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Detection and differentiation of five diarrhea related pig viruses utilizing a multiplex PCR assay

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ABSTRACT

Porcine viral diarrhea, mainly caused by porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine group A rotaviruses (RVA), porcine group C rotaviruses (RVC) and porcine circovirus 2 (PCV2), is a serious global problem, resulting in substantial economic losses to the swine industry. For fast and reliable diagnosis of the causative agent associated with viral diarrhea in pigs, an inexpensive and easy to perform gel-based multiplex PCR assay was developed in this study to detect and differentiate the different viruses by amplicon size. The assay was able to distinguish between all five viral agents without cross-reacting with other non-target pig viruses. The detection limits of the assay per reaction were 5 copies for PEDV, TGEV, RVC and PCV2 and 50 copies for RVA for the singleplex assays and 50 copies when all five viruses were multiplexed. Sixty-nine field samples were used to validate the developed multiplex assay. The overall prevalence of positive samples was 44.9% (31/69). PCV2 was detected in 37.7% of the samples, PEDV and RVC each in 4.3%, TGEV in 2.9%, and RVA was detected in 1.4% of the samples tested. A total of 5.8% of the samples were co-infected by two or more viruses, and the results of the multiplex assay were in agreement to those obtained by single PCR assays. These findings suggest that the developed cost-effective multiplex assay is specific, sensitive, and will serve as a valuable diagnostic tool for the rapid differential detection of these five viruses and for molecular epidemiological studies and diarrhea disease management.

1. Introduction

Porcine viral diarrhea is a common and important cause of morbidity and mortality particularly in young pigs and became a critical threat to the swine industry. In 2010–2012, severe diarrhea outbreaks in pigs were reported in many provinces in China, causing almost 100% morbidity among piglets and mortality of 80–100% (Wang et al., 2016). Porcine epidemic diarrhea virus (PEDV) was subsequently identified as causal agent for this epidemic situation. PEDV, which has been classified in the order Nidovirales, family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirusis*, causes a highly contagious intestinal disease, characterized with acute onset of profuse liquid diarrhea, inappetence, vomiting and lethargy commonly connected with high morbidity and mortality rates, especially when young suckling pigs in naive pig populations become infected (Jung and Saif, 2015). PEDV has become one of the most common viral causes of porcine diarrhea in China (Zhang and He, 2010). A study investigating cases of porcine enteric disease between 2005 and 2007 demonstrated that PEDV accounted for 46% of all cases (Gan et al., 2010). Besides PEDV, transmissible gastroenteritis virus (TGEV), porcine group A rotavirus (RVA) and porcine group C rotavirus (RVC) are also common viral agents involved in pig enteric disease.

Transmissible gastroenteritis virus (TGEV) infection causes severe enteritis and is highly contagious in pigs (Ding et al., 2017; Penzes et al., 2001). TGEV is an enveloped, positive-sense, single stranded RNA virus with a genome of 28.5 kb, belonging to the *Coronaviridae* family in the order Nidovirales (Laude et al., 1990). Clinical signs of the disease include watery diarrhea, vomiting, dehydration and high mortality in piglets before weaning (Ding et al., 2017; Saif, 1999).

Rotaviruses (RVs) belong to the *Reoviridae* family and are a leading cause of severe diarrhea in many animals. RVs, non-enveloped, 11-segmented double stranded (ds) RNA virus, are classified into eight groups or species (A–H) based on serological assays and sequencing of the viral protein 6 (VP6), five of which, RVA, RVB, RVC, RVE, and RVH, have been

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Table 1
Primers used for single and multiplex PCR detection.

Primer	Primer sequence (5′→3′)	Expected product (bp)	Target gene	Position	Reference sequence
PEDV-F	AACACGGCGACTACTCAGC	394	Nucleoprotein	26206-26599	KC189944
PEDV-R	GCCTTCTTTAGCAACCCAG				
TGEV-F	GTGGTGTTAGGTGATTATTTTCC	319	Spike glycoprotein	20521-20839	AJ271965
TGEV-R	TATGGTTTAACCTGCACTCACTA				
RVA-F	TATGCAATACCAGTAGGACCAG	242	Inner capsid	1080-1321	EU372771
RVA-R	GCTCTACGTAGCGAGTATGAAATC				
RVC-F	CATCCGTGAAGAGAATGGTG	159	Inner capsid	1193-1313	AB889519
RVC-R	GCATTAGCCCCTACGCAAGC				
PCV2-F	AAGAAGCGGACCCCAAC	508	Replicase	71-578	GQ996404
PCV2-R	AGGTGGCCCCACAATGA				

detected in pigs (Matthijnssens et al., 2012; Wakuda et al., 2011). Traditionally, RVA has been considered the primary cause of diarrhea in pigs, while other species including RVC had been described sporadically in pigs. However, high RVC prevalence rates of 19.5–46.0% in pig herds worldwide underline the important role of this pathogen in porcine diarrhea (Amimo et al., 2013; Marthaler et al., 2014; Otto et al., 2015). Therefore, it has been suggested that all future porcine RV epidemiological studies should include RVC (Marthaler et al., 2014).

Porcine circovirus 2 (PCV2) is a small non-enveloped virus with a single-stranded circular DNA genome of 1766–1768 nucleotides, which belongs to the genus *Circovirus*, family *Circoviridae* (Segales et al., 2005). PCV2 is the primary causative pathogen of several disease manifestations in pigs collectively known as porcine circovirus-associated disease (PCVAD). PCVAD includes respiratory disease, reproductive failure, diarrhea, and systemic diseases which all contribute to significant financial losses in the global pig production if uncontrolled (Ge et al., 2012; Grau-Roma et al., 2011; Segales et al., 2005). In addition, PCV2 can be associated with immune suppression making the pig more susceptible to other pathogens such as PEDV. Specifically, PCV2 induces lymphocyte depletion of infected pigs with loss of lymphoid follicles and a reduction of cellularity in parafollicular T-cell dependent areas (Ogawa et al., 2009), thus aggravating the clinical sign of PEDV (Jung and Saif, 2015; Liu et al., 2015). Furthermore, diarrhea caused by each of these viruses in pigs is clinically indistinguishable. It is therefore imperative and critical to establish a convenient and sensitive tool to actively survey pig herds for these five viruses before and during diarrhea outbreaks.

Standard diagnostic methods for these viruses include virus isolation, fluorescence assay, electron microscopy, in-*situ* hybridization, and enzyme-linked immunosorbent assay. However, these techniques are time consuming with relatively low specificity and sensitivity and therefore not suitable for early diagnosis or epidemiological surveys of viruses associated with enteric disease. Molecular assays such as the polymerase chain reaction (PCR), which include single and multiplex PCR assays, have proven to be a convenient and sensitive approach to detect viruses associated with diarrhea in pigs (Ben Salem et al., 2010; Collins et al., 2008; Jiao et al., 2013; Kim et al., 2007). However, none of the presently available assays allows for simultaneous detection of these five viruses in one assay.

In this study, we describe the development of a multiplex PCR assay for simultaneous detection of PEDV, TGEV, RVA, RVC and PCV2 and demonstrate its suitability in identifying potential infection of these viruses in clinical samples. A comparison between this multiplex assay and single PCRs was also carried out by using positive control and clinical samples.

2. Materials and methods

2.1. Viruses and samples

PCV2b (Jiangsu strain) (Professor Gao Song, Yangzhou University), PEDV and TGEV vaccine strain (Harbin Weike Biotechnology Development Company, Cat. no. 030718), RVA and RVC each harbored in positive pig fecal samples identified by PCR and sequencing before the formal experiment, were maintained in the authors' laboratory. Negative controls consisted of PCV1 (HZ2006, EF533941) (Professor Zhou Jiyong, Zhejiang University), porcine bocavirus (PBoV) (Zhejiang strain), porcine astrovirus (PAstV) (Zhejiang strain), a classic porcine parvovirus (PPV1) vaccine strain (Beijing Haidian Zhonghai Animal Health Science & Technology Co., Cat. no. 0040401) and a classical swine fever virus (CSFV) vaccine strain (Qianyuanhao Biological Co., Cat. no. 050656).

Sixty-nine fecal samples were collected in sixty-nine healthy growing-finishing pigs from pig farms in Zhejiang Province, China in 2013. The samples were stored at -80 ℃ before use.

2.2. Nucleic acid extraction

The samples were processed as described previously (Jiang et al., 2014). Briefly, faecal samples were re-suspended 1:10 (w/v) in PBS, vortexed for 30 s and centrifuged at 1500 g for 10 min to obtain the supernatant. Viral genomic DNA and RNA was extracted from cell cultures infected with each virus or frozen clinical samples using the AxyPrepTM Body Fluid Viral DNA/RNA Miniprep Kit 50-prep (AXYGEN, Hangzhou, China) following the manufacturer's protocol. The extracted DNA and RNA was stored at -80 ℃ until use.

2.3. Primers design

Five primer pairs each specific for one of the viruses of interest were designed by Primer Premier 5.0 (Primer Biosoft International, Palo Alto, CA, USA) within highly conserved regions and are outlined in Table 1. Highly conserved regions were obtained by aligning sequences of each virus with Clustal W (DNAStar Inc., Madison, WI, USA) and GenBank nucleotide sequence database. The specificity of the primers was confirmed by a BLAST search in GenBank databases from the National Center for Biotechnology Information (NCBI), along with the multiplex function of Primer Premier 5.0. All primers were synthesized from a commercial source (GENEWIZ Inc., Suzhou, China).

2.4. Reverse transcription

Viral cDNA synthesis was performed using random hexamer primers with RT Master Mix Kit (Vazyme Biotech Co., Ltd) according to the manufacturer's protocol and the cDNA was used immediately for amplification or stored at −80 ℃.

2.5. Single PCRs and viral reference preparation

Single PCRs were set up in a 15 μL mixture volume containing 7.5 μL of Master Mix (Vazyme Biotech Co., Ltd), 0.1 μL of each 10 μM primer, 1 μL template cDNA/DNA and distilled water to 15 μL. For the negative

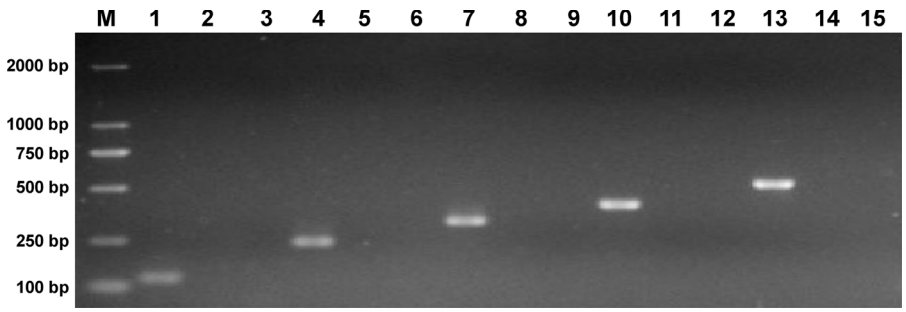

Fig. 1. Specificity of primers used in single PCR assays demonstrated by using different viral templates. M: DL2000 DNA Marker; Lanes 1, 4, 7, 10, 13 are RVC, RVA, TGEV, PEDV, and PCV2, respectively; Lane 2, 5, 8, 11, 14 are a pooled mixture of five non-target viruses; Lane 3, 6, 9, 12, 15 are RVA + TGEV + PEDV + PCV2, RVC + TGEV + PEDV + PCV2, RVC + RVA + PEDV + PCV2, RVC + RVA + TGEV + PCV2, RVC + RVA + TGEV + PEDV, respectively.

control reaction distilled water was used as a template. The amplifications were performed using a PCR amplifier (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: one cycle at 95 ℃ for 3 min, 40 cycles at 95 ℃ for 20 s, 55 ℃ for 30 s, 72 ℃ for 30, and a final incubation step of 72 ℃ for 3 min. PCR products were analyzed by electrophoresis using 1.5% agarose gel in 1 × TAE buffer. The amplification products of correct size were then separately cloned using the pMD18-T (TaKaRa) and constructs were extracted using Plasmid Miniprep Kit (Axygen). The constructs were verified by PCR and DNA sequencing (GENEWIZ). The DNA concentrations of the constructs were determined using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Multiplex PCR protocol

After confirming that all of the individual primer pairs allowed for specific amplification of all five target viruses in a single PCR, all forward and reverse virus-specific primers were mixed together to perform a multiplex PCR. To obtain high efficiency, parameter variables such as reagent concentration, PCR cycling, annealing temperature were optimized experimentally. The multiplex PCRs were carried out in a 20 μL mixture containing 10–15 μL of Master Mix (Vazyme), 25–100 nM of the RVC, RVA, TGEV, PEDV and PCV2 primer pairs, 1 μL of DNA template and distilled water to 20 μL. Distilled water was also used as a blank control. The amplifications were performed in a PCR amplifier (Bio-Rad) with the following conditions: one cycle at 95 ℃ for 3 min, 40 cycles at 95 ℃ for 20 s, 40–65 ℃ for 30 s, 72 ℃ for 30, and a final incubation step of 72 ℃ for 3 min. The obtained PCR products were analyzed by electrophoresis through 2% agarose gel in 1 × TAE buffer.

2.7. Sensitivity of the single and multiplex PCR assays

To determine the sensitivity of the assays, we performed 10-fold serial dilutions of recombinant plasmids containing specific viral target fragments. After measuring the plasmid concentration using the Nano Drop 2000 spectrophotometer, the copy number of plasmids per μL in each dilution was calculated using the following formula: Y (copies/μL) = 6.02×10^{23} × [plasmid concentration (ng/μL)] × 10^{-9})/[plasmid DNA length (bp) × 660 (g/mol/base)]. For comparison, single PCRs were also performed in parallel. The detection sensitivity was determined for each individual as well as for all equally premixed templates for the multiplex format.

2.8. Assay specificity of the multiplex PCR

To evaluate the specificity of each primer pair for its target gene, single PCR assays using the primers in Table 1 were carried out to test all the five target or non-target viruses. To verify the specificity of the multiplex PCR assay, various combinations of positive and negative controls were tested under the optimized condition. Distilled water was included in each PCR assay as a blank control. Resulting size-specific PCR products were analyzed by electrophoresis.

2.9. Detection of viruses in field samples by single or multiplex PCR assays

A total of 69 fecal samples were used for clinical evaluation of the multiplex PCR for the detection of PEDV, TGEV, RVA, RVC and PCV2 under optimized conditions once determined. For comparison, single PCRs were also performed in parallel. Specific viral target fragment from PCR positive samples was cloned into the plasmid pMD18-T and/or sequenced by GENEWIZ.

3. Results

3.1. Specificity of the primers in single PCR assays

The designed primer pair for each virus was first tested individually in a single PCR. The amplified DNA products were detected on 2.0% agarose gel. Visible bands were checked if they had the expected size for each pathogen. Specifically, amplicons were expected to be 508 bp for PCV2, 394 bp for PEDV, 319 bp for TGEV, 242 bp for RVA and 159 bp for RVC. Clearly visible and distinguishable bands were observed for all targets (Fig. 1). Primer dimer formation or non-specific amplifications were not observed, and there was no cross reactivity among the target viruses and other commonly encountered viruses (Fig. 1). These results indicate the designed primer pairs are specific for each target virus and were suitable for further analysis.

3.2. Optimization of the multiplex PCR

The optimal annealing temperature was 55 ℃ (Fig. 2). Other optimal conditions established for the multiplex PCR were as follows: 15 μL of Master Mix (Vazyme), 100 nM of the RVC primer pair, 75 nM of the RVA primer pair, 50 nM of TGEV primer pair, 25 nM of PEDV and the PCV2 primer pairs for a total of, 40 cycles of amplification reaction (data not shown).

3.3. Specificity of the multiplex PCR assay

The specificity of the multiplex assay was examined by testing 10

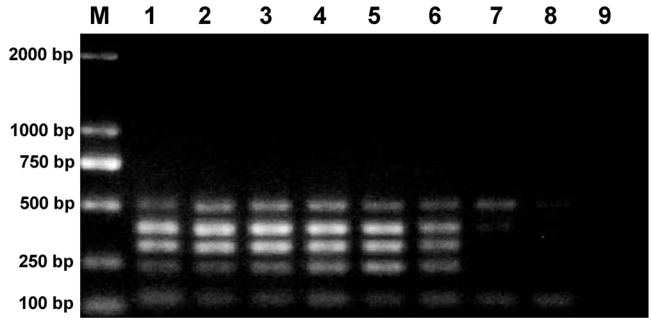

Fig. 2. Effect of annealing temperature on the multiple PCR reaction. M: DL2000 DNA Marker; Lanes 1–9 are 1: 40.0 ℃; 2: 41.7 ℃; 3: 44.7 ℃ ; 4: 49.4 ℃; 5: 55.0 ℃; 6: 59.7 ℃; 7: 63.0 ℃; 8: 65.0 ℃; 9: negative control.

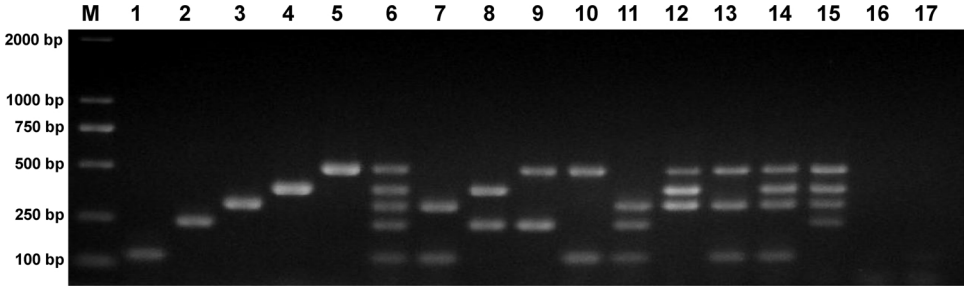

Fig. 3. Specificity of the multiplex PCR assay developed for the detection of five viruses with different template combinations. M: DL2000 DNA Marker; Lanes 1–9 are 1: RVC; 2: RVA; 3: TGEV; 4: PEDV; 5: PCV2; 6: RVC + RVA + TGEV + PEDV + PCV2; 7: RVC + TGEV; 8: RVA + PEDV; 9: TGEV + PCV2; 10: RVC + PCV2; 11: RVC + RVA + TGEV; 12: TGEV + PEDV + PCV2; 13: RVC + TGEV + PCV2; 14: RVC + TGEV + PEDV + PCV2; 15: RVA + TGEV + PEDV + PCV2; 16: non-target viruses; 17: Blank control.

different pathogens, including the five target viruses and five non-target viruses. As shown in Fig. 3, the size-specific PCR products for the five target viruses can be identified clearly through electrophoresing. When single or different combinations of the five viruses were tested in the multiplex PCR, the corresponding amplicons were produced correctly, indicating good amplification compatibility among the five viruses. In contrast, no target virus-specific amplicons were produced with the negative controls, which included non-target viruses PCV1, PPV1, CSFV, PBoV and PAstV, and a blank distilled water control. These results indicate that the multiplex PCR assay is specific for detection and differentiation of the five viruses.

3.4. Sensitivity of the single PCR and multiplex PCR assays

Ten-fold serial dilutions of viral plasmid templates with known copy numbers ranging from 5×10^0 to 5×10^5 copies/μL were used to evaluate the sensitivity of the single and multiplex assays. When only one virus was present in the multiplex PCR, 5 genome equivalent copies/μL of PEDV, TGEV, RVC or PCV2 could be detected and 50 genome equivalent copies/μL were detected for RVA. The detection level corresponded to those of the single PCR assays. The multiplex PCR assay was also able to detect at least 50 genome equivalent copies/μL of each virus in a premix of all five viruses (Fig. 4).

3.5. Evaluation of the multiplex assay with field samples

The multiplex and single PCR assays were used to screen 69 fecal samples for the five viruses (Table 2). Of these 31/69 (44.9%) tested positive by the multiplex PCR assay, which was slightly lower compared to 35/69 (50.7%) positive samples obtained by the single PCRs. PCV2 was detected most often with 37.7% (26/69) positive samples, followed by PEDV, RVC, TGEV, and RVA, accounting for 4.3% (3/69), 4.3% (3/69), 2.9% (2/69), and 1.4% (1/69) of positive samples, respectively. Coinfection was detected in 5.8% of all samples. Overall, 94.2% agreement was observed for the two PCR systems with a kappa correlation of 0.884.

4. Discussion

Outbreaks of watery diarrhea concurrent with high mortality rates in suckling piglets are usually the first signs that PEDV might be circulating within a pig herd (Diel et al., 2016) and PEDV has become the most common cause of viral diarrhea in pigs (Chae et al., 2000; Wang et al., 2016; Zhang et al., 2013). However, a diagnosis of PEDV cannot be made only based on the clinical signs and histological changes observed in the gastrointestinal tract because other enteric pathogens of pigs, including TGEV, RVA, and RVC target the same age group and can cause similar clinical manifestations (Kim et al., 2007; Zhang and He, 2010). Moreover, PCV2 is recognized to cause diarrhea in grow finish pigs but could also be associated directly or indirectly (via immune suppresion) with diarrhea in young pigs. Infection of pigs with more than one enteric virus are frequently reported (Ben Salem et al., 2010; Kim et al., 2007; Masuda et al., 2016; Zhang et al., 2013) and can result in synergistic or additive effects leading to more severe and prolonged diarrhea (DaPalma et al., 2010; Saif, 1999; Zhao et al., 2013). Given the high demands on diagnostic sensitivity, specificity and rapid turnaround time of results, a multiplex assay based on the PCR technology is therefore the method of choice for diagnostic purposes as PCRs are cost, time and labor efficient. While previous multiplex PCRs appeared to be effective in detecting two, three or four of the here investigated five

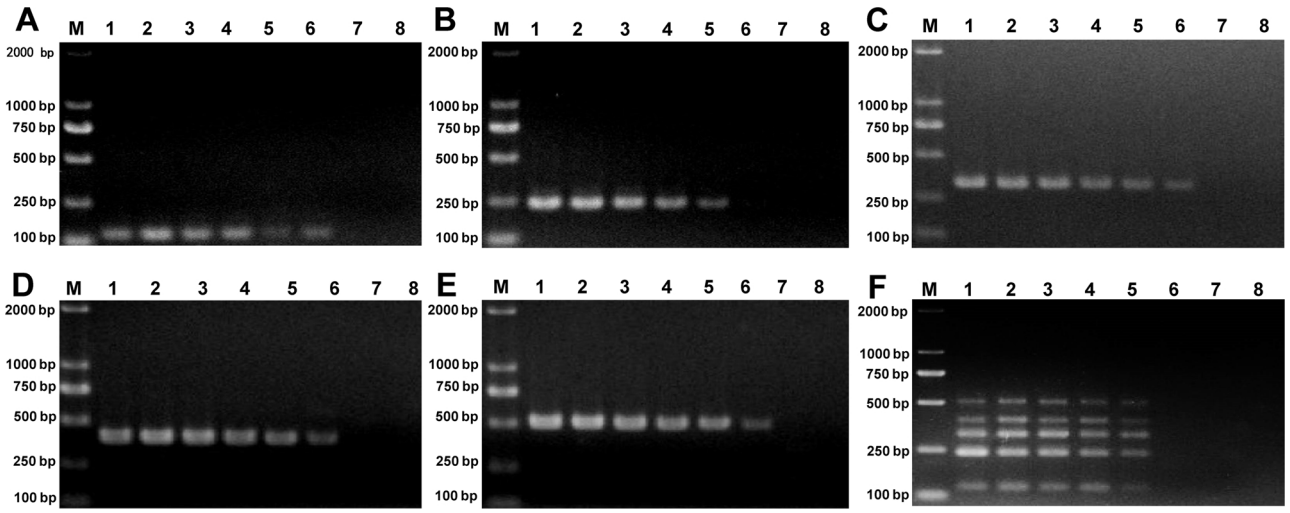

Fig. 4. Sensitivity of the multiplex PCR with single or five premixed viral templates. A: RVC; B: RVA; C: TGEV; D: PEDV; E: PCV2; F: five premixed viral template. M: DL2000 DNA Marker. Lanes 1–7 are 1: 5×10^5 copies/μL; 2: 5×10^4 copies/μL; 3: 5×10^3 copies/μL; 4: 5×10^2 copies/μL; 5: 5×10^1 copies/μL; 6: 5×10^0 copies/μL; 7: 5×10^{-1} copies/μL; 8: negative control.

Table 2
Detection of clinical samples by using the multiplex PCR vs single PCRs.

Method	No. samples	RVC [no. positive (%)]	RVA [no. positive (%)]	TGEV [no. positive (%)]	PEDV [no. positive (%)]	PCV2 [no. positive (%)]	RVA + PCV2 [no. positive (%)]	RVC + PCV2 [no. positive (%)]	PEDV + PCV2 [no. positive (%)]	TGEV + PEDV + PCV2 [no. positive (%)]	Total [no. positive (%)]
Multiplex PCR	69	2(2.9)	0(1.4)	2(1.4)	1(1.4)	22(31.9)	1(2.9)	1(1.4)	2(4.3)	0(0)	31 (44.9)
Single PCR	69	1(1.4)	1(1.4)	2(2.9)	1(1.4)	23(33.3)	1(1.4)	3(4.3)	2(2.9)	1(1.4)	35 (50.7)

viruses (Ben Salem et al., 2010; Jung et al., 2003; Ogawa et al., 2009; Zhao et al., 2013), a 5-plex PCR assay targeting all the five diarrhea viruses has been developed successfully in this study.

Despite increasing applications of multiple target detection, multiplex PCR detection is still challenging because of potential complex interactions among multiple primer sets. When developing a multiplex PCR assay, the most crucial step is primer design which usually define the specificity and sensitivity of the assay. The five selected target viruses in this study, even relatively conserved PCV2, have been found to mutate quickly and have several distinct genotypes or variants circulating in the global pig population (Ben Salem et al., 2010; Ge et al., 2012; Jung and Saif, 2015). Therefore, during primer design it is important to select conserved areas to enable detection of most strains circulating under field conditions. In this study, we utilized the in GenBank availabe extensive sequence information of these five viruses to design a set of five primer pairs with different product lengths and at most two mismatches with all available reference strains. The BLAST search indicated a perfect match with target sequences in GenBank. Furhtermore the developed multiplex PCR specifically amplified all five target viruses and has the potential to detect all known variants, although due to the lack of a panel of reference this could not be tested. Sequencing of some amplicons from PCR positive samples further confirmed the specificity of the multiplex assay.

Apart from the design of primers, the other reaction components and parameters such as concentration/ratio of primers, annealing temperature, are also important factors that affect PCR amplification efficiency and detection sensitivity, especially for multiplex assays where different co-amplifications are performed simultaneously. The developed multiple PCR system was able to detect the five viruses with high sensitivity. Currently, several different sources of templates have been used for testing the sensitivity of PCR assays for RNA viruses, such as standard plasmid containing viral target fragment, standard RNA transcribed in vitro, or RNA extracted from virus-infected cells (Kim et al., 2007; Masuda et al., 2016; Ogawa et al., 2009). Considering its convenience and stability, standard plasmid was used as the template to test the sensitivity of the multiplex and single assays described here for both DNA and RNA viruses. Unlike previous study that showed a 10–100 fold reduction in sensitivity of the multiplex PCR compared to single assays (Zhao et al., 2013), the multiple PCR assay under the current protocol had an equal detection limit (5 copies of PEDV, TGEV, RVC, and PCV2, and 50 copies of RVA per reaction) compared with those of the single PCRs. Even when the five viruses were all present, the multiplex assay achieved a sensitivity of 50 copies which is much better than previoulsy reported. Specifically, the sensitivity of conventional multiplex PCR assays targeting 2–4 of these five viruses ranged from 10^4 to 10^3 copies (Wu et al., 2014; Zhao et al., 2013), and multiplex real time PCR assays achieved sensitivities ranging from 10 to 100 copies (Kim et al., 2007; Zhou et al., 2016). The high sensitivity and specificity of our assay could be attributed to optimal design of primer pairs and extensive optimization of the multiplex assay, thus enabling the detection of the viruses during the early phase of infection when the relative viral load is lower.

We further evaluated the single and multiplex assays using 69 clinical samples. The multiplex assay detected a total of 44.9% positive samples. These results are similar to the single assays (50.7%), having a overall agreement of 94.2%. This further indicates that the multiplex assay is almost as specific and sensitive as the single PCR assays. Moreover, it can accurately and rapidly detect multiple viruses in one sample, which could substantially reduce turn-around time and reagent costs.

All of the five viruses were detected in the present study. However, while PCV2 clearly predominated with a detection rate of 37.7%, the other viruses were only sporadically detected with positive rates of less than 5%. The positive rates for each of the five viruses in the present study are in agreement with previous reports in which 1.8% and 6.7% of fecal samples were found to be positive for PEDV (Jiao et al., 2013; Zhang, 2016), 1.3% and 1.6% for TGEV (Jiao et al., 2013; Zhang and He, 2010), 0.8%, 5.3% and 7.37% for RVA (Huo et al., 2013; Machnowska et al., 2014; Zhang and He, 2010), 3% and 4.4% for RVC (Collins et al., 2008; Zhou et al., 2016), and 42.2% for PCV2 (Zhou et al., 2016). However, the incidences of these viruses was much higher in other reports with 50.4% and 81.9% for PEDV (Chae et al., 2000; Zhang et al., 2013), 22.8% and 46% for TGEV (Vemulapalli et al., 2009; Yang et al., 2006), 51.2% and 62% for RVA (Marthaler et al., 2014; Otto et al., 2015), 31%, 53% and 78% for RVC (Marthaler et al., 2014; Molinari et al., 2016; Otto et al., 2015). However, it should be noted that most of these prior studies analyzed samples from diarrheic pigs, whereas samples used in this study were collected from healthy pigs. Additionally, sampling time, age, breeding conditions and vaccination procedures vary among different pig herds studied and different detection sensitivity of these assays may account for these differences. Further studies will be required to fully validate the multiplex assay by testing more field samples from healthy as well as diarrheic pigs.

In addition, despite relatively low infection rate, coinfections were observed in the present study. Among all pathogens, PCV2 was most frequently identified together with other pathogens. Although PCVAD is multifactorial in causality and coinfection of PCV2 and other enteric virus may aggravate the sign of diarrhea (Jung and Saif, 2015), a high percentage of clinically healthy pigs infected with PCV2 or other diarrhea-causing viruses in this study may imply subclinical infection due to low amounts of antigens associated with no to minimal lesions, as has been frequently observed in prior studies (Amimo et al., 2015, 2013; Opriessnig et al., 2007; Zhang et al., 2013; Zhou et al., 2016). These results suggest the clinically diarrheic diseases are extremely complex and highlight the importance of sensitive and early detection of these viruses for prevention and control of their spread.

In conclusion, this paper describes the development and validation of a multiplex PCR assay, which proved to allow rapid, sensitive, specific and simultaneous detection of PEDV, TGEV, RVA, RVC and PCV2 in fecal samples. Multiplexing by disease or sample type syndrome simplifies the diagnostic procedure, reduces the overall cost, but also reduces test turnaround time and potential cross contamination when testing large numbers of samples. The multiplex PCR provides a rapid and cost-effective etiological diagnostic tool for viral diarrhea in pigs.

Acknowledgments

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