Intranasal vaccination with replication defective adenovirus-5 encoding influenza hemagglutinin elicits protective immunity to homologous challenge and partial protection to heterologous challenge in pigs.

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Running Title: Intranasal Ad5-HA reduces influenza viral load

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Abstract

Influenza A virus (IAV) is widely circulating in the swine population and causes significant economic loss. To combat IAV infection the swine industry utilizes adjuvanted whole inactivated virus (WIV) vaccines using a prime-boost strategy. These vaccines can provide sterilizing immunity towards homologous virus but often have limited efficacy against a heterologous infection. There is a need for vaccine platforms that induce mucosal and cell-mediated immunity cross-reactive to heterologous virus that can be produced in a short time frame. Non-replicating adenovirus 5 vector (Ad5) vaccines are one option, as they can be rapidly produced and given intranasally to induce local immunity. Thus, we compared the immunogenicity and efficacy of a single intranasal dose of an Ad5-vectored hemagglutinin (HA) vaccine to traditional intramuscular administration of WIV vaccine. Ad5-HA vaccination induced a mucosal IgA response towards homologous IAV and primed an antigen-specific IFN-γ response against both challenge viruses. The Ad5-HA vaccine provided protective immunity to homologous challenge and partial protection against heterologous challenge, unlike the WIV vaccine. Nasal shedding was significantly reduced and virus was cleared from the lung by day 5 post-infection following heterologous challenge of Ad5-HA vaccinated pigs. However, the WIV vaccinated pigs displayed vaccine associated enhanced respiratory disease (VAERD) following heterologous challenge, characterized by enhanced macroscopic lung lesions. This study demonstrates that a single intranasal vaccination with an Ad5-HA construct can provide complete protection to homologous challenge and partial protection to heterologous challenge, as opposed to VAERD, which can occur with adjuvanted WIV vaccine.
1. Introduction

Influenza A virus (IAV) infection in swine can lead to significant economic loss through decreased weight gain and increased time to market. IAV also increases the susceptibility to secondary bacterial infection leading to pneumonia and in severe cases death (8, 16, 18). Due to the high rate of antigenic drift and antigenic shift, there are multiple antigenically diverse strains of IAV currently circulating throughout the swine population (33, 35). Furthermore, the introductions of human and avian IAV into the swine population continue to increase the number of distinct circulating IAV strains (2, 11, 20, 35). The ever-changing diversity of circulating IAV is problematic for vaccine mediated protection because the vaccine has to be repeatedly updated to provide sufficient protection to circulating strains.

Vaccines currently used in the swine industry for the control of IAV are whole inactivated virus (WIV) preparations. WIV vaccines used are typically multivalent mixtures prepared with an adjuvant and administered intramuscularly using a prime-boost vaccination strategy. Adjuvanted, WIV vaccines can elicit sterilizing immunity against homologous virus (14, 30, 31). However, WIV vaccines are often ineffective at protecting against heterologous strains beyond a reduction in clinical presentation of disease (1, 6, 17, 24, 31). Moreover, recent evidence indicates that WIV vaccines may, in some circumstances, result in the development of vaccine associated enhanced respiratory disease (VAERD) when a vaccinated pig is infected with an antigenically divergent virus (6, 14, 31). VAERD is characterized by the presence of cross-reactive, non-neutralizing antibodies to heterologous virus and enhanced lung pathology in WIV vaccinated pigs following heterologous infection compared to non-vaccinated pigs (6, 14,
Thus, there is a need for alternative vaccine platforms that protect against heterologous infection without resulting in VAERD. Aside from the possible enhancement of disease, WIV vaccines can also be plagued by relatively long production times (40).

The large amount of time needed to license, approve and produce a WIV vaccine for swine severely hinders its use during a novel IAV outbreak. An alternative platform to WIV that has quick production potential is a replication defective human adenovirus 5 vector (Ad5) encoding IAV genes. The Ad5 virus is a complete virion that was made replication defective by the removal of two segments of the Ad5 viral genome (10). Deletion of two Ad5 genomic sequences permits the insertion of an IAV antigen sequence for recombinant expression (reviewed by (29)). A recent report indicates that a novel Ad5 construct can be created in less than 21 days once an antigen sequence is identified (25). The Ad5 construct can be rapidly replicated using a small bioreactor system, with viral titers of $10^{10}$ to $10^{11}$ plaque forming units (PFU) per ml in as little as 3 days (supplemental data). Considering traditional WIV vaccine production for humans has been reported as 5 to 6 months and is at least as long for fully licensed commercial veterinary vaccines, the Ad5 construct is considerably faster (40). In addition to fast production potential, the Ad5 virus makes an excellent intranasal vaccine platform due to its natural predisposition for respiratory tract infection (28). The Ad5 platform allows for the delivery and presentation of IAV antigen to the site of natural infection and because Ad5 is an infectious particle, it initiates local immune activation in the absence of an adjuvant (28). Subcutaneous and intramuscular vaccinations with Ad5 constructs containing the hemagglutinin (HA) of IAV (Ad5-HA) have been validated as effective
means of eliciting protection against IAV in mice, poultry and swine (4, 25, 37-39). The advantages of rapid production time and the option of intranasal administration make the Ad5-HA platform an attractive alternative to the currently used vaccines in swine. The Ad5-HA as a vaccine for IAV was recently improved by Steitz et al. (25), in which codon optimized IAV HA was incorporated into the Ad5 vector to improve protein expression, a change that increased immunogenicity. Thus, we sought to evaluate the efficacy of a single intranasal vaccination with an Ad5 vector encoding codon optimized HA against homologous and heterologous challenge in swine. We report herein vaccination primes a cross-reactive antigen-specific immune response, provides complete protection to homologous challenge, and limits duration of viral shedding and load following heterologous challenge.

2. Material and methods

2.1 Animals and Vaccines

Forty-eight, three-week-old crossbred pigs were procured from a high-health status herd known to be free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV). The pigs were randomly distributed into 6 treatment groups of 8 pigs each (Table 1). Pigs were housed in BSL2 containment and animal care was in compliance with the institutional animal care and use committee (IACUC) of the National Animal Disease Center (NADC). Replication defective adenovirus-5 containing the codon-optimized HA from A/CA/04/09 pH1N1 and the empty vector (referred to as Ad5-HA and Ad5-empty, respectively) were generated as previously described (25). The E1 and E3 gene segments of the adenovirus genome have been removed, rendering it replication defective. Sixteen pigs were vaccinated with 2 ml containing $10^{10}$ plaque forming units
(PFU) of Ad5-HA and 16 pigs received Ad5-empty at the same concentration in phosphate buffered saline (PBS) via the intranasal route at 5 weeks of age (Table 1). One group of 8 pigs was vaccinated intramuscularly at 5 weeks of age with 128 HA units of ultraviolet-inactivated A/CA/04/09 pH1N1 (CA09; human isolate) mixed with an oil-in-water adjuvant (Emulsigen-D, MVP Technologies, Omaha, NE) at a v:v ratio of 4:1 virus to adjuvant (referred to as kaCA) as previously described. The same 8 pigs were boosted 21 days later with the same preparation. Sera and nasal washes were collected every 7 days from all pigs beginning on the day of vaccination (day 0) for the measurement of antigen specific antibody using a previously described method. Blood was collected and peripheral blood mononuclear cells (PBMC) were isolated for IFN-γ ELIspot assay on days 21 and 42 post vaccination (dpv). Prior to challenge one pig in the Ad5-HA group to be challenged with CA09 died from causes unrelated to the experiment (Table 1). At 42 dpv, pigs were challenged by intranasal inoculation with Madin Darby canine kidney cell (MDCK) propagated CA09 or A/swine/MN/02011/08 (H1N2, MN08) at a final volume of 2 ml per pig. Back titrations of CA09 and MN08 challenge viruses were $10^{4.5}$ and $10^{5.5}$ TCID$_{50}$ per ml, respectively. Nasal swabs were collected to evaluate viral shedding at 0, 1, 3 and 5 days post-infection (dpi) as previously described (6). On dpi 5 all pigs were humanely euthanized with a lethal dose of pentobarbital (Fort Dodge Animal Health, Fort Dodge, IA). Postmortem sample collection included serum, nasal swab, nasal wash, bronchoalveolar lavage fluid (BALF), lung, and trachea. Collection of BALF consisted of lavaging with 50 ml of minimal essential media (MEM) as previously described (31).

2.2 Microbiology
Prior to the start of the study all pigs were screened for antibody against IAV nucleoprotein (NP) to verify a lack of previous exposure and immunity (Influenza A Ab Test, IDEXX, Westbrook, MA). BALF collected at 5 dpi were screened for aerobic bacteria by plating 100 µl of lavage on blood agar and Casmin (NAD enriched) agar plates and incubating at 37 °C for 48 h.

2.3 Antibody detection and characterization assays

For use in the hemagglutination inhibition (HI) assay, sera were heat inactivated at 56 °C for 30 min, then treated with a 20% kaolin (Sigma–Aldrich, St. Louis, MO) phosphate-buffered (PBS) suspension and absorbed with 0.5% turkey red blood cells (RBCs) to remove nonspecific hemagglutination inhibitors and natural serum agglutinins.

The MN08 and CA09 viruses were used as antigen in the HI assays following standard techniques with turkey RBCs (41). Reciprocal titers from HI assays were divided by 10 and log₂ transformed, analyzed, and reported as the geometric mean. Total IgG and IgA antibody against MN08 and CA09 were detected by enzyme-linked immunosorbent assay (ELISA) using whole virus preparations diluted in carbonate bicarbonate buffer to a hemagglutinin (HA) concentration of 100 HAU per 50 µl and are referred to as Isotype ELISAs. Isotype ELISAs were performed on serum, nasal wash, and BALF as previously described (15, 31) with some modifications. Briefly, 100 µl of virus was coated onto Nunc Immuno 96-well plates (Nunc, Rochester, NY) and incubated at room temperature overnight. Sera were heat inactivated at 56 °C, while nasal wash and BALF were diluted in a 10 mM dithiothreitol / PBS buffer at a 1:1 ratio for mucus dissociation and incubated at 37 °C for 1 h. All samples were assayed in triplicate. The mean optical density (OD) of triplicate wells was calculated and antibody titers were reported as
average OD for all pigs in each respective group.

2.4 IFN-γELISPOT assay

On days 21 and 42 post vaccination, whole blood was collected in sodium citrate CPT tubes (BD Vacutainer, Franklin Lakes, NJ) and PBMC were separated according to manufacturer’s recommendations. Total PBMC were processed as previously described (7), enumerated and adjusted to 5 x 10^5 cells per 0.1 ml. The IFN-γ ELISpot assay was performed according to manufacturer’s recommendations (Porcine IFN-γ ELISpot, R&D Systems, Minneapolis, MN). Wells were seeded with 0.1 ml of PBMC suspension and stimulated with 50 µl of 5 X 10^6 TCID_{50} /ml live CA09 or MN08 virus, 5 µg/ml of concanavalin A, or MDCK sham media. Final volume was brought to 0.25 ml. Following an 18 hour incubation in a 37 °C humidified 5% CO₂ incubator the assay was completed according to manufacturer’s recommendations. Plates were scanned and analyzed with UV-5 CTL-ImmunoSpot instrumentation and software (Cellular Technology Ltd, Shaker Heights, OH). The mean count of triplicate wells for each treatment for each pig was determined and used to calculate the mean for each vaccine group.

2.5 Pathology

At necropsy, lungs were removed and evaluated for the percentage of the lung affected by purple-red consolidation typical of IAV infection in swine. The percent of the surface area affected with pneumonia was visually estimated for each lung lobe, and total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume (9). Tissue samples from the trachea and right middle lung lobe were fixed in 10% buffered formalin for microscopic examination. Tissues were...
processed by routine histopathologic procedures and slides stained with hematoxylin and
eosin. Microscopic lesions were evaluated by a board certified veterinary pathologist
blinded to treatment groups. Scoring of lesions was based on parameters adapted from
Gauger et al. (6). Individual scores were assigned to four parameters: bronchial and
bronchiolar epithelial changes, bronchitis/bronchiolitis, peribronchiolar lymphocytic
cuffing, edema and interstitial pneumonia. Scores were based on percentage of airways
with lesions that included epithelial changes and inflammation on a 5-point scale: 0: No
lesions, 1: 0-25%, 2: 26 to 50%, 3: 51-75%, 4: greater than 75% of airways affected with
airway epithelial damage and inflammation. Peribronchiolar cuffing by lymphocytes was
graded on a 4-point scale: 0: None, 1: Mild, loosely formed cuff of lymphocytes, 2:
Moderate, well-formed cuffs of lymphocytes, and 3: Prominent, thick well-formed cuffs.
Degree of edema and fibrin exudation was scored on the following 4-point scale: 0: none,
1: focal small area of edema in section (less than 15% of section), 2: 15-49% of section
including interlobular and/or pleural edema and alveolar lumina and septa, 3: greater than
50% of section including interlobular and/or pleural edema and most alveolar lumina and
septa. Interstitial pneumonia was graded on 5-point scale: 0: No lesions, 1: Mild, focal
to multifocal interstitial pneumonia, 2: Moderate, locally extensive to multifocal
interstitial pneumonia, 3: Moderate, multifocal to coalescing interstitial pneumonia, and
4: Severe, coalescing to diffuse interstitial pneumonia. Trachea sections were scored
similar to the bronchi and bronchioles and were based on epithelial changes and degree of
inflammation. Tracheal epithelial changes were graded on a 5-point scale: 0: No lesions,
1: Early epithelial changes characterized by focal to multifocal loss of cilia and epithelial
degenerative changes, 2: Mild epithelial flattening with loss of cilia and goblet cells, 3:
Moderate epithelial flattening with decreased thickness of respiratory epithelium, loss of cilia and goblet cells, 4: Flattened epithelium with areas of mucosa covered by a single layer of cuboidal epithelium and epithelial loss (necrosis). Degree of tracheitis was graded on a simple 4-point scale: 0: None, 1: Mild, 2: Moderate, and 3: Severe. IAV antigen was detected in lung tissues using a previously described immunohistochemical (IHC) method with modifications (36). Tissue sections were deparaaffinized and hydrated in distilled water. Slides were quenched in 3% hydrogen peroxide for 10 min, rinsed three times in de-ionized water and treated in 0.05% protease for 2 min. Slides were then rinsed three times in de-ionized water and once in Tris-buffered saline (TBS).

Monoclonal antibody (MAb) HB65 (ATCC, Manassas, VA), specific for the nucleoprotein (NP) of IAV, was applied at 1:100 dilution and slides were incubated at room temperature for 1 h. Bound MAbs were stained with peroxidase-labeled anti-mouse IgG followed by chromogen using the DAKO LSAB2-HRP Detection System (DAKO, Carpinteria, CA) according to the manufacturer’s instructions. The slides were rinsed in deionized water and counterstained with Gill’s hematoxylin. Antigen detection was assessed using two scores: 1) airway epithelial labeling and 2) alveolar/interstitial labeling. In airway epithelium a 5-point scale was used: 0: None, 1: Few cells with positive labeling, 2: Mild scattered labeling, 3: Moderate scattered labeling, 4: Abundant scattered labeling (greater than 50% epithelium positive in affected airways). In the interstitium/alveoli, a 4-point scale was used: 0: None, 1: Minimal focal signals, 2: Mild multifocal signals, 3: Abundant signals.

**2.6 Virus isolation from nasal swabs and bronchoalveolar lavage fluid (BALF)**

BALF was collected at dpi 5 and stored at -80 °C. Nasal swabs collected at dpi 0, 1, 3
and 5 were stored at -80 °C and subsequently thawed and vortexed for 15 s, followed by centrifugation for 10 min at 640 X g. Nasal swab supernatants were passed through 0.45 µm syringe filters to remove bacterial contaminants. Ten-fold serial dilutions in serum-free MEM supplemented with TPCK-trypsin (1 µg/ml; Sigma, St. Louis, MO) and antibiotics were made for each BALF and nasal swab filtrate sample. One hundred microliters of each dilution was plated in triplicate onto confluent MDCK cells in 96-well plates. After 72 hours of incubation, MDCK monolayers were fixed with 4% phosphate buffered formalin for 30 minutes. Fixed cells were stained using a previously described immunocytochemistry technique that utilizes an anti-IAV nucleoprotein monoclonal antibody (HB65). Positive staining was used for the determination of virus titer. A final TCID$_{50}$ per milliliter titer was calculated for each sample using the method of Reed and Muench (23).

2.7 Statistical Analyses

Log$_2$-transformed HI titers and log$_{10}$-transformed NS viral titers data were analyzed using a mixed linear model for repeated measures (Proc Mixed, SAS for Windows, Version 9.2, SAS Institute Inc., Cary, NC, USA). Covariance structures within pigs across time were tested and modeled using the REPEATED statement to determine the optimal covariance structure. Linear combinations of the least squares means estimates were used in a priori contrasts after testing for a significant ($P < 0.05$) treatment group effect. Comparisons were made between each group at each time point using a 5% level of significance ($P < 0.05$) to assess statistical differences. The endpoint data for microscopic tracheal and lung lesions, macroscopic lung lesions, log$_{10}$-transformed BALF viral titers, and immunohistochemistry (IHC) staining of the lung were analyzed by analysis of
3. Results

3.1 Microbiological Assays

All sera collected from pigs prior to the start of the study were negative for IAV antibody as evaluated by the nucleoprotein (NP) antibody ELISA. At the completion of the study, *Arcanobacterium pyogenes* was isolated from the BALF of 1 pig in the Ad5-empty/NC group and 1 pig in the Ad5-empty/MN08 group. *Streptococcus* was isolated from the BALF from one pig in the Ad5-HA/MN08 group.

3.2 IAV-specific antibody in pre-challenge nasal wash and sera.

Sera from kaCA vaccinated pigs contained HI antibodies to CA09 virus; however, HI antibody cross-reactive to MN08 virus was not detected in the sera of kaCA vaccinated pigs. Sera from Ad5-HA vaccinated pigs did not contain HI antibody to CA09 or MN08 virus (data not shown).

Immunoglobulin isotype-specific ELISAs were used to evaluate IAV-specific IgA and IgG in the sera and nasal wash (NW). The Ad5-empty vaccine did not induce IgA or IgG titers against MN08 or CA09 at any time point pre-challenge in the NW or sera. However, CA09-specific IgA was detected in the NW from Ad5-HA vaccinated pigs, only on 14 dpv. IgG to heterologous MN08 virus was not detected in the NW or sera collected at any time point post-vaccination from Ad5-HA vaccinated pigs. Likewise, IgA antibody to MN08 antigen was not detected in pre-challenge sera from the Ad5-HA vaccinated pigs. Conversely, the kaCA vaccinated pigs had detectable IgG antibody to
CA09 and MN08 in pre-challenge sera similar to what has been previously described (6).

A summary of antibody results is described in Table 2.

3.3 Cell mediated immunity

All immunized pigs exhibited an antigen-specific IFN-γ recall response to both homologous CA09 and heterologous MN08 antigen although responses to homologous antigen were significantly increased over heterologous antigen (69.8 ± 8.8 vs 28.5 ± 6.5 respectively at 21 dpv; Fig. 1A and 1B). In Ad5-HA vaccinated pigs, the number of antigen-specific IFN-γ SC decreased over time, as numbers were greater at 21 dpv compared to 42 dpv for both viral antigens. The numbers of CA09-specific IFN-γ SC were 69.8 ± 8.8 at 21 dpv compared to 26.0 ± 8.8 at 42 dpv in Ad5-HA vaccinated pigs.

The kaCA vaccine primed an antigen-specific IFN-γ response to both CA09 and MN08 viruses as well. The average number of antigen-specific IFN-γ SC detected in the kaCA vaccination group was greater than that detected following Ad5-HA vaccination, which was not surprising given that kaCA vaccinated pigs were exposed to not only HA, but additional IAV proteins as well. Although the kaCA group received a boost at 21 dpv, the numbers of IFN-γ SC cells detected on dpv 42 were at or below levels detected at 21 dpv, which was prior to the boost (Fig. 1C).

3.4 Macroscopic and microscopic lung lesions

Macroscopic and microscopic lung lesion scores in the Ad5-HA/CA09 group were indistinguishable from scores in the Ad5-HA/NC group, and significantly lower than scores in the Ad5-empty/CA09 group (Fig. 2 and 3, respectively). The Ad5-HA/MN08 group had macroscopic and microscopic lesion scores that were similar to the Ad5-empty/MN08 group, but scores in the Ad5-HA/MN08 group were significantly lower.
than the kaCA/MN08 group (Fig. 2). The kaCA/MN08 group had the highest macroscopic and microscopic lung lesion scores across all vaccination groups (Fig. 2 and 3). Microscopic tracheal lesions were less severe in the Ad5-HA/MN08 group compared to either kaCA/MN08 or Ad5-empty/MN08 (1.8 ± 0.1 vs 2.9 ± 0.2 and 2.4 ± 0.2 respectively). The Ad5-HA vaccinated pigs challenged with MN08 or CA09 had lower lung IAV antigen scores compared to the Ad5-empty or kaCA group, which is suggestive of less viral antigen (Fig. 3C). Furthermore, there was a relationship between decreased virus and lung lesions in Ad5-HA/MN08 pigs, in that lung viral loads were reduced on dpi 5 but macroscopic and microscopic lung lesions were not significantly different than those observed in the Ad5-empty/MN08 group. This is in contrast to the Ad5-HA/CA09 pigs, which had a reduction in virus and a reduction in lung lesions when compared to Ad5-empty/CA09 group.

3.5 Virus titers in BALF and nasal swabs following challenge

Virus was not isolated from any of the nasal swab (NS) samples collected from the Ad5-HA/CA09 group, but was isolated from NS collected from Ad5-empty/CA09 group (Table 3), indicating protection from homologous challenge. Conversely, virus was detected in the NS collected on dpi 1 and dpi 3 from pigs in the MN08 challenge group, regardless of vaccination. NS viral titers reached the highest detected level at dpi 3 and remained elevated until dpi 5 for Ad5-empty group, regardless of the IAV challenge strain. NS samples collected on dpi 5 from the kaCA/MN08 group had lower viral titers compared to dpi 3, equal to titers on dpi 1, while Ad5-HA/MN08 NS virus titers on dpi 5 were reduced to levels less than the dpi 1 titers. On dpi 5, virus titers in BALF were 4.9 ± 0.2 TCID₅₀ (log₁₀) for the Ad5-empty/CA09 group and 4.7 ± 0.3 TCID₅₀ (log₁₀) for Ad5-
empty/MN08 group. Conversely, virus was not detected in the BALF of Ad5-HA vaccinated pigs following challenge with homologous or heterologous virus. Virus was not isolated from the NS or BALF collected from the empty/non-challenged controls at any time in the study. Results are summarized in Table 3.

3.6 Humoral response to challenge virus in BALF at 5 dpi.

An isotype-specific ELISA, using whole virus as antigen, was utilized to quantify IAV-specific IgG and IgA antibody in the BALF 5 days following challenge. The pigs vaccinated with Ad5-HA had detectable BALF IgA antibody specific to CA09 regardless of the challenge virus (Fig. 4A). MN08-specific IgA was also detected in the BALF of the Ad5-HA group challenged with MN08 (Fig. 4A), although they were vaccinated with HA from CA09. Anti-CA09 IgG antibodies were present in the BALF of Ad5-HA vaccinated pigs challenged with either CA09 or MN08 (Fig. 4B). However, MN08-specific IgG was only present in the BALF of Ad5-HA group challenged with MN08 (Fig. 4B), while anti-MN08 IgG was not present in Ad5-HA group challenged with CA09 group. The BALF from the kaCA/MN08 pigs had detectable IgG and IgA specific to both CA09 and MN08 viruses (data not shown).

4. Discussion

The commercial IAV vaccines currently available for use in swine are based on the WIV platform. Vaccination with WIV can elicit sterilizing immunity against a homologous strain, primarily through production of antibody directed towards the receptor binding domain of the immuno-dominant surface glycoprotein HA (3). Due to the highly variable nature of HA, the WIV vaccine provides limited protection against heterologous viruses with demonstrated antigenic drift. Furthermore, recent reports
suggest that WIV vaccines can result in VAERD when the vaccine strain and infecting
virus share some antigenic similarities, but vaccination does not elicit neutralizing
antibodies to the infecting virus (6, 14, 31). With the high rate of antigenic drift observed
in IAV, and the diversity of IAV currently circulating in the U.S. swine population,
heterologous mismatch is likely to occur between vaccine and infecting strain in the field.
The HA in the MN08 virus belongs to the human-like δ-cluster of HA genes, which was
introduced into the swine population from human seasonal IAV whereas the CA09 HA is
a drift variant of the classical swine lineage HA, most closely related to the γ-cluster
viruses (12, 32, 34). Protein sequence homology between the CA09 HA and the MN08
HA is approximately 77%. Therefore, a vaccine platform that provides protection to a
broad range of IAV antigenic types, but does not result in VAERD, is highly desirable.
We report herein that a single intranasal vaccination with Ad5-HA induces full protection
against homologous challenge and partial protection against a heterologous challenge by
limiting the duration and amount of viral shedding. In addition, our data indicates that
vaccination with Ad5-HA does not result in VAERD upon heterologous challenge when
using the same vaccine strain-challenge strain combination that induced VAERD with the
WIV. Lastly, Ad5-HA vaccination primed for an immune response that resulted in more
rapid production of mucosal antibody cross-reactive to heterologous virus, which likely
played a role in protection.
Heterologous MN08 virus was isolated from the nose of Ad5-HA/MN08 pigs on
dpi 1 and 3, thus vaccination did not completely prevent heterologous infection, while
reduced nasal titers on dpi 5 indicate that prior Ad5-HA vaccination increased the rate of
heterologous viral clearance. While the mechanism of heterologous virus clearance is not
completely clear, establishment of an infection prior to clearance provides evidence that cell-mediated immune mechanisms likely played an important role. The role of cell-mediated immune responses may be at the level of killing virally infected cells and/or providing more rapid help to naïve B cells. In addition, it’s possible that cross-reactive B cell clones already present in the respiratory tract quickly expand following infection and provide some level of protection.

Conserved regions within CA09 and MN08 HA likely contain T cell epitopes that would be recognized upon heterologous challenge. In the current study we assessed the quantity of antigen specific IFN-γ SC as a measure of cell-mediated immunity (CMI) induced by Ad5-HA intranasal vaccination. Pigs vaccinated with the Ad5-HA were only exposed to the CA09 HA antigen, and therefore, although whole virus was used as recall antigen in the IFN-γ ELISpot, responses were likely specific only to the HA of the virus used as recall antigen. Following Ad5-HA vaccination, PBMCs were primed to produce IFN-γ in response to both CA09 and MN08 virus (Fig. 1A & 1B). However, HI-specific antibody was never detected in the blood of Ad5-HA vaccinated pigs, regardless of virus used in the assay (CA09 or MN08). Ad5-HA vaccination did provide protection upon heterologous challenge, evidenced by reduced NS viral titers at 5 dpi and clearance of viable virus from the BALF at 5 dpi. Thus, our data indicates that the priming of CMI towards HA likely contributed to the clearance of heterologous challenge virus. The ELISpot assay did not discern if the IFN-γ SC were CD4+, CD8+ or CD4+/CD8+ double-positive T cells (a population of memory T cells in pigs (42)) and therefore it is difficult to pinpoint if more rapid viral clearance is the result of increased activity of cytotoxic T lymphocytes (CTL) or T helper cells. Previous research in mice indicates that CD4+ Th1
cells alone can decrease the severity of IAV infection (26). When primed CD4+ Th1 cells were passively transferred to naive mice that were subsequently infected with IAV, the infection was quickly cleared (26). Thus, the enhanced clearance of virus in the Ad5-HA/MN08 pigs may be due to activation of CD4+ Th1 cells that were primed towards a conserved HA epitope.

Further evidence suggesting that a primed CMI provides protection to heterologous infection is that memory CD4+ T cells have been shown to be more adept at providing B-cell help when compared to naïve CD4+ T cells, although the exact mechanism by which this occurs has not been clearly defined (reviewed in (27)).

Antibody levels in the lung lavage of pigs in the current study provide additional support to this finding. Antibody detected in the BALF following Ad5-HA vaccination would be expected to react to CA09 HA, which was the case regardless of the challenge strain (Fig. 4). However, MN08-specific antibody was detected in the BALF only following MN08 challenge, but not CA09 challenge (Fig. 4C & 4D). This data suggests that Ad5-HA vaccination alone (CA09 HA) did not induce the production of mucosal antibody that cross-reacted with MN08 because if this had been the case, we would have expected that lung lavage collected from Ad5-HA/CA09 would cross-react with MN08 antigen.

However, this was not the case. MN08-specific antibody was only detected in the lung lavage of pigs in the Ad5-HA/MN08 group (Fig. 4C & 4D). The detection of MN08-specific antibody in the BALF of Ad5-HA/MN08 pigs was associated with a decrease in virus titers in the BALF at the same time point (5 dpi; Table 3) as well as a decrease in lung IAV antigen scores compared to Ad5-empty/MN08 challenged controls (Fig. 3C). Detection of cross-reactive antibody to MN08 in conjunction with a decrease in IAV in
the lungs of Ad5-HA vaccinated pigs (Table 3) suggests an involvement of antibody in the clearance of virus. We speculate that mucosal antibody participated in clearance of heterologous virus, and its production was a consequence of MN08 virus challenge and subsequent re-activation of Ad5-HA primed CMI. This does not exclude the contribution of CTL involvement in clearance of virally infected cells from the respiratory tract, and further work is warranted to investigate the mechanism of more rapid viral clearance. Regardless of the mechanism, the clearance of heterologous virus reduced the duration and amount of viral shedding, a situation that would likely result in the reduction of transmission within and between swine herds. A vaccine that reduces heterologous viral transmission and disease would significantly lessen the economic impact experienced during an outbreak of a novel IAV strain in a herd. Previous work by Gauger et al. (6) indicates that adjuvanted WIV vaccination can cause vaccine associated enhanced respiratory disease (VAERD) in pigs when a heterologous mismatch between vaccine and challenge virus occurs (6). Gauger et al. and others have reported an association between VAERD and the presence of non-neutralizing antibody to the heterologous virus (6, 14, 31). Similarly, in kaCA/MN08 pigs we detected cross-reactive non-neutralizing antibodies along with an increased percentage of pneumonia at necropsy. Our data and that of others indicate that the involvement of non-neutralizing antibodies in the development of VAERD warrants further investigation (6, 13, 31). The kaCA vaccine did prime an antigen-specific IFN-γ SC response to both CA09 and MN08, which was greater in magnitude than that observed following Ad5-HA vaccination. However, the Ad5-HA vaccine only encoded for a single IAV antigen whereas kaCA would have included additional IAV antigens for
increased antigen-specific recall responses upon re-exposure to live virus. The route of vaccine administration may also contribute to the differences observed between vaccine groups in the number of peripheral IFN-γ SC. Previous work in mice has shown that intramuscular immunization increases the numbers of antigen-specific T cells in the periphery whereas intranasal immunization results in T cells localized in the lung (21, 22). While viral titers were reduced in both the Ad5-HA/MN08 pigs and the kaCA/MN08 pigs by 5 dpi, the Ad5-HA/MN08 pigs had a greater reduction compared to kaCA/MN08 (Table 3). Conversely, the kaCA/MN08 pigs had enhanced lung lesions while the Ad5-HA/MN08 pigs were not significantly different than Ad5-empty/MN08 (Fig. 2). The reduction in virus in the kaCA group may not be the result of a protective immune response, but instead, the effect of the severe inflammatory environment that occurs with VAERD (5). Most importantly, our data indicate that Ad5-HA vaccines can partially protect against heterologous virus without the development of VAERD.

In summary, although commercial WIV vaccines in swine can provide sterilizing immunity against homologous viruses, they provide limited protection against a heterologous virus and may lead to VAERD (31). With a single intranasal Ad5-HA vaccination, pigs were protected against homologous challenge and viral shedding and length of time infected following challenge with heterologous virus was significantly reduced. We clearly demonstrate that intranasal vaccination with an Ad5 vector provides multiple advantages over WIV. Some of the benefits of intranasal Ad5-HA vaccines include rapid production times, stimulation of the immune response similar to a natural route of infection, no requirement for added adjuvant, effective in a single dose and reduced viral shedding without causing VAERD when a viral mismatch occurs (19, 25,
The many benefits of intranasal vaccination with Ad5-HA suggest this platform is a strong candidate as an alternative to the traditional WIV vaccines used in the swine industry.

**Acknowledgements**

We thank Gwen Nordholm, Michelle Harland, and Zahra Olson for excellent technical assistance and Jason Huegel and Jason Crabtree for assistance with animal work. We also thank Dr. Susan Brockmeier for assistance with bacteriology results.

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**Figure Legends**

Fig. 1. Ad5-HA vaccination elicits IFN-γ responses to both homologous and heterologous virus. Pigs were intranasally vaccinated with Ad5-empty or Ad5-HA (CA09) on day 0. Peripheral blood mononuclear cells (PBMC) were isolated on day 21 or 42 post-vaccination from pigs vaccinated with Ad5-empty or Ad5-HA and an ELISpot assay was used to determine the number of IFN-γ secreting cells (SC) in $5 \times 10^5$ PBMC following stimulation *in vitro* for 18 h with (A) A/CA/04/09 or (B) A/SW/MN/2011/08 live IAV. (C) PBMC were also collected from pigs in kaCA group and the number of IFN-γ SC determined by ELISpot. Results are reported as the mean ± SEM and statistical
differences between non-vaccinated and vaccinated groups challenged with the same virus are indicated with connecting bars and asterisk (P < 0.05).

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Fig. 3. Microscopic pneumonia scores and IAV antigen scores 5 days post infection. Tissue was collected from pigs vaccinated intranasally with Ad5-empty (white bars) or Ad5-HA (black bars) 42 days prior to challenge or intramuscularly with kaCA (black bars) at 42 and 21 days prior to challenge. Pigs were challenged intranasally with A/CA/04/09 (CA09), A/SW/MN/2011/08 (MN08), or PBS (NC). (A) Trachea and (B) lung histopathology scores of hematoxylin and eosin stained formalin fixed tissues collected 5 days following challenge with CA09 or MN08. (C) Lung IAV antigen scores identified using an anti-NP (HB65) antibody on formalin fixed tissue, 5 dpi with CA09 or MN08 IAV as described in Materials and Methods. Results are reported as the mean ±
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References


40. **WHO** 2009, posting date. Pandemic influenza vaccine manufacturing process and timeline. [Online.]

41. **WHO** 2002, posting date. WHO Manual on Animal Influenza Diagnosis and Surveillance. 2nd. [Online.]

Table 1: Description of experimental treatment groups

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Vaccine</th>
<th>Challenge Virus</th>
<th>N</th>
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<tr>
<td>Ad5-empty/NC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ad5-empty</td>
<td>Sham</td>
<td>8</td>
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<tr>
<td>Ad5-empty/CA09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ad5-empty</td>
<td>CA09</td>
<td>8</td>
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<tr>
<td>Ad5-empty/MN08&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>MN08</td>
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<td>Ad5-HA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>CA09</td>
<td>7</td>
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<tr>
<td>Ad5-HA/MN08</td>
<td>Ad5-HA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>MN08</td>
<td>8</td>
</tr>
<tr>
<td>kaCA/MN08&lt;sup&gt;f&lt;/sup&gt;</td>
<td>kaCA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>MN08</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Non-Challenged  
<sup>b</sup>A/CA/04/09  
<sup>c</sup>A/swine/MN/2011/08  
<sup>d</sup>Codon optimized HA from A/CA/04/09  
<sup>e</sup>killed, adjuvanted CA09

Table 2: Summary of antibody results

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Sample Site</th>
<th>Isotype</th>
<th>Viral Antigen CA09</th>
<th>Viral Antigen MN08</th>
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<tr>
<td>Ad5-HA</td>
<td>Nasal Wash</td>
<td>IgG</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgA</td>
<td>Yes&lt;sup&gt;f&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>Ad5-HA</td>
<td>Sera</td>
<td>IgG</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>kaCA</td>
<td>Sera</td>
<td>IgG</td>
<td>Yes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgA</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>f</sup>Day 14 post-vaccination only  
<sup>b</sup>Days 14-42 post-vaccination (weekly bleeds)
Table 3. Viral titer in nasal swab (NS) and bronchial-alveolar lung lavage (BALF) collected at indicated days post-infection (dpi).

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Challenge Virus</th>
<th>NS dpi 1</th>
<th>NS dpi 3</th>
<th>NS dpi 5</th>
<th>BALF dpi 5</th>
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<tbody>
<tr>
<td>Ad5-Empty</td>
<td>NC</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0a</td>
</tr>
<tr>
<td>Ad5-Empty</td>
<td>CA09</td>
<td>2.1 ± 0.5b</td>
<td>2.6 ± 0.3b</td>
<td>2.7 ± 0.2bc</td>
<td>4.9 ± 0.2b</td>
</tr>
<tr>
<td>Ad5-HA</td>
<td>CA09</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0a</td>
</tr>
<tr>
<td>Ad5-Empty</td>
<td>MN08</td>
<td>3.3 ± 0.4b</td>
<td>3.4 ± 0.2c</td>
<td>3.3 ± 0.3c</td>
<td>4.7 ± 0.3b</td>
</tr>
<tr>
<td>Ad5-HA</td>
<td>MN08</td>
<td>3.2 ± 0.4b</td>
<td>3.2 ± 0.3c</td>
<td>1.0 ± 0.5a</td>
<td>0.0 ± 0.0a</td>
</tr>
<tr>
<td>kaCA</td>
<td>MN08</td>
<td>2.8 ± 0.6b</td>
<td>4.1 ± 0.2d</td>
<td>1.9 ± 0.5b</td>
<td>1.1 ± 0.8a</td>
</tr>
</tbody>
</table>

Data is reported as the mean ± SEM TCID<sub>50</sub>/ml (log<sub>10</sub>)

Different letters indicate a significant difference (P<0.05) between treatments within time point for specific sample (NS or BALF).
Fig. 1

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