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One-step triplex PCR/RT-PCR to detect canine distemper virus, canine parvovirus and canine kobuvirus

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ABSTRACT. To rapidly distinguish Canine distemper virus (CDV), canine parvovirus (CPV), and canine kobuvirus (CaKoV) in practice, a one-step multiplex PCR/RT-PCR assay was developed, with detection limits of $10^{2.1}$ $TCID_{50}$ for CDV, $10^{1.9}$ $TCID_{50}$ for CPV and 10^3 copies for CaKoV. This method did not amplify nonspecific DNA or RNA from other canine viruses. Therefore, the assay provides a sensitive tool for the rapid clinical detection and epidemiological surveillance of CDV, CPV and CaKoV in dogs.

KEY WORDS: canine distemper virus (CDV), canine kobuvirus (CaKoV), canine parvovirus (CPV), one-step triplex PCR/RT-PCR

Infection with canine distemper virus (CDV), canine parvovirus (CPV), or canine kobuvirus (CaKoV) seriously threatens the health of dogs, with mortality rates of 50–100%, causing enormous economic losses in the canine industry [12].

CDV is widely documented as causing severe immunosuppression and persistent central–nervous–system infection in dogs [15, 16, 20, 21]. Although widespread vaccination against canine distemper has been conducted for many decades, it is still an important disease in dogs [5]. CPV is one of the major pathogens responsible for acute gastroenteritis in dogs [2]. It has become a major cause of hemorrhagic gastroenteritis and myocarditis in puppies [1]. Since CaKoV first description in 2011 [8, 11], it has been detected throughout the world [3, 4, 10, 17, 18]. In China, the only two recent reports about CaKoV involved in diarrheal dogs [9, 10], which suggested that the CaKoV might be one of the important diarrheal pathogens in dogs. The CDV, CPV, canine adenovirus type 1 (CAdV-1), and canine coronavirus (CCoV) all are known as the important diarrheal pathogens in dogs, but the CDV and CPV are more prevalent in dogs in China.

The mixed infections with CDV, CPV and CaKoV commonly occur in dogs, and all these three viruses could cause diarrhea, which made differential diagnoses based on clinical symptoms difficult. Therefore, it is necessary to develop a rapid early diagnostic method to allow effective preventive and control measures to be taken as soon as possible. Although conventional PCR and reverse transcription (RT)-PCR methods have been established to detect single viruses [7, 14] and multiplex PCR to detect several viruses simultaneously [6], no multiplex PCR has been developed to detect CDV, CPV and CaKoV. However, using single PCRs to detect each virus is time–consuming and laborious. Therefore, we developed a one-step triplex PCR/RT-PCR method to simultaneously detect CDV, CPV and CaKoV in dogs.

CDV, CPV, CaKoV, CAdV-1, canine adenovirus type 2 (CAdV-2), canine parainfluenza virus (CPIV) and CCoV were provided by the Harbin Veterinary Research Institute (Harbin, China). Fecal samples (n=66) from dogs with clinical symptoms of diarrhea were collected from several provinces in China and sent our laboratory for analysis. This study was approved by the Harbin Veterinary Research Institute–Experimental Animal Welfare Ethics Committee (HVRI-EAWEC), and conducted under the guidance of the HVRI–EAWEC. The animals from which specimens were collected were handled in accordance with animal protection law of the People' Republic of China. The primers used to detect CDV and CPV were described previously [13]. The primers used to detect CaKoV were designed based on 3D gene of CaKoV. All the primer sequences are shown in Table 1.

The genomes of CDV, CPV and CaKoV were extracted with the AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Corning, Suzhou, China), according to the manufacturer's instructions.

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Table 1. Sequences of the one-step triplex PCR/RT-PCR primers

Primers	Sequences (5′-3′)	Length of products (bp)
CDV-F	TGCGGTCTTACATTTGCATC	669
CDV-R	ACTCCAGAGCAATGGGTAGGG	
CaKV-F	CCCTGGAACACCCAAGGCCGCT	504
CaKV-R	TCTGGTTGCCATAGATGTGGTG	
CPV-F	ATGGTTGGTGACTCTTTGTTT	297
CPV-R	ACATTTGATTGACACTTCCC	

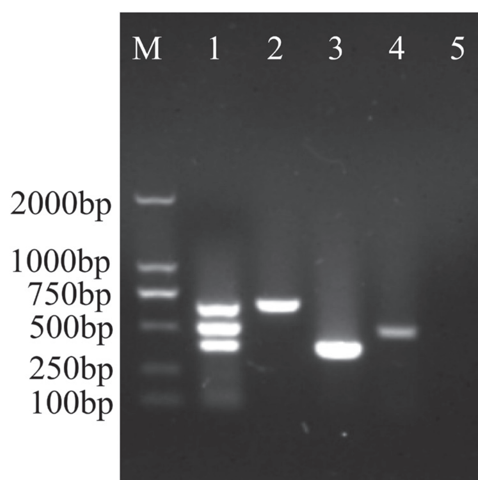

Fig. 1. Performance of one-step multiplex PCR/RT-PCR. M: DL 2,000 DNA Marker; 1: CDV+CaKoV+CPV; 2: CDV; 3: CPV; 4: CaKoV; 5: Negative control.

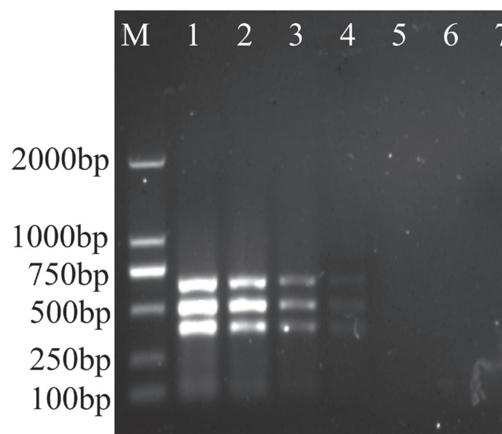

Fig. 2. Sensitivity of one-step multiplex PCR/RT-PCR. M: DL 2,000 DNA Marker; 1–6: 10^1–10^6 dilution; 7: Negative control.

The one-step multiplex PCR/RT-PCR was performed in a 25 μl reaction mix with the PrimeScript™ One Step RT-PCR Kit Ver. 2 (Takara, Dalian, China), according to the manufacturer's instructions. The components of the reaction were: 0.75 μl of CDV, 1 μl of CPV or 2.5 μl of CaKoV RNA; 2.5 pmol each of primers CDV-F, CDV-R, and CPV-F, and 10 pmol each of primers CPV-R, CaKoV-F, and CaKoV-R; 12.5 μl of 2 × 1 Step Buffer; 0.5 μl of PrimeScript 1 Step Enzyme Mix (Takara); and 4.75 μl of RNase–free dH_2O. The cycling parameters were: 50°C for 30 min; predenaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec; and a final extension step at 72°C for 10 min. The specificity of the one-step multiplex PCR/RT-PCR was assessed against other major canine viruses: CAdV-1, CAdV-2, CPIV and CCoV. To evaluate the sensitivity of this method, 10–fold serially diluted DNA/RNAs (the original concentrations were 10^7 copies for CaKoV (RNA copies for CaKoV were determined by one step SYBR Green Real-time RT-PCR assay (data not shown)), $10^{6.1}$ $TCID_{50}$ for CDV and $10^{5.9}$ $TCID_{50}$ for CPV) were used as the templates in the multiplex PCR/RT-PCR. The assay detected CDV, CPV and CaKoV, evident as bands of different sizes on agarose gel electrophoresis (Fig. 1). No PCR products were amplified from CAdV-1, CAdV-2, CPIV and CCoV. The limits of detection were 10^3 copies for CaKoV (Fig. 2), $10^{2.1}$ $TCID_{50}$ for CDV and $10^{1.9}$ $TCID_{50}$ for CPV.

The viral DNA/RNA of fecal samples collected from dogs with clinical symptoms of diarrhea were extracted and tested in parallel with both the one-step multiplex PCR/RT-PCR and the commercial Rapid CDV/CPV Ag Test Kit (Bionote, Gyeonggi, Republic of Korea) for CDV or CPV, according to the manufacturer's instruction, and a traditional simplex RT-PCR for CaKoV, as described previously with minor modification [4]. Of the 66 fecal samples tested, four (6.06%) were positive for CDV, eight (12.12%) were positive for CPV, and four (6.06%) were positive for CaKoV. Among these, two were mixed infections of CDV and CPV and one was a mixed infection of CPV and CaKoV, detected with the one-step multiplex PCR/RT-PCR. The results for the commercial CDV/CPV Ag Test Kit and the traditional simplex RT-PCR were three (4.55%) samples positive for CDV, seven (10.61%) positive for CPV, and four (6.06%) positive for CaKoV; among these positive samples, one was a mixed infection of CPV and CaKoV. For the CDV and CPV, the positive rate of clinical samples tested by one-step multiplex PCR/RT-PCR was higher than the commercial CDV/CPV Ag Test Kit. The main reason may be that the nucleic acid detection methods are more sensitive than the antigen detection methods, But statistically, we cannot conclude that the multiplex PCR/RT-PCR established in present study is more sensitive than the commercial CDV/CPV Ag Test Kit or the traditional simplex RT-PCR. The more clinical tests need further to be done to evaluate it. For one sample, the CPV was positive tested by CPV Ag Test Kit, but negative by the

multiplex PCR/RT-PCR. The result is false positive which has proved by virus isolation.

At present, it is difficult to diagnose diseases caused by mixed infections of several viruses in dogs, especially when the clinical symptoms of each infection are similar. In this study, we established a triplex PCR/RT-PCR method to simultaneously detect CDV, CPV and CaKoV that is not only highly specific and sensitive, but simplifies the operation procedure. This method also avoids the waste of materials, and reduces the false positive rate caused by contamination [19]. The assay amplified fragments of CDV, CPV and CaKoV with detection limits of $10^{2.1}$ $TCID_{50}$, $10^{1.9}$ $TCID_{50}$ and 10^3 copies, respectively, and did not detect CAdV-1, CAdV-2, CPIV or CCoV, which has high sensitivity and specificity. For the clinical detection, the one-step triplex PCR/RT-PCR and simplex assays showed the similar results. Therefore, the one-step triplex PCR/RT-PCR assay developed here provides a sensitive, rapid, and simple tool for the detection of viruses infecting dogs with diarrheal diseases, and has broad applications in laboratory and clinical diagnostic practices.

CONFLICT OF INTEREST. Authors have no conflict of interest to declare.

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