RESEARCH REPORT

PORK SAFETY

Title:	Broadly protective nasal mucosal vaccine for influenza A virus of swine – NPB #16-060)
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Industry Summary:

Influenza A virus of swine (IAV-S) is widespread and causes significant losses to swine producers. Besides its economic burden, the virus also poses a great threat to public health due to its zoonotic potential. Therefore, successful control of IAV-S will not only reduce the economic impact of this viral pathogen to the swine industry but also alleviate the threat to public health. The substantial viral genomic diversity is the main challenge for the development of a broadly protective vaccine against IAV-S. One effective approach to overcome the extraordinary genetic diversity of RNA viruses is to computationally design a consensus vaccine immunogen based on a large number of field virus sequences. It has been demonstrated in the case of several important RNA viruses such as human immunodeficiency virus (HIV), human influenza virus and porcine reproductive and respiratory syndrome viruses (PRRSV) that vaccines based on consensus sequences elicit broader levels of cross-reactive immune responses than vaccine based on naturally occurring sequences. In this project, we sought to determine if a consensus hemagglutinin (HA) protein would elicit a broader level of protection than a naturally occurring HA protein. To address this question, we computationally designed a consensus hemagglutinin subtype 3 (H3) vaccine antigen based on a set of 1,112 H3 sequences of IAV-S deposited on GenBank from 2011 to 2015. Pigs vaccinated with the consensus H3 antigen elicited a broad spectrum of neutralizing antibodies. Importantly, these vaccinated pigs were protected against challenge with a heterologous H3 IAV-S strain. Collectively, our data provide a proof-of-evidence that the consensus immunogen approach can be employed to develop a broadly protective vaccine against IAV-S.

Keywords: IAV-S, Consensus H3, Baculovirus expression, heterologous protection

Scientific Abstract:

The substantial genetic diversity represents the greatest challenge for the development of a broadly protective vaccine against IAV-S. We sought to expand the antigenic coverage of IAV-S vaccines by computationally designed a consensus HA antigens using a large set of natural HA sequences. In this particular project, we constructed a consensus H3 gene (designated H3-CON) based on a set of 1,112 natural H3 sequences of IAV-S deposited on GenBank from 2011 to 2015. The H3-CON protein was expressed by using the baculovirus expression system, followed by affinity purification by immobilized metal affinity chromatography. The purified H3-CON protein was emulsified in an oil-in-water adjuvant, and injected to pigs twice with 3-week interval. For comparative purposes, the HA protein of a naturally occurring H3N2 IAV-S strain TX98 was also expressed and purified. The H3-TX98 protein was emulsified and injected to pigs in the same manner as the H3-CON. Pigs vaccinated with H3-CON antigen elicited a broader spectrum of neutralizing antibodies than those vaccinated with H3-TX98 antigen. After challenge infection with a heterologous H3 IAV-S strain, pigs vaccinated with H3-CON antigen shed less virus than those vaccinated with H3-TX98 antigen. Collectively, the data indicate that H3-CON antigen elicited broader cross-neutralizing antibodies and better heterologous protection than did TX98 antigen. Therefore, the consensus immunogen approach might be an attractive approach to broaden the antigenic coverage of an IAV-S vaccine.

Introduction:

Influenza A virus of swine (IAV-S) is an important pathogen of swine (Rajao et al., 2014). The virus is widespread worldwide, causing tremendous economic loss to swine producers. Clinically, pigs infected with IAV-S often display signs of an acute respiratory disease which is rapidly resolved after 7-10 days. However, when associated with other pathogens of the porcine respiratory disease complex, IAV-S infection in pigs often leads to severe pneumonia and even to death (Vincent et al., 2014). In addition, the zoonotic potential of IAV-S poses a threat to public health. Direct transmission of IAV-S from pig-to-human has been documented (Ma et al., 2008). The 2009 pandemic H1N1 is an example of such an event. Due to their susceptibility to both human and avian influenza viruses, swine can act as a "mixing vessel" where genomic reassortment between different influenza viruses can occur, which may lead to the emergence of new influenza viruses with high pandemic potential (Ma et al., 2008). **Therefore, successful control of IAV-S in pigs will not only reduce the economic impact of this pathogen to the swine industry but also alleviate the threat to public health.**

The control of IAV-S is greatly challenged by the constant evolution of the virus in the field. The viral genome comprises 8 RNA segments which encode for at least 12 different viral proteins. Hemagglutinin (HA) and Neuraminidase (NA) are the two viral glycoproteins that are incorporated into the viral envelope and are used as a basis for classification of IAV-S subtypes. Currently, there are 3 major subtypes of IAV-S co-circulating in North Americas. These subtypes include: H1N1, H1N2 and H3N2. The HA subtype 1 (H1) can be phylogenetically classified into 6 distinct genetic clusters: H1δ1, H1δ2, H1α, H1β, H1γ and H1pdm09 (Anderson et al., 2013). Of the 6 H1 clusters, the H1δ1, H1δ2 are derived from human seasonal H1 viruses. Genetic distances between H1δ1, H1δ2 clusters and the remaining 4 H1 clusters could be up to 30% (Anderson et al., 2013). The HA subtype 3 can be divided into 4 clusters: H3-I, H3-II, H3-III and H3-IV, and the majority of the sequences fall into cluster IV (Anderson et al., 2013). The profound genetic/antigen diversity of IAV-S represents the most formidable challenge for the development of a broadly protective vaccine against IAV-S.

IAV-S vaccines are commonly used in the United States for control of IAV-S. A survey conducted by USDA indicated that approximately 70% of the breeding stocks were vaccinated with IAV-S vaccines (USDA, 2007). Currently, commercial IAV-S vaccines are formulated from multiple whole-inactivated virus (WIV) strains that are blended in oil-in-water adjuvants. The commercial WIV vaccines are effective in protecting vaccinated pigs against challenge infection with antigenically matched viral strains. However, the vaccine efficacy is dramatically reduced when the vaccinated pigs were challenged with mismatched virus strains (Kitikoon et al., 2013; Vincent et al., 2010; Vincent et al., 2008). Even worse, pigs vaccinated with a WIV vaccine, followed by a challenge infection with an antigenically mismatched virus strain display severe respiratory disease compared to naïve-challenge controls (Gauger et al., 2011; Vincent et al., 2008), a phenomenon called vaccine-associated enhanced respiratory disease (VAERD). Because the process of producing and licensing a WIV vaccine is time-consuming and expensive, the commercial WIV is not updated fast enough to cope with the continual evolving of IAV-S in the field. **Thus, there exists an urgent need for a new generation of IAV-S vaccine that is able to provide broad levels of heterologous protection while should not pose the risk of VAERD.**

The HA is the most abundant envelope protein which is responsible for binding of the virions to the host cells. Consequently, HA is an important target for the development of IAV-S vaccines. In this project, we employed a computational approach to design a synthetic HA antigen (designated as H3-CON) which has reduced genetic distances to the contemporary IAV-S strains. We report here the construction of the H3-CON antigen and the evaluation of its immunogenicity in pigs.

Objective:

Objective 1: Construct and evaluate the immunogenicity of the consensus swine influenza virus HA sequences.

Objective 2: Evaluate the cross-protection conferred by the consensus HA immunogens.

Materials & Methods
Cells and IAV-S strains
Madin-Darby Canine Kidney (MDCK) cells were used for propagation of IAV-S strains and for measurement of serum-virus neutralization. The cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 25mM HEPES and 0.2% (W/V) bovine serum albumin faction V. Six different IAV-S H3N2 strains were obtained from NVSL (Table 1). The percentage of amino acid sequence similarity among these strains at the HA protein level is listed in Table 2. These strains were used for evaluation of cross-neutralization and for challenge infection.

Table 1: List of H3N2 strains used in the project

Strain	Designation	GenBank Accession	Use for
A/swine/Minnesota/A01125993/2012	MN5993	AFU08620	Challenge infection
A/swine/Kansas/A01377243/2014	KS7243	AIA24431	VNA*
A/swine/Minnesota/A01392534/2013	MN2534	AHA11511	VNA
A/swine/Michigan/A01259002/2012	MI9002	AGC96222	VNA

| A/swine/Colorado/A01203748/2012 | CO3748 | AFU10042 | VNA |
| A/swine/Texas/4199-2/1998 | TX98 | AEK70342 | Clone HA protein for immunization
VNA |

VNA: Virus neutralization assay

Table 2: Percentage of amino acid similarity (%) among H3N2 strains used in this study

	TX98	MN5993	CO3748	MN2534	KS7234	MI9002
H3-CON	92.23	96.64	90.81	93.64	95.05	97.88
H3-TX98	100.00	89.58	90.64	90.28	88.69	91.34
MN5993	89.58	100.00	88.87	90.81	93.29	94.88

Design of a consensus HA vaccine antigen: We collected from GenBank 1,112 HA subtype 3 (H3) sequences of the IAV-S originating in the U.S. from 2011 to 2015. These sequences were aligned by using the MUSCLE 3.8 program and a phylogenetic tree was constructed following the maximum likelihood method in MEGA software (version 6.06). We observed that the numbers of sequences within each of the phylogenic clusters are not equal as the cluster H3 IV contains more than 90% sequences. Moreover, many sequences are closely similar (99% similarity). To avoid the bias of the consensus sequence towards the cluster containing the larger number of sequences, we first removed redundant sequences (e.g. sequences with equal or greater than 99% similarity) using Jalview. After that, we obtained a set of 230 non-redundant sequences. Based on this set of non-redundant sequences, we generated a consensus sequence designated as H3-CON.

Protein expression and purification: We used the Bac-to-Bac® Baculovirus Expression System (Invitrogen) to express two different HA antigens: H3-CON and H3-TX98. To facilitate the secretion of the expressed proteins to culture medium, the original signal sequence of each H3 protein was replaced by the honeybee melittin (HBM) signal sequence. Additionally, the transmembrane and cytoplasmic domains were removed. Finally, the bacteriophage T4 tetramerization sequence and 6X histidine tag were fused in frame to the C-terminus of the HA proteins. The T4 tetramerization helps stabilize conformational epitopes on the stalk-domain of HA protein while the 6X his tag facilitates the purification of the proteins by immobilized metal affinity chromatography. The coding sequences of these two modified HA antigens were separately cloned to the pFastBac1 plasmid. Recombinant baculoviruses expressing the HA antigens were generated following the manufacturer's protocol.

Immunization: A total of 24 3-week-old pigs were purchased from Midwest Research Swine. These pigs were tested negative for IAV-S and PRRSV. They were randomly assigned into four groups, each of which will be accommodated in a separate room in the biosecurity level -2 animal research facilities at UNL. Groups 1 and 2 were immunized with the H3-CON and H3-TX98 proteins, respectively (Table 3). These proteins were emulsified in 20% (V/V) Emulsigen ®-DL 90 (Phibro Animal Health Corporation, Omaha NE) in a way that each dose of vaccine contained 100µg protein in 2mL volume. The vaccines were given twice at 0 and 21 day post-vaccination (dpv). Group 3 was inoculated intra-tracheally with 2mL live virus containing $10^{5.0}$ TCID50 of IAV-S strain A01125993 (MN5993). This group was included to the study for comparative purposes because it is well documented that live-virus immunization often results in better protection. Group 4 was inoculated with

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2mL of cell culture medium DMEM to serve as a non-immunization control. Whole blood with or without anticoagulant were collected from all pigs every week after immunization, for isolation of serum and peripheral blood monocytes (PBMCs) which were used for measurement of systemic humoral and cellular immune responses, respectively. At day 44 post-immunization, all pigs were challenged by intra-tracheal inoculation with 2mL live virus containing $10^{5.0}$ TCID50 of IAV-S strain MN5993. After challenge infection, nasal swabs were daily collected from all pigs for evaluation of viral shedding. At day 49 post-immunization (e.g. day 5 post-challenge infection), all pigs were humanely euthanized and necropsied. Samples of lung were collected for evaluation of lung pathology.

Table 3: Experimental design

Group	Immunized with	Challenge with
1	H3-CON protein	
2	TX98-HA protein	MN25993
3	MN5993 live-virus	
4	DMEM	

Results

1. Protein expression and purification

The two HA proteins: H3-CON and H3-TX98 were expressed using the baculovirus expression system. After purification by immobilized metal affinity chromatography, the proteins were analyzed by electrophoresis on SDS-PAGE, followed by Coomassie staining. We obtained good amount of H3-CON and H3-Tx98 proteins with high levels of purity (Fig. 1).

Figure 1: Analysis of HA proteins by SDS-PAGE electrophoresis. *Various concentrations of bovine serum albumin (BSA) were loaded in the SDS-PAGE gel to establish a standard curve based on which the relative concentrations of H3-CON and H3-TX98 proteins were estimated.*
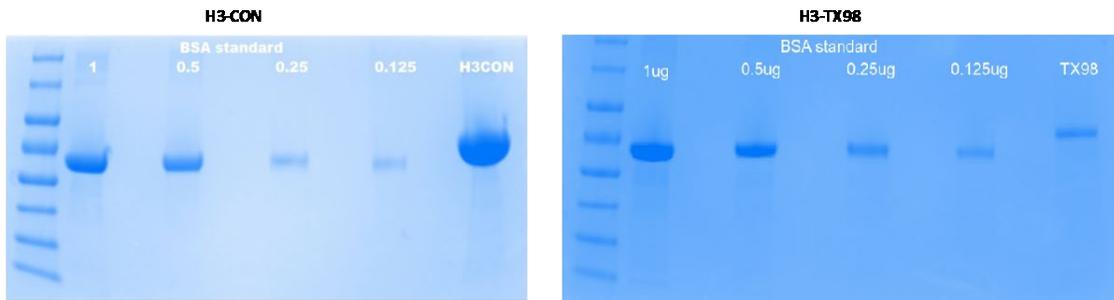
2. Antibody responses

We first measured antibody responses against MN5993, the virus strain used for challenge infection. Neutralizing antibodies were detected in group 3 (e.g. immunized by infection with live-virus MN5993 strain) at day 7 post-vaccination whereas these antibodies were not detected in pigs immunized with group 1 (e.g.

Figure 2: Kinetics of neutralizing antibodies measured against the H3N2 strain MN5993.
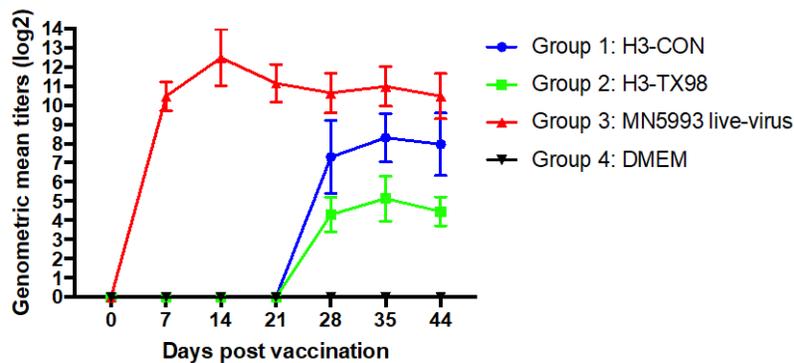
immunized with H3-CON) and group 2 (immunized with H3-TX98) until day 28 post-vaccination (e.g. 7 days after the second dose of immunization). As expected, pigs in group 3 developed the highest levels of neutralizing antibodies against MN5993, the homologous virus strain. Interestingly, we observed that pigs in group 1 mounted higher levels of neutralizing antibodies against MN5993 than those in group 2 (Figure 2).

We next measured cross-neutralizing antibodies against different divergent H3N2 strains. Pigs in group 1 elicited cross-neutralizing antibodies against several divergent H3N2 strains. Specifically, antisera from group 1 neutralized 4 different H3N2 strains (e.g. MN5993, TX98, KS7243, and MI9002) with the geometric mean titers greater than 1:100. In contrast, antisera collected from pigs in groups 2 and 3 did not significantly cross-

neutralize other virus strains although these antisera had high titers of homologous neutralizing antibodies (measure against MN5993 and TX98 virus, respectively) (Table 4).

Table 4: Cross-neutralizing antibody titers measured against different H3N2 strains

Treatment group	Virus used in the virus-neutralization assay					
	MN5993	TX98	KS7243	MN2534	MI9002	CO3748
Group 1: H3-CON	320	101	508	20	640	22
Group 2: H3-TX98	36	1,810	50	32	25	10
Group 3: MN5993 Live virus	2,032	18	10	10	10	10
Group 4: DMEM	10	10	10	10	10	10

Note: Data are expressed as the geometric mean titers of 6 pigs in each treatment group. For calculation of geometric means, samples with undetected neutralization titer were assigned a value of 10, the limit of detection of the virus neutralization assay.

3. Viral shedding

All pigs were challenged by infection with a heterologous strain MN5993 strain. Nasal swabs were collected daily from before challenge infection to day 5 post-challenge infection. As expected, pigs immunized by infection with live-virus MN5993 strain were fully projected against reinfection with the homologous virus strain,

Figure 3: Viral shedding after challenge infection. Pigs were challenged by intra-tracheal infection with MN5993 strain at day 44 post vaccination. Nasal swabs were collected right before challenge infection to day 5 post challenge (e.g. from 0 to 5 dpc). Viral RNA was determined by using the validated real-time RT-PCR kit (Qiagen). The results are expressed at the mean CT cycles of the group at each sampling point. The higher the CT values, the lower the amount of viral RNA copy. For calculation of mean, samples with undetected levels of viral RNA were assigned a CT value of 40.00.
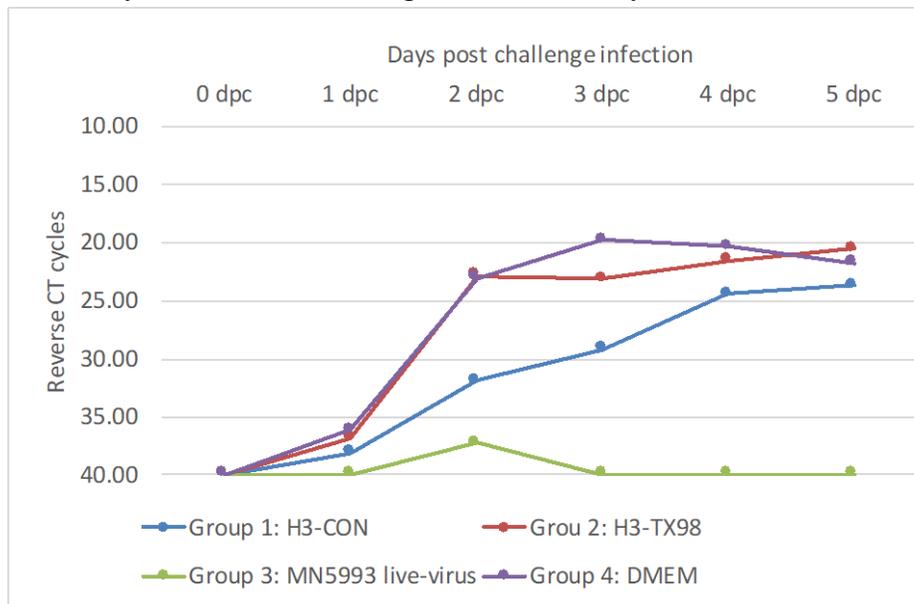
evidenced by the observation that viral RNA was only detected at 2 dpc. Immunization with H3-TX98 protein (group 3) did not result in significant protection as pigs in this group showed the same levels of viral shedding as those in the control group (group 4). In contrast, immunization with H3-CON protein (group 1) resulted in a significant protection as pigs in this group has significant lower levels of viral shedding than those in the H3-TX98 and control groups.

4. Lung pathology

At day 5 post-challenge infection, all pigs were humanely euthanized and necropsied. Samples of lung were collected for evaluation of lung pathology. However, no significant lung lesion was observed, even the DMEM group which was naïve IAV-S at the time of challenge infection. Perhaps, this is because the MN5993 strain used for challenge infection is not a virulent IAS-V strain.

Discussion:

The consensus vaccine immunogen approach has been proven effective for expanding vaccine antigenic coverage. In this project, we observed that the H3-CON protein elicited broader levels of cross-neutralizing antibody responses than the H3-TX98 and MN5993. Additionally, we observed that pigs immunized with H3-CON was better protected against challenge infection than those immunized with H3-TX98, evidenced by the significant lower levels of vial shedding in the H3-CON group than in the H3-TX98 group. However, the IAV-S strain MN5993 used in this study for challenge infection did not seem to be a pathogenic strain as it did not cause any significant clinical signs and lung damage to the pigs. Therefore, we can only use viral shedding as a main parameter for evaluation of protection. In the future, we will need to evaluate the protection against other IAV-S with high pathogenicity to determine protective efficacy of the H3-CON antigen.

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