A multiplex RT-PCR assay for rapid and simultaneous detection of four RNA viruses in swine

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A B S T R A C T

A multiplex reverse transcription polymerase chain rection (mRT-PCR) was developed for simultaneous detection of four RNA viruses in swine. The conserved target sequences directed to classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis coronavirus (TGEV) were selected based on alignments of genomic sequences and then specific primers were designed. The mRT-PCR assay was developed and evaluated for its specificity and sensitivity. The expected product from the single viral template was amplified by mRT-PCR and no spurious PCR amplification occurred from the genomic RNA or DNA of other pathogens. For single virus or different combinations of two viruses the detection limit of mRT-PCR was consistent with a single RT-PCR wtith 1×10^3 copies. For different combinations of the three viruses or four viruses, sensitivity of PEDV detection partially decreased. All of positive clinical specimens by the mRT-PCR were identically confirmed using Taqman RT-qPCR. Therefore, the mRT-PCR is a useful tool for epidemiological studies and laboratory diagnosis of single virus and/or mixed infections in swine.

1. Introduction

Classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis coronavirus (TGEV), are currently the most important pathogens that have caused tremendous economic losses for swine industries worldwide. Under typical conditions of intensive swine production, it is ordinary for swine to be simultaneously infected with two or more viral pathogens (Hu et al., 2015; Liu et al., 2011). Multiple infections lead to atypical clinical signs. For instance, CSFV and PRRSV cause respiratory failure or abortions and/or stillbirths in pigs which can be easily confused (Lim et al., 2016; Young et al., 2010). Similarly, signs of the PEDV infection were clinically and pathologically indistinguishable from those caused by TGEV (Ducatelle et al., 1982; Haelterman, 1972). Usually, viral isolation based on cell culture is the standard laboratory methods for diagnosis of viral diseases (Hofmann and Wyler, 1988; Terpstra and Wensvoort, 1988), but is time consuming and complicated. When clinical signs can be variable and may not be pathogen-specific, a number of costly virus-specific tests are performed to diagnose accurately viral pathogens. Therefore, development of rapid and simultaneous detection assay for diagnosis of swine virus pathogens is important and urgent.

Several investigators have previously developed PCR and real-time PCR based multiplex assays for the differential detection of related multiple pathogens or pathogen causing diseases with similar symptoms (Aguero et al., 2004; Hu et al., 2015; Liu et al., 2011; Settypalli et al., 2016; Thonur et al., 2012; Wu et al., 2013; Yue et al., 2009). To our knowledge, mRT-PCR for simultanous detection of CSFV, PRRSV, PEDV and TGEV infection of swine has not been reported. In this study, we developed a multiplex RT-PCR assay for the rapid and simultaneous detection of CSFV, PRRSV, PEDV and TGEV in a single reaction. The mRT-PCR assay is used for the scale screening and detection of viruses in various swine clinical specimens.

2. Materials and methods

2.1. Viruses and cells

CSFV shimen strain, PRRSV JAX1 P80 strain, TGEV Miller M6 strain, BVDV NADL strain, PCV2 10JS-2 strain, RSV A2 strain, Influenza A virus (A/chicken/Hubei/489/2004(H5N1)) were kept in our laboratory and PEDV CV777 strain was obtained from China Center for Type Culture Collection (CCTCC) (Wuhan, China). PK-15, Marc-145

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and Vero cells were obtained from CCTCC. CSFV and TGEV were propagated in PK-15 cells and PRRSV was propagated in Marc-145 cells and PEDV was propagated in Vero cells.

2.2. clinical specimens

A total of 117 clinical specimens from 67 animals, including sera, lymph nodes, spleens, lungs, renal, and intestinal, were collected from pigs with respiratory and high fever syndromes or with diarrhea from commercial herds in Hubei, Henan and Guangxi provinces, China, between February 2017 and November 2018. Collected specimens were stored at −80 °C for virus detection.

2.3. Primer design and RNA extraction

Genome sequences of CSFV, PRRSV, PEDV and TGEV were obtained from GenBank database, and aligned using Bioedit software (Tom Hall, Ibis Biosciences, Carlsbad, CA). Based on the alignments, specific primers for CSFV, PRRSV, PEDV and TGEV were designed and analyzed using the Primer Premier 5.0™ software. BLAST search was also conducted to verify the specificities of the primers. The targeted genes and primer sequences are listed in Table 1. Oligonucleotide sequences were synthesized from a commercial source (Sangon, China). The primers were aliquoted to a final concentration of 10 μM and stored at −20 °C.

Total RNA was extracted from the supernatants of infected cell cultures, tissue homogenates or sera, using Trizol reagent (Invitrogen, CA, USA) as recommended by the manufacturer. RNA was eluted using 15 μL diethylpyrocarbonate (DEPC)-treated water. The purity of extracted total RNA was determined by measuring the absorbance ratio at wavelength 260 nm over 280 nm and the RNA concentration is based on the absorbance at 260 nm using a NanoDrop 2000c spectrophotometer (Thermo Scientific, FL, USA). RNA samples with 1.9–2.1 of the 260 nm/280 nm ratio were used to RT-PCR template.

2.4. Reverse transcription and multiplex PCR amplification

mRT-PCR consists of two steps: reverse transcription (RT) and PCR amplification. RT was performed in a 20 μL reaction mixture containing 4 μL M-MLV RT 5×Buffer (Promega), 1 μL of M-MLV reverse transcriptase (200 U/μL) (Promega), 0.5 μL Ribolock RNase Inhibitor (40U/μL, 2500 U) (Invitrogen™), 1 μL Random primer d(N)9 (Invitrogen™), 1 μL dNTPs (10 mM) (Invitrogen™), and 11.5 μL of a mixture of RNA (¯2 μg) in DEPC-treated water. The RT reaction was carried out at 37 °C for 1 h and then inactivated at 95 °C for 10 min.

The mRT-PCR was optimized by varying single parameters with fixed other parameters. The optimized multiplex PCR was performed in a 20 μl volume, in which the reaction mixture contained 2 μl 10 × PCR buffer with 2 mM MgCl₂, 0.8 mM of each dNTP, four sets of primers (0.2 μM of each primer) (Table 1), 1 μl of cDNA template and 1 U *Taq* DNA Polymerase (Invitrogen™). PCR was carried out in the thermal cycler. The cycling protocol consisted of an initial denaturing at 94 °C for 5 min; then 35 cycles of denaturing at 94 °C for 30 s, annealing at

55 °C for 30 s, and extension at 72 °C for 50 s; and a final extension step at 72 °C for 7 min. Amplicons were detected by electrophoresing 5 μL aliquots through 2% agarose gels in 1 × TAE (40 mM Tris-aceate [pH 8.0], 1 mM EDTA) with ethidium bromide.

2.5. Sensitivity and specificity of mRT-PCR

To investigate sensitivity and sensitivity of mRT-PCR for simultaneous detection of four viruses, a mixture of viral RNAs, ranging from 10^6 to 10^1 copies, from a single virus or different combinations of four viruses, was tested. Specificity of mRT-PCR was evaluated by examining the amplified products of CSFV, PRRSV, PEDV and TGEV from viral RNA templates or from genomic RNAs of other pathogens, including PCV2, BVDV, RSV and H5N1. Amplification of single PCR using specific primer pair for corresponding virus to independent assay was used for control. Specificity of mRT-PCR was further confirmed by amplicon sequencing.

2.6. Detection of CSFV, PRRSV, PEDV and TGEV in clinical specimens

A total of 117 clinical specimens from 67 animals were collected for this study. The specimens were kept at 4–10 °C during transportation and temporary storage or kept at −80 °C for long-term storage. Upon receipt, the specimens were separated into two halves: one half was subjected to mRT-PCR, single RT-PCR and TaqMan RT-qPCR assays, while the other half was used for confirming data or virus isolation. All clinical specimens were confirmed by a TaqMan qRT-qPCR (Chen et al., 2004; Lurchachaiwong et al., 2008; Risatti et al., 2003; Wang et al., 2014). The selected positive specimens were used for amplicon sequencing.

3. Results

3.1. Development of a mRT-PCR assay for rapid and simultaneous detection of CSFV, PRRSV, PEDV and TGEV

Target sequences directed to CSFV, PRRSV, PEDV and TGEV were selected based on alignments of complete genome sequences. Specific primers for simultaneous detection of four viruses were designed (Table 1). To evaluate specificity of primers, the specific primers for each virus was first analyzed using the respective viral cDNA as template with a single-target PCR. The specific product was observed on amplification of CSFV, PRRSV, PEDV or TGEV and the amplicons exhibited the expected sizes with 116, 197, 435 and 720 bp, respectively, while no product was observed from negative control (Fig. 1A). Identities of the amplicons were further confirmed by sequencing. For mRT-PCR, the primers for each virus were put together in one reaction for their suitability to selectively amplify their respective targets. Similarly, desired size product was obtained for CSFV, PRRSV, PEDV or TGEV reference strain, respectively. The mRT-PCR was carried in the optimized condition. When different combinations of cDNA templates of three or four viruses were used in the optimized mRT-PCR reaction, the

Table 1
Specific primers designed to detect four viruses by multiplex RT-PCR.

Virus (accession Number)	Primers	Oligonucleotide sequences (5' to 3')	Targe[a]	Product size (bp)
CSFV (AF092448.2)	5'UTR-F	GCTCCCTGGGTGGTCTAAGTC	5'UTR	116
	5'UTR-R	GGGTTAAGGTGTGTCTTGGGC		
PRRSV (FJ548853.1)	M-F	ACCTCCAGATGCCGTTTGTG	M	197
	M-R	GCTTTTCTGCCACCCAACAC		
PEDV (AF353511.1)	M-F	GGTGTCAAGATGGCTATTCTATGG	M	435
	M-R	TGAAGCATTGACTGAACGACCA		
TGEV (DQ811785.1)	N-F	GACAAACTCGCTATCGCATGG	N	720
	N-R	AGTGGTATTTGTGTGTGAACGTGA'		

[a] 5′UTR, 5′ untranlated region; PRRSV M, membrane gene (ORF6); PEDV M, membrane gene; TGEV N, nucleoprotein gene.
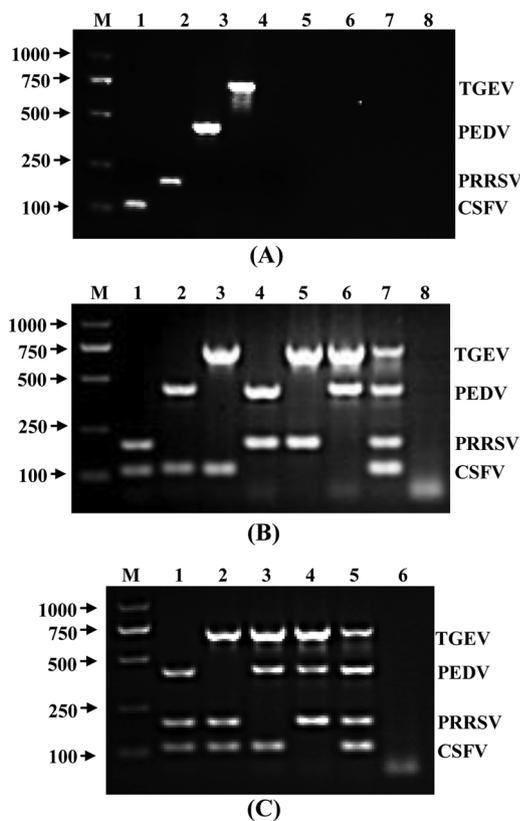
Fig. 1. Development of a multiplex RT-PCR assay for simultaneous detection of CSFV, PRRSV, PEDV and TGEV. A) Evaluation of primers specific for CSFV, PRRSV, PEDV and TGEV using a single RT-PCR. M, Marker; Lane 1, CSFV; 2, PRRSV; 3, PEDV; 4, TGEV; 5, Negative control for CSFV primer; 6, Negative control for PRRSV primer; 7, Negative control for PEDV primer; 8, Negative control for TGEV primer. B) Detection of two viral RNAs by mRT-PCR. M, Marker; Lane 1, CSFV/PRRSV; 2, CSFV/PEDV; 3, CSFV/TGEV; 4, PRRSV/PEDV; 5, PRRSV/TGEV; 6, PEDV/TGEV; 7, CSFV/PRRSV/PEDV/TGEV; 8, Negative control. **C)** Detection of triple viral RNAs by mRT-PCR. M, Marker; Lane 1, CSFV/PRRSV/PEDV; 2, CSFV/PRRSV/TGEV; 3, CSFV/PEDV/TGEV; 4, PRRSV/PEDV/TGEV; 5, CSFV/PRRSV/PEDV/TGEV; 6, Negative control.

respective products were amplified and could be differentiated by agarose gel electrophoresis (Fig. 1B and C), suggesting that the mRT-PCR developed could be used for simultaneous detection of CSFV, PRRSV, PEDV and TGEV.

3.2. Specificity of mRT-PCR

To evaluate specificity of mRT-PCR, viral RNA or DNA extracted from CSFV, PRRSV, PEDV, TGEV, BVDV, PCV2, RSV or H5N1 reference strain was tested by mRT-PCR with the four primer sets or the virus-specific primer pair, respectively. Data showed that the amplified products of expected sizes were obtained from the single viral template using the four primer sets and no spurious PCR amplification occurred from the genomic RNA or DNA of other pathogens (such as BVDV, PCV2, RSV and H5N1) or negative control RNAs extracted from the cell

culture (Fig. 2). The amplified products were obtained from each virus using the virus-specific primer pairs (Fig. 2). We also tested several bacterial pathogens by mRT-PCR and no amplicons were produced from the genomic DNA of *E. coli*, *S. Typhimurium* or *S. Enteritidis* (Data not shown).

3.3. Sensitivity of mRT-PCR

Sensitivity of single virus and combined viruses detection in mRT-PCR assay was evaluated using serially diluted viral RNAs. Viral RNAs from each virus reference strain were used for mRT-PCR assay after the copy numbers were quantified by the Taqman RT-qPCRs. For single virus the detection limit of mRT-PCR was 1×10^3 copies (Fig. 3A) and the detection limit of mRT-PCR was also 1×10^3 copies for each virus in different combinations of two viruses (data not shown). For different combinations of three viruses, the detection limit of multiplex RT-PCR was 1×10^3, 1×10^5 and 1×10^3 copies for PRRSV, PEDV, TGEV, or 1×10^3, 1×10^3 and 1×10^4 copies for CSFV, PRRSV, PEDV, respectively (Fig. 3B). When simultaneous existence of four viruses were detected using mRT-PCR, the detection limit of CSFV, PRRSV, PEDV and TGEV was 1×10^3, 1×10^3, 1×10^5 and 1×10^3 copies, respectively (Fig. 3C).

3.4. mRT-PCR detection of clinical samples

To test mRT-PCR for diagnosis of virus infection in swine, a total of 117 clinical specimens from 67 animals were assayed and confirmed by a Taqman RT-PCR detection. The mRT-PCR results showed that 10 specimens positive for CSFV, 23 positive for PRRSV, 16 positive fo PEDV, 2 positive for TGEV, 5 positive for CSFV/PRRSV and 1 positive for PEDV/PRRSV were detected by mRT-PCR. Results from mRT-PCR assay were consistent with that of single RT-PCR. However, the specimens for virus infection or co-infections were positively detected by Taqman RT-qPCR but negatively by mRT-PCR (Table 2). Our results indicated that the mRT-PCR developed is a rapid and validated method for simultaneous detection of clinical specimens. Previous reports showed that co-infection of PRRSV/CSFV and procine teschovirus (PTV) (Liu et al., 2011) or PRRSV/CSFV and pseudorabies virus (PRV) (Hu et al., 2015) was detected by mRT-PCR in clinical specimens from commercial herds in China. In our experiments, five clinical specimens exhibited co-infection of PRRSV and CSFV and one specimen co-infected with PEDV and PRRSV based on mRT-PCR detection. No clinical specimens simultaneously containing three or four viruses were identified by mRT-PCR or Taqman RT-qPCR (Table 2).

4. Discussion

Virus infection caused the tremendous economic losses for swine industries worldwide. Diagnosis of virus infection is typically accomplished by viral isolation followed by immunostaining analysis and serological methods. However, viral isolation is complex, labor intensive and time-consuming. Serological methods have low specificity and/or sensitivity. Nucleic acid detection, particularly based on PCR/RT-PCR, has expanded exponentially in recent years. By simultaneously amplifying more than one target in the same reaction, multiplex PCR increasingly is used as a rapid and convenient assay in the clinical
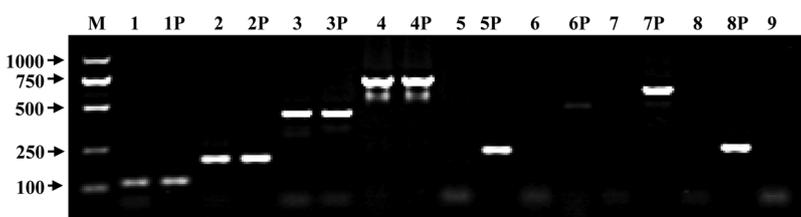

Fig. 2. Specificity of mRT-PCR. M, Marker; Lane 1–8, CSFV, PRRSV, PEDV, TGEV, BVDV, PCV2, RSV, or H5N1 by mRT-PCR amplification; Lane 1 P to 8 P, CSFV, PRRSV, PEDV, TGEV, BVDV, PCV2, RSV, or H5N1 by virus-specific RT-PCR amplification as a positive control. Lane 9, Negative control.
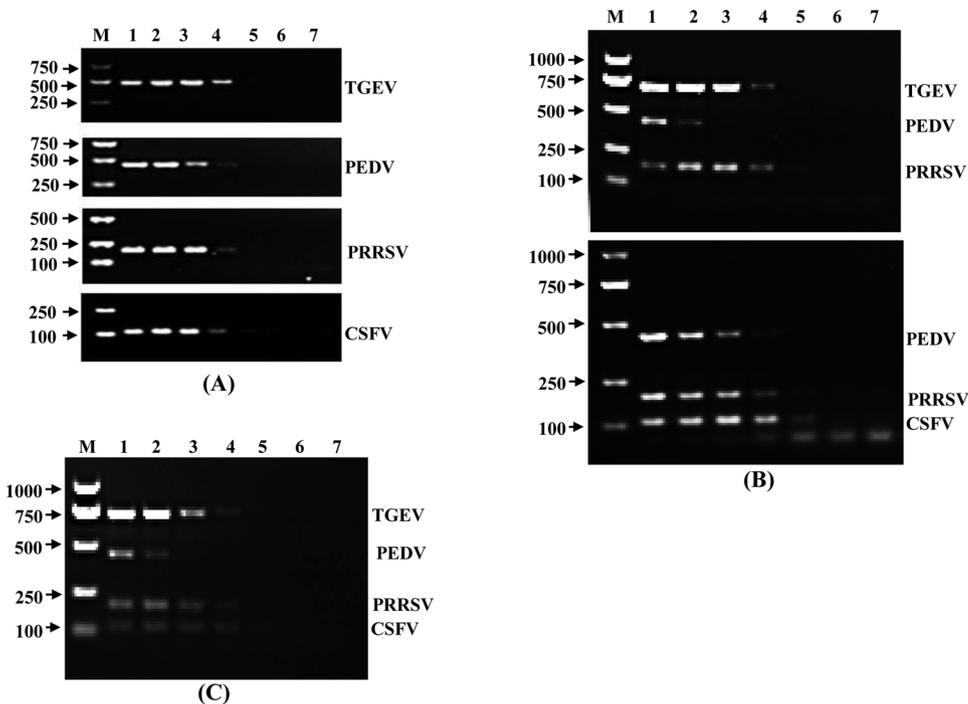
Fig. 3. Sensitivity of mRT-PCR. A) Amplification of single-target virus by mRT-PCR. of B) Amplification triple-target viruses by mRT-PCR. C) Amplification of four-target viruses by mRT-PCR. M, Marker; Lane 1, 10^6 copies; 2, 10^5 copies; 3, 10^4 copies; 4, 10^3 copies; 5, 10^2 copies; 6, 10^1 copies; 7, Negative control. Corresponding virus was marked on the right.

Table 2
Numbers of positive samples in 117 clinical specimens from sick pigs detected for earch of the four viruses by multiplex RT-PCR and TaqMan qRT-PCR[a].

Virus detected	Positive samples in multiplex RT-PCR	Positive samples in single RT-PCR	Positive samples in Taqman qRT-PCR
CSFV only	10	10	11
PRRSV only	23	23	25
PEDV only	16	16	17
TGEV only	2	2	2
CSFV/PRRSV	5	5	7
PEDV/PRRSV	1	1	1
No virus	60	60	54

[a] Taqman qRT-PCR was developed by our laboratory based on previous reports (Chen et al., 2004; Lurchachaiwong et al., 2008; Risatti et al., 2003; Wang et al., 2014).

diagnosis (Henegariu et al., 1997; Hu et al., 2015; Liu et al., 2011; Wu et al., 2013). In this study, we developed a multiplex RT-PCR assay for simultaneously detecting four viral pathogens in clinical specimens. Compared to a single RT-PCR or RT-qPCR, mRT-PCR is a rapid, convenient and effective assay and is more advantageous to detect clinically mixed infection.

For any effective detection method of viral pathogens, sensitivity and specificity are very important. Sensitivity of any PCR depends on multitude of factors including efficiency of the primer, reaction conditions and components (Elnifro et al., 2000; Reid et al., 1998). mRT-PCR contained multiple primer pairs in the same PCR reaction. Ideally, the primer pairs should enable similar amplification efficiencies for their respective targets and be not complementary for earh other, particularly at their 3′ ends (Cha and Thilly, 1993; Dieffenbach et al., 1993; Henegariu et al., 1997). Here, we optimizedly designed specific primers with G + C content of 42–62% and nearly identical annealing temperatures (58–61 °C) for mRT-PCR (Table 1). Our results demonstrated that the amplified products in a single reaction could be easily differentiated on the basis of size and that no significant mispriming to any template or dimers occurred. Sensitivity of mRT-PCR is identical to a single RT-PCR for each virus or different combinations of two viruses. When three or four viruses existed in same reaction, sensitivity of mRT-

PCR for PEDV detection decreased significantly compared to a single RT-PCR.

When assessing diagnostic performances, the mRT-PCR developed in this study could be successfully used to detect CSFV, PRRSV, PEDV and TGEV in clinical specimens. The virus positive specimens detected using mRT-PCR were consistent with that of single RT-PCRs. The partial virus negative samples detected by mRT-PCR were virus positive by Taqman RT-qPCR assay, suggesting that mRT-PCR seems to be less sensitive than Taqman RT-qPCR. However, by adding multiple primer pairs to the same reaction system and amplifying multiple cDNA templates, the mRT-PCR can amplify multiple targeted genes at one time so as to achieve the simultaneous detection of multiple pathogens. The mRT-PCR assay has the additional benefits of using a smaller sample size and fewer reagents. Therefore, the mRT-PCR developed in this study may be useful in epidemiological studies, laboratory diagnosis and surveillance of four RNA viruses in swine.

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