The S Gene Is Necessary but Not Sufficient for the Virulence of Porcine Epidemic Diarrhea Virus Novel Variant Strain BJ2011C

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ABSTRACT The recently emerged highly virulent variants of porcine epidemic diarrhea virus (PEDV) have caused colossal economic losses to the worldwide swine industry. In this study, we investigated the viral virulence determinants by constructing a series of chimeric mutants between the highly virulent strain BJ2011C and the avirulent strain CHM2013. When tested in the 2-day-old piglet model, wild-type (WT) BJ2011C caused severe diarrhea and death of the piglets within 72 h. In contrast, its chimeric derivative carrying the S gene from CHM2013 (BJ2011C-S_{CHM}) was avirulent to the piglets. Moreover, reciprocal substitution of the BJ2011C S gene (CHM2013-S_{BJ}) did not enable CHM2013 to gain any virulence. However, when the whole structural protein-coding region of BJ2011C (CHM2013-SP_{BJ}) was swapped, CHM2013 started to gain the ability to efficiently colonize the intestinal tract and caused diarrhea in piglets. A further gain of virulence required additional acquisition of the 3' untranslated region (UTR) of BJ2011C, and the resultant virus (CHM2013-SP + 3UTR_{BJ}) caused more severe diarrhea and death of piglets. Together, our findings suggest that the virulence of PEDV epidemic strains is a multigenic event and that the S gene is only one of the necessary determinants.

IMPORTANCE The recently emerged highly virulent PEDV variants are the major cause of the global porcine epidemic diarrhea (PED) pandemic. The S gene of the variants undergoes remarkable variations and has been thought to be the virulence determinant for the enhanced pathogenesis. Our studies here showed that the S gene is only part of the story and that full virulence requires cooperation from other genes. Our findings provide insight into the pathogenic mechanism of the highly virulent PEDV variants and have implications for future vaccine development.

KEYWORDS 3' UTR, PEDV, S gene, structural protein, virulence determinants

Porcine epidemic diarrhea virus (PEDV) is the etiological agent of porcine epidemic diarrhea disease (PED) and mainly infects the gastrointestinal tract in pigs, leading to a variety of clinical symptoms, such as diarrhea, vomiting, anorexia, dehydration, and weight loss (1–3). In the field, pigs of all ages are susceptible to PEDV infection, but the most severe outcome often occurs in suckling piglets under 1 week of age (4, 5). PEDV was first identified in Europe in the late 1970s and subsequently spread to other parts of the world (6, 7). In Asia, PEDV is present in many pork-producing countries, including Korea, China, Japan, Vietnam, and the Philippines (8–13), but caused only infrequent, sporadic, and regional epidemics prior to 2010 (2, 10, 14, 15). However, around 2010, highly virulent PEDV strains suddenly emerged, with a mortality rate in neonatal piglets of up to 100%, and they have since decimated numerous swine farms in China, leading to colossal economic losses (5, 16, 17). Three years later, the highly virulent PEDV landed on the American continent for the first time and killed millions of pigs within a short time (3, 18, 19); it rapidly spread across the United States and to other countries

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in North and South America (20–23). The highly virulent PEDV variants have since become a well-recognized global threat to pork production (19, 24).

PEDV belongs to the genus *Alphacoronavirus* within the family *Coronaviridae* (25). It has a 28-kb positive-sense, single-stranded RNA genome that contains at least 7 open reading frames (ORFs). ORF1a and ORF1b encode viral replicase proteins, whereas ORF2 to ORF6 encode viral structural/accessory proteins, including the spike protein (S), ORF3 protein, envelope protein (E), matrix protein (M), and nucleocapsid protein (N) (2, 26–28). Among the structural proteins, the S protein mediates PEDV invasion into host cells, with its ectodomain containing a receptor-binding subunit, S1, and a membrane fusion subunit, S2 (29–31). During PEDV entry, S1 binds to a yet-known receptor on the host cell surface for viral attachment, and then S2 transits to the postfusion conformation for membrane fusion (32–36). The S protein is the main target of neutralizing antibodies (37), and the neutralizing epitopes have been mapped to the sialic acid-binding region and the receptor-binding domain of S1 (37, 38). The S2 subunit is also capable of inducing neutralizing antibodies, and a peptide derived from the heptad repeat 2 region of S2 has been reported to be able to suppress PEDV entry and induce neutralizing antibodies (39, 40).

The highly virulent PEDV variants are genetically divergent from the classical strains and belong to the GII group (10, 18). The S gene represents one of the most genetically variable regions within the PEDV genome (10, 14, 20); deletions, insertions, and amino acid substitutions within S have been found in field strains compared to strain CV777 (18, 20, 41–43). In particular, the S proteins of the highly virulent novel PEDV variants in China possess characteristic insertions and deletions, including a 4-amino-acid (aa) (GENQ) insertion between positions 55 and 56, a 1-aa (N) insertion between positions 135 and 136, and a 2-aa (DG) deletion between positions 155 and 156 (10, 20, 41). In addition, many amino acid substitutions are found scattered across the S protein (10, 42). Currently, the classical vaccines (e.g., DR13, SM98, and CV777) do not provide efficient cross-protection against challenge by novel PEDV variants, and a lack of efficient cross-neutralization has been reported (10, 44–46). Given the important role in induction of neutralizing antibodies, it seems likely that the genetic variations of the S gene contribute to the modulation of viral virulence.

Reverse genetics technology provides a useful tool to molecular biology against the pathogenesis of PEDV (47). Although several reverse genetics systems have been established for PEDV (48–51), its application to identification of virulence determinants has been rare (47, 49, 52). A recent study reported that a mutant (icPC22A-S1Δ197) bearing a 197-aa deletion in the N-terminal domain of S from an infectious cDNA clone of the highly virulent PEDV PC22A is attenuated in piglets (52). However, this was a loss-of-function study, and a gain-of-function approach is required to define the region(s) within the viral genome that is related to the fatal virulence of PEDV variants. We have recently established a reverse genetics system for two PEDV isolates with different virulence levels (BJ2011C and CHM2013) (53). Based on this platform, here, we investigated the role of the S gene and other regions of the PEDV genome in viral pathogenesis.

RESULTS

We recently isolated two clinical PEDV strains, namely, CHM2013 and BJ2011C, from swine farms with PED outbreaks (53). These two strains share 96.8% overall similarity at the nucleotide level, and their detailed genetic differences are shown in Table 1. Phylogenetically, CHM2013 is closely related to the Korean vaccine strain SM98 of the GI group, whereas the isolate BJ2011C is a representative of the PEDV epidemic variants and belongs to the GIIb group, a cluster that contains many recent pandemic strains (53). Moreover, we have successfully developed a DNA-launched reverse genetics system for the two isolates based on the bacterial artificial chromosome (BAC) (53) (Fig. 1A). The rescued viruses BJ2011C and CHM2013 replicate with kinetics similar to those of their respective parental viruses in cell culture. When tested in the 2-day-old pig model, BJ2011C caused severe diarrhea of piglets with extensive damage to the

TABLE 1 Nucleotide and amino acid identities of different regions of the BJ2011C genome with CHM2013

Genomic region	Length (bp) (CHM2013/BJ2011C)	Identity (nucleotide/amino acid) (%)
5' UTR	291/292	97.9/—
ORF1a	12,303/12,309	97.1/97.4
ORF1b	8,037/8,037	97.7/99.1
S	4,143/4,161	94.0/92.4
ORF3	465/675	96.6/95.5
E	231/231	96.1/94.8
M	693/681	98.1/97.8
N	1,326/1,326	95.7/96.6
3' UTR	334/334	97.6/—

intestinal epithelium, leading to 100% fatality to the piglets. In contrast, the CHM2013-inoculated piglets all survived with no or only very minor tissue damage (53). Having this tool at hand, we proceeded to investigate the virulence determinants of PEDV BJ2011C.

Construction and recovery of PEDV chimeric viruses. The S genes of novel PEDV variants have been proposed as virulence factors. To test this hypothesis, we made

FIG 1 Construction of chimeric viruses of PEDV. (A) DNA-launched reverse genetics BAC system for PEDV strains BJ2011C and CHM2013. The critical restriction enzymes used for molecular cloning and their relative positions in the genome are indicated. CMV, cytomegalovirus; HDV, hepatitis D virus; BGH, bovine growth hormone polyadenylation signal. (B) Diagram of chimeric infectious cDNA clones with S genes exchanged. (C) Diagram of chimeric infected cDNA clones with exchanged structural protein (SP)-coding regions. (D) Diagram of chimeric clones with SP regions and 3' UTRs swapped. The boxes represent the genomic fragments of parental backbone viruses CHM2013 (blue) and BJ2011C (green).

FIG 2 *In vitro* characterization of PEDV chimeric viruses. (A) Virus-induced CPE and immunofluorescence staining. Vero cells were infected with the indicated viruses at an MOI of 0.01. The virus-induced CPE was monitored and recorded with an inverted microscope. At 24 h postinfection, the cells were fixed and stained with mouse monoclonal antibody 4E3 to PEDV N protein. Nuclei were stained with DAPI (blue). (B) Multistep growth curves of PEDV wild-type and chimeric viruses on Vero cells at an MOI of 0.01. Virus titers at different time points, as indicated, were determined by endpoint dilution assay. The error bars indicate standard deviations.

chimeric infectious cDNA clones between the avirulent strain CHM2013 and the epidemic, highly virulent strain BJ2011C by swapping the corresponding S genes, and the resulting constructs were named pBJ2011C-S_{CHM} and pCHM2013-S_{BJ} (Fig. 1B). Along with the two mutants, we also expanded the swapped regions to the whole structural protein-coding region (SP) and the 3' untranslated region (3' UTR), which have overall lower similarity to ORF1a and ORF1b (Table 1), to generate the following constructs (Fig. 1C and D): pBJ2011C-SP_{CHM}, pCHM2013-SP_{BJ}, pBJ2011C-SP + 3UTR_{CHM}, and CHM2013-SP + 3UTR_{BJ}. The chimeric viruses were successfully rescued by transfecting the corresponding BAC plasmids into Vero cells. Characteristic syncytium formation, a phenotype that was the same as that of the parental viruses, was observed 48 h after transfection (Fig. 2A). The recombinant viruses, which were subsequently named BJ2011C-S_{CHM}, CHM2013-S_{BJ}, BJ2011C-SP_{CHM}, CHM2013-SP_{BJ}, BJ2011C-SP + 3UTR_{CHM}, and CHM2013-SP + 3UTR_{BJ}, were verified by sequencing the corresponding swapped regions, and the virus infections were confirmed by immunofluorescence staining of the infected Vero cells with monoclonal antibodies to the nucleocapsid protein (Fig. 2A).

Growth properties of chimeric viruses. The growth properties in Vero cells of the rescued viruses were measured by multistep growth assay with a multiplicity of

infection (MOI) of 0.01 (Fig. 2B). The result showed that CHM2013 had faster growth kinetics than BJ2011C and reached a plateau around 24 h postinfection (hpi) (Fig. 2B). Interestingly, the mutant CHM2013-S_{BJ} had kinetics similar to those of BJ2011C. Moreover, the mutants CHM2013-SP_{BJ} and CHM2013-SP + 3UTR_{BJ} behaved much like CHM2013-S_{BJ}. On the other hand, when the BJ2011C S gene was replaced with that of CHM2013, the resulting mutant, BJ2011C-S_{CHM}, acquired the growth properties of CHM2013, a deviation from its parental virus, BJ2011C, and the same was true for the mutants BJ2011C-SP_{CHM} and BJ2011C-SP + 3UTR_{CHM}. Thus, the S gene is a critical factor in driving viral growth properties in cell culture.

The S gene is necessary, but not sufficient, for the virulence of BJ2011C. To test the pathogenicity of these chimeric viruses, 43 2-day-old piglets were randomly divided into 9 groups with 5 pigs in each group, except for the mock-infected control group, which contained 3 piglets. The pigs were orally inoculated with the respective viruses at a dose of 5×10^5 50% tissue culture infective doses (TCID₅₀) or mock infected with Dulbecco's modified Eagle's medium (DMEM). In the BJ2011C group, all five piglets exhibited obvious dehydration and weight loss (Fig. 3A). Severe diarrhea began at 24 h postchallenge (Fig. 3C), and the pigs all died within 72 h (Fig. 3D). The postmortem examination revealed thin, transparent, and hemorrhagic intestinal walls (Fig. 4), and hematoxylin and eosin (H&E) staining revealed extensive damage to different segments of the intestine characterized by fragmentation and shedding of intestinal villi (Fig. 5). In contrast, the piglets challenged with CHM2013 showed normal weight gain (Fig. 3A) and did not show any obvious clinical symptoms (Fig. 3B and C). In addition, there were no obvious gross lesions (Fig. 4) or histopathological changes (Fig. 5) observed. These results were in accordance with our recent report on the pathogenic properties of the two viruses (53). For the chimeric viruses, we first looked at the mutants with swapped S genes. We found that BJ2011C lost the pathogenic property (Fig. 3A, B, and C) when its S gene was replaced with the S gene of CHM2013 (BJ2011C-S_{CHM}), and all 5 piglets survived the challenge (Fig. 3D). This mutant behaved just like CHM2013 in terms of the average weight gain caused (Fig. 3A), clinical scores (Fig. 3B), fecal scores (Fig. 3C), and gross and histopathological lesions (Fig. 4 and 5). More importantly, CHM2013 did not gain any pathogenic properties when its S gene was replaced with that of BJ2011C (CHM2013-S_{BJ}) (Fig. 3, 4, and 5). Thus, we conclude that the S gene is necessary but not sufficient to confer virulence on BJ2011C.

The structural-protein-coding region and 3' UTR contribute to the virulence of BJ2011C. We next looked into the pathogenic features of the other 4 chimeric viruses with the whole SP region and 3' UTR exchanged. The animal studies showed that replacement of the SP region with BJ2011C (CHM2013-SP_{BJ}) enabled CHM2013 to gain the pathogenic property (Fig. 3). This was evidenced by an average weight loss (Fig. 3A), increased clinical scores (Fig. 3B), and severe diarrhea (Fig. 3C) in the pigs infected with the mutant CHM2013-SP_{BJ}. However, the infection was not lethal, and all the piglets survived to the end of the study (Fig. 3D). A further gain of virulence for CHM2013 required addition of the 3' UTR of BJ2011C to the 3' UTR together with the BJ2011C SP region BJ2011C SP region (Fig. 3). Upon inoculation with CHM2013-SP + 3UTR_{BJ}, the piglets showed obvious dehydration and bodyweight loss and developed severe diarrhea (Fig. 3A, B, and C). Moreover, two piglets died 5 days postinfection (Fig. 3D), an indication of acquisition of the lethal feature. Postmortem examination revealed that the pigs in the CHM2013-SP + 3UTR_{BJ} and CHM2013-SP_{BJ} groups exhibited typical PEDV-like lesions, characterized by thin and transparent intestinal walls (Fig. 4). Their stomachs contained little milk curd, and the small and large intestines were distended by watery contents that contained floccules of white to yellow undigested milk. Significantly, the histopathological lesions induced by the two viruses were apparent (Fig. 5). Thus, replacement of SP and the 3' UTR enabled the low-virulence CHM2013 to gain the pathogenic property, an indication of gain of function.

On the other hand, the piglets challenged with BJ2011C-SP_{CHM} and BJ2011C-SP + 3UTR_{CHM} behaved like the CHM2013 and mock-infected groups, showed stable weight

FIG 3 Pathogenicity analysis of PEDV chimeras. (A) Average weight gain of piglets on the day of death or euthanasia at the final time points. (B) Clinical mental state scores of piglets of different groups. Criteria for evaluation: 0, normal; 1, mild lethargy (slow to move; head down); 2, moderate lethargy (stands but tends to lie down); 3, heavier lethargy (lies down; occasionally stands); 4, severe lethargy (recumbent; moribund). (C) Fecal scores of piglets of different groups. Evaluation standards: 0, normal; 1, soft (cowpie); 2, very soft and tends to be liquid; 3, liquid with some solid content; 4, watery diarrhea with no solid content. (D) Survival rates of piglets of each group. The survival curves of piglets infected with rescued viruses in each group are shown. Asterisks indicate significant differences between BJ2011C and CHM2013 (***, $P < 0.001$). Pound signs indicate significant differences between chimeric viruses CHM2013-SP_{BJ} and CHM2013 (#, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$). Thetas indicate significant differences between chimeric viruses CHM2013-SP + 3UTR_{BJ} and CHM2013 (θ, $P < 0.05$; θθ, $P < 0.01$; θθθ, $P < 0.001$). The error bars indicate standard deviations.

gain (Fig. 3A), and did not exhibit diarrhea symptoms (Fig. 3B and C). In addition, the gross and histopathological lesions induced by these viruses were very minor (Fig. 4 and 5). These results suggest that replacement with either S, SP, or SP plus the 3' UTR of CHM2013 enables BJ2011C to lose its pathogenic property. Together, we conclude that, in addition to the S gene, other parts of the SP region and 3' UTR contribute critically to the high virulence of PEDV strain BJ2011C.

The SP region contributes to both viral colonization and postcolonization pathogenicity. We next investigated how the SP region and 3' UTR contribute to the virulence of BJ2011C by analyzing the infection efficiency of the chimeric viruses as reflected by virus shedding (Fig. 6) and tissue viral load (Fig. 7). The feces were sampled with fecal swabs, and virus shedding was evaluated qualitatively by regular PCR detection of viral-genome RNA (Fig. 6). Consistent with the previous report, BJ2011C had high infection efficiency, and all the pigs challenged with the virus started to shed the virus on day 1. The shedding measurement of this group on days 2 to 7 was

FIG 4 Gross lesions of piglets inoculated with the recombinant viruses. The intestinal lesions were examined on the day of death or euthanasia at the final time points. The necropsy images show transparent intestines observed in piglets inoculated with BJ2011C, CHM2013-SP_{BJ}, and CHM2013-SP + 3UTR_{BJ} but not in the CHM2013-, CHM2013-S_{BJ}-, BJ2011C-S_{CHM}-, BJ2011C-SP_{CHM}-, and BJ2011C-SP + 3UTR_{CHM}-infected or mock-inoculated piglets.

terminated because of the quick death of the infected piglets. In contrast, no detectable virus shedding could be found in the mock- or CHM2013-challenged group. For the loss of function of groups (BJ2011C-S_{CHM}, BJ2011C-SP_{CHM}, and BJ2011C-SP + 3UTR_{CHM}), replacement of the S gene, SP, or SP plus 3' UTR had a profound effect on virus shedding. Specifically, the fecal-swap PCRs were all negative for the BJ2011C-SP_{CHM} and BJ2011C-SP + 3UTR_{CHM} groups, except for the BJ2011C-S_{CHM} group, in which the PCR was transiently positive on day 3. On the other hand, the results from the gain of function of the CHM2013-S_{BJ}, CHM2013-SP_{BJ}, and CHM2013-SP + 3UTR_{BJ} groups showed that the mutant with replacement of the S gene alone (CHM2013-S_{BJ}) did not shed virus, whereas replacement with either SP or SP plus the 3' UTR enabled CHM2013 to gain the shedding property.

To evaluate the tissue viral loads of infected piglets, different segments of the intestine, including the duodenum, ileum, jejunum, cecum, colon, and rectum, were taken and individually processed for quantification of virus abundance by real-time reverse transcription (RT)-PCR (Fig. 7). Consistent with the virus shedding results, we could detect BJ2011C, CHM2013-SP_{BJ}, and CHM2013-SP + 3UTR_{BJ} in all the intestinal segments from infected piglets. For the BJ2011C-S_{CHM} group, the virus was detected only in the ileum and cecum. However, no virus genomes were detected in any intestinal segments in the groups challenged with BJ2011C-SP_{CHM}, BJ2011C-SP + 3UTR_{CHM}, CHM2013, CHM2013-S_{BJ}, or DMEM (mock control). These results suggest that the BJ2011C SP region is critical for the virus to establish efficient infection in the intestinal tract.

Together, the results of the two assays suggest that, in addition to the S gene product, other structural proteins and probably the 3' UTR are required for efficient viral colonization of the intestinal tract. Furthermore, considering the fact that the BJ2011C SP region also enabled CHM2013 to induce typical PEDV-like lesions, as described above (Fig. 3 and 6), these results combined indicate that the BJ2011C SP region plays dual roles: it contributes critically, not only to viral colonization, but also to postcolonization pathogenicity, thus representing a key virulence factor. The phenotype of the CHM2013-SP + 3UTR_{BJ} mutant suggests that the 3' UTR plays a synergistic role in these processes.

FIG 5 Histopathological examination of intestines of recombinant PEDV-infected piglets. Different segments, including duodenum, jejunum, cecum, colon, and rectum, of intestines from each group were taken and then processed for H&E staining, and representative images are shown.

DISCUSSION

In recent years, highly virulent PEDV variants have caused substantial economic losses to the worldwide swine industry, but the molecular determinants associated with the fatal virulence have remained poorly understood. Using reverse genetics technology, we found that (i) the S gene of the highly virulent PEDV strain BJ2011C is necessary but not sufficient to confer fatal virulence toward 2-day-old piglets and (ii) the SP region and 3' UTR promote the efficiency of viral colonization of the intestinal tract and

FIG 6 Virus shedding in feces. Daily virus shedding in feces of different groups was measured by reverse transcription (RT-PCR) detection of virus genomes in fecal swabs. The length of the target fragment is 618 bp.

also contribute critically to postcolonization pathogenicity. Together, our results suggest that the pathogenicity of the novel PEDV variant is a multigenic property. The relevant significance and insights are discussed below.

Insight into the role of the S gene in PEDV pathogenesis. The S proteins of the highly virulent variants of PEDV have multiple amino acid insertions, deletions, and substitutions relative to the classic PEDV strains (e.g., CV777) (20). Since S protein is critically involved in PEDV entry, assembly, and induction of neutralizing antibodies (30, 33, 38, 40, 54–56), the mutations likely have an influence on the virulence of PEDV. This idea is partly supported by recent studies of PEDV S protein large deletion mutants that were isolated from either cell culture (strain TC-PC177; cell adapted) (57) or swine farms (strain TTR-2) (58). Both strains are avirulent to piglets. Moreover, an engineered mutant (icPC22A-S1Δ197) bearing a 197-aa deletion in the N terminus of S, based on an infectious cDNA clone of the highly virulent PEDV PC22A strain, caused only mild to moderate diarrhea with no mortality, indicating that the S protein is a critical factor (52). However, since this was a loss-of-function approach, a caveat is that deletion of the N-terminal 197 aa may cripple the function of the S protein. Indeed, the mutant had greatly reduced growth in cell culture (52). In this study, we bypassed this problem by constructing S-swapped chimeric mutants between the highly virulent BJ2011C and the avirulent CHM2013. We found that the BJ2011C-S_{CHM}-inoculated piglets all survived

FIG 7 Quantification of viral loads in different intestine segments from PEDV-infected piglets. The segments were collected and used for total RNA extraction. Virus abundance was quantified by TaqMan real-time RT-PCR targeting the PEDV N gene. Asterisks indicate significant differences between BJ2011C and CHM2013 (***, $P < 0.001$). Pound signs indicate significant differences between chimeric viruses CHM2013-SP_{BJ} and CHM2013 (###, $P < 0.001$). Thetas indicate significant differences between chimeric viruses CHM2013-SP + 3UTR_{BJ} and CHM2013 (θθθ, $P < 0.001$). Sigmas indicate significant differences between chimeric viruses BJ2011C-S_{CHM} and CHM2013 (σσσ, $P < 0.001$). The error bars indicate standard deviations.

and showed very mild clinical signs (Fig. 3), transient virus shedding (Fig. 6), low virus loads (Fig. 7), and light histopathological lesions (Fig. 5), suggesting that the BJ2011C S gene is important for viral virulence. On the other hand, replacement with the BJ2011C S gene did not increase the virulence of CHM2013; the pigs challenged with the mutant CHM2013-S_{BJ} behaved much like the CHM2013-challenged control group (Fig. 3), suggesting that the BJ2011C S gene itself is not sufficient to confer a virulent phenotype. Thus, these findings suggest that the S gene contributes to the fatal virulence of BJ2011C but is only one of the necessary determinants.

A synergistic mechanism for PEDV pathogenesis. Our studies also found that, in addition to the S gene, other parts of the SP region are critical for viral virulence. This was concluded from the gain-of-function studies, in which CHM2013, carrying the SP region of BJ2011C, acquired virulence. First, swapping the BJ2011C SP region enabled CHM2013 to regain colonization competence, as we were able to detect the chimeric virus in infected piglets (Fig. 7). This is in stark contrast to the parental virus, CHM2013, for which we could not detect any sign of infection of the small intestine (Fig. 7). Interestingly, it has been reported that newborn piglets do not require much virus for infection leading to disease (59). This highlights the remarkable ability of the highly virulent PEDV strains to breach the physical, chemical, and immunity barriers, leading to successful colonization. In support of this view, our results showed that the mutations within the SP region of BJ2011C have a profound effect on establishing efficient infection in the intestinal tract. Second, the BJ2011C SP region affects more than just viral colonization, as the CHM2013-SP_{BJ} mutant reproduced the typical “PED-like” symptoms (Fig. 3, 4, and 5), although all the piglets survived to the end of the experiment. Thus, the SP region plays an important role in postcolonization pathogenicity. Together, these studies indicate that the SP region plays at least dual roles in causing viral pathogenesis. These findings justify further studies on dissection of the structural-protein-coding region (ORF3/E/M/N) to determine which protein synergizes with S during infection for viral pathogenesis. They also justify follow-up studies on separating the dual roles of the SP region using an *in vivo* colonization-competent avirulent strain, i.e., S indel variants (60), as the acceptor or donor.

It is interesting that the 3′ UTR also plays an important role in virulence. When the BJ2011C 3′ UTR was added to the background of the *in vivo* colonization-competent CHM2013-SP_{BJ} mutant, it increased the virulence of the mutant. The resulting mutant, CHM2013-SP + 3′UTR_{BJ}, not only resulted in a higher clinical score, but also caused the death of piglets (Fig. 3). Although we did not do single 3′ UTR swapping, the avirulent phenotype of the BJ2011C-S_{CHM} and BJ2011C-SP_{CHM} mutants (Fig. 3, 4, and 5) suggests that the 3′ UTR of BJ2011C is unlikely to play a decisive role in virulence but rather synergizes with structural proteins in viral pathogenesis. On the other hand, the fact that the CHM2013-SP + 3′ UTR_{BJ} mutant did not completely recapitulate the full virulence property of BJ2011C suggests that the sequences in the 5′ two-thirds of the genome, including the 5′ UTR, ORF1a, and ORF1b, also contribute to the virulence of BJ2011C. Thus, it is clear that the high virulence of PEDV BJ2011C is a multigenic event and requires the coordination of multiple viral genes.

In conclusion, by using the reverse genetics platform, we for the first time provided strong evidence to suggest that the structural protein-coding region and 3′ UTR are virulence determinants of the highly virulent PEDV variants. We also found that the S gene is necessary but not sufficient to confer virulence on BJ2011C. Overall, our results added details to the molecular mechanism of PEDV virulence, which provides a foundation for a better understanding of the pathogenesis of novel PEDV variants and has implications for future vaccine development.

MATERIALS AND METHODS

Cells, plasmids, and viruses. Vero CCL-81 cells were maintained in Gibco DMEM containing 10% fetal bovine serum at 37°C with 5% CO₂. The full-length infectious cDNA clone plasmids pBAC-BJ2011C and pBAC-CHM2013, derived from the highly virulent PEDV strain BJ2011C and the avirulent strain CHM2013, have been reported previously (53). The complete genomic sequences have been determined and deposited in GenBank (GenBank accession no. [KX066126.1](#) and [KM887144.1](#)).

TABLE 2 Primers used in this study

Primer	Sequence (5'-3')	Full-length plasmid constructed
WD0032F	ATCTGGGAATGTTTTCTAGGTGTTGTGAGTGCAACTATGCGAGGT	pCHM2013-SU _{BJ}
WD0032R	TAGAACTGCAGCATGTGGTGTGCTTAGAGCCTAAGATCATGGCTG	
WD0033F	CACCACATGCTGCAGTTCTACTGAC	
WD0033R	AAGTAGGTAAAGACTTCATTTGTTTACGTTGACCAATGATTAG	
WD0034F	ATGAAGTCCTTAACCTACTCTGGT	pBJ2011C-SU _{CHM}
WD0034R	ACATCATTAACAGTAGGGCCCAATGTTTAAATGCTAGGAACTGGC	
WD0039F	GTTCTTAAGAGCATTTTAATTAA GGTAGGTGCTTGTGAGGAGT	
WD0039R	GCCCTTGTGATGGCAACATTAAACCTGTTAACATT	
WD0040F	TGCCATCACAAGGGCCAAGA	pCHM2013-SP + 3UTR _{BJ}
WD0040R	AATTAAGACCTCATTTGTTTACGTTGACCAATGATTAG	
WD0041F	ATGAGGTCTTAATTTACTTCTGGTTC	
WD0041R	ACATCATTAACAGTAGGGCCCAATGTTTATTATCGGGAAA	
WD0035F	TAGCATAAAACATTGGGCCCCACTGCTAATAATGATGTTACAACAGGTC	pBJ2011C-SP + 3UTR _{CHM}
WD0035R	CACTAAGGAGTGTAGCGTTACACAGTTGGTGCTCCA	
WD0036F	TGTAACGCTAACACTCCTTAGTGGT	
WD0036R	CGTTCCTGGTGTGCTTCTCGTCACTCGAGGGCTTCGCCCTGTG	
WD0037F	TAGCATAAAACATTGGGCCCCACTGTTAATGATGTTACAACAGGT	pCHM2013S _{BJ}
WD0037R	CCATTAACGCATAAGCCAGCGATGGCAAGAACATTACAA	
WD0038F	CTTATGCGTTAATGGTGAAT	
WD0038R	TTGCATGCAAGGCGCGCCTGCATGCTGCTATTGTCTTCCAATC	
WD0061F	TAGCATAAAACATTGGGCCCCACTGCTAATAATGATGTTA	pBJ2011C-S _{CHM}
WD0061R	AGACATCTTTGACAACGTGTGCTACTGCACGTGGACCTTTTCAA	
WD0062F	CACAGTTGTCAAAGATGTCTCGAAG	
WD0062R	TTGAGGTCACGTTCTTTCGAAGTGGCCCTGGATTGTCTT	
WD0063F	TAGCATAAAACATTGGGCCCCACTGTTAATGATGTTACAACAGGT	pCHM2013-SP _{BJ}
WD0063R	TTGAAAAAGTCCAAGAAACATCAAGCTTCGTAAGGTTGAAGTCTA	
WD0064F	TGTTTCTTGGACTTTTCAATACAC	
WD0064R	TTGAGGTCACGTTCTTTCGAAGTGGCTCTGGATTGTCTTCTTAG	
WD0067F	CAAATCCAGAGCCACTTCGAAAGAACGTGACCTCAAAGACATCCCA	pBJ2011C-SP _{CHM}
WD0067R	ATAAGCCGGTCTAACATTGTTTAAATTCCTGTGTCGAAGATCTCG	
WD0068F	ACAATGTTAGACCGGCTTATCTGG	
WD0068R	CTGTTCCCTGGTGTCTCCTCGTCACTCGAGGGCTTCGCCCTGTG	
WD0069F	AAATCCAGGGCCACTTCGAAAGAACGTGACCTCAAAGACATCCCA	
WD0069R	CCAGAATAAGCCAGTCAAACATTGTTAATTCCTGTATCGAAGA	
WD0070F	ACAATGTTTGACTGGCTTATTCTG	
WD0070R	TTGCATGCAAGGCGCGCCTGCATGCTGCTATTGTCTTCCAATC	

Strategies for constructing chimeric full-length cDNA clones of PEDV. The full-length infectious cDNA clone plasmids pBAC-BJ2011C and pBAC-CHM2013 were used for constructing chimeric infectious cDNA clones. In general, the S gene, SP, and SP plus 3' UTR of the respective viruses were swapped by taking advantage of unique restriction enzymes, and all the molecular cloning was facilitated through homologous recombination instead of the conventional ligation strategy by using the ClonExpress MultiS one-step cloning kit (Vazyme, Nanjing, China). To replace the BJ2011C S gene with that of CHM2013, a set of unique restriction enzymes, PacI, ApaI, and BstBI, were utilized, and the construction was done in two steps. The first was to exchange the CHM2013 S1-coding region upstream ApaI sites. The ORF1b region (divided into two parts: 13017 to 17063 and 17064 to 20633) of BJ2011C and the CHM2013 S-coding region (20627 to 21027) upstream ApaI site were individually amplified and purified. The fragments were designed to have a 20-bp overlap relative to each other and to the plasmid backbone, pBAC-BJ2011C, and then to be cloned into a PacI/ApaI doubly digested backbone via a 3-way homologous recombination strategy, leading to generation of an intermediate plasmid, pBJ2011C-SU_{CHM}. In the subsequent step, the rest of the CHM2013 S gene coding region (downstream ApaI site, 21028 to 24769) and structural coding region (ORF3/E/M/N; 24795 to 27184) of BJ2011C were individually PCR amplified and subsequently cloned into ApaI/BstBI-digested plasmid pBJ2011C-SU_{CHM}, leading to the plasmid pBJ2011C-S_{CHM}. To replace the SP plus 3' UTR, the SP plus 3' UTR of CHM2013 was divided into two fragments and cloned into the plasmid pBJ2011C-SU_{CHM} via the ApaI and AclI restriction sites, leading to the generation of plasmid pBJ2011C-SP + 3UTR_{CHM}. To generate pBJ2011C-SP_{CHM}, the 3' UTR of CHM2013 in the plasmid pBJ2011C-SP + 3'UTR_{CHM} was replaced with the BJ2011C 3' UTR via BstBI and AclI restriction sites. A similar strategy was used to generate the chimeric infectious cDNA clones of CHM2013, including pCHM2013-S_{BJ}, pCHM2013-SP_{BJ}, and pCHM2013-SP + 3'UTR_{BJ}. The unique restriction enzymes used included AclI, ApaI, BstBI, and XhoI. All the intermediate and final plasmids were verified by DNA sequencing. The primers used for construction of the chimeric clones are listed in Table 2.

Recovery of chimeric viruses. The recombinant BAC plasmids were prepared using the E.Z.N.A. Endo-Free Plasmid Maxi kit (Omega Bio-Tek; catalogue number D6926-03) and transfected into Vero cells with Attractene transfection reagent (Qiagen, Germany; catalogue number 301005) following the manufacturer's protocol. Briefly, 2 µg of plasmids was diluted in Opti-MEM (ThermoFisher) without serum, proteins, or antibiotics to a total volume of 100 µl, followed by addition of 2 µl Attractene

transfection reagent. The DNA-transfection reagent solution was mixed well by gently tapping the tube. The transfection complexes were incubated at room temperature for 15 min to allow the complexes to form and then were added dropwise onto the cells. At 24 h posttransfection, the cells were washed twice with DMEM and then supplemented with serum-free DMEM containing 10 μ g/ml trypsin for 4 to 6 days, during which the cytopathic effect (CPE) was monitored daily.

Immunofluorescence assay. The immunofluorescence assay method has been described previously (61). Vero cells grown on coverslips were infected with PEDV or mock infected. At 24 h postinfection, the cells were fixed with 3.7% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS-2% bovine serum albumin (BSA). After being blocked in PBS-2% BSA for 30 min, the cells were incubated with mouse monoclonal antibody 4E3 to PEDV N protein (53) at a dilution of 1:1,000 at 37°C for 1 h. The coverslips with the cells were washed with PBS 3 times for 5 min each time, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG Fab' fragment (ThermoFisher) at a dilution of 1 to 2,000 for 1 h at room temperature in a humid chamber. Nuclear DNA was stained with DAPI (4',6'-diamidino-2-phenylindole) (Molecular Probes) for 10 min. Images were collected with a Nikon A1 confocal microscope.

Multistep growth analysis. To analyze the growth kinetics of the rescued virus, Vero cells in 24-well plates were infected with the indicated viruses at an MOI of 0.01. After adsorption for 2 h, the unbound viruses on the cell surfaces were inactivated by a brief acid wash. The acid was removed by washing the cells three times with DMEM, and then the cells were supplemented with DMEM containing 10 μ g/ml trypsin at 37°C with 5% CO₂. The cell supernatants and the infected cells were collected at the indicated times postinfection. Virus titers were determined by endpoint dilution assay and expressed as TCID₅₀ per milliliter, according to the Reed-Muench method (62).

Animal experiments. Forty-three 2-day-old piglets were randomly divided into 9 groups with 5 pigs in each group, except for the mock-infected group containing only 3 piglets. The piglets were fed with fresh liquid milk every 4 h. Before challenge, the piglets were confirmed negative for PEDV, transmissible gastroenteritis virus (TGEV), porcine delta coronavirus (PDCoV), and rotavirus (RV) by PCR. The original weights of the piglets were measured and recorded. For the challenge groups, the piglets were orally inoculated with 5 ml of parental and recombinant PEDV at a dose of 1×10^5 TCID₅₀/ml. The control group was mock infected with 5 ml DMEM. After challenge, rectal swab samples were also collected on a daily basis to monitor virus shedding in feces by regular RT-PCR targeting the conserved N gene of PEDV. The primers used for the assay have been reported previously (53).

The animals were monitored every 4 h for clinical signs of disease and weight gain. At 7 days postinfection, the surviving animals were euthanized by injecting pentobarbital sodium at a dose of 150 mg/kg of body weight via the ear intravenous route. The euthanasia was carried out in a soundproof room to avoid panic in the surviving pigs. At necropsy, different sections of intestinal tissues were collected for histopathological examination and stained with H&E for quantitation of virus abundance by TaqMan real-time RT-PCR (53).

Ethics statement. The animal experiments were performed according to the Chinese Regulations of Laboratory Animals and the Guidelines for the Care of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China) and the Laboratory Animal Requirements of Environment and Housing Facilities (GB 14925-2010; National Laboratory Animal Standardization Technical Committee). The license number associated with this research protocol, which was approved by the Laboratory Animal Ethical Committee of China Agricultural University, is CAU20170921-2.

Statistical analysis. Statistical analyses were performed using a one-way or two-way analysis of variance (ANOVA) test in GraphPad Prism 5. *P* values of <0.05 were considered statistically significant; *P* values of <0.001 were considered extremely significant.

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REFERENCES

1. Sueyoshi M, Tsuda T, Yamazaki K, Yoshida K, Nakazawa M, Sato K, Minami T, Iwashita K, Watanabe M, Suzuki Y, Mori M. 1995. An immunohistochemical investigation of porcine epidemic diarrhoea. *J Comp Pathol* 113:59–67. [https://doi.org/10.1016/S0021-9975\(05\)80069-6](https://doi.org/10.1016/S0021-9975(05)80069-6).
2. Song D, Park B. 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus Genes* 44:167–175. <https://doi.org/10.1007/s11262-012-0713-1>.
3. Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D, Cooper VL, Pillatzki A, Gauger P, Schmitt BJ, Koster LG, Killian ML, Yoon KJ. 2013. Emergence of porcine epidemic diarrhoea virus in the United States: clinical signs, lesions, and viral genomic sequences. *J Vet Diagn Invest* 25:649–654. <https://doi.org/10.1177/1040638713501675>.
4. Shibata I, Tsuda T, Mori M, Ono M, Sueyoshi M, Uruno K. 2000. Isolation of porcine epidemic diarrhoea virus in porcine cell cultures and experimental infection of pigs of different ages. *Vet Microbiol* 72:173–182. [https://doi.org/10.1016/S0378-1135\(99\)00199-6](https://doi.org/10.1016/S0378-1135(99)00199-6).
5. Sun RQ, Cai RJ, Chen YQ, Liang PS, Chen DK, Song CX. 2012. Outbreak of porcine epidemic diarrhoea in suckling piglets, China. *Emerg Infect Dis* 18:161–163. <https://doi.org/10.3201/eid1801.111259>.
6. Pensaert MB, de Bouck P. 1978. A new coronavirus-like particle associated with diarrhoea in swine. *Arch Virol* 58:243–247. <https://doi.org/10.1007/BF01317606>.
7. Choudhury B, Dastjerdi A, Doyle N, Frossard JP, Steinbach F. 2016. From the field to the lab; an European view on the global spread of

- PEDV. *Virus Res* 226:40–49. <https://doi.org/10.1016/j.virusres.2016.09.003>.
8. Takahashi K, Okada K, Ohshima K. 1983. An outbreak of swine diarrhea of a new type associated with coronavirus-like particles in Japan. *Nihon Juigaku Zasshi* 45:829–832. <https://doi.org/10.1292/jvms1939.45.829>.
 9. Kusanagi K, Kuwahara H, Katoh T, Nunoya T, Ishikawa Y, Samejima T, Tajima M. 1992. Isolation and serial propagation of porcine epidemic diarrhea virus in cell cultures and partial characterization of the isolate. *J Vet Med Sci* 54:313–318. <https://doi.org/10.1292/jvms.54.313>.
 10. Wang D, Fang L, Xiao S. 2016. Porcine epidemic diarrhea in China. *Virus Res* 226:7–13. <https://doi.org/10.1016/j.virusres.2016.05.026>.
 11. Kwon CH, Kwon BJ, Jung TS, Kee YJ, Hur DH, Hwang EK, Rhee JC, An SH. 1993. Isolation of porcine epidemic diarrhea virus (PEDV) in Korea. *Korean J Vet Res* 33:249–254.
 12. Duy DT, Toan NT, Puranaveja S, Thanawongnuwech R. 2011. Genetic characterization of porcine epidemic diarrhea virus (PEDV) isolates from southern Vietnam during 2009–2010 outbreaks. *Israel J Vet Med* 41:9.
 13. Paraguison-Alili R, Domingo CY. 2016. Phylogenetic tracking of current porcine epidemic diarrhea virus (PEDV) strains in the Philippines. *Arch Virol* 161:2601–2604. <https://doi.org/10.1007/s00705-016-2938-0>.
 14. Sun M, Ma J, Wang Y, Wang M, Song W, Zhang W, Lu C, Yao H. 2015. Genomic and epidemiological characteristics provide new insights into the phylogeographical and spatiotemporal spread of porcine epidemic diarrhea virus in Asia. *J Clin Microbiol* 53:1484–1492. <https://doi.org/10.1128/JCM.02898-14>.
 15. Chae C, Kim O, Choi C, Min K, Cho WS, Kim J, Tai JH. 2000. Prevalence of porcine epidemic diarrhoea virus and transmissible gastroenteritis virus infection in Korean pigs. *Vet Rec* 147:606–608. <https://doi.org/10.1136/vr.147.21.606>.
 16. 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. <https://doi.org/10.3201/eid1808.120002>.
 17. Wang XM, Niu BB, Yan H, Gao DS, Yang X, Chen L, Chang HT, Zhao J, Wang CQ. 2013. Genetic properties of endemic Chinese porcine epidemic diarrhea virus strains isolated since 2010. *Arch Virol* 158:2487–2494. <https://doi.org/10.1007/s00705-013-1767-7>.
 18. Huang YW, Dickerman AW, Pineyro P, Li L, Fang L, Kiehne R, Opriessnig T, Meng XJ. 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. *mBio* 4:e00737–13. <https://doi.org/10.1128/mBio.00737-13>.
 19. Jung K, Saif LJ. 2015. Porcine epidemic diarrhea virus infection: etiology, epidemiology, pathogenesis and immunoprophylaxis. *Vet J* 204:134–143. <https://doi.org/10.1016/j.tvjl.2015.02.017>.
 20. Lee C. 2015. Porcine epidemic diarrhea virus: an emerging and re-emerging epizootic swine virus. *Viol J* 12:193. <https://doi.org/10.1186/s12985-015-0421-2>.
 21. Mole B. 2013. Deadly pig virus slips through US borders. *Nature* 499:388. <https://doi.org/10.1038/499388a>.
 22. Cima G. 2013. Fighting a deadly pig disease. Industry, veterinarians trying to contain PED virus, new to the US. *J Am Vet Med Assoc* 243:469–470.
 23. Vlasova AN, Marthaler D, Wang Q, Culhane MR, Rossow KD, Rovira A, Collins J, Saif LJ. 2014. Distinct characteristics and complex evolution of PEDV strains, North America, May 2013–February 2014. *Emerg Infect Dis* 20:1620–1628. <https://doi.org/10.3201/eid2010.140491>.
 24. 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. <https://doi.org/10.7774/cevr.2015.4.2.166>.
 25. Brian DA, Baric RS. 2005. Coronavirus genome structure and replication. *Curr Top Microbiol Immunol* 287:1–30.
 26. Lai MM, Cavanagh D. 1997. The molecular biology of coronaviruses. *Adv Virus Res* 48:1–100.
 27. Kocherhans R, Bridgen A, Ackermann M, Tobler K. 2001. Completion of the porcine epidemic diarrhoea coronavirus (PEDV) genome sequence. *Virus Genes* 23:137–144. <https://doi.org/10.1023/A:1011831902219>.
 28. Duarte M, Tobler K, Bridgen A, Rasschaert D, Ackermann M, Laude H. 1994. Sequence analysis of the porcine epidemic diarrhea virus genome between the nucleocapsid and spike protein genes reveals a polymorphic ORF. *Virology* 198:466–476. <https://doi.org/10.1006/viro.1994.1058>.
 29. de Haan CA, Haijema BJ, Schellen P, Wichgers Schreur P, te Lintelo E, Vennema H, Rottier PJ. 2008. Cleavage of group 1 coronavirus spike proteins: how furin cleavage is traded off against heparan sulfate binding upon cell culture adaptation. *J Virol* 82:6078–6083. <https://doi.org/10.1128/JVI.00074-08>.
 30. Shirato K, Matsuyama S, Ujiike M, Taguchi F. 2011. Role of proteases in the release of porcine epidemic diarrhea virus from infected cells. *J Virol* 85:7872–7880. <https://doi.org/10.1128/JVI.00464-11>.
 31. Wicht O, Li W, Willems L, Meuleman TJ, Wubolts RW, van Kuppeveld FJ, Rottier PJ, Bosch BJ. 2014. Proteolytic activation of the porcine epidemic diarrhea coronavirus spike fusion protein by trypsin in cell culture. *J Virol* 88:7952–7961. <https://doi.org/10.1128/JVI.00297-14>.
 32. Liu C, Ma Y, Yang Y, Zheng Y, Shang J, Zhou Y, Jiang S, Du L, Li J, Li F. 2016. Cell entry of porcine epidemic diarrhea coronavirus is activated by lysosomal proteases. *J Biol Chem* 291:24779–24786. <https://doi.org/10.1074/jbc.M116.740746>.
 33. Liu C, Tang J, Ma Y, Liang X, Yang Y, Peng G, Qi Q, Jiang S, Li J, Du L, Li F. 2015. Receptor usage and cell entry of porcine epidemic diarrhea coronavirus. *J Virol* 89:6121–6125. <https://doi.org/10.1128/JVI.00430-15>.
 34. Shirato K, Maejima M, Islam MT, Miyazaki A, Kawase M, Matsuyama S, Taguchi F. 2016. Porcine aminopeptidase N is not a cellular receptor of porcine epidemic diarrhea virus, but promotes its infectivity via aminopeptidase activity. *J Gen Virol* 97:2528–2539. <https://doi.org/10.1099/jgv.0.000563>.
 35. Liu C, Luo R, He Q, van Kuppeveld FJM, Rottier PJM, Bosch BJ. 2017. Aminopeptidase N is not required for porcine epidemic diarrhea virus cell entry. *Virus Res* 235:6–13. <https://doi.org/10.1016/j.virusres.2017.03.018>.
 36. Bosch BJ, van der Zee R, de Haan CA, Rottier PJ. 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J Virol* 77:8801–8811. <https://doi.org/10.1128/JVI.77.16.8801-8811.2003>.
 37. Chang SH, Bae JL, Kang TJ, Kim J, Chung GH, Lim CW, Laude H, Yang MS, Jang YS. 2002. Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. *Mol Cells* 14:295–299.
 38. Li C, Li W, Lucio de Esarte E, Guo H, van den Elzen P, Aarts E, van den Born E, Rottier PJM, Bosch BJ. 2017. Cell attachment domains of the porcine epidemic diarrhea virus spike protein are key targets of neutralizing antibodies. *J Virol* 91:e00273-17. <https://doi.org/10.1128/JVI.00273-17>.
 39. Zhao P, Wang B, Ji CM, Cong X, Wang M, Huang YW. 2018. Identification of a peptide derived from the heptad repeat 2 region of the porcine epidemic diarrhea virus (PEDV) spike glycoprotein that is capable of suppressing PEDV entry and inducing neutralizing antibodies. *Antiviral Res* 150:1–8. <https://doi.org/10.1016/j.antiviral.2017.11.021>.
 40. Okda FA, Lawson S, Singrey A, Nelson J, Hain KS, Joshi LR, Christopher-Hennings J, Nelson EA, Diel DG. 2017. The S2 glycoprotein subunit of porcine epidemic diarrhea virus contains immunodominant neutralizing epitopes. *Virology* 509:185–194. <https://doi.org/10.1016/j.virol.2017.06.013>.
 41. Gao Y, Kou Q, Ge X, Zhou L, Guo X, Yang H. 2013. Phylogenetic analysis of porcine epidemic diarrhea virus field strains prevailing recently in China. *Arch Virol* 158:711–715. <https://doi.org/10.1007/s00705-012-1541-2>.
 42. Chen J, Liu X, Shi D, Shi H, Zhang X, Li C, Chi Y, Feng L. 2013. Detection and molecular diversity of spike gene of porcine epidemic diarrhea virus in China. *Viruses* 5:2601–2613. <https://doi.org/10.3390/v5102601>.
 43. Lin CM, Saif LJ, Marthaler D, Wang Q. 2016. Evolution, antigenicity and pathogenicity of global porcine epidemic diarrhea virus strains. *Virus Res* 226:20–39. <https://doi.org/10.1016/j.virusres.2016.05.023>.
 44. Wang X, Chen J, Shi D, Shi H, Zhang X, Yuan J, Jiang S, Feng L. 2016. Immunogenicity and antigenic relationships among spike proteins of porcine epidemic diarrhea virus subtypes G1 and G2. *Arch Virol* 161:537–547. <https://doi.org/10.1007/s00705-015-2694-6>.
 45. Annamalai T, Lin CM, Gao X, Liu X, Lu Z, Saif LJ, Wang Q. 2017. Cross protective immune responses in nursing piglets infected with a US spike-insertion deletion porcine epidemic diarrhea virus strain and challenged with an original US PEDV strain. *Vet Res* 48:61. <https://doi.org/10.1186/s13567-017-0469-7>.
 46. Sato T, Oroku K, Ohshima Y, Furuya Y, Sasakawa C. 2018. Efficacy of genogroup 1 based porcine epidemic diarrhea live vaccine against genogroup 2 field strain in Japan. *Virol J* 15:28. <https://doi.org/10.1186/s12985-018-0940-8>.
 47. Teeravechyan S, Frantz PN, Wongthida P, Chailangkarn T, Jarumponpan 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. <https://doi.org/10.1016/j.virusres.2016.05.003>.
 48. Fan B, Yu Z, Pang F, Xu X, Zhang B, Guo R, He K, Li B. 2017. Character-

- ization of a pathogenic full-length cDNA clone of a virulent porcine epidemic diarrhea virus strain AH2012/12 in China. *Virology* 500:50–61. <https://doi.org/10.1016/j.virol.2016.10.011>.
49. Jengarn J, Wongthida P, Wanasen N, Frantz PN, 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. <https://doi.org/10.1099/vir.0.000184>.
 50. Beall A, Yount B, Lin CM, 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. <https://doi.org/10.1128/mBio.01451-15>.
 51. Li C, Li Z, Zou Y, Wicht O, van Kuppeveld FJ, Rottier PJ, Bosch BJ. 2013. Manipulation of the porcine epidemic diarrhea virus genome using targeted RNA recombination. *PLoS One* 8:e69997. <https://doi.org/10.1371/journal.pone.0069997>.
 52. Hou Y, Lin CM, Yokoyama M, Yount BL, Marthaler D, Douglas AL, Ghimire S, Qin Y, Baric RS, Saif LJ, 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. <https://doi.org/10.1128/JVI.00227-17>.
 53. 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. <https://doi.org/10.1371/journal.pone.0173998>.
 54. Fu F, Li L, Shan L, Yang B, Shi H, Zhang J, Wang H, Feng L, Liu P. 2017. A spike-specific whole-porcine antibody isolated from a porcine B cell that neutralizes both genogroup 1 and 2 PEDV strains. *Vet Microbiol* 205:99–105. <https://doi.org/10.1016/j.vetmic.2017.05.013>.
 55. Zhang Y, Yao Y, Gao X, Wang Y, Jia X, Xiao Y, Wang T, Li X, Tian K. 2016. Development of a neutralizing monoclonal antibody against porcine epidemic diarrhea virus S1 protein. *Monoclon Antib Immunodiagn Immunother* 35:37–40. <https://doi.org/10.1089/mab.2015.0049>.
 56. Liu J, Shi H, Chen J, Zhang X, Ji Z, Yuan J, Shi D, Cao L, Zhu X, Dong H, Wang X, Zhang J, Feng L. 2017. Neutralization of genotype 2 porcine epidemic diarrhea virus strains by a novel monoclonal antibody. *Virology* 507:257–262. <https://doi.org/10.1016/j.virol.2017.04.026>.
 57. Oka T, Saif LJ, Marthaler D, Esseili MA, Meulia T, Lin CM, Vlasova AN, Jung K, Zhang Y, Wang Q. 2014. Cell culture isolation and sequence analysis of genetically diverse US porcine epidemic diarrhea virus strains including a novel strain with a large deletion in the spike gene. *Vet Microbiol* 173:258–269. <https://doi.org/10.1016/j.vetmic.2014.08.012>.
 58. Masuda T, Murakami S, Takahashi O, Miyazaki A, Ohashi S, Yamasato H, Suzuki T. 2015. New porcine epidemic diarrhoea virus variant with a large deletion in the spike gene identified in domestic pigs. *Arch Virol* 160:2565–2568. <https://doi.org/10.1007/s00705-015-2522-z>.
 59. Thomas JT, Chen Q, Gauger PC, Giménez-Lirola LG, Sinha A, Harmon KM, Madson DM, Burrough ER, Magstadt DR, Salzbrenner HM, Welch MW, Yoon KJ, Zimmerman JJ, Zhang J. 2015. Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naïve conventional neonatal and weaned pigs. *PLoS One* 10:e0139266. <https://doi.org/10.1371/journal.pone.0139266>.
 60. Chen Q, Gauger PC, Stafne MR, Thomas JT, Madson DM, Huang H, Zheng Y, Li G, Zhang J. 2016. Pathogenesis comparison between the United States porcine epidemic diarrhoea virus prototype and S-indel-variant strains in conventional neonatal piglets. *J Gen Virol* 97:1107–1121. <https://doi.org/10.1099/jgv.0.000419>.
 61. Du J, Ge X, Liu Y, Jiang P, Wang Z, Zhang R, Zhou L, Guo X, Han J, Yang H. 2016. Targeting swine leukocyte antigen class I molecules for proteasomal degradation by the nsp1alpha replicase protein of the chinese highly pathogenic porcine reproductive and respiratory syndrome virus strain JXwn06. *J Virol* 90:682–693. <https://doi.org/10.1128/JVI.02307-15>.
 62. Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* 27:493–497. <https://doi.org/10.1093/oxfordjournals.aje.a118408>.