Vaccine-associated enhanced respiratory disease is influenced by haemagglutinin and neuraminidase in whole inactivated influenza virus vaccines

Daniela S. Rajão, Hongjun Chen, Daniel R. Perez, Matthew R. Sandbulte, Phillip C. Gauger, Crystal L. Loving, G. Dennis Shanks and Amy Vincent

1Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, IA, USA
2Department of Veterinary Medicine, University of Maryland, College Park, and Virginia-Maryland Regional College of Veterinary Medicine, College Park, MD, USA
3Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, USA
4Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA, USA
5Australian Army Malaria Institute, Enoggera, QLD, Australia

Multiple subtypes and many antigenic variants of influenza A virus (IAV) co-circulate in swine in the USA, complicating the effective use of commercial vaccines to control disease and transmission. Whole inactivated virus (WIV) vaccines may provide partial protection against IAV with substantial antigenic drift, but have been shown to induce vaccine-associated enhanced respiratory disease (VAERD) when challenged with an antigenic variant of the same haemagglutinin (HA) subtype. This study investigated the role of the immune response against HA, neuraminidase (NA) and nucleoprotein (NP) may play in VAERD by reverse engineering vaccine and challenge viruses on a common backbone and using them in a series of vaccination/challenge trials. Mismatched HA between vaccine and challenge virus was necessary to induce VAERD. However, vaccines containing a matched NA abrogated the VAERD phenomenon induced by the HA mismatch and this was correlated with NA-inhibiting (NI) antibodies. Divergence between the two circulating swine N2 lineages (92% identity) resulted in a loss of NI cross-reactivity and also resulted in VAERD with the mismatched HA. The NP lineage selected for use in the WIV vaccine strains did not affect protection or pathology. Thus the combination of HA and NA in the vaccine virus strains played a substantial role in vaccine protection versus immunopathology, suggesting that vaccines that target the HA protein alone could be more prone to VAERD due to the absence of cross-protective NI antibodies.

INTRODUCTION

Influenza A viruses (IAVs) cause one of the most important respiratory diseases of swine, resulting in a substantial burden to the swine industry each year. There is remarkable genetic diversity among swine IAV, particularly after the introduction of human viruses into pigs in North America. The H3N2 with a triple reassortant internal gene (TRIG) constellation was introduced in 1998 (Anderson et al., 2013; Vincent et al., 2008b), followed by the introduction in 2002 of genes from human seasonal H1 (called δ in swine) and N2 [designated N2(B) to differentiate from the 1998 N2 (A)] combined with the TRIG backbone (Vincent et al.,
2009). Increased genetic diversity occurred again when the 2009 H1N1 human pandemic (H1N1pdm09) virus was introduced into pigs in North America (Howden et al., 2009). Although only three subtypes are endemic in pigs (H1N1, H1N2 and H3N2), many distinct genetic clades of haemagglutinin (HA) and neuraminidase (NA) currently co-circulate (Anderson et al., 2013), resulting in substantial antigenic diversity.

Typical IAV infection in pigs results in mild to moderate respiratory disease, with animals showing a range of fever, lethargy, coughing and nasal discharge (Janke, 2013). We previously reported a swine model that displayed aggravated pneumonia based on a mismatch between the HA of the vaccine strain used in a whole inactivated virus (WIV) vaccine and the challenge strain. The HA in the WIV and challenge strains are of the same subtype, but lack cross-neutralizing serum antibodies (Gauger et al., 2011, 2012; Vincent et al., 2008a). Although the HA mismatch is required, the role of other viral proteins in aggravated pneumonia remains unknown. The resulting enhancement is called vaccine-associated enhanced respiratory disease (VAERD), which is characterized by severe respiratory disease with prolonged fever and clinical signs, and pathological changes of increased areas of lung consolidation and moderate to severe bronchointerstitial pneumonia and necrotizing bronchiolitis (Gauger et al., 2011). The mechanisms that result in VAERD remain unknown, but the pathology appears to be immune mediated, similar to cases of severe disease in human viral infections, such as dengue virus or respiratory syncytial virus (RSV). These cases have been associated with the presence of poorly neutralizing antibodies, immune-complex formation or antibody-dependent enhancement (ADE) (Halstead & O’Rourke, 1977; Murphy et al., 1986).

Antigenic differences between the surface glycoproteins (HA and NA) in the priming influenza WIV vaccine compared with the subsequent infecting IAV can result in a poorly cross-reactive antibody response. Moreover, although primed cytotoxic T-lymphocytes (CTLs) directed against highly conserved epitopes of the nucleoprotein (NP) or matrix (M) (Assarson et al., 2008) are associated with heterosubtypic immunity in humans, CTLs may play a role in pulmonary immunopathology when pre-existing humoral immunity fails to protect against a subsequent infection with a different HA subtype (Parzych et al., 2013; Wiley et al., 2001). Here, reverse-engineered isogenic viruses that differed only in the HA, NA and/or NP were used in vivo to determine the potential role of the immune response against each protein in immunopathology associated with VAERD. We hypothesized that an immune response elicited by a vaccine virus with antigenic divergence in the HA protein compared to the subsequent challenge virus would induce VAERD due to cross-reacting adaptive immune mediators, such as non-neutralizing HA antibody and/or CTL recognition of conserved epitopes. Conversely, mismatching the NP or matching the NA may lessen the immune-mediated enhancement by reducing CTL recognition of NP epitopes or increasing the cross-reacting antibody response against the NA. Therefore, we compared an avian lineage NP (av) to the swine lineage NP and the 1998 N2(A) to the 2002 N2(B) in the context of our VAERD model based on mismatched HA.

**RESULTS**

**NP did not contribute to VAERD**

To investigate whether the NP gene would interfere with the VAERD outcome, pigs vaccinated with δH1N2(B) or δH1N2(B):avNP, along with non-vaccinated (NV) pigs, were challenged with pH1N2(B) in Study 1. The percentages of macroscopic lung lesions and scores for microscopic lung and trachea lesions were evaluated at 5 days post-infection (p.i.). To our surprise, there was no evidence for enhanced disease in the δH1N2(B) or δH1N2(B):avNP vaccinated groups compared with the NV/pH1N2(B)-positive control group in Study 1, in contrast to VAERD-affected pigs in our previous studies using pH1N1 as the challenge strain (Gauger et al., 2011). Both vaccinated groups had reduced lung involvement as compared with the NV/pH1N2(B) challenge control group (Fig. 1a, b), and there were no differences in clinical signs and febrile response (data not shown). The δH1N2(B):avNP vaccine had no statistical difference compared with the δH1N2(B) vaccine and therefore the data for both groups were combined as δH1N2(B)/pH1N2(B) in all figures.

**Matched NA abrogated VAERD**

To address these unexpected results, Study 2 investigated the impact of mismatched NA in combination with mismatched HA. By mismatching the NA between the vaccine and challenge strains, the VAERD effect was restored with the mismatched H1 vaccines. The groups challenged with pH1N2 (B) showed a milder febrile response compared with the groups challenged with pH1pN1. However, vaccinated pigs challenged with heterologous virus (δH1N2(B)/pH1pN1 and δH1N2(A)/pH1N2(B) groups) demonstrated prolonged elevated mean rectal temperatures in comparison with their respective NV challenge control groups (data not shown), with clinical disease consistent with our previous VAERD studies. The mean macroscopic percentage of pneumonia was 20% in the δH1N2(A)/pH1N2(B) and 25% in the δH1N2(B)/pH1pN1 groups, compared with 8–9% in the NV/pH1N2(B) and NV/pH1pN1 challenge groups (Fig. 1a), and mean microscopic scores were also higher for those vaccinated groups (Fig. 1b). The NV/pH1N2(B) challenge control groups from Studies 1 and 2 were not statistically different, and thus were combined in all subsequent analyses.

**Virus replication in upper and lower respiratory tract was not increased in VAERD-affected pigs**

To measure nasal viral shedding and replication in the lungs, virus titres were determined from nasal swabs...
collected at 0, 1, 3 and 5 days p.i. and bronchoalveolar lavage fluid (BALF) was collected at 5 days p.i. In Studies 1 and 2, virus shedding from the upper respiratory tract as well as virus replication in the lungs were significantly reduced in both vaccinated groups challenged with pH1N2 (B), despite the enhanced lung pathology observed in the group vaccinated with mismatched HA and NA [δH1N2 (A)/pH1N2(B)] (Fig. 2). Consistent with our findings in previous studies (Gauger et al., 2011; Vincent et al., 2012), the groups that showed enhanced pathology did not show increased viral shedding or replication in the lungs. Despite the lung pathology observed in NV pigs challenged with pH1pN1, nasal viral shedding and replication in the lungs was low for all groups challenged with this virus (Fig. 2), yet enhanced lesions were still observed for mismatched vaccine. The overall lower viral titres in nasal swabs for both challenge viruses and in BALF for pH1pN1 could be a result of lower replication for these reverse genetics (RG) viruses; challenge inoculum titres were close to the expected values of $1 \times 10^5$ TCID$_{50}$ ml$^{-1}$ (data not shown).

**Neuraminidase-inhibiting antibodies were associated with protection from IAV infection and absence of VAERD**

Serum antibody responses were evaluated in vaccinated pigs prior to infection. Vaccinated pigs seroconverted to the vaccine viruses as measured by haemagglutination inhibition (HI), serum neutralization (SN) and neuraminidase inhibition (NI) assays (Fig. 3a–d), and IgG ELISA (Fig. 3e, f), whereas non-vaccinated pigs remained negative. Robust SN antibody response against δH1N2(B) was detected at 0 days p.i. in both vaccinated groups challenged with pH1N2(B) (Fig. 3b), and NI antibody titres against the
Fig. 3. Antibody response in vaccinated pigs measured at the day of challenge. Geometric mean $\log_2$ transformed (a) HI titres and (b) SN titres to the vaccine antigen with the hemagglutinin from MN/08 [H1N2(B)]; NI titres against (c) the antigen with the NA from the 1998 lineage [N2(A)] or (d) the antigen with NA from the 2002 lineage [N2(B)]. Mean ODs in whole-virus ELISAs for serum IgG against (e) H1N2(A) or H1N2(B) vaccine antigens and (f) pH1N2(B) or pH1pN1 challenge viruses used in each group. Serum (g) antibody avidity and (h) complement activation in pigs vaccinated with H1N2(B), tested against H1N2(B), H1N2(A), pH1N2(B) or pH1pN1. Data presented as mean and SEM. Statistically significant differences between vaccine treatment groups and non-vaccinated groups (NV) are identified by asterisks (*$P<0.05$; **$P<0.005$; ***$P<0.0005$).
vaccine N2 strain were high in all vaccinated pigs (Fig. 3c, d). Similar to our previous studies, HI antibodies against the vaccine viruses containing the HA from MN08 did not cross-react to the HA of CA09 contained in the challenge virus (data not shown). In contrast, cross-reacting IgG antibodies against the challenge viruses were detected in all vaccinated groups (Fig. 3e, f).

VAERD-affected pigs demonstrated low avidity, complement-fixing antibodies and dysregulated cytokine responses

To understand the specificity of the cross-reacting IgG antibodies, avidity assays were run with urea-treated sera. Pigs vaccinated with δH1N2(B) showed overall lower avidity to the antigens with heterologous HA, and the NA did not seem to influence antibody avidity, although there was a trend for increased avidity against the pH1N2(B) antigen (Fig. 3g). Antibodies in the vaccinated pigs were shown to strongly activate complement against matched HA and NA, and partially against mismatched HA- and/or NA-containing viruses (Fig. 3h).

Cell-mediated immune responses were detected in the IFN-γ ELISpot assay; however, the responses were equivalent between the vaccine groups and between the virus strains [δH1N2(B), δH1N2(B):avNP or pH1N2(B)] used as recall antigens (data not shown). Overall, there was a dysregulation of pro- and anti-inflammatory cytokines in pigs that developed VAERD. Levels of IL-1β were higher in the groups that showed enhanced pneumonia in comparison with NV-challenged controls, and levels of IL-2 showed a similar trend, while IFN-α and IL-10 were reduced in vaccinated pigs challenged with pH1N2(B). Only cytokines with significant differences between groups are shown in Fig. 4.

DISCUSSION

VAERD is an immunopathologic process produced when pigs are vaccinated with adjuvanted WIV influenza vaccine

Fig. 4. Cytokine levels [IFN-α (a), IL-1β (b), IL-2 (c) and IL-10 (d)] in BALF at 5 days p.i. from pigs vaccinated with δH1N2(A), δH1N2(B) or non-vaccinated and challenged with either pH1pN1 or pH1N2(B) viruses, and non-challenged controls (NV/NC). Data presented as mean cytokine concentration (pg ml⁻¹) and SEM per group. Asterisks indicate statistically significant differences between vaccine treatment groups and non-vaccinated challenge groups (NV) (*P<0.05; **P<0.005; ***P<0.0005).
with HA mismatched from the challenge virus (Gauger et al., 2011, 2012). Vaccination is the most effective control measure against IAV infection, and current vaccination strategies and vaccine design are based on stimulating HA-specific immunity. Nevertheless, the ever-changing nature of influenza HA can lead to immune escape and vaccine mismatch due to the lack of relatedness between vaccine and circulating strains, potentially resulting in VAERD. Most vaccines available in the USA contain inactivated virus formulated with oil-in-water-based adjuvants, and this formulation could contribute to the pathology observed in VAERD. In our previous studies (Gauger et al., 2011; Rajao et al., 2014; Vincent et al., 2008a), there was considerable mismatch in the HA and NA viral genes between vaccine and challenge viruses, although our focus was on the HA antigenic mismatch. However, VAERD has been reproduced in pigs with different combinations of vaccine and challenge viruses, although our most frequent model uses MN/08 and CA/09 either as vaccine or challenge. MN/08 and CA/09 were the parental viruses from which the HA and NA were derived to generate the reverse genetics viruses in this study. By reverse engineering viruses with HA, NA and NP mismatches, we demonstrated that cross-reacting immune responses against NA and perhaps other viral proteins abrogated the VAERD phenomenon induced with HA and NA mismatch, although the immune response targeted to the NP protein did not seem to have a major impact on the clinical outcome.

Although the immune response against mismatched HA protein alone was shown to be enough to cause VAERD (Rajao et al., 2014), here we demonstrated that NI immunity to the homologous NA was sufficient to abrogate it. The presence of high-titre NI antibodies (Fig. 3c, d) induced by the vaccines was associated with an absence of VAERD with the same HA mismatch that we demonstrated to initiate VAERD in previous studies (Gauger et al., 2011; Vincent et al., 2008a). Surprisingly, not only did the vaccines containing matched NA with mismatched HA prevent the occurrence of VAERD, they also conferred partial protection against infection and disease (Figs 1 and 2). However, it did not appear that the NP selected for use in the vaccine strains played a major role in the mechanisms of protection or pathology, since both vaccine groups had equivalent immune responses and were partially protected from challenge. NA-specific antibodies do not neutralize infection but instead are thought to prevent release of new progeny to limit transmission and reduce severity of disease (Johansson & Cox, 2011; Sylte & Suarez, 2009). Pre-existing homologous NA immunity has been shown to contribute to reduced viral shedding and severity of symptoms in humans infected with an HA that is significantly antigenically drifted (Rott et al., 1974).

During the events of the 2009 influenza pandemic, the use of seasonal vaccines and presence of non-neutralizing antibodies against the H1N1pdm09 were correlated with increased risk of more severe influenza-like illness in infected people (Skowronski et al., 2010; To et al., 2012). This suggests that our model of immunization followed by influenza challenge in swine could have relevance to human influenza infection with non-seasonal influenza viruses and/or pandemics. Recent genomic studies of the 1918 pandemic H1N1 viruses suggest that there were at least two H1 viruses circulating during the pandemic that may have differed antigenically (Worobey et al., 2014), raising the possibility of antigen-dependent enhancement. Differential exposure in early life to distinct influenza viruses, even heterosubtypic strains, has been hypothesized to explain the unusual young adult mortality peak in 1918 (Shanks & Brundage, 2012; Worobey et al., 2014). The evidence here that VAERD due to mismatched HA immunity could be blocked by homologous NA immunity is an especially intriguing observation given that there were three separate waves of the 1918 influenza pandemic, one in early 1918 with little mortality despite much morbidity, followed by the second and third waves in late 1918–early 1919 that killed millions (Taubenberger & Morens, 2006), suggesting that they were caused by different virus strains with antigenically distinct HA and NA. Indeed, our swine influenza model shows that differences in viral antigenicity can cause large effects following infectious challenge, including severe immunopathology, which could explain the high mortality and/or severe disease outcomes observed during these human pandemics.

The activation of non-neutralizing antibodies upon influenza virus infection can be associated with antibody-dependent cellular cytotoxicity (Jegaskanda et al., 2013), which in favourable outcomes may provide broader cross-protection and a potential mechanism for universal influenza vaccines (Jegaskanda et al., 2014). However, if the non-neutralizing antibodies lead to increased infectivity of virus in a non-Fc receptor dependent manner, similar to what was observed for cross-reactive anti-HA2 antibodies in VAERD-affected pigs (Khurana et al., 2013), resulting in either increased virus replication or excessive inflammatory signals, the outcome may be immune-pathology. Low avidity, non-neutralizing antibodies may also result in immune complex formation and activation of the complement cascade (O’Brien et al., 2011), shown to be associated with increased disease severity in young adults during the 2009 pandemic (Monsalvo et al., 2011). Moreover, excessive levels of cytokines were correlated to severe lung pathology in mice infected with the 1918 pandemic and an H5N1 IAV (Perrone et al., 2008), and secretion of pro-inflammatory cytokines after IAV infection has also been associated with increased neutrophil infiltration in infected pigs (Van Reeth et al., 1998), as well as enhanced pneumonia in VAERD-affected pigs (Gauger et al., 2012). Our findings suggest that non-neutralizing, low-avidity antibodies to the mismatched challenge strain stimulated by WIV vaccination are capable of activating the complement cascade and, in association with the cytokine dysregulation trend observed here, could be detrimental to the host in the absence of neutralizing protective immunity. Additionally, although the reverse-engineered 6H1N2(B) vaccine in Study 2 was weaker in inducing homologous antibody responses as measured in the HI and SN assays (Fig. 3), pigs in this group still
exhibited low but detectable antibodies in the ELISA against the challenge strain and, more importantly, still developed VAERD upon heterologous challenge. This suggests that even relatively low levels of non-neutralizing antibodies can induce the mechanisms that lead to VAERD.

Influenza continues to cause a significant burden to the swine industry and public health, despite the availability and consistent use of vaccination and antiviral drugs, and the search continues for more effective or universal vaccine strategies. Along with the impact of the NA in our VAERD model, there is growing evidence on the advantages of considering the NA match in vaccine antigens to improve overall immunogenicity and range of cross-protection against antigenically distinct HA viruses (Johansson & Cox, 2011; Marcelin et al., 2012; Sylte & Suarez, 2009). We showed that immune responses specific to influenza virus NA, and perhaps other internal viral proteins, resulted in significant cross-protection independent of the HA antigenic relatedness, preventing the development of disease enhancement. These data may provide an explanation for human health consequences after emergence of pandemic viruses with HA and NA mismatch to previously circulating strains, and also provide important information for future vaccine strain and platform selection in human and animal populations.

**METHODS**

**Vaccine and challenge viruses.** WT viruses included A/swine/Minnesota/02011/2008 ΔH1N2(2A) (MN/08) and A/California/04/2009 H1N1pdm09 (CA/09). To test the role of the NP gene in the context of an isogenic backbone with matched and mismatched HA, a well-characterized swine-lineage IAV backbone, A/turkey/Ohio/313053/2004 (H9N2) (OH/04) (Pena et al., 2011; Tang et al., 2005), was used to engineer identical viruses that differed only in the HA, NP or NA genes. The H1 genes from MN/08 (ΔH1) and CA/09 (pH1), the N1 from CA/09 (pN1) and the 2002 N2(B) from OH/04 in combination with the remaining genes from OH/04 were cloned in the bidirectional reverse genetics system with the pDP-2002 vector derived from the pHW-2000 vector as previously described (Hoffmann et al., 2000). The NP from A/ guinea foal/HK/WF10/1999 (H9N2) (WF10) (Wan et al., 2008) was selected on the basis of overall divergence from the OH/04 NP gene (93% identity), specifically differing at amino acid positions 423 and 425 in the putative T-cell receptor epitope NP418-426 (Gras et al., 2010). The WF10 NP (avNP) was cloned, and a recombinant virus rescued with the MN/08 ΔH1 and remaining genes from OH/04. The resulting RG-engineered viruses were ΔH1N2(B), pH1pN1, pH1N2(B) and ΔH1N2(B):avNP. All viruses used are listed in Table 1.

To understand the role of the matched and mismatched N2 genes based on the results of the study above, a follow-up experiment was conducted. The NA sequences of the WT MN/08 and of the OH/04 isolate used as the RG backbone were aligned with an additional 98 N2 sequences of IAV representing both lineages currently circulating in the USA. A maximum-likelihood tree was inferred using MEGA 6.06 (Tamura et al., 2013), with a general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites and statistical support of 1000 bootstraps. The 1998-lineage N2(A) from MN/08 has 92% amino acid identity with the 2002-lineage N2(B) of the OH/04 (Fig. 5). The RG ΔH1N2(B) used as a challenge virus from Study 1 was therefore used as a vaccine strain and compared to ΔH1N2(A) WIV in pigs challenged with RG pH1pN1 or pH1N2(B).

Vaccine strains were propagated in embryonated chicken eggs and all vaccines were adjuvanted WIV. The WIV were prepared using UV-irradiated viruses at 128 HA units with the addition of a commercial oil-in-water adjuvant (Emulsigen D, MVP Laboratories) at a v/v ratio of 4:1 virus to adjuvant. Challenge viruses were propagated in Madin–Darby canine kidney (MDCK) cells. All viruses used as vaccine and challenge strains in both in vivo pig studies are summarized in Table 2.

**In vivo studies.** Three-week-old, cross-bred pigs were obtained from a high-health status herd free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV). Pigs were treated with ceftriaxone crystal-line free acid (Pfizer Animal Health) and enrofloxacin (Bayer Animal Health) to reduce bacterial contaminants prior to the start of the studies. Pigs were housed in biosafety level 2 (BSL2) containment and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center.

Study 1 was designed to evaluate a potential role for NP in VAERD. Fifteen pigs were vaccinated with ΔH1N2(B) or ΔH1N2(B):avNP. Two groups remained non-vaccinated (NV). Five pigs from each vaccine group and five NV pigs were euthanized prior to challenge to evaluate immune responses to vaccine alone. Ten pigs from each of the two vaccinated groups and 10 from the NV group were challenged with virus pH1N2(B). Ten NV pigs were not challenged (NV/NC), to serve as negative controls. All remaining pigs were euthanized at 5 days p.i. for post-mortem evaluation. Based on the results of Study 1, Study 2 was designed to investigate the role of NA in the VAERD model. Ten pigs each were vaccinated with ΔH1N2(B) and challenged with pH1pN1 or vaccinated with ΔH1N2(A) and challenged with pH1N2(B). All pigs were euthanized at 5 days p.i. for post-mortem evaluation.

In both studies, pigs were vaccinated intramuscularly at approximately 5 and 7 weeks of age and challenged at 10 weeks of age. Pigs were infected with 3 ml of 1 × 10^7 TCID_{50} ml^{-1} of each challenge virus, delivered as 2 ml intratracheal and 1 ml intranasal inoculations during the same

**Table 1.** Viruses used in Studies 1 and 2 as vaccine or challenge antigens

<table>
<thead>
<tr>
<th>Virus</th>
<th>HA</th>
<th>NA</th>
<th>Internal Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH1N2(B)</td>
<td>pH1 (CA/09)*</td>
<td>N2(B) (OH/04)</td>
<td>OH/04</td>
</tr>
<tr>
<td>ΔH1N2(B)</td>
<td>ΔH1 (MN/08)</td>
<td>N2(B) (OH/04)</td>
<td>OH/04</td>
</tr>
<tr>
<td>ΔH1N2(B):avNP</td>
<td>ΔH1 (MN/08)</td>
<td>N2(B) (OH/04)</td>
<td>OH/04 with avNP (WF10)</td>
</tr>
<tr>
<td>pH1pN1</td>
<td>pH1 (CA/09)</td>
<td>pN1 (CA/09)</td>
<td>OH/04</td>
</tr>
<tr>
<td>ΔH1N2(A)†</td>
<td>ΔH1 (MN/08)</td>
<td>N2(A) (MN/08)</td>
<td>MN/08</td>
</tr>
</tbody>
</table>

*CA/09, A/California/04/2009 (H1N1); MN/08, A/swine/Minnesota/02011/2008 (H1N2); OH/04, A/turkey/Ohio/313053/2004 (H3N2); WF10, A/guinea fowl/HK/WF10/1999 (H9N2).
†ΔH1N2(A) was a WT isolate.
challenge procedure. Inoculation was performed under anesthesia, using an intramuscular injection of a cocktail of ketamine [8 mg (kg body weight)$^{-1}$; Phoenix], xylazine (4 mg kg$^{-1}$; Lloyd) and Telazol (6 mg kg$^{-1}$; Zoetis Animal Health). Nasal swabs (Fisherbrand Dacron swabs, Fisher Scientific) were collected on 0, 1, 3 and 5 days p.i. Serum samples were collected prior to vaccination, prior to boost, before challenge (0 days p.i.) and at necropsy (5 days p.i.). Pigs were humanely euthanized with a lethal dose of pentobarbital (Fatal Plus, Vortech Pharmaceuticals). Post-mortem samples included BALF, trachea and right cardiac or affected lung tissue.

**Diagnostic microbiology.** Prior to the start of the studies, all pigs were screened for antibodies against influenza A nucleoprotein by ELISA (MultiS ELISA, IDEXX) to confirm the absence of influenza exposure. BALF samples were screened for aerobic bacteria on blood and Casmin (NAD-enriched) agar plates, and nucleic acid was extracted from BALF using the MagMax Viral RNA/DNA Isolation kit (Life Technologies) to detect potential confounding respiratory pathogens. PCR were conducted for PCV2 (Applied Biosystems), *Mycoplasma hyopneumoniae* (VetMax, Applied Biosystems) and PRRSV (VetMax NA and EU PRRS-specific PCR, Life Technologies), according to the manufacturers’ recommendations.

**Viral replication and shedding.** Nasal swab specimens were used for virus isolation, as previously described (Vincent et al., 2012). BALF and virus-isolation-positive nasal swab samples were 10-fold serially diluted in serum-free Opti-MEM (Gibco, Life Technologies) supplemented with 1 µg ml$^{-1}$ tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin and antibiotics and used for virus titration in MDCK cells as previously described (Kitikoon et al., 2006). Virus titre was calculated for each sample as TCID$_{50}$ ml$^{-1}$ according to the method of Reed & Muench (1938).

**Pathological examination of the trachea and lungs.** At necropsy, lungs were evaluated for the percentage of the lung affected by consolidation typical of IAV infection. The percentage of the surface affected with pneumonia was calculated as previously described (Gauger et al., 2011; Halbur et al., 1995). Trachea and lung tissue samples were fixed in 10% buffered formalin, routinely processed and stained with hematoxylin and eosin. Microscopic lesions were evaluated by a veterinary.
pathologist blinded to treatment groups and scored according to previously described parameters (Gauger et al., 2012). An individual composite score for each pig was computed for lung and trachea microscopic lesions on a scale from 0 to 21.

**IAV antibody detection.** Sera were heat-inactivated at 56 °C for 30 min and treated to remove non-specific HA inhibitors and natural serum agglutinins, according to standard techniques (WHO, 2002). HI assays were performed with MN/08 δH1N2(A) and CA/09 antigens and turkey red blood cells (RBCs) using standard techniques (WHO, 2002). Antibody titres were reported as geometric means. Sera were heat-inactivated at 56 °C for 30 min for the SN assay. Samples were then twofold serially diluted from 1:1 to 1:20,480 in 96-well plates in serum-free minimal essential media (MEM, ThermoFisher Scientific) supplemented with TPCK-trypsin and antibiotics. SN assays were performed with δH1N2(B) and CA/09 antigens as previously described (Gauger & Vincent, 2014). Titres were reported as the geometric means.

ELISAs were performed as previously described (Vincent et al., 2012) to detect total isotype-specific antibodies against δH1N2(B), pH1pN1, δH1N2(B):avNP (data not shown) or pH1N2(B) in serum and BALF. Assays were conducted using concentrated virus diluted to 100 HA units per 50 µL. The OD was measured at 405 nm wavelength with an automated ELISA reader. Antibody levels were reported as the mean OD of duplicates and the avidity index, expressed as a percentage, was calculated for each animal as the ratio of the mean OD with urea to the mean OD without urea.

**NI assay.** Based on the implications of matching or mismatching the NA to the vaccine strain in the VAERD model, test antigens with targeted N2(A) or N2(B) were paired with irrelevant non-H1 subtypes and tested in NI assays. Viruses were derived by RG (Hoffmann et al., 2008) and cultivated in MDCK cells. The H9N2 HA of A/guinea fowl/HK/WF10/1999 (Wan et al., 2008) and the MN/08 N2(A), representing the swine 1998-lineage (Nelson et al., 2012). The second virus, H3N2(C), contained all eight gene segments from the OH/04 strain, including the N2(B) gene representing the swine 2002-lineage (Nelson et al., 2012).

Serum NI titres were measured using an assay described previously (Lambre et al., 1990; Sandbulte & Eichelberger, 2014). Briefly, NA enzyme activity of a virus was quantified by sialic acid cleavage from fetuin on 96-well plates during an overnight incubation at 37 °C. Peanut agglutinin horseradish peroxidase conjugate (PNA-HRP) was then added for 2 h at room temperature, binding to fetuin molecules stripped of sialic acid. Signal was obtained with O-phenylenediamine dihydrochloride (OPD) substrate, and read at 490 nm. Test antigens were titrated to determine the highest dilution able to yield maximum signal. For NI antibody titration, serial dilutions of serum were mixed with virus at equal volumes in duplicate fetuin-coated wells during overnight incubation at 37 °C. The NI titre of each serum sample was defined as the highest dilution that resulted in ≥50% inhibition of NA activity.

**IgG avidity ELISA.** Modified ELISA assays were performed based on previously described methods (Monsalvo et al., 2011; Vincent et al., 2012) to measure the avidity of the total serum IgG identified in the whole virus ELISA above. Independent assays were conducted using concentrated δH1N2(B), δH1N2(A), pH1N2(B) or pH1pN1 diluted to 100 HA units per 50 µL. Sera from δH1N2(B)-vaccinated pigs in Studies 1 and 2 were used in a 1 : 1000 dilution. Samples were incubated with PBS or increasing urea concentrations (3 M, 6 M, 7 M, 8 M and 9 M) for 10 min at room temperature before incubation with secondary antibody. The OD was measured at 405 nm wavelength with an automated ELISA reader. Antibody levels were reported as the mean OD of duplicates and the avidity index, expressed as a percentage, was calculated for each animal as the ratio of the mean OD with urea to the mean OD without urea.

**Complement fixation test.** To test the ability of the lower-avidity cross-reacting serum IgG antibodies identified in the ELISA assays to fix complement, a complement fixation test (CFT) was performed as previously described (Constantine & Lana, 2003), with modifications. Briefly, heat-inactivated serum from δH1N2(B)-vaccinated pigs from Studies 1 and 2 were diluted twofold from 1:10 to 1:640 in gelatin veronal buffer (GVB; Sigma-Aldrich) in 96-well ‘U’-bottom plates and mixed with virus antigens at 256 HA units. Guinea pig complement (Sigma-Aldrich) was added and incubated overnight at 4 °C. Haemolysins (Rockland)-sensitized sheep RBCs (1% suspension) were then added and incubated at 37 °C for 30 min. Titres were defined as the reciprocal of the highest serum dilution showing complement fixation (less than 50% haemolysis). Antibody titres were reported as geometric means. Viruses used as antigens were δH1N2(B), δH1N2(A), pH1N2(B) or pH1pN1 to test the individual roles of matched or mismatched HA and NA antibodies to fix complement.

**IFN-γ ELISPOT assay.** At 0 days p.i. in Study 1, blood was collected using sodium citrate CPT tubes (BD Vacutainer), and PBMC were separated according to the manufacturer’s recommendations. The IFN-γ ELISPOT assay was performed according to the manufacturer’s recommendations (Porcine IFN-γ ELISPOT assay; R&D Systems). Briefly, wells were seeded with 5×10^5 PBMC in 96-well membrane plates provided in the ELISPOT kit and stimulated with 50 µL containing live δH1N2(B), δH1N2(B):avNP or pH1N2(B) viruses at an m.o.i. = 1, or 3 µg ml^−1 of Concanaavalin A as a positive control, or conditioned media as a negative control. Plates were scanned and the spots counted performed using a CTL-ImmuNoSpot S5 UV Analyzer and ImmunoSpot 5 software. The reported values were calculated from the mean number of spots counted for triplicate wells with each virus minus conditioned media stimulation.

**Cytokine assays.** Levels of pro-inflammatory and anti-inflammatory cytokines were determined in cell-free BALF using a multiplex ELISA according to the manufacturer’s recommendations (SearchLight; AusPhon Biosystems), as dysregulation of these cytokines was previously associated with the development of VAERD (Gauger et al., 2012; Rajao et al., 2014). Samples were analysed in duplicate and results were averaged. Data are reported for only cytokines showing differences as the mean±SEM for pigs in each treatment group.

Table 2. Vaccination and challenge treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine virus</th>
<th>Challenge virus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NV/NC*</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NV/pH1N2(B)*</td>
<td>None</td>
<td>pH1N2(B)</td>
</tr>
<tr>
<td>δH1N2(B)/pH1N2(B)</td>
<td>δH1N2(B)</td>
<td>pH1N2(B)</td>
</tr>
<tr>
<td>δH1N2(B):avNP/pH1N2(B)</td>
<td>avNP</td>
<td>pH1N2(B)</td>
</tr>
<tr>
<td><strong>Study 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NV/NC*</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NV/pH1pN1</td>
<td>None</td>
<td>pH1pN1</td>
</tr>
<tr>
<td>δH1N2(B)/pH1pN1</td>
<td>δH1N2(B)</td>
<td>pH1pN1</td>
</tr>
<tr>
<td>NV/pH1N2(B)*</td>
<td>None</td>
<td>pH1N2(B)</td>
</tr>
<tr>
<td>δH1N2(A)/pH1N2(B)</td>
<td>δH1N2(A)</td>
<td>pH1N2(B)</td>
</tr>
</tbody>
</table>

*Data from Studies 1 and 2 for NV/NC and NV/pH1N2(B) groups were combined for analysis.

http://gvj.microbiologyresearch.org

1497
Statistical analysis. Macropscopic pneumonia, composite microscopic lung and trachea scores, cytokine ELISA concentrations, IFN-γ spot counts, OD from antibody detection ELISA assays, avidity indices, log₁₀-transformed virus titres and log₂-transformed HI, CFT and NI reciprocal titres were analysed using ANOVA, with P<0.05 considered significant (Prism software, GraphPad). Response variables shown to have significant effects by treatment group were subjected to pairwise mean comparisons using the Tukey–Kramer test.

ACKNOWLEDGEMENTS

We thank Michelle Harland, Gwen Nordholm, Zahra Olson and Katie Gibson for technical assistance; Jason Hugel, Jason Crabtree and Tyler Standley for assistance with animal studies; and Dr Susan Brockmeier for assistance with microbiologic screening. We also thank Dr John Brundage for his helpful insight at all stages of these studies. This study was supported by USDA-ARS, USDA-APHIS and DOD-AFHSB. D. S. R. was a CNPq-Brazil fellowship recipient. USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. The opinions expressed are those of the authors and do not necessarily reflect those of the Australian Defence Force or the US Department of Defense.

REFERENCES


