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Original article

A multiplex PCR for simultaneous detection of classical swine fever virus, African swine fever virus, highly pathogenic porcine reproductive and respiratory syndrome virus, porcine reproductive and respiratory syndrome virus and pseudorabies in swines

L. Hu[1], X.Y. Lin[1], Z.X. Yang[1], X.P. Yao[1], G.L. Li[1], S.Z. Peng[1], Y. Wang[1,2]

[1] Animal Quarantine Laboratory,College of Veterinary Medicine, Sichuan Agricultural University, Chengdu Sichuan 611130, China

[2] Key laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu Sichuan 611130, China

Abstract

In this assay, we developed and evaluated a multiplex PCR (mPCR) for its ability in detecting multiple infections of swine simultaneously. Four pairs of primers were used to detect five viruses. Specific primers were designed for classical swine fever virus (CSFV), African swine fever virus (ASFV) and pseudorabies (PRV). A pair of primers was designed prudently for two different types of porcine reproductive and respiratory syndrome virus that respectively were porcine reproductive and respiratory syndrome virus (PRRSV), highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV). The detection limits of the mPCR were 1.09×10^4, 1.50×10^3, 2.10×10^3, 1.30×10^3 and 8.97×10^2 copies/reaction for CSFV, ASFV, HP-PRRSV, PRRSV and PRV, respectively. A total of 49 clinical specimens were tested by the mPCR, and the result showed that co-infection by two or three viruses was 51%. In conclusion, the PCR is a useful tool for clinical diagnosis of not only single infections but also mixed infections in swines.

Key words: Multiplex PCR, ASFV, CSFV, PRV, PRRSV, HP-PRRSV

Introduction

It is ordinary for swine to be simultaneously infected with two or more pathogens under typical conditions of intensive swine production (Cao et al. 2005). Because the clinical signs can be variable and may not be pathogen-specific, these multiple infections are difficult to diagnose. For instance, classical swine fever virus (CSFV), African swine fever virus (ASFV), pseudorabies (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) all cause reproductive and/or respiratory failure in pigs which can be

Correspondence to: Y. Wang, e-mail: yaanwangyin@tom.com, tel.: +86 1320 835 98 88

Table 1. Sources of pathogens.

Reference samples	Numbers	Source	Negative controls	Numbers	Source
RRV(Bartha-K61, Ea, HB-98)	3	vaccine	TGEV	1	SAU
CSFV(Shimen, tissue culture origin)	2	vaccine	PEDV	1	SAU
Gene fragments of ASFV	1	MVRI	BVDV	1	SAU
PRRSV(CH-1a, R98, CH-1R)	3	synthesized	PPV(cp99, NDAL-2, PKZ)	3	vaccine
HP-PRRSV(TJM-F92, NVDC-JXA1-R, HUN4-F 112)	3	vaccine	PCV2(ZJ/C, WH, SH)	3	vaccine
			uninfected samples	2	SAU

TGEV : Transmissible gastroenteritis virus; PEDV: Porcine epidemic diarrhea virus; BVDV: Bovine viral diarrhea virus; PPV: Porcine Parvovirus; PCV2: Porcine Circovirus type 2; SAU: Sichuan Agricultural University; MVRI: Military Veterinary Research Institute; NAFU: Northwest A F University.

easily confused. However, highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) mutated from the PRRSV of the deletion of 30 amino acids on NSP2. It has a higher pathogenic, and could not be accurately differentiated from PRRSV (Zhou et al. 2011). ASF is a highly contagious disease of pigs and it is specified in Class A of animal diseases by the World Organization for Animal Health (OIE), which is also specified as the Class I pathogen of animal diseases in China. It is the only known DNA arbovirus and often confused with the symptoms of CSFV (King et al. 2003). Even though it has not been found in China, it is particularly important to develop a rapid method to diagnose and distinguish it. As clinical signs do not reveal the causal pathogen, it is difficult to diagnose accurately and timely. So far besides viral isolation in cell culture, a lot of costly virus-specific tests are sometimes performed. It is time-consuming and labour-wasting. A multiplex PCR (mPCR) assay can resolve these limitations (Elnifro et al. 2000).

In several previous studies, DNA or RNA from several viruses could be amplified by mPCR which incorporates multiple primers simultaneously in a single reaction (Giridharan et al. 2005, Yue et al. 2009). In recent research, they are concerned about using mPCR to differentiate the highly virulent Chinese-type PRRSV (HP-PRRSV), PRRSV, PPV, CSFV, PRV and PCV2 (Jiang et al. 2010). They all cause fulminating infectious diseases in China. One or more viruses can infect swine and cause the same syndrome such as reproductive and/or respiratory failure and high fever. As far as the authors knows, mPCR has been used to detect ASFV, CSFV, PRRSV and PRV infections of swine in a single reaction before (Wernike et al. 2013), but the authors could not distinguish HP-PRRSV and PRRSV. In this study, we developed and evaluated a multiplex PCR for not only simultaneous detection of nucleic acid from four viruses: CSFV, ASFV, PRRSV, PRV, but also differentiated HP- PRRSV and PRRSV.

Materials and Methods

Virus

The gene fragments of ASFV were synthesised in Chengdu, China. Reference strains, field isolates and negative controls used in this study are described in Table 1. All vaccines were from commercial sources.

Clinical samples

Forty-nine clinical specimens, including lymph nodes, spleens, lungs and tonsils, were collected from piglets with respiratory and/or reproductive problems accompanied by progressive weight loss in local farms in Sichuan Province, China, during January 2013 to April 2014, and were stored in the authors' laboratory.

Nucleic acid extraction and reverse transcription

Viral genomic DNA and RNA were extracted from each vaccine using the TIANamp Virus RNA/DNA Kit following the manufacturer's protocol (TIANamp).

Reverse transcription was performed by PrimeScript™ RT reagent Kit(Perfect Real Time) in a final volume of 10 uL containing 2 uL of 5×PrimeScript buffer (including dNTP Mixture and Mg^{+}), 0.5 uL Prime Script RT Enzyme Mix I (contains RNase Inhibitor), 25 pmol OligodT Primer, 200 pmol Random 6 mers, 3 uL template, 2 uL RNase Free dH_2O. After incubation for 15 min at 37°C, the mixture was heated for 5 sec at 85°C for inactivation of reverse transcriptase. The mixture was then chilled on ice. The reaction products were stored at -20°C.

Table 2. Specific primer pairs used to amplify each target gene.

Virus	Target gene	Primer sequence (5'-3')	Expected Product (bp)
CSFV	E2	F: TCAACCGATGGGATAGGGC R: ACAAGTCCAGTTACCCCCCA	525
ASFV	VP72	F: TGGCCCTCTCCTATGCAA R: TGCGTCCGTAATAGGAGT	190
PRV	gB	F: TGAGCGTCTTCGTCGTGA R: TGGTCACCTTCGAGCACAA	642
PRRSV/HP-PRRSV	NSP2	F: AGTTCCTGCACCGCGTAGA R: TCGATGATGGCTTGAGCTGA	366/276

Primer design

Genomic sequences of ASFV, PRV, HP-PRRSV, PRRSV and CSFV were obtained from GenBank and aligned using the MegAlign worktable of DNAStar software (DNAStar, Madison, USA). According to the alignments and relevant references (Lee et al. 2007, Yu et al. 2012, Zhou et al. 2012), primers specific for both HP-PRRSV and PRRSV were designed on the basis of a NSP2 gene. The nucleotide sequences based upon the conserved region of gB were chosen for detection of PRV. Primers for CSFV were based on E2 gene. Primers for ASFV were based upon the conserved region of VP72. Oligo 6.0 is used to design primer sequences. BLAST and the multiplex function of DNAStar are used to elucidate the potential cross-reactivity of the oligonucleotides and target specificity. Oligonucleotide sequences of primer sets and their main characteristics are summarised in Table 2. Primers were obtained from a commercial source (Chengdu, China).

Single-target PCR

The PCR was carried out in a 25 uL mixture containing 2.5 uL of 10× PCR buffer (200 mM Tris-HCl at pH 9.0, 200 mM KCl, 100 mM $(NH_4)_2SO_4$, 15 mM $Mgcl_2$), 2 uL of 2.5 mM dNTP mixture, 1uL of each10 pmol primer (Table 2), 1U Taq DNA Polymerase (TaKaRa), 2uL of DNA or cDNA template (the vaccine of PRV, HP-PRRSV, PRRSV, CSFV and the synthetic fragments of ASFV) and 16 uL of water. The negative controls included the reagents without DNA or cDNA template. Amplification with a pre-heated thermal cycler (GeneAMP PCR System 9700) consisted of one cycle at 94°C for 3 min, followed by 30 cycles at 94°C for 45s, 60°C for 45s and 72°C for 1 min. The PCR ended with a final extension step of 10 min at 72°C. Amplicons were detected by electrophoresing 5 uL aliquots through 1% agarose gels in 1× TAE (40 mM Tris-aceate [pH 8.0], 1 mM EDTA).

Sequencing

The PCR was in a total volume of 50 uL containing 5 uL of 10 × PCR buffer, 4uL of 2.5 mM dNTP mixture, 1.5 uL of each10 pmol primer, 2.5 U Taq DNA Polymerase, 2uL of DNA or cDNA template, 35 uL of water, and amplicons were detected by electrophoresing through 0.8% agarose gels, other reaction conditions as single-target PCR. The restriction fragments were extracted from agarose gels using the E.Z.N.A.™ Gel Extraction kit following the manufacturer;s protocol (OMEGA). Each specific viral target fragment was cloned into the plasmid pMD19-T (TaKaRa), and each amplicon was extracted using the Plasmid Mini Kit(Sangon), sequenced by Sangon, China.

Optimization of multiplex conditions

Plasmid constructs containing specific viral target fragments were used as templates for optimization of mPCR. The mPCR was optimized by varying single parameters while other parameters were maintained as described earlier in the paper. The evaluated parameters and ranges in concentrations included: primer for each target from 0.5 to 30 pmol, Optimum amplification profiles were obtained with a primer concentration of 5 pmol for all sets of primers. The effects of annealing temperature (from 49.5 to 62°C), number of cycles (from 25 to 40) and other conditions also were determined experimentally. The best results were obtained with a temperature of 52.5°C for 35 cycles. Amplicons were detected by electrophoresing 5uL aliquots through 2% agarose gels in 1× TAE (40 mM Tris-aceate [pH 8.0], 1m MEDTA).

mPCR

The mPCR was carried out by mixing all four primer pairs and performed similar to that described in single-target PCR with some optimization. Ampli-

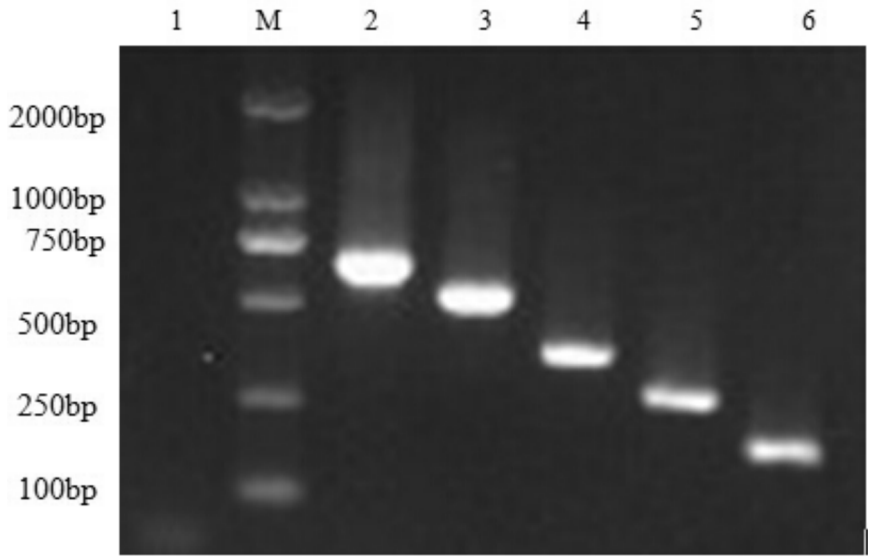

Fig. 1. *Single-target* PCR. 1:ddH_2O, negative; M: DL2000 DNA Marker; 2: PRV(642bp); 3:CSFV(525bp); 4:PRRSV(366bp); 5:HP-PRRSV(276bp); 6:ASFV(190bp).

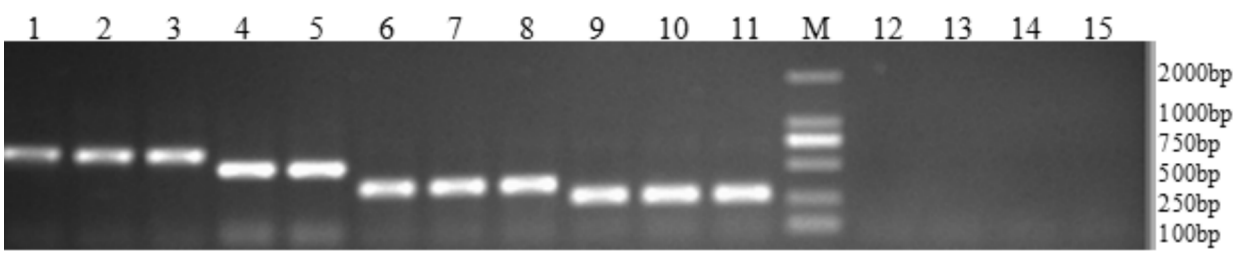

Fig. 2a. Specificity of mPCR.1:PRV(Ea strain); 2: PRV(HB-98 strain); 3: PRV (strain Bartha-K61); 4: CSFV(Shimen strain); 5: CSFV(tissue culture origin vaccine strain); 6: PRRSV(R98 strain); 7: PRRSV(CH-la strain); 8: PRRSV(CH-1R strain); 9: HP-PRRSV(NVDC-JXA1-R strain) 10: HP-PRRSV(TJM-F92 strain); 11: HP-PRRSV (HUN4-F 112 strain); M:DL2000DNA-Marker; 12: BVDV negative; 13: uninfected sample1; 14: uninfected sample 2; 15: ddH_2O, negative.

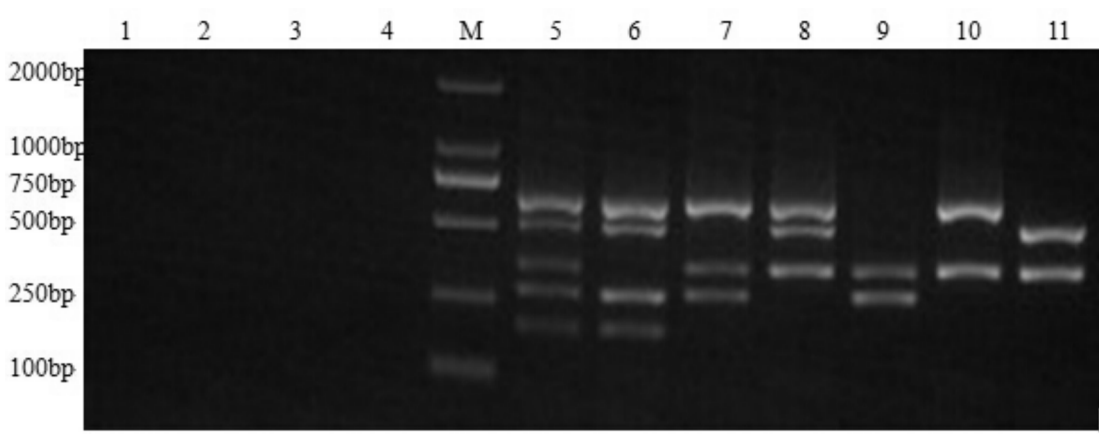

Fig. 2b. Cross-reactivity of mPCR.1:PCV2, negative; 2:TGEV, negative; 3:PEDV, negative; 4:PPV, negative; M:DL2000 DNA Marker; 5:CSFV, ASFV, PRV, PRRSV, HP-PRRSV, positive; 6:CSFV, ASFV, PRV, HP-PRRSV, positive; 7: PRV, PRRSV, HP-PRRSV, positive; 8: CSFV, PRV, PRRSV, positive; 9: PRRSV, HP-PRRSV, positive; 10: PRV, PRRSV, positive; 11: CSFV, PRRSV, positive.

Table 3. Numbers of positive samples in 49 sick piglets from January 2013 to April 2014 detected for each of the five viruses by single polymerase chain reaction (PCR) and multiplex PCR.

Viruses Number of cases	Single-target PCR/RT-PCR	Multiplex PCR
CSFV only	2	2
PRV only	3	3
PRRSV only	18	18
HP-PRRSV only	1	1
ASFV only	0	0
PRRSV + PRV	6	6
HP-PRRSV+PRRSV	14	14
PRRSV + CSFV	3	3
PRRSV + CSFV+PRV	1	1
PRRSV + HP-PRRSV+PRV	1	1

CSFV: classical swine fever virus; PRRSV: porcine reproductive and respiratory syndrome virus; HP-PRRSV: highly pathogenic porcine reproductive and respiratory syndrome virus; PRV: pseudorabies virus; ASFV: African swine fever virus.

cons were detected by electrophoresing 5uL aliquots through 2% agarose gels in 1× TAE (40 mM Tris-aceate [pH 8.0], 1 mM EDTA).

Specificity of multiplex PCR assays

The positive samples of PRV (Ea strain, HB-98 strain, strain Bartha-K61), HP-PRRSV (NVDC-JXA1-R strain, TJM-F92 strain, HUN4-F 112 strain), PRRSV (R98 strain, CH-la strain, CH-1R strain), CSFV (Shimenstrain, tissue culture originvaccine strain) were detected by the mPCR. To test its cross-reactivity, different combinations of plasmid constructs were used in the mPCR. PPV, TGEV, PEDV, PCV2 and uninfected samples were tested to validate the specificity of the mPCR.

Sensitivity of single and multiplex PCR assays

The sensitivity of the mPCR and the corresponding single-target PCRs were evaluated comparatively on serial ten-fold dilutions of plasmid.

Screening of clinical specimens by mPCR

A total of 49 clinical specimens including lymph nodes, spleens, and tonsils of sick piglets were subjected to mPCR detection.

Results

Single-target PCR and specificity of multiplex PCR

The specificity of primer pair for each virus was first analyzed in sPCR which had produced amplicons of 525, 190, 642, 276 and 366 bp for CSFV, ASFV, PRV, HP-PRRSV and PRRSV. Each viral target gene could be specifically amplified using its defined primer pair and the PCR products showed the expected size rangesas shown in Fig. 1. Positive samples of PRV, CSFV, HP-PRRSV, PRRSV were tested by mPCR, and each viral target gene could be specifically distinguished, while no amplicons were produced with uninfected samples and ddH_2O (Fig. 2a). When different combinations of plasmid constructs were used in the multiplex PCR, the respective virus amplicons were produced and could be differentiated by agarose gel electrophoresis. No amplicons were produced with other viruses which include PCV2, PPV, TGEV and PEDV (Fig. 2b).

Sensitivity of single and multiplex PCR

PCR sensitivity experiments were performed on serial ten-fold dilutions of plasmid constructs containing the specific viral target fragments. The minimum quantities detected by single-target PCRs were 2.10×10^2, 1.30×10^2, 1.09×10^3, 1.50×10^2 and 8.97×10 copies for HP-PRRSV, PRRSV, CSFV, ASFV and PRV, respectively (Fig. 3). The detection limits of the mPCR were 2.10×10^3, 1.30×10^3, 1.09×10^4, 1.50×10^3 and 8.97×10^2 copies for HP-PRRSV, PRRSV, CSFV, ASFV and PRV, respectively (Fig. 4). It's easy to see that mPCR was slightly less sensitive than the single-target PCRs.

Screening of clinical specimens by mPCR

To test the ability of mPCR for diagnosis of porcine viruses, a total of 49 clinical specimens which were confirmed by a routine PCR/RT-PCR (GB/T

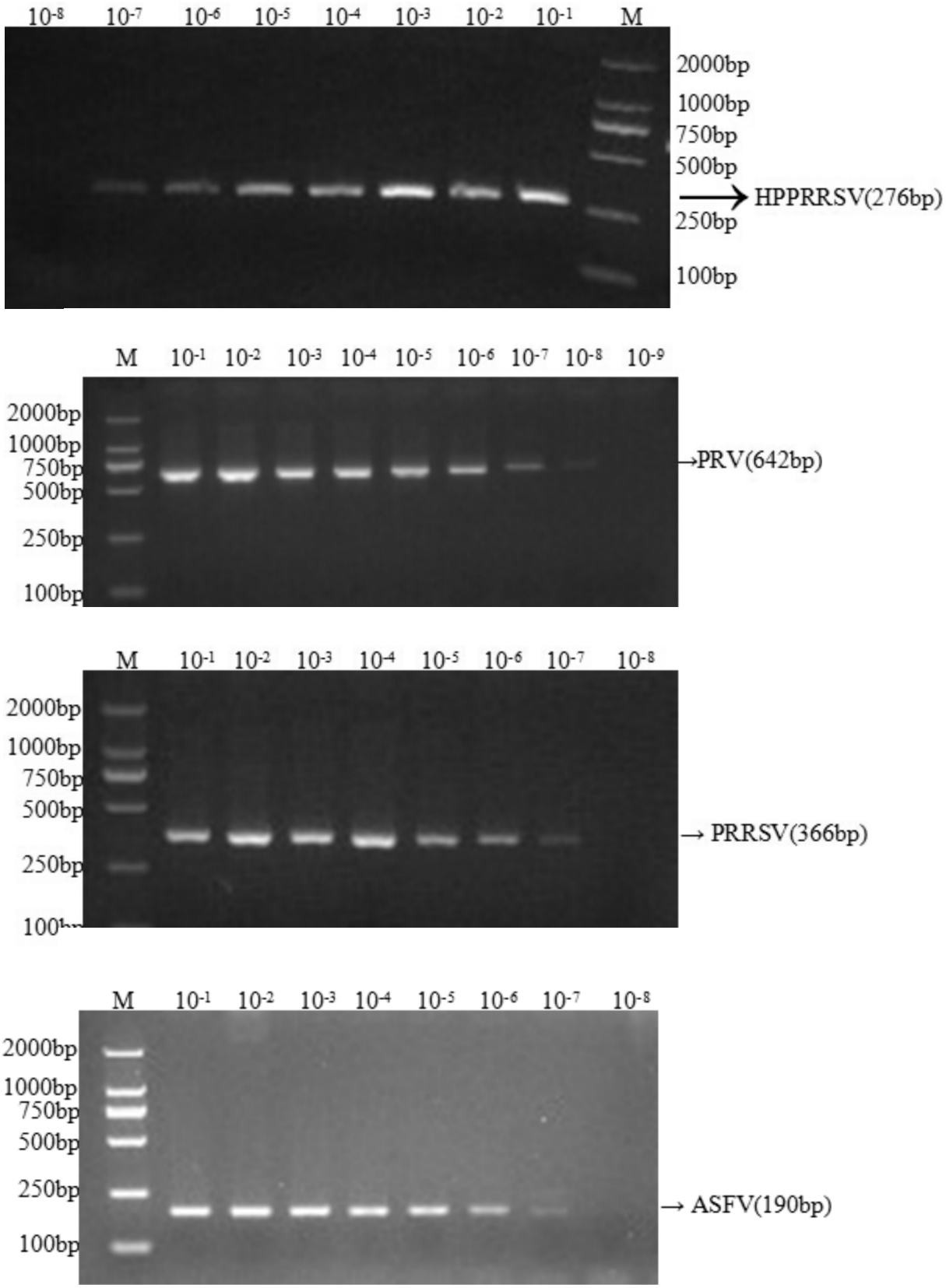
10-8 10-7 10-6 10-5 10-4 10-3 10-2 10-1 M
2000bp
1000bp
750bp
500bp
HPPRRSV(276bp)
250bp
100bp
M 10-1 10-2 10-3 10-4 10-5 10-6 10-7 10-8 10-9
2000bp
1000bp
750bp
500bp
250bp
100bp
PRV(642bp)
M 10-1 10-2 10-3 10-4 10-5 10-6 10-7 10-8
2000bp
1000bp
750bp
500bp
250bp
PRRSV(366bp)
M 10-1 10-2 10-3 10-4 10-5 10-6 10-7 10-8
2000bp
1000bp
750bp
500bp
250bp
100bp
ASFV(190bp)

Fig. 3.

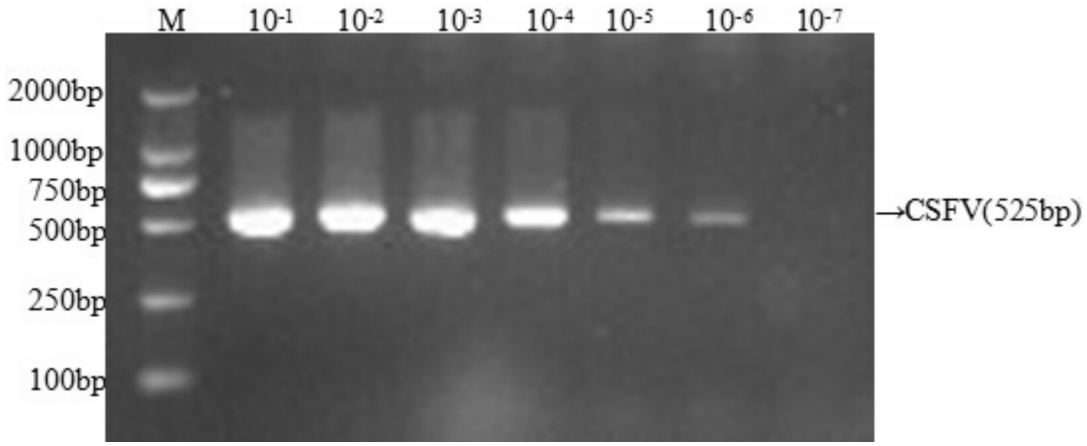

Fig. 3. Sensitivity of single-target PCR for each viral target gene.

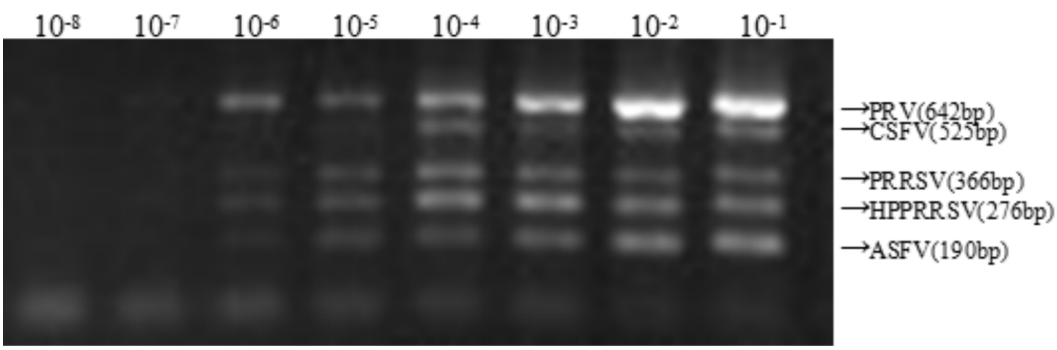

Fig. 4. Sensitivity of the multiplex PCR for simultaneous amplification of all viral target DNAs.

16551-2008, GB/T 18090-2008, GB/T 18641-2002) before and were stored in the authors' laboratory including lymph nodes, spleens, and tonsils of sick piglets, were tested for CSFV, ASFV, PRV, HP-PRRSV and PRRSV by the mPCR. The results in Table 3 were similar to those found before. The 49 specimens that were positive by sPCR were also positive by mPCR assays. Co-infection with two or three viruses was demonstrated in 25 samples (51%); however, no specimens were co-infected with all fiver viruses.

Discussion

In recent years, the swine epidemic shows some new features in which multiple infection (Liu et al. 2011) predominates due to different degrees of intensity and differences in management levels of the farms in the Chinese swine industry in addition to the abuse of some vaccines. In particular, the mixed infection among CSFV, PRRSV, HP-PRRSV and PRV are often clinically manifested as porcine reproductive and respiratory syndrome, high fever etc. (Dong et al. 2006, Zhao et al. 2008, Zhou and Yang 2010, Zanella et al. 2012). Therefore, it is difficult to accurately and timely distinguish these infections only based on clinical manifestations and pathological changes. As we all know, the isolation and identification depending on the virus and the single-target PCR detection method can waste not only reagents but also time and effort. The multiplex PCR method is more advantageous to detect multiple pathogens at one time and especially effective for the differential diagnosis of clinically mixed infection while maintaining rapid, sensitive and specific features of the conventional PCR.

African swine fever leads to high mortality, due to it being ahighly infectious disease, which is similar to the classical swine fever virus in terms ofsymptoms, epidemiology and pathological changes (Gallardo et al. 2013). The condition has not yet been seen in China, but its prevalence in neighbouring countries poses a growing threat to China. Due to the fact that China is free from the virus, the related research is limited. The primers of the experiment were selected from the conserved sequence based on foreign related studies(Aguero et al. 2004). While detecting PRV, CSFV, PRSSV, PPV, PCV2 and JEV, ASFV may be monitored for. Thus, GeXP-PCR can provide technical reserves for a diagnosis of ASFV.

The multiplex PCR technique is a process of adding multiple pairs of specific primers to the same PCR reaction system and amplifying multiple DNA templates or different areas of the same template, which can amplify multiple targeted gents at one time so as to achieve the simultaneous detection of multiple pathogens. The multi-site amplification is carried out in the same condition and reaction system, there are amplifying competition and mutual influence among different primers. Besides, change of temperature (Zhao et al. 2010), G+C content and specificity of primers (Ogawa et al. 2009, Durzyńska et al. 2011) may also affect the results. For better results, the primers of this experiment were designed carefully. The primer sequences of the viruses were in the highly conserved area and the size of the amplified fragments were significant difference. The G + C content and specificity of the primers wre compared carefully. Optimization of the reaction conditions was primarily accomplished by searching the optimal ratio of concentrations and annealing temperature of the primers based on the reaction system to avoid a large amount of preferential amplification of a product. At the same time, the designed primer in the experiment was processed by the single-target PCR amplification using the vaccine of each virus as the template and by the cloning and sequencing after the gel extraction, and the sequence analysis was performed on the sequencing result using DNAstar and BLAST. The results showed that the sequence homology between the amplified fragments and the sequence published on Genbank was up to 99%. To test the ability of the multiple PCR for diagnosis of porcine viruses, a total of 49 clinical specimens were tested using the multiple GeXP-PCR. The results showed that the assay could be used for diagnosis of diseases and detection of mixed infection of the clinical samples. However, the method cannot be used for distinguishing vaccine immunity from virulent infection, such that the detection result only reflects the real situation of infection to some extent. Other methods and ways should be cooperated in the clinical applications to make a comprehensive judgment. Overall, the method is high sensitive, specific and rapid, and it has a broad application prospect.

Conclusion

The multiplex PCR developed for HP-PRRSV, PRRSV, CSFV, PRV and ASFV in the present study could be used in epidemiological studies and laboratory identification of clinical specimens. It is a useful tool to detect and identify one or more of these viruses in swines. This PCR should reduce time, labour and cost, and allows differential diagnosis of five viral diseases in pigs. On a whole, this method is specific, rapid and has a high sensitivity, which suggests a broad application prospect.

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