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Short communication

Multiplex PCR for the simultaneous detection of porcine reproductive and respiratory syndrome virus, classical swine fever virus, and porcine circovirus in pigs

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ABSTRACT

A multiplex polymerase chain reaction (PCR) was designed for the simultaneous detection of three viruses involved in reproductive and respiratory failure in pigs: porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV) and porcine circovirus type 2 (PCV-2). Each target produced a specific amplicon with a size of 718 bp (PRRSV), 288 bp (CSFV), or 466 bp (PCV-2). The sensitivity of the multiplex PCR using purified plasmid constructs containing the specific viral target fragments was 2.0×10^4, 2.5×10^3, and 6.0×10^2 copies for PRRSV, CSFV, and PCV-2, respectively. Non-specific reactions were not observed when other viruses, bacteria, and PK-15/Marc-145 cells were used to assess the multiplex PCR. Among 82 clinical samples from Fujian province, co-infection by PRRSV and CSFV was 12.19%, co-infection by PRRSV and PCV-2 was 21.95%, CSFV and PCV-2 was 13.41%, and co-infection by the three viruses was 3.66%. In conclusion, the multiplex PCR should be useful for routine molecular diagnosis and epidemiology. The multiplex PCR was effective in detecting various combinations of one or more of these viruses in pig specimens.

1. Introduction

Under typical conditions of intensive swine production, it is common for swine to be concurrently infected with porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), and porcine circovirus type 2 (PCV-2). These multiple infections are difficult to diagnosis because the clinical signs can be variable and may not be pathogen-specific.

Classical swine fever (CSF) is a highly contagious, often fatal, disease caused by CSFV. Clinical signs of CSF are more severe when pigs are co-infected with PCV-2, or PRRSV [1–3]. PCV-2 is the primary agent causing postweaning multisystemic wasting syndrome (PMWS), PCV-2 is also associated with respiratory disease, reproduction failure in many parts of the world [4]. PRRSV is the cause of porcine reproductive and respiratory syndrome, which is characterised by reproductive failure in sows and respiratory disease in piglets [5]. Concurrent infections of PCV-2 and PRRSV are common in swine [6–10]. Concurrent infections with PCV-2 were linked to the secondary immune suppression noted in the PMWS. Lymphoid depletions and immune system dysfunction at both humoural and cellular levels in pigs affected by PMWS may increase the vulnerability of the affected animals to other viruses [11]. Dual infections are not limited to PCV-2, Swine can also be concurrently infected with PRRSV [12] and CSFV [13].

The standard laboratory methods for diagnosis of viral diseases were mainly based on viral isolation in cell culture, which is time consuming. The PCR is an alternative rapid virus detection method and several single PCR-based methods have been reported for a number of porcine pathogens. Individual PCR or reverse transcriptase (RT)-PCR assays have been developed for detection and identification of PRRSV, CSFV, and PCV-2 [14–16]. However, using conventional PCR technology to detect several viruses individually is labour intensive and expensive. These limitations can be overcome by using a multiplex PCR assay, which incorporates multiple primers that amplify RNA or DNA from several viruses simultaneously in a single reaction [17].

Therefore, the ultimate objective of this work was the development, optimisation and evaluation of a rapidly and highly sensitive multiplex PCR assay for the simultaneous detection and

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differentiation two RNA virus-PRRSV, CSFV, and one DNA virus-PCV-2 infections.

2. Materials and methods

2.1. Viruses and cells

PRRSV FJ strain (North American genotype), PCV type 2 FJ strain and PPV FJ strain were isolated by College of Life Sciences of Longyan University. Transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhoea virus (PEDV) vaccine (Harbin Weike Biotechnology Development Company), bovine viral diarrhoea virus (BVDV) and pseudorabies virus (PRV) (were provided by College of Life Sciences of Longyan University) and PK-15 cells and Marc-145 cells free of PRRSV, CSFV and PCV-2 (China Center for Type Culture Collection).

2.2. Clinical specimens

Fifty-eight clinical specimens including lymph nodes, tonsils, lungs and spleens collected from commercial herds, as well as 24 aborted foetuses, from 20 local farms in Fujian Province, China, during the period May 2011 to June 2012.

2.3. Nucleic acid extraction

Viral genomic DNA and RNA were extracted from cell cultures infected with each virus or viral genomic DNA and RNA were extracted from clinical specimens using the Roche TriPure Isolation Reagent Viral RNA/DNA Extraction Kit Ver. 3.0 (TaKaRa) following the manufacturer's protocol.

2.4. Primer design

Three previously described sets of primers that specifically amplify PRRSV, CSFV, PCV2 [18–20] (Table 1) were obtained from a commercial source (Sangon).

2.5. Reverse transcription

For the first-strand complementary DNA synthesis, 8 μl of a mixture of RNA preparation were included in a total reaction volume of 20 μl containing 4 μl of 5× reverse transcriptase (RT) buffer, 2 μl of dNTP; 1 μl of each RT-primer for CSFV, and PRRSV (10 pmol, see Table 2); 1 μl of M-MLV reverse transcriptase (200 U/μl), 1 μl of RNase Inhibitor (40 U/μl) (TaKaRa), and DEPC–water. The RT-reaction was carried out at 42 °C for 1 h and was inactivated at 70 °C for 10 min. Then the mixture was then chilled on ice.

Table 1
Specific primer pairs used to amplify each target gene.

Virus	Target gene	Primer sequence (5′–3′)	Expected product (bp)
PRRSV	ORF5	catttcatgacacctgagaccaa agagcatatatcatcaactggcgt	718
CSFV	E2	caggtatgcgatctcgtcaacca gggcacagcccaaatccgaagt	288
PCV-2	Cap	ctgttttcgaacgcagtgcc cccgcaccttcggatata	466

PRRSV: porcine reproductive and respiratory syndrome virus. CSFV: classical swine fever virus; PCV-2: porcine circovirus type 2.

Table 2
Numbers of positive samples from 58 sick piglets and 24 aborted foetuses detected for each of the three viruses by single PCR/RT-PCR and multiplex PCR.

Assay	Target viruses		
	PRRSV	CSFV	PCV-2
Single PCR/RT-PCR	43	36	38
Multiplex PCR	42	36	38

PRRSV: porcine reproductive and respiratory syndrome virus. CSFV: classical swine fever virus; PCV-2: porcine circovirus type 2.

2.6. Single PCR

In the single PCRs, a single primer pair was used to detect the target virus. The reaction mixture contained DNA/cDNA (1 μl), 10× PCR buffer (Mg2+ Plus) (2.5 μl), 2 μl of 2.5 mM dNTP, 1 μl of each 10 pmol primer (Table 1) and 0.2 μl Taq DNA Polymerase (5 U/μl) (TaKaRa). Distilled water was added to a total volume of 25 μl. The negative controls included the reagents without cDNA template. The PCR was performed at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 56.2 °C for 45 s, and 72 °C for 60 s, with a final extension at 72 °C for 7 min and holding at 4 °C. The PCR products were detected by electrophoresing 5 μl aliquots through 2% agarose gels in 1× TAE (40 mM Tris–aceate [pH 8.0], 1 mM EDTA).

2.7. Multiplex PCR

Plasmid containing specific viral target fragments served as templates for optimisation of multiplex PCR and for sensitivity analysis of single or multiplex PCR. The constructs were verified by PCR and DNA sequencing (Sangon). The DNA concentrations of the each construct were determined spectrophotometrically. The limit of detection for each target was defined as the highest dilution detected after a 10-fold serial dilution in plasmid constructs of the target sequence. The specificity of single PCR and multiplex PCR assays using primers for PRRSV, CSFV, and PCV-2 was determined by subjecting the following purified viruses (and bacterium) to independent assays: PRRSV, CSFV, PCV-2, BVDV, TGEV, PEDV, PRV, PPV, and *Escherichia coli*.

The multiplex PCR contained a mixture of all primer pairs and was performed similarly to the single PCRs with some optimisation. The evaluated parameters and ranges in concentrations included: primer for each target from 0.05 mM to 0.6 mM, dNTP from 0.01 mM to 0.6 mM. The effects of annealing temperature (from 55 °C to 61 °C) and number of cycles (from 25 to 35) also were determined experimentally. Amplicons were detected by electrophoresing 5 μl aliquots through 2% agarose gels in 1× TAE. Each specific viral target fragment was cloned into the plasmid pMD18-T (TaKaRa), and each amplicon was sequenced by Shanghai Sangon Biotechnology Co., Ltd.

2.8. Detection of CSFV, PRRSV, and PCV-2 in clinical specimens by multiplex PCR and routine PCR/RT-PCR

A total of 82 clinical specimens from pig farms (see Section 2.2) were tested for PRRSV, CSFV, and PCV-2 by the multiplex PCR and routine PCR/RT-PCR.

3. Results

3.1. Optimisation of multiplex PCR

The multiplex PCR with the primers for PRRSV, CSFV, and PCV-2 produced amplicons of 718 bp, 288 bp, and 466 bp, and the optimal