Immunogenicity of a recombinant parapoxvirus expressing the spike protein of *Porcine epidemic diarrhea virus*

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The parapoxvirus Orf virus (ORFV), has long been recognized for its immunomodulatory properties in permissive and non-permissive animal species. Here, a new recombinant ORFV expressing the full-length spike (S) protein of *Porcine epidemic diarrhea virus* (PEDV) was generated and its immunogenicity and protective efficacy were evaluated in pigs. The PEDV S was inserted into the ORFV121 gene locus, an immunomodulatory gene that inhibits activation of the NF-κB signalling pathway and contributes to ORFV virulence in the natural host. The recombinant ORFV-PEDV-S virus efficiently and stably expressed the PEDV S protein in cell culture *in vitro*. Three intramuscular (IM) immunizations with the recombinant ORFV-PEDV-S in 3-week-old pigs elicited robust serum IgG, IgA and neutralizing antibody responses against PEDV. Additionally, IM immunization with the recombinant ORFV-PEDV-S virus protected pigs from clinical signs of porcine epidemic diarrhoea (PED) and reduced virus shedding in faeces upon challenge infection. These results demonstrate the suitability of ORFV121 gene locus as an insertion site for heterologous gene expression and delivery by ORFV-based viral vectors. Additionally, the results provide evidence of the potential of ORFV as a vaccine delivery vector for enteric viral diseases of swine. This study may have important implications for future development of ORFV-vectored vaccines for swine.

INTRODUCTION

Orf virus (ORFV) is the type member of the genus *Parapoxvirus* of the family *Poxviridae* (Mercer *et al.*, 2007). ORFV is a highly epitheliotropic virus, and keratinocytes and epithelial cells in the oral mucosa are the most important – if not the only – cell type(s) to support ORFV replication in natural hosts (sheep and goats) (Jenkinson *et al.*, 1990). The ORFV genome consists of a dsDNA molecule of approximately 138 kbp in length and contains 131 putative genes (Delhon *et al.*, 2004). ORFV has been long known for its immunomodulatory properties (Weber *et al.*, 2007; Weber *et al.*, 2013). Many genes with immunomodulatory functions have been identified in the ORFV genome, including a homologue of interleukin 10 (IL-10) (Fleming *et al.*, 1997), a chemokine binding protein (CBP) (Seet *et al.*, 2003), a secreted inhibitor of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2 (Deane *et al.*, 2000), a vascular endothelial growth factor (VEGF) (Wise *et al.*, 1999), an interferon (IFN)-resistance gene (McInnes *et al.*, 1998) and more recently, three inhibitors of the NF-κB signalling pathway (ORFV002, ORFV024 and ORFV121) (Diel *et al.*, 2010, 2011a, b). Among these, the IL-10 homologue, the VEGF gene and the NF-κB inhibitor ORFV121 have been shown to contribute to ORFV virulence in the natural host (Diel *et al.*, 2011b; Fleming *et al.*, 2007; Meyer *et al.*, 1999).

ORFV has been historically used as a preventive or therapeutic agent in veterinary medicine (Weber *et al.*, 2007). The potential of ORFV as a recombinant vaccine delivery vector has been explored, and recombinant ORFV vectors...
immunization studies in pigs show that intramuscular (IM) immunization with the ORFV-PEDV-S elicited S-specific IgG, IgA and NA responses. Notably, animals immunized with the ORFV-PEDV-S via the IM route were protected from clinical signs of PED, and presented reduced virus shedding in faeces after oral challenge with a virulent PEDV strain.

RESULTS

Generation and characterization of the ORFV-PEDV-S recombinant virus

The full-length spike protein of PEDV was inserted into the ORFV121 (Diel et al., 2011b) gene locus of the ORFV genome by homologous recombination. ORFV121 (Diel et al., 2011b), was deleted from the ORFV genome and replaced by a DNA fragment encoding the full-length PEDV S protein and the reporter gene encoding the GFP under the control of individual early/late VV7.5 poxviral promoters (Fig. 1a). The recombinant virus was selected and purified by limiting dilution followed by plaque assays based on expression of the GFP (Fig. 1c). Deleted ORFV121 gene sequences were not detected in the purified recombinant virus (Fig. 1b). In contrast, PEDV S sequences were detected in the recombinant virus but not in the wild-type ORFV genome (Fig. 1b). Complete genome sequence of the ORFV-PEDV-S recombinant virus confirmed the insertion of the full-length coding sequences of PEDV S (data not shown).

Replication properties of the ORFV-PEDV-S recombinant virus were assessed in vitro. No differences in replication kinetics and viral yields were observed when multiple-step or one-step growth curves of ORFV-PEDV-S were compared to those of the wild-type OV-IA82 virus in primary ovine foetal turbinate (OFTu) cell cultures (data not shown). Replication kinetics of ORFV-PEDV-S were also assessed in porcine kidney cells (PK-15). Notably, replication of ORFV-PEDV-S in PK-15 cells was markedly impaired when compared to its replication in natural host OFTu cells (Fig. 2a, b). Similar replication kinetics were observed in swine testicle cells (ST, data not shown). These results indicate a marked growth defect for ORFV-PEDV-S in swine cells, demonstrating only minimal virus replication in cells of porcine origin.

Recombinant ORFV-PEDV-S expresses PEDV S in vitro

Expression of PEDV S by ORFV-PEDV-S recombinant virus was assessed by immunofluorescence (IFA) and Western blot assays. Expression of PEDV S by the recombinant ORFV-PEDV-S was assessed during virus infection in OFTu cells by using an S-specific mAb in an indirect IFA assay. High levels of PEDV S were detected in ORFV-PEDV-S-infected cells (Fig. 1c). Similar results were observed in PK-15 cells (data not shown). High levels of PEDV S
(~150 kDa) were detected in ORFV-PEDV-S-infected cells by Western blot assays (Fig. 1d).

Intracellular or surface expression of PEDV S by the recombinant ORFV-PEDV-S was assessed by IFA assays. As shown in Fig. 3a, PEDV S expression was detected in permeabilized (Triton X-100) and in non-permeabilized cells, indicating abundant expression/localization of PEDV S on the surface and intracellular compartment of ORFV-PEDV-S-infected cells.

The stability of PEDV S gene inserted into the ORFV121 locus of the ORFV-PEDV-S genome was assessed by IFA and confirmed by PCR and DNA sequencing following serial passages of the recombinant virus in vitro. Expression of PEDV S was consistently detected in ORFV-PEDV-S-infected cells after 1, 5 or 10 passages of the recombinant virus in OFTu cell cultures (Fig. 3b). Additionally, the full-length PEDV S was amplified from the recombinant virus on passages 1, 5 and 10 and sequencing of the p.10 full-length S revealed 100% identity with the S sequences inserted in the recombinant virus (data not shown). Together, these results demonstrate the stability of PEDV S inserted into the ORFV121 locus.

**Intramuscular immunization with ORFV-PEDV-S induces serum IgG and IgA antibody responses against PEDV in 3-week-old pigs**

The immunogenicity of ORFV-PEDV-S recombinant virus was evaluated in pigs following transcutaneous (TC) or IM immunizations. Three-week-old pigs were immunized with ORFV-PEDV-S via the TC or IM routes on day 0 and received booster immunizations on days 21 and 45 post-

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**Fig. 1.** Generation of recombinant ORFV-PEDV-S virus. (a) Schematic representation of the ORFV genome depicting ORFV121 insertion site and flanking regions (ORFV120 and ORFV122) used to generate the recombinant ORFV-PEDV-S virus. pZGFP-121PEDV-S transfer plasmid containing the full-length PEDV S gene plus the GFP selection reporter under the control of individual early/late VV7.5 poxviral promoters. Recombinant ORFV-PEDV-S genome depicting insertion of the PEDV S and the GFP reporter gene into the ORFV121 gene locus. (b) Agarose gel (1%) demonstrating PCR amplification of an internal region of the S gene (~150 bp) from the genome of the recombinant ORFV-PEDV-S gene and absence of ORFV121 gene sequences in the recombinant ORFV-PEDV-S virus. Wild-type ORFV DNA was used as a negative and positive control on the PCR amplifications with S- and ORFV121-specific primers, respectively. (c) IFA demonstrating expression of PEDV S in cell cultures infected with the recombinant ORFV-PEDV-S virus. Left panel shows expression of the reporter GFP protein by the recombinant virus. Right panel shows expression of PEDV S detected with a mouse mAb against PEDV S. (d) Western blot demonstrating expression of the full-length PEDV S (~150 kDa) by the recombinant ORFV-PEDV-S virus in cell culture in vitro. Cell lysates from cells infected with wild-type ORFV were used as negative controls. Blot was developed with an anti-His tag mAb.
primary immunization (Table 1). Serological responses elicited against PEDV were monitored by indirect S IgG- and IgA-isotype ELISAs. All animals immunized via the IM route with the recombinant ORFV-PEDV-S virus developed IgG and IgA antibody responses against PEDV S (Group 3; Fig. 4a, b), whereas no antibody responses were detected in the animals immunized via the TC route (Group 2) or in animals from control groups (Group 1 and Group 4), immunized with ORFV-GFP vectors (Group 1 and Group 4; Fig. 4a, b). Antibodies were first detected on animals from Group 3 (IM) on day 28 post-immunization (p.i.), following the booster immunization on day 21 (Fig. 4a, b). Notably, while a second booster immunization elicited anamnestic antibody responses in animals immunized by the IM route, no serological responses were detected in animals immunized by the TC route (Fig. 4a, b). Similar results were observed when serological responses were monitored using a whole virus (PEDV) indirect ELISA (data not shown). These results demonstrate that IM immunization with ORFV-PEDV-S elicits robust antibody (IgG and IgA) responses in immunized pigs.

**Intramuscular immunization with ORFV-PEDV-S induces neutralizing antibody responses against PEDV in pigs**

The ability of ORFV-PEDV-S to induce NA responses against PEDV was assessed using a fluorescent focus neutralization (FFN) assay (Okda et al., 2015). Similar to the serological responses detected with the IgG and IgA isotype ELISAs, virus neutralization assays revealed that IM immunization with ORFV-PEDV-S elicited NA responses against PEDV in all immunized animals (Fig. 4c). No NA responses were detected in animals immunized via the TC route or in control sham-immunized animals (Groups 1 and 4; Fig. 4c).

**Intramuscular immunization with ORFV-PEDV-S protects pigs from clinical PED**

To evaluate the protective efficacy of the recombinant ORFV-PEDV-S, pigs from Groups 2, 3 and 4 (Table 1) were challenged orally with the virulent PEDV strain CO13 on day 60 p.i. ($2 \times 10^5$ TCID$_{50}$ animal$^{-1}$). Clinical signs of PED and virus shedding in faeces were monitored after challenge infection. Notably, characteristic clinical signs of PEDV infection were observed in 2/4 (50%) animals from the sham-immunized group (Group 4) and in 3/4 (75%) animals from the TC immunized group (Group 2), while no animals immunized via the IM route (0/4) (Group 3) developed clinical signs of PED. Daily average clinical scores were recorded for each group, and are presented in Fig. 5a. Control sham-immunized animals (Group 4) and TC immunized animals (Group 2) presented significantly higher clinical scores when compared to sham-immunized non-challenged animals (Group 1) or to ORFV-PEDV-S immunized (IM) PEDV challenged animals (Group 3) (Fig. 5).

Virus shedding in faeces was evaluated after challenge infection using real-time reverse transcription PCR (RT-qPCR). Rectal swabs collected from all animals on days 0, 3, 5, 7, 9, 11 and 14 post-challenge (p.c.) were tested by PEDV RTPCR and the duration of virus shedding as well as PEDV genome copy numbers were compared between treatment groups. All challenged animals (Groups 2, 3 and 4) shed PEDV in faeces, while no virus shedding was detected in non-challenged animals (Group 1) (Table 2). Notably, both duration and magnitude of PEDV shedding were markedly decreased in animals from Group 3, immunized via the IM route with the ORFV-PEDV-S virus (Table 2; Fig. 5b). While 3/4 animals in Group 2 (75%) and 4/4 animals in...
Group 4 (4/4; 100 %) were positive on day 3 p.c., only 1/4 animal in Group 3 (25 %) was positive for PEDV (Table 2). Animals in Group 3 shed PEDV in faeces between days 7 and 9 p.c.; however, they ceased shedding virus earlier than animals from Groups 2 and 4 (Table 2). While on day 11 p.c. only 1/4 (25 %) animal from Group 3 was positive, all 4/4 (100 %) animals in Groups 2 and 4 were still shedding PEDV.

Quantitation of PEDV genome copy numbers in faecal swabs revealed a lower amount of the virus being shed in faeces by animals from Group 3, compared to animals from Groups 2 and 4 (Fig. 5b). Together these results indicate that immune responses elicited by IM immunization with the recombinant ORFV-PEDV-S led to protection from clinical PED and reduced virus shedding in faeces.

Serological responses after challenge

The serological responses post-challenge with PEDV were evaluated by S IgG and IgA ELISAs, virus neutralization assays and N IgG ELISA. Only animals in Group 3 presented detectable anti-S and PEDV NAs in serum (Fig. 5a–c and 6a–c) at the day of challenge (day 60 p.i.). Serological responses of each group differed markedly following challenge infection. Animals from Group 2 (which did not seroconvert after TC immunization) and from Group 4 (control sham-immunized group) developed lower levels of S-specific IgG and IgA and NAs (days 10 and 14 p.c.) when compared to animals in Group 3 (which seroconverted after IM immunization with ORFV-PEDV-S) (Fig. 6a–c). Notably, levels of NAs detected in animals from Group 3 were 3–4-fold higher than those in animals in Groups 2 and 4 (Fig. 6c). In contrast, serum IgG responses against PEDV N were more robust in animals from Groups 2 and 4 when compared to animals from Group 3 (Fig. 6d). These differences were more pronounced at early times p.c. (day 7 p.c.; P<0.05), with comparable levels of anti-NAs being detected at later times post-challenge (Fig. 6d). These results indicate an efficient priming of B cells by IM immunization with the recombinant ORFV-PEDV-S, and further demonstrate a robust and typical secondary response, with high level antibody production following challenge with PEDV.

DISCUSSION

The immunogenicity and protective efficacy of ORFv-based vectored vaccine candidates have been demonstrated in

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**Table 1. Experimental design of animal immunization challenge infection**

<table>
<thead>
<tr>
<th>Group*</th>
<th>Vector construct</th>
<th>Route†</th>
<th>Immunization days</th>
<th>Challenge infection ]</th>
<th>Inoculum</th>
<th>Day p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ORFV-GFP</td>
<td>TC+IM</td>
<td>0, 21, 45</td>
<td>MEM</td>
<td>PEDV CO13</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>ORFV-PEDV-S</td>
<td>TC</td>
<td>0, 21, 45</td>
<td>PEDV CO13</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ORFV-PEDV-S</td>
<td>IM</td>
<td>0, 21, 45</td>
<td>PEDV CO13</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ORFV-GFP</td>
<td>TC+IM</td>
<td>0, 21, 45</td>
<td>PEDV CO13</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

*Each group consisted of four 3-week-old weaned piglets.
†All control animals (Groups 1 and 4) were immunized with half dose via the TC route and half dose via the IM route.
‡Virus suspension was applied topically on a 4 cm² area of scarified skin.
§Virus suspension was injected intramuscularly.
||Each animal was challenged orally with a virus suspension containing 2×10⁵ TCID₅₀ of PEDV strain CO13.

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multiple animal species (Amann et al., 2013; Dory et al., 2006; Fischer et al., 2003; Rohde et al., 2011, 2013; Voigt et al., 2007). Notably, ORFV recombinants, based on the highly attenuated ORFV strain D1701, expressing the PRV gC and gD or the CSFV E2 glycoproteins have been shown to protect pigs against challenge with PRV and CSFV, respectively (Dory et al., 2006; Fischer et al., 2003; van Rooij et al., 2010; Voigt et al., 2007). Here we explored the potential of ORFV as a vaccine delivery vector for an enteric pathogen of swine. Using a well-characterized ORFV strain (OV-IA82) (Delhon et al., 2004; Diel et al., 2010, 2011a, b) and the locus of a recently identified virulence determinant of ORFV (ORFV121) as insertion site, we generated a recombinant ORFV expressing the full-length PEDV S protein.

The S is the major envelope glycoprotein of PEDV and has been shown to be the main target of NAs (Chang et al., 2002; Cruz et al., 2008; Sun et al., 2007). Additionally, subunit PEDV vaccine candidates based on the S protein have been shown to induce protective immune responses in pigs (Oh et al., 2014). Here, the coding sequences of the full-length PEDV S were inserted into the ORFV121 gene locus (Diel et al., 2011b) of the ORFV genome. The recombinant ORFV-PEDV-S was successfully generated (Fig. 1b–d), and sequencing of the resultant recombinant virus genome confirmed the insertion of PEDV S and deletion of the ORFV121 gene from the ORFV genome. These results demonstrate the feasibility of ORFV121 gene locus as an insertion site for heterologous antigens in ORFV-based recombinant vectors. Notably, the DNA fragment inserted into the ORFV121 locus is ~5.2 kbp in length (full-length S and GFP coding sequences, and promoter sequences), demonstrating that the ORFV121 locus may accommodate large fragments of heterologous DNA. Successful expression of both PEDV S and the GFP proteins in cells infected with the recombinant ORFV-PEDV-S (Fig. 1c), confirmed the

![Diagram (a) Serum IgG (S) vs Days post-immunization](image)

![Diagram (b) Serum IgA (S) vs Days post-immunization](image)

![Diagram (c) FFN vs Days post-immunization](image)

**Fig. 4.** Immunogenicity of recombinant ORFV-PEDV-S in pigs. (a) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV S protein. (b) Isotype ELISA demonstrating serum IgA antibody responses specific to the PEDV S protein. (c) Virus NA responses elicited by immunization with recombinant ORFV-PEDV-S. S/P, sample to positive ratio; GMT, geometric mean titre. Arrow heads represent immunization/booster immunizations (days 0, 21 and 45). Error bars represent SEM. Statistical significance was determined using one-way ANOVA and Tukey’s honest significant difference (HSD). *Statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 1, 2 and 4.
large payload capacity of the ORFV121 gene locus and of ORFV-based vectors.

The expression and genetic stability of PEDV S carried by the recombinant ORFV-PEDV-S virus were assessed in infected cell cultures. IFAs performed in cell cultures infected with the recombinant ORFV-PEDV-S and permeabilized or not with Triton X-100 revealed high levels of PEDV S expression intracellularly and on the cell surface (Fig. 3a). While intracellular expression of PEDV S may allow for antigen processing and presentation through the MHC I pathway, expression of the protein on the surface of infected cells may allow for direct recognition and uptake of the protein by antigen presenting cells (APCs) or by B lymphocytes, thus potentially leading to stimulation of both cellular and humoral immune responses (Blum et al., 2013). An important requirement for viral vectors is the stability of heterologous genes within their genome (Wang et al., 2010). As shown in Fig. 3b, serial passage of ORFV-PEDV-S in cell cultures in vitro did not affect expression of PEDV S. High levels of the PEDV S protein were detected in cell cultures infected with passages 1, 5 and 10 of the recombinant ORFV-PEDV-S, demonstrating the genetic stability of the insert into the ORFV121 gene locus.

The immunogenicity of the recombinant ORFV-PEDV-S was evaluated in pigs following IM or TC immunizations. While IM immunization has been shown effective for other ORFV-vectored antigens in pigs (Dory et al., 2006; Voigt et al., 2007), proof-of-concept TC immunization was used given its efficacy in inducing mucosal immune responses in other animal species (Lawson et al., 2012). Notably, animals immunized via the IM route developed robust antibody responses (IgG, IgA and NA) against PEDV, whereas no seroconversion was detected in animals immunized via the TC route (Fig. 4a–c). These results corroborate the findings of previous studies (Dory et al., 2006; Voigt et al., 2007), demonstrating that the IM route is an effective route to deliver ORFV-vectored antigens in swine. Although no NAs against ORFV were detected in any of the immunized animals (data not shown), it is possible that local innate inflammatory responses elicited by skin scarification may have affected the delivery and/or expression of PEDV S by ORFV-PEDV-S in the skin, thus, potentially preventing the development of immune responses against PEDV S in animals immunized via the TC route. Additionally, the dose delivered following skin scarification and topical application of the recombinant vector may not have been sufficient to prime and boost the immune system against PEDV S in animals from the TC immunized group. Given the natural tropism of ORFV for keratinocytes in natural hosts (sheep and goats), it would still be interesting, however, to explore more precise methods of TC delivery of ORFV-vectored antigens in the future.

To assess the protective potential of ORFV-PEDV-S-elicited immune responses, animals from Groups 2 (TC) and 3 (IM), and sham-immunized Group 4 (TC+IM), were challenged orally with a virulent PEDV strain CO13 \((2 \times 10^5 \text{ TCID}_{50})\). Notably, while 3 out of 4 (3/4; 75 %) animals from Group 2 (which did not seroconvert after TC immunization; Fig. 4a-c) and 2/4 (50 %) animals from control Group 4 developed characteristic signs of PED, none of the animals from Group 3 (IM, which developed serum antibody responses to PEDV) were affected. Additionally,
animals from Group 3 presented reduced virus shedding in faeces when compared to animals from Groups 2 and 4 (Fig. 5a, b, Table 2). Results from the RT-qPCR performed in rectal swabs show a delayed onset and short duration shedding of PEDV by animals from Group 3 (Fig. 5b, Table 2). Together, these results demonstrate that IM immunization with the recombinant ORFV-PEDV-S protected pigs from clinical PED and reduced virus shedding following oral challenge infection. Although results here show a strong correlation between PEDV-specific antibodies in serum, protection from clinical disease and decreased virus shedding in faeces, the precise immunological mechanisms underlying these findings were not defined in our study. In future studies, it would be interesting to assess, for example, whether IM immunization with the recombinant ORFV-PEDV-S elicits secretory IgA (sIgA) responses at mucosal surfaces, or perhaps, homing of effector B or T lymphocytes to the intestinal mucosa.

Serological responses that followed challenge infection with PEDV varied significantly between immunized groups. While animals from Group 3 presented a robust serological response, typical of a secondary immunological response, characterized by high levels of S-specific and NA responses to PEDV (Fig. 6a–c), animals from Groups 2 and 4 developed delayed antibody responses, typical of primary exposure to PEDV (Fig. 6a–c). In contrast, antibody responses to the N protein (structural protein not present in the ORFV-PEDV-S construct) were lower in animals from Group 3 (day 7 p.c.), suggesting an early inhibition of PEDV infection/replication in animals from Group 3 (Fig. 6d). Together, these results suggest that IM immunization with the recombinant ORFV-PEDV-S virus efficiently primed B cells, which rapidly and effectively responded upon exposure to the virus in the intestinal mucosa, leading to anamnestic antibody responses in immunized animals. Additional studies involving larger numbers of animals with sequential sampling of mucosal and lymphoid tissues would be necessary, however, to dissect the immunological mechanisms underlying the phenotype observed here.

Although correlate(s) of protection for PEDV remain unknown, neutralizing secretory IgA (sIgA) antibodies are thought to play a major role in protection (de Arriba et al., 2002a; Liu et al., 2012; Poonsuk et al., 2016; Saif, 2015). sIgA seem especially important in providing lactogenic immunity and protection during the first days of life of newborn piglets (Chattha et al., 2014; Saif, 2015). One of the main obstacles in eliciting lactogenic immunity to PEDV, however, is the need for local gut stimulation of IgA secreting cells (plasmablasts) and their subsequent migration to the mammary gland where they produce sIgA antibodies which are secreted in the colostrum and milk and ultimately transferred to suckling piglets (gut-mammary-sIgA axis) (Langel et al., 2016). This has been achieved by natural infection, oral vaccination of pregnant sows with live PEDV (de Arriba et al., 2002b; Goede et al., 2015; Song et al., 2007) or, more recently, by a subunit vaccine candidate (S1; Makadiya et al., 2016). Results here show partial protection of nursery pigs to oral PEDV challenge after IM immunization with the recombinant ORFV-PEDV-S. Whether this vector construct is capable of eliciting lactogenic immunity and protection in neonatal piglets remains

Table 2. Virus shedding in faeces after challenge infection with PEDV

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal ID</th>
<th>Virus shedding (day post-challenge)*</th>
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*Virus shedding was assessed by real-time PCR in faecal swabs.
†Experiment was terminated on day 14 p.c.
to be determined. Nevertheless, the fact that IM immunization with the recombinant ORFV-PEDV-S led to protective immune responses in naïve animals indicates that this virus vector could be a useful tool for the control of PEDV in endemic areas. The recombinant ORFV-PEDV-S could be used to immunize naïve gilts prior to their introduction to PEDV positive farms or to boost the immunity of pregnant gilts/sows that have been naturally exposed to the virus.

In summary, here we show the successful generation of a recombinant ORFV containing the full-length S gene of PEDV into the *ORFV121* gene locus. Characterization of the recombinant ORFV-PEDV-S virus *in vitro* demonstrates efficient and stable expression of the heterologous protein in cell cultures infected with the recombinant virus. Immunization challenge studies in pigs, show that IM delivery of the recombinant ORFV-PEDV-S elicits robust serum antibody responses in immunized animals that correlated with protection against clinical PED and decreased virus shedding in faeces. These results may have important implications for future development of ORFV-vectored vaccines for prevention of infectious diseases of swine.

**METHODS**

**Cells and viruses.** Primary OFTu, porcine kidney (PK15; ATCC PTA-8244) and Vero-76 cells (ATCC® CRL-1587™) were cultured at 37°C with 5% CO₂ in minimum essential medium (MEM) supplemented with 10% FBS, 2 mM L-glutamine and containing penicillin.

![Graphs](http://jgv.microbiologyresearch.org)

**Fig. 6.** Serological responses following challenge infection with PEDV. (a) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV S protein. (b) Isotype ELISA demonstrating serum IgA antibody responses specific to the PEDV S protein. (c) Virus NA responses. (d) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV N protein. S/P, sample to positive ratio; GMT, geometric mean titre. Error bars represent SEM. Statistical significance was determined using one-way ANOVA and Tukey's HSD. *Statistically significant at the 0.05 level when the mean of Group 3 is compared to the mean of Groups 1, 2 and 4; **statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 2 and 4; ***statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Group 4.
ORFV strain Ia82, a well-characterized ORFV strain (Delhon et al., 2004; Diel et al., 2010, 2011a, b), was used as the parental virus to construct the recombinant ORFV expressing the PEDV S protein (ORFV-PEDV-S) and in all experiments involving the use of wild-type ORFV. Wild-type and recombinant ORFV viruses were amplified in primary OFTu cells. PEDV strain USA/CO/2013 (CO13) was obtained from the National Veterinary Services Laboratory (NVSL) and propagated in Vero-76 cells in the presence of 1.5 µg ml⁻¹ L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO).

Construction of ORFV-PEDV-S recombination plasmid. The full-length coding sequence of the spike gene of PEDV strain CO13 (GenBank accession no. KF267450) was analysed, and restriction endonuclease sites required for insertion into the ORFV genome [ORFV121 locus (Diel et al., 2011b)] were removed through silent nucleotide substitutions. In addition, early poxviral transcription termination signals (TTTTTNT) present within the coding sequence of PEDV S were removed by introducing silent nucleotide mutations. Coding sequences of the His-tag epitope (6xHis) were added to the 5' and 3' ends of the S coding sequence. The sequence of the VV7.5 early/late poxviral promoter (Chakrabarti et al., 1997) was added to the 5' end of the PEDV S coding sequence. Additionally, HindIII and SalI restriction sites were added to the 5' and 3' ends of the VV7.5-PEDV-S construct, respectively. A single DNA fragment containing the full-length PEDV S coding sequences under the control of the VV7.5 early/late poxviral promoter was chemically synthesized (GenScript, Piscataway, NJ) and subcloned into the plasmid vector pZippy-EGFP (Ning et al., 2011) using HindIII and SalI restriction enzymes (pZGP-PEDV-S).

To insert the PEDV S coding sequences into the ORFV121 genome locus (Diel et al., 2011b), a recombination cassette was constructed. ORFV121 left (LF, 1016 bp) and right (RF, 853 bp) flanking regions were PCR amplified from the ORFV strain Ia82 genome with primers 121LF-Fw(Spel)-5'-ATTCTTTATGCGGCGGCGCGCACGTCTCGAGGAGTCGTC-3'; 121LF-Rv-(HindIII)-5'-CAGAATTCGCAAGCTTGGTTGTTGTTGTTGGCCACAGAGTTGAG-3'; 121RF-Fw-(NotI)-5'-TCACAGGTAACCTGTTCTTCCACGATAGTTGAG-3'; and 121RF-Rv-(BglII)-5'-CAGAATTCGCAAGCTTGGTTGTTGTTGGCCACAGAGTTGAG-3'. PCR amplicons were analysed by electrophoresis in 1 % agarose gels. Insertion and integrity of the PEDV full-length spike sequences were confirmed by whole genome sequencing using the Nexera XT DNA library preparation kit (Illumina, San Diego, CA) followed by sequencing on the Illumina Mi-Seq sequencing platform (Illumina, San Diego, CA).

Immunofluorescence. Expression of PEDV S by the ORFV-PEDV-S recombinant virus was assessed by IFA. OFTu cells were infected with the ORFV-PEDV-S recombinant virus (m.o.i. of 1) and fixed with 3.7 % formaldehyde at 24 h post-infection. After fixation cells were washed three times with PBS and permeabilized with 0.2 % PBS-Triton X-100 for 10 min at room temperature (RT). Unpermeabilized cells were kept as controls to assess expression of PEDV S on the membrane of ORFV-PEDV-S-infected cells. Cells were washed three times with PBS and incubated with PEDV S-specific mouse mAb (SD37-11; 1: 25 in PBS-1 % BSA) for 1 h at RT. After primary antibody incubation, cells were washed as above and incubated with goat anti-mouse IgG (H+L) secondary antibody (1: 250 in PBS-1 % BSA; Alexa Fluor 594 conjugate; Life Technologies, Carlsbad, CA) for 1 h at RT. Cells were washed three times with PBS and visualized under a fluorescence microscope.

Western blot. Expression of PEDV S by the ORFV-PEDV-S recombinant virus was assessed by Western blot. OFTu cells cultured in 6-well plates were infected ORFV-PEDV-S recombinant virus (m.o.i. of 10) and harvested at 48 h post-infection. Cells infected with parental ORFV strain Ia82 were used as controls. Cells were lysed with M-PER mammalian extraction reagent (Thermo Scientific, Waltham, MA) containing protease inhibitors (RPI, Mount Prospect, IL). Fifty micrograms of whole cell protein extracts was resolved by SDS-PAGE in 7 % acrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with 5 % non-fat dry milk TBS-Tween 20 (0.1 %; TBS-T) solution for 1 h at RT and probed with anti-His epitope tag antibody (His.His, Thermo Scientific, Waltham, MA) overnight at 4 °C. Blots were washed three times with TBS-T for 10 min at RT and incubated with a goat anti-mouse IgG-HRP conjugate secondary antibody for 2 h at RT. Blots were washed three times with TBS-T for 10 min and developed by using a chemiluminescent substrate (Clarity, ECL; Bio-Rad, Hercules, CA).

Antigens for ELISAs. A truncated form of PEDV S protein was expressed as a recombinant protein in Escherichia coli, and used in indirect ELISAs to assess antibody responses in animals immunized with the ORFV-PEDV-S virus. A fragment of the PEDV S protein corresponding to nucleotides 1891–2400 was amplified from the genome of PEDV strain CO13 using standard reverse transcriptase and PCR amplification methods. Primers used for PCR amplification were PED-SFlo-1891 (5'-TTCGCGGACATCCAGAGGACACTC-3') and PED-SFlo-1892 (5'-CGGCGGATCCAGCCTAACCATTGGAAG-3').
PED-Spiks-2400/XholJR-S'-5'GACATCGAGTAAAGCTGTTAAATGTTGTCG-3'. The PCR amplicon was cloned into the bacterial expression plasmid pET-28a (EMD Millipore – Novagen, Billerica, MA) using the restriction enzymes indicated on each primer sequence (underlined). The recombinant S protein was expressed in E. coli as a 6xHis-tagged fusion protein (Okda et al., 2015) and purified using nickel-charged agarose resin (Qiagen, Valencia, CA) according to the manufacturer’s recommendations. Affinity purified recombinant protein was refolded and used as antigen on indirect ELISAs. DNA sequencing was used to confirm the identity and in frame cloning of PEDV S with 6xHis-tag.

**Animal immunization challenge studies.** The immunogenicity and protective efficacy of the ORFV-PEDV-S recombinant virus were assessed in pigs. The efficacy of the vector was evaluated by two immunization routes: TC and IM. The TC route was explored here because it has been shown to induce mucosal and systemic immunity in mice and humans (Lawson et al., 2012), and because of the natural tropism of ORFV for keratinocytes in the natural host (Jenkinson et al., 1990). Sixteen 3-week-old pigs, seronegative for PEDV, were randomly allocated to four experimental groups as follows: Group 1, sham-immunized/mock-challenged (n=4); Group 2, ORFV-PEDV-S-immunized/PEVD challenged (n=4); Group 3, ORFV-PEDV-S-immunized/PEVD challenged (n=4) and Group 4, sham-immunized/PEVD challenged (n=4) (Table 1). Animals from Groups 1 and 4 were immunized with a control ORFV vector expressing GFP (ORFV-GFP) and received half dose via the TC (1 ml) and the other half via the IM route (1 ml). Animals from Group 2 were immunized with the ORFV-PEDV-S recombinant virus via the TC route only, while animals in Group 3 were immunized by the IM route. IM immunization was performed by injection of 2 ml of a virus suspension containing 10^7.20 TCID_{50} ml^{-1} in MEM into the neck. For the TC immunization the skin of the inguinal region was scarified with a sterile scalpel blade and the virus suspension containing 10^7.20 TCID_{50} ml^{-1} in MEM was applied topically to the scarified skin area using a sterile cotton swab (~4 cm²). All animals were immunized on day 0 and received two booster immunizations on days 21 and 45 post-primary immunization. Booster immunizations (TC and IM) were performed by scarification or injection in sites adjacent (3-5 cm) to original immunization sites. Serum samples were collected on days 0, 7, 14, 21, 35, 42, 49, 53, 56 and 60 p.i.

Challenge infection was conducted to evaluate the efficacy of recombinant ORFV-PEDV-S virus. On day 60 p.i., animals from Group 1 received 2 ml of MEM orally (mock challenge), while animals from Groups 2, 3 and 4 were challenged orally with a virus suspension containing 2×10^5 TCID_{50} of PEDV strain CO13. Animals were monitored daily for clinical signs of PED. Clinical signs were recorded and individual daily scores assigned to all animals based on the following criteria: 0, normal faeces; 1, pasty faeces; 2, moderate diarrhoea (semi-liquid); 3, diarrhoea (liquid); 4, severe diarrhoea (very liquid) and 5, watery diarrhoea (profuse diarrhoea) (de Arriba et al., 2002b). Mean daily group scores were calculated and compared among different treatment groups. Faecal swabs were collected on days 0, 3, 5, 7, 9, 11 and 14 post-challenge to assess virus shedding in faeces. Serum samples were collected on days 0, 3, 7, 10 and 14 post-challenge to evaluate humoral and cell-mediated responses. All animals were euthanized on day 14 post-challenge. Animal immunization challenge studies were conducted at SDSU Animal Resource Wing (ARW), following the guidelines and protocols approved by the SDSU Institutional Animal Care and Use Committee (IACUC approval no. 15-063A).

**Antibody isotype ELISAs.** PEDV-specific IgG and IgA antibody responses elicited by immunization with the recombinant ORFV-PEDV-S virus were assessed by S indirect ELISAs, while responses post-challenge infection were assessed by the S and nucleoprotein (N) (Okda et al., 2015) ELISAs. Optimal assay conditions (amount of antigen, serum and secondary antibody dilutions) were determined by a checkerboard titration. Polystyrene microtitre plates (Immunolon 1B, Thermo Scientific, Waltham, MA) were coated with the appropriate antigen, S, 100 ng well^{-1}; and N, 25 ng well^{-1} (Okda et al., 2015) in bicarbonate/carbonate coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) in alternate wells. After incubation at 37°C for 1 h, plates were washed four times with PBS Tween 20 (PBS-T, 0.05%) and blocked overnight at 4°C with PBS-T 5% non-fat dry milk. Blocking reagent was removed and plates washed three times with PBS-T (300 ml). Test and control serum samples were diluted (1:50) in PBS-T 5% non-fat dry milk, and 100 µl of diluted samples were added to paired coated and uncoated control wells and incubated at room temperature for 1 h. Unbound antibodies were washed with PBS-T (three times) and plates incubated with biotinylated secondary antibodies against swine IgG or IgA (Bethyl Laboratories, TX) followed by incubation with streptavidin-HRP conjugate (Pierce, Rockford, IL). Reactions were developed with 3,3',5,5'-tetramethylbenzidine substrate (TMB) (KPL, Gaithersburg, MA) and OD values determined at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT). OD values for each test and control samples were normalized to the OD value of uncoated wells and results expressed as sample to positive (S/P) ratios that were calculated as follows: S/P=optical density (OD) of sample – OD of buffer/OD of positive control – OD of buffer. All assay formats were pre-validated at the SD Animal Disease Research and Diagnostic Laboratory (ADRDL) using serum samples from animals of known serological status.

**Fluorescent focus neutralization assay.** NA responses elicited by immunization with the recombinant ORFV-PEDV-S were assessed by FFN assay as previously described (Okda et al., 2015). Endpoint NA titres were determined as the highest dilution of serum capable of reducing 90% of PEDV fluorescent foci relative to negative control samples. A FFN titre <20 was considered negative.

**Viral RNA extraction and real-time reverse transcription PCR.** Viral nucleic acid was extracted from faecal swabs using the MagMAX Viral RNA/DNA Isolation Kit (Life Technologies, Carlsbad, CA) following the manufacturer’s instructions. Shedding of PEDV in faeces was assessed using a commercial multiplex (RT-qPCR) kit targeting the spike gene of PEDV, and other enteric coronaviruses including transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) (EZ-PED/TGE/PDCoV MPX 1.0, Tetracore, Rockville, MD). Genome copy numbers per millilitre were determined using the relative standard curve method. All calculations were performed using a four-parameter logistic regression curve. RT-qPCR tests were performed at the SDSU ADRDL.

**Statistical analysis.** Statistical analysis was performed using SPSS 14 software. One-way ANOVA with Tukey’s HSD multiple comparison test was performed on all groups, using harmonic mean sample size of 4 to account for equal group sizes. Nonparametric Kruskal–Wallis test was used to assess differences in virus shedding between treatment groups. Differences between groups were considered significant at P<0.05.

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D. G. D and E. F. F. declare that there is a patent pending related to this work (US patent pending, P11703US00).

REFERENCES


Immunogenicity of recombinant ORFV-PEDV-S in pigs


