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Short communication

Real-time PCR to detect and analyze virulent PPV loads in artificially challenged sows and their fetuses

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ABSTRACT

To establish a real-time polymerase chain reaction with SYBR® Green for detection and quantification of porcine parvovirus (PPV) in porcine tissues, two primers specific for the non-structural protein 1 gene were designed. The detection limit of this assay was 3–23 gene copies/reaction, equivalent to 0.001 $TCID_{50}$/ml. The assay was linear over a 10^6 dilution range of template concentrations. Other porcine pathogens involved in reproductive disorders (porcine circovirus 2, porcine reproductive and respiratory virus, pseudorabies virus, classical swine fever virus) were negative by this assay. This assay could detect PPV titres at least 10^5 smaller than the hemagglutination assay. To better understand the pathogenesis of PPV, the levels of viral DNA in various tissues of artificially challenged sows and their fetuses were quantified with this method. The virus was found mainly in the heart, lung, spleen, kidney, and endometrium of sows, and mainly in the heart, spleen, lung, and testis of fetuses. This study provides a new tool for the study of PPV infection and distribution in sows and their fetuses.

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1. Introduction

Porcine parvovirus (PPV) causes reproductive failure in pregnant sows, and the disease is characterized by embryonic and foetal death, mummification, stillbirths, and delayed return to oestrus (Mengeling, 2006). Although acute infection of postnatal, non-pregnant pigs is usually subclinical, PPV has also been linked to skin lesion occurrence in piglets (Kresse et al., 1985; Whitaker et al., 1990; Lager and Mengeling, 1994), interstitial nephritis in slaughter-aged pigs (Drolet et al., 2002), and non-suppurative myocarditis in lactating piglets (Bolt et al., 1997). More recently, PPV has gained importance as an agent able to enhance the effects of porcine circovirus type 2 (PCV2) infection in the clinical course of postweaning multisystemic wasting syndrome (Allan et al., 1999; Krakowka et al., 2000), an economically significant disease worldwide (Segalés et al., 2005). Because PPV causes reproductive failure and these other clinical and pathological conditions, vaccines to this virus are marketed worldwide (Mengeling et al., 1991).

The genome of PPV is composed of a linear single-stranded segment of DNA approximately 5 kb long. The two primary ORFs, the 3′ nonstructural 'NS' ORF and the 5′ structural 'VP' ORF, each encode at least two proteins (Shackelton et al., 2007). Genetic variability of PPV isolates has been demonstrated in the VP2 protein, and recent experimental studies suggest the presence of a new antigenic variant or type of PPV from field isolates (Martins et al., 2003; Zeeuw et al., 2007).

The classical methods to confirm fetal infection by PPV are virus isolation, hemagglutination, and the immunofluorescence test, which detect viral antigen in lungs (Joo et al., 1977; Mengeling et al., 1991). The use of the hemagglutination-inhibiting assay (HIA) to detect

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anti-PPV-specific antibodies in 56–70-day-old fetuses has been reported (Hogg et al., 1977). Meanwhile, the detection of viral nucleic acid in fetuses, especially after natural transplacental infection, has provided more reliable results (Molitor et al., 1991; Soares et al., 1999). The polymerase chain reaction (PCR) has been used for the detection of genetic sequences of many viral pathogens (Lennette et al., 1995). Molitor et al. (1991) first developed a PCR for the detection of PPV DNA sequences using primers that flanked a region coding for the major structural protein VP-2. Wilhelm et al. (2005, 2006) developed a SYBR® Green real-time PCR for the detection of PPV using primers for the VP-2 gene. McKillen et al. (2007) developed a molecular beacon real-time PCR for the detection of PPV using primers for the VP-2 gene. Soares et al. (1999) first developed a common PCR using primers specific to a highly conserved region of the PPV genome that codes for the non-structural protein, NS-1.

In this study, a SYBR® Green real-time PCR was developed for the detection of PPV using primers for the NS-1 gene. The purpose of this study was to establish a real-time PCR assay with SYBR® Green to detect the NS-1 gene of PPV and to provide information about PPV infection and viral tissue distribution in sows and their fetuses.

2. Materials and methods

2.1. Primers and viruses

The primer set was selected using the Oligo 6 software (Molecular Biology Insights, Inc), based on a highly conserved sequence within the NS-1 region of the PPV genome. The primers were designed to detect the available PPV sequences in the relevant NS-1 region (forward primer: 5′-AGCCAAAAATGCAAACCCCAATA-3′; reverse primer: 5′-CTCCACGGCTCCAAGGCTAAAG-3′) and to amplify a fragment with a length of 142 bp.

The PPV Chinese isolate BQ strain was isolated in swine testicle cells (Oraveerakul et al., 1992) 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). The animal came from a herd of wild sow experiencing reproductive problems characterized by foetal mummification, abortions, and stillbirths. The sick piglet, which died at 30 days of age, had symptoms of wasting, skin pallor, and dermatitis. The isolate BQ was a virulent, low-passage (passage 6). The titre was 2^{12} by the hemagglutinin (HA) test.

Other porcine pathogens, such as porcine circovirus 2 (PCV-2), porcine reproductive and respiratory virus (PRRSV), pseudorabies virus (PRV), and classical swine fever virus (CFSV), were provided by the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences.

2.2. Preparation of standard plasmid DNA

The complete coding sequence of the PPV NS-1 gene was inserted into a clone vector, pMD18-T (TaKaRa Biotechnology (Dalian) Co., Ltd). After the culture was increased in DH5α host bacteria (TaKaRa Biotechnology (Dalian) Co., Ltd), the recombinant plasmid was purified using a commercial test kit (Watson Biotechnologies, Inc). The products were kept at −20 °C for later use.

2.3. Establishment of real-time PCR

The real-time PCR amplifications of the NS-1 gene used 25-μl reaction mixtures containing 12.5 μl of SYBR® Green master mix (SYBR® Ex Taq™ kit, TaKaRa Biotechnology (Dalian) Co., Ltd), 0.5 μl of each primer (10 pM/μl), 1 μl of the recombinant plasmid, and 9.5 μl sterilized water. The reactions were carried out in an IQ-5 cycler (Bio-Bad. Co. Ltd). The conditions were set as follows: predenaturation at 95 °C for 10 s followed by 35 cycles consisting of denaturation at 95 °C for 5 s and annealing at 55 °C for 20 s. Immediately following the PCR, a melting curve was generated by raising the incubation temperature from 70 to 95 °C. The data were analyzed with the software of IQ-5. The standard plasmid DNA was 10-fold serially diluted in phosphate-buffered saline solution (PBS), and then tested five times with real-time PCR to generate a standard curve. The products of the real-time PCR were also subjected to electrophoresis in a 1% gel.

2.4. Sensitivity of the real-time PCR

A 1-ml aliquot of a culture solution of PPV with a titer of 10^7 $TCID_{50}$/ml was subjected to a 10-fold serial dilution in PBS, with nine dilutions. The DNA of each dilution was extracted (Joseph and David, 2001) and subjected to real-time PCR. A 25-μl aliquot of each dilution was also tested with the hemagglutination assay (Oraveerakul et al., 1990). The sensitivity of the real-time PCR was compared with that of the HA.

2.5. Specificity of the real-time PCR

To determine the specificity of the real-time PCR, different virus culture samples included 9 strains of PPV, 20 strains of PRRSV, 15 strains of CSFV, 22 strains of PRV, 16 strains of PRRSV, and 18 strains of PCV-2 were processed with the real-time PCR.

2.6. Reproducibility of real-time PCR

To determine the reproducibility of the real-time PCR, the standard plasmid was diluted to 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, and 1×10^0 copies and tested by three different people at five different times per person. The variation among the three testers at each time was analyzed.

2.7. Artificial challenging of sows with PPV

Six pregnant sows (first pregnancy) of the Duroc breed were obtained from a commercial breeding herd that was free of PPV, PRRSV, PRV, PCV-2; the virus-free status of the herd was confirmed three times by HIA test and PCR. Three artificially challenged sows and three control sows were

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