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A multiplex TaqMan real-time PCR for detection and differentiation of four antigenic types of canine parvovirus in China

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ABSTRACT

Canine parvovirus (CPV) is an important pathogen in domestic dogs, and the original antigenic types CPV-2 and its variants, CPV-2a, 2b and 2c, are prevalent worldwide. A multiplex TaqMan real-time PCR method was developed for the detection and differentiation of four antigenic types of CPV. A set of primers and probes, CPV-305F/CPV-305R and CPV-2-305P (for CPV-2)/CPV-2a-305P (for CPV-2a, 2b and 2c), was able to differentiate CPV-2 and its variants (CPV-2a, 2b and 2c). Another set of primers and probes, CPV-426F/CPV-426R and CPV-2-426P (for CPV-2 and 2a)/CPV-2b-426P (for CPV-2b)/CPV-2c-426P (for CPV-2c), was able to differentiate CPV-2a (2), CPV-2b, and CPV-2c. With these primers and probes, the multiplex TaqMan real-time PCR assay detected effectively and differentiated CPV-2, 2a, 2b and 2c by two separate real-time PCRs. No cross reactivity was observed with canine distemper virus, canine adenovirus, and canine coronavirus. The detection limit of the assay is 10^1 genome copies/μL for CPV-2, CPV-2a, CPV-2b, and 10^2 copies/μL for CPV-2c. The multiplex real-time PCR has 100% agreement with DNA sequencing. We provide a sensitive assay that simultaneously detects and differentiate four antigenic types of CPV and the method was also used for quantification of CPVs viral genome.

1. Introduction

Canine parvovirus (CPV) is an important pathogen in domestic dogs and several wild carnivore species. It belongs to the genus *Protoparvovirus* within the family *Parvoviridae* [1]. The virus replicates autonomously in host cells, and is genetically related to feline parvovirus (FPV) and mink enteritis virus (MEV), which infect different host animals [2]. The original CPV-2 was first identified in 1978 and has rapidly spread worldwide [3–5]. Soon two antigenic variants, CPV-2a, which emerged in 1979 and contained 5 amino acid substitutions in VP2 [6], and CPV-2b, which appeared in 1984 and had a single additional substitution in VP2 [7], replaced the original type [8]. The third variant CPV-2c with Glu426 mutant emerged in Italy initially [9] and now the three variants have been circulating in dog populations around the world [5,10,11]. CPV-2a, CPV-2b and CPV-2c are distinguished by one or two single nucleotide polymorphisms (SNPs) in the sequence of the VP2 gene. SNPs at positions 1276 and 1278 of the VP2 gene determine whether residue 426 of the VP2 protein is Asn (CPV-2a), Asp (CPV-2b) or Glu (CPV-2c) [8].

Relative to the original CPV-2, the antigenic variants of CPV-2a, CPV-2b, and CPV-2c are more highly pathogenic in dogs and have an extended host range that includes cats [8]. Infection with any type of the CPVs, dogs show similar signs, which include loss of appetite, vomiting, diarrhea, and dehydration. The CPV types cannot be distinguished by examination or the signs of disease observed from the infected dogs. So we need a method to detect and differentiate the CPV types, which is benefit for treatment of infected dogs with the homogenous polyclonal or monoclonal antibodies of CPV.

Several assays have been reported for the detection or quantitation of CPV DNA [12–18], including PCR, nested PCR, iiPCR, RPA, LAMP-ELISA, LAMP-LFD, LAMP, polymerase spiral reaction, and SYBR Green based real-time PCR, but none of these assays enable differentiation CPV antigenic types and CPV-like viruses (MEV and FPV). Several other assays have been reported for the detection and differentiation of type 2-based vaccines and field strains of CPV [19] or typing of three antigenic types of CPV [20] or CPV and MEV [21], including PCR-RFLP

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assay and MGB probe real-time RT-PCR, but none of these assays enables simultaneous detection and differentiation of four antigenic types of CPV.

Molecular diagnostic methods have improved dramatically overthe past years, providing a huge potential for their application inclinical diagnostic where faster and accurate detection of infectious pathogens is required [22]. The real time TaqMan®-based quantitative PCR (qPCR) method, basing on the use of oligonucleotide pairs, relies on improved specificity because only sequence-specific amplifications are measured [23]. In this study, we aimed to develop and evaluate a multiplex TaqMan real-time RT-PCR assay for quantitative and differential detection of CPV-2, CPV-2a, CPV-2b, and CPV-2c.

2. Materials and methods

2.1. Ethics statement

The protocol of the study was carried out in accordance with guidelines of animal welfare of World Organization for Animal Health. All experimental protocols were approved by the Review Board Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences.

2.2. Viruses, cells and samples

The LN15-32 strain of CPV-2, the JL14-1 strain of CPV-2a, the BJ14-1 strain of CPV-2b, the BJ15-20 strain of CPV-2c [24], the CDV3 strain of canine distemper virus (CDV) [25], the CAV-2 strain of canine adenovirus (CAV) [26], and the CCV HB16-2 strain of canine coronavirus (CCV) were also used to test the specificity of primers and probes for related viruses and other dog viruses. F81 [8], Vero [25], and MDCK cells [26] were used to propagate and isolate CPV, CDV, CAV, and CCV, respectively. The cells were grown in DMEM supplemented with 10% FCS.

From the years of 2014–2017, a total of 114 dog fecal samples were collected from different animal hospitals in North China, including Beijing and Hebei. All the samples were tested positive for CPV by PCR and the Anigen Rapid CPV Ag Test Kit (BioNote, Gyeonggi-do, South Korea).

2.3. Primers and probes

The primers and probes were designed based on the alignment of 201 VP2 gene sequences of CPV from GenBank, including 15 strains of CPV-2, 81 strains of CPV-2a, 23 strains of CPV-2b, and 82 strains of CPV-2c (all the sequences see the supplementary file), and synthesized by (Sangon Biotech, Shanghai, China). For discriminating CPV-2 and the variants (CPV-2a, 2b and 2c), CPV-305F/CPV-305R and CPV-2-305P/CPV-2a-305P were designed based on the SNP in the VP2 gene between CPV-2 and the variants (913 G→T). For differentiating CPV-2a, CPV-2b, and CPV-2c, another set of primers and probes, CPV-426F/CPV-426R and CPV-2-426P/CPV-2b-426P/CPV-2c-426P, was designed based on the SNPs in the VP2 gene between the variants (1276 A→G and 1278 T→A). Sequence and position of the primers and probes are summarized in Table 1.

2.4. DNA/RNA extraction

DNA samples were extracted from 200 μL of cell culture supernatants or fecal samples using the Takara MiniBEST Viral RNA/DNA Extraction Kit Ver. 5.0 (Takara Biotechnology, Dalian, China), according to the manufacturer's instructions. RNA extraction of CCV and CDV and reverse transcription were performed using procedures described previously [27]. The extracted DNA samples were used as templates in the real-time PCR assays.

2.5. Multiplex real-time PCR standards

The fragments were generated from LN15-32 strain, JL14-1 strain, BJ14-1 strain, and BJ15-20 strain by PCR using primer pair VP2-F/VP2-R [28], and cloned into the pEASY-T1 vector (TransGen Biotech, Beijing, China) and sequenced to generate recombinant plasmids. These recombinant plasmids were used as standards in the multiplex real-time PCR. The plasmids were quantified as described previously [29].

2.6. Optimization of the multiplex real-time PCR

Real-time PCR was conducted in an Applied Biosytems QuantStudio™ 3 Real-Time PCR System (ABI, Foster city, USA). The reactions (30 μL) contained 1 μL of template or standard DNA plasmids, 15 μL of Taqman Multiplex Master mix (ABI, Warrington, UK), 200 nM of primers CPV-305F/CPV-305R (CPV-2 and the variants assay) or CPV-426F/CPV-426R (CPV-2a, 2b and 2c assay), 200 nM of probes CPV-2-305P/CPV-2a-305P (CPV-2 and the variants assay), or 200 nM of probes CPV-2-426P/CPV-2b-426P and 300 nM of probe CPV-2c-426P (CPV-2a, 2b and 2c assay). Two different wells were used for each test sample and each dilution of standard DNA plasmids. After activation of *Taq* DNA polymerase at 94 °C for 30 s, 40 cycles of two-step PCR were performed, consisting of denaturation at 94 °C for 5 s and primer annealing-extension at 61 °C (CPV-2 and the variants assay) or 63 °C (CPV-2a, 2b and 2c assay) for 34 s. The increase in fluorescent signal was registered during the annealing-extension step of the reaction and the data were analyzed with QuantStudioTM Design & Analysis Software (Applied Biosytems, Foster City, CA, USA).

2.7. Specificity of the multiplex real-time PCR

The multiplex real-time RT-PCR was evaluated for its specificity by testing LN15-32 strain (CPV-2), JL14-1 strain (CPV-2a), BJ14-1 strain (CPV-2b), and BJ15-20 strain (CPV-2c), and three other unrelated canine viruses including CDV, CAV, and CCV. RNA or DNA samples, which were extracted from infected or mock-infected cell cultures, and cDNA samples synthesized from the RNA and DNA were subjected to assays using the multiplex real-time PCR.

2.8. Sensitivity of the multiplex real-time PCR

Serial 10-fold dilutions of each standard DNA plasmids (type 2, 2a, 2b and 2c), ranging from 10^8 to 10^0 DNA copies/μL of the template, were subjected to detection by the multiplex TaqMan real-time PCR for assay limit.

2.9. Reproducibility of the multiplex real-time PCR

Inter-assay and intra-assay reproducibility tests were performed in triplicate by testing three different titers of cell cultures infected with LN15-32, JL14-1, BJ14-1, and BJ15-20 strains, respectively, to evaluate the reproducibility of the multiplex real-time PCR assay.

2.10. Comparative test

A total of 114 dog fecal samples, which were tested positive for CPV using a PCR method as described previously [8] and the Anigen Rapid CPV Ag Test Kit (BioNote, Gyeonggi-do, South Korea), were tested in parallel using the multiplex real-time PCR method and a DNA sequencing method as described previously [8].

3. Results

3.1. Establishment of standard curve of the multiplex real-time PCR

The generated standard curve based on serial 10-fold dilutions of