

ORIGINAL ARTICLE

Development of a multiplex RT-PCR for the detection of major diarrhoeal viruses in pig herds in China

Guangming Ding^{1,2} | Yuguang Fu¹ | Baoyu Li¹ | Jianing Chen¹ | Jianlin Wang² |
Baishuang Yin³ | Wanli Sha³ | Guangliang Liu¹ 

¹State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China

²School of Life Sciences, Lanzhou University, Lanzhou, China

³College of Animal Science and Technology, Jilin Agricultural Science and Technology University, Jilin, China

Correspondence

Guangliang Liu, State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 1 Xu-Jia-Ping, Yan-Chang-Bu, Cheng-Guan District, Lanzhou 730046, Gansu, China.
Email: LiuGuangliang01@caas.cn

Funding information

This work was supported by National Key R&D Program of China (2016YFD0500103), National Natural Science Foundation of China (31572498, 31702209, 31972689), the Elite Youth Program of CAAS, and partly by China Central Public-interest Scientific Institution Basal Research Fund (1610312018002).

Abstract

The major enteric RNA viruses in pigs include porcine epidemic diarrhoea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine rotavirus A (PRV-A), porcine kobuvirus (PKV), porcine sapovirus (PSaV) and porcine deltacoronavirus (PDCoV). For differential diagnosis, a multiplex RT-PCR method was established on the basis of the N genes of TGEV, PEDV and PDCoV, the VP7 gene of PRV-A, and the polyprotein genes of PKV and PSaV. This multiplex RT-PCR could specifically detect TGEV, PEDV, PDCoV, PRV-A, PKV and PSaV without cross-reaction to any other major viruses circulating in Chinese pig farms. The limit of detection of this method was as low as 10^0 – 10^1 ng cDNA of each virus. A total of 398 swine faecal samples collected from nine provinces of China between October 2015 and April 2017 were analysed by this established multiplex RT-PCR. The results demonstrated that PDCoV (144/398), PSaV (114/398), PEDV (78/398) and PRV-A (70/398) were the main pathogens, but TGEV was not found in the pig herds in China. In addition, dual infections, for example, PDCoV + PSaV, PDCoV + PRV-A, PRV-A + PSaV and PEDV + PDCoV, and triple infections, for example, PDCoV + PRV-A + PSaV and PEDV + PDCoV + PKV, were found among the collected samples. The multiplex RT-PCR provided a valuable tool for the differential diagnosis of swine enteric viruses circulating in Chinese pig farms and will facilitate the prevention and control of swine diarrhoea in China.

KEYWORDS

diarrhoea, multiplex RT-PCR, swine enteric viruses

1 | INTRODUCTION

Viral diarrhoea seriously endangers the pig industry throughout the world and is characterized by acute diarrhoea, vomiting, dehydration and high mortality in neonatal piglets, resulting in enormous economic losses. The most common and traditional causative agents responsible for diarrhoea in pigs are porcine epidemic diarrhoea virus (PEDV), transmissible gastroenteritis virus (TGEV) and group A porcine rotavirus (PRV-A). In particular, PEDV variant strains with extremely high virulence to piglets emerged in the pig population

in China in 2011 (Li et al., 2012; Sun et al., 2012) and then were reported in other Asian countries, the Americas and the European Union in the following years. In addition, several other swine enteric viruses have been reported. Porcine kobuvirus (PKV), a picornavirus, was first identified from pig faecal samples in Hungary in 2008 and then reported in China in 2009 (Reuter, Kecskemeti, & Pankovics, 2010; Yu et al., 2009). PKV was subsequently reported in many other countries, such as Thailand, Korea, Japan and the United States (Khamrin et al., 2010, 2009; Park et al., 2010; Verma, Mor, Abdel-Glil, & Goyal, 2013). Porcine sapovirus (PSaV), a calicivirus, was first reported in the United States and in the United Kingdom (Knowles,

Ding and Fu Equal contribution to this study.

1983; Saif, Bohl, Theil, Cross, & House, 1980) and is now circulating in wide geographical regions, including the America, Europe and Asia. Porcine deltacoronavirus (PDCoV), a newly emerged swine enteric virus, was first discovered in healthy pig herds by a research team in Hong Kong in 2012 after performing a molecular epidemiological investigation among 3,137 mammals and 3,519 birds (Woo et al., 2012). Two years later, PDCoV was reported to cause severe diarrhoea and/or vomiting and atrophic enteritis in pigs in the United States and China (Song et al., 2015).

These swine enteric viruses cause similar clinical symptoms in infected pigs, leading to difficulties in diagnosing diarrhoea. Although several standard detection methods, for example virus isolation, virus neutralization tests, and indirect immunofluorescence assay, are available for the detection of viruses, these techniques are time-consuming and not suitable for detecting large-scale samples. Currently, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) methods for the detection of these viruses have been reported (Luo et al., 2017; Ma et al., 2016; Zhu, Wang, Cui, & Cui, 2016). Due to the high pathogenicity of these viruses to suckling piglets, which have immature immune systems and few antibodies, ELISA is inefficient for detecting these viruses compared to PCR. Regarding available PCR methods for the detection of swine enteric viruses, none of them can differentially detect these six viruses in one assay.

In this study, a multiplex RT-PCR assay for differential detection of PKV, TGEV, PEDV, PDCoV, PRV-A and PSaV from one reaction vial was established based on the conserved N genes of TGEV, PEDV and PDCoV, the VP7 gene of PRV-A, and the polyprotein genes of PKV and PSaV. This assay showed high sensitivity and specificity to the target genes. Additionally, this assay was employed to analyse a total of 398 swine faecal samples collected from nine provinces of China. The results provided us with a detailed infection status of swine herds in field settings and will facilitate the design of effective vaccines and the development of powerful prevention strategies for swine diarrhoea.

2 | MATERIAL AND METHODS

2.1 | Enteric viruses

PKV, PEDV, PDCoV and PSaV were isolated and identified by our laboratory previously and preserved at -80°C . TGEV and PRV-A were prepared from commercial attenuated live vaccines. The total RNAs of PKV, TGEV, PEDV, PDCoV, PRV-A and PSaV were extracted using TRIzol reagent (Invitrogen) and subjected to reverse transcription to prepare cDNA with the Reverse Transcript System (Promega) following the manufacturer's instructions.

2.2 | Experimental design

In this study, we designed a multiplex RT-PCR assay to differentially detect major enteric RNA viruses in one reaction vial. The basic rationale for this assay is shown in Figure 1. Briefly, the total RNA of faecal samples was extracted and then subjected to a reverse transcription system with hexamer random primers to prepare cDNA, which was added to PCR mix with primers specific to PKV, TGEV, PEDV, PDCoV, PRV-A and PSaV. PCR products were visualized under UV light after DNA electrophoresis on a 1.5% agarose gel. The causative agents were determined on the basis of the corresponding length of the PCR fragments.

2.3 | Primers

Specific primer sets targeting the N genes of TGEV, PEDV and PDCoV, the VP7 gene of PRV-A, and the polyprotein genes of PKV and PSaV were designed based on highly conserved regions of gene sequences in GenBank[®] using Oligo 7 software. The sizes of the expected amplicons ranged from 200 bp to 1,000 bp: 998 bp for PKV, 820 bp for TGEV, 600 bp for PEDV, 498 bp for PDCoV, 350 bp for PRV-A and 194 bp for PSaV (Figure 1). The annealing temperatures of the primers were optimized between 52°C

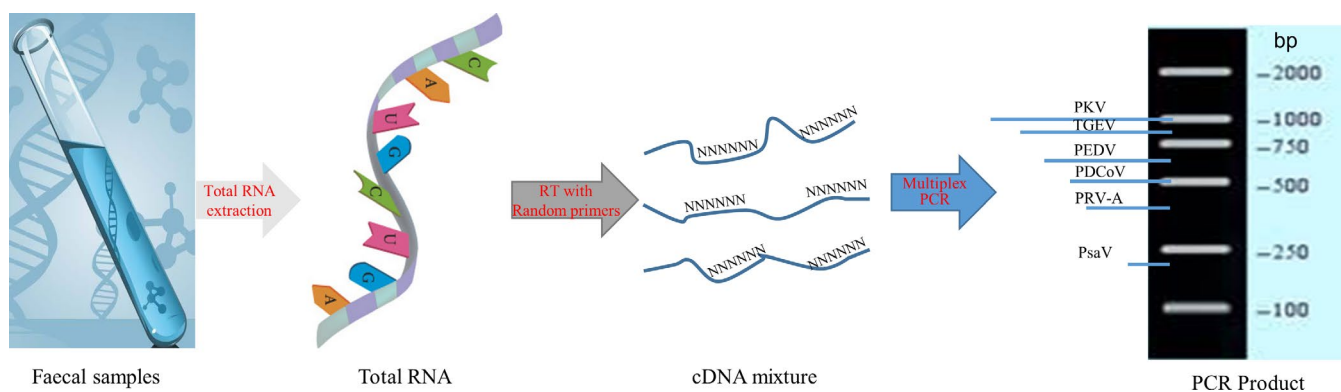


FIGURE 1 The rationale of the multiplex RT-PCR assay for the detection of swine enteric viruses. Porcine diarrhoeal samples were resuspended in $5 \times$ volume of sterile PBS and subjected to centrifugation for deletion of debris. The supernatant was filtered through a $0.45 \mu\text{m}$ filter and used for viral RNA extraction with TRIzol reagent. Next, the total RNAs were reverse transcribed using hexamer random primers and then subjected to PCR amplification with multiplex primer sets designed for major porcine diarrhoeal viruses. Amplified segments were visualized under UV light in a 1.5% agarose gel, showing different sizes for different viruses between 200 bp and 1,000 bp [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 The primer sets for detection of porcine diarrhoeal viruses using multiplex RT-PCR

Virus	Primer	Sequence	Positions	Product size	Target gene
PKoV	PKoV-F	GGCATTGACATGAATCAGGC	6946–6965	998	Polyprotein
	PKoV-R	GCGATCGTAGGTCTTCGG	7926–7944		
TGEV	TGEV-F	GGGCCAACGTAAAGAGCTTCC	27112–27132	820	Nucleoprotein
	TGEV-R	GCTCTGACCTTTCTGCAG	27914–27931		
PEDV	PEDV-F	TAGGACTCGTACTGAGGGTGT	26642–26662	600	Nucleoprotein
	PEDV-R	CTATTTTCGCCCTTGGGAATT	27222–27242		
PDCoV	PDCoV-F	GCTGACACTTCTATTAAC	24301–24319	497	Nucleoprotein
	PDCoV-R	TTGACTGTGATTGAGTAG	24804–24787		
PR-A	PRV-A-F	GTATGGTATTGAATATACC	3–21	350	VP7
	PRV-A-R	TAGACTGATCCAGTTGGC	336–353		
PSaV	PSaV-F	TACAGCAAGTGGGAC	4330–4344	194	Polyprotein
	PSaV-R	ATGACACTGGTGAACGGCAT	4488–4507		

and 54°C. The detailed information of the primer sets is listed in Table 1.

2.4 | The reaction of the multiplex RT-PCR assay

Before the establishment of the multiplex RT-PCR assay, single RT-PCR assays for each virus were developed. A total of 5 µl cDNA of each virus were used as a template and mixed with the specific primer pair (10 pmol/L each primer) for the individual viruses, 12.5 µl 2 × PCR master mix (Transgene), and nuclease-free water to 25 µl. The reaction was conducted under the following conditions: 95°C for 5 min, followed by 35 cycles of 40 s at 94°C, 60 s at 52°C, 1 min at 72°C and a final extension step at 72°C for 10 min. PCR products were stained with ethidium bromide and visualized on a 1.5% agarose gel using UV light.

For the multiplex RT-PCR assay, first, a triplex PCR assay was established with the primer sets for TGEV, PEDV and PRV-A. Then, both PSaV and PKV primer sets were added to the triplex reaction system to develop a multiplex assay to detect the five viruses. Last, the PDCoV primer pair was added to establish the final multiplex PCR assay to detect the six viruses. For a better output of the multiplex PCR assay, primer concentrations, template amount, annealing temperature and running cycles were optimized accordingly. All PCR amplifications were carried out with the same conditions in one PCR tube, and the amplified products were visualized on a 1.5% agarose gel.

2.5 | The sensitivity of the multiplex RT-PCR

The sensitivity of the multiplex RT-PCR assay was assessed by testing 10-fold (10^4 – 10^0 ng) diluted cDNA mixtures of PKV, TGEV, PEDV, PDCoV, PRV-A and PSaV. The same amount of cDNAs from each virus were combined and used as templates for PCR amplification with the multiplex reaction system. In addition, the sensitivity of a single RT-PCR assay was also evaluated with serially 10-fold diluted cDNAs of PKV, TGEV, PEDV, PDCoV, PRV-A and

PSaV, which ranged from 10^4 ng to 10^0 ng template. The cDNA of each virus was added to a single RT-PCR reaction system as a template for amplification.

2.6 | The specificity of the multiplex RT-PCR

The specificity of the multiplex RT-PCR assay was evaluated by testing the reactivity with DNA of pseudorabies virus (PrV) or cDNAs of reovirus (ReoV), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), and type A and type O foot-and-mouth disease viruses (FMDV-A, FMDV-O). These are common viruses circulating in Chinese pig farms. PRRSV was previously isolated from a pig farm by our laboratory. PrV and CSFV were retrieved from commercial attenuated live vaccines. The cDNAs of ReoV were kindly gifted by Dr. Yan Chen from Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences. The cDNAs of FMDV-A and FMDV-O were provided by the OIE/CHINA National Foot-and-Mouth Disease Reference Laboratory.

2.7 | Detection of field samples

A total of 398 faecal samples were collected from the Henan, Chongqing, Liaoning, Ningxia, Gansu, Qinghai, Shanxi, Jiangxi, and Hainan provinces, China, between October 2015 and April 2017. All samples were mixed with 300 µl sterile phosphate-buffered saline (PBS) using a vortex machine and centrifuged at $1,847 \times g$ at 4°C for 20 min. The supernatant was collected and applied for RNA extraction and cDNA preparation. The cDNAs were then subjected to PCR amplification by the established multiplex RT-PCR assay.

3 | RESULTS

3.1 | Establishment of the multiplex RT-PCR

The single RT-PCR results showed that the fragments of the target genes for each virus were successfully amplified as designed with

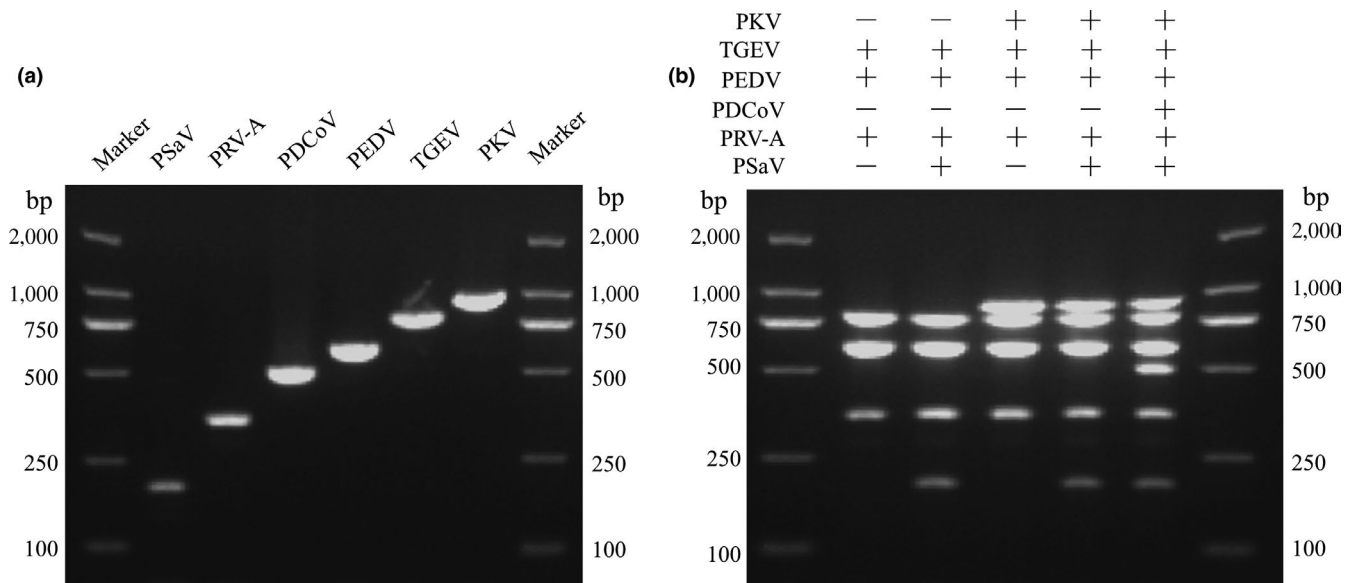


FIGURE 2 The multiplex RT-PCR assay is well established. (a) Total RNA was extracted from PSaV, PRV-A, PDCoV, PEDV, TGEV and PKV with TRIzol reagent and subjected to reverse transcription with hexamer random primers. Total cDNAs were amplified with primers targeting genes of individual viruses. (b) Multiplex RT-PCR was developed and optimized for the detection of combinations of three viruses (TGEV, PEDV, and PRV), four viruses (TGEV, PEDV, PRV, and PSaV; or PKV, TGEV, PEDV, and PRV), five viruses (PKV, TGEV, PEDV, PRV, and PSaV) and six viruses (PKV, TGEV, PEDV, PDCoV, PRV, and PSaV)

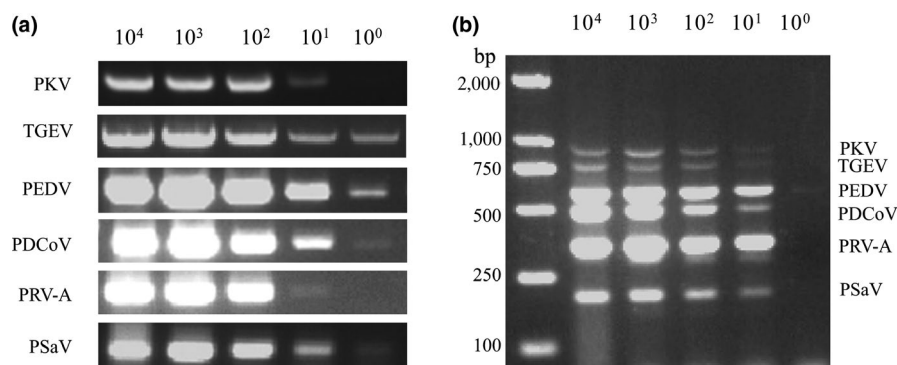


FIGURE 3 The multiplex RT-PCR assay developed in this study has high sensitivity. (a) Viral RNA of each virus were diluted in a 10-fold series between 10^4 ng and 10^0 ng and reverse transcribed with hexamer random primers. The generated cDNAs were amplified with specific primer sets. (b) The resulting cDNAs for each virus were combined at each dilution and then subjected to PCR amplification in a multiplex reaction vial with primer mixture for all six viruses

sizes between 200 bp and 1,000 bp (Figure 2a). Additionally, neither non-specific bands nor primer dimers appeared on the agarose gel (Figure 2a), indicating the high quality and specificity of the primer sets. First, the triplex assay was established and able to specifically detect the traditional diarrhoeal pathogens TGEV, PEDV and PRV-A (Figure 2b). Next, the cDNAs and primer sets of PSaV or PKV were added to the triplex assay, and the results demonstrated that all these target genes were well amplified without any interference (Figure 2b). Based on these results, both PSaV- and PKV-related reagents were combined with a triplex reaction system, which resulted in a multiplex assay that was able to detect TGEV, PEDV, PRV-A, PSaV and PKV simultaneously (Figure 2b). The final multiplex RT-PCR was eventually developed by adding PDCoV-associated reagents into the previous multiplex assay. The results indicated that

good amplification and high efficacy were obtained in this final multiplex RT-PCR assay (Figure 2b).

To achieve ideal amplification conditions, the concentrations of the primer sets of each virus were optimized. The optimum final concentrations of the mixed primer sets were as follows: 0.2 pmol/ μ l for PKV, 0.05 pmol/ μ l for TGEV, 0.3 pmol/ μ l for PEDV, 0.8 pmol/ μ l for PDCoV, 1.6 pmol/ μ l for PRV-A and 0.2 pmol/ μ l for PSaV.

3.2 | The sensitivity of the multiplex RT-PCR

To evaluate the sensitivity of the multiplex RT-PCR assay, we first investigated the sensitivity of a single RT-PCR for each virus. The results showed that 10 ng cDNA of PKV or PRV-A was detectable, while cDNA amounts as low as 1 ng could be detected for TGEV,

PEDV, PDCoV and PSaV (Figure 3a), indicating high sensitivity of the designed primer sets for each virus. When measuring the sensitivity of the multiplex RT-PCR, all primers were pooled at optimized concentrations to prepare the PCR premix, which was used to detect the pooled viral cDNAs of each virus at the indicated amounts. The multiplex RT-PCR assay results showed that this assay was able to detect 10 ng viral cDNAs of PKV, TGEV, PDCoV, PRV-A, and PSaV and as low as 1 ng cDNA of PEDV (Figure 3b), suggesting high sensitivity of the multiplex RT-PCR assay for the detection of designed swine enteric viruses.

3.3 | The specificity of the multiplex RT-PCR

To evaluate the specificity of the multiplex RT-PCR, cDNAs of ReoV, PRRSV, CSFV, FMDV-A, and FMDV-O and DNA of PrV were employed. The results illustrated that the multiplex RT-PCR could specifically detect the six swine enteric viruses, and no cross-reaction with the employed cDNA/DNA occurred (Figure 4), which was detectable using a virus-specific primer pair (Figure 4). These results indicated that the multiplex RT-PCR developed in this study was extremely specific to our expected porcine swine enteric viruses.

3.4 | Detection of field samples using the multiplex RT-PCR

A total of 398 diarrhoeal faecal samples collected from nine provinces distributed in North, Middle and South China (Figure 5a) were detected using the well-established multiplex RT-PCR assay and confirmed using single RT-PCR. Of these samples, none were detected as TGEV-positive (Table 2). The results demonstrated that PDCoV

(144/398), PSaV (114/398), PEDV (78/398) and PRV-A (70/398) were the major enteric viruses circulating in the pig population in China (Figure 5b and Table 2). In addition, dual infections, for example, PDCoV + PSaV, PDCoV + PRV-A, PRA-V + PSaV, and PEDV + PDCoV and triple infections, for example, PDCoV + PRV-A + PSaV, and PEDV + PDCoV + PKV, were detected at a relatively high frequency in the collected samples (Table 2), implying that coinfections of swine enteric viruses widely existed in pig farms in China.

4 | DISCUSSION

Swine enteric health is continuously challenged by viral infections that cause severe diarrhoea, high mortality in piglets and economic losses. The pathogenesis of TGEV, PEDV and PRV-A has been extensively characterized (Pensaert & Martelli, 2016; Vlasova, Amimo, & Saif, 2017). However, several novel viruses have been found in the faeces of pigs, such as PDCoV, PKV and PSaV. In 2017, another novel swine enteric alphacoronavirus (SeACoV) was discovered in Southern China (Gong et al., 2017; Pan et al., 2017). The aetiology of swine enteric viruses has become increasingly complicated, and the similarity of clinical signs in pigs infected with these viruses makes it difficult to determine the causative agents. Due to the lack of valid detection methods, these viruses have not been included in the lists of the differential diagnoses for swine veterinarians. A simple, rapid, efficient and high-throughput detection method is urgently needed for the differential detection of swine enteric viruses.

Although viral metagenomics and nanopore sequencing technology (Theuns et al., 2018) are valuable assets for the diagnosis of

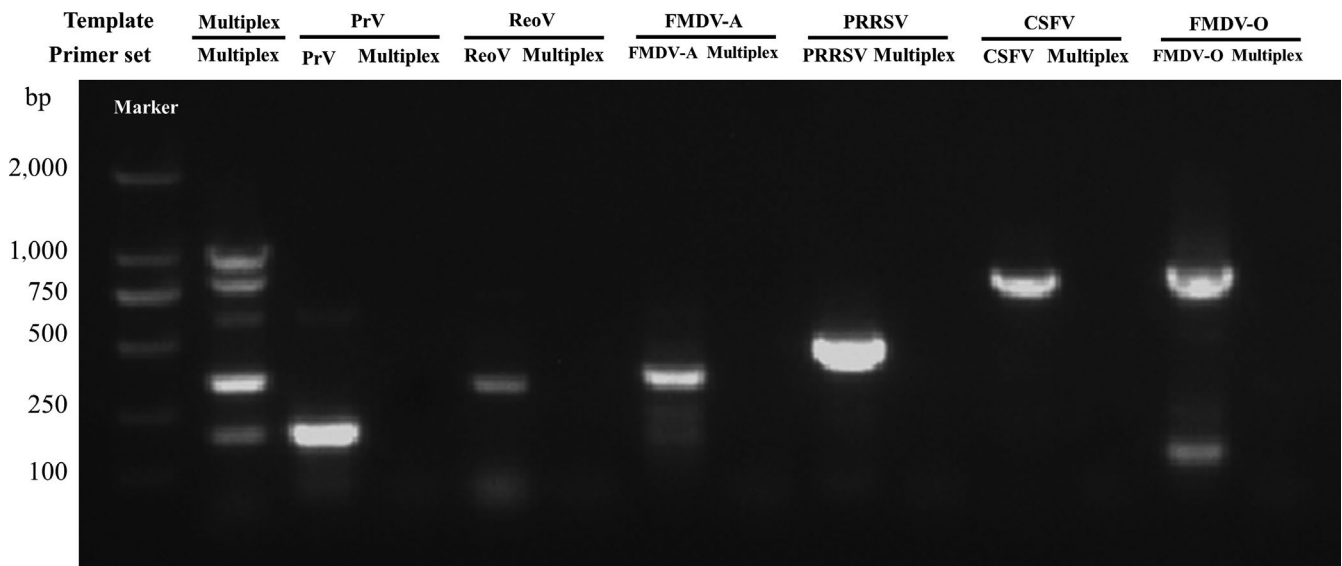


FIGURE 4 The multiplex RT-PCR assay established in this study shows high specificity. The RNAs originating from reovirus (ReoV), type A foot-and-mouth disease virus (FMDV-A), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), and type O foot-and-mouth disease virus (FMDV-O) were reverse transcribed with hexamer random primers. The resulting cDNAs and DNAs of porcine pseudorabies virus (PrV) were used as templates to validate the specificity of the multiplex RT-PCR assay developed in this study. Primer sets specific to individual viruses were employed as positive controls

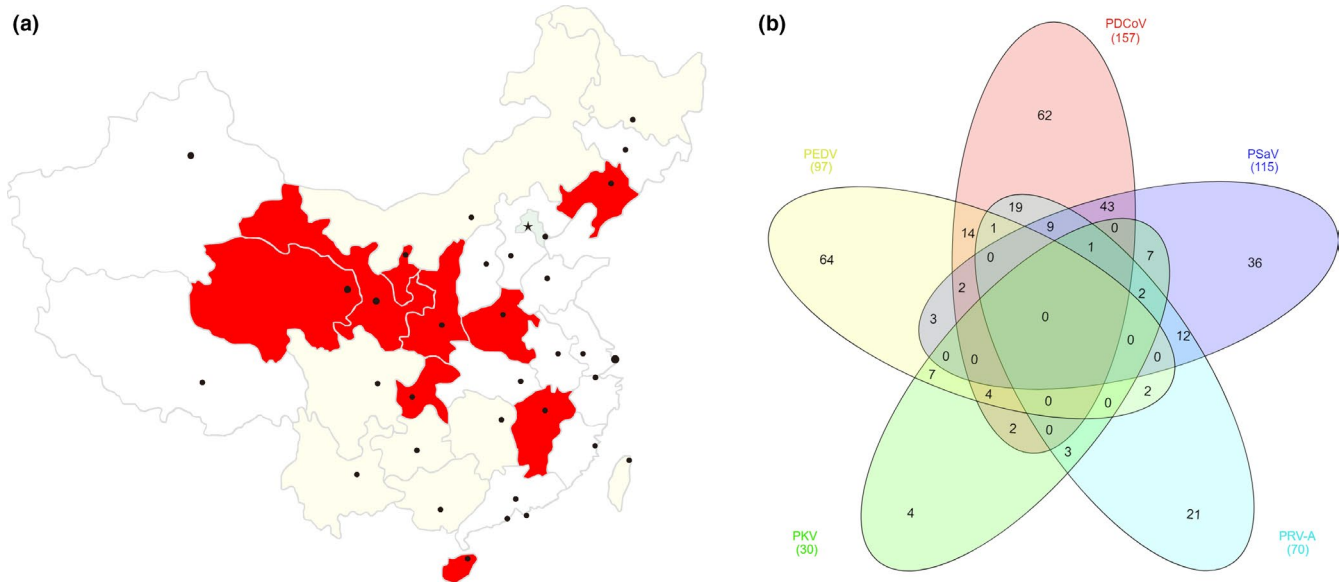


FIGURE 5 Field sample detection reveals coinfections of swine enteric viruses. (a) Geographic distribution of sample collection. A total of 382 porcine diarrhoeal samples were collected from pig farms of nine provinces distributed in North, Middle and South China between October 2015 and April 2017. All clinical samples were resuspended in 5 × volume of sterile PBS and subjected to centrifugation to remove the debris. The supernatant was used for viral RNA extraction with TRIzol reagent. Next, the total RNAs were reverse transcribed using hexamer random primers and then subjected to multiplex PCR analysis. (b) Venn diagram showing the total and proportion of positive samples for each virus. The overlapping areas indicate the total samples that were positive in 2, 3 and/or 4 different viruses by multiplex RT-PCR [Colour figure can be viewed at wileyonlinelibrary.com]

enteric diseases in pigs and for the discovery and identification of novel porcine viral enteric disease complexes, there are some disadvantages, such as cost, long diagnosis times, and high requirements for instruments and analysers, limiting their usage in the field or clinical settings for diagnosis. Multiplex PCR/RT-PCR is still widely used by most veterinary diagnostic laboratories due to simple procedures, rapid diagnostic results, high-throughput methodologies, low cost and more advantages.

As we mentioned earlier in this manuscript, TGEV is a widely accepted and well-characterized enteric pathogen (Pensaert & Martelli, 2016). Of the 382 faecal samples analysed, TGEV was not detected by our multiplex RT-PCR, which was confirmed by single TGEV RT-PCR. Song et al. also did not find TGEV-positive samples

from 356 samples of diarrhoeal specimens collected in Jiangxi Province between 2012 and 2015 (Song et al., 2015). However, Wang et al. reported an 18.5% positive rate of TGEV from 27 intestinal samples collected from four provinces of Northwest China between September 2015 and May 2016 (Wang, Ji, Zhang, Xu, & Zhang, 2018). The main reasons responsible for this difference might be the varied geographic distributions of this virus or different sample types. Using nanoparticle-assisted polymerase chain reaction, Zhu et al. found a 3.5% positive rate of TGEV infection in 114 samples from four provinces of China (Zhu et al., 2017). Investigations from Zhao et al. revealed that the TGEV infection rate was as low as 1.91% in East, Northeast and Southeast China between 2012 and 2014 (Zhao et al., 2016). Taken together, TGEV is not a prevalent or

Province	No. of samples	Number of positive samples					
		TGEV	PEDV	PRV-A	PKV	PSaV	PDCoV
Henan	63	0	11	5	0	15	15
Shaanxi	15	0	3	5	0	2	8
Liaoning	144	0	4	20	1	70	90
Gansu	30	0	8	11	0	2	11
Ningxia	40	0	1	20	5	9	12
Chongqing	62	0	27	9	16	17	16
Hainan	26	0	25	0	8	0	5
Jiangxi	8	0	8	0	0	0	0
Qinghai	10	0	10	0	0	0	0
Total	398	0	97	70	30	115	157

TABLE 2 Results of field samples detected by the multiplex RT-PCR

dominant pathogen accounting for swine viral diarrhoea in China in recent years.

Swine enteric viruses are complicated and complex. Except for the widely accepted and well-characterized PEDV, TGEV and PRV-A, there are some more poorly characterized viruses present in swine intestines, such as sapoviruses, kobuviruses, orthoreoviruses, astroviruses, enteroviruses, circular DNA viruses, bocaviruses, picobirnaviruses, porcine sapoviruses, sapeloviruses, teschoviruses and others. Importantly, some of these viruses are always detected spontaneously from the same specimen, indicating that coinfection is a common phenomenon in some pig farms. Our results showed that dual or triple infections between PEDV, PDCoV, PRV-A, PKV and PSaV commonly existed in Chinese pig herds. Zhao et al. also reported the coinfection of PKV with PEDV, TGEV and PRV-A in China (Zhao et al., 2016). Using next-generation sequencing or nanopore sequencing technologies, Chen et al. and Theuns et al. reported that coinfections of swine enteric viruses were widely presented in pig farms in the United States and Belgium (Chen et al., 2018; Theuns et al., 2018).

The coinfections of swine enteric viruses introduce two significant challenges for the prevention and control of swine viral diarrhoea. First, coinfections may speed up the evolution of individual viruses and coevolution between coinfecting viruses. Meanwhile, recombination between coinfecting viruses may also occur. New swine enteric coronaviruses were generated by recombination with PEDV and TGEV and spread across central Eastern European countries during 2012 and 2016 (Akimkin et al., 2016; Belsham et al., 2016; Boniotti et al., 2016). Recombination might create more virulent enteric virus strains or new viruses, leading to potential outbreaks or pandemics of swine viral diarrhoea. Additionally, coinfections may promote the evolution of non-pathogenic enteric viruses into high virulent and pathogenic viruses. A recent example showed the existence of PDCoV in pig herds for many years in mainland China and Hong Kong; however, the virus had no observable clinical symptoms (Pan et al., 2017; Woo et al., 2012). Unfortunately, this virus caused severe outbreaks in some states of the United States since 2014 (Ma et al., 2015; L. Wang, Byrum, & Zhang, 2014). This virulence change was most likely caused by previous exposure to PEDV or other coinfecting swine enteric viruses.

In order to effectively prevent the swine viral diarrhoea, vaccines perfectly matched the prevalent virus strains are the key strategy. On the other hand, in order to develop a preeminent vaccine covering the most virulent viruses in the field, to fully understand the virus type causing swine enteric co-infections is the key issue. Both situations have to rely on accurate clinical diagnosis and differential diagnosis. The multiplex RT-PCR developed in this study specifically targets main swine enteric viruses circulating in Chinese pig farms, providing a valuable tool for clinical diagnostic laboratories.

ACKNOWLEDGEMENTS

We thank Dr. Yan Chen from State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese

Academy of Agricultural Sciences for kindly providing the cDNA of swine reovirus. We also thank Dr. Xi Lan from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences for providing the Kobovirus and Sapovirus. Special thanks to Ms. Manita Aryal for critical proofreading of this manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL STATEMENT

Authors have declared that Ethical Statement is not applicable in the current manuscript as the samples were collected from faeces in the pig farms.

ORCID

Guangliang Liu  <https://orcid.org/0000-0001-8158-5749>

REFERENCES

- Akimkin, V., Beer, M., Blome, S., Hanke, D., Hoper, D., Jenckel, M., & Pohlmann, A. (2016). New chimeric porcine coronavirus in swine feces, Germany, 2012. *Emerging Infectious Diseases*, 22(7), 1314–1315. <https://doi.org/10.3201/eid2207.160179>
- Belsham, G. J., Rasmussen, T. B., Normann, P., Vaclavek, P., Strandbygaard, B., & Botner, A. (2016). Characterization of a novel chimeric swine enteric coronavirus from diseased pigs in central Eastern Europe in 2016. *Transboundary and Emerging Diseases*, 63(6), 595–601. <https://doi.org/10.1111/tbed.12579>
- Boniotti, M. B., Papetti, A., Lavazza, A., Alborali, G., Sozzi, E., Chiapponi, C., ... Marthaler, D. (2016). Porcine epidemic diarrhea virus and discovery of a recombinant swine enteric coronavirus, Italy. *Emerging Infectious Diseases*, 22(1), 83–87. <https://doi.org/10.3201/eid2201.150544>
- Chen, Q., Wang, L., Zheng, Y., Zhang, J., Guo, B., Yoon, K. J., ... Li, G. (2018). Metagenomic analysis of the RNA fraction of the fecal virome indicates high diversity in pigs infected by porcine endemic diarrhoea virus in the United States. *Virology Journal*, 15(1), 95. <https://doi.org/10.1186/s12985-018-1001-z>
- Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L. I., Zhang, Y., ... Cao, Y. (2017). A New Bat-HKU2-like coronavirus in swine, China, 2017. *Emerging Infectious Diseases*, 23(9), 1607–1609. <https://doi.org/10.3201/eid2309.170915>
- Khamrin, P., Maneekarn, N., Hidaka, S., Kishikawa, S., Ushijima, K., Okitsu, S., & Ushijima, H. (2010). Molecular detection of kobuvirus sequences in stool samples collected from healthy pigs in Japan. *Infection, Genetics and Evolution*, 10(7), 950–954. <https://doi.org/10.1016/j.meegid.2010.06.001>
- Khamrin, P., Maneekarn, N., Kongkaew, A., Kongkaew, S., Okitsu, S., & Ushijima, H. (2009). Porcine kobuvirus in piglets, Thailand. *Emerging Infectious Diseases*, 15(12), 2075–2076. <https://doi.org/10.3201/eid1512.090724>
- Knowles, N. J. (1983). Isolation and identification of porcine enteroviruses in Great Britain, 1979 to 1980. *British Veterinary Journal*, 139(1), 19–22. [https://doi.org/10.1016/S0007-1935\(17\)30584-5](https://doi.org/10.1016/S0007-1935(17)30584-5)
- Li, W., Li, H., Liu, Y., Pan, Y., Deng, F., Song, Y., ... He, Q. (2012). New variants of porcine epidemic diarrhoea virus, China, 2011. *Emerging*

- Infectious Diseases*, 18(8), 1350–1353. <https://doi.org/10.3201/eid1808.120002>
- Luo, S. X., Fan, J. H., Opriessnig, T., Di, J. M., Liu, B. J., & Zuo, Y. Z. (2017). Development and application of a recombinant M protein-based indirect ELISA for the detection of porcine deltacoronavirus IgG antibodies. *Journal of Virological Methods*, 249, 76–78. <https://doi.org/10.1016/j.jviromet.2017.08.020>
- Ma, Y., Zhang, Y., Liang, X., Lou, F., Oglesbee, M., Krakowka, S., & Li, J. (2015). Origin, evolution, and virulence of porcine deltacoronaviruses in the United States. *MBio*, 6(2), e00064. <https://doi.org/10.1128/mBio.00064-15>
- Ma, Y., Zhang, Y., Liang, X., Oglesbee, M., Krakowka, S., Niehaus, A., ... Li, J. (2016). Two-way antigenic cross-reactivity between porcine epidemic diarrhea virus and porcine deltacoronavirus. *Veterinary Microbiology*, 186, 90–96. <https://doi.org/10.1016/j.vetmic.2016.02.004>
- Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y. L., ... Huang, Y. W. (2017). Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. *Veterinary Microbiology*, 211, 15–21. <https://doi.org/10.1016/j.vetmic.2017.09.020>
- Park, S. J., Kim, H. K., Moon, H. J., Song, D. S., Rho, S. M., Han, J. Y., ... Park, B. K. (2010). Molecular detection of porcine kobuviruses in pigs in Korea and their association with diarrhea. *Archives of Virology*, 155(11), 1803–1811. <https://doi.org/10.1007/s00705-010-0774-1>
- Pensaert, M. B., & Martelli, P. (2016). Porcine epidemic diarrhea: A retrospect from Europe and matters of debate. *Virus Research*, 226, 1–6. <https://doi.org/10.1016/j.virusres.2016.05.030>
- Reuter, G., Kecskemeti, S., & Pankovics, P. (2010). Evolution of porcine kobuvirus infection, Hungary. *Emerging Infectious Diseases*, 16(4), 696–698. <https://doi.org/10.3201/eid1604.090937>
- Saif, L. J., Bohl, E. H., Theil, K. W., Cross, R. F., & House, J. A. (1980). Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. *Journal of Clinical Microbiology*, 12(1), 105–111.
- Song, D., Zhou, X., Peng, Q., Chen, Y., Zhang, F., Huang, T., ... Tang, Y. (2015). Newly emerged porcine deltacoronavirus associated with diarrhoea in swine in China: Identification, prevalence and full-length genome sequence analysis. *Transboundary and Emerging Diseases*, 62(6), 575–580. <https://doi.org/10.1111/tbed.12399>
- Sun, R. Q., Cai, R. J., Chen, Y. Q., Liang, P. S., Chen, D. K., & Song, C. X. (2012). Outbreak of porcine epidemic diarrhea in suckling piglets, China. *Emerging Infectious Diseases*, 18(1), 161–163. <https://doi.org/10.3201/eid1801.111259>
- Theuns, S., Vanmechelen, B., Bernaert, Q., Deboutte, W., Vandenhole, M., Beller, L., ... Nauwynck, H. J. (2018). Nanopore sequencing as a revolutionary diagnostic tool for porcine viral enteric disease complexes identifies porcine kobuvirus as an important enteric virus. *Scientific Reports*, 8(1), 9830. <https://doi.org/10.1038/s41598-018-28180-9>
- Verma, H., Mor, S. K., Abdel-Glil, M. Y., & Goyal, S. M. (2013). Identification and molecular characterization of porcine kobuvirus in US swine. *Virus Genes*, 46(3), 551–553. <https://doi.org/10.1007/s11262-013-0879-1>
- Vlasova, A. N., Amimo, J. O., & Saif, L. J. (2017). Porcine rotaviruses: Epidemiology, immune responses and control strategies. *Viruses*, 9(3), 48. <https://doi.org/10.3390/v9030048>
- Wang, L., Byrum, B., & Zhang, Y. (2014). Detection and genetic characterization of deltacoronavirus in pigs, Ohio, USA, 2014. *Emerging Infectious Diseases*, 20(7), 1227–1230. <https://doi.org/10.3201/eid2007.140296>
- Wang, X. Y., Ji, C. J., Zhang, X., Xu, D. P., & Zhang, D. L. (2018). Infection, genetic and virulence characteristics of porcine epidemic diarrhea virus in northwest China. *Infection, Genetics and Evolution*, 62, 34–39. <https://doi.org/10.1016/j.meegid.2018.04.001>
- Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., ... Yuen, K. Y. (2012). Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *Journal of Virology*, 86(7), 3995–4008. <https://doi.org/10.1128/jvi.06540-11>
- Yu, J. M., Jin, M., Zhang, Q., Li, H. Y., Li, D. D., Xu, Z. Q., ... Duan, Z. J. (2009). Candidate porcine Kobuvirus, China. *Emerging Infectious Diseases*, 15(5), 823–825. <https://doi.org/10.3201/eid1505.081518>
- Zhao, Z. P., Yang, Z., Lin, W. D., Wang, W. Y., Yang, J., Jin, W. J., & Qin, A. J. (2016). The rate of co-infection for piglet diarrhea viruses in China and the genetic characterization of porcine epidemic diarrhea virus and porcine kobuvirus. *Acta Virologica*, 60(1), 55–61. https://doi.org/10.4149/av_2016_01_55
- Zhu, Y., Liang, L., Luo, Y., Wang, G., Wang, C., Cui, Y., ... Cui, S. (2017). A sensitive duplex nanoparticle-assisted PCR assay for identifying porcine epidemic diarrhea virus and porcine transmissible gastroenteritis virus from clinical specimens. *Virus Genes*, 53(1), 71–76. <https://doi.org/10.1007/s11262-016-1405-z>
- Zhu, Y., Wang, G. H., Cui, Y. D., & Cui, S. J. (2016). Establishment of a nanoparticle-assisted RT-PCR assay to distinguish field strains and attenuated strains of porcine epidemic diarrhea virus. *Archives of Virology*, 161(9), 2543–2547. <https://doi.org/10.1007/s00705-016-2918-4>

How to cite this article: Ding G, Fu Y, Li B, et al. Development of a multiplex RT-PCR for the detection of major diarrhoeal viruses in pig herds in China. *Transbound Emerg Dis*. 2020;67:678–685. <https://doi.org/10.1111/tbed.13385>