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A multiplex PCR for rapid and simultaneous detection of porcine circovirus type 2, porcine parvovirus, porcine pseudorabies virus, and porcine reproductive and respiratory syndrome virus in clinical specimens

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Abstract A multiplex PCR (mPCR) assay was developed and evaluated for its ability to simultaneously detect multiple viral infections of swine. Specific primers were designed for each of the following four DNA or RNA viruses: porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), pseudorabies virus (PRV), and porcine reproductive and respiratory syndrome virus (PRRSV). Each target produced a specific amplicon with a size of 353 bp (PCV2), 271 bp (PPV), 194 bp (PRV), or 434 bp (PRRSV). The assay was sensitive and specific in detecting each target agent in composite cell cultures and clinical specimens. Results from mPCR were confirmed by PCR for individual viruses and by virus isolation. In conclusion, the mPCR has the potential to be useful for routine molecular diagnosis and epidemiology.

Keywords Multiplex PCR (mPCR) · Porcine circovirus type2 (PCV2) · Porcine parvovirus (PPV) · Porcine pseudorabies virus (PRV) · Porcine reproductive and respiratory syndrome virus (PRRSV)

Introduction

Under typical conditions of intensive swine production, it is common for swine to be concurrently infected with two or more viral pathogens [1]. A definitive diagnosis of multiple infections is often difficult, however, because clinical signs can be variable and differential diagnosis is required for different pathogens. When infections cause abortion and/or stillbirth, determining whether the causal agent is PPV (porcine parvovirus), PRV (pseudorabies virus), or PRRSV (porcine reproductive and respiratory syndrome virus) is difficult based only on clinical signs.

The standard laboratory methods for diagnosis of viral diseases are mainly based on viral isolation in cell culture, which is time consuming. However, in those cases where clinical signs are variable and thus fail to provide a clear indication of the most appropriate diagnostic test, a number of costly individual virus-specific tests are sometimes initially performed to expedite the testing procedure. In several previous studies, multiplex PCR (mPCR) has been used to simultaneously identify and differentiate multiple viruses in a single sample on the basis of amplicon size [2–4]. To the authors' knowledge, however, there is no report on the use of a mPCR to detect PCV2 (porcine circovirus type 2), PPV, PRV, and PRRSV in swine with a single reaction. In the study reported here, we developed an mPCR procedure to detect various combinations of these viruses in swine.

Materials and methods

Cells, viruses, and clinical specimens

The PCV l-free PK-15 cells were kindly provided by Dr. Kyoung-Jin Yoon, the Veterinary Diagnostic Laboratory of Iowa State University (Ames, IA, USA). These cells are also free of CSFV, PPV, PRRSV, BDV, and BVDV.

The viruses of PCV2 020 strain, PPV BQ strain, PRV LXB strain, and PRRSV HB strain (North American

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genotypes) were obtained from the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. PCV2 020 strain was isolated from pigs affected with postweaning multisystemic wasting syndrome in Harbin, Heilongjiang Province (northern China) and was propagated in circovirus-free PK15 and confirmed by the fluorescent antibody test. The PPV Chinese isolate BQ strain was isolated from a case of reproductive failure in 2005; the virus was isolated from the liver of a sick wild piglet on a wild boar farm located in Heilongjiang Province (northern China). PRV LXB strain and PRRSV HB strain were isolated in Hebei Province (in the middle of China). A PK15 cell line persistently infected with PCV1 was used as PCV1 stock virus. The uninfected PK15 cell line as well as bovine viral diarrhea (BVD) reference viruses were also used in the specificity assays. PCV2, PPV, and PRV were propagated in the PCV l-free PK-15 cells as described before [5, 6] and confirmed by the immunoperoxidase monolayer assay. PRRSV was propagated in the Marc-145 cells as described before, which were obtained from the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences from ATCC.

To evaluate the efficiency of the extraction method and to assess the mPCR reaction, a total of 179 clinical specimens (hearts, livers, spleens, lungs, kidneys, lymph nodes, and blood) were collected from Xinjiang, Shandong, Jilin, and Heilongjiang (Table 1) and were assayed by mPCR. In addition, tissue samples from pigs experimentally infected with PCV2, PPV, PRRSV, or PRV (all were single infections) were also assayed by mPCR. These samples came from the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences and had been determined to be positive for the indicated virus by viral culture or ELISA. Virus-negative organs from clinically healthy animals were also included.

Extraction of RNA and DNA

Frozen or fresh clinical samples were homogenized for 15–30 s with a homogenizer. Viral genomic DNA and RNA were simultaneously extracted from the homogenized tissues and from lysates of infected cell cultures with the TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.3.0 according to the manufacturer's protocol (TaKaRa).

Primer design

Four pairs of primer sets that were designed to specifically amplify PCV2, PPV, PRV, or PRRSV (Table 2) were obtained from a commercial source (TaKaRa).

Reverse transcription

The reverse transcription (RT) reaction was performed in a 20-μl PCR master mixture consisting of 4 μl 1× reaction solution (50 mM Tris–HCl, pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 6 μl dNTP, 1 μl (50 pmol/μl) PRRSV RT-primer (see Table 2), 1 μl M-MLV reverse transcriptase (200 U/μl)(Promega) and 1 μl RNase Inhibitor (40 U/μl)(TaKaRa), add 7 μl of mixture RNA and DEPC-water. The RT reaction was carried out at 42°C for 1 h and inactivated at 70°C for 10 min.

Table 1 Clinical samples used in this study

Samples source	No.	Characteristics	Specimens
Xinjiang	20	Aborted fetuses	Hearts, livers, spleens, lungs, kidneys, lymph nodes, blood
Shandong	21	Piglets	Hearts, livers, spleens, lungs, kidneys, lymph nodes, blood
Jinlin-1	108	Weaned piglet	Hearts, livers, spleens, lungs, kidneys, lymph nodes
Jinlin-2	18	Aborted fetuses	Hearts, livers, spleens, lungs, kidneys, lymph nodes
Heilongjiang	12	Aborted fetuses	Hearts, livers, spleens, lungs, kidneys, lymph nodes

Table 2 Virus-specific primers used to amplify each target gene

Virus	Target gene	Primer sequence (5′-3′)	Product (bp)
PRRSV	ORF7 (14929–15343)	RT-Primer: 5′-ATTGAATAGGTGAC-3′ PRRSV1: 5′-GCCAGTTCCAGCCAGTCAATCA-3′	434
		PRRSV2: 5′-GCCCCGATTGAATAGGTGAC-3′	
PCV2	ORF2 (1274–1607)	PCV21: 5′-AAGGGCTGGGTTATGGTATG-3′	353
		PCV22: 5′-CGCTGGAGAAGGAAAAATGG-3′	
PPV	NS1 (1475–1726)	PPV1: 5′-GAATAGGATGCGAGGAAAG-3′	271
		PPV2: 5′-GTGGAAATCTGAGAGTCTGT-3′	
PRV	gB (17202–17374)	PRV1: 5′-GAAGGGGTTGGACAGGAAGGAC-3′	194
		PRV2: 5′-ACCAGCCGCTCAAGTTCTACGA-3′	

Single PCR

Single PCR for PCV2, PPV, PRV, or PRRSV was carried out in a 50-μl mixture containing 10 μl of 1× PCR Buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3), 4 μl of 200 μM dNTP, 1 μl of each 10 pmol primer (Table 1), 1 UnitsTaq^{TM} DNA Polymerase (TaKaRa), 3 μl of DNA or cDNA template, and 30 μl of water. The negative controls are water controls, and they are reagents without addition of water. Amplification with a pre-heated thermocycler (Applied Biosystems 2720 Thermal Cycler) consisted of one cycle at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s; annealing at 50°C (PPV), 55°C (PCV2), 58.6°C (PRRSV), or 61.5°C (PRV) for 1 min; and amplification at 72°C for 1 min. The PCR reaction was ended with a final extension step of 10 min at 72°C. Amplicons were detected by electrophoresing 10-μl aliquots through 2% agarose gels in 1× TAE(40 mM Tris–aceate [pH 8.0], 1 mM EDTA).

Optimization of multiplex conditions

The mPCR reactions were optimized by varying primer concentrations and by methodical variation of each test parameter under standard PCR conditions. The primer concentration tested ranged from 0.5 to 20 pmol; the $MgCl_2$ concentration ranged from 1.5 to 4.5 mM; the dNTP concentrations ranged from 150 to 400 μM; the TaKaRa Taq^{TM} Hot Start Version DNA Polymerase (TaKaRa) quantities ranged from 0.5 to 2.5 U. The annealing temperature (55–62°C) and number of cycles (25–40 cycles) were also optimized. 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). Each specific viral target fragment was cloned into the plasmid pMD18-T (TaKaRa), and the sequence of each amplicon was confirmed by TaKaRa Biotechnology Co.

mPCR reaction

The mPCR reaction was carried out by mixing all four primer pairs and with optimized parameters. The mPCR reaction consists of two-steps: reverse transcription (RT) and PCR amplification. The RT reaction was performed in a 20-μl PCR master mixture consisting of 4 μl 1× reaction solution (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 6 μl dNTP, 1 μl 50 pmol/μl PRRSV RT-primer (see Table 1), 1 μl M-MLV reverse transcriptase(Promega Corporation), and 1 μl RNase Inhibitor (TaKRa), 7 μl of mixture (RNA/DNA), and DEPC-water. The mPCR reaction was performed in a 50-μl volume. The reaction contained 2 mM $MgCl_2$, 10 μl of 1× PCR Buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3), 200 μM each dNTP (4 μl in total), 1 μl of each 10 pmol primer, 1.5 U TaKaRa Taq^{TM} Hot Start Version DNA Polymerase (TaKaRa), and 1 μl of each DNA or cDNA template, and then added with DEPC-water to 50-μl volume in total. The negative controls are water controls, and they are reagents without addition of water. Polymerase chain reaction (PCR) was performed as follows: initial denaturing at 95°C for 5 min; followed by 35 cycles of 94°C for 45 s, 58.6°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. Negative controls were run with each test. 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).

Sensitivity of single and mPCR assays

The sensitivity of the mPCR and the corresponding single PCRs were carried out as described before [4]. The sensitivity of the mPCR and the corresponding single PCRs was evaluated comparatively on serial 10-fold dilutions of selected spiked samples containing all the target viruses.

Specificity of single and multiplex PCR assays

In the specificity studies of single PCR and multiplex PCR assays, classical swine fever virus (CSFV), swine influence virus (SIV), Porcine circovirus type 1 (PCV1), Bovine viral diarrhea-mucosal disease virus (BVDV), *Escherichia coli*, PCV2, PPV, PRV, and PRRSV were tested with primers for PCV2, PPV, PRV, and PRRSV.

Virus isolation and sequencing

To verify the results of the single PCR and multiplex PCR assays, all the positive samples were sent to Porcine Reproductive and Respiratory Syndrome team and Porcine circovirus team for isolation, and all the isolates were sequenced and analyzed.

Results

Optimization of mPCR conditions

To determine the optimum annealing temperature for mPCR, different temperatures (55–62°C) were used in the same reaction that included DNA of PCV2, PPV, PRV, and cDNA of PRRSV as template. The primers for PCV2, PPV, PRV, and PRRSV viruses produced amplicons of 194, 271, 353, and 434 bp, respectively. The optimum annealing temperature for mPCR was 58.6°C (Fig. 1).

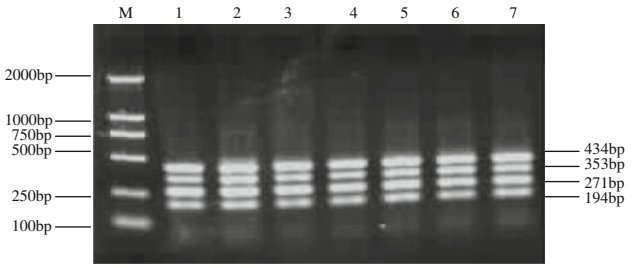

Fig. 1 The annealing temperature of mPCR. M: DL2000 DNA Marker; 1: 57.9°C; 2: 58.6°C; 3: 59.3°C; 4: 60.0°C; 5: 60.8°C; 6: 61.4°C; 7: 61.7°C

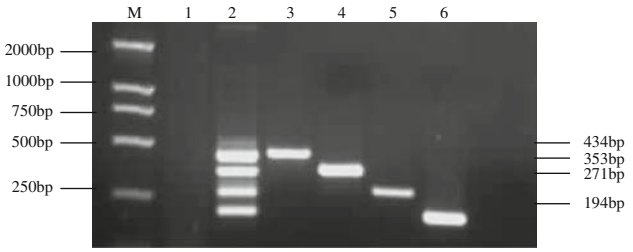

Fig. 2 mPCR. M: DL2000 DNA Marker; 1: negative control; 2: PRRSV, PCV2, PPV, PRV mPCR; 3: PRRSV PCR; 4: PCV2 PCR; 5: PPV PCR; 6: PRV PCR

Other optimum conditions for mPCR were as follows: primer concentration (10 pmol of each virus), $MgCl_2$ concentration (2.0 mM); dNTP concentrations (200 μM); Taq™ DNA Polymerase (TaKaRa Biotechnology co., Let) concentration (1.5 U), and number of cycles (35 cycles). Amplicons were detected by electrophoresing 10-μl aliquots through 2% agarose gels in 1× TAE (40 mM Tris–aceate [pH 8.0], 1 mM EDTA). After multiplex conditions were optimized, the mPCR reaction effectively amplified all the four viruses (Fig. 2).

Analytic sensitivity of mPCR

mPCR was able to detect as little as 1×10^{-5} to 1×10^{-6} ng of each viral target (Fig. 3), and single PCR was able to detect as little as 1×10^{-7} ng of each viral target DNA (Fig. 4). The following formula was used to calculate

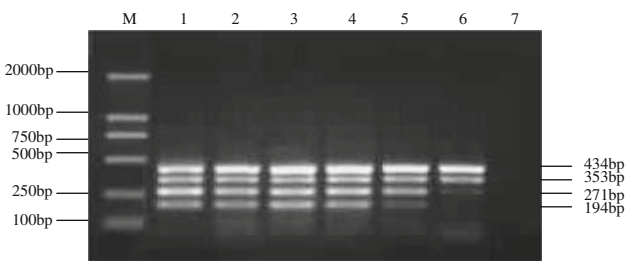

Fig. 3 Sensitivity of mPCR. M: DL2000 DNA Marker. M: DL2000 DNA Marker; 1: 10^0diluted; 2: 10^{-1} diluted; 3: 10^{-2}diluted; 4: 10^{-3} diluted; 5: 10^{-4} dilute; 6: 10^{-5}diluted; 7: 10^{-6}diluted

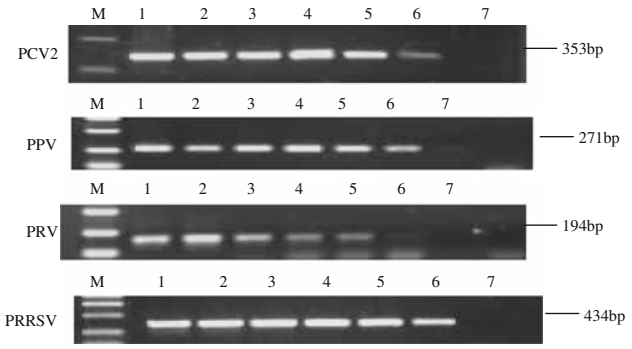

Fig. 4 Sensitivity of single PCR for each viral. M: DL2000 DNA Marker; 1: 10^0 dilute; 2: 10^{-1}; 3: 10^{-2} diluted; 4: 10^{-3} diluted; 5: 10^{-4}diluted; 6: 10^{-5} diluted; 7: 10^{-6} diluted

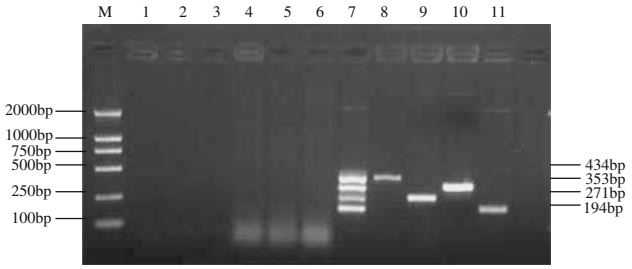

Fig. 5 Specificity of the mPCR for the differentiation. M: DL2000 DNA Marker; 1: CSFV; 2: SIV; 3: PCV1; 4: BVDV; 5: *Escherichia coli*; 6: negative control; 7: PRRSV, PCV2, PPV, PRV mPCR; 8: PRRSV PCR; 9: PPV PCR;10: PCV2 PCR; 11: PRV PCR

the number of gene copies per ml in each dilution: copies/ml = 6.02×10^{23} (copies/mol) × concentration of nucleic acid (g/reaction)/average molecular weight of nucleic acid (g/reaction). It follows that mPCR could detect as many as 42–420 gene copies/reaction for PRRSV, 52–520 for PCV2, 68–680 for PPV, and 94–940 for PRV. Similarly, the single PCR could detect as few as four gene copies/reaction for PRRSV, five for PCV2, seven for PPV, and nine for PRV.

Specificity of mPCR

The specificity of primer pairs for each virus was analyzed with single PCR and mPCR. As illustrated in Fig. 5, both single and mPCR were specific for the target viral agent because no amplification occurred with CSFV, SIV, PCV1, BVDV, *Escherichia coli*, or ddH_2O (lanes 1–6) whereas each viral target gene was specifically amplified using its defined primer pair (lanes 8–11).

Detection of virus in known-positive specimens

RNA and DNA was extracted from tissue samples singly infected with PCV2, PPV, PRRSV, or PRV. The extracted RNA and DNA were mixed in different ways and then

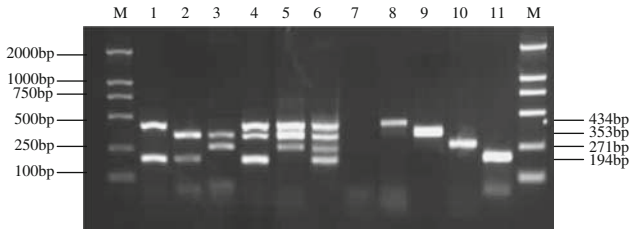

Fig. 6 mPCR reaction in positive sample. M: DL2000 DNA Marker; 1: PRRSV + PRVPCR; 2: PCV2 + PRV PCR; 3: PCV2 + PPV PCR; 4PRRSV + PCV2 + PRV PCR; 5: PRRSV + PCV2 + PPV; 6: PRRSV + PCV2 + PPV + PRV PCR; 7: negative control; 8: PRRSV PCR; 9: PCV2 PCR; 10: PPV PCR; 11: PRV PCR

subjected to mPCR. The mPCR correctly detected the viruses in the mixtures (Fig. 6). The PCR products were 353 bp (PCV2), 271 bp (PPV), 194 bp (PRV), and 434 bp (PRRSV).

Detection of viruses in clinical specimens by mPCR

A total of 179 clinical specimens were subjected to mPCR and single PCR. The results of mPCR and single PCR were identical in all the cases (Table 3). PCV2 and PRRSV were detected in single infections and in co-infections, but PPV and PRV were not detected in these samples (Table 4).

Virus isolation and sequencing

Viruses were isolated from whole clinical samples as previously described [5, 6]. PK-15 cells were used to isolate PCV2, PPV, and PRV, while Marc-145 cells were used to isolate PRRSV. PK-15 cell cultures were inoculated with whole-sample homogenates; after five successive passages and incubation for 5 days, no cytopathic effect was observed but PCV2 nucleic acid was detected in 149 samples using IPMA. Marc-145 cell were also inoculated with whole-sample homogenates; after five successive passages and incubation for 5 days, a cytopathic effect was observed, and 158 samples were PRRSV positive according to IF. The samples that were positive based on virus isolation were positive based on mPCR. Additional partial data on virus isolation and sequencing have been published in several other articles [7, 8].

Table 3 Detection of viruses in clinical samples by single PCR and mPCR

Virus	mPCR	Single PCR	Positives (%)
PRRSV	158[a]	158[a]	88.3
PCV2	149[b]	149[b]	83.2
PPV	0	0	0
PRV	0	0	0

[a] Confirmed by virus isolation and IF and sequencing

[b] Confirmed by virus isolation and IPMA (immunoperoxidase monolayer assay) and sequencing

Table 4 Detection results of multiplex infections

Samples source	No.	Character	Infection type
Xinjiang	20	Aborted fetuses	PCV2 + PRRSV
Shandong	21	Piglets	PCV2
Jinlin-1	108	Weaned Piglet	PCV2 + PRRSV
Jinlin-2	18	Aborted fetuses	PRRSV
Heilongjiang	12	Aborted fetuses	PRRSV

Discussion

Because multiplex PCR is designed to detect more than one target, it has the potential to save substantial time and effort. In fact, the concept of multiplex PCR has been successfully applied to molecular diagnostics at numerous veterinary diagnostic laboratories [2–4]. Although fluorogenic PCR technology has many advantages over conventional PCR and is more commonly used than multiplex PCR at diagnostic laboratories in developed countries, fluorogenic PCR is still being developed, but its cost prohibits its use in many developing countries. Therefore, most diagnostic laboratories in developing countries rely on conventional, gel-based PCR technology for rapid and specific detection of infectious agents. In this regard, the multiplex PCR method developed in this study provides a rapid, convenient, and reliable means for routine diagnosis of PCV2, PPV, PRV, and PRRSV in swine.

The testing of 179 clinical samples from cases of aborted fetus and piglets by multiplex PCR indicated that 71.5% of the samples were co-infected with PCV2/PRRSV. PPV and PRV were not detected in the samples. Because these clinical samples are representative of clinical samples in general in China, the results indicate that PCV2 and PRRSV have a high prevalence in China. The results also suggest that single and co-infections of PCV2 and PRRSV may play an important roles in porcine circovirus disease and reproductive failure.

The multiplex PCR assay developed in this study specifically detected and differentiated the four target swine viruses (PRV, PPV, PCV2, and PRRSV) in clinical tissue specimens. This result indicates that the primers were correctly designed to avoid any possible formation of primer dimers and that PCR conditions were optimized for detecting different targets in one reaction. Because the results of PCR for individual viral agents showed 100% agreement with those of multiplex PCR, the sensitivity of the multiplex PCR was not compromised during its development.

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