Protective efficacy of a virus-vectored multi-component vaccine against porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and swine influenza virus

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Abstract
Porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2) and swine influenza virus (SIV) are three of the most economically important swine pathogens, causing immense economic losses to the global swine industry. Monovalent commercial vaccines against each of the three viruses are routinely used in pig farms worldwide. A trivalent vaccine against all three pathogens would greatly simplify the vaccination programme and reduce the financial burden to the swine industry. In this study, by using an attenuated strain of PRRSV (strain DS722) as a live virus vector, we generated a multi-component vaccine virus, DS722-SIV-PCV2, which expresses the protective antigens from SIV and PCV2. The DS722-SIV-PCV2 trivalent vaccine virus replicates well, and expresses PCV2 capsid and SIV HA proteins in vitro. A subsequent vaccination and challenge study in 48 pigs revealed that the DS722-SIV-PCV2-vaccinated pigs had significantly reduced lung lesions and viral RNA loads when challenged with PRRSV. Upon challenge with PCV2, the vaccinated pigs had partially reduced lymphoid lesions and viral DNA loads, and when challenged with SIV the vaccinated pigs had significantly reduced acute respiratory sign scores. The results from this study demonstrate the potential of DS722-SIV-PCV2 as a candidate trivalent vaccine, and also shed light on exploring PRRSV as a potential live virus vaccine vector.

INTRODUCTION
Porcine reproductive and respiratory syndrome virus (PRRSV) is arguably the most economically-important global swine pathogen, causing immense economic losses to the global swine industry. It was estimated that PRRSV resulted in more than $660 million annual losses in the United States alone [1, 2]. The typical clinical signs of PRRSV infection include reproductive failure in pregnant sows and respiratory disease in growing pigs [3, 4]. Porcine circovirus type 2 (PCV2) is another economically important swine virus, causing porcine circovirus-associated disease (PCVAD) with a broad spectrum of clinical signs including reproductive failure, respiratory disease, enteritis and wasting [5, 6]. Swine influenza virus (SIV) causes acute respiratory diseases in breeding and nursery pigs and can predispose the infected animals to secondary bacterial infections [7, 8]. In addition to the adverse economic impact on pig producers, SIV is also a zoonotic pathogen posing a threat to humans [9, 10].

Commercial monovalent vaccines against each of the three pathogens (PRRSV, PCV2, SIV) are available and in use worldwide. Currently, the three vaccines are individually administered to pigs, making the vaccination programme more complex and increasing the financial burden to swine producers. Most importantly, co-infections of pigs with all three viruses on the same farms are frequently reported [11–13]. Therefore, a trivalent vaccine which can protect pigs against all three viruses in a single dose would be an ideal alternative strategy to control these important pathogens.

PRRSV is a member of the family Arteriviridae in the order Nidovirales, and contains a single-stranded, positive-sense...
RNA molecule of about 15 kb in size [14]. The PRRSV genome comprises short 5’- and 3’-terminal untranslated regions (UTRs) flanking a large replicase gene and eight structural genes [15]. Like other nidoviruses, PRRSV adapts a unique discontinuous RNA synthesis mechanism to generate a nested set of subgenomic mRNAs, which are then expressed into structural proteins [16, 17]. The base-pairing interactions between a short conserved transcription-regulating sequence (TRS) in the 5’-UTR with individual TRS of each structural gene is critically important for the generation of individual subgenomic mRNA during transcription. Therefore, based on the transcription mechanism of the virus, PPRSV can be explored as a potential live viral vector to express foreign antigens via the generation of separate additional subgenomic mRNA [18, 19].

PCV2 is a small, non-enveloped, single-stranded circular DNA virus belonging to the family Circoviridae [20, 21]. The genome of PCV2 contains at least two functional ORFs: ORF1 encodes the replication protein, and the ORF2 encodes an immunogenic capsid protein which has been the target antigen for vaccine development [20, 22]. Among the five recognized PCV2 genotypes (PCV2a–PCV2e), PCV2b is one of those currently responsible for most PCVAD cases [23, 24]. In addition to the classical H1N1 SIV, a triple-reassortant H3N2 SIV has been circulating in North American swine herds since the turn of the last century [25]. The haemagglutinin (HA) of SIV elicits neutralizing antibodies that protect animals from infection, and therefore is the primary antigen targeted for subunit vaccine development [26].

In this study, we utilized an attenuated strain of PRRSV, DS722 [27], as a live virus vector to express the truncated HA antigen from H3N2 SIV and the capsid antigen from PCV2b. The resulting recombinant multi-component vaccine virus, DS722-SIV-PCV2, was subsequently evaluated as a potential trivalent vaccine against PRRSV, PCV2 and SIV in a vaccination and challenge pig study. The findings from this study have implications for developing multivalent vaccines for pigs, as well as for further exploring the use of PRRSV as a potential live virus vaccine vector.

RESULTS

Successful generation of a recombinant multi-component candidate vaccine virus DS722-SIV-PCV2 expressing protective antigens from SIV and PCV2

PRRSV can serve as a potential vector to express and deliver foreign proteins via the generation of additional subgenomic mRNAs [18]. In this study, a novel trivalent candidate vaccine against PRRSV, PCV2b and SIV was constructed. The protective antigens from SIV and PCV2b were cloned into the backbone of an attenuated PRRSV strain DS722 and expressed along with the PRRSV proteins. A synthetic DNA fragment, HA-ORF2, containing a cassette of three antigen domains from the HA gene of SIV and the full-length ORF2 capsid gene of PCV2b, was commercially synthesized (Fig. 1a). The HA-ORF2 fragment was cloned into a DNA-launched infectious clone of the attenuated PRRSV pDS722 to produce a novel recombinant PRRSV clone, pDS722-SIV-PCV2, expressing PCV2b ORF2 capsid and SIV HA proteins. The authenticity of the recombinant PRRSV clone was verified by DNA sequencing.

At 2 days post-transfection of BHK-21 cells with the recombinant PRRSV pDS722-SIV-PCV2 clone, the culture supernatant (P0 virus) was harvested and used to inoculate fresh MARC-145 cells. At 5 days post-inoculation, cytopathic effects (CPEs) were observed in inoculated cells. Immunofluorescence assay (IFA) using PRRSV N-specific monoclonal antibody confirmed that the CPEs were PRRSV-specific, thus indicative of the production of infectious progeny viruses (Fig. 1b). To further confirm that the rescued recombinant virus indeed originated from the clone, the region flanking the inserted HA-ORF2 sequence in the PRRSV DS722 backbone was amplified from the P1 viruses by RT-PCR and sequenced. Sequence analysis confirmed that the HA-ORF2 foreign sequence was inserted exactly as designed between the ORF1b and ORF2a of PRRSV genomic backbone, and that the sequence of the rescued virus was identical to that of the original recombinant clone.

To analyse the expression profile of the inserted HA-ORF2 genes by the rescued recombinant virus DS722-SIV-PCV2, MARC-145 cells were infected with the P0 recombinant virus. IFA staining with specific antibodies recognizing the FALG tag linked to the HA of SIV, and capsid protein of PCV2 revealed that only the DS722-SIV-PCV2 recombinant virus-infected cells showed positive signals, while the DS722- or mock-infected cells remained negative (Fig. 1b). To evaluate the growth ability of the multi-component candidate vaccine virus, the P3 viruses of the DS722-SIV-PCV2 and its parental PRRSV DS722 were used to infect MARC-145 cells at an m.o.i. of 0.1. The results showed that the multi-component candidate vaccine virus DS722-SIV-PCV2 replicated well in the MARC-145 cells but with an overall reduced growth ability when compared to the parental PRRSV DS722 (Fig. 1c). Specifically, the DS722-SIV-PCV2 virus had significantly lower virus titres at 36–96 h post-infection (h p.i.) with the peak virus titre at approximately $1.0 \times 10^6$ TCID$_{50}$/ml.

Collectively, the results indicated that the recombinant multi-component candidate vaccine virus DS722-SIV-PCV2 expressing SIV and PCV2 antigens was successfully generated, and the DS722-SIV-PCV2 virus can replicate to a relatively high titre in MARC-145 cells, even though it has a reduced growth ability when compared to the parental virus.

Pigs vaccinated with the multi-component candidate vaccine virus DS722-SIV-PCV2 had significantly reduced lung lesions as well as significantly lower viral RNA loads in sera and lung tissues after PRRSV challenge

To evaluate the protective efficacy of the recombinant virus DS722-SIV-PCV2 as a potential trivalent vaccine, we
conducted a vaccination and challenge study in pigs (Table 1). After vaccination, the anti-PRRSV antibody responses in each pig were monitored weekly using the IDEXX HerdChek X3 ELISA kit. The data shown that all vaccinated pigs seroconverted to PRRSV antibodies at 14 days post-vaccination (dpv), while the pigs in the negative control group remained seronegative until virus challenge (Table S1, available in the online version of this article). The results suggested that a robust replication of the multi-component vaccine virus DS722-SIV-PCV2 had occurred in pigs. After challenge with PRRSV, the DS722-SIV-PCV2-vaccinated group showed significantly decreased gross lung lesion scores (mean value=4.9) compared to those of non-vaccinated control (mean value=45.4) (Fig. 2a). Consistent with the gross lung lesions, the DS722-SIV-PCV2-vaccinated pigs (mean value=0.6) also had significantly lower microscopic lung lesion scores than those of non-vaccinated controls (mean value=2.9) (Fig. 2b).

PRRSV RNA loads in serum and lung tissues are crucial parameters which are routinely used for measuring the efficacy of PRRSV vaccines [28–30]. After challenge with PRRSV strain VR2385, most DS722-SIV-PCV2-vaccinated pigs (5/7) were negative for viral RNA in sera at 6 days post-challenge (dpc), while all the pigs in the non-vaccinated group were positive for PRRSV RNA (Fig. 3a). The mean values of PRRSV RNA loads in DS722-SIV-PCV2-vaccinated group (5.0×10^{5} copies ml^{-1}) were significantly lower than those of the non-vaccinated group (1.3×10^{7} copies ml^{-1}) (Fig. 3a). Similar data were also obtained at 10 dpc (1.3×10^{5} copies ml^{-1} in DS722-SIV-PCV2-vaccinated group; 6.3×10^{6} copies ml^{-1} in non-vaccinated group). Additionally, for the viral RNA loads in the lung tissues (Fig. 3b), the DS722-SIV-PCV2-vaccinated group had significantly decreased viral RNA copy numbers (1.2×10^{4} copies g^{-1}) when compared to the non-vaccinated group (4.0×10^{5} copies g^{-1}), and most pigs (5/7) in the vaccinated group were negative for viral RNA.
Pigs vaccinated with the multi-component candidate vaccine virus DS722-SIV-PCV2 had partially reduced PCVAD lesions and viral DNA loads in sera and lymphoid tissues after PCV2b challenge

To evaluate the protective efficacy of the multi-component candidate vaccine against PCV2, the vaccinated pigs were challenged with a PCV2b strain, NC16845. Prior to challenge, all the pigs remained negative for PCV2b DNA (Table S1). At necropsy, the three parameters of PCVAD-related microscopic lesions (lymphoid depletion, histiocytic replacement, immunohistochemistry (IHC) for PCV2 antigen) in three main lymphoid tissues (lymph nodes, tonsils and spleens) were evaluated. The DS722-SIV-PCV2-vaccinated group had lower PCVAD lesion scores than the non-vaccinated group (Table 2). Specifically, with the exception of the lymphoid depletion scores of tonsils and IHC scores of lymph nodes, all other lesion scores in the DS722-SIV-PCV2-vaccinated group were numerically lower than those in the non-vaccinated group, although the differences were not statistically significant. The IHC score in tonsil tissues of DS722-SIV-PCV2-vaccinated group was significantly lower than that in the non-vaccinated group (Table 2).

The viral DNA loads in sera and lymphoid tissues were also determined. After PCV2b challenge, both groups of pigs developed viraemia from 7 dpc, and peaked at necropsy day (Fig. 4a). Overall, the mean values of the viral DNA loads in the DS722-SIV-PCV2-vaccinated group were lower than those in the non-vaccinated group at all three time points, and the difference was statistically significant at 14 dpc (Fig. 4a). Also, the DS722-SIV-PCV2-vaccinated group had significantly reduced viral DNA loads in tonsils and spleens.

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Table 1. Experimental design for the vaccination and challenge study of the candidate trivalent vaccine in pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of pigs</th>
<th>Vaccination with*</th>
<th>Challenge at 42 dpv with†</th>
<th>Necropsy day (dpc) at</th>
<th>No. of pigs at necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>PBS</td>
<td>PRRSV</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>PBS</td>
<td>PCV2</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>PBS</td>
<td>SIV</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>DS722-SIV-PCV2</td>
<td>PRRSV</td>
<td>10</td>
<td>7‡</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>DS722-SIV-PCV2</td>
<td>PCV2</td>
<td>21</td>
<td>7‡</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>DS722-SIV-PCV2</td>
<td>SIV</td>
<td>5</td>
<td>7‡</td>
</tr>
</tbody>
</table>

dpv, days post-vaccination; dpc, days post-challenge.

*Vaccine Dose: 1.0×10^6.0 TCID50/pig. Route: intramuscular injection (IM).
†PRRSV, 1.0×10^6.0 TCID50/pig, intramuscular injection (IM); PCV2, 5.0×10^4.0 TCID50/pig, IM; SIV, 1.0×10^7.0 TCID50/pig, intranasal and intratracheal inoculation.
‡One piglet died from an unrelated cause prior to challenge.

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Fig. 2. Macroscopic and microscopic lung lesion scores of pigs challenged with PRRSV. At necropsy (10 dpc), the lung tissues were scored for macroscopic lesions by a veterinary pathologist (a). Samples of the lung tissues were also fixed in neutral formalin for histological examination of microscopic lung lesion scores (b). Each plot represents the value of one pig, and the error bars indicate standard errors. Significant differences are indicated with asterisks (*** P<0.001, ****P<0.0001).
when compared to the non-vaccinated group (Fig. 4c, d),
but the difference was not significant for lymph nodes
(Fig. 4b).

Pigs vaccinated with multi-component candidate
vaccine DS722-SIV-PCV2 had significantly reduced
acute respiratory sign scores and partial reduction
of viral RNA loads after SIV challenge

To evaluate whether the multi-component candidate vaccine virus DS722-SIV-PCV2 can provide any protection against SIV, the vaccinated pigs and controls were challenged with an H3N2 SIV strain. After challenge, only one pig (14.3%) in the DS722-SIV-PCV2-vaccinated group developed fever above 40.6°C and mild respiratory symptoms, while the majority (87.5%) of the non-vaccinated pigs developed fever above 40.6°C and all (100%) non-vaccinated pigs showed acute respiratory signs such as coughing and sneezing (Table 3). After necropsy, the mean lung gross lesion and histopathological lesion scores of the DS722-SIV-PCV2-vaccinated group were slightly lower than that of the non-vaccinated group, but not statistically significant (Table 3).

The SIV viral RNA loads in nasal swabs and lung tissues were also determined. At the first day post-challenge (1 dpc), both DS722-SIV-PCV2-vaccinated and non-vaccinated groups had the highest viral RNA loads in nasal swabs compared to other time points at 3 and 5 dpc (Fig. 5a). The virus shedding gradually decreased over time, and three vaccinated and non-vaccinated pigs had cleared the virus at 5 dpc. The mean value of SIV RNA loads in the DS722-SIV-PCV2-vaccinated group were numerically lower than those in the non-vaccinated group at 1 and 5 dpc, and the viral RNA loads in the vaccinated group were significantly lower than those in the non-vaccinated group at 3 dpc (Fig. 5a).

For viral RNA loads in the lung tissues, the DS722-SIV-PCV2-vaccinated group had a slightly lower mean value than the non-vaccinated group (Fig. 5b), although the difference is not significant.

The multi-component candidate vaccine virus
DS722-SIV-PCV2 was genetically stable in low
numbers of serial passages in vitro

To investigate the genetic stability of the multi-component candidate vaccine virus, DS722-SIV-PCV2, the virus was serially passaged in MARC-145 cells. The inserted foreign gene region and its flanking sequences of the passaged candidate vaccine virus were sequenced at every two passages. Sequence analysis revealed that the candidate vaccine virus was genetically stable until passage 5. After passage 5, however, there was 100–500 bp deletion at the 3′-terminus of the HA sequence and 5′-terminus of the ORF2 sequence. The results indicated that the recombinant multi-component candidate vaccine DS722-SIV-PCV2 virus was only genetically stable during low numbers of in vitro passages.

DISCUSSION

PRRSV, PCV2 and SIV are currently three of the most economically important viral pathogens affecting the global swine industry. Vaccinations with three single vaccines against PRRSV, PCV2 or SIV given separately to each pig are routinely used on pig farms, although there are considerable costs and risks (needle breakage, transmission of pathogens etc.) associated with this conventional vaccination strategy. Most importantly, co-infections on the same farm with two or three of these viruses are common. Therefore, a trivalent vaccine which can provide protection against all three viruses would be of great benefit to the swine industry.
Live viral vaccine vectors such as alphavirus and adenovirus have numerous advantages in regard to expressing and delivering multiple foreign antigens as vaccines [31, 32]. The first member of the arteriviruses to have been tested as a potential viral vector is equine arteritis virus (EAV) [33, 34], followed by PRRSV [35, 36]. Compared to other viral vectors, PRRSV has several unique advantages. First, PRRSV uses a set of subgenomic mRNAs to express its viral proteins, this making it possible to deliver foreign antigen via generation of additional separate subgenomic mRNAs. Second, since PRRSV itself is an economically important pathogen in pigs, the use of an attenuated PRRSV as live virus vaccine vector can provide protection against PRRSV in addition to other intended pathogens. In this study, therefore, we utilized an attenuated strain of PRRSV to express the protective antigens from PCV2 and SIV as a potential trivalent vaccine against PRRSV, SIV and PCV2. Two sites in the PRRSV genome have been successfully used to insert relatively large foreign genes: the hypervariable region of non-structural protein 2 (nsp2), and the overlap region between ORF1b and ORF2a [37]. In this present study, we chose the latter site since insertion in this site showed greater genetic stability of the inserted foreign genes [18, 35, 36, 38]. To further increase the genetic stability of the inserted foreign genes, we also included about 40 nt sequences of TRS 5 and TRS 6 from the PRRSV genome as suggested in a previous study [18]. Despite this modification, the recombinant trivalent candidate vaccine virus DS722-SIV-PCV2 was genetically stable for only five passages in cell culture. After five passages, deletion of inserted foreign genes occurred, indicating that the size of the inserted foreign sequences exceeded the tolerance limitation of the PRRSV genome, as most RNA viruses possess a genomic size limitation [39]. It has been shown that the inserted green fluorescent protein (GFP) gene (about 700 bp) in the PRRSV genome can be kept phenotypically stable for 37 passages [18]. In the present study, however, the size of the inserted foreign sequences exceeded the tolerance limitation of the PRRSV genome, as most RNA viruses possess a genomic size limitation [39]. The protective efficacy of the multi-component candidate vaccine virus DS722-SIV-PCV2 against PRRSV, PCV2 and SIV challenges was evaluated in pigs. When challenged with PRRSV strain VR2385, the pigs vaccinated with DS722-SIV-PCV2 showed significantly decreased gross and microscopic lung lesions when compared to non-vaccinated controls. The DS722-SIV-PCV2-vaccinated group also had significantly reduced viral RNA loads in sera and lungs compared to the non-vaccinated group, and the majority of the vaccinated pigs cleared the challenge virus at necropsy. Although a PRRSV vector control group was not included in the study, the results indicate that the combination of vaccines may be a promising strategy for the development of a trivalent vaccine against PRRSV, PCV2 and SIV.
not included in this study, the DS722-SIV-PCV2 recombinant vaccine virus did induce a similar level of protection compared to the attenuated PRRSV DS722 against PRRSV challenge based on our previously studies [28, 40]. Apparently the DS722-SIV-PCV2 recombinant virus induced a similar level of protective immune responses in pigs compared to that of the parental virus DS722, since all PRRSV viral components in DS722-SIV-PCV2 were identical to the parental DS722 virus. Collectively, the results showed that the multi-component candidate vaccine virus DS722-SIV-PCV2 induced a robust protection against PRRSV strain VR2385, suggesting that the insertion of foreign genes did not reduce the immunogenicity of PRRSV. Therefore, the data provide a proof of concept for further development of PRRSV as a potential live vaccine virus vector.

**Fig. 4.** Viral DNA loads in sera and tissues of pigs challenged with PCV2. PCV2 DNA copy numbers in sera at 7, 14, 21 dpc (a), and in lymph nodes (b), tonsils (c) and spleens (d) at 21 dpc were determined by qPCR. The detection limit of this assay is 100 (2 log10) copies ml⁻¹ (serum) or g⁻¹ (tissue). Samples below the detection limit were considered as negative, and calculated as 1 log10. Each plot represents the mean viral DNA copy number of triplicate testing data of one pig, and the error bars indicate standard errors. Significant differences are indicated with asterisks (*P<0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of pigs with high fever/total</th>
<th>No. of pigs with acute respiratory signs/total</th>
<th>Lung gross lesion score</th>
<th>Lung histopathology lesion score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS/SIV</td>
<td>7/8 (40.9±0.2)*,⁴</td>
<td>8/8 (3.3±0.4)†,⁴</td>
<td>49.1±8.3</td>
<td>1.8±0.4</td>
</tr>
<tr>
<td>VAC/SIV</td>
<td>1/7 (40.0±0.3)⁰</td>
<td>1/7 (0.1±0.1)†</td>
<td>46.7±14.1</td>
<td>1.7±0.3</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are group mean of body temperature±SEM (°C). Different superscripts (A, B) indicate statistical significance (P<0.01).
†Numbers in parentheses are group mean of respiratory sign scores±SEM ranging from 0 (normal) to 6 (severe dyspnoea at rest). Different superscripts (A, B) indicate statistical significance (P<0.0001).
When challenged with PCV2b, the DS722-SIV-PCV2-vaccinated pigs showed reduced microscopic lesions and viral DNA loads in sera and tissues compared to non-vaccinated pigs, although the differences for most pathological and virological parameters were not statistically significant. We believe that the observed partial protection against PCV2b challenge is due to the immune responses to the capsid protein of PCV2b that was expressed by DS722-SIV-PCV2 recombinant vaccine virus. However, as discussed above, the inserted ORF2 was not genetically stable in the recombinant virus, which may explain the limited protection. When challenged with SIV, the DS722-SIV-PCV2-vaccinated pigs were protected from acute respiratory diseases while the parameters of pathological lesion and viral RNA loads were not significantly improved when compared to non-vaccinated pigs. The reason for this intriguing observation was not investigated in this study. However, we speculate that non-specific cellular immune responses triggered by replication of the recombinant vaccine virus may play a role in the observed reduction of respiratory clinical signs [41, 42].

Taken together, the data from this study showed that the multi-component candidate vaccine virus DS722-SIV-PCV2 provided a sufficient level of protection against PRRSV but only partial protection against PCV2 and SIV. This study demonstrated the potential use of PRRSV as a live viral vector to express protective antigens from other swine pathogens. The genetic stability of the recombinant PRRSV and the optimal insertion size of foreign genes in the PRRSV vector will need to be improved in the future, but the results of this study provide a solid starting point.

**METHODS**

**Cells and viruses**

Baby hamster kidney cell (BHK-21) cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Monkey kidney (MARC-145) cells were cultured in DMEM supplemented with low glucose and 10% FBS, and maintained in low-glucose DMEM with 2% FBS for PRRSV propagation. Porcine kidney (PK-15) cells were cultured in minimal essential medium (MEM) supplemented with 10% FBS, and maintained in MEM with 2% FBS for PCV2b propagation. A DNA-launched infectious clone of PRRSV, pDS722, was constructed in our lab previously [27]. The PRRSV DS722 (GenBank accession no. JX044138), a derivative of PRRSV strain VR2385 generated by DNA shuffling of the ORF5 gene, is attenuated in pigs when compared to its parental strain VR2385 [27, 43, 44]. PCV2b strain NC16845

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**Fig. 5.** Viral RNA loads in nasal swabs and lung tissues of pigs challenged with SIV. SIV RNA copy numbers in nasal swabs at 1, 3, 5 dpc (a), and in lung tissues (b) at 5 dpc were determined by RT-qPCR. The detection limit of this assay is 1000 (3 log10) copies ml⁻¹ (serum) or g⁻¹ (lung tissue). Samples below the detection limit were considered as negative, and calculated as 2 log10. Each plot represents the mean viral RNA copy number of triplicate testing data of one pig, and the error bars indicate standard errors. Significant differences are indicated with asterisks (*P<0.05).
(accession no. GU799576) was originally isolated from a pig in the United States, and the PCV2b virus stock was prepared as described previously [45, 46]. SIV strain A/swine/Minnesota/1145/2007(H3N2) (accession no. FJ410317), originally obtained from the University of Minnesota Veterinary Diagnostic Laboratory, is described elsewhere and was propagated in specific-pathogen-free (SPF) embryonated chicken eggs [47, 48].

Construction of a multi-component recombinant virus infectious clone using an attenuated PRRSV as the backbone

A nucleotide acid sequence fragment HA-ORF2, consisting of the SIV HA gene and the PCV2b ORF2 capsid gene, was commercially synthesized (Integrated DNA Technologies, Coralville, IA) (Fig. 1). Briefly, the HA-ORF2 fragment contains a truncated HA gene from SIV strain A/swine/Minnesota/1145/2007(H3N2) and a full-length ORF2 capsid gene sequence from PCV2b strain NC16845. A TRS 5 sequences (40 nt) derived from the PRRSV pDS722 infectious clone backbone was inserted before the start codon of PCV2b ORF2, and a TRS 6 sequences (45 nt) was also inserted after the stop codon of PCV2b ORF2. The introduction of an additional TRS sequence ensures that the multi-component recombinant virus generates separate subgenomic mRNAs [18]. The truncated SIV HA gene encodes three antigen domains of HA protein from SIV strain A/swine/Minnesota/1145/2007(H3N2), two from the HA1 subunit and one from the HA2 subunit. These three antigen domains have been shown to elicit broadly neutralizing antibodies, and are highly conserved in influenza A viruses [49]. Through amino acid sequence alignment with a referencing sequence [49], the three domains were verified and then connected by a flexible GSA amino acid linker. For easy detection of HA expression in the recombinant virus, a FLAG tag was fused to the N-terminus of the HA cassette. By using the Not I and PacI restriction enzyme sites engineered into the synthesized HA-ORF2 fragment, the truncated HA of SIV and the complete ORF2 of PCV2b were introduced into the genomic backbone of the DNA-launched infectious clone of PRRSV pDS722, to create the final full-length clone of the recombinant multi-component candidate vaccine virus, designated pDS722-SIV-PCV2 (Fig. 1a).

Rescue of the recombinant PRRSV expressing immunogenic antigens from SIV and PCV2b

To rescue the recombinant PRRSV expressing SIV HA antigen and PCV2 capsid antigen, plasmid DNA from the full-length recombinant clone pDS722-SIV-PCV2 was isolated using the QIAprep Spin Miniprep kit, and quantified using Nanodrop. Fresh BHK-21 cells seeded in a 6-well plate at approximately 60–80% confluency were transfected with 2 µg of the plasmid DNA per well using the Lipofectamine LTX and Plus Reagent kit (Invitrogen) according to the manufacturer’s instructions. After 48 h incubation at 37 °C with 5% CO2, cell culture supernatants were harvested and designated as passage 0 (P0) virus. The P0 virus was passaged on MARC-145 cells to confirm the viability of the rescued recombinant virus.

Indirect immunofluorescence assay (IFA)

IFA with antibodies against PRRSV, PCV2b and FLAG, respectively, was used to verify the viability of the rescued recombinant virus expressing PCV2b capsid and SIV HA antigens, essentially as described elsewhere [40, 50]. Briefly, at 48 h p.i., cells were washed with phosphate-buffered saline (PBS) and fixed in cold methanol for 15 min. After washing with PBS, the fixed cells were blocked in 1% bovine serum albumin (BSA) at room temperature for 30 min. The cells were washed with PBS and were then incubated with anti-PRRSV N monoclonal antibody SDOW17 (Rural Technologies, Inc., Brookings, SD) to verify the viability of the recombinant PRRSV, with anti-FLAG antibody to confirm the expression of SIV HA (ThermoFisher Scientific, Wal-tham, MA), or with anti-PCV2b antibody to confirm the expression of PCV2b capsid protein at 37°C for 2 h. After extensive washing with PBS, the cells were incubated with corresponding fluorescein-conjugated secondary antibodies for 1 h at 37°C. After washing with PBS twice, fluorescent signals were visualized using an Olympus inverted fluorescence microscope fitted with a digital camera.

Virus growth kinetics assay

To characterize the growth kinetics of the rescued recombinant PRRSV DS722-SIV-PCV2 in vitro, a multiple-step growth curve assay was conducted in MARC-145 cells as described elsewhere [17].

Experimental design for a challenge and vaccine efficacy study of the trivalent candidate vaccine in pigs

A total of 48 piglets of 3 weeks of age were randomly divided into 6 groups of 8 piglets per group (Table 1). All the piglets were free of PRRSV, PCV2b and SIV as confirmed by PCR and antibody assays. Piglets in each group were vaccinated intramuscularly with the recombinant trivalent candidate vaccine virus DS722-SIV-PCV2 (1.0×108 TCID50/pig) or with PBS buffer as control. Serum samples were collected from each pig prior to vaccination and weekly thereafter. At 42 days post-vaccination (dpv), the pigs were challenged with PRRSV strain VR2385, PCV2b strain NC16845 or SIV strain A/swine/Minnesota/1145/2007(H3N2), respectively. Clinical signs including body temperature and respiratory scores were recorded after challenge as described elsewhere [44].

For the SIV-challenged groups, nasal swab samples were also collected from each pig daily, and all SIV-challenged pigs were necropsied at 5 days post-challenge (dpc). For the PRRSV-challenged groups, serum samples were collected at 6 and 10 dpc, and all PRRSV-challenged pigs were necropsied at 10 dpc. For the PCV2b-challenged groups, serum samples from each pig were collected at 7, 14 and 21 dpc, and all PCV2b-challenged pigs were necropsied at 21 dpc. Lung tissue samples of pigs from all groups were collected during necropsies for gross pathology and...
histopathology evaluation and quantification of viral DNA or RNA loads. Tissue samples of tonsil, spleen and lymph nodes were also collected in PCV2b-challenged groups for evaluation. This study was approved by Iowa State University Institutional Animal Care and Use Committee (approval number 12-16-8404-S).

**Gross pathology and histopathology evaluation**

At necropsies, the lungs of all pigs were evaluated for visible gross lesions as described previously [44]. Five sections of lung tissue were collected and fixed in formalin and processed for histopathology evaluation. The microscopic lung lesions were scored based on the presence and severity of interstitial pneumonia, ranging from 0 to 6 as described elsewhere [40, 44]. For the PCV2b-challenged groups, tissue samples of tonsil, spleen and lymph nodes were collected during necropsy and processed routinely for PCVAD-related histological lesion examination and PCV2b immunohistochemistry (IHC) at Iowa State University Veterinary Diagnostic Lab as described elsewhere [46, 51].

**Quantitation of viral RNA or DNA loads in samples**

Viral RNAs or DNAs were extracted from serum or nasal swab samples using ZR Viral RNA or DNA kit (ZYMED RESEARCH, USA) following the manufacturer’s protocols. Total RNAs from lung tissues of the PRRSV-challenged pigs were extracted using TRI Reagent (MRC) following the manufacturer’s protocol. The tissues from PCV2b-challenged pigs were homogenized and prepared in 10% tissue suspension in PBS buffer. PCV2b viral DNAs were extracted using ZR Viral DNA kit.

The quantification of PRRSV RNA copy number was conducted by RT-qPCR as described previously [27, 40]. qPCR SYBR green assay was used to quantify the PCV2b DNA copy numbers with a previously validated protocol [45]. The assay was conducted using iTaq qPCR SYBR green kit (BIO-RAD) and PCV2b-specific primers (PCV2-83F: 5′-TGG TAACCATCCACCACCTT-3′, PCV2-83R: 5′-TGG TACCGATGGGGCTGCTAA-3′) was used as the standard to quantify the DNA copy number [52]. The SIV RNA copy number was quantified by two-step RT-qPCR using iScript cDNA Synthesis Kit and iTaq qPCR SYBR green kit (BIO-RAD) according to the manufacturer’s protocol. The primer pair used for SIV RNA quantification (M25: 5′-AGATGACTTCTAAACCGGAGG TCG-3′, M124: 5′-TGCAAAAAACATTCTCAAGTCTCCTG-3′) has been previously validated elsewhere [53]. Recombinant plasmid expressing the M gene of SIV was used as the standard to quantify cDNA copy number in the qPCR reaction.

**Statistical analyses**

The data were analysed using GraphPad Prism (version 6.0). Unpaired Student’s t-test was used to compare differences between two groups.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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