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

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6 Running Title: Intranasal Ad5-HA reduces influenza viral load

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36 **Abstract**

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

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59 **1. Introduction**

60 Influenza A virus (IAV) infection in swine can lead to significant economic loss
61 through decreased weight gain and increased time to market. IAV also increases the
62 susceptibility to secondary bacterial infection leading to pneumonia and in severe cases
63 death (8, 16, 18). Due to the high rate of antigenic drift and antigenic shift, there are
64 multiple antigenically diverse strains of IAV currently circulating throughout the swine
65 population (33, 35). Furthermore, the introductions of human and avian IAV into the
66 swine population continue to increase the number of distinct circulating IAV strains (2,
67 11, 20, 35). The ever-changing diversity of circulating IAV is problematic for vaccine
68 mediated protection because the vaccine has to be repeatedly updated to provide
69 sufficient protection to circulating strains.

70 Vaccines currently used in the swine industry for the control of IAV are whole
71 inactivated virus (WIV) preparations. WIV vaccines used are typically multivalent
72 mixtures prepared with an adjuvant and administered intramuscularly using a prime-boost
73 vaccination strategy. Adjuvanted, WIV vaccines can elicit sterilizing immunity against
74 homologous virus (14, 30, 31). However, WIV vaccines are often ineffective at
75 protecting against heterologous strains beyond a reduction in clinical presentation of
76 disease (1, 6, 17, 24, 31). Moreover, recent evidence indicates that WIV vaccines may, in
77 some circumstances, result in the development of vaccine associated enhanced
78 respiratory disease (VAERD) when a vaccinated pig is infected with an antigenically
79 divergent virus (6, 14, 31). VAERD is characterized by the presence of cross-reactive,
80 non-neutralizing antibodies to heterologous virus and enhanced lung pathology in WIV
81 vaccinated pigs following heterologous infection compared to non-vaccinated pigs (6, 14,

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82 31). Thus, there is a need for alternative vaccine platforms that protect against
83 heterologous infection without resulting in VAERD. Aside from the possible
84 enhancement of disease, WIV vaccines can also be plagued by relatively long production
85 times (40).

86 The large amount of time needed to license, approve and produce a WIV vaccine
87 for swine severely hinders its use during a novel IAV outbreak. An alternative platform
88 to WIV that has quick production potential is a replication defective human adenovirus 5
89 vector (Ad5) encoding IAV genes. The Ad5 virus is a complete virion that was made
90 replication defective by the removal of two segments of the Ad5 viral genome (10).
91 Deletion of two Ad5 genomic sequences permits the insertion of an IAV antigen
92 sequence for recombinant expression (reviewed by (29)). A recent report indicates that a
93 novel Ad5 construct can be created in less than 21 days once an antigen sequence is
94 identified (25). The Ad5 construct can be rapidly replicated using a small bioreactor
95 system, with viral titers of ~10^{10} to 10^{11} plaque forming units (PFU) per ml in as little as 3
96 days (supplemental data). Considering traditional WIV vaccine production for humans
97 has been reported as 5 to 6 months and is at least as long for fully licensed commercial
98 veterinary vaccines, the Ad5 construct is considerably faster (40). In addition to fast
99 production potential, the Ad5 virus makes an excellent intranasal vaccine platform due to
100 its natural predisposition for respiratory tract infection (28). The Ad5 platform allows for
101 the delivery and presentation of IAV antigen to the site of natural infection and because
102 Ad5 is an infectious particle, it initiates local immune activation in the absence of an
103 adjuvant (28). Subcutaneous and intramuscular vaccinations with Ad5 constructs
104 containing the hemagglutinin (HA) of IAV (Ad5-HA) have been validated as effective

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105 means of eliciting protection against IAV in mice, poultry and swine (4, 25, 37-39). The
106 advantages of rapid production time and the option of intranasal administration make the
107 Ad5-HA platform an attractive alternative to the currently used vaccines in swine.

108 The Ad5-HA as a vaccine for IAV was recently improved by Steitz et al. (25), in
109 which codon optimized IAV HA was incorporated in to the Ad5 vector to improve
110 protein expression, a change that increased immunogenicity. Thus, we sought to evaluate
111 the efficacy of a single intranasal vaccination with an Ad5 vector encoding codon
112 optimized HA against homologous and heterologous challenge in swine. We report
113 herein vaccination primes a cross-reactive antigen-specific immune response, provides
114 complete protection to homologous challenge, and limits duration of viral shedding and
115 load following heterologous challenge.

116 **2. Material and methods**

117 *2.1 Animals and Vaccines*

118 Forty-eight, three-week-old crossbred pigs were procured from a high-health status herd
119 known to be free of IAV and porcine reproductive and respiratory syndrome virus
120 (PRRSV). The pigs were randomly distributed into 6 treatment groups of 8 pigs each
121 (Table 1). Pigs were housed in BSL2 containment and animal care was in compliance
122 with the institutional animal care and use committee (IACUC) of the National Animal
123 Disease Center (NADC). Replication defective adenovirus-5 containing the codon-
124 optimized HA from A/CA/04/09 pH1N1 and the empty vector (referred to as Ad5-HA
125 and Ad5-empty, respectively) were generated as previously described (25). The E1 and
126 E3 gene segments of the adenovirus genome have been removed, rendering it replication
127 defective. Sixteen pigs were vaccinated with 2 ml containing 10^{10} plaque forming units

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128 (PFU) of Ad5-HA and 16 pigs received Ad5-empty at the same concentration in
129 phosphate buffered saline (PBS) via the intranasal route at 5 weeks of age (Table 1). One
130 group of 8 pigs was vaccinated intramuscularly at 5 weeks of age with 128 HA units of
131 ultraviolet-inactivated A/CA/04/09 pH1N1 (CA09; human isolate) mixed with an oil-in-
132 water adjuvant (Emulsigen-D, MVP Technologies, Omaha, NE) at a v:v ratio of 4:1 virus
133 to adjuvant (referred to as kaCA) as previously described (6). The same 8 pigs were
134 boosted 21 days later with the same preparation. Sera and nasal washes were collected
135 every 7 days from all pigs beginning on the day of vaccination (day 0) for the
136 measurement of antigen specific antibody using a previously described method (16).
137 Blood was collected and peripheral blood mononuclear cells (PBMC) were isolated for
138 IFN-γ ELIspot assay on days 21 and 42 post vaccination (dpv). Prior to challenge one pig
139 in the Ad5-HA group to be challenged with CA09 died from causes unrelated to the
140 experiment (Table 1). At 42 dpv, pigs were challenged by intranasal inoculation with
141 Madin Darby canine kidney cell (MDCK) propagated CA09 or A/swine/MN/02011/08
142 (H1N2, MN08) at a final volume of 2 ml per pig. Back titrations of CA09 and MN08
143 challenge viruses were $10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ per ml, respectively. Nasal swabs were
144 collected to evaluate viral shedding at 0, 1, 3 and 5 days post-infection (dpi) as previously
145 described (6). On dpi 5 all pigs were humanely euthanized with a lethal dose of
146 pentobarbital (Fort Dodge Animal Health, Fort Dodge, IA). Postmortem sample
147 collection included serum, nasal swab, nasal wash, bronchoalveolar lavage fluid (BALF),
148 lung, and trachea. Collection of BALF consisted of lavaging with 50 ml of minimal
149 essential media (MEM) as previously described (31).
150 *2.2 Microbiology*

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

156 *2.3 Antibody detection and characterization assays*

157 For use in the hemagglutinination inhibition (HI) assay, sera were heat inactivated at 56
158 °C for 30 min, then treated with a 20% kaolin (Sigma–Aldrich, St. Louis, MO)
159 phosphate-buffered (PBS) suspension and absorbed with 0.5% turkey red blood cells
160 (RBCs) to remove nonspecific hemagglutination inhibitors and natural serum agglutinins.
161 The MN08 and CA09 viruses were used as antigen in the HI assays following standard
162 techniques with turkey RBCs (41). Reciprocal titers from HI assays were divided by 10
163 and $\log_2$ transformed, analyzed, and reported as the geometric mean. Total IgG and IgA
164 antibody against MN08 and CA09 were detected by enzyme-linked immunosorbent assay
165 (ELISA) using whole virus preparations diluted in carbonate bicarbonate buffer to a
166 hemagglutinin (HA) concentration of 100 HAU per 50 µl and are referred to as Isotype
167 ELISAs. Isotype ELISAs were performed on serum, nasal wash, and BALF as
168 previously described (15, 31) with some modifications. Briefly, 100 µl of virus was
169 coated onto Nunc Immuno 96-well plates (Nunc, Rochester, NY) and incubated at room
170 temperature overnight. Sera were heat inactivated at 56 °C, while nasal wash and BALF
171 were diluted in a 10 mM dithiothreitol / PBS buffer at a 1:1 ratio for mucus dissociation
172 and incubated at 37 ºC for 1 h. All samples were assayed in triplicate. The mean optical
173 density (OD) of triplicate wells was calculated and antibody titers were reported as

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174 average OD for all pigs in each respective group.

175 *2.4 IFN-γ ELISPOT assay*

176 On days 21 and 42 post vaccination, whole blood was collected in sodium citrate CPT
177 tubes (BD Vacutainer, Franklin Lakes, NJ) and PBMC were separated according to
178 manufacturer's recommendations. Total PBMC were processed as previously described
179 (7), enumerated and adjusted to 5 x 10^5 cells per 0.1 ml. The IFN-γ ELISpot assay was
180 performed according to manufacturer's recommendations (Porcine IFN-γ ELISpot, R&D
181 Systems, Minneapolis, MN). Wells were seeded with 0.1 ml of PBMC suspension and
182 stimulated with 50 l of 5 X 10^6 $TCID_{50}$ /ml live CA09 or MN08 virus, 5 g/ml of
183 concanavalin A, or MDCK sham media. Final volume was brought to 0.25 ml.
184 Following an18 hour incubation in a 37 C humidified 5% CO_2 incubator the assay was
185 completed according to manufacturer's recommendations. Plates were scanned and
186 analyzed with UV-5 CTL-ImmunoSpot instrumentation and software (Cellular
187 Technology Ltd, Shaker Heights, OH). The mean count of triplicate wells for each
188 treatment for each pig was determined and used to calculate the mean for each vaccine
189 group.

190 *2.5 Pathology*

191 At necropsy, lungs were removed and evaluated for the percentage of the lung affected
192 by purple-red consolidation typical of IAV infection in swine. The percent of the surface
193 area affected with pneumonia was visually estimated for each lung lobe, and total
194 percentage for the entire lung was calculated based on weighted proportions of each lobe
195 to the total lung volume (9). Tissue samples from the trachea and right middle lung lobe
196 were fixed in 10% buffered formalin for microscopic examination. Tissues were

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197 processed by routine histopathologic procedures and slides stained with hematoxylin and
198 eosin. Microscopic lesions were evaluated by a board certified veterinary pathologist
199 blinded to treatment groups. Scoring of lesions was based on parameters adapted from
200 Gauger et al. (6). Individual scores were assigned to four parameters: bronchial and
201 bronchiolar epithelial changes, bronchitis/bronchiolitis, peribronchiolar lymphocytic
202 cuffing, edema and interstitial pneumonia. Scores were based on percentage of airways
203 with lesions that included epithelial changes and inflammation on a 5-point scale: 0: No
204 lesions, 1: 0-25%, 2: 26 to 50%, 3: 51-75%, 4: greater than 75% of airways affected with
205 airway epithelial damage and inflammation. Peribronchiolar cuffing by lymphocytes was
206 graded on a 4-point scale: 0: None, 1: Mild, loosely formed cuff of lymphocytes, 2:
207 Moderate, well-formed cuffs of lymphocytes, and 3: Prominent, thick well-formed cuffs.
208 Degree of edema and fibrin exudation was scored on the following 4-point scale: 0: none,
209 1: focal small area of edema in section (less than 15% of section), 2: 15-49% of section
210 including interlobular and/or pleural edema and alveolar lumina and septa, 3: greater than
211 50% of section including interlobular and/or pleural edema and most alveolar lumina and
212 septa. Interstitial pneumonia was graded on 5-point scale: 0: No lesions, 1: Mild, focal
213 to multifocal interstitial pneumonia, 2: Moderate, locally extensive to multifocal
214 interstitial pneumonia, 3: Moderate, multifocal to coalescing interstitial pneumonia, and
215 4: Severe, coalescing to diffuse interstitial pneumonia. Trachea sections were scored
216 similar to the bronchi and bronchioles and were based on epithelial changes and degree of
217 inflammation. Tracheal epithelial changes were graded on a 5-point scale: 0: No lesions,
218 1: Early epithelial changes characterized by focal to multifocal loss of cilia and epithelial
219 degenerative changes, 2: Mild epithelial flattening with loss of cilia and goblet cells, 3:

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220 Moderate epithelial flattening with decreased thickness of respiratory epithelium, loss of
221 cilia and goblet cells, 4: Flattened epithelium with areas of mucosa covered by a single
222 layer of cuboidal epithelium and epithelial loss (necrosis). Degree of tracheitis was
223 graded on a simple 4-point scale: 0: None, 1: Mild, 2: Moderate, and 3: Severe. IAV
224 antigen was detected in lung tissues using a previously described immunohistochemical
225 (IHC) method with modifications (36). Tissue sections were deparaffinized and hydrated
226 in distilled water. Slides were quenched in 3% hydrogen peroxide for 10 min, rinsed three
227 times in de-ionized water and treated in 0.05% protease for 2 min. Slides were then
228 rinsed three times in de-ionized water and once in Tris-buffered saline (TBS).
229 Monoclonal antibody (MAb) HB65 (ATCC, Manassas, VA), specific for the
230 nucleoprotein (NP) of IAV, was applied at 1:100 dilution and slides were incubated at
231 room temperature for 1 h. Bound MAbs were stained with peroxidase-labeled anti-mouse
232 IgG followed by chromogen using the DAKO LSAB2-HRP Detection System (DAKO,
233 Carpinteria, CA) according to the manufacturer's instructions. The slides were rinsed in
234 deionized water and counterstained with Gill's hematoxylin. Antigen detection was
235 assessed using two scores: 1) airway epithelial labeling and 2) alveolar/interstitial
236 labeling. In airway epithelium a 5-point scale was used: 0: None,1: Few cells with
237 positive labeling, 2: Mild scattered labeling, 3: Moderate scattered labeling, 4: Abundant
238 scattered labeling (greater than 50% epithelium positive in affected airways). In the
239 interstitium/alveoli, a 4-point scale was used: 0: None, 1: Minimal focal signals, 2: Mild
240 multifocal signals, 3: Abundant signals.
241 *2.6 Virus isolation from nasal swabs and bronchoalveolar lavage fluid (BALF)*
242 BALF was collected at dpi 5 and stored at -80 °C. Nasal swabs collected at dpi 0, 1, 3

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243 and 5 were stored at -80 C and subsequently thawed and vortexed for 15 s, followed by
244 centrifugation for 10 min at 640 X g. Nasal swab supernatants were passed through 0.45
245 m syringe filters to remove bacterial contaminants. Ten-fold serial dilutions in serum-
246 free MEM supplemented with TPCK-trypsin (1 μg/ml; Sigma, St. Louis, MO) and
247 antibiotics were made for each BALF and nasal swab filtrate sample. One hundred
248 microliters of each dilution was plated in triplicate onto confluent MDCK cells in 96-well
249 plates. After 72 hours of incubation, MDCK monolayers were fixed with 4% phosphate
250 buffered formalin for 30 minutes. Fixed cells were stained using a previously described
251 (13) immunocytochemistry technique that utilizes an anti-IAV nucleoprotein monoclonal
252 antibody (HB65). Positive staining was used for the determination of virus titer. A final
253 $TCID_{50}$ per milliliter titer was calculated for each sample using the method of Reed and
254 Muench (23).
255 *2.7 Statistical Analyses*
256 Log_2-transformed HI titers and log_{10}-transformed NS viral titers data were analyzed using
257 a mixed linear model for repeated measures (Proc Mixed, SAS for Windows, Version
258 9.2, SAS Institute Inc., Cary, NC, USA). Covariance structures within pigs across time
259 were tested and modeled using the REPEATED statement to determine the optimal
260 covariance structure. Linear combinations of the least squares means estimates were used
261 in *a priori* contrasts after testing for a significant (P <0.05) treatment group effect.
262 Comparisons were made between each group at each time point using a 5% level of
263 significance (P <0.05) to assess statistical differences. The endpoint data for microscopic
264 tracheal and lung lesions, macroscopic lung lesions, log_{10} –transformed BALF viral titers,
265 and immunohistochemistry (IHC) staining of the lung were analyzed by analysis of

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266 variance using a general linear model for unbalanced data. A significance level of 5%
267 was also used for comparisons between treatment groups for the microscopic lesions and
268 IHC.

269 **3. Results**

270 *3.1 Microbiological Assays*

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

276 *3.2 IAV-specific antibody in pre-challenge nasal wash and sera.*

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

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

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289 CA09 and MN08 in pre-challenge sera similar to what has been previously described (6).

290 A summary of antibody results is described in Table 2.

291 3.3 *Cell mediated immunity*

292 All immunized pigs exhibited an antigen-specific IFN-γ recall response to both

293 homologous CA09 and heterologous MN08 antigen although responses to homologous

294 antigen were significantly increased over heterologous antigen (69.8 $\pm$ 8.8 vs 28.5 $\pm$ 6.5

295 respectively at 21 dpv; Fig. 1A and 1B). In Ad5-HA vaccinated pigs, the number of

296 antigen-specific IFN-γ SC decreased over time, as numbers were greater at 21 dpv

297 compared to 42 dpv for both viral antigens. The numbers of CA09-specific IFN-γ SC

298 were 69.8 $\pm$ 8.8 at 21 dpv compared to 26.0 $\pm$ 8.8 at 42 dpv in Ad5-HA vaccinated pigs.

299 The kaCA vaccine primed an antigen-specific IFN-γ response to both CA09 and MN08

300 viruses as well. The average number of antigen-specific IFN-γ SC detected in the kaCA

301 vaccination group was greater than that detected following Ad5-HA vaccination, which

302 was not surprising given that kaCA vaccinated pigs were exposed to not only HA, but

303 additional IAV proteins as well. Although the kaCA group received a boost at 21 dpv, the

304 numbers of IFN-γ SC cells detected on dpv 42 were at or below levels detected at 21 dpv,

305 which was prior to the boost (Fig. 1C).

306 *3.4 Macroscopic and microscopic lung lesions*

307 Macroscopic and microscopic lung lesion scores in the Ad5-HA/CA09 group were

308 indistinguishable from scores in the Ad5-HA/NC group, and significantly lower than

309 scores in the Ad5-empty/CA09 group (Fig. 2 and 3, respectively). The Ad5-HA/MN08

310 group had macroscopic and microscopic lesion scores that were similar to the Ad5-

311 empty/MN08 group, but scores in the Ad5-HA/MN08 group were significantly lower

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312 than the kaCA/MN08 group (Fig. 2). The kaCA/MN08 group had the highest
313 macroscopic and microscopic lung lesion scores across all vaccination groups (Fig. 2 and
314 3). Microscopic tracheal lesions were less severe in the Ad5-HA/MN08 group compared
315 to either kaCA/MN08 or Ad5-empty/MN08 (1.8 $\pm$ 0.1 vs 2.9 $\pm$ 0.2 and 2.4 $\pm$ 0.2
316 respectively). The Ad5-HA vaccinated pigs challenged with MN08 or CA09 had lower
317 lung IAV antigen scores compared to the Ad5-empty or kaCA group, which is suggestive
318 of less viral antigen (Fig. 3C). Furthermore, there was a relationship between decreased
319 virus and lung lesions inAd5-HA/MN08 pigs, in that lung viral loads were reduced on dpi
320 5 but macroscopic and microscopic lung lesions were not significantly different than
321 those observed in the Ad5-empty/MN08 group. This is in contrast to the Ad5-HA/CA09
322 pigs, which had a reduction in virus and a reduction in lung lesions when compared to
323 Ad5-empty/CA09 group.
324 *3.5 Virus titers in BALF and nasal swabs following challenge*
325 Virus was not isolated from any of the nasal swab (NS) samples collected from the Ad5-
326 HA/CA09 group, but was isolated from NS collected from Ad5-empty/CA09 group
327 (Table 3), indicating protection from homologous challenge. Conversely, virus was
328 detected in the NS collected on dpi 1 and dpi 3 from pigs in the MN08 challenge group,
329 regardless of vaccination. NS viral titers reached the highest detected level at dpi 3 and
330 remained elevated until dpi 5 for Ad5-empty group, regardless of the IAV challenge
331 strain. NS samples collected on dpi 5 from the kaCA/MN08 group had lower viral titers
332 compared to dpi 3, equal to titers on dpi 1, while Ad5-HA/MN08 NS virus titers on dpi 5
333 were reduced to levels less than the dpi 1 titers. On dpi 5, virus titers in BALF were 4.9 $\pm$
334 0.2 $TCID_{50}$ (log_{10}) for the Ad5-empty/CA09 group and 4.7 $\pm$ 0.3 $TCID_{50}$ (log_{10}) for Ad5-

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335 empty/MN08 group. Conversely, virus was not detected in the BALF of Ad5-HA
336 vaccinated pigs following challenge with homologous or heterologous virus. Virus was
337 not isolated from the NS or BALF collected from the empty/non-challenged controls at
338 any time in the study. Results are summarized in Table 3.

339 *3.6 Humoral response to challenge virus in BALF at 5 dpi.*

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

351 **4. Discussion**

352 The commercial IAV vaccines currently available for use in swine are based on
353 the WIV platform. Vaccination with WIV can elicit sterilizing immunity against a
354 homologous strain, primarily through production of antibody directed towards the
355 receptor binding domain of the immuno-dominant surface glycoprotein HA (3). Due to
356 the highly variable nature of HA, the WIV vaccine provides limited protection against
357 heterologous viruses with demonstrated antigenic drift. Furthermore, recent reports

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358 suggest that WIV vaccines can result in VAERD when the vaccine strain and infecting
359 virus share some antigenic similarities, but vaccination does not elicit neutralizing
360 antibodies to the infecting virus (6, 14, 31). With the high rate of antigenic drift observed
361 in IAV, and the diversity of IAV currently circulating in the U.S. swine population,
362 heterologous mismatch is likely to occur between vaccine and infecting strain in the field.
363 The HA in the MN08 virus belongs to the human-like δ-cluster of HA genes, which was
364 introduced into the swine population from human seasonal IAV whereas the CA09 HA is
365 a drift variant of the classical swine lineage HA, most closely related to the γ-cluster
366 viruses (12, 32, 34). Protein sequence homology between the CA09 HA and the MN08
367 HA is approximately 77%. Therefore, a vaccine platform that provides protection to a
368 broad range of IAV antigenic types, but does not result in VAERD, is highly desirable.
369 We report herein that a single intranasal vaccination with Ad5-HA induces full protection
370 against homologous challenge and partial protection against a heterologous challenge by
371 limiting the duration and amount of viral shedding. In addition, our data indicates that
372 vaccination with Ad5-HA does not result in VAERD upon heterologous challenge when
373 using the same vaccine strain-challenge strain combination that induced VAERD with the
374 WIV. Lastly, Ad5-HA vaccination primed for an immune response that resulted in more
375 rapid production of mucosal antibody cross-reactive to heterologous virus, which likely
376 played a role in protection.

377 Heterologous MN08 virus was isolated from the nose of Ad5-HA/MN08 pigs on
378 dpi 1 and 3, thus vaccination did not completely prevent heterologous infection, while
379 reduced nasal titers on dpi 5 indicate that prior Ad5-HA vaccination increased the rate of
380 heterologous viral clearance. While the mechanism of heterologous virus clearance is not

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381 completely clear, establishment of an infection prior to clearance provides evidence that
382 cell-mediated immune mechanisms likely played an important role. The role of cell-
383 mediated immune responses may be at the level of killing virally infected cells and/or
384 providing more rapid help to naïve B cells. In addition, it's possible that cross-reactive B
385 cell clones already present in the respiratory tract quickly expand following infection and
386 provide some level of protection.

387 Conserved regions within CA09 and MN08 HA likely contain T cell epitopes
388 that would be recognized upon heterologous challenge. In the current study we assessed
389 the quantity of antigen specific IFN-γ SC as a measure of cell-mediated immunity (CMI)
390 induced by Ad5-HA intranasal vaccination. Pigs vaccinated with the Ad5-HA were only
391 exposed to the CA09 HA antigen, and therefore, although whole virus was used as recall
392 antigen in the IFN-γ ELISpot, responses were likely specific only to the HA of the virus
393 used as recall antigen. Following Ad5-HA vaccination, PBMCs were primed to produce
394 IFN-γ in response to both CA09 and MN08 virus (Fig. 1A & 1B). However, HI-specific
395 antibody was never detected in the blood of Ad5-HA vaccinated pigs, regardless of virus
396 used in the assay (CA09 or MN08). Ad5-HA vaccination did provide protection upon
397 heterologous challenge, evidenced by reduced NS viral titers at 5 dpi and clearance of
398 viable virus from the BALF at 5 dpi. Thus, our data indicates that the priming of CMI
399 towards HA likely contributed to the clearance of heterologous challenge virus. The
400 ELISpot assay did not discern if the IFN-γ SC were $CD4^+$, $CD8^+$ or $CD4^+/CD8^+$ double-
401 positive T cells (a population of memory T cells in pigs (42)) and therefore it is difficult
402 to pinpoint if more rapid viral clearance is the result of increased activity of cytotoxic T
403 lymphocytes (CTL) or T helper cells. Previous research in mice indicates that $CD4^+$ Th1

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404 cells alone can decrease the severity of IAV infection (26). When primed $CD4^+$ Th1 cells
405 were passively transferred to naive mice that were subsequently infected with IAV, the
406 infection was quickly cleared (26). Thus, the enhanced clearance of virus in the Ad5-
407 HA/MN08 pigs may be due to activation of CD4+ Th1 cells that were primed towards a
408 conserved HA epitope.
409 Further evidence suggesting that a primed CMI provides protection to
410 heterologous infection is that memory $CD4^+$ T cells have been shown to be more adept at
411 providing B-cell help when compared to naïve $CD4^+$ T cells, although the exact
412 mechanism by which this occurs has not been clearly defined (reviewed in (27)).
413 Antibody levels in the lung lavage of pigs in the current study provide additional support
414 to this finding. Antibody detected in the BALF following Ad5-HA vaccination would be
415 expected to react to CA09 HA, which was the case regardless of the challenge strain (Fig.
416 4). However, MN08-specific antibody was detected in the BALF only following MN08
417 challenge, but not CA09 challenge (Fig. 4C & 4D). This data suggests that Ad5-HA
418 vaccination alone (CA09 HA) did not induce the production of mucosal antibody that
419 cross-reacted with MN08 because if this had been the case, we would have expected that
420 lung lavage collected from Ad5-HA/CA09 would cross-react with MN08 antigen.
421 However, this was not the case. MN08-specific antibody was only detected in the lung
422 lavage of pigs in the Ad5-HA/MN08 group (Fig. 4C & 4D). The detection of MN08-
423 specific antibody in the BALF of Ad5-HA/MN08 pigs was associated with a decrease in
424 virus titers in the BALF at the same time point (5 dpi; Table 3) as well as a decrease in
425 lung IAV antigen scores compared to Ad5-emtpy/MN08 challenged controls (Fig. 3C).
426 Detection of cross-reactive antibody to MN08 in conjunction with a decrease in IAV in

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427 the lungs of Ad5-HA vaccinated pigs (Table 3) suggests an involvement of antibody in
428 the clearance of virus. We speculate that mucosal antibody participated in clearance of
429 heterologous virus, and its production was a consequence of MN08 virus challenge and
430 subsequent re-activation of Ad5-HA primed CMI. This does not exclude the contribution
431 of CTL involvement in clearance of virally infected cells from the respiratory tract, and
432 further work is warranted to investigate the mechanism of more rapid viral clearance.
433 Regardless of the mechanism, the clearance of heterologous virus reduced the duration
434 and amount of viral shedding, a situation that would likely result in the reduction of
435 transmission within and between swine herds. A vaccine that reduces heterologous viral
436 transmission and disease would significantly lessen the economic impact experienced
437 during an outbreak of a novel IAV strain in a herd.

438 Previous work by Gauger et al. (6) indicates that adjuvanted WIV vaccination can
439 cause vaccine associated enhanced respiratory disease (VAERD) in pigs when a
440 heterologous mismatch between vaccine and challenge virus occurs (6). Gauger et al. and
441 others have reported an association between VAERD and the presence of non-
442 neutralizing antibody to the heterologous virus (6, 14, 31). Similarly, in kaCA/MN08
443 pigs we detected cross-reactive non-neutralizing antibodies along with an increased
444 percentage of pneumonia at necropsy. Our data and that of others indicate that the
445 involvement of non-neutralizing antibodies in the development of VAERD warrants
446 further investigation (6, 13, 31). The kaCA vaccine did prime an antigen-specific IFN-γ
447 SC response to both CA09 and MN08, which was greater in magnitude than that
448 observed following Ad5-HA vaccination. However, the Ad5-HA vaccine only encoded
449 for a single IAV antigen whereas kaCA would have included additional IAV antigens for

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450 increased antigen-specific recall responses upon re-exposure to live virus. The route of
451 vaccine administration may also contribute to the differences observed between vaccine
452 groups in the number of peripheral IFN-γ SC. Previous work in mice has shown that
453 intramuscular immunization increases the numbers of antigen-specific T cells in the
454 periphery whereas intranasal immunization results in T cells localized in the lung (21,
455 22). While viral titers were reduced in both the Ad5-HA/MN08 pigs and the kaCA/MN08
456 pigs by 5 dpi, the Ad5-HA/MN08 pigs had a greater reduction compared to kaCA/MN08
457 (Table 3). Conversely, the kaCA/MN08 pigs had enhanced lung lesions while the Ad5-
458 HA/MN08 pigs were not significantly different than Ad5-empty/MN08 (Fig. 2). The
459 reduction in virus in the kaCA group may not be the result of a protective immune
460 response, but instead, the effect of the severe inflammatory environment that occurs with
461 VAERD (5). Most importantly, our data indicate that Ad5-HA vaccines can partially
462 protect against heterologous virus without the development of VAERD.
463 In summary, although commercial WIV vaccines in swine can provide sterilizing
464 immunity against homologous viruses, they provide limited protection against a
465 heterologous virus and may lead to VAERD (31). With a single intranasal Ad5-HA
466 vaccination, pigs were protected against homologous challenge and viral shedding and
467 length of time infected following challenge with heterologous virus was significantly
468 reduced. We clearly demonstrate that intranasal vaccination with an Ad5 vector provides
469 multiple advantages over WIV. Some of the benefits of intranasal Ad5-HA vaccines
470 include rapid production times, stimulation of the immune response similar to a natural
471 route of infection, no requirement for added adjuvant, effective in a single dose and
472 reduced viral shedding without causing VAERD when a viral mismatch occurs (19, 25,

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473 28). The many benefits of intranasal vaccination with Ad5-HA suggest this platform is a
474 strong candidate as an alternative to the traditional WIV vaccines used in the swine
475 industry.
476

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482 purpose of providing specific information and does not imply recommendations or
483 endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity
484 employer.
485

486 **Figure Legends**
487 Fig. 1. Ad5-HA vaccination elicits IFN-γ responses to both homologous and heterologous
488 virus. Pigs were intranasally vaccinated with Ad5-empty or Ad5-HA (CA09) on day 0.
489 Peripheral blood mononuclear cells (PBMC) were isolated on day 21 or 42 post-
490 vaccination from pigs vaccinated with Ad5-empty or Ad5-HA and an ELISpot assay was
491 used to determine the number of IFN-γ secreting cells (SC) in $5x10^5$ PBMC following
492 stimulation *in vitro* for 18 h with (A) A/CA/04/09 or (B) A/SW/MN/2011/08 live IAV.
493 (C) PBMC were also collected from pigs in kaCA group and the number of IFN-γ SC
494 determined by ELISpot. Results are reported as the mean $\pm$ SEM and statistical

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495 differences between non-vaccinated and vaccinated groups challenged with the same
496 virus are indicated with connecting bars and asterisk ($P \leq 0.05$).
497
498 Fig. 2. Macroscopic lung lesions on day 5 post-infection were reduced by Ad5-HA
499 vaccination and enhanced in kaCA vaccinated pigs. Pigs were vaccinated intranasally
500 with Ad5-empty or Ad5-HA 42 days prior to challenge or intramuscularly with kaCA at
501 42 and 21 days prior to challenge. Pigs were challenged intranasally with A/CA/04/09
502 (CA09), A/SW/MN/2011/08 (MN08), or PBS (NC). The percentage of macroscopic lung
503 lesions in the (A) Ad5-HA or Ad5-empty vaccinated and (B) kaCA vaccinated pigs were
504 evaluated 5 days post infection with the indicated virus. Results are reported as the mean
505 $\pm$ SEM and statistical differences between non-vaccinated and vaccinated groups
506 challenged with the same virus are indicated with connecting bars and asterisk ($P \leq 0.05$).
507
508 Fig. 3. Microscopic pneumonia scores and IAV antigen scores 5 days post infection.
509 Tissue was collected from pigs vaccinated intranasally with Ad5-empty (white bars) or
510 Ad5-HA (black bars) 42 days prior to challenge or intramuscularly with kaCA (black
511 bars) at 42 and 21 days prior to challenge. Pigs were challenged intranasally with
512 A/CA/04/09 (CA09), A/SW/MN/2011/08 (MN08), or PBS (NC). (A) Trachea and (B)
513 lung histopathology scores of hematoxylin and eosin stained formalin fixed tissues
514 collected 5 days following challenge with CA09 or MN08. (C) Lung IAV antigen scores
515 identified using an anti-NP (HB65) antibody on formalin fixed tissue, 5 dpi with CA09 or
516 MN08 IAV as described in Materials and Methods. Results are reported as the mean $\pm$

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517 SEM and statistical differences between non-vaccinated and vaccinated groups

518 challenged with the same virus are indicated with connecting bars and asterisk ($P \leq 0.05$).

519

520 Fig. 4. Ad5-HA vaccination elicits IAV-specific IgG and IgA in the lung lavage. Pigs

521 were vaccinated with Ad5-empty or Ad5-HA (CA09) intranasally 42 days prior to

522 infection with A/CA/04/09 (CA09) or A/SW/MN/2011/08 (MN08) IAV. ELISA plates

523 were coated with CA09 or MN08 as antigen and levels of (A & C) IgA and (B & D) IgG

524 antibody in BALF samples (diluted as described in materials and methods) collected 5

525 days post infection with the indicated challenge virus are shown. Results are reported as

526 the mean of optical densities (O.D.) $\pm$ SEM for each group. Statistical differences

527 between non-vaccinated and vaccinated groups challenged with the same virus are

528 indicated with connecting bars and asterisk ($P \leq 0.05$).

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Table 1: Description of experimental treatment groups

Abbreviation	Vaccine	Challenge Virus	N
Ad5-empty/NC[a]	Ad5-empty	Sham	8
Ad5-empty/CA09[b]	Ad5-empty	CA09	8
Ad5-empty/MN08[c]	Ad5-empty	MN08	8
Ad5-HA/CA09	Ad5-HA[d]	CA09	7
Ad5-HA/MN08	Ad5-HA	MN08	8
kaCA[e]/MN08	kaCA	MN08	8

686 [a]Non-Challenged
687 [b]A/CA/04/09
688 [c]A/swine/MN/2011/08
689 [d]Codon optimized HA from A/CA/04/09
690 [e]killed, adjuvanted CA09
691
692
693

Table 2: Summary of antibody results

Vaccine	Sample Site	Isotype	Viral Antigen	
			CA09	MN08
Ad5-HA	Nasal Wash	IgG	No	No
		IgA	Yes[a]	No
Ad5-HA	Sera	IgG	No	No
		IgA	No	No
kaCA	Sera	IgG	Yes[b]	Yes[b]
		IgA	No	No

694 [a]Day 14 post-vaccination only
695 [b]Days 14-42 post-vaccination (weekly bleeds)
696
697
698
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Table 3. Viral titer in nasal swab (NS) and bronchial-alveolar lung lavage (BALF) collected at indicated days post-infection (dpi).

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

703 Data is reported as the mean ± SEM $TCID_{50}$/ml (log_{10})
704 Different letters indicate a significant difference ($P<0.05$) between treatments within time
705 point for specific sample (NS or BALF).
706

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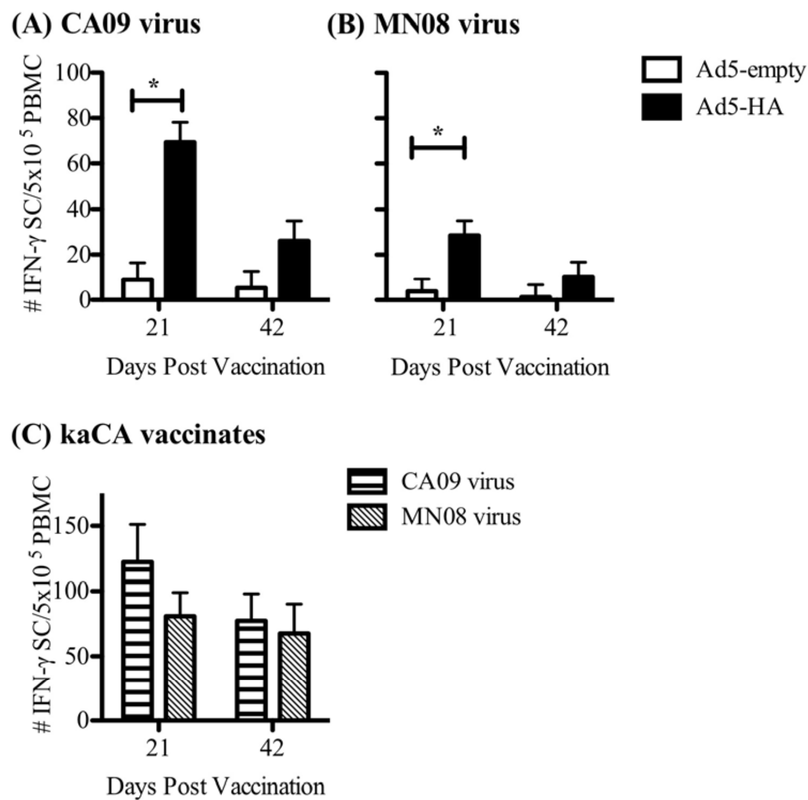

Fig. 1

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 $5x10^5$ 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 $\pm$ SEM and statistical differences between non-vaccinated and vaccinated groups challenged with the same virus are indicated with connecting bars and asterisk ($P \leq 0.05$)..

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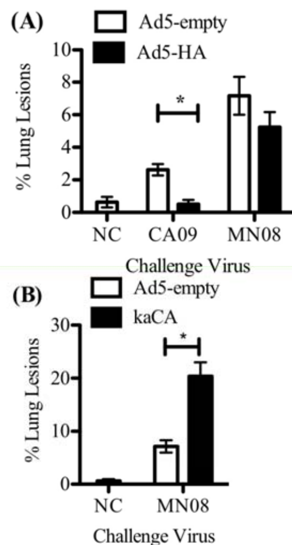

Fig. 2

Fig. 2. Macroscopic lung lesions on day 5 post-infection were reduced by Ad5-HA vaccination and enhanced in kaCA vaccinated pigs. Pigs were vaccinated intranasally with Ad5-empty or Ad5-HA 42 days prior to challenge or intramuscularly with kaCA 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). The percentage of macroscopic lung lesions in the (A) Ad5-HA or Ad5-empty vaccinated and (B) kaCA vaccinated pigs were evaluated 5 days post infection with the indicated virus. Results are reported as the mean $\pm$ SEM and statistical differences between non-vaccinated and vaccinated groups challenged with the same virus are indicated with connecting bars and asterisk ($P \leq 0.05$).

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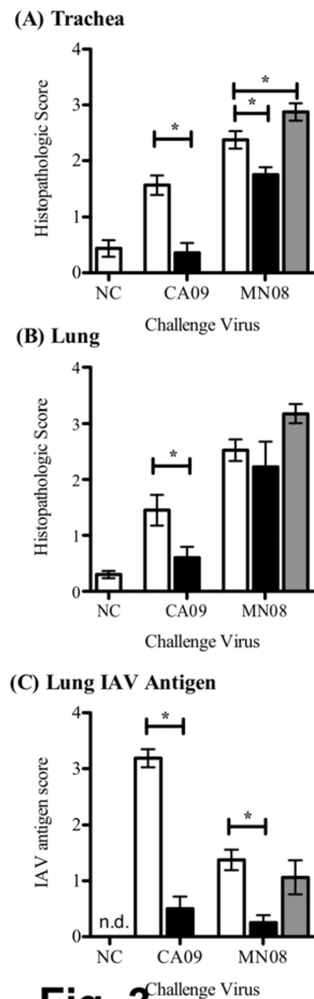

Fig. 3. Microscopic pneumonia scores and IAV antigen scores 5 days post infection. Tissue was collected from pigs vaccinated intranasally with Ad5-empty or Ad5-HA 42 days prior to challenge or intramuscularly with kaCA at 42 and 21 days prior to challenge. Pigs were challenged intranasally with A/CA/04/09 (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 ± SEM and statistical differences between non-vaccinated and vaccinated groups challenged with the same virus are indicated with connecting bars and asterisk ($P \leq 0.05$).

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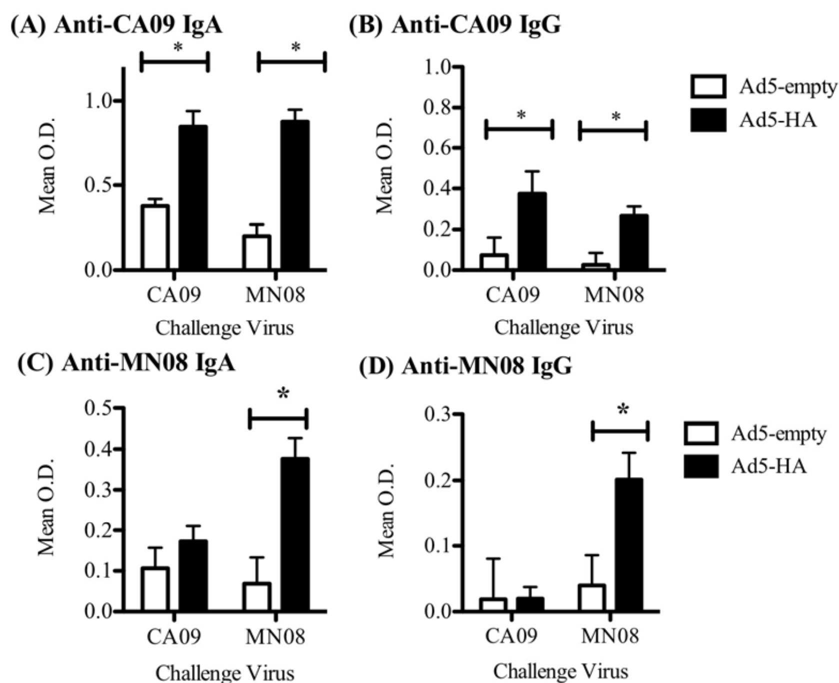

Fig. 4

Fig. 4. Ad5-HA vaccination elicits IAV-specific IgG and IgA in the lung lavage. Pigs were vaccinated with Ad5-empty or Ad5-HA (CA09) intranasally 42 days prior to infection with A/CA/04/09 (CA09) or A/SW/MN/2011/08 (MN08) IAV. ELISA plates were coated with CA09 or MN08 as antigen and levels of (A & C) IgA and (B & D) IgG antibody in BALF samples (diluted as described in materials and methods) collected 5 days post infection with the indicated challenge virus are shown. Results are reported as the mean of optical densities (O.D.) $\pm$ SEM for each group. Statistical differences between specific groups are indicated with connecting bars and asterisk ($P \leq 0.05$).