Short Communication

A novel vaccine against Porcine circovirus type 2 (PCV2) and Streptococcus equi ssp. zooepidemicus (SEZ) co-infection

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ABSTRACT

To develop a vaccine against Porcine circovirus type 2 (PCV2) and Streptococcus equi ssp. zooepidemicus (SEZ) co-infection, the genes of porcine IL-18, capsid protein (Cap) of PCV2 and M-like protein (SzP) of SEZ were inserted into the swinepox virus (SPV) genome by homologous recombination. The recombinant swinepox virus rSPV-ICS was verified by PCR and indirect immunofluorescence assays. To evaluate the immunogenicity of rSPV-ICS, 28 PCV2 and SEZ seronegative Bama minipigs were immunized with rSPV-ICS (n=8), commercial PCV2 vaccine and SEZ vaccine (n=8) or wild type SPV (n=8). The results showed that SzP-specific antibody and PCV2 neutralizing antibody of the rSPV-ICS immunized group increased significantly compared to the wild type SPV treated group after vaccination and increased continuously over time. The levels of IL-4 and IFN-γ in the rSPV-ICS immunized group were significantly higher than the other three groups, respectively. After been co-challenged with PCV2 and SEZ, 87.5% piglets in rSPV-ICS immunized group were survived. Significant reductions in gross lung lesion score, histopathological lung lesion score, and lymph node lesion score were noticed in the rSPV-ICS immunized group compared with the wtSPV treated group. The results suggested that the recombinant rSPV-ICS provided piglets with significant protection against PCV2-SEZ co-infection; thus, it offers proof-of-principle for the development of a vaccine for the prevention of these swine diseases.

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1. Introduction

Porcine circovirus type 2 (PCV2) is the main pathogen of Porcine circovirus associated disease (PCVAD) (Rose et al., 2012). PCV2 preferentially targets the lymphoid tissues, which leads to lymphoid depletion and immunosuppression in pigs (Wikstrom et al., 2011). PCVAD would be exacerbated by concurrent infections with other pathogens, such as porcine reproductive and respiratory syndrome virus (PPRSV) and Streptococcus equi ssp. zooepidemicus (SEZ) (Metwally et al., 2010; Opreiessing et al., 2008). The capsid protein (Cap) which binds to host cell receptors, is the primary immunogenic protein of PCV2, and thus has been the target for development of vaccines of PCV2.

SEZ infects a wide range of animals, including equine, swine, bovine, ovine, avian, canine, feline, and harbor seals (Akinened et al., 2005; Blum et al., 2010). In China, SEZ is the main pathogen of swine streptococcosis (Wei et al., 2012). Humans may also be infected by SEZ via consuming contaminated food or having close contact with the infected animals (Kuusi et al., 2006). M-like protein (SzP) is a cell surface-anchored protein that conveys
phagocytosis resistance and is an important protective antigen, so M-like protein is therefore an ideal target for developing a vaccine against SEZ infections.

In previous studies, we constructed several recombinant SPV vaccines expressing protective antigens of swine pathogens, such as the recombinant rSPV-MRP expressing muramidase-released protein (MRP) of Streptococcus suis, the recombinant rSPV-szp expressing M-like protein (SzP) of SEZ and the recombinant rSPV-cap expressing capsid protein (Cap) of PCV2 (Huang et al., 2012; Lin et al., 2011, 2012), respectively. The results showed that all of the recombinant SPV could express the foreign gene for more than 30 passages and could provide the immunized animals with significant protections. In this study, a recombinant swinepox virus co-expressing porcine IL-18, Cap and SzP was constructed and the potential of using the recombinant swinepox virus as a porcine vaccine candidate against PCV2 and SEZ co-infections also had been explored.

2. Materials and methods

2.1. Viruses, cells and animals

Wild type swinepox virus (wtSPV, Kasza strain, VR-363TM) and PCV-free PK-15 cells (CCL-33TM) used in this study were purchased from the American Type Culture Collection (ATCC). Twenty-eight 21 day old PCV2 and SEZ seronegative gnotobiotic Bama minipigs were purchased from Shanghai Academy of Agricultural Sciences. They were randomly divided into four groups and housed in four separate rooms. All experimental protocols were approved by the Laboratory Animal Monitoring Committee of Jiangsu Province and performed accordingly.

2.2. Construction of the transfer vector

The transfer vector pUSG11/P28ICS was constructed using the pUC19 plasmid (Takara) backbone (Fig. 1). A 1.1 kb flanking region upstream of SPV016 (GenBank: AF410153), containing SPV020, SPV019, SPV018 and SPV017 was amplified from the SPV genomic DNA using primers LF1/LF2 (Table 1); a 1.4 kb flanking region downstream of SPV022, containing SPV021 and SPV020 (Huang et al., 2012), was amplified using primers RF1/RF2 (Table 1). These two PCR amplicons were cloned into pUC19 to construct the plasmid pUS01. A 774 bp GFP gene with the promoter P11 sequence was amplified from the pEGFP-N1 plasmid (Takara) using primers 11G1/11G2 (Table 1). A 78 bp DNA fragment containing the promoter P28 sequence and a multiple cloning site (MCS) was formed by annealing of oligonucleotides 28M1/28M2 (Table 1). These two fragments were cloned into the plasmid pUS01 to construct the plasmid pUSG11/P28. Finally, the 576 bp porcine IL-18 gene (GenBank: AF191088.1) was amplified from the total RNA extracts of swine spleen cells by RT-PCR using primers IL181/IL182 (Table 1); the 699 bp cap gene (GenBank: JN382185.2) was amplified from the PCV2 genomic DNA using primers CAP1/CAP2 (Table 1); the partial 1, 041 bp szp gene (GenBank: EU624402) was amplified from the SEZ ATCC35246 genomic DNA using primers SZIP1/SZIP2 (Table 1); These three fragments were cloned into the plasmid pUSG11/P28 to create the recombinant plasmid pUSZ11/P28ICS.

2.3. Construction and identification of the recombinant swinepox virus

The recombinant swinepox virus rSPV-ICS was constructed by homologous recombination of wtSPV with pUSG11/P28ICS as previously described (Lin et al., 2011). Briefly, PK-15 cells grown in a 6-well plate were infected with the SPV (m.o.i. of 0.05) for 1 h, and subsequently transfected with 4.0 μg of the pUSG11/P28ICS plasmid using ExfectTM Transfection Reagent (Vazyme Biotech Co., Ltd.). The cells were collected after 4 days of incubation in 2 ml EMEM medium with 2.0% FBS. The virus was released by freezing and thawing of the cell suspension three times. The proper dilution of the lysate was used to infect PK-15 cells grown in a 6-well plate, for the further purification of the recombinant virus. The rSPV-ICS was identified by PCR, western blot and indirect immunofluorescence (Lin et al., 2011).

2.4. Animal experimental design

Twenty-eight 21 day old PCV2 and SEZ seronegative gnotobiotic Bama minipigs were purchased from Shanghai Academy of Agricultural Sciences. The piglets were randomly divided into four groups and housed in four separate rooms. Group1 (n = 8) was vaccinated intramuscularly with rSPV-ICS by 5.0 × 10⁶ TCID50 per piglet. Group2 (n = 8) was vaccinated intramuscularly, following the recommended dose, with 1 ml commercial PCV2 vaccine (Boehringer Ingelheim Vetmedica Inc.) and 1 ml commercial SEZ vaccine (Nanjing Tianbang Bio-industry Co., Ltd.) per piglet. Group 3 (n = 8) and group 4 (n = 4) were the challenge control (intramuscularly treated with 5.0 × 10⁶ TCID50 wtSPV) and empty control (without treatment), respectively. All piglets were boosted with same dose 14 days later. At 37 days post primary inoculation, Groups 1, 2, and 3 were challenged with PCV2 (1.0 × 10⁵ TCID50, oronasally) and SEZ (4.0 × 10⁵ CFU, intramuscularly). The animals were monitored for 14 days post-challenge; rectal temperatures, clinical signs, average daily weight gain (ADG) and mortality were observed daily by observers blinded to their vaccination status (Lin et al., 2012). At the end of experiment, the pigs of each group were euthanized for necropsy and pathological examination.

2.5. Serology

Before and after vaccination, sera samples were collected at 7-day intervals for detection of SzP-specific antibodies (Lin et al., 2011) and PCV2 neutralizing antibodies (Wang et al., 2006) as previously described.

2.6. rSPV-ICS induced immune reactions

The levels of serum IFN-γ and IL-4 induced by rSPV-ICS were investigated to evaluate the cellular immune
response. Immune responses were mainly evoked by Th1 and Th2 T-cell subgroups. Th1 cells, which produce IFN-γ, IL-2 and TNF-β, evoke cell-mediated immunity and phagocyte-dependent inflammation. Th2 cells, which produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, evoke strong antibody responses and eosinophil accumulation. The immune response type was assessed indirectly by measuring the levels of IFN-γ and IL-4 in serum. They were detected using ELISA kits (ExCell Bio, China) according to the manufacturer's instructions. Standard curves were generated using control IFN-γ and IL-4 serially diluted twofold in PBS and coated onto ELISA plates overnight at 37 °C. The levels of serum IFN-γ and IL-4 were calculated according to their corresponding standard curves.

2.7. Gross pathology and histopathology evaluation

The total extent of macroscopic lung lesions, the histopathological changes in lung and lymph nodes for each treatment were estimated and calculated as previously described (Straw et al., 1986). Observers were blinded to vaccination status.
Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Restriction enzyme</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF1</td>
<td>GAATTCTAAATCTACTTCCGAA</td>
<td>EcoR I</td>
<td>LF</td>
</tr>
<tr>
<td>LF2</td>
<td>GGTACCTAACTACTAGTTCCACAC</td>
<td>Kpn I</td>
<td>RF</td>
</tr>
<tr>
<td>RF1</td>
<td>CTCGAGGGCCGGATATTGATTGATTA</td>
<td>Xho I</td>
<td>P11; GFP</td>
</tr>
<tr>
<td>RF2</td>
<td>AAGCTTATTTTATCCTATGTTGCCC</td>
<td>Hind III</td>
<td>Not 1</td>
</tr>
<tr>
<td>11G1</td>
<td>CTCGAGGGCCGGATATTGATTGATTA</td>
<td>Not 1</td>
<td>P28 promoter; MCS</td>
</tr>
<tr>
<td>11G2</td>
<td>CTCGAGGGCCGGATATTGATTGATTA</td>
<td>Xho I</td>
<td>Not 1; Kpn 1</td>
</tr>
<tr>
<td>28M1</td>
<td>GAGCTTATTTTATCCTATGTTGACCTAG</td>
<td>P11</td>
<td>Porcine IL-18</td>
</tr>
<tr>
<td>28M2</td>
<td>GAGCTTATTTTATCCTATGTTGACCTAG</td>
<td>P11</td>
<td>Porcine IL-18</td>
</tr>
</tbody>
</table>

Italic = restriction enzyme site.

2.8. Statistical analysis

All data were analyzed using one-way ANOVA and values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Construction and identification of the recombinant swinepox virus

A cloning system was developed to generate the recombinant swinepox virus rSPV-ICS. The foreign genes were inserted into the SPV genome by homologous recombination and the recombinant SPV was screened using the GFP reporter (Fig. 2).

The genome of rSPV-ICS was analyzed by PCR using primers IL181/IL182, Cap1/Cap2 and SzP1/SzP2, which amplified the gene fragments present in the recombinant virus, but not in the wtSPV (Fig. 3). To ensure the viral genomic DNA of rSPV-ICS clones without mutation, one pair of oligonucleotide primers rSPV1/rSPV2 (Table 1) annealing in the flanking regions were designed to amplify 2543 bp of all inserted genes. The amplified products were sequenced, and the results proved the genetic homogeneity and genetic stability of all recombinant virus. Western blot analysis verified the gene products of approximately 41 kDa (SzP), 28 kDa (Cap) and 22 kDa (IL-18) in size, which were detected in rSPV-ICS infected PK-15 cells (Fig. 4).

An indirect immunofluorescence assay was used to verify the expression and localization of IL-18, Cap and SzP.

Fig. 2. The genome of rSPV-ICS. LF and RF respectively indicate left flanking sequences and right flanking sequences of SPV. GFP was the reporter gene.

Fig. 3. PCR analysis of rSPV-ICS. Lane M: DL5000 DNA marker. Lane 1: wtSPV (template), IL181/IL182 (primers). Lane 2: rSPV-ICS, IL181/IL182. Lane 3: wtSPV, Cap1/Cap2. Lane 4: rSPV-ICS, Cap1/Cap2. Lane 5: wtSPV, SzP1/SzP2. Lane 6: rSPV-ICS, SzP1/SzP2.

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A significant red fluorescence was observed in rSPV-cap infected cells, whereas no specific red fluorescence was detected in the wtSPV infected cells (Fig. 5). The majority of the Cap was distributed throughout the cytoplasm. Of more than 30 passages of rSPV-ICS infected cells, PCR, western blot and IFA analysis of the lysate were able to identify the genes and gene products, respectively.

3.2. Clinical evaluation after vaccination

Prior to PCV2 and SEZ challenge, no clinical signs were observed in the four groups. The rectal temperature of the four groups was all below 40.0 °C and no pox spot or papule was observed on the skin surface including injection sites. The average daily weight gain (ADG) difference of the four groups was not significant.

3.3. Detection of SzP-specific antibody

ELISA analysis showed that a SzP-specific antibody response was detected in the rSPV-ICS immunized group 7 days after the first immunization. The antibody level of the rSPV-ICS immunized group gradually increased to maximum titers of 2180 at 35 days after the first vaccination. The difference of the rSPV-ICS immunized group and the commercial SEZ vaccine immunized group was not significant in 4 weeks. The antibody level of the rSPV-ICS immunized group (2180) was significantly higher than the commercial SEZ vaccine immunized group (420) at 35 days post-vaccination (P < 0.05). No significant SzP-specific antibody was observed between the wtSPV treated group and the empty control group.

3.4. Detection of PCV2 neutralizing antibody

For detecting the PCV2 neutralizing activity of sera from immunized piglets, PK15 cells were challenged with PCV2. The results showed that the sera of the rSPV-ICS immunized group protected PK15 cells from PCV2 challenge in vitro, with neutralizing titers up to 1:49 on 35 days after first immunization, whereas sera from the wtSPV treated or empty control group showed no neutralization activity. The antibody titers of the rSPV-ICS vaccinated group were significantly higher than the commercial PCV2 and SEZ vaccines vaccinated group (P < 0.05) by day 14, 28 and 35 post-vaccination.

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3.6. Clinical signs after challenge

All piglets in the wtSPV treated group showed severe clinical symptoms, including rough hair, decreased mobility, arthritis, septicemia, and died within 4 days. In contrast, eight piglets immunized with the commercial PCV2 vaccine and the commercial SEZ vaccine showed no obvious clinical symptoms and were survived during the trial period. However, one piglet in the rSPV-ICS immunized group showed moderate arthritis and died on 4 days post-challenge. The survival rate of the rSPV-ICS vaccinated group was 87.5%. The results indicated that rSPV-ICS provided piglets with a strong protection against PCV2 and SEZ co-challenge.

3.7. Gross pathology and histopathology studies

Gross lesions were observed of the lungs in the wtSPV treated group, including severe pulmonary congestion and atrophy. A significant reduction in gross lung lesion scores was noted in the rSPV-ICS immunized group compared with the wtSPV treated group ($P < 0.05$). Meanwhile, no significant difference was observed between the rSPV-ICS immunized group and the commercial PCV2 vaccine and the commercial SEZ vaccine immunized group (Table 2).

For the morphometric analysis of histopathological pulmonary changes, severe interstitial congestion and thickening, alveolar neutrophil infiltration, and interstitial lymphocyte infiltration were observed. A significant reduction in histopathological lung lesion scores was noted in the rSPV-ICS immunized group compared with the wtSPV treated group ($P < 0.05$). Meanwhile, no significant difference was observed between the rSPV-ICS immunized group and the commercial PCV2 vaccine and the commercial SEZ vaccine immunized group. No obvious histopathological pulmonary lesions were observed in the empty control group (Table 2).

Multifocal lymphoid congestion and necrosis accompanied by inflammatory cells infiltration were observed in the lymph nodes of the wtSPV treated group. Morphometric analysis of histopathological changes in the lymph node showed a significant reduction of histopathological lymph node lesion scores in the rSPV-ICS immunized group compared with the wtSPV treated group ($P < 0.05$). Meanwhile, no significant difference was observed between the rSPV-ICS immunized group and the commercial PCV2 vaccine and the commercial SEZ vaccine immunized group. No histopathological lymph node lesions were observed in the empty control group.

4. Discussion

PCV2 infection could lead to immunosuppression in pigs, and, therefore, more prone to develop co-infections,

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**Table 2**

Lung lesion scores and lymph node lesion scores of different treatment groups (group mean ± standard error).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Lung lesion</th>
<th>Lymph node lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gross score</td>
<td>Microscopic score</td>
</tr>
<tr>
<td>rSPV-ICS</td>
<td>8</td>
<td>0.72 ± 0.47</td>
<td>1.33 ± 0.32</td>
</tr>
<tr>
<td>SEZ + PCV2</td>
<td>8</td>
<td>0.63 ± 0.30</td>
<td>1.28 ± 0.23</td>
</tr>
<tr>
<td>wtSPV</td>
<td>8</td>
<td>8.57 ± 0.73</td>
<td>6.63 ± 0.52</td>
</tr>
<tr>
<td>Empty control</td>
<td>4</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

* Ranges from 0 (normal) to 10 (severe lesion).

b Ranges from 0 (no lesion visible) to 9 (severe lesion).

c Ranges from 0 (normal) to 5 (severe lymphoid depletion and granulomatous replacement).

* Indicates significantly ($P < 0.05$) higher lesion scores of the wtSPV treated group compared to the rSPV-ICS immunized group and the commercial PCV2 and SEZ vaccines immunized group.

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such as SEZ (Wikström et al., 2011). Majority of currently available vaccines against PCV2 or SEZ are inactivated vaccines or subunit vaccines. Recombinant swinepox virus (SPV) immunization represents a novel vaccine strategy, because of their ease of production, safety, and stability, and also can induce both cellular and humoral immunity (Xu et al., 2013). SPV possesses a large double stranded DNA genome and a host range limited to swine. SPV only propagate strictly in the cytoplasm, which avoids the possibility of the integration of its genome into the host’s chromosome. And the processing and presentation of the viral epitope occurs in a way that is similar to natural infection. Many studies have already demonstrated the efficacy of recombinant SPV expressing protective antigens of swine pathogens.

Cytokine adjuvants have been widely used to promote the induction of immune responses and enhance the immune-protectiveness of vaccines (Barouch et al., 2004; Buglione-Corbett et al., 2013; Decker and Safdar, 2011). IL-18, also known as IFN-γ inducing factor (IGIF), is a pleiotropic cytokine that plays an important role in both innate and acquired immunity (Li et al., 2009; Schneider et al., 2010). IL-18 mRNA is expressed in a wide range of cells, including various types of immune competent cells and non-immune cells. Similar to IL-1 in structure, its function is consistent with IL-12 (Takeda et al., 1998), that mainly reflected in the T cell and the enhancement of cell-mediated immunity. In particular, IL-18 augments cytotoxicity of NK cells and enhances the activity of T and NK cells. IL-18 is a unique cytokine that enhances innate immunity and both Th1 and Th2 type immune responses. Therefore, we chose porcine IL-18 as the adjuvant for this recombinant swinepox virus vaccine.

In this study, we constructed a recombinant SPV (rSPV-ICS) co-expressing the porcine IL-18, the Cap of PCV2 and the S2p of SEZ, and tested its immunogenicity in Bama minipigs. The result showed that vaccinated with rSPV-ICS can induce capacity immune responses as vaccinated with commercial PCV2 vaccine and SEZ vaccine. The S2p-specific antibody response and PCV2 neutralizing antibodies of the rSPV-ICS vaccinated piglets were significantly higher than the wtSPV treated piglets and the commercial PCV2 and SEZ vaccines immunized group. The immunoprotection of rSPV-ICS vaccinated piglets against co-challenge of PCV2 and SEZ was 87.5%, indicating that this novel vaccine was effective. The protective efficacy of rSPV-ICS could be enhanced significantly by simultaneous expression of porcine IL-18. The serum IL-4 and IFN-γ levels in immunized piglets indicated that rSPV-ICS had stimulated both Th1 and Th2-mediated immune responses.

In conclusion, the results of immune response and pathogens challenge showed that the rSPV-ICS vaccine was able to elicit strong cellular and humoral response and was able to protect piglets from PCV2 and SEZ co-infection. This new construction will avoid immunosuppression caused by PCV2 infection and prevent the occurrence of streptococcal disease, so it would be beneficial to swine producers. The effectiveness of this novel vaccine in actual field application remains to be determined in the future.

Acknowledgements

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References


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