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RESEARCH ARTICLE

Rapid Detection of Variant and Classical Porcine Epidemic Diarrhea Virus by Nano-Nest PCR

Kai Wang[1], Junhui Zhu[1], Hao Dong[1], Zhihua Pei[1], Tiezhong Zhou[§2] and Guixue Hu[§2]*

[1]College of Animal Science and Technology, Jilin Agricultural University, Xincheng Street No. 2888, Changchun 130118, P. R. China; [2]College of Animal Husbandry & Veterinary, Jinzhou Medical University, Renmin Street, No. 48, Section 5, Jinzhou 121001, P. R. China
*Corresponding author: guixue1964@126.com

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ABSTRACT

The aim of the study was to develop an assay which can distinguish between classical and variant strains of porcine epidemic diarrhea virus (PEDV), and understand the recent epidemiology knowledge of porcine epidemic diarrhea (PED) in China. Two pairs of primers based on the S gene differentiated between variant and classical PEDV strains were redesigned. A nest PCR associated with a novel method of nanoparticle constructed nano-nest PCR was developed. Seventy-eight cases collected in 9 different areas in China from 2015 to 2016 were tested using the nano-nest PCR assay. The results indicated that the lowest detection limit of the nano-nest PCR assay was 2.21×10^{-7} ng/μL which was 100-fold more sensitive than common RT-PCR. This assay was highly specific to PEDV, which did not amplify DNA or cDNA of pseudorabies virus, porcine reproductive and respiratory syndrome virus, classical swine fever virus, porcine rotavirus, porcine transmissible gastroenteritis virus. The average positive rate was 74.36% (58/78) for the PED, and the positive rate of the variant strains of PEDV was 79.31% (46/58). Our results demonstrated that the nano-nest PCR allows for accurate and sensitive detection of variant and classical PEDV infection. PEDV infection was mainly the variant strains of PEDV in China.

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INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is a member of the genus *Alphacoronavirus* and family *Coronaviridae*. PEDV can cause severe diarrhea and vomiting, thereby inducing dehydration and high mortality in pigs (Duarte *et al*., 1993). In 1970, PEDV was identified in England, and since then, it has spread throughout Europe and Asia (Song and Park, 2012). Recently, there was a PEDV outbreak in China, which resulted from the deletion of the PEDV spike protein. Consequently, this led to many failed immunizations and caused a significant loss of piglets in China.

PEDV has an envelope, positive-sense single stranded RNA genome, which is approximately 28 kb long. The virus contains seven nonstructural and structural proteins. The four structural proteins are 150-220 kDa spike (S), 7 kDa envelope (E), 20-30 kDa membrane (M) and 58 kDa nucleocapsid (N), and the three nonstructural proteins are replicates 1a, 1b and 3b (Kocherhans *et al*., 2001). Since 2010, PEDV has re-emerged with mutations: a 15 bp insertion and 6 bp deletion within the S gene. These mutations are within the neutralizing epitope region and could affect the host's immune response (Sun *et al*., 2014). As a major functional protein, the S protein is associated with virulence, antigenicity and tissue tropism. The novel S gene of PEDV has hampered protection of the vaccine strain, CV777. However, the classical and variant strains both existed in China, which may account for the increase in porcine illnesses in the Chinese piglet industry.

Some methods have been developed to detect PEDV, including ELISA, RT-PCR, RT-LAMP and real time PCR (Kweon *et al*., 1997; Jung and Chae, 2005; Ren and Li, 2011; Zhao *et al*., 2014; Li *et al*., 2015; Yu *et al*., 2015). However, these methods have some disadvantages, which accounts for their limited practical usage in detecting PEDV. Nanoparticles have been created as a new bio-nanotechnology and have been applied in PCR diagnostics and gene therapy. Nanoparticles have improved the

[§]These authors contributed equally

sensitivity of PCR but could also alter nucleic molecular structure and influence biochemical activities (An and Jin, 2012). Nanoparticles and nested PCR are superior to common PCR, especially in terms of sensitivity, accuracy and efficiency. Li *et al.* (2005) reported that the addition of gold nanoparticles (AuNPs) in PCR reactions could improve specific amplification.

MATERIALS AND METHODS

Virus and samples collection: PEDV classical (CV777, GenBank: AF353511.1) and variant strains, porcine transmissible gastroenteritis virus (TGEV), porcine rotavirus (PoRV), classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV) and pseudorabies virus (PRV) were stored at Jilin Agricultural University in China. The Vero cells were cultured with Minimum Essential Medium (Gibco, USA) supplemented with 8% fetal calf serum (Hyclone, USA) and antibiotics (100 units/ml of penicillin and 100 μg/mL of streptomycin). Porcine intestinal specimens were collected from 9 different areas in China between 2015 and 2016. The samples were homogenized with 10% PBS and centrifuged for 8 min at 10000×g, and the suspensions were stored at -80°C.

Preparation of template: Total RNAs of the classical and variant virus strains were extracted with Simply P Total RNA kits (BioFlux, Hangzhou, China) and was subsequently reverse transcribed using the TranScript First-Strand cDNA Synthesis SuperMix kits (TranScript Biotech company, Beijing, China) according to the manufacturer's instructions. For virus DNA were extracted from with a Viral DNA Kit (OMEGA, USA) in accordance with manufacturer's instructions and stored at -20°C.

Nano-nest PCR: The outer (P1-S-F, P1-S-R) and inner primers (P2-S-F, P2-S-R) for the nano-nest PCR were designed based upon classical and variant PEDV strains (Table 1). The nano-nest PCR assay was constructed with a nanoPCR kit (GREDBIO, Weihai, China) and a mixture containing 0.4μL cDNA, 12.5μL nanoPCR Mixture, 0.4μL nanoTaq DNA polymerase, 1.5μL of each outer prime (a 20 μM concentration of each) and nuclease-free water up to 25μL. Thermocycling conditions of the first round PCR consisted of a 5min hold at 95°C for denaturation, 35 cycles of 40s at 94°C, 1 min at 57°C and 1min at 72°C,with a final extension cycle 10min at 72°C. The second round PCR were similar to the first, except the template was 3μL of a 50-fold dilution of the first PCR products. The second nano-nest PCR thermocycling conditions consisted of 30 cycles of 40s at 94°C, 40s at 58°C and 40s at 72°C, with a final hold of 10 min at 72°C. The amplicons were electrophoresed on a 2% agarose gel.

Analytic specificity and sensitivity of the nano-nest PCR assay: The specificity of nano-nest PCR was evaluated using PoRV, TGEV, CSFV, PRV and PRRSV. The PCR products were analyzed by electrophoresis on a 1% agarose gel. To compare the sensitivity of nano-nest PCR and conventional RT-PCR, the total RNA concentration of the PEDV was quantified using spectrophotometry (Thermo, NanoDrop 2000, USA) and diluted 10-fold with RNAse-free ddH_2O from 22.1 ng/μL to 2.21×10^{-10} ng/μL.

Detection of PEDV in clinical samples: Seventy-eight porcine intestinal samples were detected by nano-nest PCR and conventional RT-PCR assays, then PEDV-positive samples were categorized as classical or variant strains PEDV. For the positive samples, the first nano-nest PCR products were sequenced and a second confirmatory nano-nest PCR test. Next, the sequence data were entered into a BLAST search of the GenBank, which determined sequence homology and confirmed accuracy of the nano-nest PCR. 6 variant strains and 3 classical strains of PEDV from 9 different areas in China were chosen randomly for phylogenetic analysis.

Table 1 Sequences of primers used in this study

Primer name	Sequence (5′-3′)	Product size (bp)
P1-S-F	TTTAGGCGGTTCTTTTCA	817
P1-S-R	TTACAAACRCCATCSATC	
P2-S-F	CAGTTTCCHAGCATYAAA	295
P2-S-R	TACCATCCTCACCAGCAC	

The outer primer determined the location of the relatively conserved region and the inner primer was designed by incorporating the classic and variant strains' mutated region. P1-S-F, P1-S-R: the outer primers of nano-nest PCR and primers of conventional PCR, P2-S-F, P2-S-R: inner primer of nano-nest PCR. F: forward primer; R: reverse primer.

RESULTS

Nano-nest PCR: Based on the classical and variant PEDV strains, the same sequence designed a fragment of 817bp outer primers and the different region set inner primers of 295bp. When the outer primers were simultaneously amplified, the classical and variant strains of PEDV displayed conserved regions. However, the inner primer only identified the variant strain and did not amplify the classical strain (Fig. 1). Thus, the nano-nest PCR assay was able to distinguish between the classical and variant strains of PEDV.

Specificity and sensitivity of the nano-nest PCR assay: The specificity of the nano-nest PCR was evaluated using TGEV, PoRV, CSFV, PRRSV, PRV. The cDNA or DNA of the five viruses demonstrated non-specific amplification, as only the PEDV cDNA was detected. Furthermore, the assay could distinguish between classical and variant strains of PEDV. These results suggest that the nano-nest PCR assay is specific (Fig. 2).

The sensitivity of the nano-nest PCR measured with 10-fold, serially diluted PEDV RNA. The nano-nest PCR products were compared with the same diluted RNA template constructed from the common RT-PCR (Fig. 3). The detection range of the nano-nest PCR was 2.21×10^{-7} ng/μL to 2.21×10^{-5} ng/μL for the common RT-PCR. The results indicated that the sensitivity of the nano-nest PCR was 100-fold higher than the common RT-PCR.

Application of the nano-nest PCR to clinical samples: Seventy-eight clinical samples were detected via nano-nest PCR and RT-PCR. All of the clinical samples, 74.36% (58/78) and 56.41% (44/78) were detected as PEDV-positive by nano-nest PCR and common RT-PCR,

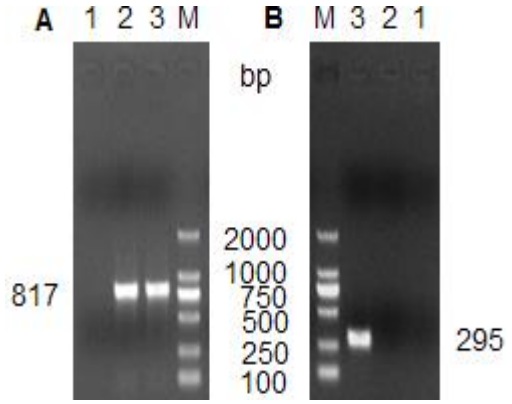

Fig. 1: Size of the first products (A) and second products (B) of the differential diagnosis technology of the PEDV nano-nest PCR was distinguished the classical strains and variant strains of PEDV by gel electrophoresis. M: DL2000; 1: negative control; 2; classical strains of PEDV (CV777); 3: variant strains of PEDV.

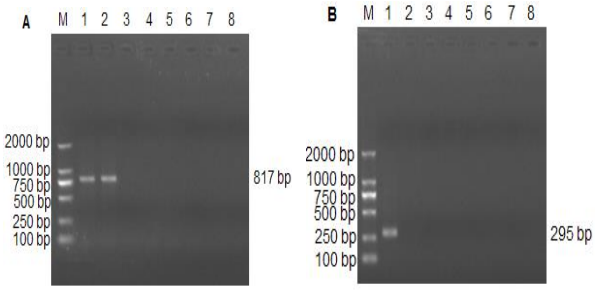

Fig. 2: The specificity of the assay. Specificity of the first products (A) and second products (B) were analyzed by established using nano-nest PCR. The specificity of the nano-nest PCR assay was tested by other porcine viruses. M: DL2000; 1: variant strains of PEDV; 2: classical strains of PEDV (CV777); 3: TGEV; 4: PoRV; 5: CSFV;6: PRRSV; 7: PRV; 8: negative control.

Table 2: PEDV popular trends between 2015 and 2016 detected by nano-nest PCR

Assay	No. of clinical samples	No. of positive samples	Positive (%)	Variant (%) (V/P)	Classical (%) (C/P)
conventional PCR	78	44	56.41	-	-
nano-nest PCR	78	58	74.36	79.31 (46/58)	20.69 (12/58)

V: No. of variant strains; C: No. of classical strains; P: No. of PEDV positive samples

respectively. All of the PEDV-positive samples, the positive rate of the PEDV variant strains was respectively 79.31% (46/58) by nano-nest PCR (Table 2). The first PCR products were assessed with automated sequencing reactions and the sequence alignment indicated that the results corresponded to the results obtained from the second nano-nest PCR testing. These results indicate that nano-nest PCR can discriminate between classical and variant strains of PEDV. Additionally, the sensitivity of the nano-nest PCR was higher than the common RT-PCR. 6 PEDV variant strains（CH-GD-2015, CH-FJ-2016, CH-ZJ-2015, CH-HB-2015, CH-HN-2016, CH-JL-2015) and 3 classical strains of PEDV （CH-SD-2016, CH-LN-2015 and CH-HLJ-2016）from 9 different areas in China were chosen randomly for sequence analysis by the Biological software DNAMAN 6.0 (Lynnon biosoft, America). The results showed that when the base sequence TTG, GGGTGTC(T)AA and AAT were inserted separately in the position 163bp-164bp, 175bp-176bp and 416bp-417bp, and TGGAAA was deleted in the position 478bp-479bp for the classical strains of PEDV, the classical strains of PEDV was changed to the variant strains of PEDV (Fig.4). Phylogenetic analysis based on the high variable region of S gene indicated that all of the isolates were grouped into two large branches; 6 variant strains of PEDV formed a branch with variant strains HB-2012-1, 3 classical strains formed another branch together with classical strains CV777 (Fig. 5).

DISCUSSION

Several assays have been developed as diagnostic tools for detecting PEDV, which include common RT-PCR, real-time PCR and RT-LAMP assays (Jung and Chae, 2005; Zhao *et al*., 2014; Yu *et al.*, 2015). Common RT-PCR is less sensitive, and real-time PCR requires complex instrumentation. Furthermore, RT-LAMP assays need strictly designed primers and can be easily contaminated. Nanotechnology offers the opportunity to improve PCR efficiency. Nano-nest PCR can be extensively applied in clinical diagnosis due to its simplicity, rapidity and sensitivity. The nanoparticles make nano-nest PCR a revolutionary disease diagnosis technology. Development of nanoPCR methods have been used in the determination of numerous porcine viruses including pseudorabies virus (PRV) (Ma *et al*., 2013), porcine bocavirus (PBoVs) (Wang *et al*., 2014), and PEDV (Yuan *et al*., 2015).

Nanofluids increase thermal conductivity and create temperature stability, which is important for quickly reaching target temperatures in PCR assays, therefore gold nanoparticles (AuNPs) PCR has extensively been used as a microbial detection tool. The nano-nest PCR provides a slight increase in sensitivity, is more cost effective method than real-time PCR and but can be easily applied in clinical practice. In addition, nano-nest PCR was able to differentiate between the classical and variant strains of the PEDV, which provided a more precise treatment plan. Because this assay can accurately distinguish between the two strains of PEDV, they can reduce unnecessary investments in vaccine development and increase protection against a PEDV infection. In this study, the sensitivity of the nano-nest PCR assay was 100-fold higher than common RT-PCR. It could detect a concentration of 2.21×10^{-7} ng/μL of PEDV RNA. Besides that, TGEV, PoRV, CSFV, PRRSV, PRV were not detected by the nano-nest PCR, it proved that the assay in this study had good specificity.

PEDV is responsible for economic losses in the pig industry. Since 2010, PEDV variant strains have been reported in most provinces of China (Sun *et al*., 2014). Because various gene mutations occur that produce variant strains of PEDV, new types of PEDV outbreaks have emerged in Asia including Japan and South Korea (Lin *et al*., 2014; Lee, 2015; Suzuki *et al*., 2015). It has been well-documented that since 2013, PEDV has spread through 31 states in the US, Mexico and Canada (Oka *et al*., 2014; Ojkic *et al*., 2015). PEDV can be genetically separated into G1 (classical) and G2 (field epidemic or pandemic) groups by genetic and phylogenetic analysis (Lee, 2015). The outbreaks of classical PEDV in Europe

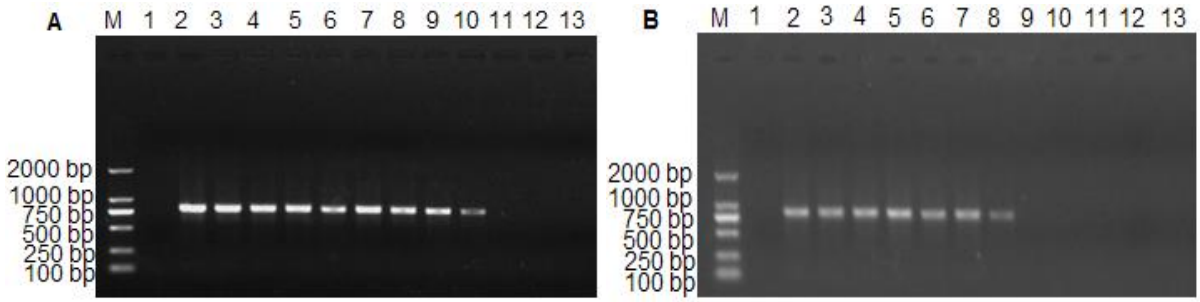

Fig. 3: The sensitivity of the assay. Sensitivity of the first round of nano-nest PCR (A) compared with as the same first round of conventional nest PCR assay (B) for detection of classical and variant strains of PEDV RNA in a dilution series. The first PCR products were analyzed by agarose gel electrophoresis. M: DL2000; 1: negative control; 2: 22.1 ng/μL; 3: 2.21 ng/μL; 4: 2.21×10^{-1} ng/μL; 5: 2.21×10^{-2} ng/μL; 6: 2.21×10^{-3} ng/μL; 7: 2.21×10^{-4} ng/μL; 8: 2.21×10^{-5} ng/μL; 9: 2.21×10^{-6} ng/μL; 10: 2.21×10^{-7} ng/μL; 11: 2.21×10^{-8} ng/μL; 12: 2.21×10^{-9} ng/μL; 13: 2.21×10^{-10} ng/μL.

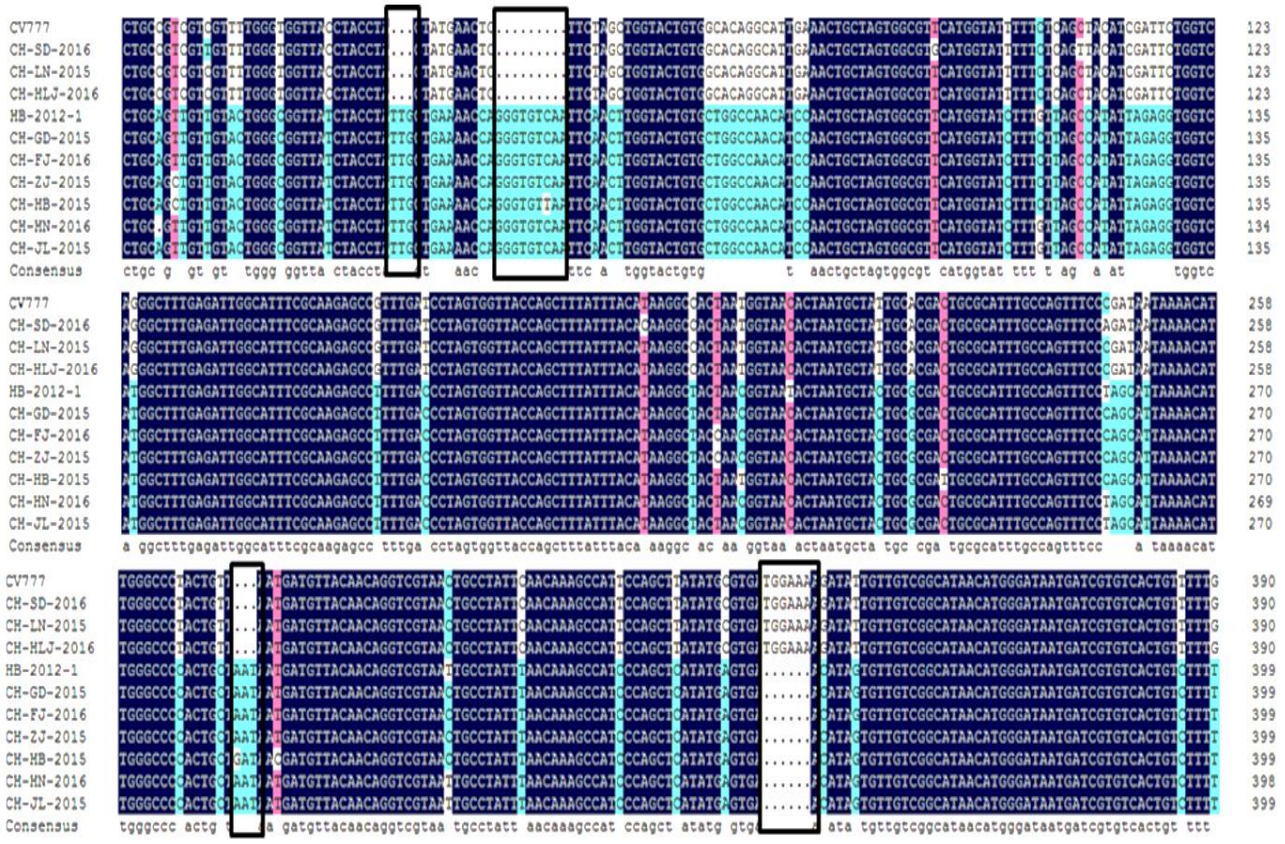

Fig. 4: Sequence analysis by the Biological software DNAMAN 6.0. The genes were amplified by external primers, after sequence analysised, the effective region was intercepted. TTG, GGGTGTC(T)AA and AAT were inserted separately in the position 163bp-164bp, 175bp-176bp and 416bp-417bp, or TGGAAA was deleted in the position 478bp-479bp, the classical and variant strains of PEDV can been differenced by the inserted 15 genes and the deleted 6 genes. The inserted or deleted genes were marked with the symbol "□"

indicate that classical PEDV has circulated worldwide in the swine industry and continues to persist (Hanker *et al*., 2015; Stadler *et al*., 2015). Here, seventy-eight cases from 9 different areas in China, from 2015 to 2016 were tested using the nano-nest PCR. The results showed that the average positive rate was 74.36% (58/78), and the positive rate was 79.31% (46/58) for the variant strains of PEDV. Common PCR assays were also used to evaluate the seventy-eight cases, and the positive rate of PEDV was 56.41% (44/78). The study proved that the sensitivity of the nano-nest PCR assay is higher than common RT-PCR for the detection of clinical samples. Our results demonstrated that PEDV infection was very widespread in China.

In summary, the nano-nest PCR allows for accurate and sensitive detection of variant and classical PEDV infection. PEDV infection was mainly the variant strains of PEDV in China.

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Authors contribution: KW and JHZ conceived of the study. HD participated in verification of data. ZHP helped draft the manuscript. TZZ and GXH participated in its design and coordination. All authors critically reviewed and edited the manuscript.

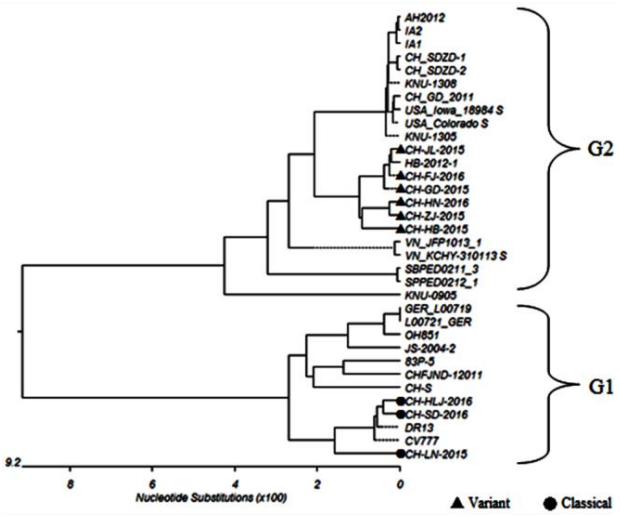

Fig. 5: The phylogenetic tree of nucleotide sequences in the high variable region of PEDV S gene. 6 variant strains of PEDV（CH-GD-2015, CH-FJ-2016, CH-ZJ-2015, CH-HB-2015, CH-HN-2016, CH-JL-2015) and 3 classical strains of PEDV （CH-SD-2016, CH-LN-2015 and CH-HLJ-2016）from 9 different areas in China were chosen randomly for sequence analysis by the Biological software DNAMAN 6.0. All of the isolates were grouped into two large branches (G1 and G2), variant strains of PEDV were labelled with a "▲", classical strains of PEDV were marked with the symbol "●".

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