

# Differentiation of PEDV Classical Attenuated Vaccine Strains from Wild-type Strains using One-Step Real-Time Fluorescent Reverse Transcription PCR Assay Targeting ORF1 Nucleotides Deletion Region

**Zhilin Wang**

Lanzhou Veterinary Research Institute

**Xuerui Li**

Lanzhou Veterinary Research Institute

**Youjun Shang**

Lanzhou Veterinary Research Institute

**Jinyan Wu**

Lanzhou Veterinary Research Institute

**Zhen Dong**

Lanzhou Veterinary Research Institute

**Wanning Wang**

Lanzhou Veterinary Research Institute

**Yongsheng Liu**

Lanzhou Veterinary Research Institute

**Xi Lan** (✉ [lanxi@caas.cn](mailto:lanxi@caas.cn))

Lanzhou Veterinary Research Institute <https://orcid.org/0000-0001-7073-8818>

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## Research

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# Abstract

## Background

Porcine epidemic diarrhea virus (PEDV) is a pathogen causing serious disease and resulting in severe economic losses in the swine industry. In recent years, although China has adopted a large-scale vaccine immunization strategy, many types of PEDV strains, including classical attenuated vaccine strains, have been discovered in the immunized pig herds. Therefore, monitoring the prevalence of different types of PEDV strains is particularly important for the production of pigs and the safety evaluation of related attenuated vaccines

## Methods

In the study, a one-step real-time fluorescent reverse transcription PCR (one-step real-time RT-PCR) assay targeting 24-nucleotide deletion in the ORF1 region of three PEDV classical attenuated vaccine strains (derived from classical strains) was established, which could effectively distinguish PEDV classical attenuated vaccine strains and wild-type strains.

## Results

In our study, the RNA detection limits for PEDV wild-type strains and classical attenuated vaccine strains were  $3.0 \times 10^3$  copies and  $3.0 \times 10^2$  copies, respectively. This assay was highly specific for PEDV, with no cross-reactivity for other viruses, causing diarrheal disease. A total of 117 swine fecal samples were analysed by this established real-time RT-PCR assay, indicating that classical attenuated vaccine strains were present in the swine herds in Gansu province, China. Additionally, a pair of primers and two probes of the established assay can be placed in one reaction tube to distinguish PEDV classical attenuated vaccine strains and wild-type strains.

## Conclusion

Our results provided an effective and cheap technology platform for clinical rapid identification testing and epidemiological investigations of PEDV wild-type strains and classical attenuated vaccine strains

## Background

Porcine epidemic diarrhea virus (PEDV), an enveloped virus belonging to the genus *Alpha coronavirus* and the family *Coronaviridae*, can induce acute diarrhea and vomiting in newborn suckling and weaning pigs[1]. In 1971, PED was first reported among feeder and fattening pigs in England[2]. Since then, the diarrheal disease caused by PEDV has broken out in many regions of the world, including China[3-5]. After 2010, the highly pathogenic PEDV variant strains cause the morbidity rate of newborn suckling piglets to reach 100%, with mortality rates of 80–100%, which result in significant economic losses in the global swine industry[6-8]. Since the 1990s, many Chinese pig farms have adopted vaccination measures to prevent the spread of the disease[9]. At present, CV777-based live attenuated vaccine and

PEDV(CV777) and transmissible gastroenteritis virus (TGEV)-based dual attenuated vaccines have been extensively applied for PED prevention in China[10, 11]. There is a short period of carrying attenuated vaccine virus in the orally immunized pig population, and the possibility that the wild-type strains and the attenuated vaccine strains will exist simultaneously in the pig herds[12, 13]. In recent study, multiple strains were also found in many immunized pig population in China[4, 14, 15], and phylogenetic analysis of the whole genome also showed that there are multiple variation sites in PEDV genome[16, 17]. The antigenicity of vaccines derived from classical vaccine strains may be altered due to these genetic mutations, resulting in low vaccination efficiency and inability to protect pigs from variant PEDV strains[16]. Those have an impact on accuracy of the epidemiological survey data of the disease to a certain extent.

Porcine epidemic diarrhea virus (PEDV) is a positive-sense, single-stranded virus RNA with a genome of about 28 kb in length[1]. The PEDV genome comprises seven open reading frames in the order of ORF1a, ORF1b, spike (S) gene, accessory gene ORF3, envelope (E) gene, membrane (M) gene and nucleocapsid (N) gene[3, 18, 19]. The ORF1a and ORF1b region at the 5'end of the genome occupy 2/3 of the entire genome, and the two viral replicase polyproteins (pp1a and pp1ab) encoded by them are cleaved into 16 non-structural proteins (nsp1-nsp16), which are involved in the synthesis and transcription of viral RNA[20]. Among them, the nsp3 is the largest non-structural protein and also a multifunctional protein, which may be closely related to virus replication and transcription[21, 22]. A recent variation analysis of 49 PEDV entire-genome sequences in GenBank showed that the N-terminal domain of PEDV nsp3 is a hypervariable region and relatively conservative, and the characteristic mutation sites in this region may potentially serve as markers for the classification of PEDV different genotypes[16, 23]. Currently, many molecular detection methods targeting N, M, S, ORF3 genes have been developed in the laboratory[24-27], such as traditional PCR[13, 28], nanoparticle-assisted RT-PCR[29], and real-time RT-PCR[12, 27, 30] methods to detect or distinguish the wild-type strains (classical wild strains and variant strains) and attenuated vaccine strains, these methods is useful for understanding the epidemic status of different strains. In previous studies, researchers rarely used the ORF1 region as the genetic characteristics to distinguish PEDV attenuated vaccine strains and wild-type strains. In present study, we found that N-terminal domain of ORF1 region nsp3 in the three PEDV classical attenuated vaccine strains (PEDV attenuated vaccine strain KC189944, attenuated CV777 and DR13) and five Vero-cell-adapted isolates (JS2008, SDM, SQ2014, SC1402, HLJBY) bore a 24-nucleotide deletion (see Fig.1 and Table S1 ) compared with 38 PEDV wild-type strains whose sequences were available in GenBank[31]. These three attenuated vaccine strains and five Vero cell-adapted isolates were artificially cell-passaged and were not original strains in nature. Unless they are used as live attenuated vaccines for vaccination and spread to the pig population, it is impossible to detect exactly the same strains in the field. Due to these discoveries, we developed a convenient, cheap, efficient, secure and reliable one-step real-time RT-PCR assay to distinguish PEDV classical attenuated vaccine strains and wild-type strains.

## Methods

### Viruses and clinical samples

PEDV attenuated vaccine strain CV777 and Vero-cell-adapted isolate JS2008 were passaged in Vero E6 cell. Nucleic acid products and clinical samples of PEDV wild-type strain DX, transmissible gastroenteritis virus (TGEV), porcine circovirus type 2(PCV-2), Porcine deltacoronavirus(PDCoV), Porcine parvovirus (PPV), and porcine kobuvirus (PKV) were preserved in our laboratory. All 117 swine fecal samples with suspected PEDV infection were obtained from seven swine farms in Lanzhou, Dingxi, Baiyin, Jiayuguan, Linxia and Tianshui, Gansu province, China, between October 2015 and June 2018. All samples were stored at -80°C until use.

### DNA/RNA extraction

All clinical samples were centrifuged at 4000 g for 15 min and the supernatants were stored at -80°C. Viral RNA and DNA were extracted using the TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Version 5.0 (Takara, Dalian, China) according to the manufacturer's instructions. Viral RNA or DNA of each sample was eluted in 30 µL of RNase-free water. All RNA and DNA samples were stored at -80°C until use.

### Design of primers and probes

According to the presence of a 24-nucleotide deletion (Fig.1) in the ORF1 regions of three PEDV classical attenuated vaccine strains and five Vero-cell-adapted isolates, a pair of primers and two probes were designed (synthesized by Sangon Biotech, Shanghai, China) to differentiate PEDV classical attenuated vaccine strains from wild-type strains (Table 1). Additional primers and probes (Table S2) (synthesized by Sangon Biotech, Shanghai, China) were synthesized to detect PEDV, TGEV, PKV, PPV, PDCoV, and PCV-2 in the Fecal samples.

**Table1. Primers and probes used in the established real time RT-PCR assay**

Name	Primers or probes Sequence(5'-3')	Location	Length (bp)
F3365	GGTGGCAGATGTGGCTAACT	ORF1,3365-3384 <sup>a</sup>	
R3445	AATAAAGGACAAAGTTGCGGC	ORF1,3445-3425 <sup>a</sup>	185bp
P3390-W	FAM-AGGATGATGGTCTTAATGTAGCTCCTGAA-BHQ1	ORF1,3390-3418 <sup>b</sup>	
P3388-V	ROX-TGAGGCTGATGATGTAGAGTCTGAAGT-BHQ2	ORF1,3388-3414 <sup>a</sup>	
F-V	CACCGATCCTAATCTGCCCG	ORF1,3217-3236 <sup>a</sup>	
R-V	TGGACCAACTCTACCAGCAC	ORF1,3612-3632 <sup>a</sup>	415bp
F-W	ACACTATACTATCCCACCG	ORF1,2915-2932 <sup>b</sup>	
R-W	CACCCAAAGATCCCAAGA	ORF1,3690-3708 <sup>b</sup>	793bp

379. PEDV classical attenuated vaccine strain CV777, GenBank accession number KT323979.1

## Generation of RNA standards

The first strand PEDV classical attenuated vaccine strain CV777 and wild strain DX cDNA were synthesized by reverse transcription with PrimeScript™ first strand cDNA synthesis kit (Takara, Dalian, China). A PCR fragment of the PEDV ORF1 region was amplified using primers F-V/R-V (Table 1) from PEDV classical attenuated vaccine strain CV777 cDNA and named PEDV-V/qRT-PCR. Another PCR fragment of the PEDV ORF1 region was amplified using primers F-W/R-W (Table 1) from PEDV DX cDNA and named PEDV-W/qRT-PCR. The PCR reaction was performed with PrimeSTAR® Max DNA Polymerase kit (Takara, Dalian, China) and the reaction system as follows: 25µL PrimeSTAR® Max Premix (2X), 1.5µL each primer (50µM), 2µL cDNA, and ddH<sub>2</sub>O to a total volume of 50 µL in each PCR tube, and cycled as follows: 35 cycles of 98°C for 10 s, 55°C for 15s, and 72°C for 10s. Both recombinant plasmid DNA were constructed by cloning of two PCR fragments into the pET-30a vector (Gencreate, Nanjing, China) and were sequenced by TsingKe (Xian, China), respectively. Both recombinant plasmids were linearized with *NdeI* (Takara, Dalian, China), purified using the TaKaRa MiniBEST DNA Fragment Purification Kit Version 4.0 (Takara, Dalian, China), and both PEDV RNAs were transcribed *in vitro* using the RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI, USA). The length and integrity of transcribed both standard PEDV RNAs *in vitro* were verified by Agarose gel electrophoresis. The concentration of both RNA standards were measured using a ND-2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and the RNA copy number was calculated as follows:  $(6.02 \times 10^{23} \text{ molecules/mole}) \times (\text{RNA concentration}) / (340 \times \text{number of bases})$ .

## The one-step real-time RT-PCR assay

Real-time RT-PCR assay was carried out using a Bio-Rad CFX Manager (Bio-Rad, USA) instrument. The primers and probes were designed to amplify the PEDV gene ORF1 region in this study or the PEDV S1 domain [12]. The reactions were carried out using the One Step PrimeScript® RT-PCR Kit (Perfect Real Time) (Takara, Dalian, China) with reaction system as follows: 2×One Step RT-PCR Buffer 12.5µL, TaKaRa Ex Taq HS (5 U/µL) 0.5µL, PrimeScript RT Enzyme Mix 0.5µL, each primer (10µM) 0.5µL, each probe (10µM) 1µL, 1µL of viral RNA/DNA or 4µL sample RNA, and ddH<sub>2</sub>O to a total volume of 25 µL in each PCR tube, and cycled as follows: 42°C for 5 min, 95 °C for 10s, then 40 cycles of 95 °C for 5 s and 60 °C for 31s.

## The one-step RT-PCR assay

Eight pairs of primers (Table S2) were used for RT-PCR with the PrimeScript™ One Step RT-PCR Kit Ver. 2.0 (Takara, Dalian, China) to detect of major diarrhoeal viruses (PEDV, TGEV, PKV, PPV, PDCoV, and PCV-2) in 117 samples of suspected PEDV infection. This RT-PCR reaction system as follows: PrimeScript one Step Enzyme Mix 2µL, 2×one Step Buffer 25 µL, each primer 10pmol, 1µL of viral RNA/DNA or 4µL sample RNA/DNA, and ddH<sub>2</sub>O to a total volume of 50 µL in each PCR tube, and cycled as follows: 50°C for 45min,

94°C for 2 min, followed by 35 cycles of 95°C for 5 s, 55°C for 30s, and 72°C for 60s, with a final extension at 72°C for 10 min.

### **Specificity and sensitivity analysis of PEDV one-step real-time RT-PCR assay**

All viral RNA and DNA samples were quantitated using a ND-2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA) in our laboratory. Ten nanograms of RNA or DNA extracted from PEDV classical attenuated vaccine strain CV777, Vero-cell-adapted isolate JS2008, PEDV DX, TGEV, PKV, PPV, PDCoV, and PCV-2 in three replicates was used as template for specificity analysis of PEDV one-step real-time RT-PCR assay. PEDV one-step real-time RT-PCR assay was performed using a Bio-Rad CFX Manager instrument (Bio-Rad, USA).

For sensitivity analysis of PEDV one-step real-time RT-PCR assay, ten-fold serial dilutions of both RNA standards were used as template (range:  $3.0 \times 10^{10}$ – $3.0 \times 10^1$  copies). One microliter of each PEDV RNA standard serial dilution (range:  $3.0 \times 10^1$ – $3.0 \times 10^{10}$  copies) was applied to evaluate the dynamic detection range of one-step real-time RT-PCR assay. Each experiment was repeated three times and regression analysis was performed using Bio-Rad CFX Manager (Bio-Rad, USA) to determine detection limits.

### **Analysis of clinical samples using PEDV real-time RT-PCR and RT-PCR assay**

A total of 117 fecal samples were collected from seven pig farms with the background of immunizing with CV777-based monovalent or bivalent attenuated vaccines, six of which from six piglets with oral attenuated vaccine CV777 and showed no clinical symptoms of diarrhea, and the remaining 111 fecal samples were obtained from 111 piglets with clinical status of diarrhea. All fecal samples were used to evaluate the reliability of the established PEDV one-step real-time RT-PCR assay. Another real-time RT-PCR and RT-PCR assays were compared, and all results were shown in Table 3. All positive PCR products were sequenced by TsingKe (XiAn, China).

## **Results**

### **Specificity, sensitivity, and repeatability analysis of PEDV one-step real time RT-PCR assay**

For the specificity of PEDV one-step real time RT-PCR assay, only PEDV wild-type strain DX showed a fluorescent signal using the primers F3365/R3445 and probe P3390-W (Fig.2.A). Only PEDV classical attenuated vaccine strain CV777 or Vero-cell-adapted isolate JS2008 showed fluorescent signals using the primers F3365/R3445 and probe P3388-V (Fig.2.B). However, TGEV, PKV, PPV, PDCoV, and PCV-2 showed no fluorescent signals in this assay, indicating good specificity (Fig.2). The primers F3365/R3445, probes P3390-W, and P3388-V could be combined in a tube for simultaneously detecting PEDV attenuated vaccine strain CV777 and PEDV wild-type strain DX, without cross-reactivity for each other (Fig.3).

The detection limits for PEDV wild-type strains and classical attenuated vaccine strains were  $3.0 \times 10^3$  copies/reaction (Fig.4) and  $3.0 \times 10^2$  copies/reaction, respectively (Fig.5). Using 10-fold serial dilutions of RNA standard as template, each experiment was repeated three times, to establish standard curves for PEDV wild-type strains (Fig.4.B) and classical attenuated vaccine strains (Fig.5.B). As shown in Table 2, the detection limit of the PEDV wild-type strains were  $3.0 \times 10^3$  RNA copies/reaction for PEDV wild-type strain DX standard RNA, and the detection limit of the PEDV classical attenuated vaccine strains were  $3.0 \times 10^2$  RNA copies/reaction for PEDV classical attenuated vaccine strain CV777 standard RNA. The repeatability of this established assay was evaluated using two ORF1 region standard RNAs of PEDV classical attenuated vaccine strain CV777 and wild-type strain DX, respectively, with coefficients of variation 0.50-6.8 (Table 2).

**Table 2. The sensitivity and repeatability of established PEDV real-time RT-PCR assay**

Standard RNA	Copy number	Cq (mean±S.D.)	CV%	Standard RNA	Copy number	Cq (mean±S.D.)	CV%
ORF1 of PEDV DX <sup>a</sup>	$3.0 \times 10^8$	13.30±0.52	3.90	ORF1 of PEDV CV777 <sup>b</sup>	$3.0 \times 10^8$	12.49±0.85	6.8
	$3.0 \times 10^7$	16.17±0.59	3.65		$3.0 \times 10^7$	16.41±0.09	0.55
	$3.0 \times 10^6$	18.37±0.26	1.42		$3.0 \times 10^6$	19.19±0.33	1.72
	$3.0 \times 10^5$	21.67±0.33	1.52		$3.0 \times 10^5$	22.76±0.24	1.05
	$3.0 \times 10^4$	24.72±0.38	1.54		$3.0 \times 10^4$	25.42±0.27	1.06
	$3.0 \times 10^3$	26.15±0.26	0.94		$3.0 \times 10^3$	29.15±0.29	0.99
	$3.0 \times 10^2$	None	None		$3.0 \times 10^2$	32.12±0.16	0.50
	$3.0 \times 10^1$	None	None		$3.0 \times 10^1$	None	None
	$3.0 \times 10^0$	None	None		$3.0 \times 10^0$	None	None
	NCT	None	None		NCT	None	None

a, Related to the ORF1 region of PEDV wild-type strain DX, the Standard RNA as template was used for detection of PEDV wild-type strains.

b, Related to the ORF1 region of PEDV classical attenuated vaccine strain CV777, the Standard RNA as template was used detection of PEDV classical attenuated vaccine strains

### Evaluation of real time RT-PCR and RT-PCR assays using clinical samples

117 fecal samples from 117 pigs with suspected PEDV infection were subjected to PEDV one-step real-time RT-PCR (a), real-time RT-PCR (b), and RT-PCR assays (c). PEDV wild-type strains were detected in 83.76, 83.76, and 79.80.% of all samples, respectively, and PEDV classical attenuated vaccine strains were detected in 6.84, 6.84 and 5.98% of all samples, respectively (Table 3). As shown in Table 3, of the 17 samples that tested negative in the RT-PCR assay(c), 11 samples were PEDV-negative and the remaining 6 samples were PEDV-positive (five wild-type strains and one classical attenuated vaccine strain) as detected by the one-step real-time RT-PCR (a) and real-time RT-PCR assays (b). As shown in Fig.6, through the analysis of the established one-step real-time RT-PCR assay, the viral load of 106 PEDV-positive samples was concentrated between  $10^{3.265}$ - $10^6$  copies/reaction. Sequencing results for all PEDV-positive samples indicated that the PEDV one-step real time RT-PCR assay (a) had high specificity and sensitivity. In addition, TGEV and PKV were detected in the 11 PEDV-negative samples and 8 PEDV-positive samples (detailed data no shown), indicating that the co-infections of multiple diarrhea viruses have occurred in some pig farms.

**Table 3. Results for 117 samples in the three assays.**

location in Gansu Province	Samples number	One-step qRT-PCR(a)		qRT-PCR(b)		RT-PCR(c)	
		wild strain	vaccine strain	wild strain	vaccine strain	wild strain	vaccine strain
DingXi	25	24	0	24	0	23	0
JiayuGuan	19	15	2	15	2	14	2
LinXia	23	17	3	17	3	16	2
TianShui	13	9	2	9	2	9	2
BaiYin	20	19	0	19	0	19	0
LanZhou	17	14	1	14	1	13	1
total	117	98	8	98	8	93	7
The positive rate		83.76%	6.84%	83.76%	6.84%	79.80%	5.98%

1. Method established in this study
2. Method from [Su et al., 2018\[12\]](#)
3. Method from [He et al., 2019\[13\]](#)

## Discussion

Despite the current vaccination policy in China, multiple types of PEDV strains still appeared in pig farms, especially the variant strains that began in southern China in 2010, causing huge losses to the pig industry[6, 13]. A recent phylogenetic tree study of Chinese PEDV strains and some PEDV representative strains of other countries revealed that the full-length genomic sequences can be divided into two independent subgroups, namely GI (classical strains, GI-a and GI-b) and GII (variant strains, GII-a, and GII-b)[17, 32]. The PEDV strains of the GI-b subgroup contained Vero-cell-adapted vaccine strains (PEDV attenuated vaccine KC189944, attenuated CV777 and DR13) and five Vero-cell-adapted isolates (JS2008, SDM, SQ2014, SC1402, HLJBY)[15, 17, 32-36]. By alignment analysis of ORF1 region sequence in the 46 PEDV strains released by GenBank (Fig.1 and Table S1 ), we found that three classical attenuated vaccine strains (KC189944, attenuated strain CV777 and DR13, GI-b) and Vero-cell-adapted isolates (JS2008, SC1402, SQ2014, HLJBY and SDM, GI-b) have a novel 24-nucleotide deletion in the N-terminal domain of ORF1 region nsp3 compared with 38 wild-type PEDV strains (GI-a and GII). PEDV Vero-cell-adapted vaccine strains belonging to GI-b subgroup not only have nucleotides variation in the ORF3[16], but there are also 24-nt deletions in the ORF1 region (Fig.1 and Table S1). Based on these discoveries, a one-step real-time RT-PCR assay was developed to distinguish PEDV classical attenuated vaccine strains and wild-type strains. The 24-nucleotide-deletion pattern in the ORF1 region of PEDV Vero cell-adapted strains could be the marker of adaptation to Vero cell culture, and it is a valuable tool for monitoring of the persistence of classical attenuated vaccine strains and epidemiologic research of PEDV infection in swine herds as well as stability and safety analysis of classical attenuated vaccines. However, there are some situations that need our attention. For example, since the ORF1 sequence of some candidate vaccines has not been reported, especially the vaccine candidates from highly virulent strains (genotype 2a) emerged after 2010, we are not sure whether the ORF1 of these vaccine candidates derived from non-classical attenuated vaccine strains have the same 24 nucleotides deletion pattern. If this pattern exists in all cell-adapted strains during continuous passages in Vero cells, it can be used as a genotyping marker just like nucleotide deletions of Spike or ORF3 genes in the Vero-cell-adapted strains[37, 38]. Moreover, multiple PEDV strains can co-exist in some co-infection events, and it is possible that the wild-type virus repairs the 24nt deleted region of the classical attenuated vaccine strain. If the 24 nucleotides deletion pattern of the attenuated vaccine strains is repaired, the method we established will not apply. Nevertheless, Commercial vaccines based on classical strains are extensively applied in Chinese pig farms and play a very important role in controlling PEDV infections, while vaccines derived from non-classical attenuated vaccine candidates are being developed and not yet commercially available. Therefore, our method is safe, accurate, and reliable, and can be applied to the identification of classical attenuated vaccine strains and the safety evaluation of attenuated vaccines in pig farms

The one-step real-time RT-PCR assay could only detect PEDV, with no cross-reactivity with other enteroviruses. The limits of detection for PEDV wild-type strains and classical attenuated vaccine strains in the one-step real-time RT-PCR assay were  $3.0 \times 10^3$  copies and  $3.0 \times 10^2$  copies, respectively. Compared with the other two methods (Table 3), the detection results of 117 fecal samples showed that the our established assay had highly PEDV positive diagnosis agreement with real-time RT-PCR(100%) and RT-PCR assays(95.6%) (Table 3), respectively, indicating that the established one-step real-time RT-PCR

assay have high sensitivity, rapidity, and accuracy. The detection results of 117 fecal samples in our established one-step real-time RT-PCR assay, 98 PEDV wild-type strains were detected, including PEDV classical wild strains and variants, 8 PEDV classical attenuated vaccine strains were detected, and TGEV and PKV were detected in the 11 PEDV-negative samples and 8 PEDV-positive samples (data not shown). The sequencing results of 8 positive samples tested as classical attenuated strains in this study showed that they have the same nucleotide deletion positions as the ORF1 and ORF3 fragments in the classical attenuated vaccine CV777. These results showed that PEDV attenuated vaccines have existed in Gansu province, China and the co-infections of multiple diarrhea viruses have occurred in some pig farms. Therefore, it is necessary to take measures to dynamically monitor the co-infection of other enteric pathogens with PEDV in pig farms. Besides, We did not successfully isolate live virus using Vero-E6 cells in the positive samples tested as PEDV classical attenuated vaccine strains. This may be due to low live virus content or only nucleic acid fragments in the sample.

## Conclusion

This one-step real-time RT-PCR assay was developed to distinguish between classical attenuated vaccine strains that were artificially inoculated and wild-type strains during epidemiological surveillance, and to provide technical support for collecting more accurate epidemiological data of PEDV infection. Since, various PEDV strains may coexist in the same environment, even the same pig, which may cause the vaccine strain to revert to virulence by genetic recombination with other types of PEDV strains. Thus, monitoring the spread of attenuated vaccine strains and changes in virus titer could provide an important reference for evaluating immune protection effect of attenuated vaccine strains and the prevention and control of PEDV. Additionally, our method only use a pair of primers and two probes that placed in one reaction tube to detect different types of PEDV strains, without cross-reactivity between each other. When distinguishing different types of PEDV strains in a sample, a tube of amplification enzyme, a pairs of primers, and a tube of PCR reaction tube can be save, and this also reduced the cost of detecting sample in the commercial application. Moreover, this method allows quantitative calculation of viral load, and has practical value for epidemiological investigations of wild-type strains and classical attenuated vaccine strains. Simultaneously, combined with other methods for detecting PEDV, it can be effective for the genotyping and prevalence of various strains of PEDV. Finally, it also represents an alternative detection tool for preliminary identification of clinical samples.

## Abbreviations

PEDV: Porcine epidemic diarrhea virus; PED: Porcine epidemic diarrhea; PCV-2: Porcine circovirus type 2; PDCoV: Porcine deltacoronavirus; PKV: Porcine kobuvirus; PPV: Porcine parvovirus; TGEV: Transmissible gastroenteritis virus; N: Nucleocapsid; S: Spike; ORF3: Open reading frame 3; E: Envelope; M: Membrane; nsp3: non-structural protein; S-INDEL: Insertion and deletion in the S gene; CV: Coefficients of variation; SD: Standard deviation; RT-PCR: Reverse transcription-polymerase chain re-action; PCR: Polymerase chain reaction

# Declarations

## Ethics approval and consent to participate

All animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the study was approved in writing by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese

Academy of Agricultural Sciences (No. LVRIAEC 2016–003). The Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences approved the collection of fecal samples after obtaining verbal consent from the swine farm owners

## Consent for publication

Not applicable.

## Author's contribution

Xi Lan collected the samples in Gansu Province and designed the study; Zhilin Wang performed the research; Zhilin Wang, Xuerui Li, Youjun Shang, Zhen Dong, Wanning Wang, and Yongsheng Liu analyzed data; Zhilin Wang drafted the manuscript. All authors read and agreed the final manuscript

## Conflict of interest

The authors declare that they have no competing interests. The work is an original paper and is not under consideration in other journals.

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## Availability of data and material

The data of this study are available from the corresponding author on reasonable request.

## Additional file

All data generated or analysed during this study are included in this published article (and its additional file Table S1 and Table S2)

Table S1. The name, accession number, and search website of the PEDV strain in the study.

Table S2. Additional primers and probes of detecting PEDV, TGEV, PKV, PPV, PDCoV, and PCV-2 in the study.

## Acknowledgements

Not applicable.

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## Figures

ORF1 Sequence strain	3080	3090	3010	3107	3131	3140	3150
				.....24nt-deletion.....			
CV777 KT323979.seq	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
DR13 vaccine JQ023162.seq	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
JS2008 KC210146.seq	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
JS2008 KC109141.seq	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
"SDM vero JX560761.seq"	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
"HLJBY KP403802.seq"	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
"KC189944.seq"	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
"SC1402 KP162057.seq"	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
SQ2014 KP728470.1seq	TGGCTAACTCTGAAACTGAGGCTGATG				ATGTAGAGTCTGAAGTTGAGGAAGTT		
AVCT12 LC053455.seq	TGGCTAACTCTGAACCTGGGGATGATG	GTCTTCCTGTGGCACCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CHM2013 KM887144.seq	TGGCTAACTCTGAACCTGGGGATGATG	GTCTTCCTGTGGCACCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
SM98 GU937797.seq	TGGCTAACTCTGAACCTGGGGATGATG	GTCTTCCTGTGGCACCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
LZC EF185992.seq	TGGCTAACTCTGAACCTGGGGATGATG	GTCTTCCTGTGGCACCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
EAS1 KR610991.seq	TGGCTAACTCTGAACCTGGGGATGATG	GTCTTCCTGTGGCACCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
GDS01 KM089829.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CHGD-01 JX261936.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
PEDV-7C KM609204.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
ZJC24 JX524137.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
SH MK841494.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
PC21A KR078299.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
DR13 JQ023161.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CH/HNAY/2015 KR809885.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
XY2013 KR818832.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CHHNQX-314 KR095279.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CH/HNYF/14 KP890336.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
PEDV-10F KM609206.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
PEDV-14 KM609207.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
JSHA2013 KR818833.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
JS-HZ2012 KC210147.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CHZJX-12012 KF840537.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
KJ777677.seq"	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
BJ-2011-1 JN825712.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
PEDV-8C KM609205.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
AH2012 KC210145.1.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CH/JX-2/2013 KJ526096.1.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
PEDV-CHZ KM609209.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
LNCT2 KT323980.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CH/FJND-3/2011 JQ282909.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CH/GDZQ/2014 KM242131.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
PEDV-WS KM609213.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CH/ZMDZY/11 KC196276.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CH/YNKM-8/2013 KF761675.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
FL2013 KP765609.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
LC JX489155.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
AJ1102 JX188454.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
YN1 KT021227.seq	TGGCTAACTCTGAGCTGAGGATGATG	GGTCTTAATGTAGCTCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		
CV777 AF353511.seq	TGGCTAACTCTGAACCTGGGGATGATG	GTCTTCCTGTGGCACCTGAAACAA			ATGTAGAGTCTGAAGTTGAGGAAGTT		

Figure 1

ORF1 regions alignment results of 38 PEDV wild-types strains and 8 Vero cell-adapted strains whose sequences were available in GenBank, and 24-nucleotide deletions in the ORF1 regions of three PEDV classical attenuated vaccine strains and five Vero-cell-adapted isolates.

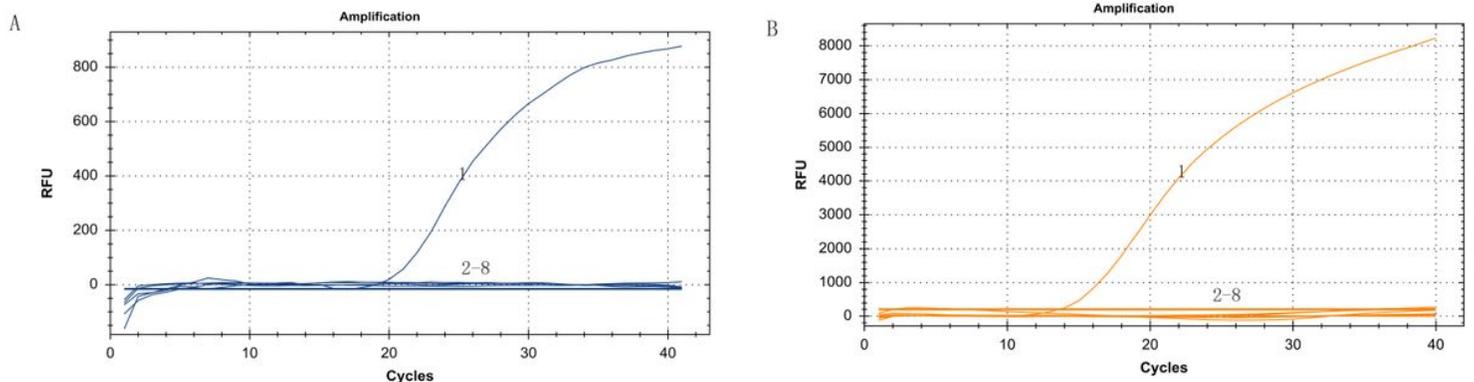


Figure 2

A. Specificity for PEDV wild-type strain of the one-step real time RT-PCR assay. line 1: PEDV DX; lines 2–8: PEDV classical attenuated vaccine strain CV777 or Vero-cell-adapted isolate JS2008, TGEV, PKV, PPV, PDCoV, and PCV-2 and negative control, respectively. B. Specificity for PEDV classical attenuated vaccine strains of the one-step real time RT-PCR assay. line 1: PEDV classical attenuated vaccine strain CV777 or Vero-cell-adapted isolate JS2008; lines 2–8: PEDV DX, TGEV, PKV, PPV, PDCoV, and PCV-2, and negative control, respectively.

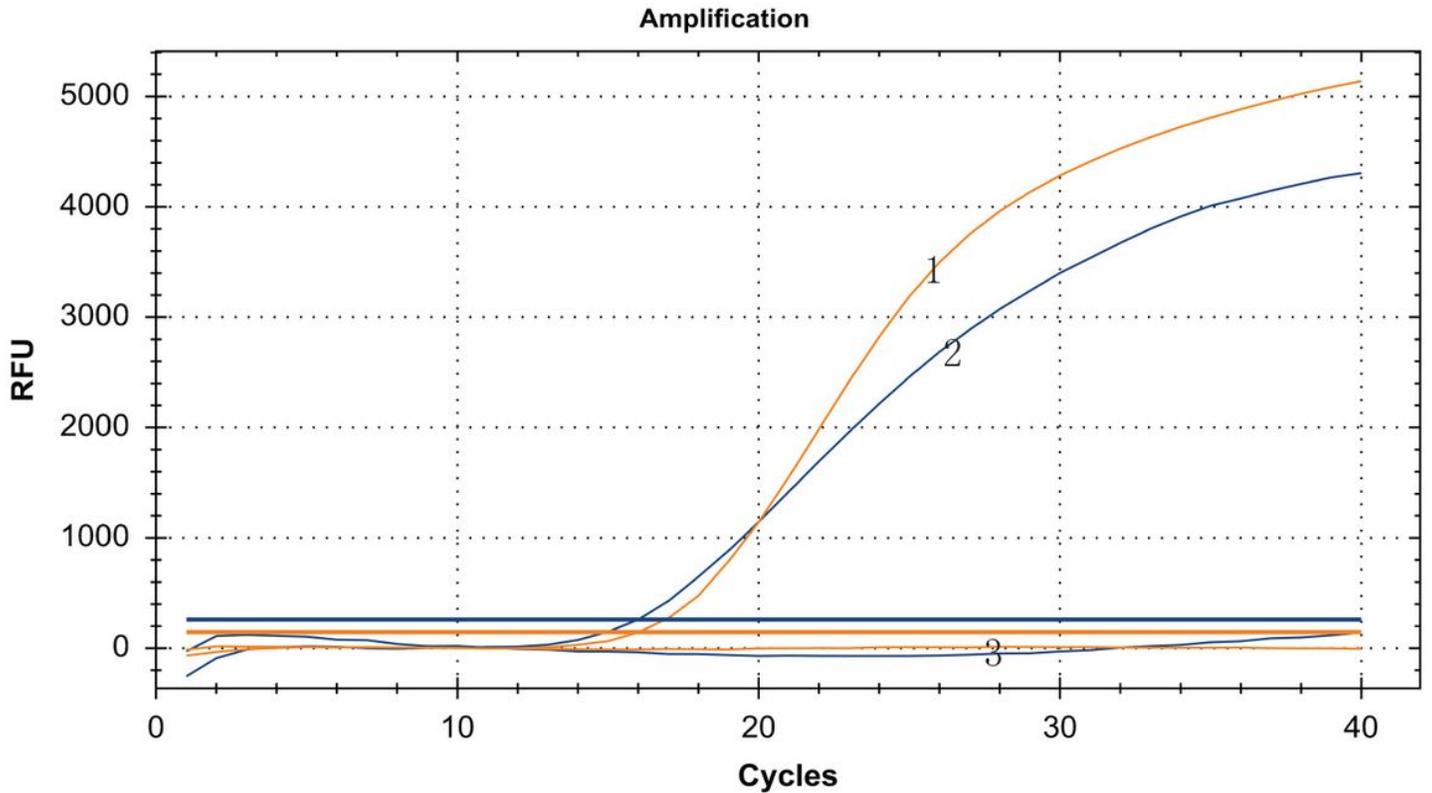


Figure 3

Specificity of primers and probes in a PCR reaction tube: 1–2: PEDV classical attenuated vaccine strain CV777 RNA, Vero-cell-adapted isolate JS2008, wild-type strain DX RNA, two probes, and a pair of primers were placed in a PCR reaction tube ; 3: ddH<sub>2</sub>O, two probes, and a pair of primers.

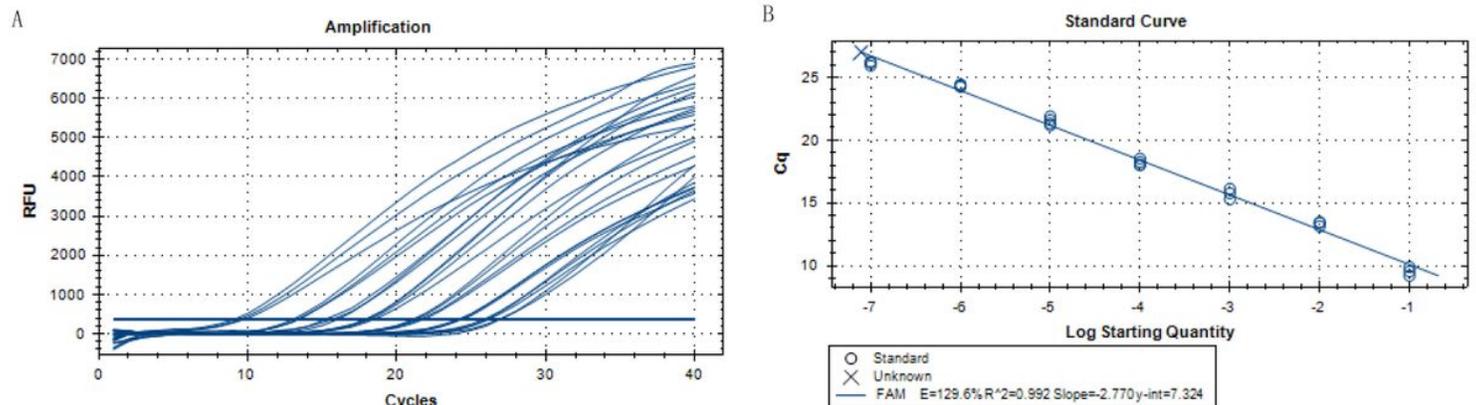
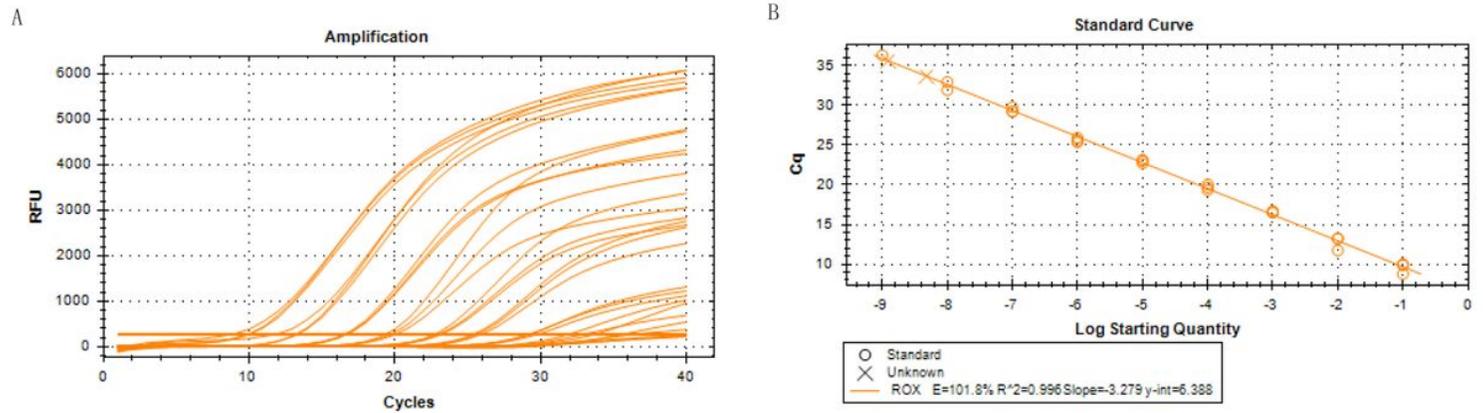


Figure 4

Sensitivity for PEDV wild-type strains of the one-step real-time RT-PCR. A. Fluorescence development over time using a dilution range of  $10^7$ – $10^1$  copies of the RNA standard (dilution gradient of  $10^{-1}$ – $10^{-7}$ ). B. Standard curves of detection of PEDV wild-type strains in the one-step real-time RT-PCR assay. Linear regression of the data provided a formula between RNA copy number (The abscissa represent 10-fold dilution gradient ) and Cq (Ct).



**Figure 5**

Sensitivity for PEDV classical attenuated vaccine strains of the one-step real-time RT-PCR. A. Fluorescence development over time using a dilution range of  $10^9$ – $10^1$  copies of the RNA standard (dilution gradient of  $10^{-1}$ – $10^{-9}$ ). B. Standard curves of detection of PEDV classical attenuated vaccine strains in the one-step real-time RT-PCR assay. Linear regression of the data provided a formula of between RNA copy number and Cq(Ct).

## PEDV viral load

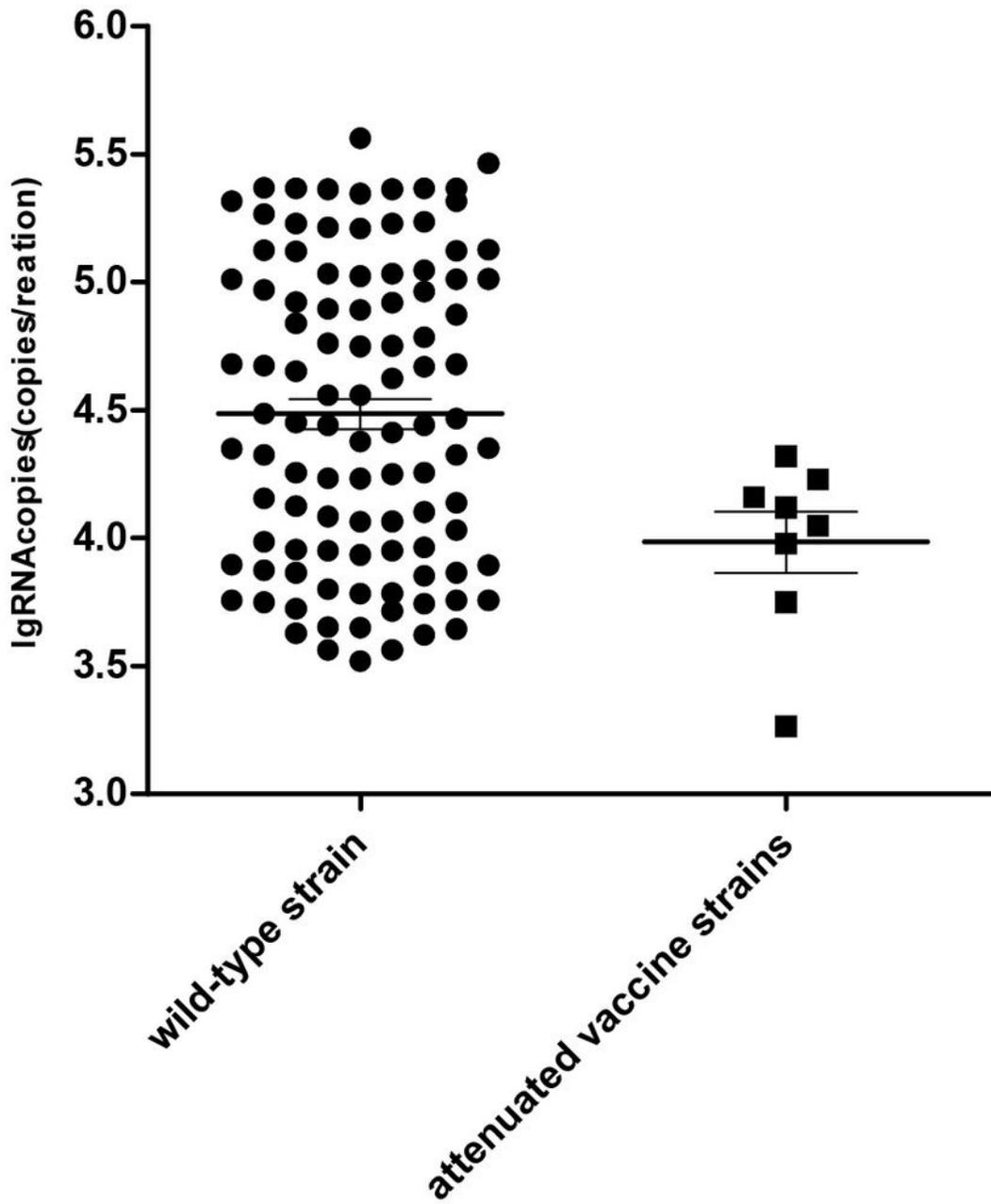


Figure 6

PEDV viral load of each PEDV-positive clinical sample in the established real time RT-PCR assay in this study

## Supplementary Files

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