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Simultaneous detection of porcine circovirus type 2, classical swine fever virus, porcine parvovirus and porcine reproductive and respiratory syndrome virus in pigs by multiplex polymerase chain reaction

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ABSTRACT

A multiplex polymerase chain reaction (PCR) was designed for the simultaneous detection of four viruses involved in reproductive and respiratory failure in pigs: porcine circovirus type 2 (PCV-2), porcine parvovirus (PPV), classical swine fever virus (CSFV) and porcine reproductive and respiratory syndrome virus (PRRSV). Each of the four pairs of oligonucleotide primers exclusively amplified the targeted fragment of the specific viruses. The sensitivity of the multiplex PCR using purified plasmid constructs containing the specific viral target fragments was 2.58×10^7, 2.64×10^5, 2.66×10^7 and 2.73×10^5 copies for PRRSV, PCV-2, CSFV and PPV, respectively. Using the multiplex PCR, co-infections with these four viruses were identified in 26/76 (34.2%) piglets born from sows with reproductive failure in China. This method is a rapid, sensitive and cost-effective diagnostic tool for the routine surveillance of viral infections in pigs.

Introduction

Classical swine fever virus (CSFV), porcine circovirus type 2 (PCV-2), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine parvovirus (PPV) are economically important viruses that cause reproductive and/or respiratory failure in pigs. Rapid and reliable detection of these viruses is essential for epidemiological surveillance and disease management.

Classical swine fever (CSF) is a highly contagious, often fatal, disease caused by CSFV. CSFV C-strain vaccine is widely used for CSF prevention and control in China. However, CSF immunisation failures have been reported, due to maternal antibody interference and immunotolerance caused by improper immunisation, resulting in atypical or chronic CSF (Li et al., 2007). This often leads to difficulty in identifying wild-type virus-infected pigs in vaccinated pig herds. Since wild-type CSFV has about 95% nucleic acid homology with the vaccine C-strain, most currently available detection methods do not discriminate between wild-type CSF viruses and vaccine strains.

Clinical signs of CSF are more severe when pigs are co-infected with PCV-2, PPV or PRRSV (Allan et al., 2000; Kennedy et al., 2000; Harms et al., 2001; Rovira et al., 2002). PCV-2 is the primary agent causing postweaning multisystemic wasting syndrome (PMWS), which is characterised by progressive weight loss in 4- to 16-week-old pigs (Allan et al., 1998; Allan and Ellis, 2000). PCV-2 is also associated with respiratory disease, reproductive failure and a variety of other manifestations in many parts of the world (Segales et al., 2004). PPV causes reproductive failure in pigs characterised by embryonic death and resorption, mummified fetuses, stillbirths and prolonged farrowing intervals (Mengeling and Cutlip, 1975; Joo et al., 1976; Mengeling et al., 2000). PRRSV is the cause of porcine reproductive and respiratory syndrome, which is characterised by reproductive failure in sows and respiratory disease in piglets (Rossow, 1998).

Individual PCR or reverse transcriptase (RT)-PCR assays have been developed for detection and identification of CSFV, PCV-2, PPV and PRRSV (Van Woensel et al., 1994; Larochelle and Magar, 1997; Soares et al., 1999; Cheon and Chae, 2000). 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 (Elnifro et al., 2000). A recent study reported the development of specific primers to detect and differentiate CSF wild-type viruses from the vaccine C-strain (Li et al., 2007). In the present study, these primers were used to

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Table 1
Oligonucleotide primer sequence and characteristics.

Virus	Primer	Sequence (5′–3′)	Tm	Target products (bp)
PCV-2	CVF	CGAGAAAGCGAAAGGAACAGA	59.9	ORF 1 (371)
	CVR	GGTAACCATCCCACCACTT	57.3	
PRRSV	RRSF	GAGTTTCAGCGGAACAATGG	58.0	ORF 6 (451)
	RRSR	GCCGTTGACCGTAGTGGAG	58.5	
PPV	PVF	AGTTAGAATAGGATGCGAGGAA	55.7	NS1 gene (265)
	PVR	AGAGTCTGTTGGTGTATTTATTGG	55.2	
CSFV	CSFF[a]	AACATGGATGGTGTAACTGGT	54.1	E^{rns} protein gene (343)
	CSFR[a]	TTCTCTATAGTGTTGGTCATTCC	53.4	

PCV-2: porcine circovirus type 2; PPV: porcine parvovirus; CSFV: classical swine fever virus; PRRSV: porcine reproductive and respiratory syndrome virus; ORF: open reading frame.
[a] CSFV primers according to Li et al. (2007).

develop a multiplex PCR for specific detection of wild-type CSFV, PCV-2, PRRSV and PPV.

Materials and methods

Virus strains and clinical samples

PRRSV (ZJ2005, DQ269472), CSFV (Hangzhou strain) and PPV vaccine (Beijing Haidian Zhonghai Animal Health Science and Technology Co., Cat. no. 0040401) reference strains were maintained in the authors' laboratory, while PCV-2 (HZ0202, AY217743) was provided by Professor Zhou Jiyong, Zhejiang University. Negative controls consisted of PCV-1 (HZ2006, EF533941), provided by Professor Zhou Jiyong, CSFV vaccine (Qianyuanhao Biological Co., Cat. no. 050656), bovine viral diarrhoea virus (BVDV) (Oregon C24 strain), transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhoea virus (PEDV) vaccine (Harbin Weike Biotechnology Development Company, Cat. no. 030718), pseudorabies virus (PRV) (China Animal Husbandry Industry Co., Cat. no. 0412014-3) and PK-15 cells free of PCV-2, PPV, CSFV and PRRSV (China Institute of Veterinary Drug Control).

Seventy-six clinical specimens, including lymph nodes, tonsils, lungs and spleens, were collected from forty-nine 4- to 12-week-old piglets with respiratory and/or reproductive problems accompanied by progressive weight loss, as well as 27 aborted fetuses, from 11 local farms in Zhejiang Province, China, during the period June 2006 to July 2007.

Nucleic acid extraction

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

Primer design

Genomic sequences of PCV-2, PPV, PRRSV and CSFV were obtained from genomic data bases. Oligonucleotide primers for CSFV were according to Li et al. (2007). Sequences of other viruses were aligned using the Megalign application (DNAStar) and alignments were used for primer design (Table 1). The potential cross-reactivity of the oligonucleotides and target specificity were elucidated using BLAST,[1] along with the multiplex function of Primer Premier 5.0. Oligonucleotide sequences of primer sets and their main characteristics are summarised in Table 1. Primers were synthesised commercially (Sangon).

Reverse transcription

For the first-strand complementary DNA synthesis, 2 μL of the viral RNA/DNA preparation were included in a total reaction volume of 20 μL containing 4 μL of 5× reverse transcriptase (RT) buffer (50 mM Tris–HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol pH 8.3), 0.5 mM each deoxynucleotide triphosphate (dNTP), 1 μM antisense primers of CSFV and PRRSV, respectively, 20 U of RNase inhibitor and 5 U of avian myeloblastosis virus reverse transcriptase (Takara). After incubation for 60 min at 42 °C, the mixture was heated for 3 min at 95 °C to denature the products. The mixture was then chilled on ice.

Single polymerase chain reaction

The total PCR reaction volume of 25 μL contained 2 μL of virus cDNA or RNA/DNA, 0.5 μM of each primer, 2.5 μL of 10× PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% NP-40), 2 mM $MgCl_2$, 0.2 mM each dNTP, 1.25 U of Taq polymerase (Sangon) and 17.25 μL distilled water. The PCR reaction was performed under the following conditions in a thermal cycler (Bioer): initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s; then final extension at 72 °C for 7 min. PCR products were analysed by electrophoresis in 1% agarose for single PCR or 2.5% agarose for multiplex PCR, followed by ethidium bromide staining and visualisation under ultraviolet light.

Multiplex polymerase chain reaction

Plasmid constructs containing specific viral target fragments were used as templates for optimisation of multiplex PCR and to measure the limit of detection for each virus by single and multiplex PCR. First round amplification products (Table 1) were separately cloned using the pGEM-T Easy Vector System II (Promega) and constructs were extracted using the Rapid Plasmid Daily-Prep Kit (V-Gene). The constructs were verified by PCR and DNA sequencing (Sangon). The DNA concentrations of the constructs were determined spectrophotometrically (Molecular Devices). The limit of detection for each target was defined as the highest dilution detected of a series of serially diluted (1:10) plasmid constructs of the target sequence.

The multiplex PCR was optimised and standardised using various concentrations of $MgCl_2$ (1–2 mM) and primers (0.2–1 mM), annealing temperatures (50–62 °C) and numbers of cycles (30, 35, 40, 45) using the positive control plasmid constructs (data not shown). The optimum product yield was achieved with 1.5 mM $MgCl_2$, 0.5 mM primer for PCV-2, PPV and PRRSV and 0.25 mM primer for CSFV, an annealing temperature of 55 °C and 35 cycles.

Results

Specificity of single and multiplex polymerase chain reaction

The specificity of primer pairs for each virus was first analysed in single PCR by agarose gel electrophoresis. Each viral target gene could be specifically amplified using its defined primer pair (data not shown). When different combinations of the four viruses

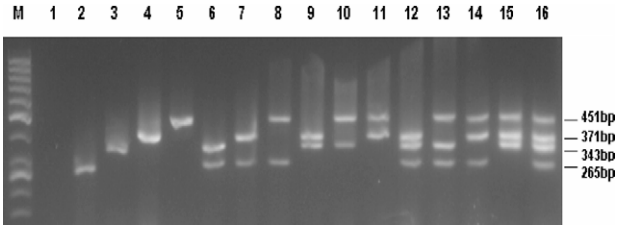

Fig. 1. Specificity of the multiplex polymerase chain reaction (PCR) assay developed for the detection of porcine circovirus type 2 (PCV-2), porcine parvovirus (PPV), classical swine fever virus (CSFV) and porcine reproductive and respiratory syndrome virus (PRRSV). Agarose gel showing simultaneous multiplex PCR amplification of different combinations of viruses with the four sets of primers. Lane 1: negative cell control; lane 2: PPV alone; lane 3: CSFV alone; lane 4: PCV-2 alone; lane 5: PRRSV alone; lane 6: PPV + CSFV; lane 7: PPV + PCV-2; lane 8: PPV + PRRSV; lane 9: CSFV + PCV-2; lane 10: CSFV + PRRSV; lane 11: PCV-2 + PRRSV; lane 12: PPV + CSFV + PCV-2; lane 13: PPV + CSFV + PRRSV; lane 14: PPV + PCV-2 + PRRSV; lane 15: CSFV + PCV-2 + PRRSV; lane 16: PPV + CSFV + PCV-2 + PRRSV; lane M: 50 bp DNA ladder.

[1] See: http://www.ncbi.nlm.nih.gov.