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Rapid manipulation of the porcine epidemic diarrhea virus genome by CRISPR/Cas9 technology

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is a highly pathogenic enteric coronavirus causing lethal watery diarrhea in suckling piglets. Reverse genetics is a valuable tool to study the functions of viral genes and to generate vaccine candidates. In this study, a full-length infectious cDNA clone of the highly virulent PEDV strain AJ1102 was assembled in a bacterial artificial chromosome (BAC). The rescued virus (rAJ1102) exhibited similar proliferation characteristics *in vitro* to the wildtype AJ1102. Using CRISPR/Cas9 technology, a recombinant virus rAJ1102-ΔORF3-EGFP in which the ORF3 gene was replaced with an EGFP gene, was successfully generated, and its proliferation characteristics were compared with the parental rAJ1102. Importantly, it just took one week to construct the recombinant PEDV rAJ1102-ΔORF3-EGFP using this method, providing a more efficient platform for PEDV genome manipulation, which could also be applied to other RNA viruses.

1. Introduction

Porcine epidemic diarrhea (PED) is a highly contagious acute diarrheal disease characterized by watery diarrhea, vomiting and dehydration, with an 80%–100% mortality rate in neonatal piglets (Jung and Saif, 2015). PED was first recognized in the United Kingdom and Belgium in the 1970s (Wood, 1977), and sporadically or endemically appeared in Europe and Asia before 2010 (Van Reeth and Pensaert, 1994). In late 2010, a large-scale outbreak of PED, which was caused by a highly virulent PED virus (PEDV) variant (GII genogroup), occurred in China (Li et al., 2012; Sun et al., 2016). In April 2013, the Chinese-like PEDV variant also emerged in the United States and rapidly spread across the country (Stevenson et al., 2013; Vlasova et al., 2014). Currently, PEDV has been reported in many countries and causes high economic losses (Jung and Saif, 2015; Lee, 2015; Song et al., 2015; Wang et al., 2019; Zhang and Yoo, 2016).

PEDV is an enveloped, single-stranded positive-sense RNA virus, belonging to the genus Alphacoronavirus within the *Coronaviridae* family in the order *Nidovirales* (Song and Park, 2012). The complete genome of PEDV is approximately 28 kb, encoding at least seven open reading frames (ORFs). The two large ORFs, 1a and 1b, occupy almost two-thirds of the genome, encoding two large replicase proteins, pp1a and pp1b, which are post-translationally cleaved into 16 nonstructural proteins (nsps), nsp1–nsp16. The remaining ORFs, ORF2–6, encode spike (S) protein, envelop (E) protein, membrane (M) protein, nucleocapsid (N) protein, and one accessory protein, ORF3 (Duarte et al., 1993).

Reverse genetics systems are valuable tools to study the functions of viral genes and to generate recombinant viruses with defined genetic changes as vaccine candidates. In 2013, Li et al. first reported a reverse genetics system for the Korean classical PEDV vaccine strain DR13 based on a targeted RNA recombination method (Li et al., 2013). Following this, Jengarn et al. engineered an infectious cDNA clone of the Thailand classical PEDV strain AVCT12 into a bacterial artificial chromosome (BAC) using eight contiguous cDNA fragments (Jengarn et al., 2015). In 2016, Beall et al. constructed infectious cDNA clones of a highly pathogenic US PEDV strain PC22A using *in vitro* ligation of contiguous cDNA fragments, and performed *in vitro* transcription to generate infectious viral RNA (Beall et al., 2016). Using a similar strategy, Fan et al. developed an infectious cDNA clone for a Chinese PEDV variant strain, AH2012/12 (Fan et al., 2017). Li et al. developed a reverse genetics system for two Chinese PEDV strains with differing virulence by ligation of cDNA fragments into BACs one by one (Li et al., 2017). Using established reverse genetics systems, the functions of

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some PEDV proteins, such as S protein and ORF3, in modulating PEDV pathogenicity have been examined (Beall et al., 2016; Hou et al., 2017, 2019; Kaewborisuth et al., 2018; Wang et al., 2018). Several recombinant PEDV vaccine candidates have also been generated using reverse genetics systems (Hou et al., 2019; Kao et al., 2018; Wang et al., 2018). Although infectious clone systems for PEDV using various strategies (Teeravechyan et al., 2016) have become established, approaches to generate a new mutant PEDVs with defined genetic changes using infectious clones remains a tedious process, usually requiring constructing and ligating a set of contiguous cDNA fragments. A simple and rapid method for manipulation of the full-length infectious clone is desirable.

In this study, a full-length infectious clone of PEDV strain AJ1102 was generated and a simple method to construct recombinant PEDV was developed based on the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology, providing a more efficient platform for PEDV genome manipulation.

2. Materials and methods

2.1. Cells, virus and antibodies

Vero cells (ATCC CCL-81) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) in a 37 °C, 5% CO_2 humidified atmosphere. PEDV strain AJ1102, a highly virulent PEDV variant isolated from a neonatal piglet with acute diarrhea in China in 2011 (Bi et al., 2012), was propagated in Vero cells supplemented with trypsin (10 μg/mL). The mAb against PEDV N protein was produced at Huazhong Agricultural University as described previously (Ding et al., 2014).

2.2. Construction of the full-length infectious clone of PEDV AJ1102 (F12)

A low-copy number BAC vector (pBeloBAC11) was used to construct the infectious cDNA clone of AJ1102 (F12), the 12th passage of AJ1102 strain. The pBeloBAC11 was modified to incorporate the CMV promoter, PEDV 5′ UTR, N-terminal of ORF1a (the first 800 nucleotides; nts), restriction enzyme site of PacI, C-terminal end of the N gene (nt 26,888-27,701), PEDV 3′ UTR, a 28-residue poly(A) tail, hepatitis delta virus (HDV) ribozyme self-cleavage site and bovine growth hormone (BGH) termination sequences (Fig. 1A), generating an intermediate BAC plasmid, pBAC-M-PEDV. Total viral RNA was extracted from Vero cells infected with PEDV strain AJ1102 (F12) by using a TaKaRa MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa). The cDNA was synthesized from genomic RNA using SuperScript IV Reverse Transcriptase (Invitrogen) in accordance with the manufacturer's instructions. Five overlapping DNA fragments (A: nt 1,050–7,173; B: nt 7,151–13,036; C: nt 12,975–17,617; D: nt 17,598–22,217; E: 22,199–26,929) were amplified with SuperFi™ Green PCR Master Mix (Invitrogen) in accordance with the manufacturer's instructions, and then assembled into the modified BAC vector pBAC-M-PEDV. First, fragments A and B were ligated into pBAC-M-PEDV by homologous recombination with an Infusion Clone Kit (TaKaRa), resulting in pBAC-AB. Using the same method, fragments C, D, and E were then ligated into pBAC-M-PEDV, resulting in the plasmid pBAC-CDE. Finally, plasmids pBAC-AB and pBAC-CDE were double digested with *Pac*I and *Sac*II, then ligated together with T4 DNA ligase (ThermoFisher Scientific) to create the construct pBAC-AJ1102. The primers used to construct the full-length cDNA clone are listed in Table 1.

2.3. Recovery of recombinant viruses

Confluent Vero cells in six-well culture plates were transfected with BAC plasmids harboring the complete or edited genome (6 μg/well) using Lipofectamine® 3000 (Invitrogen). At 6 h post-transfection, the cells were rinsed twice with DMEM and supplemented with 2 mL of DMEM containing 10 μg/mL of trypsin (Sigma), then placed in a 37 °C, 5% CO_2 incubator to facilitate the recovery of infectious virus. Cells were observed daily for the appearance of CPE.

2.4. Generation of sgRNAs

The sgRNA templates were amplified with forward primers sgRNA-ΔORF3a/b and reverse primer scaffold oligo using SuperFi™ Green PCR Master Mix (Invitrogen) at 98 °C for 3 min and 34 cycles of 98 °C 30 s, 55 °C 30 s, 72 °C 30 s, followed by a final extension at 72 °C for 5 min. The amplification products were then purified using a DNA extraction kit (Omega Bio-tek) and transcribed at 37 °C overnight with a T7 transcription kit (NEB) according the manufacturer's instructions. The sgRNAs were phenol chloroform extracted and eluted in RNase free water.

2.5. Cleavage of the pBAC-AJ1102 and construction of the recombinant BAC

The specific cleavage reaction was conducted in a 50 μL-mixture containing 5 μg pBAC-AJ1102, 5 μL of Cas9 nuclease (NEB), 20 μg sgRNAs (10 μg for each sgRNA) and 5 μL of 10 × NEB Cuterbuffer 3.1 at 37 °C for 2.5 h. The cleaved pBAC-AJ1102 was purified with a DNA Cycle Pure Kit (Omega Bio-tek) and verified by electrophoresis in 0.8% agarose gel. The recombinant pBAC-AJ1102-ΔORF3-EGFP was constructed by homologous recombination using an Infusion Clone Kit (Clontech) in a mixture containing the cleaved pBAC-AJ1102 and a DNA fragment with an EGFP gene, E gene, partial sequences of S gene and M gene, and two 20 bp homologous arms.

2.6. Indirect immunofluorescence assay

Vero cells seeded in 12-well plates were infected with wtAJ1102 and rAJ1102 for 24 h. The cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with cold methanol for 10 min at room temperature, followed by washing three times with PBS, and blocked with 5% bovine serum albumin for 1 h. Afterwards, the cells were washed thrice with PBS and then incubated with mAb against PEDV N protein at room temperature for 1 h. After three washes, the cells were stained with fluorescence isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Cell nuclei were stained with 0.01% 4′,6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature. After three washes with PBS, fluorescent images were visualized with a fluorescence microscope (Nikon).

2.7. Western blot analysis

Vero cells seeded in six-well plates were infected or mock infected with viruses for 24 h and harvested with lysis buffer (4% SDS, 3% DTT, 0.065 mM Tris-HCl, [pH 6.8], 30% glycerine) supplemented with protease inhibitor (PMSF, Beyotime). The cell lysates were boiled at 100 °C for 10 min before separation by SDS-PAGE and electroblotting onto polyvinylidene difluoride membranes (Millipore). The membranes were first blocked with 10% skimmed milk and then inoculated with the corresponding primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody, and visualized using chemiluminescent substrate (Bio-Rad).

2.8. Viral plaque assay

Monolayers of Vero cells in 12-well plates were incubated with 500 μL of 10-fold serially diluted parental or rescued PEDV for 1 h at 37 °C with periodic gentle rocking. Following this, the cells were washed thrice with DMEM to remove unabsorbed viruses, then overlaid with 1 mL of DMEM containing 1.5% methylcellulose and 10 μg/mL

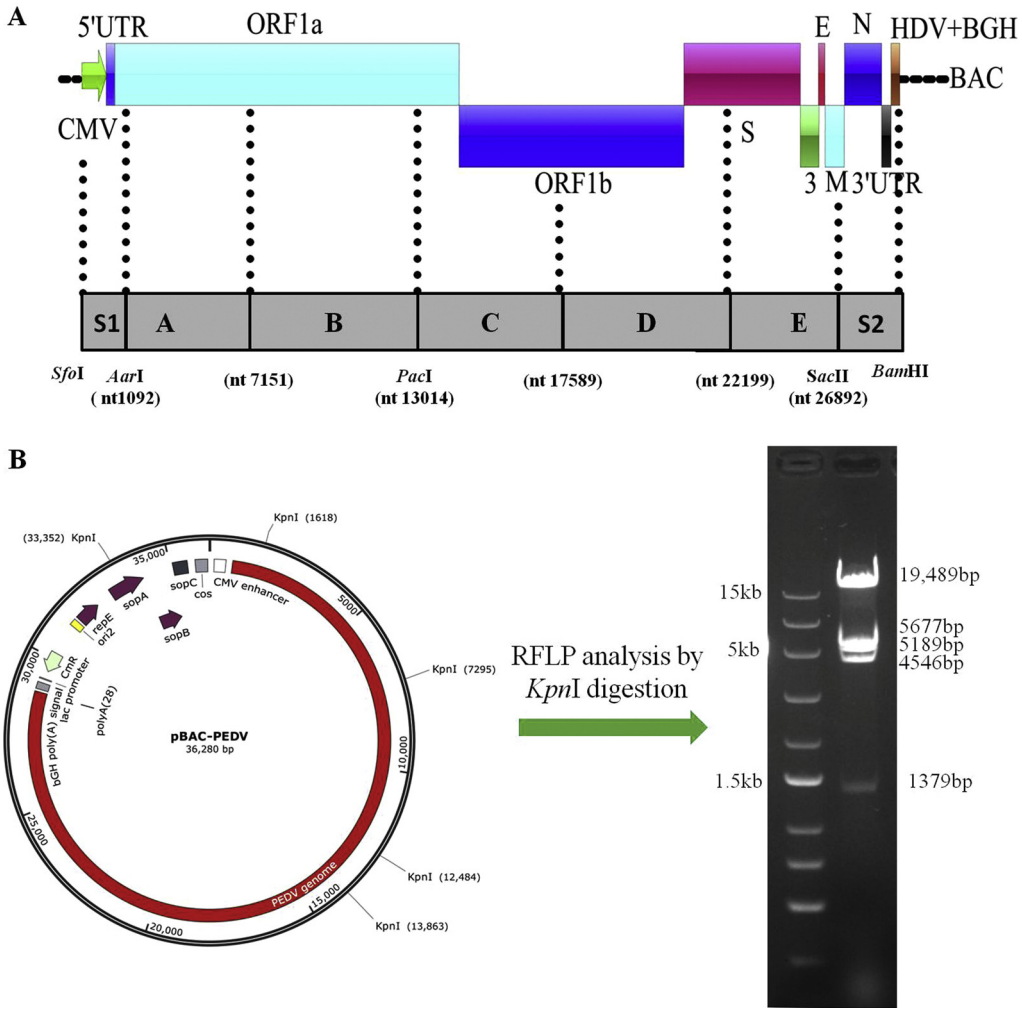

Fig. 1. Construction of a full-length cDNA clone of PEDV strain AJ1102. (A) Schematic diagram depicting the construction of a full-length cDNA clone of PEDV strain AJ1102. Restriction enzyme sites in the PEDV genome were employed to ligate the full-length PEDV AJ1102 genome. (B) Restriction fragment length polymorphism analysis with KpnI restriction digestion of pBAC-AJ1102. Sizes of the digested bands are indicated.

Table 1
Primer sequences used in this study.

Primer ID	Sequence (5'–3')
PEDV-A-F	TCGCTTGCTCTGGTAAATTCC
PEDV-A-R	TTGTACTTCCAAAATGTGTCACC
PEDV-B-F	GGTGACACATTTTGGAAGTACAA
PEDV-B-R	ACTCCTCACAAGCACCTACC
PEDV-C-F	GGTAGGTGCTTGTGAGGAGT
PEDV-C-R	GAGGCAAAAGGTGTGCGAAT
PEDV-D-F	ATTCGCACACCTTTTGCCTC
PEDV-D-R	GTTGGCACCACTATGACCA
PEDV-E-F	TGGTCATAGTGGTGCCAAC
PEDV-E-R	CCACGACCCTGGTTATTTCC
sgRNA-ORF3a	TTCTAATACGACTCACTATAGGCTATTAGTCAAACTTCTAGTTTTAGAGCTAGA
sgRNA-ORF3b	TTCTAATACGACTCACTATAGGGCTAGCTTTCAGGTCAACGTTTTAGAGCTAGA
PEDV-ORF3-upF	AGAGGCTATTAGTCAAACTT
PEDV-ORF3-upR	AAAGCAAAAAAGACCCAGTT
EGFP-F	GAAAAGGTCCACGTGCAGTGATGGTGAGCAAGGGCGAGGA
EGFP-R	TTACTTGTACAGCTCGTCCA
PEDV-ORF3-downF	TGGACGAGCTGTACAAGTAAGATTCAATTCAACTAGACGAG
PEDV-ORF3-downR	CACTGCACGTGGACCTTTTC
scaffold oligo	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

trypsin. After incubation at 37 °C for 48 h, the cells were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

2.9. Virus titrations by $TCID_{50}$ assay

Vero cells in 12-well plates were inoculated with parental or recombinant PEDV at a multiplicity of infection (MOI) of 0.1 in triplicate wells. One hundred microliters of virus mixture was added to each well. After 1 h inoculation at 37 °C for virus attachment, the inocula were aspirated and cells were washed once with DMEM, then 1 mL of DMEM medium containing 10 μg/mL trypsin was pipetted into each well. Cell supernatants and infected cells were collected at various time-points for virus titration by $TCID_{50}$ assay.

3. Results

3.1. Construction of the full-length cDNA clone of PEDV AJ1102 (F12)

To facilitate assembly of the complete cDNA, an intermediate BAC plasmid, termed pBAC-M-PEDV, was constructed as the backbone to harbor the complete genome of PEDV strain AJ1102 (F12). Then, five overlapping DNA fragments (A–E) were amplified and assembled into pBAC-M-PEDV (Fig. 1A). The resulting plasmid, pBAC-AJ1102, was digested with *Kpn*I for restriction fragment length polymorphism analysis (RLFP) (Fig. 1B) and re-sequenced. The results showed the full-length cDNA clone of PEDV AJ1102 (F12) was successfully constructed, and there was no deletion or insertion compared with the original PEDV strain AJ1102 (F12) sequence (GenBank accession number MK584552) (data not shown).

3.2. Recovery, identification, and proliferation characteristics of rAJ1102

After transfection of pBAC-AJ1102 into Vero cells, evident CPE, characterized by cell fusion and syncytium formation was observed at 24 h post-transfection. The rescued rAJ1102 was confirmed by indirect immunofluorescence assay (IFA) using mouse anti-PEDV N protein monoclonal antibody (mAb) (Fig. 2A). The growth curves of the rescued rAJ1102 and the parental PEDV AJ1102 (F12) were further compared. As shown in Fig. 2B, rAJ1102 demonstrated similar proliferation properties to the parental PEDV strain, the titers of which peaked at 36 h post-infection (hpi). Plaque assay was also conducted and the results showed that there is no significant difference in plaque morphology between rAJ1102-infected cells and cells infected with the parental PEDV AJ1102 (F12) (Fig. 2C).

3.3. Editing the genomic cDNA of PEDV in the BAC plasmid using CRISPR/Cas9

To prove the idea that CRISPR/Cas9 technology is a simple tool that can be used to edit the genomic cDNA of PEDV in BAC plasmids, the ORF3 gene was chosen as a target because previous studies have demonstrated that the ORF3 gene is nonessential for PEDV replication and could be replaced with other foreign genes (Beall et al., 2016). An enhanced green fluorescent protein (EGFP) gene was designed to replace the ORF3 gene. To this end, two sgRNAs (sgRNA-ΔORF3a and sgRNA-ΔORF3b) targeting the upstream and downstream sequences of the ORF3 gene, respectively, were synthesized. Each sgRNA contained a cleavage site which was located at three nucleotides upstream of the protospacer adjacent motif (PAM) (Fig. 3A). The sgRNAs were generated by T7 *in vitro* transcription of templates amplified with primers of sgRNA-ORF3a/b and scaffold oligo (Table 1) by overlapping PCR (Fig. 3B). To cleave the BAC plasmid pBAC-AJ1102, a mixture containing *S. pyogenes* Cas9 protein (1 μM), sgRNA-ΔORF3a and sgRNA-ΔORF3b (300 nM), pBAC-AJ1102 (5 μg) and the Cas9 nuclease buffer (1 ×) was incubated at 37 °C for 2.5 h. As shown in Fig. 3C, pBAC-AJ1102 was cleaved at the designed sites, producing a linearized BAC (34.1 kb) and a 2.2 kb fragment containing ORF3 gene, E gene and partial sequences of S gene and M gene. At the same time, a 2.3 kb fragment containing the cDNA of EGFP, E gene, partial sequences of S gene and M gene, and a 20 bp homologous arm at both terminals (homologous arm 5'-AGAGGCTATTAGTCAAACTT-3' at 5' terminal, homologous arm 5'-AACTGGGTCTTTTTTGCTTT-3' at 3' terminal), was generated by fusion PCR with primers listed in Table 1. Then the linearized BAC fragment was purified and ligated with the 2.3 kb fragment by homologous recombination (Fig. 3A). After transformation of DH10B competent cells, the recombinant BAC plasmid, pBAC-AJ1102-ΔORF3-EGFP, was obtained. Sequence analysis confirmed that the ORF3 gene was successfully replaced by the EGFP gene (Fig. 3D). To evaluate the success rate and reproducibility of this method, three independent experiments were performed. For each experiment, ca. 20 clones were picked. The percentage of positive clones in three independent experiments was 47% (8/17), 38% (8/21), and 47.8% (11/23), respectively, and the mean positive rate was 44%, demonstrating that this method is an efficient technology for manipulation of the full-length infectious clone of PEDV genome.

3.4. Recovery, identification, and proliferation properties of rAJ1102-ΔORF3-EGFP

The plasmid, pBAC-AJ1102-ΔORF3-EGFP, was transfected into Vero cells and evident CPE characterized by syncytium formation could be observed at 24 h after transfection (Fig. 4A). Furthermore, EGFP was expressed in cells transfected with pBAC-AJ1102-ΔORF3-EGFP, while not in mock-transfected cells (Fig. 4A). The rescued virus, termed rAJ1102-ΔORF3-EGFP, was passaged in Vero cells and the 3rd passage virus was inoculated onto cell monolayers. Nearly all cells were infected and fluorescence could be observed (Fig. 4B). To further identify the recombinant virus rAJ1102-ΔORF3-EGFP, Vero cells were infected with the parental PEDV AJ1102, the rescued virus rAJ1102 or rAJ1102-ΔORF3-EGFP. At 24 h after infection, cells were collected for Western blot with antibodies against PEDV N protein and EGFP. As shown in Fig. 4C, all three viruses expressed N protein, while only rAJ1102-ΔORF3-EGFP-infected cells expressed EGFP, which further confirmed that EGFP was successfully inserted into the PEDV genome.

To further characterize the proliferation properties of rAJ1102-ΔORF3-EGFP, the growth curve of rAJ1102-ΔORF3-EGFP was determined in Vero cells. As shown in Fig. 4D, rAJ1102-ΔORF3-EGFP reached a peak titer ($TCID_{50}$ = $10^{-6.7}$/mL) at 36 hpi. Compared with the growth curve of rAJ1102 (Fig. 4D), rAJ1102-ΔORF3-EGFP attained similar titers in Vero cells, consistent with a previous study that demonstrated that ORF3 is not essential for PEDV replication *in vitro* (Jengarn et al., 2015).

4. Discussion

Coronaviruses are single-stranded positive-sense RNA viruses, and contain the largest genome among the RNA viruses, making them difficult to be manipulated directly. In this study, an infectious clone of PEDV strain AJ1102 was constructed based on the BAC system, and a strategy to edit the genome in an *in vitro* plasmid system was developed by using CRISPR/Cas9 technology. The results clearly showed this is a simple and rapid method to manipulate the full-length cDNA clone of PEDV, providing a more efficient platform for basic studies and vaccine development for PEDV.

CRISPR/Cas9 technology has been applied to engineer the genomes of many organisms or large DNA plasmids with high efficiency and specificity (Jiang et al., 2015; Mali et al., 2013; Moradpour and Abdulah, 2019; Wang et al., 2015). For example, Wang et al. used the Cas9/sgRNA complex to cut a 22 kb plasmid, and then a 783 bp DNA fragment was inserted into the linearized plasmid seamlessly through Gibson assembly to select 4 clones which were all positive (Wang et al., 2015). Jiang et al. used RNA-guided Cas9 to cut bacterial chromosomes

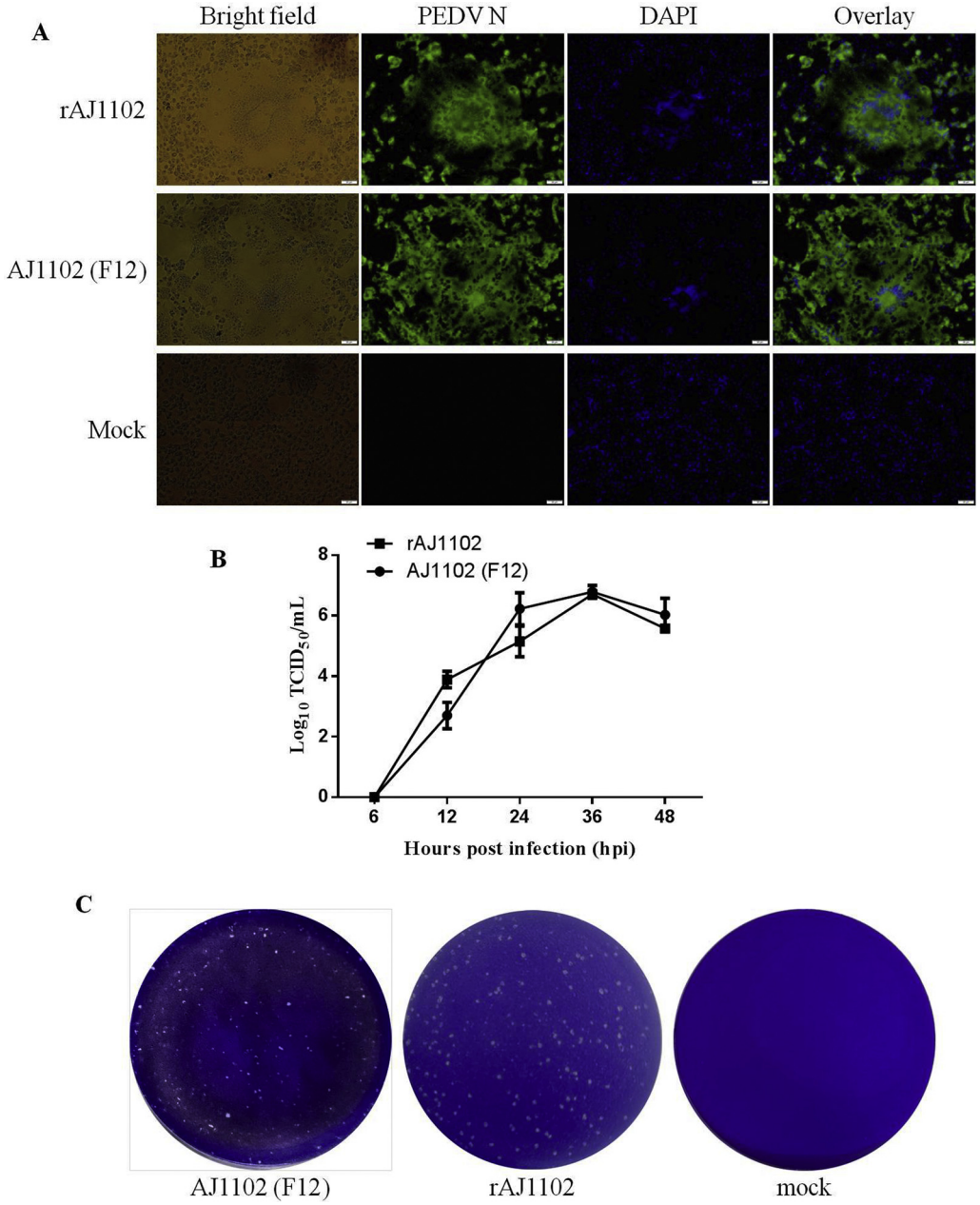

Fig. 2. Identification and growth curve of rAJ1102. (A) Vero cells were infected with the rescued rAJ1102 or the parental AJ1102 (F12) at an MOI of 0.1. At 24 h post-infection (hpi), the rescued PEDV was identified by indirect immunofluorescence with mouse anti-PEDV N monoclonal antibody. (B) Vero cells were infected with rAJ1102 or parental AJ1102 (F12) at an MOI of 0.1. The infected cells were collected at the indicated time-points and viral titers were determined by $TCID_{50}$ assay. (C) Viral plaques were stained with 0.1% crystal violet at 48 hpi.

and the cleaved target genome segment was ligated to a PCR amplified vector backbone through Gibson assembly (Jiang et al., 2015). The cloning efficiency ranged from 21.5%–65.8% (21.5% for 100 kb and 65.8% for 50 kb fragment) (Jiang et al., 2015). In the present study, CRISPR/Cas9 nuclease cleavage was combined with the in-fusion clone method to edit the viral genome *in vitro* using the infectious clone of PEDV as a model. Three independent experiments showed that the recombinant efficiency is 38%–47.8%. To the best of our knowledge, this is the first time this approach has been used to manipulate an infectious clone of RNA virus by CRISPR/Cas9 technology. Relative to previous strategies used to manipulate infectious clones of large RNA viruses by constructing and ligating a set of contiguous cDNA fragments, this method has demonstrable advantages in improving the quality and efficiency of constructing recombinant RNA viruses. First, two sgRNAs targeting the genes or regions of interest can be easily designed and generated using a T7 *in vitro* transcription kit. Second, cleavage of the target genes or regions can be completed within 3 h in a simple nuclease buffer system containing BAC plasmid, Cas9 nuclease, and sgRNAs. Third, the cleaved BAC plasmids can be easily ligated with a DNA fragment with a 20 bp homologous arm using an infusion clone kit to obtain the desired BAC plasmid containing the mutation, insertion or deletion of interest. In our experience, the whole process from sgRNA generation, BAC plasmid cleavage, homologous recombination, transformation, BAC plasmid extraction, transfection and final recovery of recombinant virus, could be completed within one week. Most importantly, a DNA template library with different mutations can be prepared to construct numerous recombinant PEDVs simultaneously. In other words, a high throughput method to construct recombinant viruses can be developed. This will dramatically reduce the time and cost for manipulation of the PEDV genome.

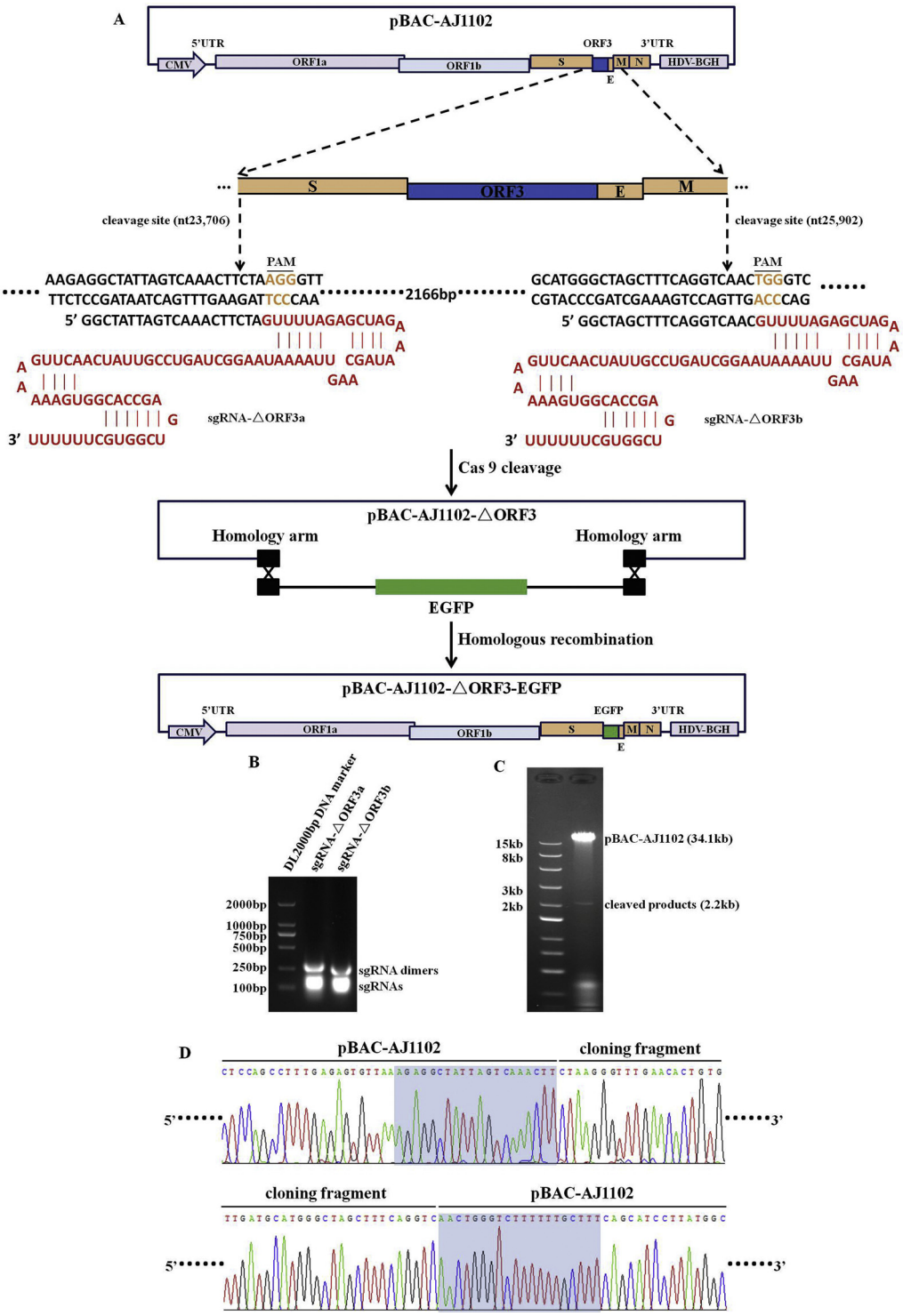

Fig. 3. Cleavage of pBAC-AJ1102 with CRISPR/Cas9 *in vitro*. (A) Schematic diagram for cleavage of pBAC-AJ1102 with CRISPR/Cas9. The cleavage sites were three nucleotides upstream of protospacer adjacent motif (PAM) sequences. The nt positions correspond to the complete genome sequence of AJ1102 (GenBank accession number MK584552). (B) Generation of sgRNA-ΔORF3a and sgRNA-ΔORF3b for specific cleavage of pBAC-AJ1102. (C) Electrophoresis of the cleaved pBAC-AJ1102 in 0.8% agarose gel to verify specific cleavage. The expected cleavage products were about 34.1 kb and 2.2 kb. (D) Sequencing results demonstrated that the amplified fragment was successfully inserted into the pBAC-AJ1102. The shaded regions are the homologous sequences. The upper panel is the sequencing results of 5′ terminal of the insertion portion. The lower panel is the sequencing results of 3′ terminal of the insertion portion.

In addition to the obtained rAJ1102-ΔORF3-EGFP, the CRISPR/Cas9 technique was used to construct another recombinant PEDV, in which a continuous 12-nt motif was successfully inserted into the C-terminal of the S gene of PEDV strain AJ1102 (F12) (data not shown), further demonstrating that CRISPR/Cas9 is a more efficient technology for manipulation of the PEDV genome. Furthermore, this strategy was also used to edit the full-length infectious clones of other RNA viruses with larger genomes, including transmissible gastroenteritis virus (TGEV), another swine enteric coronavirus, and porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family *Arteriviridae* (unpublished data). Collectively, this study has established a more efficient platform for PEDV genome manipulation, and the method described in this study is also applicable to other RNA viruses.

Accessory proteins are genus-specific for coronaviruses and only a single accessory protein gene, the ORF3 gene, has been identified in the PEDV genome. PEDV ORF3 encodes an ion channel protein, but it is not essential for PEDV replication (Beall et al., 2016; Wang et al., 2012; Wongthida et al., 2017). Thus, the ORF3 gene was chosen as a model to be replaced with an EGFP gene using the method described in this study. Consistent with previous studies, the rescued rAJ1102-ΔORF3-EGFP possessed similar proliferation properties *in vitro* compared with the wildtype AJ1102 virus. The insertion of reporter genes such as

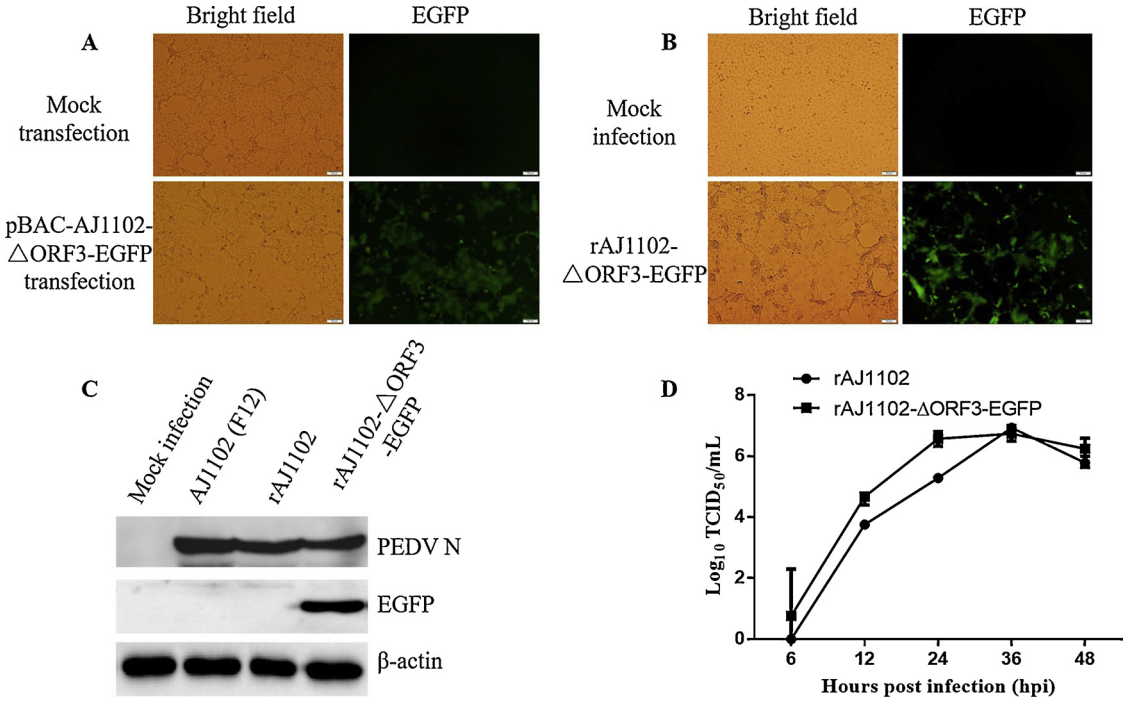

Fig. 4. Identification and growth kinetics of rAJ1102-ΔORF3-EGFP. (A) Vero cells were transfected with recombinant plasmid pBAC-AJ1102-ΔORF3-EGFP. Cytopathic effect and fluorescence were visualized at 24 h post-transfection. (B) Vero cells were infected with the 3rd passage of the rescued virus (rAJ1102-ΔORF3-EGFP). Cytopathic effect and fluorescence were visualized at 24 h post-infection (hpi). (C) Vero cells were infected with rAJ1102-ΔORF3-EGFP, rAJ1102 or parental AJ1102 (F12) (MOI = 0.1). At 24 hpi, cells were collected for Western blots with antibodies against PEDV N protein and EGFP. (D) Vero cells were infected with rAJ1102 or rAJ1102-ΔORF3-EGFP at an MOI of 0.1. Infected cells were collected at the indicated time-points (6, 12, 24, 36, 48 hpi). Viral titers were determined by $TCID_{50}$ assay in Vero cells and growth curves were drawn.

EGFP will be very useful for the study of various molecular and virological aspects of PEDV infection *in vitro* and *in vivo*. For example, rAJ1102-ΔORF3-EGFP can be exploited to establish convenient virus neutralization assays that provide answers within hours rather than days, and this reporter PEDV can also be used to investigate the transportation dynamics of PEDV in intestinal tract of piglets *in vivo*.

In summary, the CRISPR/Cas9 technology was successfully used to generate a recombinant reporter PEDV that demonstrates *in vitro* replication properties similar to the parental strain. Application of this technique can generate such recombinant viruses within a week, thus establishing a rapid and efficient platform for manipulation of not only the PEDV genome, but also of other RNA viruses.

Ethics approval and consent to participate

Not applicable.

Declaration of competing interests

The authors declared no potential conflicts of interest.

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References

Beall, A., Yount, B., Lin, C.M., Hou, Y., Wang, Q., Saif, L., Baric, R., 2016. Characterization of a pathogenic full-length cDNA clone and transmission model for porcine epidemic diarrhea virus strain PC22A. mBio 7, e01451–15.

Bi, J., Zeng, S., Xiao, S., Chen, H., Fang, L., 2012. Complete genome sequence of porcine epidemic diarrhea virus strain AJ1102 isolated from a suckling piglet with acute diarrhea in China. J. Virol. 86, 10910–10911.

Ding, Z., Fang, L., Jing, H., Zeng, S., Wang, D., Liu, L., Zhang, H., Luo, R., Chen, H., Xiao, S., 2014. Porcine epidemic diarrhea virus nucleocapsid protein antagonizes beta interferon production by sequestering the interaction between IRF3 and TBK1. J. Virol. 88, 8936–8945.

Duarte, M., Gelfi, J., Lambert, P., Rasschaert, D., Laude, H., 1993. Genome organization of porcine epidemic diarrhoea virus. Adv. Exp. Med. Biol. 342, 55–60.

Fan, B., Yu, Z., Pang, F., Xu, X., Zhang, B., Guo, R., He, K., Li, B., 2017. Characterization of a pathogenic full-length cDNA clone of a virulent porcine epidemic diarrhea virus strain AH2012/12 in China. Virology 500, 50–61.

Hou, Y., Lin, C.M., Yokoyama, M., Yount, B.L., Marthaler, D., Douglas, A.L., Ghimire, S., Qin, Y., Baric, R.S., Saif, L.J., Wang, Q., 2017. Deletion of a 197-Amino-Acid region in the N-Terminal domain of spike protein attenuates porcine epidemic diarrhea virus in piglets. J. Virol. 91, e00227–17.

Hou, Y., Meulia, T., Gao, X., Saif, L.J., Wang, Q., 2019. Deletion of both the tyrosine-based endocytosis signal and the endoplasmic reticulum retrieval signal in the cytoplasmic tail of spike protein attenuates porcine epidemic diarrhea virus in pigs. J. Virol. 93, e01758–18.

Jengarn, J., Wongthida, P., Wanasen, N., Frantz, P.N., Wanitchang, A., Jongkaewwattana, A., 2015. Genetic manipulation of porcine epidemic diarrhoea virus recovered from a full-length infectious cDNA clone. J. Gen. Virol. 96, 2206–2218.

Jiang, W., Zhao, X., Gabrieli, T., Lou, C., Ebenstein, Y., Zhu, T.F., 2015. Cas9-Assisted Targeting of CHromosome segments CATCH enables one-step targeted cloning of large gene clusters. Nat. Commun. 6, 8101.

Jung, K., Saif, L.J., 2015. Porcine epidemic diarrhea virus infection: etiology, epidemiology, pathogenesis and immunoprophylaxis. Vet. J. 204, 134–143.

Kaewborisuth, C., He, Q., Jongkaewwattana, A., 2018. The accessory protein ORF3 contributes to porcine epidemic diarrhea virus replication by direct binding to the spike protein. Viruses 10, E399.

Kao, C.F., Chiou, H.Y., Chang, Y.C., Hsueh, C.S., Jeng, C.R., Tsai, P.S., Cheng, I.C., Pang, V.F., Chang, H.W., 2018. The characterization of immunoprotection induced by a cDNA clone derived from the attenuated Taiwan porcine epidemic diarrhea virus pintung 52 strain. Viruses 10, E543.

Lee, C., 2015. Porcine epidemic diarrhea virus: an emerging and re-emerging epizootic swine virus. Virol. J. 12, 193.

Li, C., Li, Z., Zou, Y., Wicht, O., van Kuppeveld, F.J., Rottier, P.J., Bosch, B.J., 2013. Manipulation of the porcine epidemic diarrhea virus genome using targeted RNA recombination. PLoS One 8, e69997.

Li, J., Jin, Z., Gao, Y., Zhou, L., Ge, X., Guo, X., Han, J., Yang, H., 2017. Development of the full-length cDNA clones of two porcine epidemic diarrhea disease virus isolates with different virulence. PLoS One 12, e0173998.

Li, W., Li, H., Liu, Y., Pan, Y., Deng, F., Song, Y., Tang, X., He, Q., 2012. New variants of

porcine epidemic diarrhea virus, China, 2011. Emerg. Infect. Dis. 18, 1350–1353.
Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013. RNA-guided human genome engineering via Cas9. Science 339, 823–826.
Moradpour, M., Abdulah, S.N.A., 2019. CRISPR/dCas9 platforms in plants: strategies and applications beyond genome editing. Plant Biotechnol. J. https://doi.org/10.1111/pbi.13232. [Epub ahead of print].
Song, D., Moon, H., Kang, B., 2015. Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. Clin. Exp. Vaccine Res. 4, 166–176.
Song, D., Park, B., 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. Virus Genes 44, 167–175.
Stevenson, G.W., Hoang, H., Schwartz, K.J., Burrough, E.R., Sun, D., Madson, D., Cooper, V.L., Pillatzki, A., Gauger, P., Schmitt, B.J., Koster, L.G., Killian, M.L., Yoon, K.J., 2013. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J. Vet. Diagn. Invest. 25, 649–654.
Sun, D., Wang, X., Wei, S., Chen, J., Feng, L., 2016. Epidemiology and vaccine of porcine epidemic diarrhea virus in China: a mini-review. J. Vet. Med. Sci. 78, 355–363.
Teeravechyan, S., Frantz, P.N., Wongthida, P., Chailangkarn, T., Jaru-Ampornpan, P., Koonpaew, S., Jongkaewwattana, A., 2016. Deciphering the biology of porcine epidemic diarrhea virus in the era of reverse genetics. Virus Res. 226, 152–171.
Van Reeth, K., Pensaert, M., 1994. Prevalence of infections with enzootic respiratory and enteric viruses in feeder pigs entering fattening herds. Vet. Rec. 135, 594–597.
Vlasova, A.N., Marthaler, D., Wang, Q., Culhane, M.R., Rossow, K.D., Rovira, A., Collins, J., Saif, L.J., 2014. Distinct characteristics and complex evolution of PEDV strains, North America, May 2013-February 2014. Emerg. Infect. Dis. 20, 1620–1628.
Wang, D., Ge, X., Chen, D., Li, J., Cai, Y., Deng, J., Zhou, L., Guo, X., Han, J., Yang, H., 2018. The S gene is necessary but not sufficient for the virulence of porcine epidemic diarrhea virus novel variant strain BJ2011C. J. Virol. 92, e00603–18.
Wang, J.W., Wang, A., Li, K., Wang, B., Jin, S., Reiser, M., Lockey, R.F., 2015. CRISPR/Cas9 nuclease cleavage combined with Gibson assembly for seamless cloning. BioTechniques 58, 161–170.
Wang, K., Lu, W., Chen, J., Xie, S., Shi, H., Hsu, H., Yu, W., Xu, K., Bian, C., Fischer, W.B., Schwarz, W., Feng, L., Sun, B., 2012. PEDV ORF3 encodes an ion channel protein and regulates virus production. FEBS Lett. 586, 384–391.
Wang, Q., Vlasova, A.N., Kenney, S.P., Saif, L.J., 2019. Emerging and re-emerging coronaviruses in pigs. Curr. Opin. Virol. 34, 39–49.
Wongthida, P., Liwnaree, B., Wanasen, N., Narkpuk, J., Jongkaewwattana, A., 2017. The role of ORF3 accessory protein in replication of cell-adapted porcine epidemic diarrhea virus (PEDV). Arch. Virol. 162, 2553–2563.
Wood, E.N., 1977. An apparently new syndrome of porcine epidemic diarrhoea. Vet. Rec. 100, 243–244.
Zhang, Q., Yoo, D., 2016. Immune evasion of porcine enteric coronaviruses and viral modulation of antiviral innate signaling. Virus Res. 226, 128–141.