Genetic manipulation of porcine epidemic diarrhoea virus recovered from a full-length infectious cDNA clone

Juggragarn Jengarn, Phonphimon Wongthida, Nanchaya Wanasen, Phanramphoei Namprachan Frantz, Asawin Wanitchang and Anan Jongkaewwattana

Correspondence
Anan Jongkaewwattana
anan.jon@biotec.or.th

Virology and Cell Technology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani 12120, Thailand

Porcine epidemic diarrhoea virus (PEDV) causes acute diarrhoea and dehydration in swine of all ages, with significant mortality in neonatal pigs. The recent rise of PEDV outbreaks in Asia and North America warrants an urgent search for effective vaccines. However, PEDV vaccine research has been hampered by difficulties in isolating and propagating the virus in mammalian cells, thereby complicating the recovery of infectious PEDV using a full-length infectious clone. Here, we engineered VeroE6 cells to stably express porcine aminopeptidase N (pAPN) and used them as a platform to obtain a high-growth variant of PEDV, termed PEDV AVCT12. Subsequently, the full-length cDNA clone was constructed by assembling contiguous cDNA fragments encompassing the complete genome of PEDV AVCT12 in a bacterial artificial chromosome. Infectious PEDV could be recovered, and the rescued virus displayed phenotypic properties identical to the parental virus. Interestingly, we found that PEDV AVCT12 contained a C-terminal deletion of the spike gene, resulting in disruption of the ORF3 start codon. When a functional ORF3 gene was restored, the recombinant virus could not be rescued, suggesting that ORF3 could suppress PEDV replication in vitro. In addition, a high-growth and genetically stable recombinant PEDV expressing a foreign protein could be rescued by replacing the ORF3 gene with the mCherry gene. Together, the results of this study provide a means to generate genetically defined PEDV as a promising vaccine candidate.

INTRODUCTION

Porcine epidemic diarrhoea virus (PEDV) is the causative agent of porcine epidemic diarrhoea, a highly contagious disease of pigs characterized by acute watery diarrhoea and vomiting. The disease has a mortality as high as 100% in newborn piglets, and infected pre-weaning pigs often succumb to severe dehydration (Jung & Saif, 2015; Song & Park, 2012). Since its first appearance in Europe, PEDV outbreaks have occurred persistently in Asia and recently in North America, resulting in enormous economic loss worldwide to the swine industry (Pasick et al., 2014; Vlasova et al., 2014; Wang et al., 2014). PEDV is an enveloped RNA virus possessing a single-stranded, positive-sense genome with a 5' cap and a 3' poly(A) tail and belonging to the genus Alphacoronavirus in the family Coronaviridae (Masters, 2006; Park et al., 2012). Based on the entire genome sequence of the well-characterized CV777 strain, the PEDV genome is approximately 28 kb, bearing at least seven overlapping ORFs encoding non-structural proteins including replicase1a, 1b and ORF3, and structural proteins including spike (S), envelope (E), matrix (M) and nucleocapsid (N) proteins (Kocherhans et al., 2001).

For as yet unknown reasons, efforts to productively propagate PEDV in mammalian cells have remained largely unsuccessful. Although it was shown that PEDV could be propagated in African green monkey kidney (Vero) cells with treatment using trypsin (Hofmann & Wyler, 1988; Shirato et al., 2011; Wicht et al., 2014), the described method appears inapplicable to most isolates (Oka et al., 2014). As multiple passages are usually required to obtain high-growth PEDV, only a few cell-adapted strains have been reported (Kusanagi et al., 1992; Kweon et al., 1999). During the past decade, studies have identified porcine aminopeptidase N (pAPN) or CD13 as a receptor of PEDV by showing that expression of pAPN in non-permissive cells enabled them to support productive PEDV infection (Li et al., 2007; Oh et al., 2003). Moreover, high levels of
pAPN expression were reported to be a critical prerequisite for PEDV infection, as engineered swine testicular (ST) cells expressing enhanced levels of pAPN could substantially support productive PEDV replication compared with the non-permissive WT counterpart (Nam & Lee, 2010).

Despite their large genome size, infectious particles of many coronaviruses can be recovered successfully in vitro using various technological platforms (Almazán et al., 2014; Totura & Baric, 2012). One of the most effective strategies is either by direct introduction of a full-length cDNA infectious clone into permissive cells or by in vitro transcription of the clone followed by transfection of the full-length RNA. In fact, reverse genetics systems developed to recover several coronaviruses, including mouse hepatitis virus (Yount et al., 2002), transmissible gastroenteritis virus (TGEV) (Ortego et al., 2002; Yount et al., 2000), avian infectious bronchitis virus (Casais et al., 2001) and, more recently, severe acute respiratory syndrome coronavirus and Middle East respiratory syndrome coronavirus (MERS-CoV) (Almazán et al., 2006, 2013; Scobey et al., 2013; Yount et al., 2003), have been reported using this approach. However, the recovery of infectious PEDV using the full-length cDNA approach has been challenged by several technical difficulties, one of which is the fact that available cell lines do not adequately support productive replication of the virus, and the full-length cDNA clone of PEDV is not stable in conventionally used plasmid vectors. Notably, Li et al. (2013) recently described the reverse genetics of PEDV based on targeted RNA recombination technology. Although applicable, this approach could be complicated by several steps including the generation of a PEDV derivative carrying spikes derived from another coronavirus, and use of the RNA of this so-called chimeric virus as an intermediate for recombination with a parental RNA to restore the PEDV spike. This strategy is generally chosen to rescue recombinant coronaviruses in vitro when the full-length infectious cDNA clones are not yet available (Masters & Rottier, 2005).

In this study, we engineered Vero E6 cells to stably express pAPN, hereafter referred to as VeroE6–APN cells, and successfully used them to obtain a high-growth PEDV, called PEDVAVCT12. We then synthesized contiguous cDNA fragments for assembly of the full-length PEDV genome in a bacterial artificial chromosome (BAC). Transfection of the PEDVAVCT12 cDNA clone into VeroE6–APN cells resulted in the production of infectious PEDV. The growth characteristics of the recombinant PEDV were indistinguishable from those of the parental virus. We also used the developed reverse genetics system to demonstrate that the ORF3 protein could impair PEDV replication in vitro. Moreover, we showed that PEDVAVCT12 can be engineered to harbour a foreign gene, which may serve as a new platform for candidate PEDV vaccine development.

RESULTS

Generation of Vero E6 cells permanently expressing pAPN for PEDV propagation

To establish a reverse genetics system to rescue infectious PEDV in vitro, a platform supporting virus replication was needed. Given that expression of pAPN was found to render a number of cells permissive for PEDV infection (Ortego et al., 2002; Yount et al., 2000), we thus speculated that Vero E6 cells stably expressing pAPN would do likewise. To this end, we constructed a retroviral vector bearing pAPN followed by an internal ribosomal entry site (IRES) and the EGFP gene, designated pQCXIH-pAPN-EGFP (Fig. 1a). Retroviruses were subsequently produced and used to transduce Vero E6 cells. The transduced cells were sorted for EGFP expression by flow cytometry, and the sorted cells were serially diluted to obtain single clones of VeroE6–APN cells. Flow cytometry analysis revealed that more than 99% of VeroE6–APN cells expressed EGFP, indicating stable expression of the transduced genes (data not shown). As antibodies specific for pAPN are not available commercially, we instead determined the level of pAPN expression by assessing its aminopeptidase activity. To do so, cell-surface APN activity of VeroE6–APN cells was assessed using an assay based on the hydrolysis of leucine p-nitroanilide (Leu-pNA) (Cristofoletti et al., 2006). Results from independent experiments (n=4) indicated that VeroE6–APN cells displayed substantially higher APN activity compared with VeroE6 cells (Fig. 1b). Among all VeroE6–APN clones, the one with the highest APN activity was selected for PEDV isolation from reverse transcription (RT)-PCR-positive samples (n=5) extracted from the small intestines of infected piglets from different Thai farms during outbreaks in 2010. We observed that four out of five isolates gave rise to syncytium formation in VeroE6–APN cells 3–5 days post-infection (p.i.). To confirm the infection, the syncytia were subjected to immunofluorescence analysis by mAbs directed against PEDV N protein and high expression of PEDV N protein was noted (Fig. 1c). Of note, when PEDV was pre-heated at 56 °C for 30 min prior to adsorbing onto VeroE6–APN cells, no syncytium formation was detected (data not shown). Taken together, these results demonstrated that expression of pAPN could promote PEDV replication in VeroE6–APN cells.

Genome characteristics of PEDVAVCT12

The PEDV isolate exhibiting the most extensive syncytium formation observed visually in infected VeroE6–APN cells (PEDVAVCT12) was subjected to RNA extraction followed by whole-genome sequencing. Full-length genome analysis revealed that this isolate exhibited a 52 nt deletion at the C-terminal region of the S gene, causing a shortening of the S protein by 7 aa. Furthermore, this truncation of the S gene also resulted in disruption of the ORF3 gene reading frame (Fig. 2). In addition, we found that this unique S gene sequence was also present in the intestinal sample prior to
inoculation of VeroE6–APN cells. Moreover, we did not observe deletion of the S gene in other isolates tested in this study (data not shown). When the genome sequence of PEDVAVCT12 was compared with others in GenBank, we discovered that this shortening of S and loss of ORF3 were similarly reported in the published sequence of PEDV strain CHM2013 (GenBank accession no. KM887144). A whole-genome alignment showed that PEDVAVCT12 and CHM2013 shared approximately 99 % nucleotide sequence identity (data not shown). Although not directly proven in this study, this finding suggests that PEDVAVCT12 was originally derived from a CHM2013-like strain.

Construction of a PEDV infectious cDNA clone
To generate a construct spanning the entire genome of PEDVAVCT12, we synthesized eight overlapping DNA fragments, designated A–H, flanked by appropriate restriction sites introduced at specific sites of the PEDV genome (Fig. 3a). Notably, the PEDV genome contains sequences that are toxic to Escherichia coli, leading to instability of the insert during the cloning process. To resolve this issue, we employed the BAC vector pSMART-BAC, which contains transcriptional terminator sequences flanking both directions of the cloning site, thus allowing the cloning of toxic or unstable inserts. To generate a backbone to harbour the full-length PEDV cDNA clone, we designed the BAC system following a strategy used previously for the recovery of MERS-CoV (Almazán et al., 2013). To this end, pSMART-BAC was modified to incorporate the synthesized sequences of the cytomegalovirus (CMV) immediate-early promoter, PEDV 5′ UTR, EGFP, the C-terminal end of the N gene (nt 26 372–27 657), PEDV 3′ UTR, a 25-residue poly(A) tract, hepatitis delta virus

![Fig. 1. Characteristics of VeroE6–APN cells. (a) Schematic representation of the pQCXIH-pAPN-EGFP retroviral vector constructed to stably express EGFP and pAPN proteins in VeroE6–APN cells. CMV, cytomegalovirus; PSV40, simian virus 40 promotor. The GenBank accession no. of pAPN is indicated. (b) pAPN activity of parental Vero E6 and VeroE6–APN cells was assessed based on the hydrolysis of leucine p-nitroanilide. The absorbance of p-nitroanilide was measured at a wavelength of 405 nm (A405). ***, P<0.001. (c) Immunofluorescence staining of VeroE6–APN cells infected by PEDV extracted from infected piglet’s intestines using mouse mAbs specific for PEDV N protein. Nuclei were stained with DAPI.](image)
(HDV) ribozyme self-cleavage site, and bovine growth hormone termination and poly(A) sequence (BGH) (Fig. 3b). It is important that the synthetic construct generates mRNA bearing 3' and 5' UTRs identical to the natural PEDV RNA. Therefore, the plasmid was checked by transfection into human embryonic kidney (HEK) 293T cells and the mRNA of GFP was subsequently extracted and subjected to 3' and 5' RACE analysis to verify the sequences of the 3' and 5' UTRs transcribed under the synthesized CMV promoter (data not shown). Once the plasmid was verified, the GFP gene was then exchanged with the full-length PEDV fragment generated from the sequential cloning of fragments A–H according to the strategy depicted in Fig. 3(c), resulting in the generation of the full-length infectious cDNA clone, pSMART-BAC-PEDVAVCT12.

**Rescue of infectious PEDV from the cDNA clone in VeroE6–APN cells**

Recovery of reverse genetics-derived PEDV (RgPEDV AVCT12) was carried out by transfecting pSMART-BAC-PEDV AVCT12 into VeroE6–APN cells. Within 72 h post-transfection, we started to observe syncytium formation in the transfected culture. The recovered viruses were subsequently characterized for their genotypic properties. To this end, we made use of the MluI restriction site introduced at nt 26372 of the cDNA clone (Fig. 3) to distinguish RgPEDV AVCT12 from the parental PEDV AVCT12. As shown in Fig. 4(a), RT-PCR products amplified from RNA of RgPEDV AVCT12 could be digested by MluI, whilst those of PEDV AVCT12 were unaffected. In addition, nucleotide sequencing of the RT-PCR products also indicated that the MluI restriction site was present in the genome of RgPEDV AVCT12 but not in that of PEDV AVCT12 (Fig. 4a). When RgPEDV AVCT12 and the parental PEDV AVCT12 were propagated in VeroE6–APN cells, progressive cell fusion was detected as early as 48 h p.i. and both of them replicated to titres approaching 5–6 log TCID50 ml⁻¹ (Fig. 4b). Moreover, the presence of PEDV was confirmed by immunofluorescence staining of RgPEDV AVCT12-infected VeroE6–APN cells as well as Western blot analysis of supernatants obtained from VeroE6–APN cells infected by the parental PEDV AVCT12 and RgPEDV AVCT12 using mAbs specific for the PEDV N protein (Fig. 4c).

**PEDV AVCT12 bearing functional ORF3 cannot be rescued by reverse genetics**

Given that the genome of PEDV AVCT12 lacks the start codon of the ORF3 gene (Fig. 2a) and some cell-adapted PEDV strains display internal truncation of the ORF3 gene (Park et al., 2008), we speculated that functional ORF3 might play an important role in regulating PEDV replication in
Fig. 3. Construction of full-length infectious clone of PEDV<sub>AVCT12</sub>. (a) Schematic representation of the synthesized full-length genome of PEDV<sub>AVCT12</sub> showing relevant restriction sites used for assembly of the plasmid. The underlined restriction sites represent those introduced to the original sequence for cloning purposes. Those in italics are restriction sites removed from the original sequence. Synthesized fragments (A–H) and how they were designed are indicated. (b) Schematic representation of pSMART-BAC-GFP used as the backbone for pSMART-BAC-PEDV<sub>AVCT12</sub> construction. pSMART-BAC-PEDV<sub>AVCT12</sub> was constructed by replacing the GFP fragment with the pre-assembled A–H fragment through NheI/MluI double digestion T<sub>5</sub>, transcription terminator. Figures are not drawn to scale. (c) Schematic representation of the sequential cloning steps to assemble the full-length PEDV genome from eight synthetic fragments.
Fig. 4. Characteristics of PEDV recovered by reverse genetics. (a) RgPEDV<sub>AVCT12</sub> was rescued and subsequently propagated in VeroE6–APN cells. At passage 5, viral RNA was extracted and subjected to RT-PCR analysis using primers covering the MluI site at position 26 372. The RT-PCR products of PEDV<sub>AVCT12</sub> and RgPEDV<sub>AVCT12</sub> were subsequently analysed by MluI digestion. Sequencing results of RT-PCR products displaying the difference between RgPEDV<sub>AVCT12</sub> and PEDV<sub>AVCT12</sub> at position 26 372 are also indicated. (b) VeroE6–APN cells were infected with RgPEDV<sub>AVCT12</sub> or parental PEDV<sub>AVCT12</sub> at an m.o.i. of 0.002 TCID<sub>50</sub> per cell. At 12, 24, 48 and 72 h p.i., supernatants were collected and used to determine TCID<sub>50</sub> in VeroE6–APN cells. (c) Immunofluorescence staining of cells and Western blot of supernatants obtained from VeroE6–APN cells infected by RgPEDV<sub>AVCT12</sub> using mouse mAbs directed against PEDV N protein. Nuclei were stained with DAPI.
**vivo**. To test this hypothesis, we engineered the PEDV<sub>AVCT12</sub> infectious clone to express full-length ORF3 derived from the Chinese strain JS-2004-2 (RgPEDV<sub>wtORF3</sub>) using the strategy depicted in Fig. 5(a). As a control, we also constructed RgPEDV<sub>trORF3</sub> by introducing an internal deletion (aa 82–98) to ORF3. The schematic representation in Fig. 5(b) displays the construction of ORF3 in each recombinant virus. Recovery of RgPEDV<sub>wtORF3</sub> and RgPEDV<sub>trORF3</sub> was carried out using the same conditions as that of RgPEDV<sub>AVCT12</sub>. We found that transfection of VeroE6–APN cells by infectious cDNA clones of PEDV<sub>wtORF3</sub> and PEDV<sub>trORF3</sub> gave rise to remarkably different results. Whilst RgPEDV<sub>trORF3</sub> could be rescued with replication kinetics comparable to those of RgPEDV<sub>AVCT12</sub>, RgPEDV<sub>wtORF3</sub> did not generate noticeable syncytia after transfection, which is concordant with the barely detectable titre monitored throughout the experiment (Fig. 5b). The inability to recover PEDV bearing the full-length, and presumably functional, ORF3 in this study suggested that ORF3 plays a crucial role in negatively modulating PEDV replication in *vivo*.

**Recovery and characterization of PEDV expressing mCherry fluorescent protein**

To test whether the recovery system established in this study could be used to generate PEDV expressing a foreign protein, we introduced the mCherry gene into the ORF3 cassette using the same strategy as in Fig. 5(a) and attempted to rescue the recombinant PEDV by transfecting the modified infectious clone into VeroE6–APN cells. Extensive syncytium formation expressing mCherry fluorescent protein was observed after transfection (Fig. 6a). To analyse the growth of PEDV<sub>mCherry</sub>, VeroE6–APN cells were infected with RgPEDV<sub>AVCT12</sub> or PEDV<sub>mCherry</sub> and viral genome release into the supernatant was compared by quantitative RT-PCR (qRT-PCR) to assess replication kinetics. As shown in Fig. 6(b), RgPEDV<sub>AVCT12</sub> and RgPEDV<sub>mCherry</sub> displayed comparable replication kinetics. It is also noteworthy that strong expression of mCherry was retained in PEDV<sub>mCherry</sub>-infected cells after 10 passages in VeroE6–APN cells (data not shown). Taken together, our results suggested that the PEDV infectious cDNA clone developed here could be used to construct recombinant PEDV stably expressing foreign proteins in infected cells.

**DISCUSSION**

In this study, we demonstrated the success of PEDV recovery in *vivo* using an infectious cDNA clone strategy. Moreover, we showed that the infectious cDNA clone could be further modified to accommodate foreign protein expression, thereby serving as an efficient platform for live PEDV vaccine design. It is now generally accepted that effective vaccines against enteric pathogens will have to elicit strong mucosal immune responses, especially secretory IgA antibodies (sIgA) (Mantis & Forbes, 2010; Mantis et al., 2011). As administration of killed virus or soluble antigens via commonly used vaccination routes such as intramuscular, intradermal or subcutaneous injection is unlikely to stimulate sufficient sIgA, currently available PEDV vaccines are of low efficacy or offer only partial protection from infection. Several lines of evidence have indicated that sIgA passively transferred from sows to neonatal piglets during the nursing period play a pivotal role in protection from not only PED but also from other enteric diseases (Bohl & Saif, 1975; Bohl et al., 1972; Oh et al., 2014). Indeed, a so-called ‘gut–mammary glands–sIgA axis’ model was postulated more than 40 years ago to address how lactogenic immunity could control infection of TGEV, a close relative of PEDV (Bohl et al., 1972). According to this notion, natural infection or oral administration of live vaccines can induce IgA plasma cell precursors in Peyer’s patches and mesenteric lymph nodes, giving rise to robust generation of IgA plasma cells that secrete pathogen-specific sIgA antibodies into the colostrum and milk (Chattha et al., 2015). Recently, proof of this concept was shown in a study by Goede et al. (2015) in which 100 % of piglets (n>50) born to sows pre-exposed to a mild strain of PEDV could be protected from lethal challenge of the highly virulent strain. Collectively, these studies strongly indicate that oral vaccination with well-characterized and properly attenuated live PEDV is a promising, if not ideal, approach to contain outbreaks of PEDV. The capacity to manipulate the PEDV infectious cDNA clone as demonstrated in this study is likely to revolutionize PEDV vaccine research.

Limited success of efficient PEDV propagation in traditional cell lines is one of the key hurdles in PEDV research. The best result so far was described in a study in which different derivatives of Vero cell lines were tested for their ability to recover US PEDV isolates. However, out of the 88 PEDV-positive samples tested, only nine (10.22 %) exhibited cytopathic effects in cells and positive RT-PCR results in supernatants (Oka et al., 2014). Here, we have developed a platform to facilitate the *in vitro* culture of PEDV by engineering Vero E6 cells to permanently express pAPN as a substrate for PEDV propagation. This modified cell line can augment PEDV replication, based on the observation that the majority of PEDV-positive intestinal samples used in our study gave rise to progressive syncytium formation in VeroE6–APN but not in parental VeroE6 cells (data not shown). As Vero cells are of monkey origin and do not express pAPN, it is possible that PEDV may use an alternative pathway to initiate replication in Vero cells. Interestingly, we demonstrated that ectopic expression of pAPN in Vero E6 cells could enhance the replication efficiency of PEDV in this modified cell line. In addition, the fact that PEDV freshly isolated from the intestines of infected piglets grew better in VeroE6–APN than in VeroE6 cells suggests that PEDV may prefer pAPN to available alternative receptors for Vero E6 cell entry. Recently, a number of studies have shown that cells of porcine origin, such as small intestinal epithelial cells and alveolar macrophages, are susceptible to PEDV infection. However, it has
Fig. 5. ORF3 suppresses PEDV growth in VeroE6–APN cells. (a) Schematic representation of the strategy used to introduce a foreign gene into the full-length cDNA clone of PEDV AVCT12. PCR products of foreign genes (wtORF3, trORF3 or mCherry) were inserted into the reading frame of ORF3 using the BsiWI/XhoI restriction sites in the synthetic fragment, and the fragment bearing the insert subsequently replaced the original fragment in pSMART-BAC-PEDV AVCT12 at the AsISI/SalI restriction sites. (b) Parallel cultures of VeroE6–APN cells were infected with viruses recovered from the full-length cDNA clone of PEDV AVCT12, PEDV wtORF3 and PEDV trORF3. At 24, 48, 72 and 98 h p.i., samples of the supernatants were taken, and virus titres (calculated as relative TCID$_{50}$ ml$^{-1}$) were determined by one-step quantitative RT-PCR. Schematic representations of each ORF3 construct are also presented.
been shown that growth of PEDV in these cells is markedly slower than in Vero cells (Cong et al., 2015; Park & Shin, 2014). Moreover, whilst ST cells permanently expressing high levels of pAPN (ST-pAPN-H/Q cells) could be efficiently infected by PEDV, the plaque size of PEDV-infected ST-pAPN-H/Q cells is apparently smaller than that of infected Vero cells (Nam & Lee, 2010). As the growth kinetics of PEDV-infected ST-pAPN-H/Q cells have not been clearly presented elsewhere, we cannot evaluate PEDV growth in ST-pAPN-H/Q cells in comparison with the VeroE6-APN cells generated in this study.

It is also important to emphasize that reverse genetics of PEDV has recently been reported using the targeted RNA recombination approach (Li et al., 2013). In contrast to the full-length cDNA clone strategy described here, this approach is usually employed when it is technically complicated to construct a full-length infectious cDNA clone of the parental virus (Masters & Rottier, 2005). In fact, construction of the full-length genome of PEDV in this study was beset by many technical difficulties. Similar to what was reported with the construction of other coronavirus infectious clones (González et al., 2002; St-Jean et al., 2006; Yount et al., 2002), we found that the fragment encompassing nt 12,810–15,657 (fragment D) was toxic to the bacterial host, as the synthetic fragment could not be cloned using the conventional plasmid pUC57. We successfully used the pET28b plasmid to clone this fragment, but plasmid yield was low. To maintain the stability of this fragment in the full-length infectious clone as well as to obtain sufficient amounts of material for further applications, we resorted to a BAC system (pSMART-BAC), in which transcription terminator sequences have been introduced at both flanks of the cloning site to minimize the generation of unwanted mRNA with toxic sequences. In fact, BAC vectors are highly efficient for recovering viruses
associated with sequence toxicity in the bacterial host (Almazán et al., 2014; Usme-Ciro et al., 2014). Additionally, we also encountered problems associated with insertion of a bacterial transposon into the PEDV genome. In particular, we discovered that the sequence spanning positions 9741–9749 (ATGGTACCG) is a target site for transposon insertion. To resolve this problem, we utilized DH10B, a host commonly used to maintain large plasmids, to minimize the insertion of transposon elements into the PEDV genome. BLAST analyses also indicated that this target site is highly conserved in PEDV genome. It is thus important to take this issue into account should infectious clones of other PEDV strains be constructed. Whilst it is possible that insertion of transposable elements can occur elsewhere in the full-length PEDV genome, we did not detect them in our study.

Recovery of infectious PEDV was performed by transfecting the full-length cDNA clone directly into VeroE6–APN cells. In our study, we found that, whilst trypsin treatment is critical for syncytium formation among transfected cells, the optimal concentration of trypsin (2 μg ml⁻¹) was not as high as reported previously (Park et al., 2011; Shirato et al., 2011; Wicht et al., 2014). Although PEDV_AVCT12 can grow to high titre in VeroE6–APN cells, this property was not observed when VeroE6 cells were used to propagate the virus using the same amount of trypsin. In fact, it has been reported previously that propagation of PEDV in different Vero cell lines requires at least 10 μg trypsin ml⁻¹, and Vero E6 cells cannot tolerate trypsin at this concentration (Oka et al., 2014). We thus speculate that VeroE6 cells may require much higher concentrations of trypsin to support PEDV replication than VeroE6–APN cells and, consequently, no syncytium formation was observed in PEDV_AVCT12-infected Vero E6 cells under the conditions used in this study.

Another factor that could contribute to the cell adaptability of PEDV_AVCT12 is the lack of ORF3 due to deletion of its start codon. It is important to note that the transcription regulatory sequences that regulate the expression of subgenomic ORF3 mRNA are not affected by the deletion (Fig. 2). However, the ORF Finder program suggested that only small proteins less than 20 aa in size can be transcribed alternatively from the ORF3 mRNA of PEDV_AVCT12. In addition, we showed that when a full-length ORF3 cloned from a field isolate was replaced with the impaired ORF3 of PEDV_AVCT12, we could not rescue the virus, even in VeroE6–APN cells. However, when an ORF3 with an internal truncation was introduced into the PEDV_AVCT12 genome, we found that the recombinant virus displayed growth characteristics comparable to those of PEDV_AVCT12. These data collectively suggested that ORF3 exerts an inhibitory effect against PEDV replication in vitro. Despite an extensive body of research, knowledge regarding the function of ORF3 is still limited. Wang et al. (2012) proposed that ORF3 functions as an ion channel that may regulate virus replication. In contrast to our results, they showed that disruption of ORF3 expression by small interfering RNA resulted in decreased virus production in infected Vero cells. However, the high growth of ORF3-null PEDV shown in this study and by Li et al. (2013) disputes the essential role of ORF3 in PEDV replication. It is also worth pointing out that our results so far suggest a strong correlation between ORF3 and cell-culture adaptability. However, we could not draw any conclusion regarding how ORF3 may correlate with virulence. The fact that we detected disruption of the ORF3 gene in the isolate extracted from infected intestines of a sick piglet suggests that disruption of ORF3 alone may be necessary but not sufficient to give rise to a low pathogenic phenotype of the virus.

In addition to the parental PEDV infectious clone, we also successfully generated an mCherry-encoding PEDV. Our results indicate that not only did this recombinant virus grow with growth kinetics comparable to those of the WT virus, it also exhibited strong expression of mCherry, which could be observed in infected cells even after multiple passages, suggesting that PEDV can tolerate the addition of a foreign gene. Similarly, a study by Li et al. (2013) also successfully generated recombinant PEDV expressing foreign proteins including GFP and Renilla luciferase. Interestingly, it has also been shown in this study that, besides the ORF3 reading frame, a foreign gene can be introduced under the transcription regulatory sequences of the S gene. Whilst it has not been shown whether other transcription regulatory sequence positions can be manipulated likewise, the current data suggest that the construction of recombinant PEDV carrying multiple foreign proteins is possible. Whilst reverse genetics-derived PEDV expressing foreign antigens are valuable for vaccine development, this platform could be hampered by problems associated with genetic instability of the insert. Given that coronaviruses, like other positive-sense RNA viruses, are highly prone to genetic recombination (Denison et al., 2011; Huang et al., 2013), the loss of foreign inserts is very common (Bentley et al., 2013; Sola et al., 2003). Although mCherry was stably expressed by recombinant PEDV in this study (more than 10 passages), it is not known whether recombinant PEDV expressing other foreign genes would behave likewise. Interestingly, it has been demonstrated that the location of a foreign insert within the coronavirus genome is a critical factor for the genetic stability of recombinant coronaviruses (Bentley et al., 2013; de Haan et al., 2003). In line with these findings, recombinant PEDV with the luciferase gene at the ORF3 position was also found to display substantially higher activity than that with the foreign gene located before the S gene (Li et al., 2013). It thus remains to be investigated whether other foreign genes with various sizes could replace ORF3 in the PEDV genome without compromising the stability of the recombinant viruses.

In conclusion, this study demonstrated that reverse genetics can be used to rapidly rescue infectious PEDV. Construction of a stable infectious clone of PEDV and its further modification can be performed with the BAC
system. Moreover, recovery and propagation of infectious virus can be carried out in VeroE6–APN cells. This PEDV reverse genetics system serves as a useful technological platform that can be implemented for not only vaccine development but also for other applications such as antiviral screening and the study of PEDV pathogenesis.

**METHODS**

**Cells and viruses.** Vero E6 cells were purchased from the American Type Culture Collection (ATCC CRL-1587) and grown in Opti-MEM medium supplemented with 10 % FBS (Gibco-BRL), penicillin (100 U ml$^{-1}$) and streptomycin (50 μg ml$^{-1}$). VeroE6–APN cells were constructed by retrovirus transduction (see below) and cultured in the same medium with the addition of hygromycin (1 mg ml$^{-1}$). GP2-293 cells, a packaging cell line that produces retrovirus particles, and HEK 293T cells were grown in Opti-MEM supplemented with 10 % FBS and 1 % antibiotic/antimycotic solution. Unless otherwise indicated, all cells were maintained at 37 °C with 5 % CO$_2$. To propagate PEDV in VeroE6–APN cells, PEDV-positive intestinal samples, verified by RT-PCR, were diluted 10-fold in PBS and centrifuged at 5000 g for 10 min at 4 °C. Supernatants were filtered through a 0.2 μm filter and adsorbed onto VeroE6–APN cells for 1 h at 37 °C. After washing, infected cells were maintained in FBS-free Opti-MEM medium with trypsin (2 μg ml$^{-1}$). The PEDV isolate displaying the most extensive syncytium formation in infected cells was subjected to RNA extraction and subsequently whole-genome sequencing. The primers used are available upon request.

**Retrovirus construction and generation of VeroE6–APN cells.** The pCDNA3 expressing pAPN cDNA, a kind gift of Dr Yi Jing Li (Li et al., 2007), was amplified and cloned using NotI restriction sites. In addition, the IRES-EGFP fragment was amplified from a plasmid expressing sNA–IRES–EGFP developed previously (Wanitchang et al., 2011) using primers bearing NotI restriction sites. Both fragments were subsequently cloned into the retroviral pQCXIIH vector (Clontech), giving rise to the pQCXIH-pAPN-EGFP retroviral vector (Fig. 1a). Retroviruses were subsequently produced from GP2-293 cells and used to transduce Vero E6 cells as described previously (Zuo et al., 2011). Briefly, pQCXIIH-pAPN-EGFP plasmid (3 μg) was co-transfected with a vesicular stomatitis virus glycoprotein envelope expression construct (2 μg) into GP2-293 packaging cells. Infectious supernatants were collected, filtered through a 0.2 μm filter, diluted 1 : 1 with Opti-MEM containing 20 μg Polybrene ml$^{-1}$ and applied to Vero E6 cells for 12 h. Selection with hygromycin (1 mg ml$^{-1}$) was initiated 48 h later. Transduced cells were sorted for EGFP expression using flow cytometry and the cells were serially diluted to obtain a single clone.

**Aminopeptidase assay.** Cell-surface APN activity of VeroE6–APN cells was determined based on the hydrolysis of Leu-pNA as described previously (Cristofoletti et al., 2006; Tsukamoto et al., 2008). Briefly, VeroE6–APN cells were suspended in PBS (pH 7.4) at 1 x 10$^6$ cells ml$^{-1}$, and then 100 μl cell suspension was mixed with an equal volume of substrate solution (final concentration of Leu-pNA: 1.5 mM in PBS). Cells were incubated for 30 min at 37 °C. The amount of p-nitroaniline generated was assessed in the supernatant by reading the absorbance at 405 nm.

**Plasmids for full-length PEDV cDNA assembly.** The sequence of PEDV$_{AVCT12}$ was used as a reference for synthesizing eight overlapping DNA fragments flanked by the appropriate restriction sites as follows: NheI–A–SpI1 (nt 295–4439), SpI1–B–BsuWI (nt 4434–7440), BsuWI–C–BstSI (nt 7735–12 815), BstSI–D–AgeI1 (nt 12 810–15 662), AgeI1–E–NolI (nt 15 657–18 083), NolI–F–ApaI (nt 18 076–21 033), ApaI–G–SalI (nt 21 028–25 190), and SalI–H–MluI1 (nt 25 187–26 372) (Fig. 3a). Whilst all fragments were cloned in the pUC57 cloning vector, fragment D was cloned in the pET28b vector. As a backbone to harbour the full-length PEDV cDNA clone, pSMART-BAC (Lucigen) was modified to include synthesized sequences of the CMV immediate-early promoter, PEDV 5’ UTR (nt 1–295), EGFP, the C-terminal region of the PEDV N gene and 3’UTR (nt 26 369–27 991), poly(A) signal (25 nt), HDV ribozyme self-cleavage site and BGH (Fig. 3b). The EGFP gene was designed to be flanked by NheI and MluI restriction sites to facilitate subcloning of the full-length PEDV cDNA in this plasmid system (Fig. 3b).

**Full-length PEDV infectious cDNA assembly.** Assembly of the fragments spanning the entire PEDV genome was performed according to the strategy depicted in Fig. 3(c). In the first step, fragments AB, CD and GH were constructed through the common restriction sites, namely SpI1, BstSI and SalI (at positions 4438, 12 810 and 25 185, respectively). Subsequently, fragments CDE and FGH were generated by joining E with CD and F with FH through the AgeI1 (position 15 657) and ApaI (position 21 032) restriction sites. In the final step, a four-fragment ligation was performed with fragments AB, CDE and GH and the modified pSMART-BAC through NheI (position 295), BstWI (position 7735), NolI (position 18 077) and MluI1 (position 26 372) digestion and ligation. To maintain the stability of the insert, the ligation mixture was transformed into E. coli DH10B. The genetic integrity of pSMART-BAC-PEDV$_{AVCT12}$ was verified by restriction analyses, PCR assays and full-length nucleotide sequencing.

**Insertion of the mCherry or ORF3 gene into the PEDV$_{AVCT12}$ infectious clone.** The mCherry or ORF3 gene was inserted into the PEDV$_{AVCT12}$ genome by replacing the ORF3 gene following the strategy depicted in Fig. 5(a). Briefly, a fragment spanning the AsoI (position 24 769) and SalI (position 25 185) restriction sites (numbering based on the published CV777 sequence; GenBank accession no. AF535311.1) was synthesized. To facilitate foreign gene insertion, BstWI and XhoI restriction sites were added after the transcriptional regulatory sequence of the ORF3 gene. The full-length mCherry or ORF3 gene was PCR amplified using primers bearing BstWI and XhoI restriction sites, subsequently double digested and inserted into the synthesized fragment. The fragment bearing the foreign gene was then digested with AsoI and SalI, and ligated with pSMART-BAC-bearing the FGH fragment pre-digested with AsoI and SalI. The fragment harbouring the foreign gene was then swapped with the original fragment in the pSMART-BAC-PEDV$_{AVCT12}$ via PacI and MluI restriction sites.

**Recovery and titration of infectious PEDV.** To rescue infectious PEDV, VeroE6–APN cells were grown to 90 % confluency in a six-well plate and transfected with 2 μg pSMART-BAC-PEDV$_{AVCT12}$ using 6 μl Fugene HD (Promega) according to the manufacturer’s instructions. At 24 h post-transfection, cells were treated with trypsin (2 μg ml$^{-1}$) and incubated at 37 °C until virus-induced syncytia were apparent. The trypsin concentration was increased to 4 μg ml$^{-1}$ if syncytium formation was not observed within 72 h after transfection. Clarified supernatants obtained after removing cell debris at 800 g for 5 min were passaged once on fresh VeroE6–APN cells and the recovered viruses were used as virus stocks for further analyses. To determine viral titre, TCID$_{50}$ was determined in VeroE6–APN cells based on syncytium formation at 72 h p.i. TCID$_{50}$ titres were calculated as described previously (LaBarre & Lowy, 2001).

**Quantification of viral RNA by one-step qRT-PCR.** Viral RNAs from infected cell-culture supernatants were extracted with a Viral Nucleic Acid Extraction kit II (Geneaid), according to the manufacturer’s instructions. One-step qRT-PCR was performed in a 20 μl reaction mixture with 8.75 μl extracted RNA with primers specific for
the PEDV M gene, designed using the Primer-BLAST program, using an iTaq universal SYBR Green One-step kit (Bio-Rad) under the following conditions: initial reverse transcription at 50 °C for 30 min, followed by PCR activation at 95 °C for 1 min and 40 cycles of amplification (15 s at 95 °C and 1 min at 60 °C). For the relative quantification of viral titre, RNA extracted from virus with a known TCID50 titre was used as a standard. Data analyses were carried out using the CFX Manager software (Bio-Rad).

**Evaluation of PEDV proteins in PEDV-infected VeroE6–APN cells.** VeroE6–APN cells (1 × 10⁶) were infected with parental PEDV or reverse genetics-derived PEDV until progressive syncytium formation was observed. Subsequently, cells were lysed in 200 µl mammalian cell lysis buffer for 30 min on ice. After centrifugation at 10,000 × g for 5 min, the samples were analysed by SDS-PAGE (10 % acrylamide) and transferred onto nitrocellulose membranes (Bio-Rad), followed by blocking with 5 % non-fat milk in TBS-T (buffer with 0.1 % Tween-20) for 1 h. After sequential incubation with mouse mAb against PEDV N protein (clone S-1-5; Medgene Lab) and secondary anti-mouse antibody conjugated to HRP (Santa Cruz Biotechnology), signals were visualized with a chemiluminescence system (Bio-Rad). Evaluation of PEDV-infected cells by immunofluorescence staining was performed as described previously (Wanitchang et al., 2013). Briefly, VeroE6–APN cells were seeded in eight-well Lab-Tek chamber slides (Thermo Scientific) and infected 12 h later with PEDV_AVT2. After incubation for 72 h, infected cells were fixed and permeabilized with ice-cold acetone at −20 °C for 10 min. Cells were washed and blocked with 10 % (v/v) PBS plus 1 % BSA in PBS for 30 min at room temperature. Cells were subsequently incubated with mouse anti-PEDV N mAb (Abcam) diluted in 1 % BSA plus 10 % (v/v) FBS in PBS for 30 min at room temperature. Cells were subsequently incubated with mouse anti-PEDV N mAb for 1 h at room temperature, followed by washing with PBS and incubation with Texas Red-conjugated anti-mouse antibody (Abcam) diluted in 1 % BSA for 1 h. After five washes with PBS, coverslips were mounted onto glass slides using VECTASHIELD mounting medium with DAPI (Vector Laboratories) and examined using an Olympus IX51 fluorescence microscope (Olympus).

**Data presentation and statistical analysis.** All results are expressed as means ± SD and were analysed with GraphPad Prism software. Analyses for statistically significant differences were performed using Student’s t-test.

**ACKNOWLEDGEMENTS**

This work was co-funded by the National Science and Technology Agency (NSTDA) and Betagro Science Center (BSC) under CPMO grants P-11-00087, P-12-01765 and P-14-50863. We acknowledge the following individuals for their help with this work: Dr Roongtham Thanathom Chailangkarn for immunofluorescence analysis. We are also grateful to Dr Samaporn Teeravechyan for her critical reading of the manuscript.

**REFERENCES**


