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Simultaneous detection of five pig viruses associated with enteric disease in pigs using EvaGreen real-time PCR combined with melting curve analysis

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ABSTRACT

In recent years, a series of porcine diarrhea viruses such as porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), rotaviruses of group A (RVA), rotaviruses of group C (RVC), and porcine circovirus 2 (PCV2) caused enormous economic losses all over the world. While any of these viruses is capable to cause disease alone, there is often concurrent infection with more than one virus on pig farms. In this study, a multiplex real-time PCR method based on EvaGreen fluorescent dye and melting curve analysis was established to simultaneously detect these five viruses in a single closed tube. Five distinct melt peaks were obtained with different melting temperature (Tm) value corresponding to each of the five viruses. This method was highly sensitive to detect and distinguish TGEV, RVA, RVC, PEDV and PCV2 with the limits of detection ranging from 5 to 50 copies/μL. The intra-assay and inter-assay reproducibility were good with coefficient of variation of Tm and cycle threshold values less than 0.32% and 2.86%, respectively. Testing of 90 field samples by the single and multiplex real-time PCR assays demonstrated a concordance of 91.1%. Thus, the EvaGreen multiplex real-time PCR is a rapid, sensitive and low-cost diagnostic tool for differential detection and routine surveillance of TGEV, RVA, RVC, PEDV and PCV2 in pigs.

1. Introduction

Diarrhea in pigs is increasing in the global pig population and often characterized by rapid onset, rapid transmission, high morbidity, high mortality and wide distribution, causing disastrous economic losses around the world (Fairbrother et al., 2005; Zhang et al., 2018). There are many causative agents of pig diarrhea including parasites, viruses, bacteria, and others, but viruses are most important (Saif, 1999). Among all viruses in pigs, porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), rotaviruses of group/species A (RVA), rotaviruses of group/species C (RVC), and porcine circovirus 2 (PCV2) are common and harmful pathogens. Generally, PEDV, TGEV and RVA can be most commonly found in cases of viral diarrhea, with often high detection rates (Ogawa et al., 2009; Song et al., 2006). Recent studies reported that RVC and PCV2 also play a significant role in swine diarrhea (Marthaler et al., 2013; Theuns et al., 2016; Zhao et al., 2013). Pigs are susceptible to these five pathogens and common clinical signs include vomiting, watery diarrhea and dehydration. Moreover, pigs can be infected with several different viruses at the same time (Schoborg and Borel, 2014; Zhao et al., 2016). Sensitive and accurate diagnosis of involved viral agents in cases of diarrhea is therefore of great significance to the surveillance, prevention and treatment of enteric disease in pigs.

Traditional diagnostic methods such as immunofluorescent assays (IFA), enzyme linked immunosorbent assays (ELISA) and gene chips have been developed to detect these viruses (Jiang et al., 2010; Liu et al., 2011; Wu et al., 2014). However, these methods are often laborious, insensitive, time-consuming and it is rather difficult or impossible to detect different viral infections simultaneously (Liu et al., 2011; Xu et al., 2012). Thus it is of great importance to develop an accurate and effective way to detect and distinguish these viruses in a single step. PCR technology has become one of the popular tools for detecting viruses in recent years. Conventional multiplex PCR technology can detect several viruses in a single sample simultaneously and the resulting amplicons can be separated by electrophoresis (Liu et al., 2019; Ogawa et al., 2009; Yue et al., 2009). However, conventional multiplex PCR not only has the risk of product contamination (false positive results), but also fails to monitor in real time. A multiplex real-time PCR properly overcomes these disadvantages. In general, the multiplex real-time PCR is efficient and rapid for detecting pathogens in

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samples qualitatively and quantitatively (Armani et al., 2012; Hyeon et al., 2010). The multiplex real-time PCR is generally divided into probe-based assays and fluorescent-dye-based assays. Compared to probe-based assays, dye-based assays are low-cost. The most widely used dye is SYBR Green I, which has been shown to be one of the most effective tools in the rapid differential detection of a variety of viral pathogens (Perez et al., 2012). However, utilizing SYBR Green I still has some limitations, including inhibition of PCR at high concentration and poor repeatability and specificity easily affected by melting temperatures (Arvidsson et al., 2008; Polz and Cavanaugh, 1998), thus impeding their use for multiplexing. EvaGreen is a new type of saturated dye that has been shown superior to SYBR Green I because it has extremely powerful sensitivity, specificity and repeatability (Aloisio et al., 2018; Eischeid, 2011; Zheng et al., 2016)

In this study, a multiplex real-time PCR assay combined with EvaGreen®-generated melting curve analysis is described, which relies on the difference of the resulting amplicon melting temperature (Tm) specific for each virus to detect and distinguish PCV2, PEDV, TGEV, RVA, and RVC in a single tube. For practical application, this assay was validated including its specificity, sensitivity and robustness using both positive standards and clinical samples.

2. Materials and methods

2.1. Virus and clinical specimens

PCV2b (Jiangsu strain) (Professor Gao Song, Yangzhou University), PCV1 (EF533941) (Professor Zhou Jiyong, Zhejiang University), porcine bocavirus (PBoV), porcine astrovirus (PAstV), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), Japanese encephalitis virus (JEV), RVA and RVC each contained in pig samples that have been identified by PCR and sequencing before the formal experiment, were maintained in the author's laboratory. PEDV and TGEV vaccine strains (Harbin Weike Biotechnology Development Company, Cat. no. 030,718), a classic porcine parvovirus (PPV) vaccine strain (Beijing Haidian Zhonghai Animal Health Science & Technology Co., Cat. no. 0,040,401) and a classical swine fever virus (CSFV) vaccine strain (Qianyuanhao Biological Co., Cat. no. 050,656) were also used in this study. Ninety fecal samples collected from clinically healthy growing-finishing pigs from pig farms located in the Zhejiang Province, China in 2013 were also used.

2.2. Nucleic acid extraction

The samples were processed as described previously (Jiang et al., 2014). Briefly, the 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 were simultaneously extracted from cell cultures infected with each virus or the supernatant from the clinical samples using AxyPrepTM Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, China) according to the manufacturer's directions. The concentration and purity of the extracted total RNA/DNA was determined using a spectrophotometer (NanoDrop, USA).

2.3. Primer design

The nucleotide sequences of PCV2, TGEV, PEDV, RVA, and RVC were downloaded from the NCBI GenBank sequence database and then aligned using the CLUSTAL W program of the DNAStar software. Primers were designed in conserved regions of the viral sequences by using Primer Premier 5.0 focused on producing amplicons with different Tms that allow for differentiating the viruses. The resulting five pairs of primers (PCV2, TGEV, PEDV, RVA, and RVC) are shown in Table 1.

2.4. First strand cDNA synthesis

Viral cDNA synthesis was performed using random hexamer primers with RT Master Mix Kit (Vazyme, China) following the manufacturer's recommendations and the cDNA/DNA was used immediately for amplification or stored at −80 °C until use.

2.5. Standard plasmid template construction

The PCR amplification was operated with the primers shown in Table 1 in a total reaction volume of 20 μL containing 10 μL Master Mix, 0.5 μL of each 10 μM primer, 1 μL of cDNA/DNA as a template and 4 μL distilled water. The PCR reaction was conducted under the following conditions: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s with a final extension for 5 min at 72 °C. Amplification products were analyzed by agarose gel electrophoresis, purified with a PCR purification kit (Axygen), cloned into the plasmid pUCm-T Vector (Axygen), and the plasmid constructs containing the corresponding insert was confirmed by DNA sequencing. Based on the size of the amplified fragment for each target, the amount of purified plasmids was calculated after measuring the concentration. The calculation formula is as follows: $(A_{260}\ (ng/\mu L) \times 10^{-9} \times 6.02 \times 10^{23})/(DNA\ length \times 650) = copies/\mu L$ (Lamien et al., 2011). The PCV2, PEDV, TGEV, RVA and RVC plasmids were diluted 10-fold in a range of $5.0 \times 10^{\circ}$ to 5.0×10^{7} copies/μL.

2.6. Single and multiplex EvaGreen real-time PCR

Single real-time PCR assay for PCV2, PEDV, TGEV, RVA, and RVC was performed in a 20 μL reaction volume containing 15 μL Master Mix (Vazyme), 1 × EG (Biotium, USA), 200 nM of the forward and reverse primers, and 0.5 μL of plasmid DNA or sample cDNA/DNA. Based on the results obtained in the single real-time PCR assays, a series of experiments were then performed to optimize the multiplex EvaGreen real-time PCR protocol, including adjustment of reagent concentration and PCR cycling parameters. After optimization, the multiplex real-time PCR reaction (20 μL) contained 15 μL master mix, 250 nM final concentration of the primer pair for TGEV and RVA, 75 nM for RVC and PEDV, 200 nM for PCV2, and 0.5 μL of the DNA template. The reaction conditions for both single and multiplex assays were 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s, 57 °C for 30 s and 72 °C for 30 s. The melting procedure was performed as follows: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s. Melting curves were automatically generated by the software and Tm values were obtained. Nuclease-free distilled water was used instead of template DNA in each set of reactions as a negative control, and standard plasmid positive control was include in every run. The EvaGreen real-time PCR results were defined as positive if the fluorescence intensity value (derivative) of the characteristic melting peak (Tm) was higher than 5×10^{3}. All real-time reaction was run on a 7300 real-time PCR machine (Applied Biosystems, USA). Each assay was performed in duplicate or triplicate.

2.7. Standard curves and sensitivity of the EvaGreen real-time PCR

Recombinant plasmids with 10-fold serial dilutions ranging from $5.0 \times 10^{\circ}$ to 5.0×10^{7} copies/μL were used to prepare standard curves and to determine the detection limit of the EvaGreen real-time PCR for PCV2, PEDV, TGEV, RVA, and RVC, respectively.

3. Results

3.1. Specificity of the EvaGreen real-time PCR

To determine the specificity of the newly developed EvaGreen real-time PCR, five targeted and several non-targeted viruses including PBoV, PAstV, PPV, CSFV, PRRSV, JEV, PRV and PCV1 were included to

Table 1
Primers used in multiplex real-time PCR assays.

Primers	Sequence (5´→3´)	Predicted Amplicon Tm (°C)	Amplicon size (bp)	Target gene	Location	Reference
PEDV-F	GGCGGATACTGGAATGAGCAA	87.7	110	Nucleoprotein (N)	26441	KC189944
PEDV-R	CGGTCGGCGTGAGGTCCTGTT				26549	
TGEV-F	ATGGTGTTAGGTGATTATTTCC	78.6	106	Spike glycoprotein (S)	20521	AJ271965
TGEV-R	AATACAATGCTTTAAGATTTTCCA				20626	
RVA-F	TGAAGTGAGGACCAGGCTAA	80.6	97	Inner capsid (VP6)	1210	eu372771
RVA-R	ACGAAATCACACCCTTACTTG				1306	
RVC-F	TGTTGCATCCGTGAAGAGAATGGT	84.3	126	Inner capsid (VP6)	1159	KM099258
RVC-R	GCATTAGCCCCTACGCAAGC				1284	
PCV2-F	ATCCGAAGGTGCGGGAGA	92.2	162	Capsid protein (CP)	1572	KC859451
PCV2-R	TGACGTATCCAAGGAGGCG				1733	

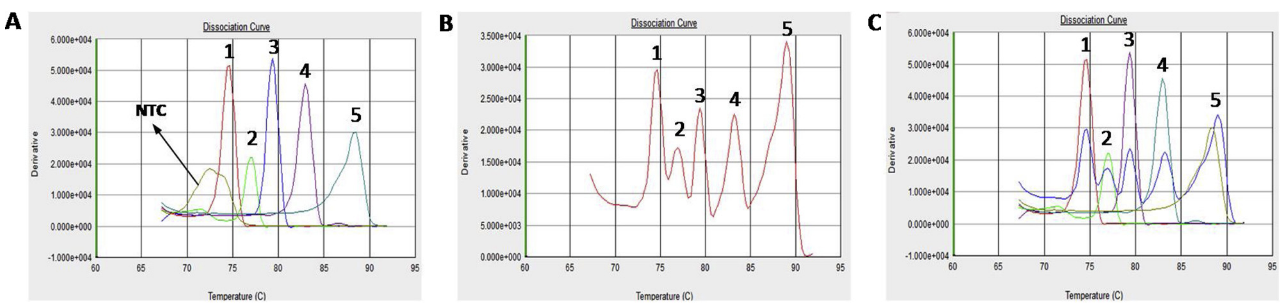

Fig. 1. Specificity of the EvaGreen multiplex real-time PCR assay demonstrated by melting curve analysis. (A) Results obtained using each swine virus alone. Only one specific product peak is visible for TGEV, RVA, RVC, PEDV and PCV2 respectively and there are no specific amplifications with the negative control (NTC), PBoV, PAstV, PPV, CSFV, PRV, PRRSV, JEV and PCV1. (B) Results obtained using all five target plasmid DNAs mixed in a single tube. In the presence of five viral targets, the melting curve had five characteristic peaks corresponding to the Tm values of the five amplicons of the five viruses. (C) Results obtained by merging different melting peaks for TGEV, RVA, RVC, PEDV and PCV2 in the case of single and five viral targets. Melting peaks 1–6 refer to TGEV, RVA, RVC, PEDV and PCV2 respectively.

test the assay. As showed in Fig. 1A, amplification products from the five target virus templates generated five clearly distinct sharp melting peaks in the EvaGreen multiplex real-time PCR system, with melting temperature (Tm) values of 75.0 ± 0.33 °C for TGEV, 77.6 ± 0.25 °C for RVA, 79.8 ± 0.34 °C for RVC, 83.3 ± 0.20 °C for PEDV and 88.5 ± 0.25 °C for PCV2, which were similar to those obtained with the single real-time PCR assays (data not shown). When tested together, the five targets were amplified and clearly separated by five different melting peaks (Fig. 1B), with almost identical Tm values compared to those in the corresponding single target PCR runs (Fig. 1C). Moreover, there was no target virus specific melting peak in the non-target negative control group (Fig. 1A). These results indicated that the EvaGreen multiplex real-time PCR was specific for the detection and identification of the five in this study selected pig viruses.

3.2. Sensitivity and linearity of the multiplex assay

The sensitivity of the assay was evaluated by testing 10-fold dilutions of TGEV, RVA, RVC, PEDV, and PCV2, ranging from $5 \times 10^{\circ}$ copies/μL to 5×10^7 copies/μL. The minimum plasmid concentrations detected was 5 copies/μL for TGEV, 5 copies/μL for RVA, 50 copies/μL for RVC, 5 copies/μL for PEDV and 50 copies/μL for PCV2 respectively (Fig. 2). In the single real-time assays, the limits of detection were 5 copies/μL for TGEV, RVA, RVC, and PEDV, and 50 copies/μL for PCV2 (data not shown). The sensitivity of the multiplex assay for each virus reached 50 copies/μL when all five viruses were mixed in equimolar amounts as templates (Fig. 3). Furthermore, all produced standard curves had an excellent linear correlation with a range of 5×10^1 copies/μL to 5×10^7 copies/μL ($R^2 > 0.99$) (Fig. 2), suggesting that the quantification component of the assay is robust. When performing the EvaGreen multiplex real-time PCR, the amplification efficiency of TGEV, RVA, RVC, PEDV, and PCV2 was 96.8%, 100.7%, 90.2%, 98.2% and 99.3%, indicating a satisfactory amplification (Fig. 2). Taking into account the conditions of the actual site environment and the potential of concurrent infection with any of these viruses, various virus combinations were also selected to determine the sensitivity of the assay. As shown in Fig. 4, when one or two viruses were fixed at 5×10^5 copies per reaction, the detection limit for the other or third virus was still up to 50 or 500 copies, as demonstrated by cases of concurrent TGEV and PCV2 infection versus PEDV, PEDV and PCV2 versus PEDV, PEDV versus TGEV, PCV2 versus PEDV, PCV2 versus TGEV, TGEV versus PEDV, RVA versus RVC, and RVC versus RVA.

3.3. Reproducibility

Intra-assay and inter-assay repeatability experiments for the multiplex system were performed using a single viral template with three concentrations (5.0×10^6 copies, 5.0×10^4 copies and 5.0×10^2 copies), analyzing both Tm and threshold cycle (Ct) values. The result showed that the coefficients of variation (CV) of Tm and Ct values were less than 0.3% and 2.9%, respectively (Table 2). When DNA templates containing all five viruses with three concentrations were used to perform the EvaGreen multiplex real-time PCR for determination of the reproducibility of assays, the intra-assay and inter-assay CV of Tm values were also found to be low (Fig. 5). From the above, the low CV among intra- and inter-assay tests indicated a good repeatability of the assay.

3.4. Application of the EvaGreen multiplex real-time PCR assay for clinical samples

To evaluate the EvaGreen multiplex real-time PCR assay for accurate diagnosis, 90 faecal samples from healthy pigs were tested and compared with single EvaGreen real-time PCR assay results. Among the 90 clinical samples, 51 (56.7%) samples were positive by the multiplex assay which included 2.2%, 2.2%, 4.4%, 28.9% and 45.6% positive for TGEV, RVA, RVC, PEDV and PCV2, and 21/90 (23.3%) samples were co-infected with two more viruses. The single assays detected one or more of the selected viruses in 57/90 samples (63.3%) positive of which 2.2%, 3.3%, 8.9%, 34.4% and 52.2% were positive for TGEV, RVA,

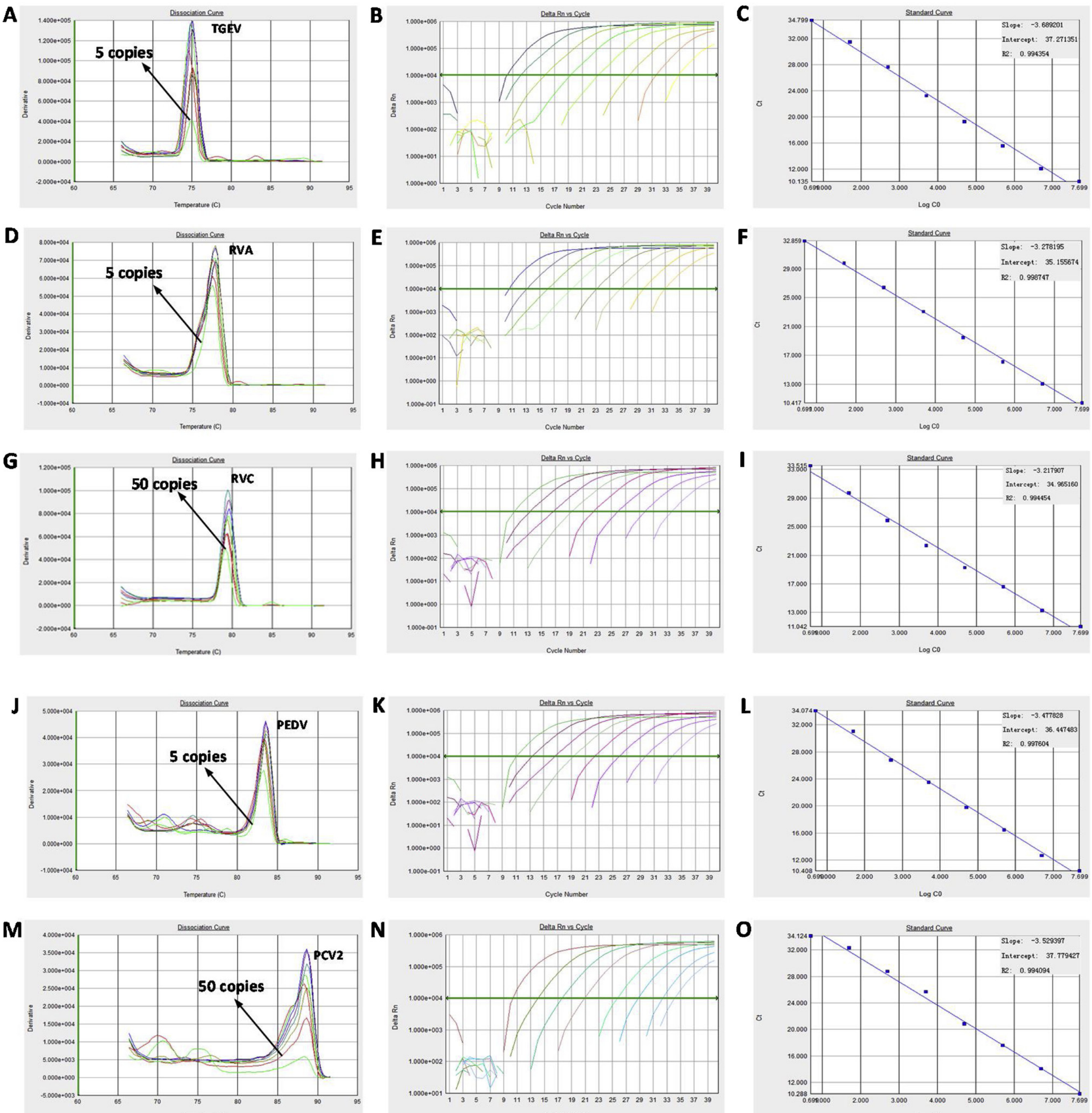

Fig. 2. Sensitivity, amplification curve, and standard curves of the multiplex real-time PCR. (A)-(O) are sensitivity, amplification curves and standard curves for TGEV, RVA, RVC, PEDV and PCV2, respectively. Melting curves obtained with the minimum DNA copies was considered to be the detection limit (sensitivity) of the test.

RVC, PEDV and PCV2, and 28 (31.1%) were infected with two or more viruses (Table 3). Overall, concordance between single and multiplex assays was 91.1% with a kappa correlation of 0.816.

4. Discussion

TGEV, RVA, RVC, PEDV, and PCV2 are five common pig pathogens which can cause diarrhea in pigs (Jarvis et al., 2016; Ogawa et al., 2009). Once infected with one or more of these five pathogens, especially in young piglets, the morbidity can be up to 100%, with an average mortality rate of 50%, resulting in high financial losses to farmers (Cao et al., 2015; Ogawa et al., 2009; Otto et al., 2015). However, the potential involvement of any of the viruses in similar clinical conditions requires the establishment of diagnostic tests for each agent. Thus, the availability of a multiplex PCR able to detect each and all these agents in clinical samples is preferred due to greatly enhanced diagnosis by reducing labor, cost and time. In this study, a novel EvaGreen multiplex real-time PCR assay was successfully established to detect TGEV, RVA, RVC, PEDV, and PCV2 simultaneously in one tube with five pairs of specific primers.

For the melting curve based multiplex real-time PCR, the specificity is defined by characteristic amplicon Tm value of each target (Perez et al., 2012; Zheng et al., 2016). Many factors may influence amplicon Tm and among them nucleotide composition and amplicon length of the target are fundamental (Aloisio et al., 2018; Wang et al., 2008). To enable broad application of the multiplex assay under field conditions, the most conserved capsid protein, spike glycoprotein or nucleoprotein gene was therefore selected as diagnostic region for primer design of

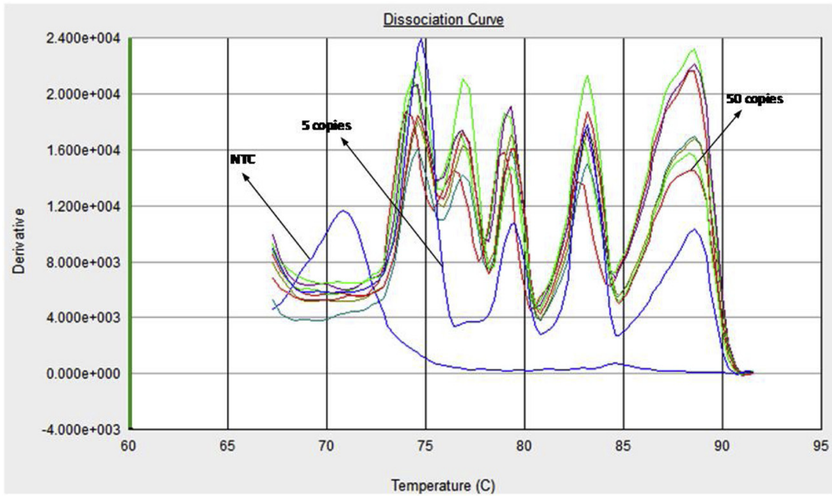

Fig. 3. Sensitivity of the multiplex real-time PCR assay when five viral templates are present in equimolar amount ranging from $5 \times 10^{\circ}$ copies/μL to 5×10^{7} copies/μL.

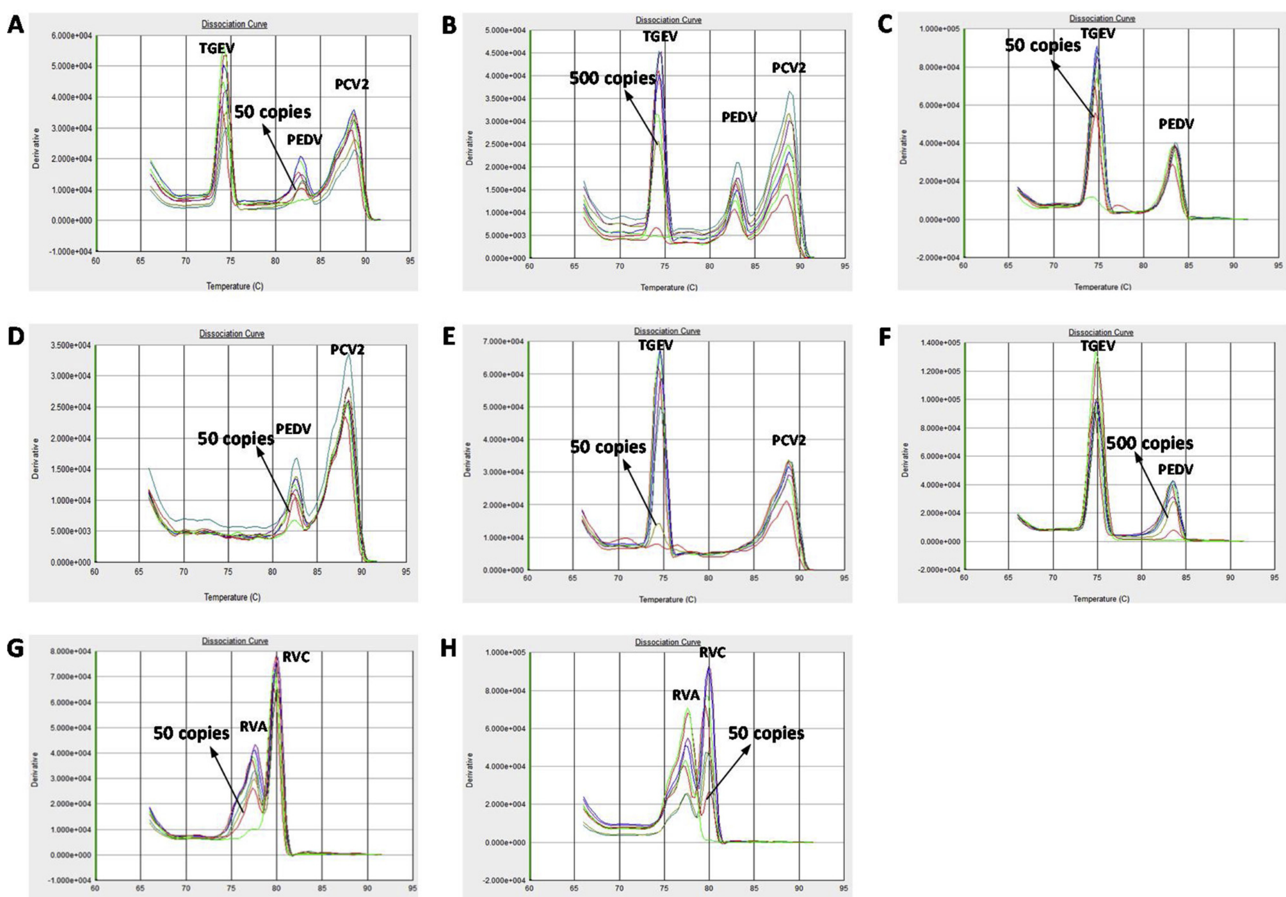

Fig. 4. Sensitivity of the multiplex real-time PCR for different template combinations of five viruses. (A) Sensitivity for PEDV with a background of 5×10^{5} copies/μL TGEV and PCV2. (B) Sensitivity for TGEV with a background of 5×10^{5} copies/μL PEDV and PCV2. (C) Sensitivity for TGEV with a background of 5×10^{5} copies/μL PEDV. (D) Sensitivity for PEDV with a background of 5×10^{5} copies/μLPCV2. (E) Sensitivity for TGEV with a background of 5×10^{5} copies/μL PCV2. (F) Sensitivity for PEDV with a background of 5×10^{5} copies/μL TGEV. (G) Sensitivity for RVA with a background of 5×10^{5} copies/μL RVC. (H) Sensitivity for RVC with a background of 5×10^{5} copies/μL RVA.

each target virus included in this study, resulting in 97–100% coverage of respective sequences available in NCBI, when at most three mismatches are allowed at primer binding positions that do not significantly affect the performance of the primers. While PEDV, TGEV and PCV2 are highly conserved in their corresponding target regions with nucleotide sequence homology of 99–100%, 96–100% and 97–100%, respectively, the target regions from RVA and RVC have 91–100% and 90–100% sequence similarity among respective strains available in NCBI. This may result in slight Tm variation in the resulting amplicons, as demonstrated by a relatively low Tm standard deviation

Table 2
Intra-assay and inter-assay repeatability analysis of the multiplex real-time PCR when a single pig diarrhea virus was present.

Virus concentration (Copies)		Intra-assay					Inter-assay				
		Tm/Ct value			Average	CV	Tm/Ct value			Average	CV
5×10^6	TGEV	74.8/14.05	74.8/14.07	74.8/14.07	74.80/14.06	0.00/0.08	74.8/14.05	75.1/14.87	74.9/14.38	74.93/14.43	0.20/2.86
	RVA	77.8/13.58	77.8/13.55	77.8/13.55	77.80/13.56	0.00/0.13	77.8/13.58	77.9/13.34	77.8/13.98	77.83/13.63	0.07/2.37
	RVC	79.9/15.11	79.6/15.34	79.6/15.34	79.70/15.26	0.22/0.87	79.9/15.11	79.8/15.56	79.6/15.79	79.77/15.49	0.19/2.23
	PEDV	83.4/14.42	83.4/14.55	83.5/14.55	83.40/14.51	0.00/0.52	83.4/14.42	83.3/14.23	83.5/14.34	83.40/14.33	0.12/0.67
	PCV2	88.6/14.83	88.3/15.01	88.6/14.94	88.50/14.93	0.20/0.61	88.6/14.83	88.5/15.14	88.6/14.94	88.57/14.97	0.07/1.05
5×10^4	TGEV	74.9/23.62	75.1/23.22	75.1/23.32	75.03/23.39	0.15/0.89	74.9/23.62	75.1/23.34	75.0/23.42	75.00/23.46	0.13/0.61
	RVA	77.8/23.24	77.8/23.07	77.7/23.14	77.77/23.15	0.07/0.37	77.8/23.00	77.9/23.14	77.7/23.19	77.80/23.11	0.13/0.43
	RVC	79.9/26.59	79.9/26.76	80.1/26.22	79.97/26.52	0.14/1.04	79.9/26.59	79.8/25.38	80.2/26.26	79.97/26.08	0.26/2.40
	PEDV	83.5/23.17	83.5/23.29	83.5/23.64	83.50/23.37	0.00/1.05	83.5/23.17	83.5/22.90	83.6/23.68	83.53/23.25	0.07/1.70
	PCV2	88.9/25.78	88.6/25.49	88.6/25.13	88.70/25.47	0.20/1.28	88.9/25.78	88.6/25.49	88.7/25.17	88.73/25.48	0.17/1.20
5×10^2	TGEV	75.1/30.98	75.1/31.39	74.9/31.78	75.03/31.38	0.15/1.27	75.1/30.98	75.0/31.39	74.9/31.58	75.00/31.32	0.13/0.98
	RVA	77.5/29.68	77.5/29.34	77.5/30.48	77.50/29.83	0.00/1.96	77.5/29.68	77.8/29.34	77.5/30.27	77.60/29.76	0.22/1.58
	RVC	79.9/30.28	79.6/30.47	79.6/31.55	79.70/30.77	0.22/2.23	79.9/30.28	80.1/30.47	79.6/31.43	79.87/30.73	0.32/2.01
	PEDV	83.6/31.79	83.6/31.77	83.6/31.29	83.60/31.62	0.00/0.90	83.6/31.79	83.7/31.77	83.6/31.84	83.63/31.80	0.07/0.11
	PCV2	88.6/31.27	88.6/32.12	88.6/33.10	88.60/32.16	0.00/2.85	88.6/31.27	88.7/32.12	88.6/31.97	88.63/31.79	0.07/1.43

of less than 0.59 (data now shown). To better distinguish the five viruses, the primers were designed to generate amplicons with large amplicon Tm differences between two successive peaks, which allowed for easily separating the melting peaks even when different viral strains with slightly variable amplicon Tm values were tested.

A key consideration in performing EvaGreen multiplex real-time PCR assays is the possibility of primer dimer formation, which can lead to false positive results influencing the specificity and sensitivity of the multiplex real-time PCR detection (Belák, 2007; Huang et al., 2010; Vandesompele et al., 2002). To reduce or avoid the effect of primer dimers on experimental results, the primer pairs generated were strictly assessed for species specificity through analyzing potential mismatches in primers by Blast database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) search and primer interactions by using the multiplex program of Primer Premier 5.0. The best output-scores for each primer pair were chosen by means of a software package, and optimal primer pair combinations were established experimentally to achieve high performance.

In this study, primer dimers still existed after replacing the primers and optimizing the annealing temperature and the concentration of each primer. (Chun et al., 2007; Xu et al., 2017). Nevertheless, the primer dimers correspond to lower melting peaks that could be distinguished from the target products and the sensitivity of the multiplex assay was not significantly affected. Distinct peaks and Tm values corresponding to the five viruses were obtained after amplification and melting curve analysis by the EvaGreen multiplex real-time PCR. When testing other viral pathogens of pigs, there was no specific amplification and no target virus-specific melting peak. In addition, some positive samples detected by the assay were further confirmed by gel electrophoresis and DNA sequencing. These results indicated that the method had good specificity.

High sensitivity of diagnostic applications is essential for early detection of viruses. In the present study, the detection limits of TGEV, RVA RVC, PEDV and PCV2 by the EvaGreen multiplex real-time PCR assay were 5, 5, 50, 5 and 50 copies/μL, respectively, being equal to or 10 times less sensitive than the single assays. This sensitivity loss may be due to primer dimer formation, which possibly occurred in the presence of non-target primer pairs at low DNA concentration. What was more significant was that when the five virus templates coexisted in the multiplex reaction, the minimum detection limit for each virus still reached 50 copies. The sensitivity of the EvaGreen multiplex real-time PCR established in this study was similar to that of a conventional multiplex PCR assay developed by our research group (Liu et al., 2019), but much higher than that of other similar assays, which had the minimum detection limit of 1.74×10^4, 2.1×10^3, 2.17×10^3 and 1.26×10^4 copies/μL for TGEV, PEDV, PCV2 and RVA, respectively (Zhao et al., 2013). In addition, the multiplex assay described here was even more sensitive than SYBR Green based real-time PCRs for detection of TGEV, PCV2 and PEDV (30, 250, 432 copies/μL for TGEV, PCV2 and PEDV, respectively) (Wang et al., 2008; Xiu et al., 2012; Zhang et al., 2011), and was comparable to a probe-based multiplex real-time PCR which had a detection limit around 90 copies/ μL for TGEV and 70 copies/ μL for PEDV (Kim et al., 2007). To simulate field settings several mixed viral template combinations with a background of high concentration of target viruses were chosen to further validate the sensitivity of the EvaGreen multiplex real-time PCR assay. The sensitivity of PEDV decreased to 500 copies/μL in combination with TGEV, RVA decreased to 50 copies/μL in combination with RVC, TGEV decreased to 500 copies/ μL in combination with PEDV and PCV2. This may be caused by multiple targets competing for enzymes and nucleotides in one reaction (Zheng et al., 2016). Overall the obtained data demonstrated that the diagnostic method applied in this study is highly

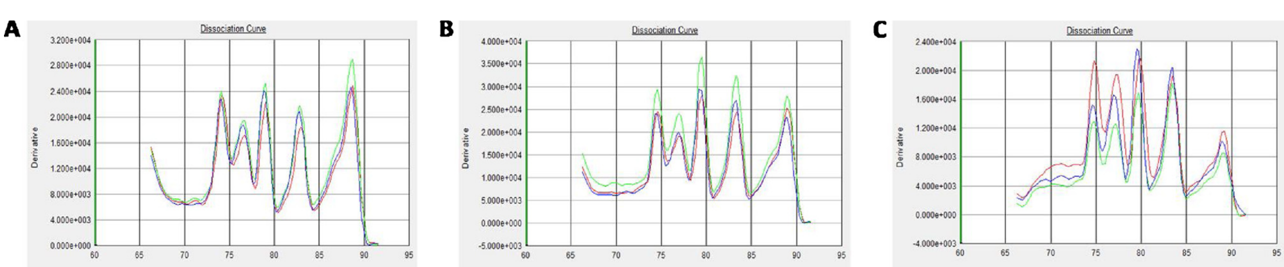

Fig. 5. Reproducibility of the EvaGreen multiplex real-time PCR assay. (A) Results obtained using all five target DNAs mixed as templates with a concentration of 5×10^6 copies/μL in a single tube. (B) Results obtained using all five target DNAs mixed as templates with a concentration of 5×10^4 copies/μL in a single tube. (C) Results obtained using all five target DNAs mixed as templates with a concentration of 5×10^2 copies/μL in a single tube. Each concentration was assayed three times.

Table 3
Detection of five pig viruses in clinical pig samples by using multiplex real-time PCR and single real-time PCR.

Method	No. samples	TGEV [no. positive (%)]	RVA [no. positive (%)]	RVC [no. positive (%)]	PEDV [no. positive (%)]	PCV2 [no. positive (%)]	TGEV + PCV2 [no. positive (%)]	RVA + PCV2 [no. positive (%)]	RVC + PCV2 [no. positive (%)]	PEDV + PCV2 [no. positive (%)]	RVA + PEDV + PCV2 [no. positive (%)]	RVC + PEDV + PCV2 [no. positive (%)]	Total [no. positive (%)]
Multiplex real-time PCR	90	1(1.1)	1(1.1)	0(0)	8(8.9)	20(22.2)	1(1.1)	1(1.1)	1(1.1)	15 (16.7)	0(0)	3(3.3)	51(56.7)
Single real-time PCR	90	1(1.1)	1(1.1)	1(1.1)	7(7.8)	19(21.1)	1(1.1)	1(1.1)	2(2.2)	18(20)	1(1.1)	5(5.6)	57(63.3)

sensitive. This might be due to a better primer design, an optimized reaction system and an opportune fluorescent dye-EvaGreen®. SYBR Green dyes are defective for multiplexing because of their preference for the amplicons of high GC contents and long segments, while EvaGreen is inserted evenly into the DNA double chain without saturation effect (Rao et al., 2014).

To quantitatively detect the viruses, the linear standard curves were obtained by plotting the Ct values against the log of the concentration (copies/μL). All graphs showed good efficiencies, indicated by the slopes of the standard curves (Fig. 2). R^2 had the desired value over 0.99 for the five viruses. In addition, the coefficients of variation, Tm and Ct values of less than 0.32% and 2.86% respectively, indicated a good reproducibility of the here presented multiplex real-time PCR. Taken together, the here described multiplex real-time PCR can accurately quantitate the titers of the five viruses. However, it should be noted that quantitative detection is not possible when more than one target virus is present in a sample; although a total titer could be estimated using the multiplex assay standard curve. To address this possible issue, the melting curve analysis-based multiplex assay combined with the corresponding single real-time PCR could better fulfill this task if accurate quantitation of each virus is needed in some cases.

Upon completion of the establishment of the multiplex PCR assay with plasmid standard templates, negative and positive control, the assay was initially validated with 10 positive and 10 negative samples that have been previously identified by a conventional PCR and the involved viruses have been confirmed by amplicon sequencing (Liu et al., 2019). The diagnostic accuracy of the multiplex PCR for detection of the five viruses was found to have 100% sensitivity and specificity (data not shown). For further evaluating the applicability of the developed EvaGreen multiplex real-time PCR assay, 90 clinical samples were tested by both the multiplex and single real-time PCR and representative positive samples have been confirmed by conventional PCR and amplicon sequencing. The overall agreement between them was 91.1%. The positive rates of TGEV, RVA, RVC, PEDV and PCV2 detected by the multiplex assay were 2.2%, 2.2%, 4.4%, 28.9% and 45.6%, respectively, which were a bit lower than those of the single assays. Considering the slightly lower sensitivity of the multiplex assay, particularly in cases with one target virus in significant excess over the other target when amplifying multiple target viruses, this result was explainable. Previous researches indicated that the TGEV, RVA, RVC, PEDV and PCV2 detection rate was 46%, 62%, 53%, 35.7% and 85% by real-time PCR assays (Marthaler et al., 2014; Shi et al., 2017; Vemulapalli et al., 2009; Yang et al., 2007). The relatively low detection rate obtained in this study may be due to the fact that the clinical samples were collected from healthy pigs, not diarrheic pigs. In addition, regional differences, feeding differences, pig-age differences and sanitary conditions of the pigsty also affected the infection rate of swine diarrhea viruses (Chae et al., 2000; Lowe et al., 2014; Shibata et al., 2000). In the following study, more samples from healthy pigs as well as diarrheic pigs should be investigated to fully validate the multiplex assay. Nevertheless, the high total positive rate in clinically healthy samples highlights the importance of early detection for the prevention and control of viral diarrhea.

5. Conclusion

In conclusion, the multiplex assay developed in this study, which is a combined EvaGreen based multiplex real-time PCR with melting curve analysis, has been proven to be a fast, robust, economical, efficient, specific and sensitive method for qualitatively detecting single or concurrent infection with TGEV, RVA, RVC, PEDV and PCV2 in clinical samples in a single closed tube, eliminating the need for fluorescence probes. Moreover, the assay could quantitate each of these five viruses in cases when a single target virus was present. This method is therefore an alternative approach suitable for laboratory diagnosis, routine surveillance and epidemiological investigations of viral diarrhea in pigs.

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