plasmid, DNA-vaccine, and FluSureXP) (Fig. 2A). In terms of temperatures, we observed that DNA-vaccinated was statistically significantly lower than FluSureXP groups at day 4 post infection ($p<0.01$), but it was not lower than the non-infected group. At day 5 post infection, viral load reduction was not observed in BALF or nasal swabs in neither the DNA-vaccinated nor FluSureXP-vaccinated group (Fig. 2B and 2C). The DNA vaccine by itself was not protective against intranasal challenge with A/California/04/2009 (H1N1). Similarly, commercial vaccine did not provide significant protection.

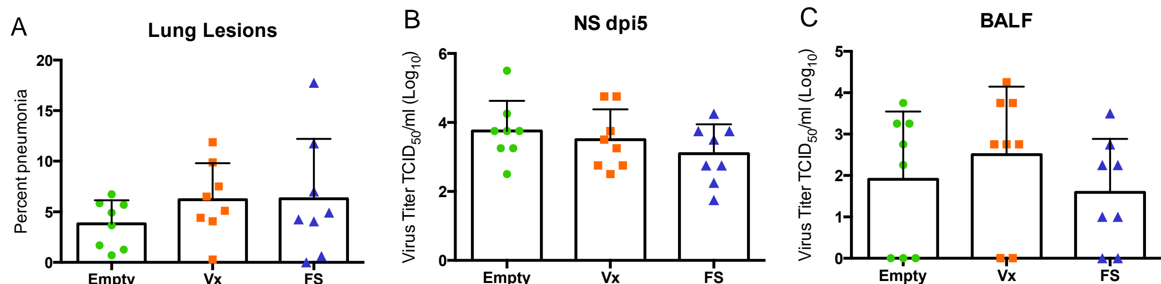

Figure 2. Vaccine efficacy evaluation. A) Comparison of lung lesions between vaccinated groups. Number of lung lesions is expressed as total percentage for the entire lung calculated based on weighted proportions of each lobe to the total lung volume. B) Nasal swabs (NS) and C) BALF were evaluated in a plaque reduction assay to evaluated viral titers.

Discussion

We developed an IAV DNA vaccine based on highly conserved influenza genome components (T cell epitopes) to promote broad cross-protection against multiple IAV strains.

In order to predict potential T cell epitopes, we initially proposed to use two prediction matrices developed based on the pocket profile method and EpiMatrix; however, we built six additional matrices for common SLA alleles using the same approach. Using these matrices, we were able to predict potentially immunogenic epitopes that are not only cross-conserved across seven IAV viruses, but also predicted to bind to multiple SLA alleles.

Newly developed matrices were successfully integrated into iVAX toolkit enabling the application of other validated tools such as Conservatrix, EpiAssembler, ClustiMer, and VaccineCAD to design a novel epitope-driven IAV vaccine. Further studies could potentially include more IAV genomes and more SLA alleles to have a more comprehensive set of matrices.

Peptides predicted using PigMatrix were able to induce specific recall responses, demonstrating their antigenicity and validating the SLA matrices. In addition, ***only 48 class I and II peptides induced similar responses*** as the quadrivalent inactivated currently available vaccine.

We selected DNA vaccines as the means of delivery for this proof or principle project because previous vaccination studies in pigs have shown that DNA vaccines elicited robust serum antibody and cellular responses after three immunizations and conferred significant protection against influenza virus challenge[14]. However, predicted epitopes could be delivered using other delivery vectors such as adenovirus as they can be produced rapidly, inexpensively, and given intranasally to induce local immunity[17].

Considering that the number of SLA matrices that we had available was less than the total number of SLA alleles for which we hope to have predictions, *perhaps it is not surprising that the first test of this prototype showed immunogenicity, but lack of protection in terms of viral load*. The results were also complicated by the fact that similar results were observed in the group vaccinated with the commercial vaccine, which demonstrated no protective effect. A repeat study will need to be performed, with epitopes representing additional SLA allele predictions, which can be expected to will improve the efficacy of the vaccine. Overall, the program was remarkably successful, considering we went from “zero to sixty” and developed our first prototype PigMatrix-epitope based vaccine. Further development of the PigMatrix tool for iVAX is likely to yield a means of accelerating and improving vaccines for the pork industry.

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