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

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Introduction

Orf virus (ORFV), 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-monocyte 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...
Based on the highly attenuated ORFV strain D1701 have been shown to induce protective immunity against several viral diseases in permissive and non-permissive animal species (Amann et al., 2013; Dory et al., 2006; Fischer et al., 2003; Henkel et al., 2005; Marsland et al., 2003; Rohde et al., 2011, 2013; van Rooij et al., 2010; Voigt et al., 2007). Notably, ORFV D1701-based recombinants expressing the pseudorabies virus (PRV) glycoproteins gC and gD induced protective immunity against PRV infection in pigs (Dory et al., 2006), while a recombinant ORFV expressing the classical swine fever virus (CSFV) E2 glycoprotein protected swine against intranasal challenge with a virulent CSFV strain (Voigt et al., 2007). These studies demonstrate the efficacy of ORFV-based vectors in eliciting protective immune responses in swine. In the present study, the potential of ORFV as a vaccine delivery vector for enteric viral diseases of swine was investigated. The porcine epidemic diarrhea virus spike (PEDV S) glycoprotein was used as a model antigen to evaluate the immunogenicity and protective efficacy ORFV-based vectors in pigs.

Porcine epidemic diarrhea virus (PEDV), a member of the genus Alphacoronavirus of the family Coronaviridae, causes severe enteric disease (porcine epidemic diarrhea; PED) in pigs (Debouck et al., 1981; Pensaert & de Bouck, 1978). PEDV infects pigs of all ages, producing high mortality rates (50–100 %) in suckling piglets and weight loss due to diarrhea in older animals (Pensaert & de Bouck, 1978). The virus replicates primarily in enterocytes of the small intestines leading to villous atrophy and malabsorptive diarrhea followed by electrolyte imbalance, metabolic acidosis and death (Madson et al., 2014). Characteristic clinical signs of PED include watery diarrhea, vomiting, anorexia and dehydration (Lee, 2015). In older pigs, the disease is usually milder resulting in low mortality rates (Lee, 2015).

The PEDV genome is a large (~28 kb) positive-sense RNA molecule that contains six ORFs, encoding the replicase proteins (pp1a and pp1ab), four structural proteins [spike (S), envelope (E), membrane (M) and nucleoprotein (N)] and one accessory protein (ORF3) (Lee, 2015; Song & Park, 2012). Among the structural proteins, the S protein is the major envelope glycoprotein responsible for virus attachment and entry (Cruz et al., 2008; Sun et al., 2007, 2008). Given its critical function in attachment and entry, the S glycoprotein is the main target for neutralizing antibodies (NA) (Chang et al., 2002; Cruz et al., 2008; Paudel et al., 2014; Suo et al., 2012), with several neutralizing epitopes being mapped to this glycoprotein (Chang et al., 2002; Cruz et al., 2006, 2008; Sun et al., 2007, 2008).

Here a novel recombinant ORFV expressing the full-length PEDV S protein (ORFV-PEDV-S) was generated, and its immunogenicity and protective efficacy were evaluated in pigs. The PEDV S coding sequences were inserted into the ORFV121 gene locus, a recently characterized immunomodulatory gene of ORFV that contributes to the virus virulence in the natural host (Diel et al., 2011b). Results from 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 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).

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 RT-qPCR 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

**Fig. 2.** Replication properties of the recombinant ORFV-PEDV-S. (a) Multistep growth curve of the recombinant ORFV-PEDV-S in primary OFTu and porcine kidney (PK15) cells. (b) Single-step growth curve of the recombinant ORFV-PEDV-S in primary OFTu and porcine kidney (PK15) cells. Cells were collected at indicated time points and virus titres determined by the Spearman and Karber’s method and expressed as log₁₀ tissue culture infections dose 50 (TCID₅₀) per millilitre. Error bars represent SEM calculated based on the results of three independent experiments.
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**

| Group | Vector construct | Route† | Immunization days | Challenge infection|| |
|-------|-----------------|--------|------------------|-------------------|-------------------|
|       |                 |        |                  | Inoculum          | Day p.i.          |
| 1     | ORFV-GFP        | TC†+IM§| 0, 21, 45        | MEM               | 60                |
| 2     | ORFV-PEDV-S     | TC     | 0, 21, 45        | PEDV CO13         | 60                |
| 3     | ORFV-PEDV-S     | IM     | 0, 21, 45        | PEDV CO13         | 60                |
| 4     | ORFV-GFP        | TC+IM  | 0, 21, 45        | PEDV CO13         | 60                |

*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.
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

![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.](image-url)
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 (2×10⁵ TCID₅₀). 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 pigs (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

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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.
(100 U ml⁻¹), streptomycin (100 µg ml⁻¹) and gentamicin (50 µg ml⁻¹).

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 (ORFVPEDV-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-phenyl-ethyl 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. KF627450) 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 poxviral transfer vector pZippy-EGFP (Ning et al., 2011) using HindIII and SalI restriction enzymes (pZGFP-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(SpeI)-5'-ATTCTTATGCGGCCGCGCACGTCTGCCAGAGTGCTC-3'; 121LF-Rv-(HindIII)-5'-CAGAATTCGACGTTGTTGTGTGCGGACAGAGTGTTGAG-3'; 121RF-Fw-(NotI)-5'-ATTCTTAATCGGCGGGCCAGCTCGCACGAGGAGTGCTC-3'; 121RF-Rv-(BglII)-5'-CAGAATTCGACGTTGTTGTGTGCGGACAGAGTGTTGAG-3'. The primers used for PCR amplification of PEDV-S sequences were PEDV-SPikS-1891(BamHI)-F-5'-CGGCGGATCCCGCTCGTATGAGG-3' and PEDV-SPikS-1891(BamHI)-R-3'-CGGCGGATCCCGCTCGTATGAGG-3'. The recombinant 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 mamalian 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 chemiluminescent substrate (Clarity, ECL; Bio-Rad, Hercules, CA).

Growth curves. Replication properties of ORFV-PEDV-S recombinant virus were assessed in vitro. Oftu and PK15 cells were cultured in 6-well plates, inoculated with ORFV-PEDV-S [m.o.i. of 0.1 (multistep growth curve) and m.o.i. of 10 (single-step growth curve)] and harvested at various time points post-infection (6, 12, 24, 48 and 72 h). Virus titres were determined on each time point using the Spearman-Karber's method (Hierholzer & Killington, 1996) and expressed as tissue culture infective dose 50 (TCID₅₀) per millilitre.

Antigens for ELISAs. A truncated form of PEDV S protein was expressed as a recombinant protein in Esherichia 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-SpKs-1891(BamHI)-F-5'-CGGCGGATCCCGCTCGTATGAGG-3' and PED-SpKs-1891(BamHI)-R-3'-CGGCGGATCCCGCTCGTATGAGG-3'.
PED-Spik-2400 (Xhol)R-5′-CACACCTCGAGTTAAGGCTGAAAATT TCTGTTCC-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 the naturally 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/PEDV challenged (n=4); Group 3, ORFV-PEDV-S-immunized/PEDV challenged (n=4) and Group 4, sham-immunized/PEDV 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 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 10⁷ TCID₅₀ ml⁻¹ 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 SDS 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 FN 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 FN 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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