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Protocols

Establishment and application of a multiplex RT-PCR to differentiate wild-type and vaccine strains of porcine epidemic diarrhea virus

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ABSTRACT

Due to outbreaks of porcine epidemic diarrhea (PED) and the wide use of attenuated live vaccine, both wild-type and vaccine strains (CV777) are believed to circulate in Chinese pig farms. Thus, identification of different PEDV strains is of epidemiological importance. In this study, a multiplex RT-PCR method was established based on the sequence features of spike (S) gene and ORF3 gene of PEDVs. The method could identify PEDV variant strains, classical wild-type strains and classical vaccine strains. The limit of detection of the RT-PCR was 1.51×10^4 copies/uL for plasmids and $1 \times 10^{1.7}$ $TCID_{50}$/100 u L for PEDV, respectively. There were no cross-detections among three different PEDVs and no false detections among six swine pathogens. This assay was used to test 940 samples from China of which 303 samples were PEDV positive, and 289, 5, 10 were positive for variant, classical wild, classical vaccine, respectively. One sample was positive for both variant and classical vaccine PEDV. The variant PEDVs could be detected in samples from 13 provinces, while classical PEDVs were detected from nine provinces, supporting the prevalence of variant PEDV in China. In summary, this multiplex RT-PCR was a useful tool for the clinical detection and epidemiological survey of PEDV.

1. Introduction

Porcine epidemic diarrhea (PED), whose etiology is porcine epidemic diarrhea virus (PEDV), is an acute, highly contagious enteric disease, characterized by acute and watery diarrhea, vomiting, followed by dehydration. Pigs of all ages can be infected by PEDV but the piglets are the most vulnerable. The first PEDV strain, named as CV777, was isolated in Belgium in 1978 (Pensaert and De Bouck, 1978). In China, transmissible gastroenteritis (TGE)-like outbreaks of acute diarrhea were first reported in 1973, which was later confirmed as PED (Xuan et al., 1984). Since 1995, inactivated or live attenuated PEDV CV777 strain-based vaccines have been developed (Tong et al., 1999) and applied in China. Since October 2010, the emerging of variant PEDV in China resulted in heavy economic losses in the pig-raising provinces (Li et al., 2012). Severe PED outbreaks were frequently observed in the herds that had previously immunized with CV777-based inactivated or attenuated vaccine (Wang et al., 2013).

The S gene is one of the most variable gene in the PEDV genome and considered as a useful marker in understanding genetic variations of PEDV strains (Chen et al., 2013; Tian et al., 2014; Chen et al., 2016; Sun et al., 2015). Thus, the S gene plays an important role in understanding genetic relatedness among PEDV isolates and developing diagnostic assays (Lee, 2015; Lee et al., 2010). The ORF3 is the only accessory gene in PEDV and associated with virus production and virulence (Wang et al., 2012). Studies had shown that wild-type and cell culture adapted PEDV have variations in the ORF3 gene (Li et al., 2016; Wang et al., 2012). For example, the attenuated CV777–based vaccine strain used in China has 49-nt deletions in ORF3 gene (Chen et al., 2010).

Both classical and variant strains are circulating in Asian and European countries (Gerdts and Zakhartchouk, 2017; Hanke et al., 2017; Jang et al., 2017; Lee et al., 2017). The morbidity and mortality were different if pigs were infected by different filed PEDV strains (Li et al., 2012; Song and Park, 2012; Stevenson et al., 2013). After 2010, some epidemic PEDV strains, including YN strain, a local strain, were successfully isolated from piglets with diarrhea (Chen et al., 2016; Fan et al., 2017; Zhang et al., 2015). To fight against this emerging disease, a YN strains-based attenuated vaccine candidates for oral immunization had been produced via continuous Vero cell culture passage in our

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laboratory (Chen et al., 2015; Chen et al., 2016; Lin et al., 2016). In 2017, bivalent inactivated vaccine using attenuated PEDV (AJ1102 strain, GII-b) and TGEV (WH-1 strain) was prepared and approved to undergo field trial in China (Zeng et al., 2017).

Therefore, targeted vaccine strains should be selected according to the prevalent PEDV strains in pig farms. However, the traditional RT-PCR could only distinguish the classical attenuated vaccine strains from the field strains based on ORF3 gene (Zhu et al., 2016a,b). Analyses of S gene of some US, China and Korea strains, indicate that it could be a genetic marker to differentiate variant PEDV strains (Liu and Wang, 2016; Su et al., 2018; Wang et al., 2014). Due to the prevalence of variant strains and the wide-use of live vaccine strains (Chen et al., 2017; Lin et al., 2016; Zeng et al., 2017), there are difficulties to the monitor, diagnosis and prevent PED. Therefore, there is an urgent need to develop an accurate, rapid and cost-effective diagnostic assay for the identification of different PEDV strains.

In this study, a multiplex RT-PCR assay was developed, evaluated and implemented to identify variant, classical wild and classical vaccine PEDV strains. The sensitivity and specificity were analyzed followed by further detection of 940 clinical diarrhea samples collected from January 2017 to January 2018, highlighting its potential as a useful tool to support the detection and epidemiology of PEDV.

2. Materials and methods

2.1. Viruses

Classical vaccine strain (cv-PEDV, CV777), variant field and attenuated PEDV strain (named as vf-PEDV and va-PEDV), porcine transmissible gastroenteritis virus (TGEV), porcine group A rotavirus (RVA), porcine reproductive and respiratory syndrome virus (PRRSV, JXA1-R vaccine strain), pseudorabies virus (PRV), classical swine fever virus (CSFV, attenuated vaccine strain), and porcine circovirus type 2 (PCV2) were preserved by our laboratory. The classical wild strain of PEDV was isolated from the clinical samples and identified as PEDV by sequencing.

2.2. Primer design and synthesis

Based on the sequences of PEDV S1 and ORF3, three pairs of primers were designed (Table 1). The forward primer (C/V-F) was shared by two reverse primers (C-R and V-R) in PCR amplification of S1 gene. The expected amplified fragments from S1 and ORF3 gene for variant, classical wild and classical vaccine (CV777) strains of PEDV were 720 bp, 606 bp, 606 bp and 373 bp, 373 bp and 324 bp, respectively.

2.3. Clinical samples collection and preparation

A total of 940 samples (i.e., faeces, faecal swabs, intestinal tract, environmental swabs, stomach contents, vomitus, etc.) were collected from 15 provinces (Hebei, Henan, Hubei, Jiangxi, Sichuan and other 10 provinces of China) from January 2017 to January 2018. These samples were individually collected and homogenized with phosphate-buffered saline at ratio of 1:10 (w/v) (0.1 M PBS, pH 7.4). The homogenate was frozen and thawed three times to release viruses and further vortexed for 10 min and centrifuged at 9391 xg (Eppendorf, Germany) for 10 min at 4 °C. The supernatants were applied to RNA extraction immediately or stored at -20 °C freezer for further usage.

Table 1
The primers utilized in this study.

Primer name	Nucleotide sequence (5'-3')	Primer location (nt)[a]
C/V-F	TCATCCATTAGTGATGTTGTGTTAGG	20532-20557
C-R	CGACAACRATRTTTTTTCCATCCTG	21114 -21138
V-R	GCATAGCACAACCTCCACTG	21239-21258
ORF3-F	AAGCGTCTTCTTTGAGGCG	24971-24989
ORF3-R	CGCAACAGATGTAGGTCAGC	25324-25343

[a] Numbers correspond to positions within genomes of CV777 strain (KT323979.1) and variant YN144 strain (KT021232.1), respectively. C: classical; V: variant; F: forward; R: reverse.

2.4. Extraction of RNA and cDNA synthesis

The 100 μL of clarified supernatants were added into sterile centrifuged tubes for viral RNA extraction according to the instructions of RNA extraction kit (BioFlux, China). The reverse transcription (RT) reaction was carried out in a 10 μL PCR master mixture consisting of 2 μL 5 × Prime Script® RT Master Mix (RR036A, Takara, Japan) and 8 μL of extracted RNA. The resultant cDNA was stored at -20 °C for single PCR and multiplex PCR.

2.5. The construction of standard recombinant plasmids

According to the instruction manual of the RNA extraction kit, the RNAs of variant PEDV strain (attenuated YN144), classical attenuated strain (CV777) and classical wild strain were extracted. The cDNAs were obtained by reverse transcription for further amplification of targeted genes by three pairs of primers, respectively.

The targeted S1 and ORF3 fragments from different above PEDVs were amplified from cDNAs using designed primers, gel based-purified, cloned into pMD® 18-T Vector (Takara, Japan) and transformed into *E.coil* DH5α strain. Then the positive clones were cultured for plasmid extraction using AxyPrepTM Plasmid Miniprep Kit (Axygen Biotechnology Company, China) for verification. The recombinant plasmids were further confirmed by PCR and sequencing methods. The concentrations of these plasmids were measured using Nanodrop. Recombinant plasmids contain variant PEDV (attenuated YN144) S1 gene plasmid, classical attenuated PEDV (CV777) S1 gene plasmid, wild (classical/variant) PEDV ORF3 gene plasmid and classical vaccine PEDV (CV777) ORF3 gene plasmid were named as p-va-PEDV-S1, p-cv-PEDV-S1, p-wild-PEDV-ORF3, p-cv-PEDV-ORF3, respectively. These recombinant plasmids were used for optimization and sensitivity testing in the multiplex RT-PCR.

2.6. Single-plex PCR

Single-plex PCR for four standard recombinant plasmids were carried out in a 25 μL mixture containing 12.5 μL of 2 × Es TaqMasterMix (Cwbio, China), 1 μL of each primer (10 μM), 4 μL of DNA or cDNA template and 6.5 μL of distilled water. DEPC-treated water was used as negative control. Amplification using Thermal Cycler (Eastwin, China) was started by initial denatured at 95 °C for 5 min, followed by 35 cycles of 95 °C for 35 s, annealing at 58.7 °C (primer pairs of C-F/R and V-F/R) or 56.2 °C (primer pairs of ORF3-F/R) for 35 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The PCR products were subjected to electrophoresis analysis on a 1.5% agarose gel in 1 × TAE buffer.

2.7. Optimization of the multiplex RT-PCR conditions

The multiplex RT-PCR reactions were optimized by varying a single parameter while other parameters were fixed as described by Zhao (Zhao et al., 2013). The primer concentration for each target ranged from 2 to 12 pmol. The annealing temperature (from 55.2 to 60.8 °C) and number of cycles (from 20 to 40) were also tested. The RT-PCR products were subjected to electrophoresis analysis on a 1.5% agarose gel in 1 × TAE buffer.

2.8. Sensitivity of the single-plex and multiplex RT-PCR assays

The plasmids and viruses were used in the sensitivity assay. Four

plasmids containing four specific viral target fragments were serial diluted by 10-fold. The following formula was used to calculate the number of gene copies per microlitre in each dilution: copies/μL = (6.02×10^{23}) × (plasmid concentration [ng/μL] $\times 10^{-9}$)/(DNA length [bp] ×660) (Zhao et al., 2013). Meanwhile, $1 \times 10^{4.7}$ $TCID_{50}$/100 μL of CV777 and YN144 strain virus were 10-fold serially diluted by Dulbecco Modified Eagle medium (DMEM) and used as original templates in the multiplex RT-PCR sensitivity assay to determine the detection limitation.

2.9. Specificity of multiplex RT-PCR assay

To verify the specificity of the multiplex RT-PCR, the cDNAs of PRRSV, CSFV, TGEV, RVA, and DNAs of PRV, PCV2 were used for RT-PCR using above primers (Table 1). The size-specific PCR products from four standard recombinant plasmids (720, 606, 373 and 324 bp) were subjected for sequencing. The sequence homology analysis was performed with the available PEDV sequences deposited in the GenBank using a BLAST search to confirm the specificity.

2.10. Feasibility verification of multiplex RT-PCR method

The established multiplex RT-PCR and conventional RT-PCR method were used to simultaneously detect 388 fecal samples from diarrhea pigs. The consistency and reliability were evaluated by Kappa test. If Kappa value is 0.8–0.99, it indicated almost perfect agreement between two methods (Mchugh, 2012).

2.11. Clinical application of multiplex RT-PCR method

In order to further evaluate the in-house RT-PCR method and to understand the epidemic characteristics of PEDV in some provinces of China, the developed multiplex RT-PCR was used to detect 940 samples (i.e., faeces, faecal swabs, intestinal tract, environmental swabs, stomach contents, vomitus, etc.) from January 2017 to January 2018. The presences of different PEDV were judged through the combinations of amplicon sizes.

3. Results

3.1. The optimized conditions of multiplex RT-PCR

The condition of the 25 μL multiplex RT-PCR reaction system was as followed: 12.5 μL of 2× Es Taq Master Mix, 1 μL of each ORF3-F and ORF3-R, 0.4 μL of the C/V-F and C-R, and 0.4 μL of the C/V-F and V-R primers (10 μM), and 4 μL of cDNA and 4.9 μL of distilled water. PCR reaction was performed as follows: 94 °C for 5 min, followed by 30 cycles of 95 °C for 35 s, 58.4 °C for 35 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min.

3.2. The sensitivity of single-plex RT-PCR and multiplex RT-PCR

The detection limits of the plasmids in single-plex RT-PCR were 1.27×10^3, 1.51×10^4, 2.44×10^3 and 2.82×10^3 copies/μL for p-va-PEDV-S1, p-cv-PEDV-S1, p-wild-PEDV-ORF3, p-cv-PEDV-ORF3 recombinant plasmids (Fig. 1A–D), respectively. Meanwhile, the detection limits for the multiplex RT-PCR were 1.27×10^4, 1.51×10^4, 2.44×10^3 and 2.82×10^3 copies/μL of DNA, respectively (Fig. 1E). Using viruses, the detection limits were $1 \times 10^{0.7}$ $TCID_{50}$/100 μL, $1 \times 10^{0.7}$ $TCID_{50}$ /100 μL, $1 \times 10^{1.7}$ $TCID_{50}$/100 μL and $1 \times 10^{1.7}$ $TCID_{50}$/100 μL for single-plex RT-PCR (Fig. 2A–D) and $1 \times 10^{1.7}$ $TCID_{50}$/100 μL for both variant and classical PEDV for multiplex RT-PCR (Fig. 2E). This means multiplex RT-PCR had the similar analytical sensitivity to that of single-plex RT-PCR.

3.3. Specificity of multiplex RT-PCR assay

The multiplex RT-PCR assay could detect the wild and vaccine strains of PEDV as the expected amplicon sizes were observed by electrophoresis analyses (Fig. 3). Absences of amplicons were observed when detection of PRRSV, CSFV, TGEV, RVA, PRV, and PCV2, indicating that there were no false detections.

3.4. Feasibility verification of multiplex RT-PCR method

From 388 samples, 104 and 112 were detected as PEDV positive by the established RT-PCR and conventional RT-PCR, respectively (Table 2). The Kappa value was 0.949, indicating a good consistency between the two methods.

3.5. Clinical application of the multiplex RT-PCR assay

From the total of 940 samples, 32.2% (303/940) were PEDV-positive. Among 303 PEDV positive samples, the numbers of positive samples for variant strains, classical wild strains, classical vaccine strains were 289, 5, 10, respectively. The detection of variant strains among PEDV-positive samples accounted for 95.38% (289/303), indicating that variant PEDV strains were prevalent in the diarrhea samples. Interestingly, one sample contained both classical vaccine and variant PEDV.

The infection rate of PEDV was age-dependent (Table 3). Although PEDV variant strains were detected in samples from pigs at all stages, the highest detection rate of 45.4% was observed in suckling pigs followed by that of sows. PEDV could also be detected from some items (6/60, 10.00%) used within the farms such as staff shoes and vehicles indicating that these items may play an important role in PEDV transmission in the farms.

The detection of various PEDVs varied from different provinces (Table 4 and Fig. 4). From the samples collected from 15 provinces, PEDV variant strains could be detected in 13 provinces except Yunnan and Jiangsu province (Table 4). PEDV classical vaccine strains (CV777 vaccine strain) and emerging variant wild strains were detected in Anhui, Guangdong, Hebei, Hubei, Jilin, Shanxi[2] and Sichuan Provinces, and classical wild strains and variant strains were detected in Henan and Jiangxi Province. The sample that contained classical vaccine and variant PEDV was collected from Shanxi[2] province. From the detection data, geographical distributions of PEDV were identified (Fig. 4).

4. Discussion

Due to the emerging outbreaks of PED in China and co-existence of PEDV with different genomic backgrounds that cause confusion on epidemiology and vaccine selection, a multiplex RT-PCR assay was developed to distinguish three PEDV strains. The method could detect $1 \times 10^{1.7}$ $TCID_{50}$/100 μL of PEDV and 1.51×10^4 copies/μL of cDNA. There was no cross-amplification either among variant, classical wild and vaccine strains or with the other six swine pathogens. Classical wild strains, classical vaccine strains (CV777) and variant strains can be detected quickly, sensitively and specifically.

The targets for amplification are very important in development of PCR. ORF3 gene is an important gene for distinguishing classical attenuated strains from wild strains (Park et al., 2008; Wang et al., 2012). The cell culture adapted PEDV (CV777) contains 49-nt deletions in ORF3 gene (Chen et al., 2010). It was used as a bio-marker to differentiate classical attenuated vaccine strains and classical field strains (Zhu et al., 2016a,b). The S gene, as a variable gene, is very important in understanding genetic variations of PEDV strains (Chen et al., 2013; Tian et al., 2014). Multiple variations in the S1 of S gene are the basis for distinguishing the PEDV into two genotypes: GI (the classical strains) and GII (the emerging variant strains) (Chen et al., 2016; Sun et al., 2015), so it can be used as the genetic marker to differentiate

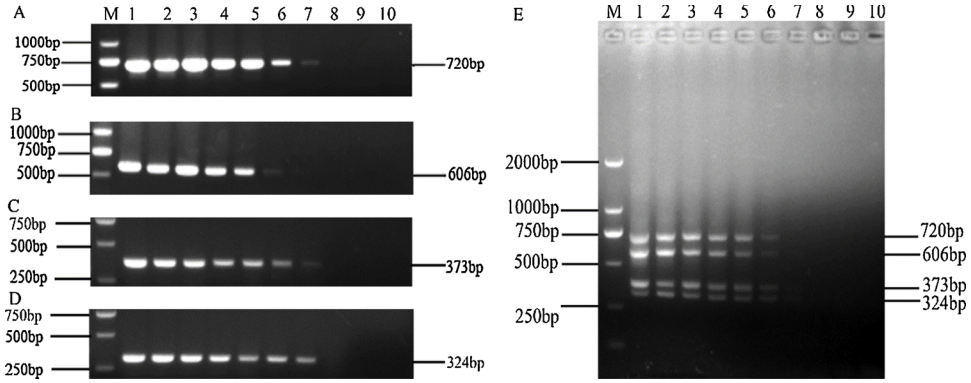

Fig. 1. The analytical sensitivity of the singleplex and multiplex RT-PCR for p-va-PEDV-S1, p-cv-PEDV-S1, p-wild-PEDV-ORF3, p-cv-PEDV-ORF3 recombinant plasmids. Four recombinant PEDV plasmids were serially diluted 10-fold to assess assay sensitivity. (A) p-va-PEDV-S1. M: DL2000 DNA Marker; lane 1–9:1.27 × 10^9–1.27 × 10^1 copies/μL; 10: negative control; (B) p-cv-PEDV-S1. M: DL2000 DNA Marker; lane 1–9: 1.51 × 10^9–1.51 × 10^1 copies/μL; 10: negative control; (C) p-wild-PEDV-ORF3. M: DL2000 DNA Marker; lane 1–9: 2.44 × 10^9–2.44 × 10^1 copies/μL; 10: negative control; (D) p-cv-PEDV-ORF3. M: DL2000 DNA Marker; lane 1–9: 2.82 × 10^9–2.82 × 10^1 copies/μL; 10: negative control; (E) Four mixed (S1 and ORF3) recombinant PEDV plasmids. M: DL2000 DNA Marker; lane 1–9: 10^9–10^1 copies/μL; 10: negative control.

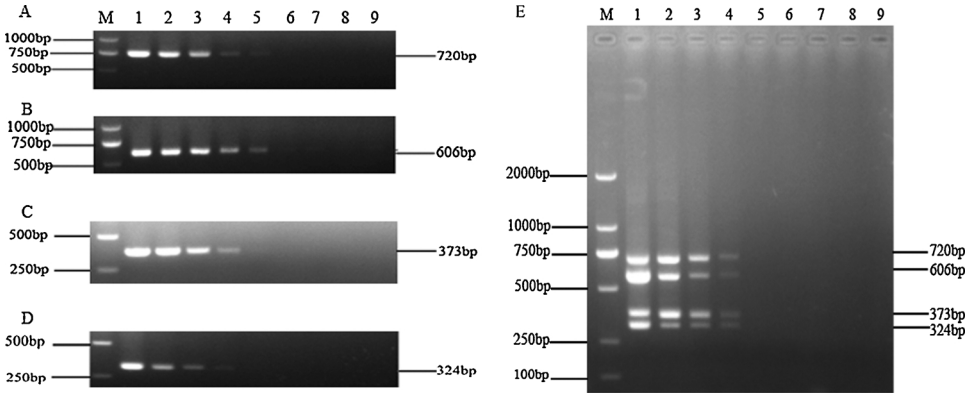

Fig. 2. The analytical sensitivity of the singleplex and multiplex RT-PCR for viral detection. Fig. 2A–D: Attenuated PEDV CV777 and variant PEDV YN144 were 10-fold diluted for RNA extraction and cDNA synthesis. M: DL2000 DNA Marker; lane 1-8: 1 × $10^{4.7}$ $TCID_{50}$/100 μL-1 × $10^{-2.3}$ $TCID_{50}$/100 μL; 9: negative control; (A) Amplicons from cDNA of YN144 with V-F/R primer pairs; (B) Amplicons from attenuated CV777 cDNA using C-F/R primer pairs; (C) and (D) Amplicons using cDNAs of attenuated YN144 and attenuated CV777 as templates with ORF3-F/R primer pairs, respectively; (E) Amplicons using mixed cDNAs of attenuated CV777 and YN144 as templates with designed primer pairs (C-F/R, V-F/R, ORF3-F/R). M: DL2000 DNA Marker; lane 1-8: 1 × $10^{4.7}$ $TCID_{50}$/100 μL-1 × $10^{-2.3}$ $TCID_{50}$/100 μL; 9: negative control.

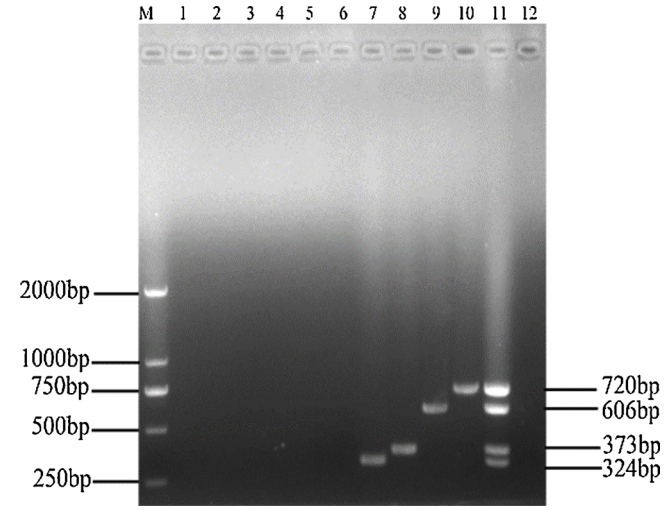

Fig. 3. The specificity test of multiplex RT-PCR. M: DL2000 DNA Marker; 1: PRRSV; 2: CSFV; 3: TGEV; 4: RVA; 5: PRV; 6: PCV2; 7: p-cv-PEDV-ORF3 recombinant plasmid; 8:p-wild-PEDV-ORF3 recombinant plasmid; 9: p-cv-PEDV-S1 recombinant plasmid; 10: p-va-PEDV-S1 recombinant plasmid; 11: Mixture of plasmids harboring S1 plus ORF3; 12: negative control.

variant PEDV strains (Liu and Wang, 2016; Su et al., 2018). Therefore, the sequence features of spike (S) gene and ORF3 gene of PEDVs were used as the genetic marker in this study, aiming to differentiate kinds of PEDV with different genetic backgrounds.

The multiplex RT-PCR and conventional RT-PCR method were used to detect 388 samples from diarrhea pigs. Eight PEDV weak positive samples that were detected by conventional method could not be identified by the established method. However, there was still a good consistence between conventional RT-PCR and the developed multiplex RT-PCR indicated by high Kappa value. The advantage of the newly developed method over conventional one is ability of accurately, effectively and simultaneously identifying different strains of PEDV, so it is a suitable method for clinical detection of PEDV.

The method was applied to clinical detection and revealed the epidemic characteristics of PEDV in China. Within the geographical scope of the sampling, the main prevalent PEDV strain was variant strain, consistent with results of recent studies (Chen et al., 2016; Li

Table 2
Consistency between multiplex and conventional RT-PCR.

Kappa test		Conventional RT-PCR		Total
		+	–	
Multiplex RT-PCR	+	104	0	104
	–	8	276	284
Total		112	276	388

Table 3
The infection of PEDV in pigs at different growth stages.

Growth stage	No.of tested samples	No.of Positive samples	Positive rate
suckling piglet	502	228	45.4%
Sow	177	36	20.3%
weaned pig	152	28	18.4%
finishing pig	49	5	10.2%

Table 4
Detection of various PEDVs samples collected from some provinces of China.

Provinces	No.of				
	Samples tested	Positive samples	PEDV variant strains	PEDV classical vaccine strains	PEDV classical wild strains
Anhui	65	3	2	1	0
Fujian	24	9	9	0	0
Guangdong	31	16	15	1	0
Hebei	143	46	43	3	0
Henan	141	30	27	0	3
Hubei	201	80	78	2	0
Yunnan	10	0	0	0	0
Jiangsu	58	0	0	0	0
Jiangxi	100	40	38	0	2
Jilin	3	3	2	1	0
Liaoning	5	3	3	0	0
Shandong	6	3	3	0	0
Shanxi[1]	36	23	23	0	0
Shanxi[2]	34	8	8	1	0
Sichuan	83	39	38	1	0
Total	940	303	289	10	5

et al., 2012; Sun et al., 2015). The classical vaccine strains and variant strains were detected in Anhui, Guangdong, Hebei, Hubei, Jilin, Shanxi[2] and Sichuan Province, and the classical wild and variant PEDV strains were detected in Henan and Jiangxi provinces. PEDV classical vaccine and variant strains were detected only in suckling piglets and sows. Therefore, all pig farmers should pay attention to the immunological prevention of suckling piglets and sows. However, the diarrhea samples detected in this study were collected mostly from pig farms located at Central, North and Southwest China, but fewer from Northeast, Northwest and South China. Therefore, more samples from these provinces should be collected in the later period of clinical detection, so as to comprehensively analyze the epidemic characteristics of PEDV in China.

The variations in the novel PEDV, which was prevalent in China, contribute to immune failure from the CV777-based vaccine. In order to effectively prevent and control PED, immunoprophylaxis measures should be taken according to the genotype and characteristics of the endemic strains in different provinces and pig farms. The farmers should use genetically-matched vaccine, on the basis of understanding the true genotype of PEDV after testing the samples by this in-house method, to provide effective protection for the pigs.

In conclusion, a differentiate detection method for PEDV variant strains, classical wild strains and classical vaccine strains was successfully established with good repeatability, susceptibility and specificity, and was further clinically evaluated in this study. In particular, rapidly simultaneous differentiation of CV777-like wild strain and emerging variant strain will provide a clue for pig farmers to select suitable vaccine aiming to better prevention of PED.

Acknowledgments

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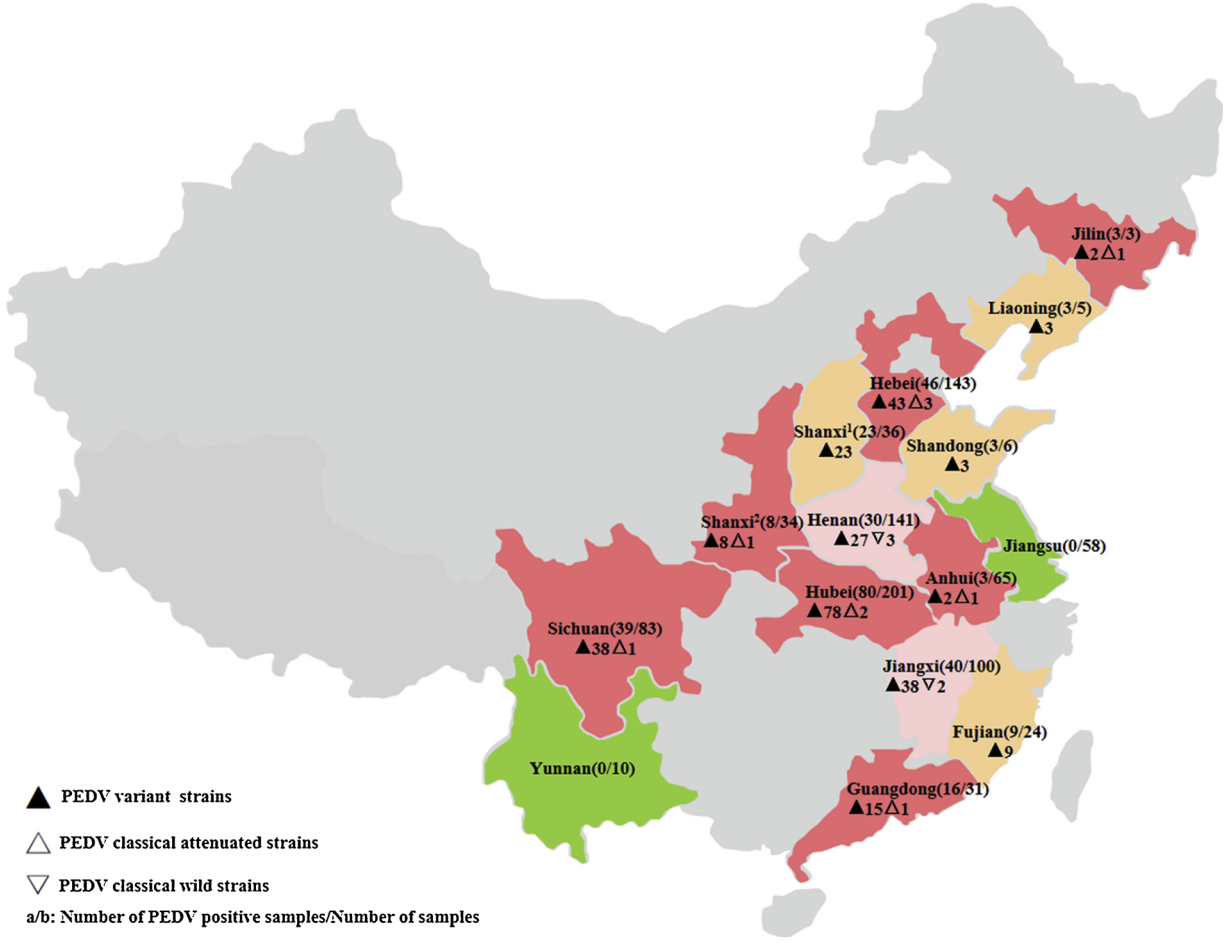

Fig. 4. Geographical distributions of PEDVs detected in China.

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