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Establishment and application of a multiplex PCR for rapid and simultaneous detection of six viruses in swine

Zhiyong Zeng[a,b,*], Zhijie Liu[a], Weicheng Wang[a], Deyuan Tang[a], Haiying Liang[c], Zhao Liu[a]

[a] College of Animal Sciences, Guizhou University, Guiyang 550025, China
[b] Key Laboratory of Animal Epidemic Disease and Veterinary Public Health in Guizhou Province, Guiyang 550025, China
[c] Teaching and Experimental Farms, Guizhou University, Guiyang 550025, China

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A B S T R A C T

A multiplex PCR assay was developed and evaluated subsequently for its effectiveness in simultaneously detecting mixed viral infections of swine. Specific primers were designed and used for testing the six swine viruses: three DNA viruses, including pseudorabies virus (PRV), porcine parvovirus (PPV), and porcine circovirus type 2 (PCV2); three common RNA viruses, including porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), and Japanese encephalitis virus (JEV). This technique has shown to be highly sensitive in that the minimum detection amounts of nucleic acids from PRV, PPV, PCV2, PRRSV, CSFV, and JEV were 6.6, 96, 12.9, 10.5, 51, and 46 pg, respectively. It also was effective for detecting one or multiple viruses in the specimens, such as the lungs, spleens, lymph nodes, and tonsils collected from clinically ill pigs. The multiplex PCR method can detect simultaneously not only infection of the six viruses, but also other swine DNA and RNA viruses. Given its rapidity, specificity, and sensitivity, the multiplex PCR is a useful tool for diagnosing clinically the mixed infections of swine DNA and RNA viruses.

1. Introduction

Along with the large-scale and intensive development of modern swine production, mixed infections of multiple pathogens are becoming more and more common in swinery (Arce et al., 2009; Cao et al., 2005). Among the viral diseases occurred on pig farms, six major viruses, including porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), porcine pseudorabies virus (PRV), porcine parvovirus (PPV), and Japanese encephalitis virus (JEV), are the major pathogens that cause reproductive failures in pigs and bring great economic losses to the swine industry (Chamberlain et al., 1988). Additionally, the five viruses (PRV, PPV, PCV2, PRRSV, and CSFV) can also damage porcine immune system to different degrees and decrease immunity (Chen et al., 2010), causing swine to be easily infected by other pathogens. Since the PCR technology was first reported to diagnose Duchenne muscular dystrophy (DMD) by Chamberlain in 1988 (Elnifro et al., 2000), it has been widely used in many fields, especially in nucleic acid research. To increase the detection efficiency of PCR, multiplex PCR methods are established to simultaneously detect and differentiate multiple DNA or RNA viruses in a single sample on the basis of amplicon size (Giammarioli et al., 2008; Huang et al., 2004; Joo et al., 1976; Li et al., 2000). In this study, a multiplex PCR method was developed to detect and distinguish the three DNA viruses (PRV, PPV, and PCV2) and three RNA viruses (PRRSV, CSFV, and JEV) of swine. The technique was demonstrated to be useful for detecting viruses in specimens from swine with mixed infections.

2. Materials and methods

2.1. Viruses and cells

PRV, PCV2, PRRSV, CSFV, and JEV used in this study were isolated by our laboratory (Zeng et al., 2011; Liu et al., 2012a, 2010; Huang et al., 2009; Xu et al., 2009). The Sichuan strain of PPV was kindly provided by Prof. Wan-Zhu Guo (College of Veterinary Medicine, Sichuan Agricultural University) (Yin et al., 2006). These viruses were maintained in −80 °C and used as standard viruses for the

* Corresponding author at: College of Animal Sciences, Guizhou University, 14 Xiahui Road, Huaxi District Guiyang, Guizhou 550025, China.
Tel.: +86 13809495107; fax: +86 851 8298005.
E-mail address: as.zyzeng@gzu.edu.cn (Z. Zeng).

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multiplex PCR. *Escherichia coli* and Swine influenza virus (SIV) were also used in the specificity assays. Vero, BHK-21, Marc-145 and PK-15 cell lines free of PCV1 were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FCS. To evaluate the efficiency of the multiplex PCR, virus samples from cells infected with each of PRV, PPV, PCV2, PRRSV, CSFV or JEV were examined.

2.2. Clinical specimens

During the period from November 2009 to September 2012, 103 clinical specimens containing the tonsils, lungs, kidneys, livers, lymph nodes, and spleens were collected from 20 aborted fetuses (numbering: LT1–20, Group 1), 22 breeding piglets (numbering: BR 1–22, Group 2), 33 nursery pigs (numbering: BY 1–33, Group 3), and 28 fattening pigs (numbering: YF 1–28, Group 4) in the swine farms of Guizhou province, China.

2.3. Primer design

According to the gene sequences of PRV gE, PPV NS1, PCV2 ORF2, PRRSV ORF7, CSFV E2, and JEV E retrieved from GenBank, six pairs of specific primers aimed at these target genes were designed using DNAStar, Oligo, Mpprimer, and MFEprimer (Li et al., 2007). These primers were synthesized by TaKaRa (Dalian, China). PCR primer pairs for each target gene, the size of each amplicon, and GenBank accession number of the corresponding gene were summarized in Table 1.

2.4. Nucleic acid extraction

Viral nucleic acids were extracted from homogenates of mixed tissue samples and cell cultures infected with each virus using the TaKaRa RNA/DNA Mini kit (TaKaRa, Dalian, China) according to the manufacture's protocol. Homogenates of mixed tissue samples (tonsils, lungs, kidneys, livers, lymph nodes, and spleens 500 mg, respectively) were prepared in DMEM and clarified by centrifugation as previously described (Cao et al., 2005). The suspensions were used immediately or stored at −80 °C. A single nucleic acid extraction protocol was adopted for the simultaneous extraction of both RNA and DNA viruses. The viral genomic DNA and RNA were extracted from 500 μl volumes of tissue suspensions. *E. coli* genomic DNA was extracted by alkaline lysis as previously described (Ehrt and Schnappinger, 2003).

2.5. Single PCR

The single PCR for PRV, PPV, and PCV2 was performed separately in a mixture of 25 μl: 12.5 μl of 2× One Step Buffer, 1 μl PrimeScript One Step Enzyme Mix (TaKaRa, Dalian, China), 1.0 μl of 10 pmol/μl each primer, 2.5 μl of each viral nucleic acid, and 7.0 μl of sterilized water. The reaction conditions were as follows: 1 cycle of 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 s, 60 °C (for PRV) or 53 °C (for PPV) or 57 °C (for PCV2) for 30 s, and 72 °C for 30 s; and a final extension step of 72 °C for 10 min. The PCR products were analyzed by electrophoresis on 1.2% agarose gels (40 mmol/L Tris-acetate, pH 8.0,1 mmol/L EDTA) containing ethidium bromide (0.5 μg/ml) and visualized by ultraviolet illumination.

2.6. Single RT-PCR

The single reverse transcription PCR (RT-PCR) for PRRSV, CSFV, and JEV was carried out in a 25 μl of a mixture containing 12.5 μl of 2× One Step Buffer, 1 μl PrimeScript One Step Enzyme Mix, 1.0 μl of 10 pmol/μl each primer, 2.5 μl of each viral nucleic acid, and 7.0 μl of sterilized water. The reaction conditions were as follows: 1 cycle of 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 57 °C (for PRRSV) or 56 °C (for CSFV) or 58 °C (for JEV) 30 s, and 72 °C for 30 s; and a final extension of 72 °C for 10 min. The PCR products were analyzed as described above.

2.7. Multiplex PCR

2.7.1. Multiplex PCR detection of infected cells

The cell cultures infected with each virus were mixed and these viral nucleic acids were extracted with the TaKaRa RNA/DNA Mini kit. A single PCR detection of the six viruses from mixed nucleic acids was first examined. Then the multiplex PCR detection with different combinations, such as JEV and PRRSV; JEV and CSFV; JEV and PRV; PPV, PCV2 and PRRSV; PRV, PPV, PCV2, PRRSV, CSFV and JEV was examined. Each assay was repeated twice.

2.7.2. Establishment of the multiplex PCR system

The multiplex PCR was performed by mixing the six primer pairs with optimized parameters, such as annealing temperatures, concentrations of upstream and downstream primers, and amounts of templates from the six viruses.

2.7.3. Optimization of the multiplex PCR conditions

To obtain the optimal reaction conditions of the multiplex PCR, a number of experiments were carried out, including annealing temperatures ranging from 50° to 60 °C, primer concentrations ranging from 5 to 20 μmol/L, and cycle numbers ranging from 30 to 35.

2.8. Sensitivity test of the multiplex PCR

The sensitivities of the multiplex PCR and the corresponding single PCR were determined as described previously (Liu et al., 2011). The viral nucleic acids were determined by BioPhotometer Plus (Eppendorf, Germany). The 5-fold serial dilutions were carried out to evaluate the sensitivity of the multiplex and 10-fold serial dilutions of single PCR.

2.9. Specificity test of the multiplex PCR

To determine the specificity of the multiplex PCR, the nucleic acids from swine influenza virus (SIV), *E. coli*, and the six viruses were extracted and amplified with the multiplex PCR, respectively. To confirm further the results of the multiplex PCR assays, the amplicons, with the size of 178, 271, 353, 433, 508 and 1015 bp, were cloned and sequenced, respectively.

2.10. Detecting the clinical samples using multiplex PCR

The above 103 clinical specimens were detected using multiplex PCR and rechecked by the corresponding single PCR.

3. Results

3.1. Detections of the six viruses by single and multiplex PCR

The six viruses were detected using the single and multiplex PCR. Results showed that all the six viruses could be simultaneously detected and the primers for PRV, PPV, PCV2, PRRSV, CSFV, and JEV produced amplicons of 178, 271, 353, 433, 508, and 1015 bp, respectively (Figs. 1 and 2).

3.2. Establishment and optimization of the multiplex PCR

To determine the optimum annealing temperatures for the multiplex PCR, different temperatures (ranging from 50° to 60 °C) were tested in the same reaction that included DNA of PCV2, PPV, and PRV