Use of a Multiplex RT-PCR Assay for Simultaneous Detection of the North American Genotype Porcine Reproductive and Respiratory Syndrome Virus, Swine Influenza Virus and Japanese Encephalitis Virus

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Abstract

A multiplex reverse transcriptase-polymerase chain reaction (multiplex RT-PCR) assay was developed and subsequently evaluated for its efficacy in the detection of multiple viral infections simultaneously, in swine. Specific primers for each of the 3 RNA viruses, North American genotype porcine reproductive and respiratory syndrome virus, Japanese encephalitis virus, and swine influenza virus, were used in the testing procedure. The assay was shown to be highly sensitive because it could detect as little as 10^{-5} ng of each of the respective amplicons in a single sample containing a composite of all 3 viruses. The assay was also effective in detecting one or more of the same viruses in various combinations in specimens, including lymph nodes, lungs, spleens, and tonsils, collected from clinically ill pigs and in spleen specimens collected from aborted pig fetuses. The results from the multiplex RT-PCR were confirmed by virus isolation. The relative efficiency (compared to the efficiency of separate assays for each virus) and apparent sensitivity of the multiplex RT-PCR method show that this method has potential for application in routine molecular diagnostic procedures.

Key words: Japanese encephalitis virus, multiplex RT-PCR, porcine reproductive and respiratory syndrome virus, swine influenza virus

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), which emerged in North America and Europe simultaneously in the late 1980s (Wensvoort et al. 1991; Collins et al. 1992), is characterized by reproductive failure in sows and respiratory disease in piglets (Goyal 1993). The causative agent is the porcine reproductive and respiratory syndrome virus (PRRSV), a member of the arteriviruses – a group of small, enveloped, positive-strand RNA viruses. PRRSV isolates can be classified into 2 distinct genotypes, North American (NA) and European (EU),

with VR-2332 and Lelystad virus (LV) being the prototype viruses of the 2 genotypes, respectively. To date, all the PRRSV strains isolated in China have been characterized as the NA genotype. PRRSV infections are characterized by immunosuppression, which results in combined infections or secondary infections with the swine influenza virus (SIV), porcine pseudorabies virus and porcine circovirus type 2. van Reeth et al. (1996) found that the clinical effects of PRRSV were exacerbated in concurrent infection with SIV. Although various subtypes of influenza viruses have been reported to infect swine populations in various countries, H1N1, H1N2 and H3N2 have been identified in all countries.

Under the typical conditions of intensive swine farming, the animals may often show simultaneous infection with 2 or more viral pathogens (Ogawa *et al.* 2009). Owing to the variable clinical signs of multiple viral infections and the consequent difficulty in the selection of the most appropriate testing method, a definitive diagnosis in the presence of multiple infections is often difficult. In several previous studies, multiplex PCR (mPCR) (Edward and Gibbs 1994) has been used to identify simultaneously and differentiate multiple viruses in a single sample on the basis of the amplicon sizes (Belák and Thorén 2001; Belák 2005). This ability of mPCR can facilitate the screening of "panels of pathogens" in a sample in which the targeted organisms are grouped on the basis of the clinical symptoms and relevance (Belák and Thoré 2001). In May 2006, a highly pathogenic PRRSV that causes continuous high fever and a high proportion of deaths in vaccinated pigs of all ages emerged and is prevalent in mainland China since then. Porcine reproductive disorders caused by other pathogens like SIV and Japanese encephalitis virus (JEV) have also been reported occasionally. Therefore, we developed and evaluated a multiplex reverse transcriptase-polymerase chain reaction (multiplex RT-PCR) assay for the simultaneous detection of nucleic acids from 3 RNA swine viruses, namely, PRRSV, SIV and JEV.

MATERIALS AND METHODS

Viruses and cells

In October 2001, a clinical isolate of SIV was obtained from one among a group of approximately 16-wk-old pigs in Henan Province, China, showing slow growth, cough and dyspnea. The isolate was propagated in white Leghorn specific-pathogen-free (SPF) chicken embryos and identified as H3N2 SIV [A/Swine/Henan/703/2001 (H3N2)]. Another clinical isolate of SIV was isolated in December 2005 from one among a group of approximately 12-wk-old pigs in Henan showing weight loss and coughing. The isolate was identified as H1N1 SIV [A/Swine/Henan/407/2005(H1N1)]. The H1N2 SIV [A/Swine/Hebei/1/2006 (H1N2)] isolate was gifted by Dr. Qiao Chuanling (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China). The

JEV 53-S strain was purchased from the Beijing Ordinary Microbiology Strain Store Center, Beijing, China, and was propagated in the PK-15 porcine kidney cell line. The PRRSV VR-2332 strain was gifted by Dr Yang Hanchun (China Agricultural University, China) and was propagated in the Marc-145 cell line. These viruses were used as standard viruses for the multiplex RT-PCR and maintained at -70°C until analysis. Uninfected PK-15, Marc-145 cell line, allantoic fluids, and classical swine fever virus (CSFV) were also used in the specificity assays.

Clinical specimens

From June 2007 to September 2008, 23 clinical specimens, including those of lymph nodes, tonsils, lungs, colons, duodenums, jejunums, hearts, kidneys, livers, spleens, and gonads, were collected from 23 4-8-wk-old sick piglets in 8 local farms and 6 aborted fetuses from different abortion cases. The samples were obtained from the Veterinary Medical Teaching Hospital, College of Veterinary Medicine, Henan Agricultural University, China.

Nucleic acid extraction and reverse transcription

Viral RNA was extracted from 200-µL aliquots of samples [10% tissue homogenates in phosphate buffered saline (PBS), which were prepared from 1 g of tissue or virus-infected cell culture supernatant] using a commercial test kit (QIAamp RNA Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions; the RNA extracts were then stored at -20°C until analysis.

The RT reaction was performed using 20-µL volumes; the reaction mixture in each volume contained $5 \times$ strand buffer, 25 mmol L^{-1} of each deoxynucleoside triphosphate (dNTP, Amersham Biosciences Corp., Piscataway, NJ, USA), 2.5 U of RNase inhibitor (Promega Corporation, Madison, WI, USA), 50 pmol mL^{-1} random hexamers, Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA), 5 µL of each RNA, and diethyl pyrocarbonate (DEPC)-water. RT was performed at 42°C for 60 min and at 75°C for 10 min.

Primer design

The nucleoprotein (NP) nucleotide sequences of the SIV strains/isolates were retrieved from GenBank and aligned using the DNAStar software (DNAStar Inc., Madison, WI, USA). The primers were selected using the Primer Premier software (ver. 5.0) and were based on a highly conserved sequence within the NP region of the SIV genome. A primer pair specific to the *ORF6* gene of PRRSV was designed to detect PRRSV. JEV was detected by identifying the nucleotide sequence of the conserved region of the *E* gene. The primer sequences for the detection of PRRSV and JEV were obtained using the PrimerSelect program in the DNAStar 5.0 software (DNAStar Inc.). The PCR primer pairs for each target gene and the size of each amplicon are summarized in Table 1.

Table 1 Specific primer pairs used to amplify each target gene

Virus	Target gene	Primer sequence (5′→3′)	Expected products (bp)
PRRSV	*ORF6*	PRRS1: ATTACCTACACGCCAGTG	418
		PRRS2: TAACAGCTTTTCTGCCAC	
JEV	*E*	E1: CTGAGATTTCAACGCTGGC	349
		E2: TGAGCGTTATTGAGCGAGC	
SIV	*NP*	NP1: ACGGTAGTTATAAGCATGA	155
		NP2: TGTCTCGGAAGATATAAGA	

Optimization of multiplex PCR conditions

The reactions were optimized by varying the primer concentrations of each target. Optimization was performed by methodical variation of each test parameter under standard PCR conditions. The tested primer concentrations ranged from 0.5 to 50 pmol. A primer concentration of 10 pmol yielded optimum amplification profiles for all the primers sets. Different $MgCl_2$ concentrations (1.5-3.5 mmol L^{-1}) were evaluated, and the most efficient concentration was selected. The annealing temperature and number of cycles were also determined experimentally. The best results were obtained at an annealing temperature of 52°C with 32 cycles.

Single RT-PCR assays

The single RT-PCR assays were performed using 25-μL volumes. The reaction mixtures contained the following reagents: 3.0 mmol L^{-1} $MgCl_2$, 1×PCR buffer II (500 mmol L^{-1} KCl and 100 mmol L^{-1} Tris HCl, pH 8.3), 200 μmol L^{-1} of each dNTP, 20 pmol of each primer, 2 μL cDNA, and 2.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). After optimization, the following standard thermocycler protocols were used: (1) For PRRSV, the cycling protocol consisted of an initial denaturation at 94°C for 3 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min; (2) for SIV, the cycling protocol consisted of an initial denaturation at 96°C for 3 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min; (3) for JEV, the cycling protocol consisted of an initial denaturation at 94°C for 3 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min.

The PCR amplification products were analyzed by agarose gel electrophoresis using 2% agarose stained with ethidium bromide and visualized under UV light.

Multiplex RT-PCR reaction

The multiplex RT-PCR consists of a 2-step procedure involving RT and PCR amplification. The RT reaction was performed using 5 μL of the three RNA mixture in 20-μL volumes. The multiplex RT-PCR reaction was performed using 25-μL sample mixtures in the Gene Amp PCR Kit and AmpliTaq Gold. The reaction mixture contained 2.5 mmol L^{-1} $MgCl_2$, 1×PCR buffer II (500 mmol L^{-1} KCl and 100 mmol L^{-1} Tris HCl, pH 8.3), 200 μmol L^{-1} of each dNTP, 10 pmol of each primer, and 2.5 U of AmpliTaq Gold. This mixture was added to the RT reaction tubes along with 100 ng of template and DEPC-water. The cycling protocol consisted of an initial denaturation at 94°C for 5 min; 32 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Negative controls were used for each test. Agarose gel electrophoresis was used to detect the multiplex RT-PCR products.

Sensitivity and specificity of single and multiplex RT-PCR assays

The sensitivity of each single RT-PCR has been previously reported (Schorr *et al*. 1994; Suarez *et al*. 1994; Paranjpe and Banerjee 1998). The sensitivities of the multiplex RT-PCR and the corresponding single RT-PCRs were compared using serial 10-fold dilutions of selected spiked samples containing all the target viruses. The specificity of the primer pair for each virus was analyzed using single PCR. The specificity of an amplicon corresponding to each target was confirmed by cloning DNA into the pGEM-T Easy vector (Promega, USA) and sequencing using an automatic DNA sequencer (ABI-377; PE Applied Biosystems, USA). The specificity of the multiplex RT-PCR was assessed in the clinical specimen. The specificity of the multiplex RT-PCR was extended to the CSFV, uninfected PK-15, Marc-145 cell line, and allantoic fluids.

Virus isolation

To verify the results of the multiplex PCR assays, viruses were isolated from all the positive samples. The PRRSV was isolated using previously described methods (Collins *et al*. 1992).

The SIV was isolated by inoculating the positive samples for the detection of SIV into 11-d-old SPF embryonated eggs. Whole-sample homogenates were diluted (1:5) in PBS containing 100 U mL^{-1} penicillin G and 100 mg mL^{-1} streptomycin sulfate. Eggs were inoculated with 0.2 mL of inoculum in the allantoic cavity, with 4 eggs per homogenate sample. The eggs were candled at 24 h post-inoculation, and dead eggs were discarded. The allantoic fluid was harvested from each egg at 72 h post-inoculation and was assayed for SIV. The presence of SIV was determined using a hemagglutination assay. Equal volumes of allantoic fluid and 0.5% chicken red blood cells were mixed together in V-bottom microtiter plates. The presence of hemagglutination was assessed after incubation for 40 min at room temperature.

JEV was isolated by inoculating the positive samples for the detection of JEV onto PK15 cells. A monolayer of PK15 cells grown in a 25-cm^2 culture flask was briefly adsorbed with 0.5 mL of the inoculum at 37°C for 2 h. After adsorption, the inoculum was replenished with 10 mL of maintenance medium. We also set up suitable mock-infected cell controls. The cells were then incubated at 37°C and observed daily for cytopathic effects. The presence of JEV was confirmed using the indirect immunofluorescence (IF) test.

RESULTS

Specificity of single and multiplex RT-PCR assays

Three RT-PCR primer sets were selected for the optimization and standardization of the multiplex RT-PCR. We also performed different amplification assays to optimize the multiplex reaction conditions. The specificity of a primer pair for each virus was analyzed using single RT-PCR. As shown in Fig.1, each viral target gene could be specifically amplified using its defined primer pair, and the RT-PCR products were found to be 418 bp (PRRSV *ORF6*), 349 bp (JEV *E*), and 155 bp (SIV *NP*) in size (Fig.1, lanes 1-3). The nucleotide sequence of each amplicon was further confirmed by DNA sequencing and found to be similar to the sequence in GenBank. All 3 viral RNAs could be simultaneously detected (Fig.1, lane 4). The 3 subtypes of swine influenza virus, A/Swine/Henan/703/2001(H3N2), A/Swine/Henan/407/2005(H1N1) and A/Swine/Hebei/1/2006(H1N2), were detected using the *NP*-specific
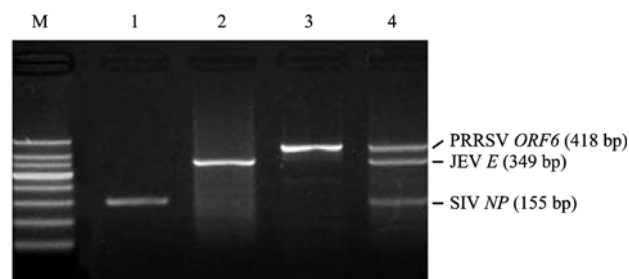

Fig. 1 Electrophoretic profiles of DNA amplicons obtained by single RT-PCR or multiplex RT-PCR. Single RT-PCR for SIV (lane 1), JEV (lane 2) and PRRSV (lane 3) viral RNA was performed using a single primer pair specific to each target gene. Lane 4, multiplex RT-PCR with mixed viral RNAs using all the mixed primer pairs. Lane M, 50-bp DNA ladder (Fermentas Life Sciences, Hanover, USA). The same as below.

primers to SIV (Fig.2, lanes 1-3). None of the amplicons had molecular sizes different from those expected for any of the known positive samples. Non-specific reactions were not observed with any of the negative controls included in the study. The CSFV, uninfected PK-15, Marc-145 cell line, and allantoic fluids did not generate amplicons (Fig.3).
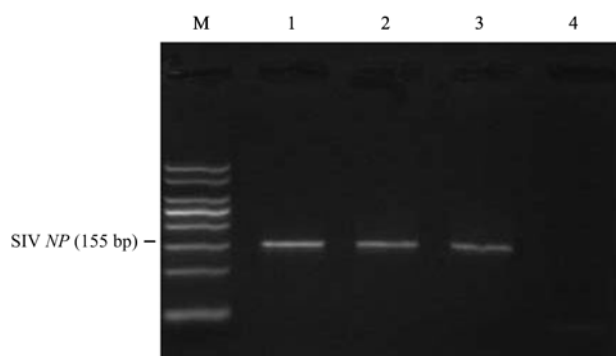

Fig. 2 Single RT-PCR for 3 subtypes of SIV. Single RT-PCR for A/Swine/Henan/703/2001(H3N2) (lane 1), A/Swine/Henan/407/2005(H1N1) (lane 2) and A/Swine/Hebei/1/2006(H1N2) (lane 3) viral RNA was performed using a primer pair specific to the SIV *NP* gene. Lane 4, negative control.
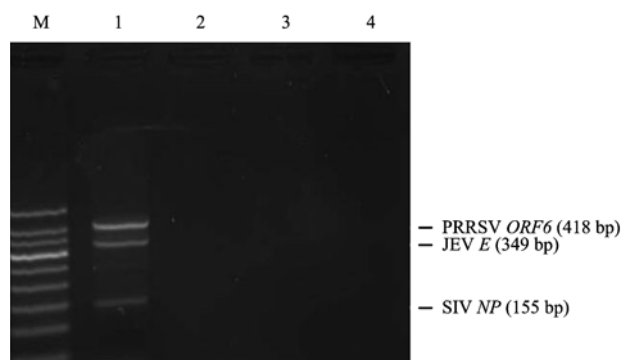

Fig. 3 Multiplex RT-PCR specificity. Primer specificity was tested by performing a multiplex RT-PCR reaction with mixed viral RNAs from all virus targets (lane 1) and with 3 negative controls, CSFV (lane 2), uninfected Marc-145 cell line (lane 3), and uninfected PK-15 cell line (lane 4).

Sensitivity of single and multiplex RT-PCR assays

The mixture of specific viral target RNAs served as the template for sensitivity analysis of single or multiplex RT-PCR. Analysis of the RT-PCR products by agarose gel electrophoresis after staining with ethidium bromide

allowed the detection of as little as 10^{-6}-10^{-7} ng of each viral target RNA in single RT-PCR (data not shown) and as little as 10^{-5} ng in multiplex RT-PCR (Fig.4). To determine the detection limit of the multiplex RT-PCR, the experiments were performed using serial 10-fold dilutions of selected spiked samples containing all the target viruses. Comparison of the sensitivities of the multiplex RT-PCR and the corresponding single RT-PCR for the detection of each of the 3 targets showed that the 2 techniques had identical sensitivities for PRRSV detection, while the sensitivity of the multiplex method was 1 log lower in the case of JEV and 2 logs lower in the case of SIV.
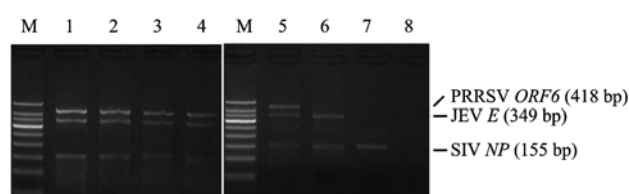

Fig. 4 Sensitivity of multiplex RT-PCR for simultaneous amplification of all viral target RNA. Each viral target RNA was purified and 10-fold series diluted at the concentration of 10^{-1}-10^{-8} ng μL^{-1} (lanes 1-8, respectively). One microliter of each concentration of each target RNA was mixed and multiplex PCR was followed.

Reproducibility of multiplex RT-PCR assay

When the RNA samples of the 3 viruses were used for the evaluation of reproducibility of the multiplex RT-PCR assay, each viral target RNA was purified and serially diluted 10-fold at the concentration of 10^{-3}-10^{-5} ng μL^{-1}. 1 mL of each concentration of each target RNA was mixed and multiplex PCR was followed. Each concentration was repeated for 3 times. Each virus was amplified for each time. The results indicated that the reproducibility of the multiplex RT-PCR assay is highly positive.

Screening of clinical specimens by multiplex RT-PCR assay

23 clinical specimens, including those of lymph nodes, tonsils, lungs, colons, duodenums, jejunums, hearts, kidneys, livers, spleens, and gonads of sick piglets, were subjected to the multiplex RT-PCR detection. The de-

tection results of multiplex RT-PCR for each of the 23 specimens showed that at least one virus was present in the lymph nodes, lungs, spleens, and tonsils. PRRSV infection was detected in 30.43% (7/23); SIV infection, in 13.02% (3/23); JEV infection, in 4.34% (1/23) of the tissue samples; PRRSV/SIV mixed infection in 21.73% (5/23) of the tissue samples; PRRSV/JEV mixed infection, in 8.69% (2/23) of the tissue samples; and SIV/JEV mixed infection, in 4.34% (1/23) of the tissue samples. The 4 remaining specimens were uninfected. All 6 aborted fetuses collected from different abortion cases demonstrated PRRSV mixed infection with SIV (4/6) or JEV (2/6) in the spleen which is shown in Table 2.

On testing 29 clinical samples obtained from aborted fetuses and piglets by multiplex PCR indicated the positive detection rates of PRRSV, SIV and JEV to be 68.96% (20/29), 44.82% (13/29) and 20.69% (6/29), respectively.

Table 2 Detection results of multiplex infections

Specimen type	No.	Character of infection types					
		PRRSV	SIV	JEV	PRRSV/SIV	PRRSV/JEV	SIV/JEV
Clinical specimens	23	7	3	1	5	2	1
Aborted fetuses	6				4	2	

Virus isolation

To verify the results of the multiplex PCR assays, viruses were isolated from all the positive samples. Marc-145 cells were inoculated with whole-sample homogenates; after 5 successive passages and incubation for 5 d, a cytopathic effect was observed. Of the 29 samples mentioned above, 20 were PRRSV-positive according to the IF test. The SPF chicken embryos were inoculated with whole-sample homogenates; after 3 successive passages and incubation for 72 h, 12 samples were found to be SIV positive according to hemagglutination test. PK-15 cell cultures were inoculated with whole-sample homogenates; after 5 successive passages and incubation for 5 d, a cytopathic effect was observed, and 4 samples were JEV positive according to the results of IF tests. The samples that tested positive on the basis of the virus isolation also tested positive after mPCR.

DISCUSSION

In this study, a multiplex RT-PCR assay was demonstrated to be capable of rapid detection and differentiation of 3 important swine RNA viruses (PRRSV, SIV and JEV) in a single reaction. The assay was shown to be highly sensitive because it could detect as little as 10^{-5} ng of each of the respective amplicons in a single sample containing a composite of all 3 viruses. Nonspecific reactions were not observed with any of the negative controls included in the study. The CSFV, uninfected PK-15, Marc-145 cell line, and allantoic fluids did not generate amplicons. This method was also applied for 23 clinical specimens and the results showed that PRRSV infection was detected in 30.43% (7/23), SIV infection in 13.02% (3/23) and JEV infection in 4.34% (1/23) of the tissue samples; as well as PRRSV/SIV mixed infection in 21.73% (5/23), PRRSV/JEV mixed infection in 8.69% (2/23) and SIV/JEV mixed infection in 4.34% (1/23) of the tissue samples. These results confirmed that the animals may often show simultaneous infection with two or more viral pathogens (Ogawa *et al.* 2009).

Precise primer design and the appropriate ratio of each primer pair are crucial for successful amplification using mPCR (Bej *et al.* 1990). We selected 3 RT-PCR primer sets for the optimization and standardization of the multiplex RT-PCR. Different amplification assays were also performed to optimize the multiplex reaction conditions. The specificity of a primer pair for each virus was analyzed using single RT-PCR. As shown in Fig.1, each viral target gene could be specifically amplified using its defined primer pair.

SIV is the major causative virus in the porcine respiratory disease complex and is characterized by cough, fever, lethargy, and anorexia commencing 1-2 d after experimental infection and lasting for 3-4 d (van Reeth and Nauwynck 2000). The SIV infects the epithelium of the respiratory tract in pigs and the epithelial cells and the mucociliary apparatus of the airways, thereby resulting in combined infections or secondary infec-

tions with other pathogens. In this study, the SIV *NP* gene, which is highly conserved, served as a remarkable target gene for detection of SIV. The results showed that the developed assay detects 3 subtypes of swine influenza virus without discriminating between them (Fig.2). Therefore, an ensuing step, which uses assays capable of typing SIV as well as revealing mixed infections caused by the 3 subtypes, is necessary.

In addition, the detection of JEV in the spleen samples from 2 aborted fetuses confirms that JEV is one of the most important infectious causative agents of reproductive failure in swine (Nidaira *et al.* 2009). Although we chose primers corresponding to the *E* gene region that was conserved in all genotypes of JEV, the actual applicability of multiplex RT-PCR in detecting all the genotypes of JEV needs to be further investigated.

Generally, the development of a multiplex RT-PCR assay is difficult. The optimization of a multiplex reaction requires adjustments in the concentrations of reagents, annealing temperature, and/or cycling conditions to obtain the best protocol for amplification of more than one target sequence. Such adjustments may contribute to the selection of less than optimal conditions for some primers (Henegariu *et al.* 1997). The most common problem is that some of the primers used in the same reaction tube may interact with each other, thereby blocking the reaction (Elnifro *et al.* 2000). However, the observed variability among viruses in a multiplex reaction is probably the result of primer design. Indeed, different primers can significantly alter the sensitivity of a multiplex RT-PCR, and in some instances, changing the sequence of a primer by even a few base pairs can have a dramatic effect on the sensitivity of the assay. The identification of primer sets that exhibit equal sensitivity for all their target sequences in a multiplex assay would require extensive testing. Another problem may be the reliable identification of the various RT-PCR products. To overcome these problems, we performed a very careful primer selection in the present study; this procedure yielded amplified products that could clearly be differentiated by agarose gel electrophoresis. One of the main steps in setting up the single-step multiplex RT-PCR protocol was the use of an enzyme mixture containing hot-start DNA polymerase. Because the template could be RNA genomes, and the first step of reverse transcription had to be performed at a low temperature, the use of a hot-start *Taq* DNA polymerase was essential to avoid non-specific amplifications caused by primer annealing at a low temperature.

In this study, the testing of 29 clinical samples from cases of aborted fetus and piglets by multiplex PCR revealed infection with detection of PRRSV, SIV and JEV in 20, 13 and 6 samples, respectively. However, by the virus isolation method, PRRSV, SIV and JEV were detected in 20, 12 and 4 samples, respectively. There are two reasons as to why the result of multiplex PCR was not in agreement with that of virus isolation method: First, PCR assay is highly sensitive and second, there is no established gold standard for the comparison between the results of multiplex PCR and virus isolation. Because of its high experimental requirements, PCR can frequently provide false-positive results and is thereby not entirely appropriate for application outside the laboratory.

In this study, we did not notice any spurious amplification reactions among the 3 pathogens with various amounts of RNA mixtures, and all the uninfected controls as well as the CSFV scored negative.

In conclusion, the multiplex RT-PCR method for rapid detection of PRRSV, SIV and JEV is a convenient and reliable method for routine diagnosis of swine disease.

Acknowledgements

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