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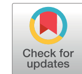
Design and characterization of a consensus hemagglutinin vaccine immunogen against H3 influenza A viruses of swine



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ABSTRACT

The substantial genetic diversity exhibited by influenza A viruses of swine (IAV-S) represents the main challenge for the development of a broadly protective vaccine against this important pathogen. The consensus vaccine immunogen has proven an effective vaccinology approach to overcome the extraordinary genetic diversity of RNA viruses. In this project, we sought to determine if a consensus IAV-S hemagglutinin (HA) immunogen would elicit broadly protective immunity in pigs. To address this question, a consensus HA gene (designated H3-CON.1) was generated from a set of 1,112 H3 sequences of IAV-S recorded in GenBank from 2011 to 2015. The consensus HA gene and a HA gene of a naturally occurring H3N2 IAV-S strain (designated H3-TX98) were expressed using the baculovirus expression system and emulsified in an oil-in-water adjuvant to be used for vaccination. Pigs vaccinated with H3-CON.1 immunogen elicited broader levels of cross-reactive neutralizing antibodies and interferon gamma secreting cells than those vaccinated with H3-TX98 immunogen. After challenge infection with a fully infectious H3N2 IAV-S isolate, the H3-CON.1-vaccinated pigs shed significantly lower levels of virus in their nasal secretions than the H3-TX98-vaccinated pigs. Collectively, our data provide a proof-of-evidence that the consensus immunogen approach may be effectively employed to develop a broadly protective vaccine against IAV-S.

1. Introduction

Influenza A virus of swine (IAV-S) is one of the most important respiratory pathogens of swine (Rajao et al., 2014a). The virus is widespread worldwide, causing tremendous economic losses to swine producers (Haden et al., 2012). 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 genetic 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 that this pathogen has on the swine industry but also alleviate the threat to public health.

The effective control of IAV-S is greatly challenged by the constant evolution of the virus in the field (Rajao et al., 2018). Hemagglutinin (HA) and Neuraminidase (NA) are the two viral envelope glycoproteins that are used as a basis for classification of IAV-S subtypes. Currently, three major subtypes of IAV-S are co-circulating in North America: H1N1, H1N2 and H3N2 (Walia et al., 2018). The HA subtype 1 (H1) can be phylogenetically classified into 6 distinct genetic clades namely: H1δ1, H1δ2, H1α, H1β, H1γ and H1pdm09 (Anderson et al., 2015, 2013). Of the 6 H1 clades, the H1δ1, H1δ2 are derived from human seasonal H1 IAV-S (Vincent et al., 2009). Genetic distances between H1δ1, H1δ2 clades and the remaining 4 H1 clades could be up to 35% (Anderson et al., 2013). The H3 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). New strains of influenza virus frequently emerge in swine herds in the U.S. (Zeller et al., 2018).

The profound genetic diversity represents the most formidable challenge for the development of a broadly protective vaccine against IAV-S. Currently, polyvalent, whole-inactivated virus (WIV) vaccines

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are commonly used in the U.S. to control IAV-S [reviewed in (Sandbulte et al., 2015)]. The commercial WIV vaccines are effective in protecting vaccinated pigs against challenge infection with antigenically matched viral strains. However, vaccine efficacy is dramatically reduced when the vaccinated pigs are challenged with mismatched virus strains (Kitikoon et al., 2013; Vincent et al., 2010, 2008). In some cases, 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). This phenomenon is referred to as vaccine-associated enhanced respiratory disease (VAERD). Because the process of producing and licensing a WIV vaccine is time-consuming and expensive, commercial WIV vaccines-are not updated fast enough to cope with the continually evolving IAV-S in the field. To ensure the antigenic match, swine producers often use autogenous vaccines produced from inactivated IAV-S isolates originating from their farms [reviewed in (Sandbulte et al., 2015)]. However, the autogenous vaccines are not required to be tested for potency and efficacy, and their efficacy sometimes might be suboptimal (Sandbulte et al., 2015). Recently, a live-attenuated influenza virus (LAIV) vaccine was licensed for clinical application in the U.S. Experimental data demonstrate that LAIV vaccine confers better heterologous protection than WIV vaccines (Abente et al., 2018; Gauger et al., 2014; Loving et al., 2013). However, there is a great concern of reversion to virulence due to the potential reassortment between LAIV and field IAV-S isolates.

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. As demonstrated in the case of human immunodeficiency virus type 1 (HIV-1), the average genetic distances between a concensus vaccine immunogen and wild-type viruses can be reduced to half of those between wild-type viruses to each other (Novitsky et al., 2002). Importantly, it has been demonstrated that consensus HIV-1 vaccine immunogens elicit broader levels of protective immunity than naturally occurring immunogens (Hulot et al., 2015; Santra et al., 2008; Weaver et al., 2006). HA is an important target for the development of IAV vaccines because it is the most abundant envelope protein responsible for binding of IAV to the host cells. There are numerous studies on the protective immunity conferred by consensus HA immunogens for human and avian IAV (Chen et al., 2008; Hyoung et al., 2017; Lingel et al., 2017; Webby and Weaver, 2015; Zhou et al., 2017). However, we are not aware of any publications on the development of consensus HA vaccine immunogens for IAV-S except one publication describing the protective immunity of DNA vaccines encoding matrix 2 ectodomain (M2e), cytotoxic T lymphocyte epitopes and consensus H3 of IAV-S in mice (Wang et al., 2012). We report here the design and characterization of a consensus HA vaccine immunogen against H3 IAV-S. We demonstrate that the consensus H3 immunogen elicited broad levels of protective immunity in pigs as compared to a naturally occurring H3 immunogen.

2. Materials and methods

2.1. Cells and viruses

The Madin-Darby Canine Kidney (MDCK) cell line was obtained from American Type Culture Collection (ATCC® CCL-34). The cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Cat. # 12800-082) supplemented with 0.2% bovine serum albumin (BSA) fraction V (Sigma, Cat. # A8412), 25 mM HEPES (Hyclone, Cat. # SH30237.01), 10% fetal bovine serum (Atlanta Biological, Cat. # S10650) and 1X antibiotic (100 U/ml penicillin and 100 μg/ml streptomycin) as previously described (World Health Organization., 2011). *Spodoptera frugiperda* Sf9 (insect cells) were cultured in protein-free ESF 921™ culture medium (Expression System, Cat. # 96-001-01) supplemented with 1X antibiotic (100 U/ml penicillin and 100 μg/ml). Six H3N2 IAV-S isolates used in this study (Table 1) were obtained from the National Veterinary Services Laboratories (NVSL).

Table 1
List of swine H3N2 isolates used in the study.

H3N2 isolates	Designation	GenBank Accession
A/swine/Minnesota/A01125993/2012	MN5993	AFU08620
A/swine/Kansas/A01377243/2014	KS7243	AIA24431
A/swine/Minnesota/A01392534/2013	MN2534	AHA11511
A/swine/Michigan/A01259002/2012	MI9002	AGC96222
A/swine/Colorado/A01203748/2012	CO3748	AFU10042
A/swine/Texas/4199-2/1998	TX98	AEK70342

2.2. Design of the H3 consensus sequence

A total of 1,112 HA subtype 3 (H3) sequences of the IAV-S originating in the U.S. from 2011 to 2015 were collected from the Influenza Virus Resources database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=genomeset) on September 20th, 2015. These sequences were aligned by using the MUSCLE 3.8 program (Edgar, 2004). A phylogenetic tree was constructed following the maximum likelihood method in MEGA software (version 6.06) (Tamura et al., 2013). Redundant sequences sharing equal or greater than 99% similarity were removed by using Jalview (Waterhouse et al., 2009). After that, a set of 230 non-redundant sequences was obtained. Finally, a consensus sequence designated H3-CON.1 was generated by using the default setting in Jalview (Waterhouse et al., 2009).

2.3. Protein expression and purification

The Bac-to-Bac® Baculovirus Expression System (Invitrogen) was used to express two different HA antigens: H3-CON.1 and H3-TX98 (GenBank accession no. AEK70342.1). The coding sequences of these two modified HA antigens were separately cloned to the pFastBac1 plasmid. The resulting plasmids were then transformed into MAX efficiency® DH10Bac™ competent cells to generate the recombinant bacmids. After that, the recombinant bacmids were transfected into Sf9 insect cells to generate recombinant baculovirus expressing the HA proteins, following the manufacturer's protocol. The recombinant baculovirus stocks were then used to infect Sf9 cells for expression of HA proteins. Recombinant proteins secreted in the culture medium were purified by immobilized metal affinity chromatography. Protein purity and integrity was analyzed by reducing and denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie staining.

2.4. Pig experiments

Pig experiments conducted in this study were approved by the University of Nebraska-Lincoln (UNL) Institutional Animal Care and Use Committee under the protocol number 1297. Two experiment were conducted. In the first experiment, a total of 18 three-week-old, IAV-S seronegative pigs were purchased from Midwest Research Swine (Glencoe, MN). The pigs were randomly assigned into three groups, each of which were 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.1 and H3-TX98 proteins, respectively. 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 100 μg protein in 2 mL volume. The vaccines were administered twice intramuscularly at day 0 and 21. Pigs in group 3 were inoculated with 2 mL DMEM medium to serve as a non-vaccination control. Whole blood with or without anticoagulant were collected from all pigs before vaccination, and at days 35 and 44 post-vaccination (pv), for isolation of serum and peripheral blood monocytes (PBMCs) which were used for measurement of humoral and cellular immune

responses, respectively. At day 44 pv, all pigs were challenged by an intratracheal inoculation with 2 mL live virus containing $10^{5.0}$ $TCID_{50}$ of the H3N2 IAV-S isolate MN5993. After challenge infection, nasal swabs were collected daily from all pigs for evaluation of viral shedding. At day 49 pv (*e.g.* day 5 post-challenge infection), all pigs were humanely euthanized and necropsied. Samples of lung were collected for evaluation of lung pathology by a board-certified pathologist blinded to the treatment groups as described previously (Gauger et al., 2012).

In the second experiment, 6 three-week-old, IAV-S seronegative pigs were purchased from Midwest Research Swine (Glencoe, MN) and were accommodated in a separate room in the biosecurity level -2 animal research facilities at UNL as mentioned above. The pigs were inoculated intratracheally with 2 mL virus inoculum containing $10^{5.0}$ $TCID_{50}$ of the H3N2 IAV-S isolate MN5993. Serum samples were collected from the pigs before infection and at day 35 post-infection for evaluation of cross-neutralizing activities against different H3N2 IAV-S isolates.

2.5. RNA in situ hybridization

RNA *in situ* hybridization (ISH) was performed on formalin-fixed, paraffin-embedded (FFPE) tissues by using the RNAscope® assay (Advanced Cell Diagnostics, ACD). The procedure was performed manually according to the manufacturer's instruction. Briefly, 4 μm sections were baked for 1 h at 60 °C, deparaffinized in xylene, followed by dehydration in an ethanol series. Afterwards, sections were treated with RNAscope® Hydrogen peroxide reagent (H_2O_2) (ACD, Cat. # 322330) for 10 min at room temperature and washed twice with distilled water. Then slides were immersed in boiling RNAscope® target retrieval reagent (ACD, Cat. # 322000) for 15 min and washed twice in distilled water. Sections were treated with Protease plus reagent (ACD, Cat. # 322330) for 30 min at 40 °C in a HybEZ hybridization oven. The sections were then incubated with an RNA probe specific to the IAV-S NP protein (V-InfluenzaA-H3N2-NP) for 2 h at 40 °C in a HybEZ hybridization oven. Signal amplification and detection reagents were applied sequentially using RNAscope® 2.5 HD Detection Reagent-Brown (ACD, Cat. # 322310) and incubated in AMP 1, AMP 2, AMP 3, AMP 4, AMP 5, and AMP 6 reagents, for 30, 15, 30, 15, 30, 15 min respectively. Slides were repeatedly washed twice using wash buffer reagent (ACD, Cat. # 310091) after each amplification step. Chromogenic detection was performed using diaminobenzidine (DAB), followed by counterstaining with Gill´s hematoxylin. The Ss-PPIB (Sus Scrofa Peptidylprolyl Isomerase B (cyclophilin B) probe was used as a positive control, and the dapB probe was used as a negative control.

2.6. Quantification of viral loads

Viral RNA from nasal swabs were extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Cat. # 52906), according to the manufacturer's instruction. Viral genomic copy numbers were quantified using a real-time reverse transcription PCR (RT-PCR) kit (Life Technologies, VetMax-Gold SIV Detection Kit, Cat. #4415200). The RT-PCR product was gel-purified and cloned into the pGEMT-easy vector (Promega, Cat. # A1360) and sequenced. After that, the RNA amplicon (UCGAGCUC UCGGACGAAAAGGCAACGAACCCGAUCGUGCCUUCCUUUGACAUGA GUAAUGAAGGAUCUUAUUUCUUCGGAGACAAUGCAGAGA) was chemically synthesized and used to establish a standard curve based on which the absolute copy numbers of viral RNA in each sample was estimated. Viral loads in nasal swabs were reported as $\log_{10}$ copies per μL of RNA loaded to the RT-PCR reaction. For statistical purposes, samples that had undetectable levels of viral RNA were assigned a value of 0.

2.7. Measurements of immune responses

The serum-virus neutralization (SVN) assay was performed on MDCK cells as previously described (Gauger and Vincent, 2014; Van Reeth et al., 2003), with modifications. Briefly, the sera were treated with receptor destroying enzyme II (HARDY Diagnostics, Cat. # 370013) at 37 °C overnight, followed by inactivation at 56 °C for 30 min. Treated sera were diluted 2-fold serially in 50 μl of virus inoculation medium (DMEM supplemented with 0.2% BSA faction V, 25 mM HEPES, 1x antibiotic and 1 μg/mL TCPK-treated trypsin (Sigma Cat. # T1426) on a 96-well plate and then incubated with an equal volume (50 μl) containing 100 $TCID_{50}$ of test virus for 1 h at 37 °C. The entire mixture of serum and virus (100 μl/well) was transferred to an Ubottom 96-well plate containing confluent MDCK cells that had been seeded 48 h earlier. The plate was further incubated for 4 days at 37 °C in a humidified atmosphere containing 5% CO2. After that, the presence of virus infection in each well were determined by using the hemagglutination assay. The neutralization titer is the highest serum dilution that does not exhibit any sign of hemagglutination. For statistical purposes, samples that do not exhibit any sign of neutralization will be assigned a titer of 2.

The frequencies of IFN-γ secreting cells (IFN-γ SCs) in PBMCs were measured by using an IFN-γ Elispot assay as previously described (Correas et al., 2017; Meier et al., 2003). Briefly, ninety-six well plates with PVDF membrane were rinsed with 30% ethanol, followed by incubation overnight at 4 °C with 50 μl/well of anti-porcine IFN-γ antibody (BD Biosciences Pharmingen, clone P2G10) at a concentration of 10 μg/mL. The plate was washed three times with sterile PBS and blocked by incubation with 250 μl/well of complete RPMI (cRPMI) medium for 2 h at 37 °C in a 5% CO_2 incubator. PBMCs of each pig were plated in duplicate in 100 μl/well of cRPMI, followed by stimulation with one of the selected H3N2 IAV-S isolates in the form of live virus diluted in cRPMI. A cocktail of PMA (Sigma Cat # P8139, final concentration 10 ng/mL) and Iomycin (Sigma Cat # I-0634, final 1 μl/mL) diluted in cRPMI was used as positive control while cRPMI was used as negative control. After 20 h incubation at 37 °C in a 5% CO_2 incubator, the cells were removed by washing three times with 250 μl/well of PBS containing 0.05% tween-20 (PBS-T20). Fifty microliters of biotin-labeled anti-porcine IFN-γ (clone P2C11) at a concentration of 2 μg/mL in PBS-T20 were added to each well and the plate was incubated at room temperature for 1 h. Spot detection was performed using alkaline phosphatase conjugated streptavidin and alkaline phosphatase substrate as previously described (Parida et al., 2012). Spots were counted and analyzed using a CTL ImmunoSpot counter (Cellular Technology).

2.8. Statistical analysis

Prior to statistical analysis, the neutralization titers were transformed into log base 2. Virus neutralization titers, and IFN-γ SCs in PBMCs were analyzed by one-way analysis of variance (ANOVA). Viral shedding was analyzed by repeated-measures two-way ANOVA. Tukey's multiple comparisons test was used for comparison among treatments. Genetic distances from wild-type H3 to the consensus H3-CON.1 and among wild-type H3 sequences were analyzed by unpaired *t*-test with Welch's correction. Results were considered statistically significant when $p < 0.05$. All statistical analysis was done in GraphPad Prism 7.0 (GraphPad Software, Inc).

3. Results

3.1. H3 consensus immunogen has reduced genetic distances to the contemporary IAV-S sequences

A total of 1,112 amino acid sequences of H3 IAV-S originating in the U.S. were collected from the Influenza Virus Recourse database on September 20th, 2015. Initial analysis showed that the numbers of sequences within each of the phylogenic clusters were not equal and that many sequences were closely similar, sharing equal or greater than 99% identify. Consequently, the consensus H3 sequence generated from this

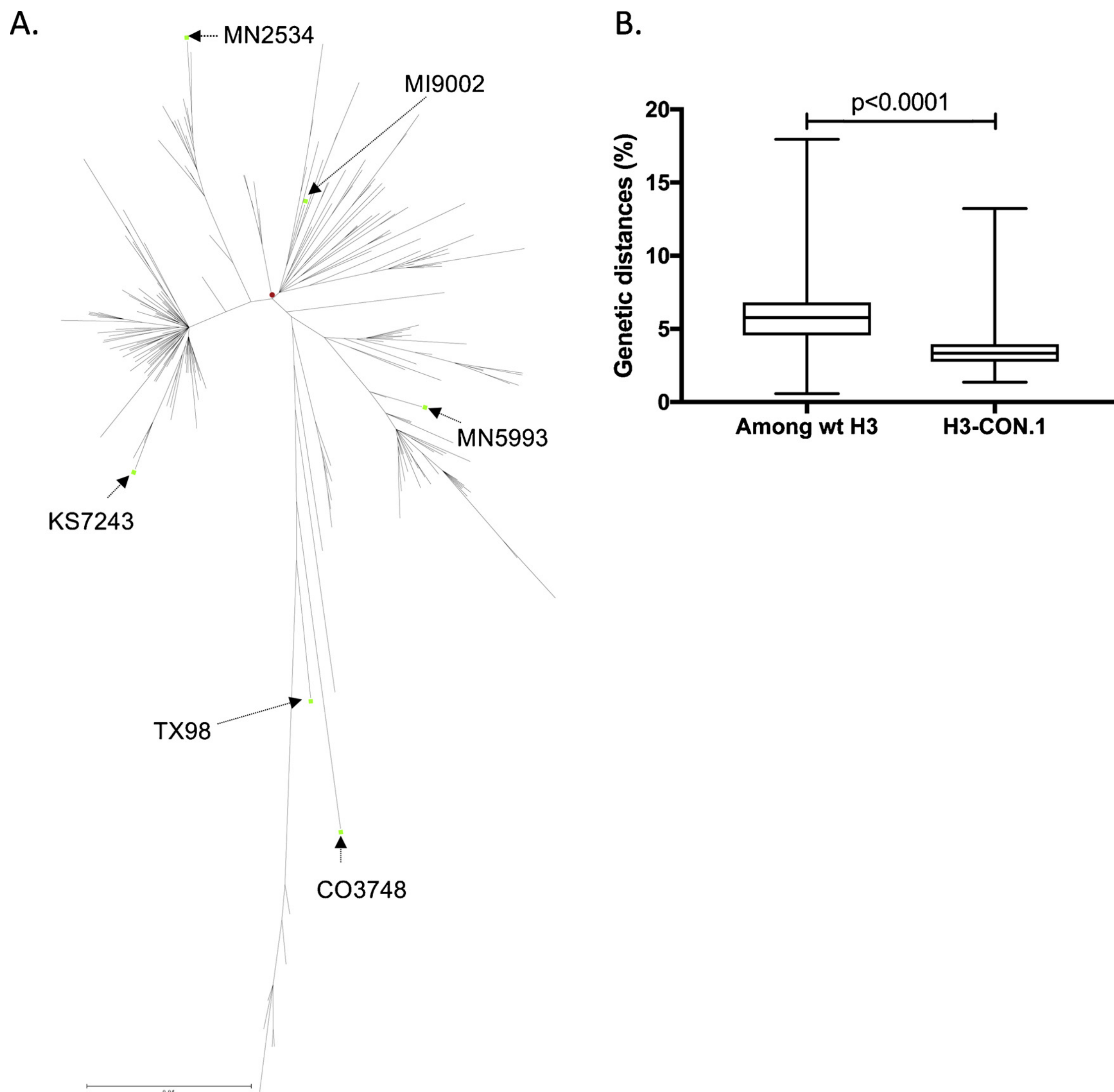

Fig. 1. Phylogenetic analysis of IAV-S H3 sequences. (A) A phylogenetic tree constructed from a set of 230 non-redundant wild-type H3 sequences, together with a consensus sequence (H3-CON.1). Bar represents nucleotide substitutions per site. Locations of the H3-CON.1 in the tree is indicated by a red dot whereas the locations of naturally occurring IAV-S H3N2 isolates used in this study are indicated by green dots. The phylogenetic tree with tip labels is presented in Fig. S1 in the supplemental material. (B) Pairwise genetic distances among wild-type H3 sequences, and between wild-type H3 and the H3-CON.1. The lower and upper boundaries of the box indicate the 25th and 75th percentiles, respectively. The solid line in the box represents the median. Whiskers below and above the box indicate the smallest and largest values, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

original data set skewed toward the phylogenetic cluster containing a larger number of sequences. To avoid this problem, redundant sequences were removed from the original data set to obtain a subset of sequences with less than 99% similarity. A consensus sequence was empirically computed in a way that it should be located closer to the center of the phylogenetic tree (Fig. 1A). Next, the pairwise genetic distances between H3-CON.1 and wild-type H3 sequences and the distances among wild-type H3 sequences were calculated. The pairwise genetic distances between H3-CON.1 and wild-type H3 sequences varied from 1.87% to 13.04%, with the median of 3.58%. The pairwise genetic distances among wild-type H3 sequences varied from 2.06% to 17.57%, with the median of 6.70% (Fig. 1B). Clearly, the pairwise genetic distances between the H3-CON.1 and wild-type H3 sequences were significantly shorter than the pairwise distances among the wild-type H3 sequences. We hypothesized that a subunit vaccine formulated in base of H3-CON.1 gene might confer broader levels of heterologous protection than a subunit vaccine formulated in based of a single, naturally occurring H3 sequence.

3.2. Expression of the HA antigens

H3-CON.1 was expressed using baculovirus expression system. For comparative purposes, HA sequence of the H3N2 strain TX98 (designated H3-TX98) was also expressed in the same manner. The TX98 strain was selected for comparative purposes because it has been used to formulate multiple WVI and LAIV candidate vaccines (Richt et al., 2006; Vincent et al., 2012). To enhance the secretion of the expressed proteins, 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 (Krammer et al., 2012) while the 6X histidine tag facilitates the purification of the expressed proteins by immobilized metal affinity chromatography (Fig. 2A). After purification, the proteins were analyzed for purity by SDS-PAGE, followed by Coomassie staining. As shown in (Fig. 2B), only one single band of approximately 72 KDa was detected in both H3-

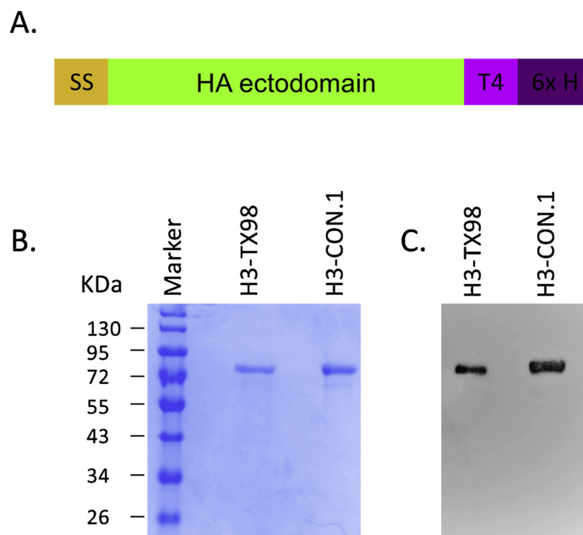

Fig. 2. Expression and purification of H3 immunogens. (A) Schematic representation of the H3 constructs. Original signal sequence of each H3 protein was replaced by the honeybee melittin (HBM) signal sequence (SS). The transmembrane and cytoplasmic domains were replaced by bacteriophage T4 tetramerization sequence (T4) and 6X histidine tag (6x H). (B & C) Analysis of the recombinant HA protein preparations. Purified proteins were resolved by reducing and denaturing SDS-PAGE. (B) The SDS-PAGE gel was stained with Coomassie blue. (C) Western blot analysis using an antibody against histidine tag. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

CON.1 and H3-TX98 protein preparations, without any visible signs of contamination with other proteins, indicating the high levels of purity of our protein preparations. As expected, the two proteins were reacted with anti-histidine tag antibody as analyzed by western blot (Fig. 2C).

3.3. H3-CON.1 immunogen elicited broadly neutralizing antibody responses in pigs

The H3-CON.1 and H3-TX98 protein preparations were used to immunize pigs. Antisera collected at day 35 after the first vaccination (corresponding to day 14 after the second vaccination) were used to evaluate the cross-neutralizing activities against divergent H3N2 IAV-S isolates. Although antisera collected from pigs vaccinated with H3-TX98 protein displayed the highest virus neutralization titer against the homologous IAV-S strains TX98, these antisera exhibited significantly lower cross-neutralizing activities against the heterologous IAV-S isolates (Fig. 3). In contrast, antisera collected from pigs vaccinated with H3-CON.1 displayed a broad spectrum of cross-neutralization. Specifically, antisera against H3-CON.1 had significantly higher cross-neutralization titers against MN5993, KS7243, MI9002 and CO3748 than antisera against H3-TX98. However, the antisera against H3-CON.1 did not exhibit significantly higher levels of cross-neutralizing activities against MN2534 than antisera against H3-TX98.

Next, antisera against MN5993, an IAV-S strain located in another branch of the phylogenetic tree (Fig. 1A), were comparatively analyzed for their cross-neutralizing activities. Of note, these antisera were collected from pigs at day 35 after experimentally infected with the IAV-S isolate MN5993. Similar to antisera against H3-TX98 protein, the antisera collected from pigs infected with MN5993 displayed high neutralization titers against the homologous virus. Surprisingly, the MN5993 antisera did not significantly neutralize any of the five tested heterologous IAV-S isolates (Fig. 3). Consequently, the MN5993 had significantly lower cross-neutralization titers against TX98, KS4243, MN2534, MI9002 and CO3748 than the H3-CON.1 antisera. Collectively, the data demonstrated that H3-CON.1 elicited broader cross-neutralizing antibodies than the H3-TX93 and MN5993 live virus.

3.4. H3-CON.1 immunogen elicited cross-reactive T cell responses

Cross-reactivity of T cells in PBMCs was measured against four divergent H3N2 isolates by using the IFN-γ ELISPOT assay. The PBMCs were collected at day 44 after the first vaccination. Pigs immunized with H3-CON.1 immunogen had greater number of IFN-γ SCs than those immunized with H3-TX98 immunogen when measured against three out of four tested IAV-S isolates namely: MN5993, MI9002 and CO3748 (Fig. 4). Interestingly, the H3-CON.1 immunized pigs had similar number of IFN-γ SCs when measured against the H3N2 isolate TX98 as compared to the H3-TX98 immunized pigs. Collectively, the data demonstrate that H3-CON.1 immunogen elicits broadly reactive T cell responses in pigs.

3.5. H3-CON.1 immunogen conferred better protection against a heterologous virus infection

At day 44 after the first vaccination, all pigs were challenged by an intratracheal inoculation with the H3N2 isolate MN5993. This IAV-S isolate was selected for challenge infection because its genetic distance to H3-CON.1 (3.36%) is equivalent to the median distance between H3-CON.1 and the wild-type IAV-S isolates (3.58%, Fig. 1B). Nasal swabs were collected daily after infection for evaluation of viral shedding. Pigs vaccinated with H3-CON.1 immunogen shed significantly lower levels of virus in their nasal secretions than those immunized with H3-TX98 immunogen (Fig. 5). However, none of them showed any clinical signs after challenge infection, including those in DMEM group which were seronegative at the time of challenge infection. Likewise, no significant lung lesion was observed. By using *in situ* hybridization, however, we were able to detect viral RNA in the bronchiolar epithelium cells of all pigs, clearly indicating that they were infected with the challenge virus (Fig. 5B).

4. Discussion

In this study, we sought to evaluate the cross-protective immunity elicited by a consensus HA antigen of IAV-S. We opted to use the baculovirus expression system (BVES) to express the HA immunogens. One major advantage of the BVES is that the expressed proteins undergo post-translation modifications that are necessary to maintain their immunogenicity [reviewed in (van Oers et al., 2015v)]. Additionally, the insect cells can grow to high cell density in suspension culture in serum-free medium; thus, making it easy for scale-up production. Of note, the BVES has been used to produce trivalent seasonal human influenza vaccine (Treanor et al., 2011). We observed that H3-CON.1 elicits broader levels of virus-neutralizing antibodies and virus-specific IFN-γ SCs than H3-TX98, a naturally occurring immunogen. Our results further support the notion that consensus immunogens can be employed to expand the antigenic-coverage for highly variable RNA viruses.

We observed in this study that virus-neutralizing antibody titers seem to correlate with frequencies of IFN-γ SCs. For instance, pigs immunized with H3-CON.1 immunogen displayed high neutralizing antibody titers against MN5993, TX98, and MI9002 and they also had high frequencies of IFN-γ SCs against these three viral isolates. On the other hand, the H3-CON.1- immunized pigs had lower virus neutralizing antibody titer against CO3748 and they also displayed lower frequencies of IFN-γ SCs against this viral isolate. However, this observation needs to be interpreted with caution since we only measured neutralizing antibody titers and frequencies of IFN-γ SCs against a small number of AIV-S isolates.

Ideally, we need to conduct multiple immunization/challenge experiments against multiple wild-type H3N2 IAV-S isolates to comprehensively evaluate the protective efficacy of the H3-CON.1 immunogen. However, this will require a lot of time and resources. Thus, in this study, we conduct one challenge study against a representative wild-

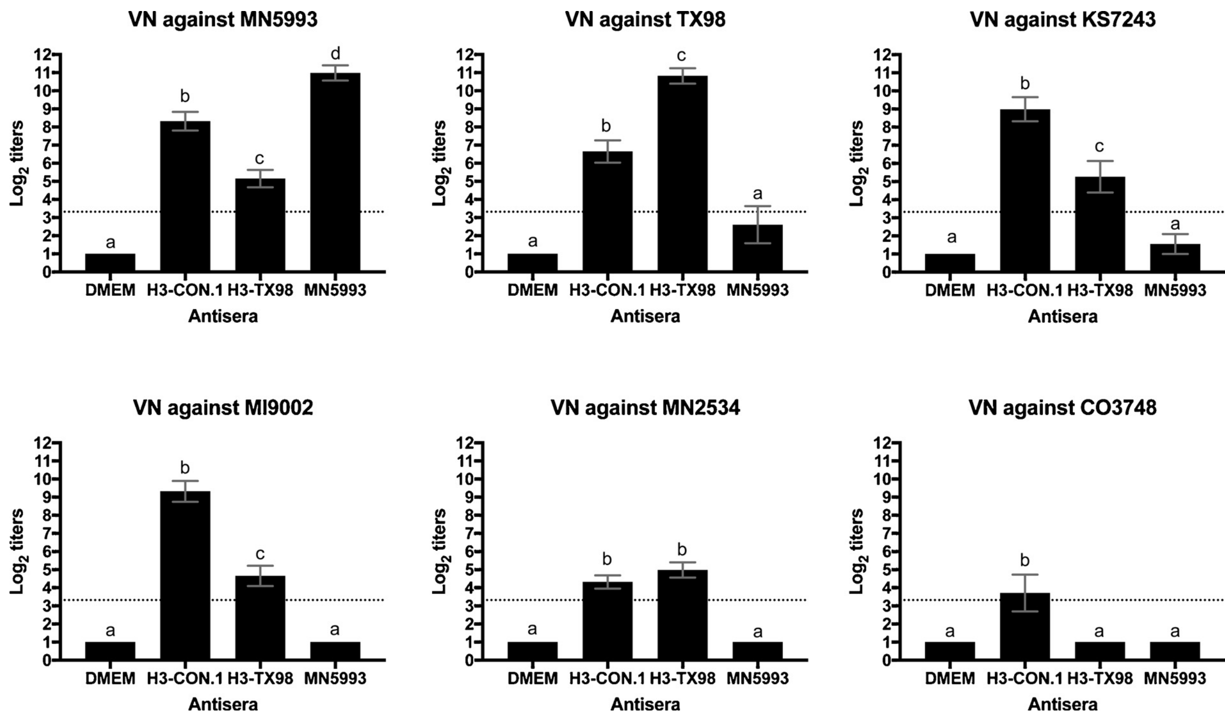

Fig. 3. Cross neutralizing antibody responses. Neutralizing activities of antisera collected at day 35 after the first vaccination were measured against six different IAV-S H3N2 isolates. Neutralization titers are expressed as means and standard error of means (SEMs) calculated from six pigs in each treatment group. The horizontal dotted line at 3.3 log2 indicates the cut-off of the assay. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. Treatments with the different superscripts are statistically different ($p < 0.05$).

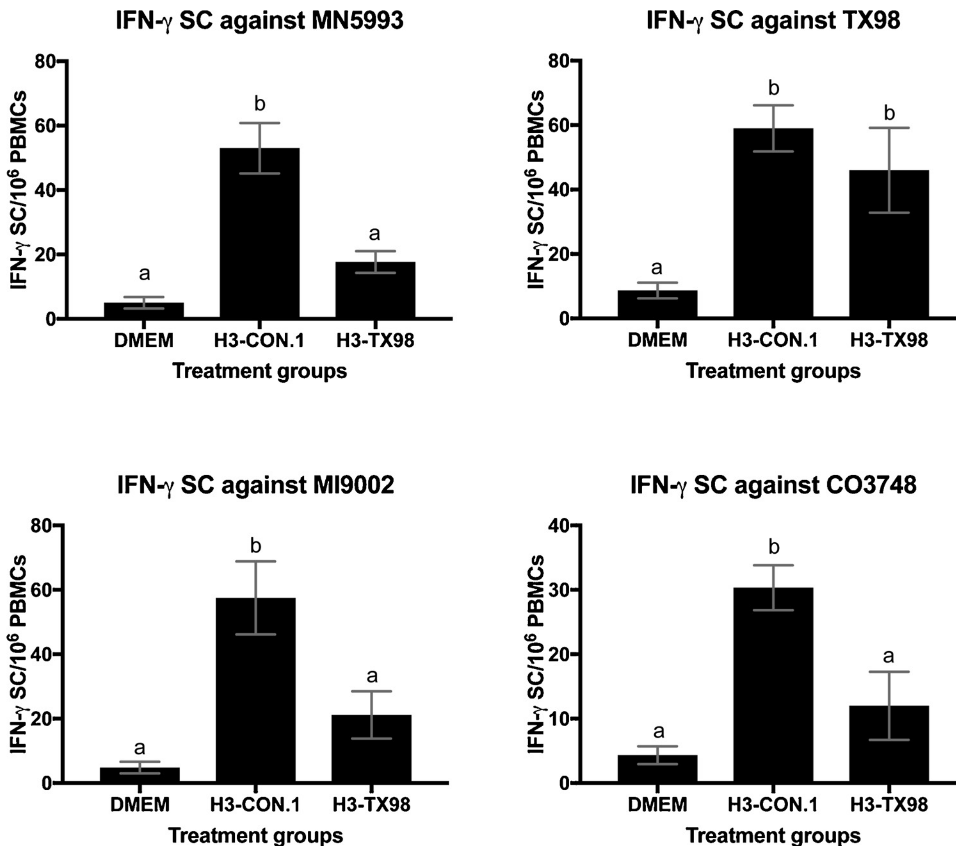

Fig. 4. Cross-reactive T cell responses. PBMCs were collected at day 44 after the first vaccination. Frequencies of IFN-γ secreting cells against four selected H3N2 isolates were measured by IFN-γ ELISPOT. Date presented in this figure are means and SEMs calculated from six pigs in each treatment group. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. Treatments with the different superscripts are statistically different ($p < 0.05$).

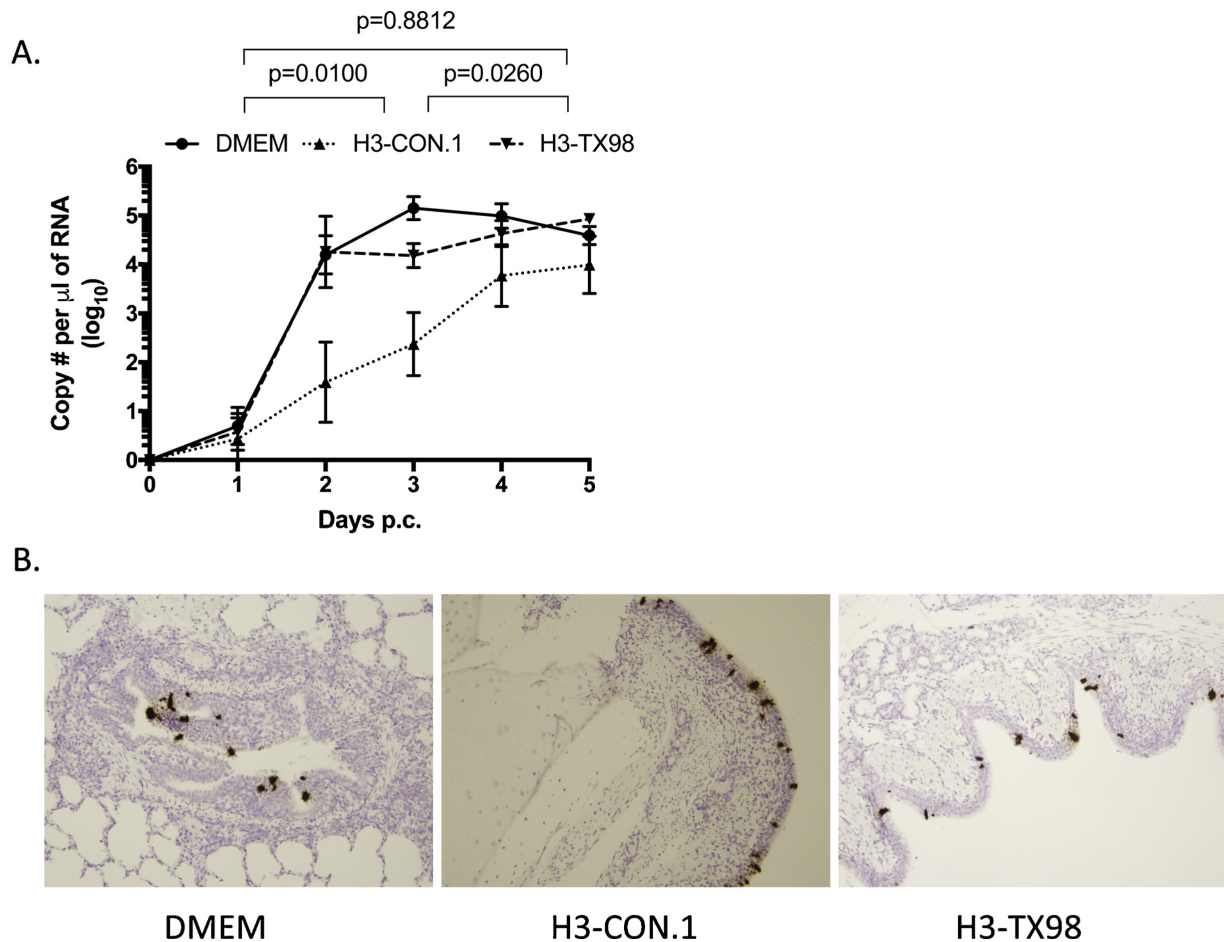

Fig. 5. Protection against challenge infection. At day 44 after the first vaccination, pigs were challenged by an intratracheal inoculation with the H3N2 isolate MN5993. (A) Nasal swabs were collected daily until day 5 post-challenge. Viral RNA was quantified by using a validated real-time RT-PCR kit. Data are expressed as mean and SEM calculated from six pigs in each treatment group. Data were analyzed by repeated-measures two-way ANOVA, followed by Tukey's multiple comparisons test. P values represent statistical difference in the levels of viral shedding among treatment groups. (B) RNA *in situ* hybridization was performed on formalin-fixed, paraffin-embedded (FFPE) lung tissues by using the RNAscope® assay (ACD, CA). Positive hybridization signal for influenza NP RNA (dark brown) is observed in bronchiolar epithelial cells, 200×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

type H3N2 isolate (namely MN5993) selected from the groups of 1,112 wild-type H3N2 isolates used to construct the H3-CON.1 sequence. The challenge isolate was selected in the way that its genetic distance to H3-CON.1 is equivalent to the median distance between H3-CON.1 and the contemporary H3N2 IAV-S isolates (3.58%, Fig. 1B). Unfortunately, the MN5993 isolate used for challenge infection does not have enough level of virulence in pigs, as it did not cause any significant clinical signs or lung lesions to the pigs, including those in the non-immunization control group. This lack of pathogenicity does not allow us to fully evaluate the protective efficacy of our candidate vaccine. Therefore, we use viral shedding as a main parameter for evaluation of protection. We observed significant lower levels of vial shedding in the H3-CON.1 group than in the H3-TX98 group, especially during the early time-points after challenge infection. Thus, under the conditions of this study, the H3-CON.1 immunogen seems to provide better protection against challenge infection than the naturally occurring H3-TX98 immunogen.

Pigs vaccinated with a WIV vaccine followed by a challenge infection with a mismatched IAV-S strain often develop VAERD (Gauger et al., 2011; Vincent et al., 2008). Moreover, VAERD was observed in pigs vaccinated with a subunit HA vaccine produced in BVES followed by a challenge infection with a mismatched IAV-S (Rajao et al., 2014b). In this study, we did not observe VAERD in pigs that were vaccinated with H3-TX98 immunogen and subsequently challenged with MN5993, whose HA sequence shares 89.5% identity with the H3-TX98 sequence. It was reported that adjuvants used for vaccine formulation impacts VAERD outcomes (Souza et al., 2018). Since the adjuvant used in our study is the same as the one used by Rajao and co-workers when they defined the VAERD phenomenon in SIV vaccination (Rajao et al., 2014b), we can rule out the contribution of adjuvant to this discrepancy. In that previous study, the HA subunit vaccine was derived from the H1N1 strain A/California/04/2009 and the challenge virus was δ1-H1N2 A/Swine/Minnesota/02011/2008 (Rajao et al., 2014b). The HA genes of these two strains share only 77.7% identify. Perhaps, the pigs vaccinated with H3-TX98 did not show VAERD following challenge with MN5993 because the difference between them is not sufficient for VAERD to occur. It should be noted that it remains unknown how much difference between the vaccine immunogens and the challenge virus in order for VAERD to occur.

Currently, two HA subtypes of IAV-S (H1 and H3) are co-circulating in the swine herds (Walia et al., 2018). Therefore, vaccines against swine IAV-S need to include immunogens for both H1 and H3 subtypes. Our data provide the evidence that the consensus H3 immunogen elicits a broad spectrum of protective immunity. The most logical next steps would be to design and evaluate the protective immunity elicited by a consensus H1 immunogen in pigs. Especially, it would be interesting to evaluate the protective efficacy of a polyvalent vaccine containing both H1 and H3 consensus immunogens.

Author contributions

Conceptualization, H.V.; Methodology, H.S. and H.V.; Validation, H.S. and H.V.; Formal Analysis, H.S., and H.V.; Investigation, H.S., J.S., D.S., S.S. and H.V.; Data Curation, H.V.; Writing – Original Draft Preparation, H.S.; Writing – Review & Editing, H.V., and D.S.; Visualization, H.S., J.S. and H.V.; Supervision, H.V.; Project Administration, H.V.; Funding Acquisition, H.V."

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108451.

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