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Short communication

Concurrent infections of pseudorabies virus and porcine bocavirus in China detected by duplex nanoPCR

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Nanoparticle-assisted polymerase chain reaction (nanoPCR) is a novel method for the simple, rapid, and specific amplification of DNA and has been used to detect viruses. A duplex nanoPCR molecular detection system was developed to detect pseudorabies virus (PRV) and porcine bocavirus (PBoV). Primers were selected to target conserved regions within the PRV gE gene and the PBoV NS1 gene. Under optimized nanoPCR reaction conditions, two specific fragments of 316 bp (PRV) and 996 bp (PBoV) were amplified by the duplex nanoPCR with a detection limit of 6 copies for PRV and 95 copies for PBoV; no fragments were amplified when other porcine viruses were used as template. When used to test 550 clinical samples, the duplex nanoPRC assay and a conventional duplex PCR assay provided very similar results (98.1% consistency); single PRV infections, single PBoV infections, and concurrent PRV and PBoV infections were detected in 37%, 15%, and 9% of the samples, respectively. The results indicate that the novel duplex nanoPCR assay is useful for the rapid detection of PRV and PBoV in pigs.

1. Introduction

Pseudorabies virus (PRV), also known as Aujeszky's virus, belongs to the subfamily Alphaherpesvirinae of the family Herpesviridae. Acute PRV infection is characterized by high fever, extreme itching, and encephalomyelitis. PRV can cause disease in swine, bovine, sheep, and wild animals (Eraldo et al., 2012). Porcine pseudorabies has become a serious global disease of pigs. Because PRV replication requires the gE gene (Enquist et al., 1999), the absence of gE gene is associated with reduced PRV pathogenicity. The gE gene can be used to determine whether animals are infected with attenuated vaccine strains or wild virus strains.

Porcine bocavirus (PBoV) belongs to the family Parvoviridae (Huang et al., 2014; Li et al., 2011a,b). PBoV was first described in 2009 when it was isolated from feces of pigs with postweaning multisystemic wasting syndrome (PMWS) in Sweden (Blomstrom et al., 2009). PBoV is a small, single-stranded DNA virus whose genome includes non-structural proteins (NS1 and NP1) and VP1/VP2 structural proteins (Wang et al., 2014). The NS1 protein is an important multifunctional phosphoprotein for parvoviruses (Sun et al., 2009). In addition, the role of the NS1 protein is similar among different paroviruses (Wang et al., 2014).

Nanoparticle-assisted polymerase chain reaction (nanoPCR) is an advanced form of PCR in which solid gold nanometal particles (1–100 nm) form colloidal nanofluids that increase thermal conductivity (Shen et al., 2009; Zhang et al., 2005). Therefore, PCR assays with nanofluids reach the target temperature more quickly than PCR assays with original liquids. This reduces the time at non-target temperatures and thereby reduces non-specific amplification and increases specific amplification (Li and Rothberg, 2004; Ma et al., 2013).

Since 2010, PRV and PBoV have been commonly associated with increased mortality in pigs in China. In this study, a duplex nanoPCR was developed for PRV and PBoV and the PRV gE gene and the PBoV NS1 gene were chosen as targets. The established duplex nanoPCR assay will be useful to investigate the molecular epidemiology of these viruses.

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Table 1
Sequences of the primers used in this study.

Virus	Gene	Primer	Primer sequence (5′–3′)	Fragment size
PRV	gE	E1	F.ACGAGCCCCGCTTCCACGCG	316 bp
		E2	R.CACCGGTCCCCGAGCAGCGG	
PBoV	NS1	S1	F.CATCCTTTAGTCAATGCGAGAA	996 bp
		S2	R.CGGTAACAGCATAGAGTCCC	

2. Materials and methods

2.1. Viruses and recombinant plasmids

The recombinant plasmids pET30a-PRV-gE and pUC57-PBoV-NS1 and additional viruses including porcine parvovirus (PPV, strain BQ-C), porcine circovirus type 2 (PCV2, strain SH), porcine reproductive and respiratory syndrome virus (PRRSV, strain HB), classic swine fever virus (CSFV), porcine Teschovirus (PTV), and African swine fever virus (ASFV) were obtained from the Harbin Veterinary Research Institute of Chinese Academy of Agricultural Science. The nanoPCR Kit (NPK02) was purchased from GRED-BIO (Weihai, China). The recombinant plasmids pET30a-PRV-gE and pUC57-PBoV-NS1 were amplified in *Escherichia coli* DH5α and purified with the AxyPrepTM Plasmid Midi and Maxi Plasmid Kits (AXYGEN Biotechnology Company, Hangzhou, China). The plasmids were stored at −20 °C.

2.2. Primer design

The full-length genes of PRV and PBoV (Table 1) were chosen according to published sequences available in GenBank (GenBank accession numbers NC_006151 and HM053694). The primers were designed with Primer Premier 5.0 software. All of the primers were synthesized by BoShi Biotech (Harbin, China), and their sequences primers are listed in Table 1.

2.3. Sample preparation

Animal experiments were approved by the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, and animal experiments were performed in accordance with animal ethics guidelines and approved protocols. The Animal Ethics Committee approval number was Heilongjiang-SQ 2013-2046. The samples were ground in a mortar and then repeatedly frozen and thawed. DNA was recovered from 200 μL of the homogenized samples according to the instructions of the TIANamp Virus genomic DNA/RNA kit (Beijing Tiangen Biotech Company, Beijing, China). The extracted DNA was stored at −20 °C before it was subjected to conventional duplex PCR and to the novel duplex nanoPCR as described in the following sections. In addition, identified PRV and PBoV bands by duplex nanoPCR in the first 54 samples were checked by sequencing.

2.4. Establishment of the conventional duplex PCR assay

The conventional duplex PCR assay was performed in a 20 μL system that included 2.4 μL of template (for comparison with the duplex nanoPCR and for positive controls); 1.2 μL of each of the recombinant plasmids pET30a-PRV-gE and pUC57-PBoV-NS1; (pET30a-PRV-gE and pUC57-PBoV-NS1); 1.0 μL each of primers E1 and E2 and 0.8 μL each of primers S1 and S2 (Table 1); 2.0 μL of dNTPs; 1.0 μL of KOD FX Neo (1 U/μL) (TOYOBO Biotechnology Company, Shanghai, China); 5.0 μL of 2× PCR buffer for KOD FX Neo (TOYOBO Biotechnology Company, Shanghai, China); and *dd*H_2O up to 20 μL. PCR reaction conditions were: 94 °C for 5 min; followed by 30 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min. The amplified products were analyzed by electrophoresis on 1.5% agarose gels.

2.5. Establishment of the duplex nanoPCR assay

2.5.1. Optimization of the duplex nanoPCR assay

The duplex nanoPCR assay is based on the conventional duplex PCR assay. The buffers were prepared as described (Zhang et al., 2005). Experiments were performed to optimize the annealing temperature and primer volume for the duplex nanoPCR assay. The duplex nanoPCR assay was performed in a 20 μL system. Each of the recombinant plasmids (pET30a-PRV-gE and pUC57-PBoV-NS1) was tested at volumes ranging from 0.2 μL to 1.4 μL (Table 1) in increments of 0.2 μL. Each pair of forward and reverse primers (E1 and E2, and S1 and S2 at 10 μM) was tested at volumes ranging from 0.2 μL to 1.2 μL (Table 1) in increments of 0.2 μL. The reaction volume also contained 0.5 μL of Taq DNA polymerase (5 U/μL) (GRED, Shandong, China), 10 μL of 2× nanoPCR Buffer (GRED, Shandong, China), and *dd*H_2O up to 20 μL. PCR reaction conditions were: 94 °C for 5 min; followed by 30 cycles of 94 °C for 40 s, annealing temperatures from 50 °C to 60 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min. Amplified products were analyzed by electrophoresis on 1.5% agarose gels.

The optimization was conducted in the following steps. First, the optimal annealing temperature was determined using primer concentrations and template concentrations as indicated for the conventional duplex PCR. The optimal annealing temperature was then used to determine the optimal primer concentrations with the template concentrations as indicated for the conventional duplex PCR. Finally, the optimal template concentrations were determined using the previously determined optimal annealing temperature and primer concentrations.

2.5.2. Sensitivity of the duplex nanoPCR assay

To determine the sensitivity of the duplex nanoPCR assay, pET30a-PRV-gE and pUC57-PBoV-NS1 were purified with the AxyPrepTM Plasmid Midi and Maxi Plasmid Kits (AXYGEN Biotechnology Company, Hangzhou, China) and quantified using UV spectroscopy (6.44×10^{10} copies/μL of pET30a-PRV-gE and 9.51×10^{10} copies/μL of pUC57-PBoV-NS1). When 10-fold serial dilutions were prepared, the concentration ranged from 6.44×10^{10} to 6×10^{0} copies/μL for pET30a-PRV-gE and from 9.51×10^{10} to 9×10^{0} copies/μL for pUC57-PBoV-NS1. Each dilution was tested by the duplex nanoPCR assay and the conventional duplex PCR assay, and *dd*H_2O was used as a negative control. Amplified products were analyzed by electrophoresis on 1.5% agarose gels.

2.5.3. Specificity of the duplex nanoPCR assay

DNA or cDNA of the following viruses and bacterium were separately subjected to the duplex nanoPCR and the conventional duplex PCR: PPV, PCV2, PRRSV, CSFV, PTV, ASFV, and *Escherichia coli*. The recombinant plasmids pET30a-PRV-gE and pUC57-PBoV-NS1 were used as positive controls. PCR products were analyzed by electrophoresis on 1.5% agarose gels.

3. Results

3.1. Optimization of the duplex nanoPCR assay

The recombinant plasmids pUC57-PBoV-NS1 and pET30a-PBoV-NS1 were used as templates in the optimization experiments. The tested annealing temperatures ranged from 50 to 60 °C, and amplification results were best with an annealing temperature of 55 °C (data not shown).

An annealing temperature of 55 °C was used to determine the optimal primer concentrations. The tested volume of primers

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