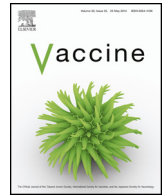




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Poly I:C adjuvanted inactivated swine influenza vaccine induces heterologous protective immunity in pigs



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ABSTRACT

Swine influenza is widely prevalent in swine herds in North America and Europe causing enormous economic losses and a public health threat. Pigs can be infected by both avian and mammalian influenza viruses and are sources of generation of reassortant influenza viruses capable of causing pandemics in humans. Current commercial vaccines provide satisfactory immunity against homologous viruses; however, protection against heterologous viruses is not adequate. In this study, we evaluated the protective efficacy of an intranasal Poly I:C adjuvanted UV inactivated bivalent swine influenza vaccine consisting of Swine/OH/24366/07 H1N1 and Swine/CO/99 H3N2, referred as PAV, in maternal antibody positive pigs against an antigenic variant and a heterologous swine influenza virus challenge. Groups of three-week-old commercial-grade pigs were immunized intranasally with PAV or a commercial vaccine (CV) twice at 2 weeks intervals. Three weeks after the second immunization, pigs were challenged with the antigenic variant Swine/MN/08 H1N1 (MN08) and the heterologous Swine/NC/10 H1N2 (NC10) influenza virus. Antibodies in serum and respiratory tract, lung lesions, virus shedding in nasal secretions and virus load in lungs were assessed. Intranasal administration of PAV induced challenge viruses specific-hemagglutination inhibition- and IgG antibodies in the serum and IgA and IgG antibodies in the respiratory tract. Importantly, intranasal administration of PAV provided protection against the antigenic variant MN08 and the heterologous NC10 swine influenza viruses as evidenced by significant reductions in lung virus load, gross lung lesions and significantly reduced shedding of challenge viruses in nasal secretions. These results indicate that Poly I:C or its homologues may be effective as vaccine adjuvants capable of generating cross-protective immunity against antigenic variants/heterologous swine influenza viruses in pigs.

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1. Introduction

The genetic diversity of swine influenza A virus (SIV) in North America has increased in the last two decades. However, the majority of the SIV infections in pigs are caused by subtypes H1N1, H1N2 and H3N2 [1]. Emergence of the H3N2 subtype containing a triple reassortment internal gene (TRIG) cassette contributed vastly to the generation of antigenic divergent reassortant viruses [2,3]. The hemagglutinin (HA) gene in these H3N2 viruses was derived from

the different seasonal human influenza viruses. Subtypes containing H1 also exhibited a high rate of divergence and are currently classified into clusters α , β , γ and δ . The emergence of the 2009 H1N1 pandemic virus (H1N1 pdm09) and its subsequent reassortments with the recent H3N2 variant increased the antigenic variation of SIV [4–7]. A combination of some of the HA gene alleles and TRIG cassettes might be contributing towards survival and propagation of emerging SIV variants in pigs [8]. Establishment of these antigenic variants in the swine population poses a zoonotic threat as they can be transmitted to humans. Current vaccine approaches are inadequate to counter the antigenic diversity of SIV because the vaccine-derived protective immunity is typically strain-specific [9,10].

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Vaccination against SIV is routinely employed in swine farms. Most of the commercial vaccines are bivalent or trivalent and contain whole inactivated virus. The SIV strains used in these vaccines vary between regions and their protective efficacies depend on the strains prevalent in those areas. Although inactivated vaccines are effective against homologous strains, only limited protection is offered against heterologous strains [11,12]. Moreover, inactivated SIV vaccines are also associated with development of vaccine-associated enhanced respiratory disease (VAERD) [13,14]. This happens when the vaccine and challenge strains belong to the same subtype but differ due to antigenic drift. Another weakness of currently employed commercial inactivated vaccines is that these products are administered by an intramuscular route and do not induce adequate mucosal immunity [15]. This is important because cross-protective activity of influenza vaccines is largely correlated to mucosal immunity. Intranasal administration of live attenuated SIV vaccines containing virus with truncated NS1 protein [16] and modified HA protein [17,18] developed both mucosal and humoral antibodies in different animal species. Similarly, an intranasal inoculation of seasonal trivalent inactivated vaccine provided mucosal immunity in mice [19]. These vaccines provided protection against both homologous and heterologous strains. Intranasal vaccine administration induced a higher secretory IgA production when compared with administration by the parenteral route. The IgA antibodies, which have higher avidity than IgG antibodies, can readily access mucosal viral antigens and are able to provide protection against heterologous strains [20]. Furthermore, use of an effective mucosal adjuvant in conjunction with intranasal vaccine administration could enhance vaccine efficiency. Poly (I:C), a synthetic double-stranded RNA, has been demonstrated as a potent adjuvant capable of enhancing the host innate immune response. Intranasal administration of a bivalent inactivated influenza virus vaccine along with poly (I:C) protected mice from heterologous strains [19].

In this study, we evaluated the immunogenicity and protective efficacy of poly (I:C) adjuvanted bivalent inactivated SIV vaccine (PAV) in commercial pigs. Intranasal administration of PAV in pigs induced IgA antibody response in respiratory tract and provided protection against challenge with the antigenic variant MN08 and the heterologous NC10 SIV.

2. Materials and methods

2.1. Cells, viruses and experimental and commercial vaccines

Madin-Darby Canine Kidney (MDCK) cells and MK1-OSU cells were grown in DMEM supplemented with 10% FBS, 1% antibiotics and 2 mM L-glutamine. MK1-OSU is a newly established spontaneously immortalized cell line derived from the distal trachea and proximal lung tissue of a 5–6 week-old pig. These cells express avian and mammalian influenza virus receptors and support the replication of swine, avian and human-origin influenza viruses (unpublished results).

Swine influenza viruses, Swine/Ohio/24366/07 H1N1 (γ -cluster) [21] and Swine/CO/99 H3N2 (cluster II), were used to prepare an experimental vaccine in MK1-OSU cells. CO99 virus was obtained from Dr. J.A. Richt, Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS. To prepare the vaccine stocks, MK1 cells were infected with these viruses in the presence of 1 μ g tolylsulfonyl phenylalanyl chloromethyl ketone-treated (TPCK) trypsin. After 48 h, the virus-infected cultures were frozen and thawed three times. The cellular debris was cleared by centrifugation and supernatants were inactivated by a UV irradiation using a UV lamp at 254 nm

Table 1

Experimental groups for the evaluation of protective efficacy of PAV in pigs.

Groups	Vaccination	Challenge	Number of pigs
1	None	None	4
2	None	H1N1 ^a	4
3	None	H1N2 ^b	4
4	PAV	H1N1	4
5	PAV	H1N2	4
6	CV	H1N1	4 ^c
7	CV	H1N2	4

^a MN08.

^b NC10.

^c One pig in this group was euthanized before challenge as it developed respiratory illness after vaccination.

for 40 min (UVS-28 EL series; Entela, Upland, CA). Virus inactivation was confirmed by the inability of these viruses to replicate in MDCK and MK1-OSU cells. Swine/Minnesota/2073/2008 (H1N1; MN08, α -cluster) and Swine/North Carolina/0036-2/2010 (H1N2; NC10, δ -cluster) were used as challenge viruses. The HA amino acid homology between MN08 and NC10 is 67%. Challenge virus stocks were prepared in MDCK cells as described previously [22].

A commercial vaccine containing killed swine H1N1, H1N2 and H3N2 viruses with adjuvant was also included in the study (PneumoSTAR SIV Complete (Novartis Animal Health US, Inc., Larchwood, IA).

2.2. Pigs

Twenty-eight three-week-old weaned pigs were obtained from a commercial herd. The pigs were derived from sows which were immunized with MaxiVac Excell(r) 5.0 (Intervet) swine influenza vaccine. The pigs were acclimatized for 5 days before vaccine inoculation. Maintenance of pigs and all experimental procedures were conducted in accordance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, South Dakota State University.

2.3. Experimental design and sample collection

Groups of three-week-old commercial influenza antibody-positive pigs were inoculated intranasally with UV-inactivated vaccine containing OH07 and CO99 (each 5×10^6 TCID₅₀ per pig) and Poly I:C (300 μ g per pig; PAV) or a commercial vaccine (CV) (at manufacturer's recommended dose) twice at 2 weeks' intervals. In a previous study Poly I:C (300 μ g/pig) was shown to enhance the immunogenicity of foot and mouth disease (FMDV) multi-epitope vaccine and Poly I:C adjuvanted vaccine provided complete protection against virulent FMDV challenge [23]. The pigs were housed in separate isolation units. At the time of booster vaccination, three weeks after the 2nd immunization before challenge, we collected the blood to examine HI titers. Three weeks after the 2nd immunization, pigs were challenged with the antigenic variant MN08 H1N1 and the heterologous NC10 H1N2 viruses (5×10^6 TCID₅₀ per pig) by the intranasal route (Table 1). Pigs were monitored daily for clinical signs after challenge until they were euthanized. Nasal swabs were collected at 3 and 6 days post-challenge (DPC) for virus titration in MDCK cells. At DPC 6, pigs were euthanized by pentobarbital administration and lungs were examined for gross pneumonic lesions and lung lysate was prepared for virus quantification.

Gross lung lesions were evaluated as described previously [22,24]. Total percent of consolidated area (purple colored) was calculated based on weighted proportions of each lobe to the total

lung volume. A score was assigned based upon the percentage of virus-affected lesions in each lung lobe.

2.4. Detection of challenge virus in nasal swabs and lungs

A 10% (w/v) homogenate of individual lung lobes (left cranial, left caudal, right cranial, right middle, right caudal and accessory lobe) was prepared in DMEM with antibiotics using a tissue homogenizer (Omni homogenizer GLH) and Omni Tip™ Plastic Generator Probes (Omni International). Debris was removed by centrifugation (10 min at ~1500 g) and the supernatant collected and filtered through 0.45 µm filters.

Serial tenfold dilutions of nasal swabs and lung homogenates were prepared in DMEM supplemented with 1 µg of TPCK trypsin/ml. MDCK cells cultured in 96-well tissue-culture plates were inoculated with the dilutions and were examined for cytopathic effects after 48–72 h incubation at 37 °C. Virus titers were calculated by the Reed and Muench method [25].

2.5. Hemagglutination inhibition (HI) test

Cross-reactive HI antibodies in the sera of PAV and CV-immunized pigs were detected by standard HI assay (WHO manual). Blood was collected at the time of booster vaccination and before challenge. HI antibodies were detected against the challenge viruses MN08 and NC10.

2.6. IgG and IgA ELISA

Challenge virus-specific IgA and IgG antibodies in lung lysate and IgG in serum were measured by ELISA as described previously [26] with few modifications. Briefly, Maxisorp 96-well plates (Thermo Scientific) were coated overnight with 100 HA units of MN08 or NC10 at RT. The plates were blocked for 2 h at RT with 100 µl of 2.5% fat free milk in phosphate buffered saline containing Tween 20 (PBST, 0.1 M Tris, 0.17 M NaCl, 0.05% Tween 20; blocking buffer) and then washed four times with PBST. Serum samples diluted to 1:500 and lung lysate samples diluted to 1:4 in blocking buffer were added (100 µl/well) in duplicate and the plates were incubated for 2 h at RT. After washing four times, the plates were incubated with HRP labeled anti-porcine IgA (Bethyl labs) and IgG antibody (KPL) for 2 h. After washing, the plates were developed using ABTS substrate and optical density (OD) was measured at 405 nm (Molecular Devices SpectraMax Plus 384).

2.7. Statistical analysis

Lung gross lesions, log₂ HI titers, log₁₀ virus titers in nasal swabs and lungs and ELISA OD values between immunized and non-immunized pigs were compared using One-Way ANOVA followed by Tukey's test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. PAV immunized pigs had cross-reactive HI and serum IgG antibodies

We measured serum HI antibodies against challenge viruses; MN08 and NC10 at 14 and 35 DPV. Pigs in the non-vaccinated group had minimal HI antibodies against either antigen at 14 or at 35 DPV. Pigs in the CV-inoculated group had mean MN08 specific HI titers of 108 and 88 at 14 and 35 days-post-vaccination (DPV) respectively, whereas PAV induced a MN08 specific titer of 146 at 14 DPV and 160 at 35 DPV in immunized pigs (Fig. 1A). NC10 virus-specific mean HI titers are shown in Fig. 1B. Both CV and PAV induced HI antibodies

against NC10. HI titers were slightly higher in the CV-immunized pigs at 14 DPV compared to PAV-immunized pigs, whereas at 35 DPV, NC10-specific HI titers were higher in the PAV group (Fig. 1B).

We also determined the IgG antibody response in serum against MN08 and NC10 in immunized pigs before challenge and at DPC 6. Low levels of IgG antibodies against MN08 and NC10 were detected in the non-vaccinated and non-vaccinated but challenge groups. Serum IgG levels against MN08 and NC10 were slightly higher in the CV-immunized group at both DPC 0 and DPC 6 than PAV group. However, this increase was not significant ($P > 0.05$) (Fig. 2A and B).

3.2. PAV induced mucosal IgG and IgA antibodies

The main goal of this study was to examine if Poly I:C adjuvant can enhance protective immunity of an inactivated swine influenza vaccine in the respiratory tract. Therefore, in this study, pigs were immunized through nasal route and we examined the vaccine-induced mucosal antibody response in the respiratory tract. We examined IgG and IgA antibodies in the lung lysate of pigs at DPC 6 (Fig. 3). Low levels of IgG antibodies were observed in non-vaccinated MN08 and NC10-challenged groups. IgG antibodies against MN08 in PAV immunized pigs were slightly higher than in CV-immunized pigs whereas IgG antibodies against the NC10 strain were less than that in the CV-immunized group (Fig. 3A).

Infection of nonvaccinated pigs with MN08 and NC10 induced IgA antibody responses in the lung. MN08- and NC10-specific IgA antibody responses in lungs of PAV immunized pigs were higher than the IgA levels in the lung lysate of CV immunized pigs (Fig. 3B). These results indicate that intranasal immunization of PAV induced a mucosal antibody response.

3.3. PAV provided protection against antigenic variant H1N1 and heterologous H1N2

To assess the protective efficacy of PAV and CV against MN08 and NC10, we compared reductions in lung lesions, virus shedding in nasal secretions and challenge virus titers in the lungs of pigs. MN08 and NC10 infection in non-vaccinated but challenged pigs produced extensive lung lesions (Fig. 4A). Immunized pigs either with PAV or CV had less lung lesions as compared to non-immunized MN08 or NC10 challenged pigs. Mean lung lesions were significantly less ($P < 0.05$) in PAV- and CV-immunized pigs as compared to non-immunized but MN08 challenged pigs. Lung lesions were also less in PAV-immunized pigs than CV-immunized and MN08 challenged pigs (Fig. 4B). Likewise, mean lung lesions in PAV-immunized and NC10 challenged pigs were less ($P = 0.052$) than the non-immunized, but NC10 challenged pigs. Lung lesions were also considerably less in PAV-immunized pigs than lung lesions in CV-immunized and NC10, challenged pigs (Fig. 4B). These data indicate that PAV provided protection against challenge with antigenic variant and heterologous swine influenza viruses.

Next, we determined the challenge virus shedding by examining the virus titers in nasal swabs collected at DPC 3 and 6 by titration in MDCK cells. Virus shedding data are shown in Fig. 5A. No challenge viruses were detected in non-challenged pigs (data not shown). At DPC 3, virus shedding in nasal secretions was significantly lower ($P < 0.05$) in CV and PAV immunized pigs challenged with MN08 as compared to non-immunized but MN08 challenged pigs. Importantly, PAV immunized pigs, challenged with heterologous NC10 SIV showed significantly less ($P < 0.05$) virus shedding at DPC 3 as compared to non-immunized but NC10 challenged pigs (Fig. 5A). At DPC 6, essentially a similar trend was observed, PAV immunized pigs shed significantly less ($P < 0.05$) challenge viruses as compared to non-immunized MN08 and NC10 challenged pigs (Fig. 5B).

We also determined the challenge virus titers in the lungs of immunized and non-immunized challenged pigs collected at DPC

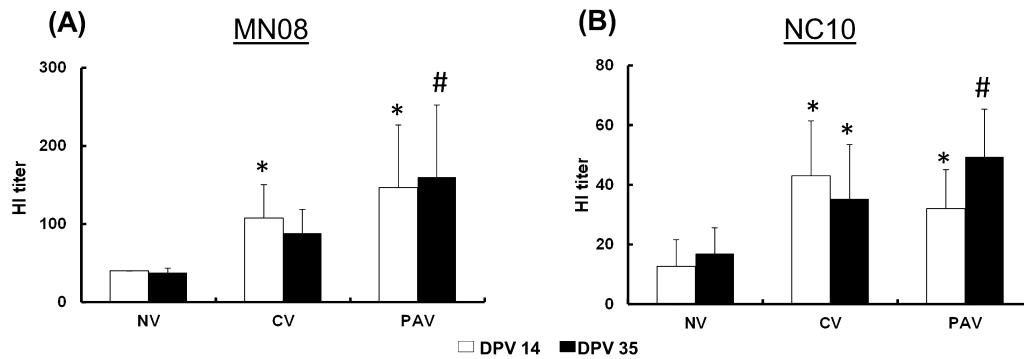


Fig. 1. Serum HI antibodies in vaccinated/challenged pigs. Mean HI titers in serum of pigs immunized either with PAV or CV were determined at DPV 14 and 35 against MN08 (A) and NC10 virus (B). Data are expressed as mean HI titers of four pigs \pm SD. *—significantly different from NV group ($P < 0.05$); #—significantly different from NV and CV groups ($P < 0.05$).

6 by titration in MDCK cells. Both MN08 and NC10 in unvaccinated challenged groups replicated to high titers in lungs (Fig. 5C). MN08 and NC10 virus titers in lungs of PAV immunized pigs were significantly less ($P < 0.05$) than non-immunized MN08 and NC10 challenged pigs (Fig. 5C). Titers of challenge viruses were also lower in CV immunized pigs as compared to non-immunized pigs (Fig. 5C).

4. Discussion

In this study, we evaluated the immunogenicity and cross-protective efficacy of PAV administered intra-nasally in maternal antibody positive conventional pigs. Intranasal administration of PAV induced challenge virus-specific HI and IgG antibodies in serum, IgA antibodies in the respiratory tract and provided protection against challenge with antigenic variant and heterologous SIV.

Vaccine administered by intranasal route induces both mucosal and systemic immune responses while administration by parenteral route elicits only systemic immune responses [19]. Poly I:C, a TLR-3 agonist, when used as adjuvant has been shown to enhance the immune response against influenza [19,27–31]. A

previous study in mice demonstrated that intranasal administration of Poly I:C adjuvanted H5N1 vaccine induced an increased IgA titer in nasal washings and provided protection from both homologous and heterologous virus challenge [27]. A similar finding was reported recently in humans when Ampligen® (synthetic poly I:C) was used as an adjuvant along with FluMist® vaccine [29]. Similarly in our study, the intranasal administration of PAV provided protection against heterologous SIV in pigs and we also observed higher IgA titers in the lungs of PAV vaccinated pigs as compared to CV vaccinated pigs. Therefore, as observed in previous studies in mice and humans [19,27,29], higher IgA titers in the lungs of PAV vaccinated pigs may be involved in inducing cross-protective immunity against antigenic variant H1N1 and heterologous H1N2 SIV.

In addition to boosting the mucosal IgA response, Poly I:C also enhances cell-mediated immunity when used as a vaccine adjuvant. Poly I:C has been shown to enhance the activity of respiratory dendritic cells and helped migration of both effector and memory T cells [32–34]. Intranasal administration of Poly I:C in mice caused T cell proliferation and recruitment of these cells in to airways [33]. In the current study, there was a small increase in serum and lung IgG titers in vaccinated/challenge pigs. Also, the levels of cross-reactive HI antibodies were low in PAV and CV-immunized

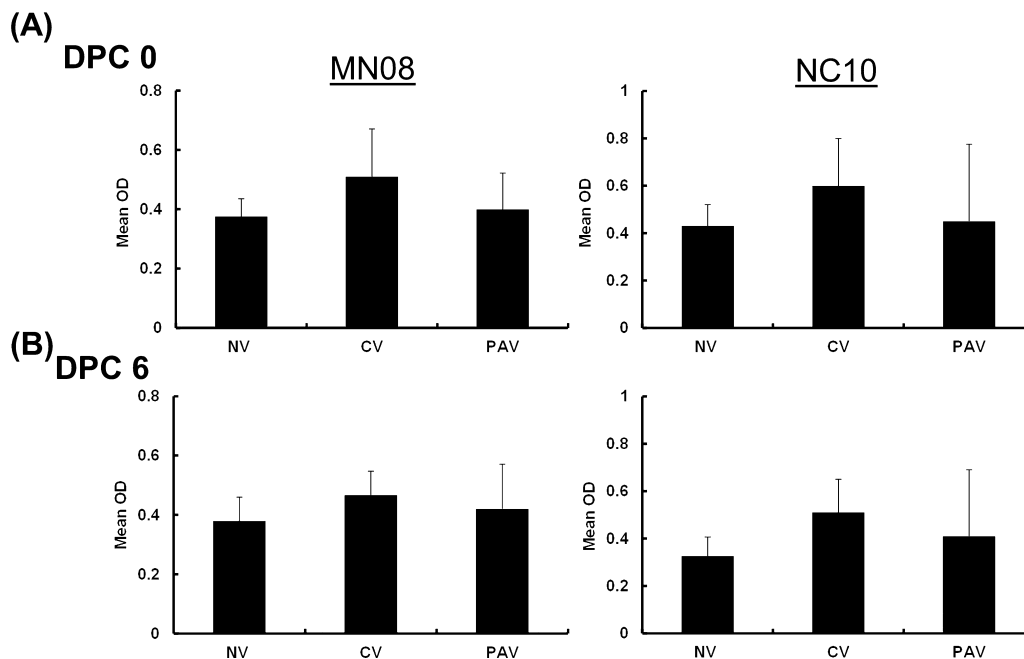


Fig. 2. Serum IgG antibodies in vaccinated/challenged pigs. Challenge virus specific-IgG antibody levels in serum of immunized/challenged pigs were determined by ELISA against MN08 and NC10 at 35 DPV (DPC 0) (A) and at DPC 6 (B). Data are expressed as mean ODs of four pigs \pm SD.

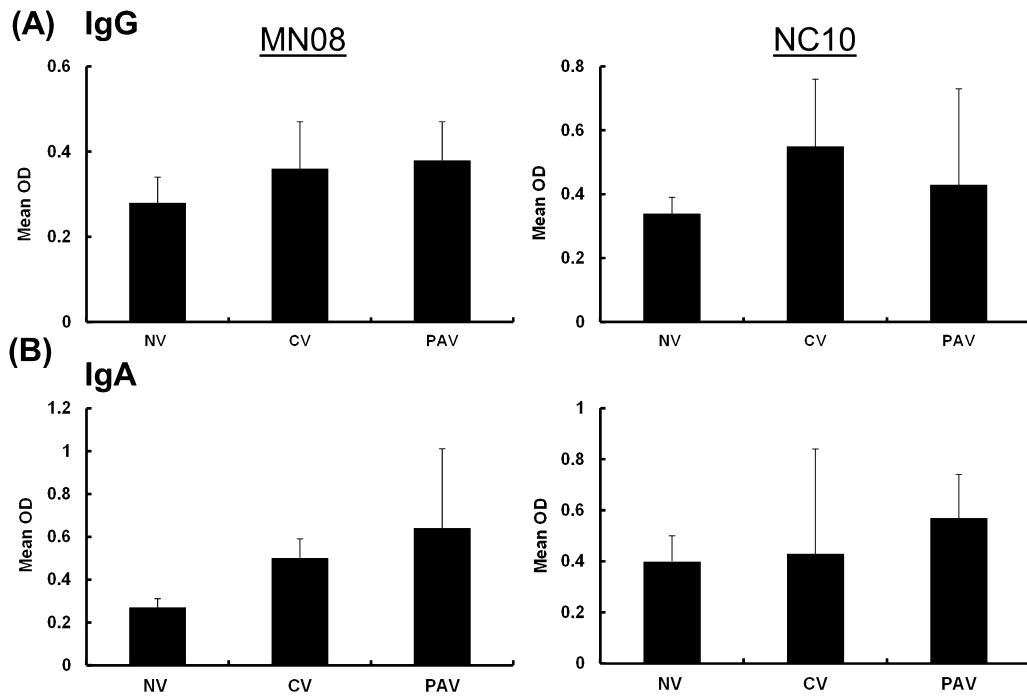


Fig. 3. Virus-specific IgG and IgA antibody levels in lungs of pigs. At necropsy on DPC 6, lungs were obtained from the groups of vaccinated and vaccinated challenged pigs and lung lysates were prepared as described in Materials and Methods. IgG (panel A) and IgA (panel B) antibody responses against MN08 and NC10 in lung lysates of these groups of pigs were examined by ELISA. Data are expressed as mean OD at 405 nm of four pigs \pm SD.

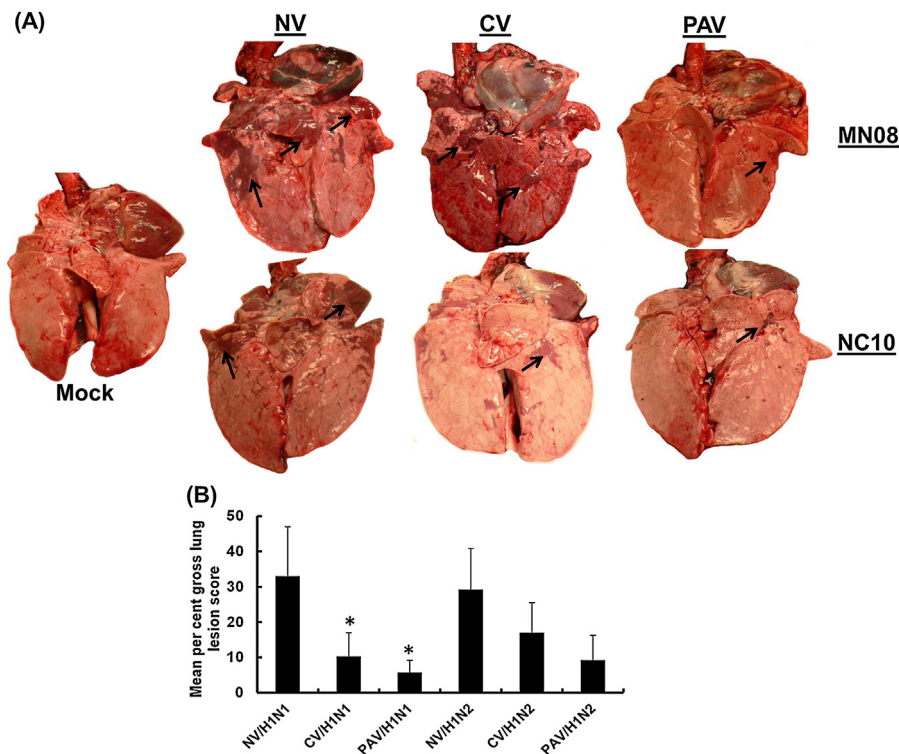


Fig. 4. Poly I:C adjuvanted vaccine provides protection against antigenic variant and heterologous virus challenge. Groups of immunized and non-immunized pigs were challenged with either MN08 or NC10 SIV. Six days after challenge (DPC 6), gross lung lesions were examined (A). Extensive areas of consolidation were observed in non-vaccinated MN08- or NC10-challenged pigs. Minimal lung lesions were observed in PAV-immunized and MN08- and NC10-challenged pigs whereas pigs immunized with CV and challenged with MN08 and NC10 viruses showed moderate levels of lung consolidation. No lung lesions were observed in mock-inoculated pigs. Arrows indicate influenza specific lesions in the lungs. Representative pictures from different groups are shown. At necropsy on DPC 6, mean gross lung lesions from all the six lung lobes were scored on the basis of percent pneumonic lesions (B). Each bar indicates the mean lung lesion scores of four pigs \pm SD. *—significantly different from NV group, ($P < 0.05$).

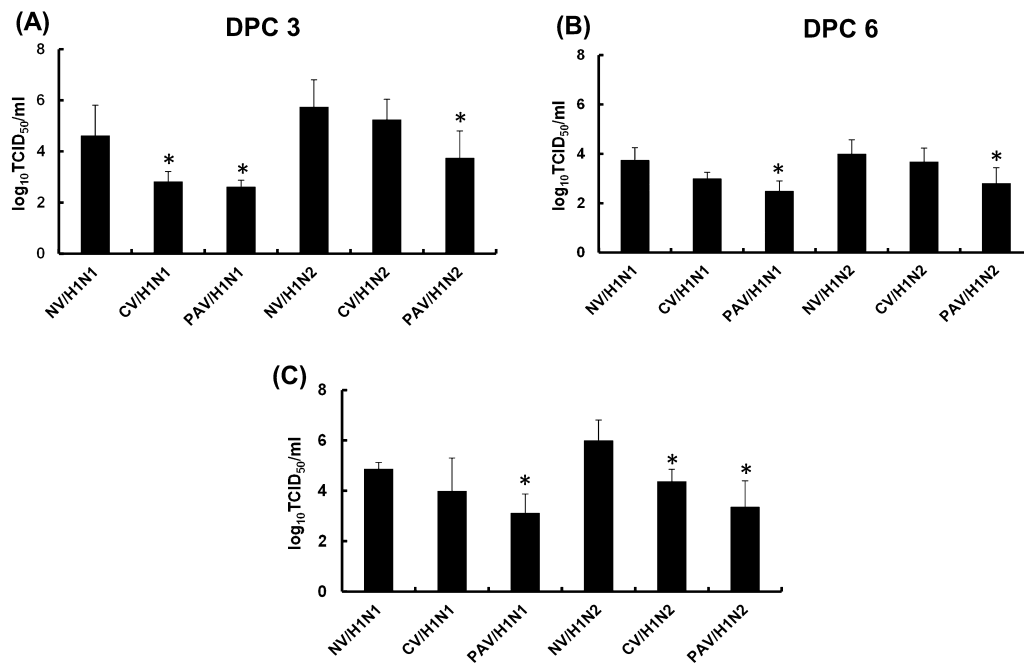


Fig. 5. Detection of challenge virus shedding in nasal secretions and lungs of pigs. Groups of immunized and non-immunized pigs were challenged with either MN08 or NC10 SIV. At DPC 3 (A) and 6 (B) nasal swabs were collected and challenge virus shedding was determined by titration in MDCK cells. At DPC 6 (C) lungs were harvested and 10% homogenate of lung tissue was prepared. Challenge virus replication was determined by titration in MDCK cells. Values indicate mean virus titers in nasal swabs/lungs of four pigs \pm SD except in CV-MN08 group where values are mean virus titer from three pigs \pm SD because one pig in this group was euthanized before challenge as it developed respiratory illness after vaccination. "*"—significantly different from NV group, ($P < 0.05$).

pigs. Although not examined in this study, the cross-protection we observed in this study indicates that in addition to cross-reactive HI and mucosal antibodies, cross-reactive T cells may also have played a role in providing protection against antigenic variant and heterologous SIV in PAV-immunized pigs. Previously, inactivated SIV vaccine was shown to induce cross-reactive T cell responses in immunized pigs [35].

The mean percent gross lesion score was significantly decreased for pigs that received PAV and challenged with either the homologous or heterologous strain compared with NV pigs. Previously, VAERD has been reported in pigs vaccinated with inactivated influenza vaccines followed by challenge with heterologous virus [14,36,37]. Although the mechanisms for VAERD is poorly defined, recent research suggests that one of the contributing factors could be production of cross-reactive IgG against non-neutralizing epitopes [13,36]. Similar to an inactivated swine influenza vaccine induced VAERD, intranasal administration of UV inactivated severe acute respiratory syndrome (SARS) vaccine in mice induced extensive eosinophilic infiltration in the lungs which could enhance the respiratory disease [38]. Also, the immune response was skewed towards Th2 cell activation resulting in production of excessive antibodies. However, administration of a combination of TLR agonists including Poly I:C along with UV inactivated SARS vaccine reduced eosinophilic infiltration, shifted the immune response to Th1 cell mediated immunity, and produced high neutralizing antibody titers [38]. In the present study, we used Poly I:C as an adjuvant to UV-inactivated SIV vaccine and observed significant protection against challenge with antigenic variant and heterologous SIV without the evidence of VAERD. Collectively, our findings and previous findings on the SARS vaccine study suggest that poly I:C has a protective effect against VAERD.

Transmission of swine influenza between pigs and humans occurs by aerosol. Virus is mostly shed through nasal discharges to the surrounding environment. An efficient vaccine could prevent virus replication in the airways and thus reduce the virus shedding. There is a positive correlation between virus shedding in nasal

secretions and the possibility of a previously uninfected pigs getting infected through aerosol. Therefore, it is pertinent that even vaccines yielding partial protection could slow the rate of influenza transmission. The pigs that received PAV shed significantly less virus through nasal discharge on both DPC 3 and 6 when infected with the antigenic variant or the heterologous strain. Additionally, the virus load in the lung at DPC 6 was significantly lower in PAV immunized group compared with NV challenged with MN08 or NC10. These findings suggest that PAV vaccination conferred effective protection against both homologous and heterologous strains.

Previous research demonstrated that Poly I:C can reduce the replication of Chikungunya virus *in vitro* [39] and pretreatment of mice with poly I:C provided significant protection against SARS and influenza infection [40]. Poly I:C was also effective as an adjuvant for Foot and Mouth disease vaccine in pigs [23,41] and for influenza virus vaccine studies in chickens [42,43]. In humans, efficacy and safety of Ampligen (synthetic poly I:C) are being tested in clinical experiments. Our preliminary findings suggest that PAV-immunized pigs were protected from influenza challenge and that the use of Poly I:C as an adjuvant with an inactivated vaccine did not cause VAERD in pigs. However, protection was incomplete. Therefore, additional studies are required for optimizing the vaccine and Poly I:C dosing to further enhance the cross-protective efficacy of PAV and its usefulness in field situations.

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