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Development and validation of a multiplex conventional PCR assay for simultaneous detection and grouping of porcine bocaviruses

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A B S T R A C T

Porcine bocavirus (PBoV), a newly described porcine parvovirus, has received attention because it can be commonly identified in clinically affected pigs including pigs with post-weaning multisystemic wasting syndrome (PWMS) and pigs with diarrhea. In recent years, novel PBoVs have been identified and were classified into three genogroups, but the ability to detect and classify these novel PBoVs is not comprehensive to date. In this study, a multiplex conventional PCR assay for simultaneous detection and grouping of PBoVs was developed by screening combinations of mixed primer pairs followed by optimization of the PCR conditions. This method exclusively amplifies targeted fragments of 531 bp from the VP1 gene of PBoV G1, 291 bp from the NP1 gene of PBoV G2, and 384 bp from the NP1/VP1 gene of PBoV G3. The assay has a detection limit of 1.0×10^3 copies/μL for PBoV G1 4.5×10^3 for PBoV G2 and 3.8×10^3 for PBoV G3 based on testing mixed purified plasmid constructs containing the specific viral target fragments. The performance of the multiplex PCR assay was comparable to that of the single PCRs which used the same primer pairs. Using the newly established multiplex PCR assay, 227 field samples including faeces, serum and tissue samples from pigs were investigated. All three PBoV genogroups were detected in the clinical samples with a detection rate of 1.3%, 2.6% and 12.3%, respectively for PBoV G1, G2 and G3. Additionally, coinfections with two or more PBoV were detected in 1.7% of the samples investigated. These results indicate the multiplex PCR assay is specific, sensitive and rapid, and can be used for the detection and differentiation of single and multiple infections of the three PBoV genogroups in pigs.

1. Introduction

Porcine bocavirus was first discovered in Swedish pigs with post-weaning multisystemic wasting syndrome (PMWS) in 2009 and was classified within the *Bocaparvovirus* genus (Blomström et al., 2009; Cadar et al., 2011; Cotmore et al., 2014). Bocavirus was discovered in both humans and animals and to date includes human bocavirus (HBoV) (Allander et al., 2005; Cashman and O'shea, 2012), porcine bocavirus (PBoV) (Blomström et al., 2009), bovine parvovirus (BPV) (Chen et al., 1986), minute virus of canines (MVC) (Binn et al., 1970), gorilla bocavirus (GBoV) (Kapoor et al., 2010a) and California sea lion bocavirus (CslBoV) (Li et al., 2011b). Viruses in the *Bocaparvovirus* genus belong to the subfamily *Parvovirinae* of the *Parvoviridae* family, which is a group of divergent linear ssDNA viruses (Tijssen et al., 2011). Porcine bocavirus has a genome of ~5 kb and has three open reading frames that encode four proteins: NS1, NP1, VP1 and VP2 (Choi et al., 2014; Zeng et al., 2011; Zhang et al., 2015b). Parvoviruses were demonstrated to have nucleotide substitution rates that are as high as those of some RNA viruses (Duffy et al., 2008; Shackelton et al., 2005) and recent results suggest that the presently circulating PBoVs exhibit considerable genetic diversity within the same sample and between different pigs (Lau et al., 2011; Liu et al., 2014; Shan et al., 2011; Zhang et al., 2014). These findings may indicate that these viruses are in the process of adaptation and can undergo rapid evolution to generate new genotypes or species. Numerous PBoVs have been discovered to date. These PBoVs have been classified into three genogroups, named provisionally G1, G2 and G3, based on phylogenetic analysis of the NS1, NP1 and VP1/2 coding genes and genomes (Gunn et al., 2015; Jiang et al., 2014; Xiao et al., 2013; Yang et al., 2012).

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Members of the genus *Bocaparvovirus* are pathogens that have been associated with various disease manifestations, including respiratory and enteric diseases (Jiang et al., 2014). All four genotypes of HBoV have been identified in children with acute gastroenteritis, whereas only HBoV1 and HBoV2 were identified in respiratory tract samples (Xu et al., 2012a). More recent research strongly supported the hypothesis that HBoV can cause severe acute respiratory tract infection in children in the absence of viral and bacterial co-infections (Broccolo et al., 2015; Moesker et al., 2015). Reports suggest that some PBoVs may be associated with respiratory signs or diarrhea, although the pathogenicity of PBoVs has also not been recognized clearly mainly due to a lack of a suitable cell culture system or animal model (Blomström et al., 2009; Zhai et al., 2010). Therefore, detection and differentiation of PBoV is important to better understand the potential associations between PBoVs and related diseases.

Recently, emerging parvoviruses in pigs were discovered through the application of random amplification and large-scale sequencing techniques, followed by bioinformatic analysis of large numbers of the sequences of the resulting clones (Allander et al., 2001, 2005; Shan et al., 2011). With public availability of these viral sequences, simple and sensitive PCR methods using specific primers have been developed for PBoV (Kapoor et al., 2010b; Lau et al., 2011). However, these methods usually focused on G1 of PBoVs mainly due to availabilty of limited numbers of reference strains and a consensus PCR assay using one primer pair targeting a conserved region to detect all the PBoVs has not been reported yet mainly due to the high level of genetic diversity. In addition, conventional PCR technology to detect several PBoV genogroups individually is labor-intensive and expensive. These limitations can be overcome by using a multiplex conventional PCR assay, which incorporates multiple primers that amplify RNA or DNA from several viruses simultaneously in a single reaction (Elnifro et al., 2000).

In the present study, a multiplex conventional PCR assay was developed by combining three pairs of primers in one reaction. Furthermore, the reaction conditions were optimized for the rapid detection and differentiation of PBoV G1, G2, and G3 on the basis of amplicon size. To validate its application, we tested this assay for specificity and sensitivity on clinical samples and compared the results with those obtained by using single PCRs.

2. Materials and methods

2.1. Viruses and samples

The following viruses were used and stored in our laboratory: PBoV G1 strain MN307 (GenBank Accession number KF025391), PBoV G2 strain IA147 (GenBank Accession number KF025392) and PBoV G3 strain IA270 (GenBank Accession number KF025390). To test specificity of the assay, the following non-targeted viruses were utilized: A commercial porcine parvovirus (PPV) vaccine strain (Beijing Zhonghai Animal Health Science and Technology Co., Ltd, China, No. 0040401), commercial transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) vaccine strains (Harbin Weike Biotechnology development company, Harbin, China, No. 030718), a classical swine fever virus (CSFV) (Hangzhou strain), porcine circovirus type 2 (PCV2) (GenBank Accession Number GQ996404) and porcine reproductive and respiratory syndrome virus (PRRSV) (GenBank accession number DQ269472). All viruses were maintained in the authors' laboratory.

A total of 227 pig samples were utilized to test the multiplex PCR. The samples included 200 faecal samples from clinically normal pigs located in the Zhejiang province and collected during 2013. In addition, 22 serum samples from normal pigs and five lung samples from pigs suffering from respiratory tract symptoms or reproductive failure were collected in different pig farms in several provinces in China during 2013–2014. All samples were stored at −80 °C until testing. The samples used in this study were collected in accordance with international standards for animal welfare.

2.2. Primers design

All genomic sequences of the PBoVs utilized in this study were obtained from the GenBank nucleotide sequence database from the National Center for Biotechnology Information (NCBI). Highly conserved regions within each PBoV genogroup genomes were identified by alignment of all available 62 partial and complete or nearly complete genome sequences with Clustal W (DNAStar Inc., Madison, WI, USA) (see Supplementary Fig. S1 in the online version at DOI: 10.1016/j.jviromet.2016.06.014). Primers corresponding to these conserved regions of the viral genomes were designed using Primer Premier 5.0 (Primer Biosoft International, Palo Alto, CA, USA). Three pairs of primers were designed to amplify PBoV G1, PBoV G2 or PBoV G3 and are outlined in Table 1. The specificity of the primers was confirmed against random nucleotide sequences obtained by a BLAST search in the GenBank database. All primers were obtained from a commercial source (Sangon Biotech. Co., Ltd, Shanghai, China).

2.3. Nucleic acid extraction

The samples were processed as described previously (Jiang et al., 2014). Briefly, tissue samples were minced and diluted 1:10 (w/v) in Dulbecco's modified Eagle's medium, homogenized and centrifuged at 1500*g* for 10 min to obtain the supernatant. Faecal samples were resuspended 1:10 (w/v) in PBS, vortexed for 30 s and centrifuged at 1500*g* for 10 min. Viral genomic DNA was extracted from frozen clinical samples using the AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit 50-prep (Axygen, Hangzhou, China) according to the manufacturer's instructions. The extracted DNA was stored at -80 °C until use.

2.4. Single PCR and plasmid template construction

The PCR reaction for PBoV G1, G2 and G3 was conducted in a 25 μL mixture including 2.5 μL 10 × PCR buffer, 1.2 μL 2.5 mM of each dNTPs, 2.5 μL 25 mM $MgCl_2$, 0.5 μL of each 10 μM primer (Table 1), 1.5 U of Taq DNA polymerase (5 U/μL) (Sangon), 2 μL of the DNA and 16 μL distilled water. The amplifications were performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: after initial denaturation at 95 °C for 3 min, 35 cycles were conducted at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The amplicons were detected by electrophoresing 5 μL aliquots through 1.5% agarose gel 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 then sequenced and confirmed the constructed recombinant plasmids which were used as standard templates for optimization of the following PCR assays.

2.5. Optimization of the multiplex PCR assay

Based on the established single PCRs, a series of experiments were performed to optimize the multiplex PCR protocol, including reagent concentration and PCR cycling parameters. The multiplex PCR was carried out in a 20 μL reaction mixture consisting of 2 μL of 10× PCR Buffer, 0.1–6 mM $MgCl_2$, 0.01–0.6 mM dNTP mix, 0.5–6 U Taq DNA polymerase, 2 μL of each plasmid DNA, and 0.05–0.6 mM each primer pair of three PBoV genogroups. The amplification was run in a Bio-Rad thermal cycler (Laboratories, Hercules, CA, USA) under the following conditions: initial denaturation at 95 °C for