Original Article

Detection and phylogenetic analysis of Porcine epidemic diarrhea virus based on the ORF3 gene in pigs in the north of Vietnam

Tran Duc Hoan[1*] Doan Thi Thao[1] Nguyen Thi Huong Giang[1] Nguyen Thi Thu Huyen[1]

Abstract

To identify further the infectivity, genetic diversity and molecular characteristics of the porcine epidemic diarrhea virus (PEDV). 286 samples of the intestinal tissue of pigs with severe diarrhea were assessed from 5 provinces in the north of Vietnam. The full-length ORF3 gene of 20 representative field strains from 36 farms in 5 provinces was sequenced and analyzed. The prevalence of PEDV was 90.56% and was detected in 259 of 286 samples, with 94.44% (34 of 36) of pig farms harboring the disease. Based on the phylogenetic analysis of the ORF3 genes, our isolates all fell into group G2 (variants) and showed a close relationship to isolates from Chinese (GH1, QH2, and SX1), Korean (DR13 and DR13 attenuated) sources, and these isolates differed genetically from other Chinese (CH and CV777), Korean (DBI865) and French (CV777) strains. Our isolates also differed from attenuated vaccine strains, CV777 (used in China) and DR13 (used in Korea). According to our derived amino acid sequence analysis, we detected one novel variant PEDV, viz: BF, with 6-nt insertion, 4-nt difference, 68-nt deletion and 17-aa deletion at position 63-79 compared to the CV777 attenuated strain. Our ORF3 gene analyses showed that the prevalent PEDV isolates were variants and the isolated strains differed genetically from the vaccine strains. These results demonstrated the existence of genetic diversity among geographically distinct PEDV strains and our study has contributed to conducting further researches into PEDV and on an efficacious vaccine design in the future.

Keywords: Detection, PEDV, ORF3 gene, Phylogenetic analysis, Variants

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Introduction

Porcine epidemic diarrhea (PED) is an acute, highly contagious disease of swine caused by the Porcine Epidemic Diarrhea virus (PEDV), which leads to severe vomiting and diarrhea along with dehydration and high mortality in new-born piglets (Pensaert and de Bouck, 1978). PEDV belongs to the family *Coronaviridae*, genus Alphacoronavirus and was first reported in England (Takahashi *et al.*, 1983). Since then a lot of research has been reported worldwide, including in Belgium, France, Japan, Korea, Italy, Thailand, the USA, Canada and Mexico (Cima, 2013; Martelli *et al.*, 2008; Pensaert and de Bouck, 1978; Puranaveja *et al.*, 2009; Takahashi *et al.*, 1983), that poses severe economic burdens in pig production. Since 2010 serious PED outbreaks have been detected in China and Vietnam (Dam *et al.*, 2014; Do *et al.*, 2011; Sun *et al.*, 2012). PEDV is an enveloped ssRNA coronavirus with a 28 kb genome, including seven open reading frames (ORFs), a 5′ untranslated region (UTR) and a 3′ UTR with a polyadenylated tail. The seven ORFs encode four structural proteins, spike (S), envelope (E), membrane (M) and nucleocapsid (N) and three non-structural proteins, replicases 1a, 1b and ORF3 (Bridgen *et al.*, 1998; Kocherhans *et al.*, 2001). The S protein contains a specific receptor binding site that plays an important role in cell membrane fusion and virus entry and is an antigenic target for neutralising antibodies (Duarte *et al.*, 1994). The M protein is the most abundant surface protein and co-expression with E protein to form pseudo-particles results in interfering genic activity (Baudoux *et al.*, 1998). The N protein is highly conserved and binds to virion RNA to provide a structural basis for the helical nucleocapsid and it is used for early diagnosis (Isabel *et al.*, 2005). For non-structural proteins, replicases 1a and 1b are multi-functional and associated with viral genome replication (Brian and Baric, 2005) and the accessory ORF3 protein is thought to influence virulence (Wang *et al.*, 2012).

The accessory ORF3 gene is highly relevant to the virulence of PEDV as previously reported (Wang *et al.*, 2012) and reduction in virulence is produced through cell culture adaptation (Park *et al.*, 2008; Park *et al.*, 2007). The ORF3 gene of attenuated vaccine isolates has a continuous deletion of 17 amino acids (aa 82–99) thus distinguishing the vaccine isolates from variant PEDV (Park *et al.*, 2008). Therefore, the ORF3 gene has been the focus of molecular epidemiology to study the characteristics of PEDV (Chen *et al.*, 2013; Li *et al.*, 2013; Song *et al.*, 2015; Temeeyasen *et al.*, 2014). As aforementioned, the ORF3 genes were chosen as the target genes for molecular characteristical analysis.

Many studies have indicated that the ORF3 gene of PEDV has an unexpected genetic variability (Chen *et al.*, 2013). Especially, wild-type and cell culture adapted PEDVs almost have complete sequence identity except for variations and truncations in the ORF3 gene observed, exclusively, in the cell culture adapted PEDV (Chang *et al.*, 2002; Chen *et al.*, 2013; Sun *et al.*, 2008; Suzuki *et al.*, 2015). Similarly, the highly adapted PEDV attenuated DR13 is differentiated from wild-type PEDVs by both reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP), which use sequence variations in the ORF3 gene of the highly adapted PEDV. Moreover, the ORF3 gene has been suggested as an important determinant for PEDV biological properties.

Based on the ORF3 gene, the phylogenetic analysis of PEDV isolates from China divide into genotypes G1 and G2, the G2 genotype including 2a PEDV and 2b PEDV subgroups (Wang *et al.*, 2016) and other strains isolated from some countries. To investigate further infectivity, genetic diversity and molecular characteristics of PEDV, we performed phylogenetic analyses based on the ORF3 gene of the latest collected Vietnamese isolates. This is the first report in Vietnam about the characterization of the PEDV ORF3 gene.

Materials and Methods

Sample collection and cDNA synthesis: The 286 intestinal tissue samples were used in the clinical diagnosis of acute diarrhea from pigs with different ages suffering from severe diarrhea on 36 farms in 5 provinces (Bacgiang, Hanoi, Haiduong, Quangninh, and Vinhphuc) of northern Vietnam from September 2018 to August 2019. The samples were prepared for RNA extraction and RT-PCR detection.

Primers design: The PCR primers for amplification of the Porcine Epidemic Diarrhea Virus (PEDV) were designed by Primer Premier 5.0 software according to the nucleotide sequence published in GenBank (accession number EU054929). The sequences of the sense and anti-sense primers are as follows: Sense: 5′-TTCTGAGTCACGAACAGCCA -3′ and Anti-sense: 5′-CATATGCAGCCTGCTCTGAA -3′.

A pair of primers based on the ORF3 gene of PEDV was designed and synthesized by Sangon Biotech Co., Ltd.

RNA extraction and cDNA synthesis: Intestinal tissue samples collected from diseased pigs were processed as 20% suspensions in PBS. RNA was extracted from 200 µl of each suspension prepared from the pig samples and the PEDV isolates using an RNA/DNA Isolation Kit for Cells and Tissues (WizPrep, Viral RNA Mini Kit, Korea) according to the manufacturer's instructions.

The synthesis of the cDNA was carried out through reverse transcription as described below. A total of 13 µl of viral RNA was mixed with 1 µl of 10 pmol Oligo (dT) primer (TaKaRa), incubated at 70°C for 10 mins, then placed on ice for 1 min. Next, 4 µl of 5×RT buffer, 1 µl of dNTP (2.5 mM) mixture, 0.5 µl of Rnase inhibitor (40 U/µl) and 0.5 µl of reverse transcriptase M-MLV (200 U/µl) were added and gently mixed. The mixture was kept at 42 °C for 1 h and the resulting cDNA stored at -20 °C until further use.

RT-PCR detection: PCR amplification was performed using Platinum Tag DNA polymerase High Fidelity (Invitrogen, CA, USA). After an initial incubation at 94°C for 5 mins, the reactions were subjected to 30 cycles of PCR as follows: 94°C for 60 s, 50°C for 60 s and 72°C for 60 s. These cycles were followed by a

terminal 10-min extension at 72°C. The PCR products were visualized by agarose gel electrophoresis.

Sequencing and analysis: PCR products were purified using the QIAquick PCR purification kit (Qiagen). The purified PCR products were sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems) according to the supplier's instructions. The sequence of the PCR products was determined using the dye terminator sequencing method and analyzed by an AB1 PRISM 310 auto-sequencer.

PEDV genome pairwise comparison: To compare the PEDV nucleotide sequence pairwise identity, twenty of Open Reading Frame 3 (ORF3) gene sequences were downloaded from GenBank (Table 2) and compared using pairwise sequencing identity using the Mega5.10 software program.

PEDV genome nucleotide homology and divergence: The recently identified nucleotide sequence was aligned and analyzed with pairwise analysis and divergence based on the sequence by using the MegAlign 6, DNASTAR software program (Madison, WI, USA), nucleotide homology percentage was compared with available different ORF3 genes from NCBI GenBank database.

Sequence alignments and phylogenetic analysis: Sequences of ORF3 were processed by the Clustal W method (Guindon *et al.*, 2009) and phylogenetic trees were constructed by the neighbor-joining method using MEGA 6 software (Tamura *et al.*, 2013). Bootstrap values were indicated for each node from 2000 replicates. In addition, 20 reference strains (Table 2) were chosen from Genbank for inclusion in the phylogenetic analysis.

Results

PCR detection: DNA fragments with expected lengths were amplified from DNA templates extracted from diseased pigs. PEDVs were detected by PCR on 94.44% (34 of 36) pig farms in 5 provinces and 90.56% (259 of 286) of samples were positive for PEDV in clinical diseased samples.

Analyses of nucleotides and amino acids sequences: The homology of PEDV BF strain nucleotide sequences compared with known PEDVs on the database was deduced by Blastn (NCBI) and analyzed with DNAstar 2.0, SeqEdit, MegAlign, Clustal W and Jotun Hein methods. The results confirmed that its sequence was highly conserved and indicated that it had several homologues at the GenBank database. The percent identity results are shown under Table 3.

The sequence result of PEDV-BF and corresponding amino acids compared with the published sequence in GenBank described in Fig. 3 displayed 98.69 – 99.63% similarity in the nucleotide sequence and 98.63 – 99.95% in amino acid sequence with the published one. It indicates that the isolated sequence was highly related to that published one.

The sequence results revealed that the ORF sequence of the PEDV BF strain was 636 bp. The open reading frame started with ATG at the beginning and ended with a TGA stop codon at base 636, encoding an about 25.2 kDa polypeptide of 224 amino acids. The deduced protein contained 17 strongly basic amino acid residues, 18 strongly acidic amino acid residues, 105 hydrophobic amino acid residues and 65 polar amino acid residues. Meanwhile the total net charge was positive. There were 4 nucleotide changes at the positions of 15, 16, 334 and 484; 6 nucleotide insertions at 627-630, 632, 634; 68 nucleotide deletions. There were 3 amino acid changes at the positions of 6, 106 and 159; 17 nucleotide deletion at 63-79. The theoretical pI of the protein was 4.61 and no signal peptide was found in the deduced protein but one glycosylation site and twenty-one phosphorylation sites were detected by sequence analysis.

Pairwise comparison: The isolate PEDV of nucleotide sequence pairwise comparison showed extensive similarity and divergence from previously isolated PEDV. The nucleotide sequences analyses of the new isolate were closely related to the strains DR13 attenuated, DR13 from Korea and QH1, QH2, NX1, SX from Shanxi, China.

To compare the difference of genes of PEDV, all genes including accessory genes were aligned and compared pairwise on the nucleotide similarity index (Table 4), the high nucleotide similarity of the PEDV BF strain was found 99 - 100% similar with PEDV ORF3 genes from Shanxi, China and Korea.

Phylogenetic tree analysis: The phylogenetic analysis revealed that, based on the ORF3 gene, the PEDV isolates were further divided into 3 clusters: clusters 1, 2 and 3 (Fig. 3). BF PEDV field isolates were in cluster 2, as were the isolates from China. Cluster 2 consisted of CV777 and isolates with a unique truncated portion, a 17-amino acid deletion at position 82–98, which represented attenuated virus vaccines and no BF PEDV isolate was included in this group. According to the analysis of the ORF3 genes, twenty PEDV isolates in this study were cluster 2, which showed similarity to the phylogenetic trees based on the sequences of amino acid. Our strains also showed a close relationship to published isolates and genetically differed from the vaccine strains which were all cluster 3, indicating that the prevailing PEDV strains were mostly variants. According to the sequences of ORF3 genes processes, our isolates exhibited 95.9-96.9% nucleotide identity and 93.8–96.4% amino acid identity compared with the CV777 strain. Meanwhile, our isolates exhibited 91.3–93.1% nucleotide identity and 84.6–89% amino acid identity compared with the CV777 attenuated/Korea strain.

Discussion

In this research, we describe the open reading frame ORF3 sequence of the PEDV BF strain, isolated from diseased pigs from five provinces, including Bacgiang, Hanoi Haiduong, Quangninh and Vinhphuc, Vietnam.

Previous studies found 79.66% of pig farms in 29 provinces to be positive for the presence of PEDV, with 72.27% of samples confirmed as PEDV-positive (Chen

et al., 2013). The results of our study showed 94.03% (34 of 36) of pig farms in 5 provinces, 92.25% (259 of 286) samples were PEDV-positive, indicating a high prevalence of PEDV in clinical diseased samples.

. The ORF3 genes of our field isolates sequenced to compare with the current isolates are closely related to field isolates in Korea and China or to vaccine isolates. The ORF3 genes have been used to differentiate between field and vaccine-derived isolates. The vaccine-derived isolates have a unique characteristic at nucleotide position 245–293, a continuous deletion of 49 NT. The results of this study demonstrated that the Vietnamese field isolates do not possess that unique 49-nucleotide deletion suggesting that they are not related to vaccine isolates.

Table 1 Numbers of PEDV positive samples and detection rates in respective age groups according to research periods, 2018~2019

Location (province)	Collection samples	Positive	Rate (%)
Bacgiang	56	52	92.86[a]
Hanoi	58	52	89.66[b]
Haiduong	55	51	92.73[a]
Quangninh	61	54	88.52[b]
Vinhphuc	56	50	89.29[b]
Overall	**286**	**259**	**90.56**

Values within columns not sharing a common superscript letter (a–b) are significantly different at $p < 0.05$.

Table 2 Summary of virus strains used in this study, Porcine Epidemic Diarrhea Virus; Accession number used for phylogenetic tree, pair wise comparison

Reference strain	Year	Location	GenBank accession no.	nt size
CH/SXYC	2016	Shanxi, China	KU977500	675
DR13	2008	Korea	EU054929	675
attenuated DR13	2012	Korea	JQ023162	675
attenuated DR13	2008	Korea	EU054930	624
J-vac	2016	Korea	EF628251	612
CH3-ORF	2013	Hubei, China	KC342813	626
DBI865 truncated ORF3	2012	Korea	HQ537432	672
CV777 truncated ORF3	2010	Heilongjiang, China	GU372744	725
CH4-ORF3	2013	Hubei, China	KC342814	626
CH/BJ/2011 truncated ORF3	2012	Heilongjiang, China	JQ027019	626
QH2-ORF3	2018	Shanxi, China	MF418657	675
GS1-ORF3	2018	Shanxi, China	MF418656	675
QH1-ORF3	2018	Shanxi, China	MF418658	672
NX1-ORF3	2018	Shanxi, China	MF418655	672
SX2-ORF3	2018	Shanxi, China	MF418657	672
SX1-ORF3	2018	Shanxi, China	MF418653	672
CV777	2016	France	Z24733	1740
CV777 attenuated	2016	Heilongjiang, China	KT323979	276
CH/HNHB-1	2016	Hebei, Henan, China	KU977480	675
CH/HNHB-2		Hebei, Henan, China	KU977481	675
CH/HNHB-3		Hebei, Henan, China	KU977482	675
CH/HNHB-4		Hebei, Henan, China	KU977483	675
CH/HNHB-5		Hebei, Henan, China	KU977484	675
CH/HNHB-6		Hebei, Henan, China	KU977485	675
CH/HNHB-7		Hebei, Henan, China	KU977486	675
CH/HNKF-1		Henan, China	KU977487	675
CH/HNKF-2		Henan, China	KU977488	675
CH/HNKF-3		Henan, China	KU977489	675
CH/HNSMX		Henan, China	KU977490	675
CH/SXYC		Shanxi, China	KU977491	675
CH/HNXC		Henan, China	KU977492	675
CH/HNXX		Henan, China	KU977493	675
CH/HNAY		Henan, China	KU977494	675
CH/HNAY-1		Henan, China	KU977495	675
CH/HNAY-2		Henan, China	KU977496	675
CH/HNAY-3		Henan, China	KU977497	675
CH/HNZMD		Henan, China	KU977498	675
CH/HNLY		Henan, China	KU977499	675
CH/HNPY		Henan, China	KU977500	675

Table 3 The nucleotide homology of PEDV BF strain ORF3 gene with other PEDV strains available on GenBank

Description	Max Score	Total Score	Per. Ident	Accession
Porcine epidemic diarrhea virus strain attenuated DR13 ORF3 gene, complete cds	1131	1131	99.36%	EU054930.1
Porcine epidemic diarrhea virus strain Ah2016f2, complete genome	1122	1122	99.04%	MN486588.1
Porcine epidemic diarrhea virus strain AH-2018-HF1, complete genome	1122	1122	99.04%	MN315264.1
Porcine epidemic diarrhea virus strain CN/Liaoning25/2018, complete genome	1122	1122	99.04%	MK796238.1
Porcine epidemic diarrhea virus strain PPC 14, complete genome	1122	1122	99.04%	MG781192.1
Porcine epidemic diarrhea virus strain SQ2014, complete genome	1122	1122	99.04%	KP728470.1
Porcine epidemic diarrhea virus strain AH-M, complete genome	1122	1122	99.04%	KJ158152.1
Porcine epidemic diarrhea virus, complete genome	1122	1122	99.04%	KC189944.1
Porcine epidemic diarrhea virus isolate CH3 nonfunctional ORF3 protein gene, complete sequence	1122	1122	99.04%	KC342813.1
Porcine epidemic diarrhea virus strain SD-M, complete genome	1122	1122	99.04%	JX560761.1
Porcine epidemic diarrhea virus strain attenuated DR13, complete genome	1122	1122	99.04%	JQ023162.1
Porcine epidemic diarrhea virus isolate DBI865 truncated ORF3 gene, complete cds	1122	1122	99.04%	HQ537432.1
Porcine epidemic diarrhea virus strain CV777 truncated ORF3 protein gene, complete cds	1122	1122	99.04%	GU372744.1
Porcine epidemic diarrhea virus strain PEDV-SX, complete genome	1116	1116	98.88%	KY420075.1
Porcine epidemic diarrhea virus isolate JSLS-1/2015, complete genome	1116	1116	98.88%	KX534205.1
Porcine epidemic diarrhea virus isolate HLJBY, complete genome	1116	1116	98.88%	KP403802.1
Porcine epidemic diarrhea virus isolate CH4 nonfunctional ORF3 protein gene, complete sequence	1116	1116	98.88%	KC342814.1
Porcine epidemic diarrhea virus strain CV777, complete genome	1110	1110	98.72%	KT323979.1
Porcine epidemic diarrhea virus strain JS2008, complete genome	1110	1110	98.72%	KC210146.1
Porcine epidemic diarrhea virus strain CH/BJ/2011 truncated ORF3 protein gene, complete cds	1110	1110	98.72%	JQ027019.1
Porcine epidemic diarrhea virus isolate SC1402, complete genome	1101	1101	98.56%	KP162057.1
Porcine epidemic diarrhea virus isolate GDS09, complete genome	697	1141	99.22%	MH726408.1
Porcine epidemic diarrhea virus isolate QH2 ORF3 gene, complete cds	693	1143	99.48%	MF418657.1
Porcine epidemic diarrhea virus isolate GS1 ORF3 gene, complete cds	693	1143	99.48%	MF418656.1
Porcine epidemic diarrhea virus isolate QH1 ORF3 gene, complete cds	673	1123	98.69%	MF418658.1
Porcine epidemic diarrhea virus isolate NX1 ORF3 gene, complete cds	673	1123	98.69%	MF418655.1
Porcine epidemic diarrhea virus isolate SX2 ORF3 gene, complete cds	673	1123	98.69%	MF418654.1
Porcine epidemic diarrhea virus isolate SX1 ORF3 gene, complete cds	673	1123	98.69%	MF418653.1

Table 4 PEDV genes nucleotide sequence pairwise comparison in percentage identity and divergence values. The nucleotide sequences of the ORF3 gene of PEDV and other PEDVs.

	Percent identity																			
Divergence	Seq->	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	BF/VN	ID	0.88	0.885	0.974	0.974	0.367	0.971	0.906	0.857	0.968	0.902	0.902	0.898	0.898	0.898	0.347	0.883	0.882	0.882	0.88
	CH/SXYC/ShxCN	0.88	ID	0.979	0.902	0.902	0.379	0.903	0.846	0.788	0.9	0.976	0.976	0.971	0.971	0.971	0.371	0.995	0.994	0.994	1
	DR13/Kr	0.885	0.979	ID	0.906	0.906	0.38	0.908	0.85	0.791	0.908	0.98	0.98	0.976	0.976	0.976	0.374	0.983	0.982	0.982	0.979
	DR13/attenuated/Kr	0.974	0.902	0.906	ID	1	0.37	0.996	0.928	0.86	0.993	0.924	0.924	0.92	0.92	0.92	0.348	0.905	0.903	0.903	0.902
	DR13/Kr	0.974	0.902	0.906	1	ID	0.37	0.996	0.928	0.86	0.993	0.924	0.924	0.92	0.92	0.92	0.348	0.905	0.903	0.903	0.902
	Jvac/Kr	0.367	0.379	0.38	0.37	0.37	ID	0.371	0.347	0.323	0.368	0.382	0.382	0.382	0.382	0.382	0.145	0.382	0.38	0.383	0.379
	CH/HubCN	0.971	0.903	0.908	0.996	0.996	0.371	ID	0.931	0.863	0.996	0.927	0.927	0.922	0.922	0.922	0.349	0.906	0.905	0.905	0.903
	DBI865/Kr	0.906	0.846	0.85	0.928	0.928	0.347	0.931	ID	0.926	0.928	0.868	0.868	0.864	0.864	0.864	0.375	0.848	0.847	0.847	0.846
	CV777/HljCN	0.857	0.788	0.791	0.86	0.86	0.323	0.863	0.926	ID	0.86	0.808	0.808	0.804	0.804	0.804	0.405	0.79	0.789	0.789	0.788
	CH/BJ/HljCN	0.968	0.9	0.908	0.993	0.993	0.368	0.996	0.928	0.86	ID	0.924	0.924	0.92	0.92	0.92	0.348	0.903	0.902	0.902	0.9
	QH2/ShxCN	0.902	0.976	0.98	0.924	0.924	0.382	0.927	0.868	0.808	0.924	ID	1	0.995	0.995	0.995	0.376	0.979	0.977	0.977	0.976
	GS1/ShxCN	0.902	0.976	0.98	0.924	0.924	0.382	0.927	0.868	0.808	0.924	1	ID	0.995	0.995	0.995	0.376	0.979	0.977	0.977	0.976
	QH1/ShxCN	0.898	0.971	0.976	0.92	0.92	0.382	0.922	0.864	0.804	0.92	0.995	0.995	ID	1	1	0.374	0.974	0.973	0.973	0.971
	NX1/ShxCN	0.898	0.971	0.976	0.92	0.92	0.382	0.922	0.864	0.804	0.92	0.995	0.995	1	ID	1	0.374	0.974	0.973	0.973	0.971
	SX/ShxCN	0.898	0.971	0.976	0.92	0.92	0.382	0.922	0.864	0.804	0.92	0.995	0.995	1	1	ID	0.374	0.974	0.973	0.973	0.971
	CV777/France	0.347	0.371	0.374	0.348	0.348	0.145	0.349	0.375	0.405	0.348	0.376	0.376	0.374	0.374	0.374	ID	0.372	0.372	0.372	0.371
	CH/HNAY/CN	0.883	0.995	0.983	0.905	0.905	0.382	0.906	0.848	0.79	0.903	0.979	0.979	0.974	0.974	0.974	0.372	ID	0.998	0.998	0.995
	CH/HNHB/CN	0.882	0.994	0.982	0.903	0.903	0.38	0.905	0.847	0.789	0.902	0.977	0.977	0.973	0.973	0.973	0.372	0.998	ID	0.997	0.994
	CH/HNKF/CN	0.882	0.994	0.982	0.903	0.903	0.383	0.905	0.847	0.789	0.902	0.977	0.977	0.973	0.973	0.973	0.372	0.998	0.997	ID	0.994
	CH/SXYC/CN	0.88	1	0.979	0.902	0.902	0.379	0.903	0.846	0.788	0.9	0.976	0.976	0.971	0.971	0.971	0.371	0.995	0.994	0.994	ID

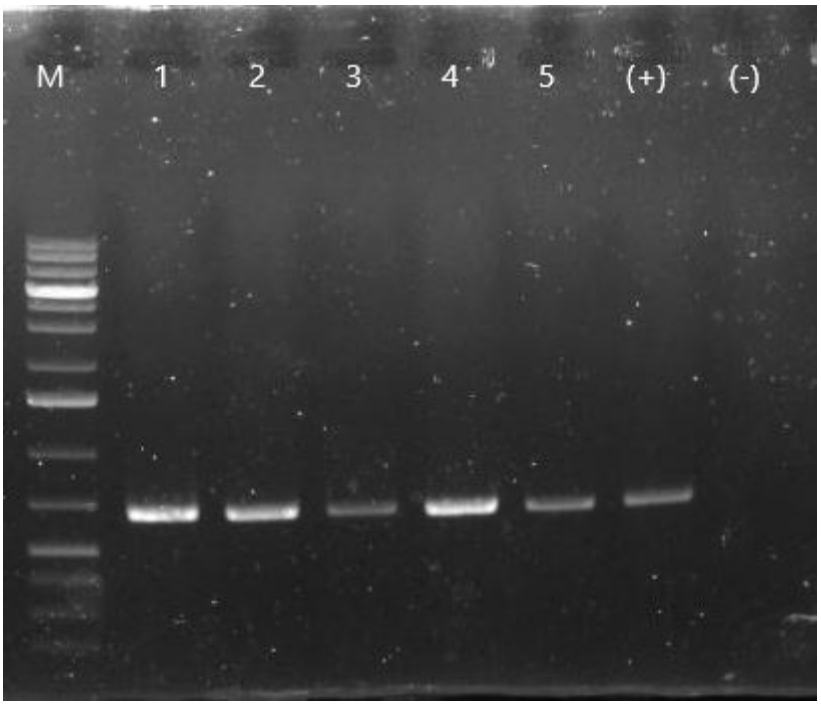
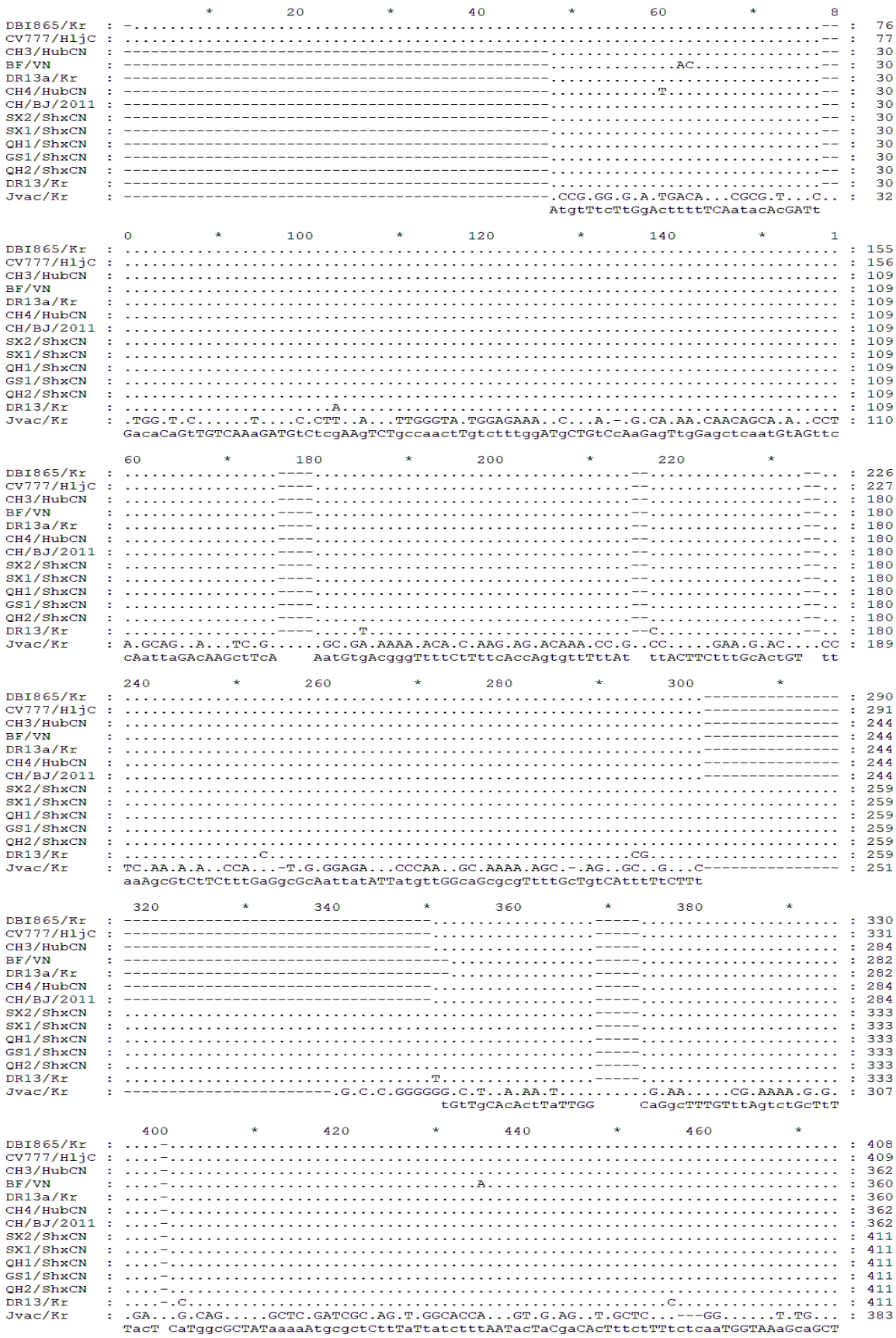


Figure 1 Identification of PEDV from the diseased pigs samples by PCR amplification
M: Marker DNA (1 kb Plus DNA Ladder, Invitrogen); Lane 1: PEDV-BG; Lane 2: PEDV-HN; Lane 3: PEDV-HD; Lane 4: PEDV-QN; Lane 5: PEDV-VP; Lane 6: Positive control; Lane 7: Negative control.


DBI865/Kr
CV777/HljC
CH3/HubCN
BF/VN
DR13a/Kr
CH4/HubCN
CH/BJ/2011
SX2/ShxCN
SX1/ShxCN
QH1/ShxCN
GS1/ShxCN
QH2/ShxCN
DR13/Kr
Jvac/Kr

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                480         *       500         *       520         *       540         *
DBI865/Kr   : ........--..................................................................... : 485
CV777/HljC  : ........--..................................................................... : 486
CH3/HubCN   : ........--..................................................................... : 439
BF/VN       : ........--..................................................................... : 437
DR13a/Kr    : ........--..................................................................... : 437
CH4/HubCN   : ........--..................................................................... : 439
CH/BJ/2011  : ......A.--..................................................................... : 439
SX2/ShxCN   : ........--..................................................................... : 488
SX1/ShxCN   : ........--..................................................................... : 488
QH1/ShxCN   : ........--..................................................................... : 488
GS1/ShxCN   : ........--..................................................................... : 488
QH2/ShxCN   : ........--..................................................................... : 488
DR13/Kr     : ........--..................................................................... : 488
Jvac/Kr     : GT.CG.....TA..GG..T.-..AC..GC...C.T----A.A..T..A.A..G...G.GCCA..G...---.A.C.AAA : 454
              taTtaTgA  cgGCaaATcCaTTgtGAttCTAgAaggtggTgACcATtAcATcACTtTtggcAAcTCTtttGtTgCttt

                560         *       580         *       600         *       620         *
DBI865/Kr   : ..................................................................... : 564
CV777/HljC  : ..................................................................... : 565
CH3/HubCN   : ..................................................................... : 518
BF/VN       : ...............................................T.................... : 516
DR13a/Kr    : ..................................................................... : 516
CH4/HubCN   : ..................................................................... : 518
CH/BJ/2011  : ..................................................................... : 518
SX2/ShxCN   : ..................................................................... : 567
SX1/ShxCN   : ..................................................................... : 567
QH1/ShxCN   : ..................................................................... : 567
GS1/ShxCN   : ..................................................................... : 567
QH2/ShxCN   : ..................................................................... : 567
DR13/Kr     : ..................................................................... : 567
Jvac/Kr     : T...GAGCTTC...TT.CAC.GG.G.--..G.A.TTAAAA.TG.G.AT.---...T.C.C-CA.A...AGAAG..A--- : 524
              cGTTagtagcaTTGacTtgtAtcTaGctATaCgTgggcggCaaGaagcTgacCTAcAtCtgttGcGAActgttGAgctt

                640         *       660         *       680         *       700         *
DBI865/Kr   : ..................................................................... : 643
CV777/HljC  : ..................................................................... : 644
CH3/HubCN   : ..................................................................... : 597
BF/VN       : ..................................................................... : 595
DR13a/Kr    : ..................................................................... : 595
CH4/HubCN   : ..................................................................... : 597
CH/BJ/2011  : ..................................................C.................. : 597
SX2/ShxCN   : ..............................................---.................... : 643
SX1/ShxCN   : ..............................................---.................... : 643
QH1/ShxCN   : ..............................................---.................... : 643
GS1/ShxCN   : ..................................................................... : 646
QH2/ShxCN   : ..................................................................... : 646
DR13/Kr     : .....T.....................G.............C.............C.................. : 646
Jvac/Kr     : ---A.GAAG..C....G.G.AAC.A---.G..G..G..G.A..AA.AGGCC.TAT...A..-..C..GG.--G.G.C.T : 594
              cttgAcggcAAgAAGCtTtAtgtCttttCaCAaCAtCAaAtTGttGgcattA   ATGctGcATtTGacTcaaTtCaAc

                720         *       740         *       760         *       780         *
DBI865/Kr   : ........................----------------------------------------------- : 672
CV777/HljC  : ........................T..T....T..C..................................... : 723
CH3/HubCN   : ........................----------------------------------------------- : 626
BF/VN       : ......................TT.TT.T.T..------------------------------------- : 636
DR13a/Kr    : ........................----------------------------------------------- : 624
CH4/HubCN   : ........................----------------------------------------------- : 626
CH/BJ/2011  : ........................----------------------------------------------- : 626
SX2/ShxCN   : ........................----------------------------------------------- : 672
SX1/ShxCN   : ........................----------------------------------------------- : 672
QH1/ShxCN   : ........................----------------------------------------------- : 672
GS1/ShxCN   : ........................----------------------------------------------- : 675
QH2/ShxCN   : ........................----------------------------------------------- : 675
DR13/Kr     : ........................----------------------------------------------- : 675
Jvac/Kr     : CT..T...ACCCA.G.C.--------------------------------------------------------- : 612
              taGAcGAGtatgcTaCaAttagtgaatga
```

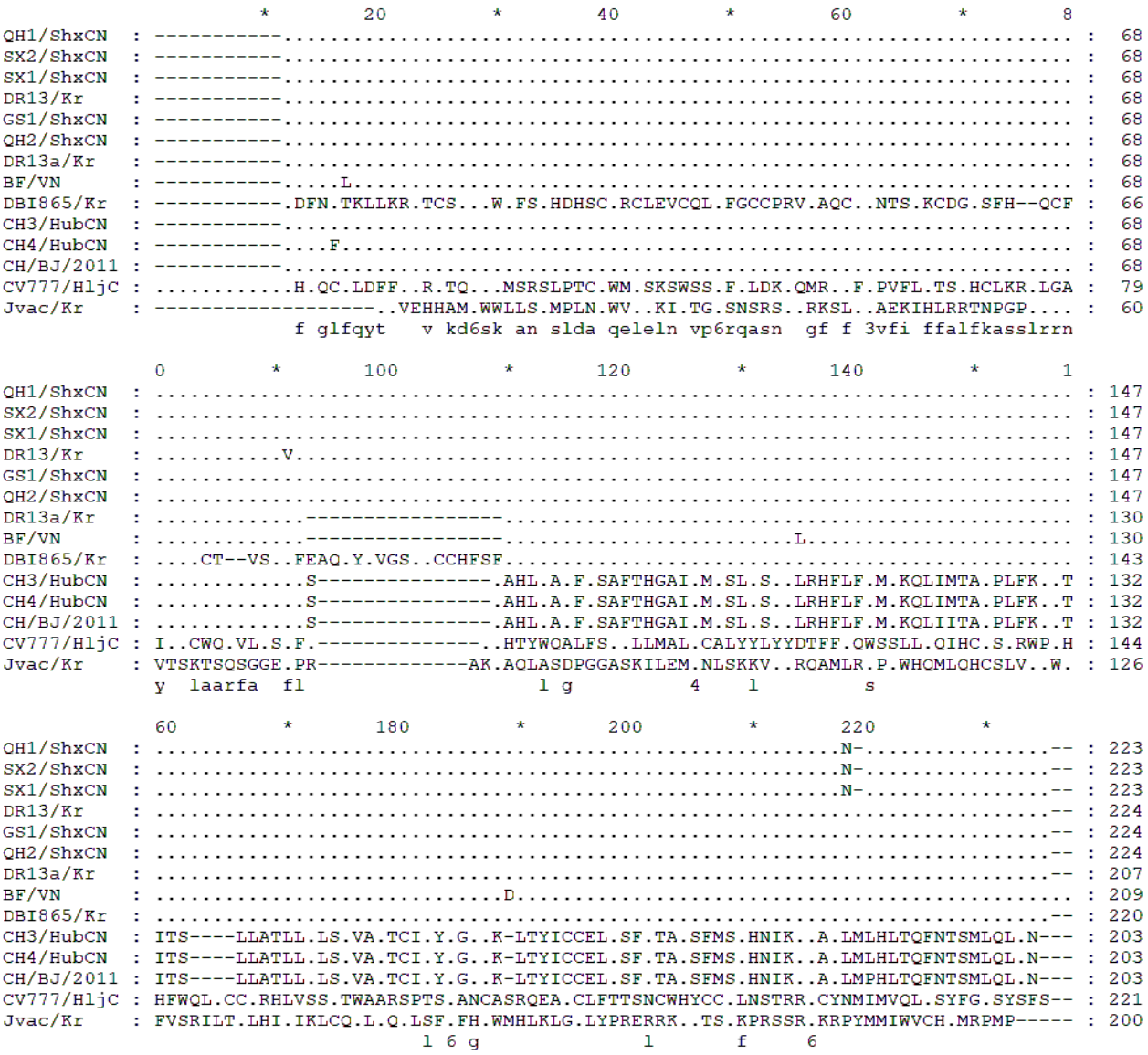

Figure 2 The ORF Nucleotides sequence comparison for the 636 nucleotide fragment of PEDV-BF and corresponding amino acids sequence with twelve PEDVs from Genbank

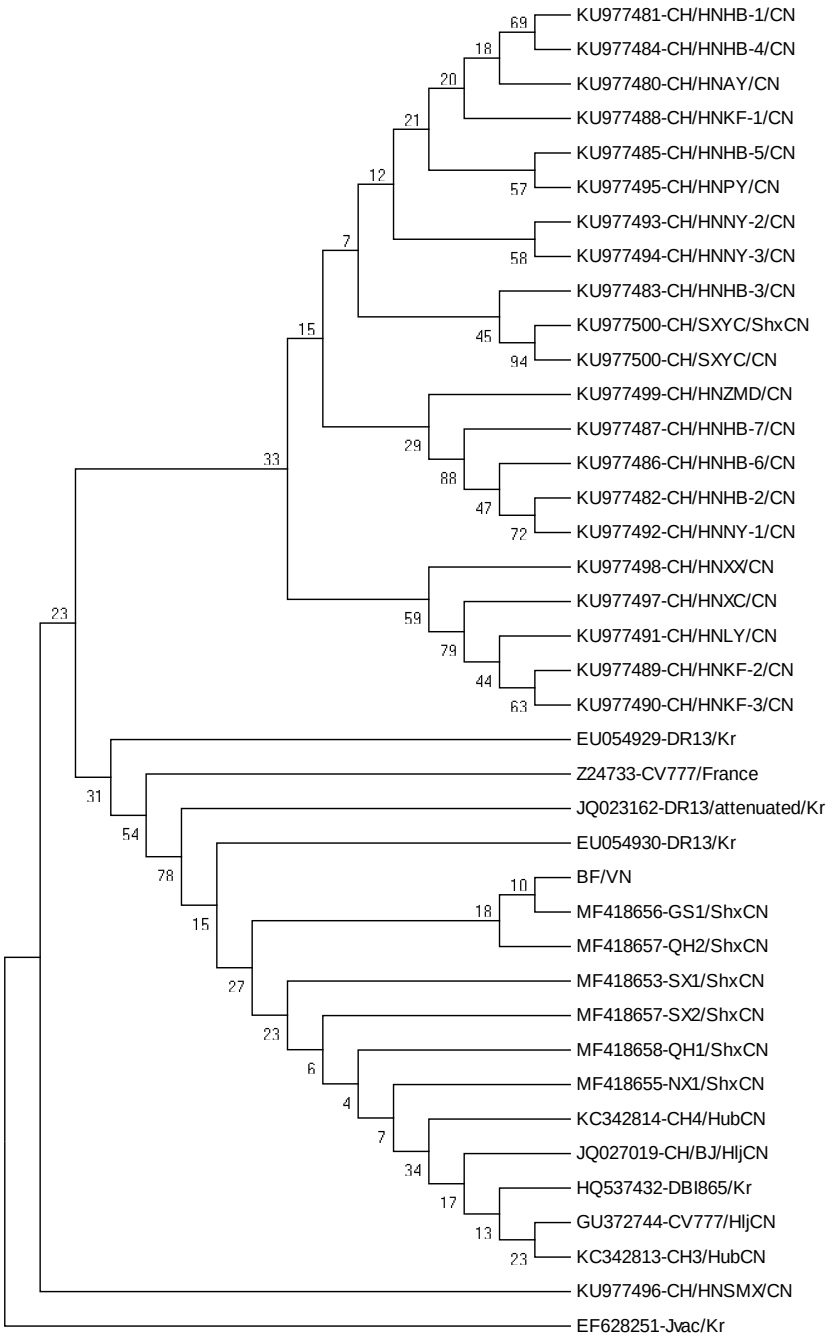

Figure 3 Phylogenetic analysis of the ORF3 nucleotide sequences of 20 PEDV isolates, including the reference strains. The trees were constructed by the neighbor-joining method in MEGA 6 software. Bootstrap values were indicated for each node from 2000 replicates. The names of the strains, years and places of isolation and GenBank accession numbers proposed are shown in Table 1.

The results of sequence analysis revealed that the ORF sequence of the PEDV BF strain was 636 bp. The open reading frame (ORF) started with ATG at the beginning and ended with a TGA stop codon at base 636, encoding about 25.2 kDa polypeptide of 224 amino acids. The nucleotide sequence of the PEDV BF strain compared with the published PEDV ORF3 sequence from GenBank revealed four change positions at 15, 16, 334 and 484, and the amino acids showed three different positions at 6, 106 and 159. It appears that the nucleotide and amino acid sequence of PEDV ORF3 are conserved between the strains of PEDV.

The ORF3 gene is highly relevant to the virulence of PEDV (Chen *et al*., 2013; Park *et al*., 2007), since it regulates virus production (Wang *et al*., 2012). The ORF3 genes of the PEDV isolates in this study and other isolates did not show the large deletion characteristic of the vaccine CV777 strain. According to the phylogenetic analysis of ORF3, the PEDV isolates in this study were divided into subtype G2 (Fig. 3). The ORF3 gene analysis not only suggested that the isolates in the northern province of Vietnam were not only vaccine-unrelated but the presence of multiple, distinct mutations indicated there is wide diversity in this virulence gene. However, further studies are needed to clarify whether the virulence is a change among these PEDV strains.

Phylogenetic analysis of the ORF3 gene showed that our isolates exhibited high similarity to variant reference strains and differed from CV777 and Jvac. Phylogenetic analysis of ORF3 did not reveal differences between our isolates and partial classical PEDV strains which were similar to other reports (Wang *et al*., 2016; Su *et al*., 2016). Further studies are required to conduct the biological functions among PEDV phylogenetic groups.

In conclusion, our study highlighted the present landscape of PEDV in the northern province of Vietnam and the isolated strains in this study were all variable and genetically diverse. These findings make it clear that a new vaccine is required to control this disease. Besides, the discovery of a novel strain, PEDV BF provides an avenue for future investigations into the biological functions of PEDV.

Conflicts of Interest: The authors declare no conflict of interest.

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