Archives of Virology (2018) 163:3345–3349
https://doi.org/10.1007/s00705-018-3982-8

BRIEF REPORT

A fast and simple one-step duplex PCR assay for canine distemper virus (CDV) and canine coronavirus (CCoV) detection

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Received: 15 November 2017 / Accepted: 8 June 2018 / Published online: 23 August 2018

Abstract

The one-step polymerase chain reaction (one-step PCR) detection assay is an innovative PCR detection method, eliminating nucleic acid extraction steps, in which samples can be directly added to PCR reagents for testing. For simultaneous detection of CDV and CCoV, a sensitive and specific one-step duplex PCR (one-step dPCR) assay was developed with two pairs of primers that were designed based on *H* and *M* gene sequences of CDV and CCoV, respectively. The one-step dPCR with optimized detection conditions has high specificity and sensitivity; independent sequencing assays further verified these results.

Introduction

Canine distemper virus (CDV) is the etiological agent of a serious and often fatal disease in dogs and many other carnivores. CDV was first isolated in 1905 and for decades was responsible for large numbers of animal deaths worldwide [1]. Although attenuated vaccines considerably reduced mortality rates and have partially controlled the disease in the last years, several outbreaks of CDV are still reported in dogs and wildlife hosts [2, 3]. CDV is an enveloped virus with a single-stranded negative-sense RNA genome, encoding six structural and two nonstructural proteins. Two surface glycoproteins, namely the Haemagglutinin (H) and Fusion protein (F), play key roles in virus attachment and entry into host cells; in addition they are the main targets for the immune response as well. The glycosylated H protein mediates receptor binding and is much more variable than other CDV proteins, which renders it a suitable target for investigating CDV genetic/antigenic diversity [4]. The analysis of CDV strains has revealed pronounced genetic diversity of the *H* gene, with eleven main geographically-distinct lineages (genotypes) described [4, 5].

Coronaviruses are single-stranded RNA viruses which infect humans and a variety of nonprimate mammals, including canines [6]. Canine coronavirus (CCoV) was first recognized as an enteric pathogen of dogs in 1971 [7]. CCoV is a common pathogen of dog populations, particularly those housed in large groups [8–10]. In some cases, CCoV infection can be fatal in young dogs, in particular when co-infected with CDV or other intestinal pathogens [11]. In recent years, an increasing number of highly virulent CCoV infections have also been documented in Europe [12–14]. CCoV particles are composed of four major structural proteins termed spike (S), envelope (E), membrane (M) and nucleocapsid (N) [15]. The M protein, the most abundant structural component, is a type III glycoprotein that induces antibody-dependent, complement-mediated, viral neutralization [16, 17].

CDV and CCoV have similar clinical symptoms, which makes clinical diagnosis difficult. At present, diagnosis of CDV and CCoV relies mainly on virus isolation and

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serological tests, but these methods either have a low level of sensitivity and specificity, a long diagnostic period [3], or depend on expensive imported reagents. Therefore, it is urgent to establish a rapid, simple and accurate method to rapidly detect both CDV and CCoV allowing early diagnosis, epidemic monitoring and epidemiological investigation of canine diarrhea. The objective of this study was to establish a new one-step dPCR assay for CDV and CCoV diagnosis in surveillance studies. This assay has been evaluated using samples from canine sera, in order to demonstrate its value for detecting CDV and CCoV in an animal hospital.

It is necessary to establish rapid early diagnostic methods to allow effective control measures to be implemented as soon as possible and reduce the harm caused by these diseases to the dog industry.

The published sequences of conserved regions within the *H* genes of CDV and the *M* genes of CCoV were obtained from GenBank (GenBank accession numbers JN381191 and AY436635, respectively). One-step dPCR primers were designed with Primer Premier 5.0 software. Primers were designed to amplify a 760-bp amplicon from the CDV *H* gene and a 540-bp amplicon from the CCoV *M* gene. All primers were synthesized by BGI (Beijing, China). The primer sequences are listed in Table 1.

RNA was extracted with a RNA simple Total RNA kit (Beijing Tiangen Biotech Company, Beijing, China) in accordance with the manufacturer's instructions. DNA was extracted with a TIANamp Virus genomic DNA/RNA kit (Beijing Tiangen Biotech Company, Beijing, China). cDNA synthesis was performed using the TranScript Firststrand cDNA Synthesis SuperMix (Beijing TransGen Biotech Company, Beijing, China). DNA and cDNA were stored at -20 °C.

cDNA representing CDV and CCoV was used as the template for PCR amplification, and 760 bp (CDV-H) and 540 bp (CCoV-M) fragments were obtained, consistent with the size of the expected gene fragment. The obtained fragments were inserted into the vector pMD18-T (TaKaRa Biotechnology Company, Dalian, China) as standards. The recombinant plasmids pMD18-T-CDV-H and pMD18-T-CCoV-M were amplified in *E. coli* DH5α, and the recombinant plasmids were purified with the QIAprep Spin Miniprep Kit (Cat No. /ID: 27106). The plasmids were verified by both PCR and restriction enzyme digestion analyses. Plasmids were also verified by DNA sequencing (data not shown).

The conventional duplex PCR assay was performed in a 25 μL system consisting of: 1.0 μL of each of the recombinant plasmids pMD18-T-CDV-H and pMD18-T-CCoV-M, respectively; 1.0 μL each of primers DP1 and DP2, as well as CP1 and CP2; 2.0 μL of dNTPs (10 mM); 1.0 μL of KOD FX Neo (1U/μL) (TOYOBO Biotechnology Company, Shanghai, China); 12.5 μL of 2 × PCR buffer for KOD FX Neo (TOYOBO Biotechnology Company, Shanghai, China); and ddH_2O up to 25 μL. PCR reaction conditions were as follows: 94 °C for 5 min followed by 30 cycles of 94 °C for 40 s, 55 °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.

The one-step dPCR assay is based on the conventional dPCR assay. Experiments were performed to optimize the template concentration, amplification temperature and primer volume for the one-step dPCR assay. The one-step dPCR assay was performed in a 25 μL system. Each of the template plasmids (pMD18-T-CDV-H and pMD18-T-CCoV-M) was tested at volumes ranging from 0.2 μL to 1.4 μL in increments of 0.2 μL. Each pair of primers (DP1 and DP2, CP1 and CP2 at 10 μM) was tested at volumes ranging from 0.4 μL to 1.2 μL in increments of 0.2 μL. The annealing temperature ranged from 52 °C to 60 °C. Amplified products were analyzed on 1.5% agarose gels. Based on the optimized conditions, the one-step duplex PCR assay was conducted in a 25 μL reaction mixture that included: 0.8 μL of the CDV and CCoV extracted cDNA and standard plasmid; 12.5 μL PCR reaction mix (Coyote Bioscience Co., Ltd), 1.0 μL DNA polymerase mix (Coyote Bioscience Co., Ltd), 0.8 μL each of primers DP1 and DP2 (10 μM), and CP1 and CP2 (10 μM); with ddH_2O up to 25 μL. PCR reaction conditions were: 94 °C for 5 min; 30 cycle of 94 °C for 40 s, 56 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min.

DNA or cDNA of CDV, CCoV, canine parvovirus (CPV), infectious canine hepatitis virus (ICHV), canine parainfluenza virus (CPIV), canine herpesvirus (CHV) were separately subjected to the one-step dPCR and the conventional duplex PCR. The one-step dPCR could not amplify target gene fragments without target DNA or cDNA as template (Fig. 1). Amplification did not occur when CPV, CPIV, CHV, ICHV, or *E. coli* were used as templates. These results indicated that these assays were highly specific.

Table 1 Specific primers used to amplify target genes

Virus and genes	GenBank accession No.	Primer name	Sequences (5' → 3')	Fragment size (bp)
CDV-H	JN381191	DP1: DP2:	GCAACACCTGTGGATCAAGT ATTGGCGACACCACAAATCG	760
CCoV-M	AY436635	CP1: CP2:	ATATGTAATAATTTTTCATGCTCAC TCGTGTGTGGCATTAATGCTT	540

Fig. 1 The specificity of the one-step dPCR assay. M: DL2000 DNA Marker; 1: CDV, CCoV; 2: CPV; 3: ICHV; 4: CPIV; 5: CHV; 6: *E. coli*; 7: Negative control

To evaluate the sensitivity of the one-step dPCR assay in comparison with that of the conventional PCR, recombinant plasmids and total RNA extracted from infected cells were quantified and serially diluted by tenfold. Each sample dilution was tested as a template using the optimized one-step dPCR assay reaction parameters. The conventional PCR assay was performed as follows: the pMD18-T-CDV-H and pMD18-T-CCoV-M were diluted separately in a 10-fold serial dilution series to achieve plasmid concentrations ranging from 5.52×10^{8} copies/μL to 5.52×10^{0} copies/μL and 6.31×10^{8} copies/μL to 6.31×10^{0} copies/μL. RNA of CDV and CCoV was quantified and diluted separately in a 10-fold serial dilution to achieve RNA concentrations ranging from 1.5×10^{0} μg/mL to 1.5×10^{-5} μg/mL and 2.1×10^{0} μg/mL to 2.1×10^{-5} μg/mL. Diluted recombinant plasmids and RNA were tested by the optimized one-step dPCR assay and the conventional duplex PCR assay using the same system and reaction parameters. For pMD18-T-CDV-H and pMD18-T-CCoV-M, the detection limit of the conventional duplex RT-PCR assay and the one-step dPCR assay was 5.52×10^{4} copies/μL and 6.31×10^{4} copies/μL respectively (Fig. 2A, 2B). For RNA of CDV and CCoV, the detection limit of the conventional duplex RT-PCR assay and the one-step dPCR assay were 1.5×10^{-2} μg/mL and 2.1×10^{-2} μg/mL respectively (Fig. 3A, B). These results indicated that the sensitivity of the one-step dPCR assay was equal to the conventional duplex PCR assay.

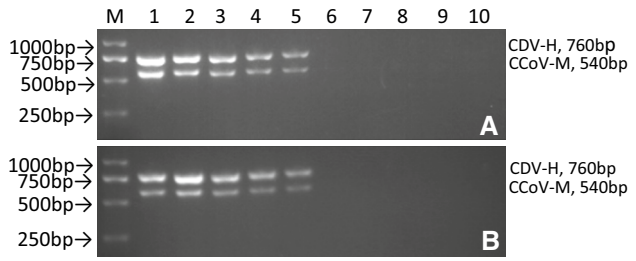

Fig. 2 Sensitivity of the one-step dPCR assay (A) and the conventional dPCR assay (B) using different CDV-H and CCoV-M plasmid dilutions. M: DL2000 marker; 1–9: pMD-18T-CDV-H concentrations ranging from 5.52×10^{8} copies/μL to 5.52×10^{0} copies/μL, pMD-18T-CCoV-M concentrations ranging from 6.31×10^{8} copies/μL to 6.31×10^{0} copies/μL; 10: Negative control

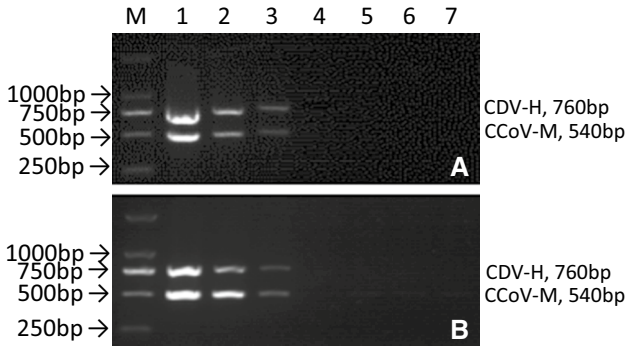

Fig. 3 Sensitivity of the one-step dPCR assay (A) and conventional dPCR assay (B) using different CDV RNA and CCoV RNA dilutions. M: DL2000 marker; 1–6: CDV RNA concentrations ranging from 1.5×10^{0} μg/mL to 1.5×10^{-5} μg/mL, CCoV RNA concentrations ranging from 2.1×10^{0} μg/mL to 2.1×10^{-5} μg/mLL; 7: Negative control

A total of 173 serum samples were collected from dogs suspected of having CDV or CCoV infections from animal hospitals in the following municipalities or provinces in China: Beijing (89 samples), Anhui (44 samples) and Shanxi (40 samples). Serum samples were collected in 2014 or 2015 from dogs with symptoms of fever, coughing, vomiting, diarrhea, ataxia, and paralysis. Samples were homogenized and centrifuged at 3000×*g* for 15 min to obtain a cell-free supernatant, and the supernatant was directly added to the tube as a template for one-step dPCR. An independent sequencing assay was used to evaluate the one-step duplex PCR assay. The results are shown in Table 2. Among the 173 clinical specimens, 23 specimens displayed a single infection with CDV; 20 specimens displayed a single infection with CCoV and 13 specimens displayed co-infection with CDV and CCoV. A prevalence (28.08%) of diarrheic dogs, shown to harbor at least one pathogen by one-step dPCR, was observed in the Beijng samples, which exceeded those in the Anhui (20.45%) and Shanxi (17.5%) samples, as shown in Table 2. At the same time, the samples were analyzed by conventional duplex RT-PCR. The results were consistent with one-step dPCR. This independent assay confirmed the one-step duplex PCR results (100% match), including DNA sequencing verification, indicating a high specificity for the one-step duplex PCR assay. The prevalence of co-infection we observed in diarrheic dogs (10.11%) in Beijing was also higher than those in the other cities tested. Despite the higher prevalence of entero-pathogens and co-infections in Beijing, the rates in the other countries are also relevant, indicating that infectious diarrhea may be a national phenomenon rather than a phenomenon specific to a particular city.

Previous studies have demonstrated several diagnostic methods, including reverse transcription polymerase chain reaction (RT-PCR) and multiplex PCR. Moreover,

Table 2 One-step dPCR testing diarrheic dogs from Beijing, Anhui and Shanxi in China

Infectious agent	Prevalence					
	Beijing		Anhui		Shanxi	
	Test by one-step dPCR	Test by dRT-PCR	Test by one-step dPCR	Test by dRT-PCR	Test by one-step dPCR	Test by dRT-PCR
CDV infection rate	14/89(15.73%)	14/89(15.73%)	5/44(11.36%)	5/44(11.36%)	4/40(10%)	4/40(10%)
CCoV infection rate	11/89(12.35%)	11/89(12.35%)	6/44(13.63%)	6/44(13.63%)	3/40(7.5%)	3/40(7.5%)
Overall infection rate	25/89(28.08%)	24/89(26.96%)	9/44(20.45%)	9/44(20.45%)	7/40(17.5%)	7/40(17.5%)
Coinfection rate	9/89(10.11%)	9/89(10.11%)	3/44(6.81%)	3/44(6.81%)	1/40(2.5%)	1/40(2.5%)
Sample included	n = 89		n = 44		n = 40	

real-time quantitative PCR is currently available for the diagnosis of CDV or CCoV. However, it is rarely reported to detect CDV and CCoV, simultaneously. The occurrence of false-positive PCR products for each of these methods hinders the identification of wild-type CDV strains [18]. Real-time quantitative PCR methods have also been reported to detect CDV or CCoV. The advantages of real-time PCR, compared with conventional RT-PCR, include a higher speed, greater sensitivity, and less handling of PCR products. However, real-time PCR instruments are expensive and may not be readily available in many laboratories [19]. Other methods such as serological assays (indirect immunofluorescence assay and enzyme-linked immunosorbent assay), virus isolation and in situ hybridization have also been developed, but are not applicable for the fast and sensitive diagnosis of CDV or CCoV [20–22]. The one-step PCR detection assay is an innovative PCR detection method, eliminating the nucleic acid extraction step, with the samples being directly added into the PCR reagents for testing.

To the best of our knowledge, this is the first one-step duplex PCR-based detection assay that can simultaneously identify CDV and CCoV pathogens which has been performed in China. Our results indicate that this specific and sensitive method can identify CDV and CCoV even at low copy number, and in complicated samples such as sera, rendering it feasible as a tool to monitor in vivo CDV and CCoV replication status. Hence, our research has paved the way for studies of CDV and CCoV infection and proliferation, as well as the pathogenesis and prevention of CDV and CCoV.

Availability of data and materials

In this study, we used the one-step PCR kit which was purchased from Coyote Bioscience Company. The website link is: http://www.coyotebio.com/?productshow2/cid/14/id/47.html.

Acknowledgements This work was partly supported by the Agricultural Science and Technology Innovation Program of China (ASTIP-IAS15) and the National Key Research and Development Program of China (Nos. 2016YFD0501003, 2017YFD0502300 and 2017YFD0501603).

Compliance with ethical standards

Conflict of interest The authors declare that they had no conflict of interest.

Ethical approval This article does not contain any studies with human participants by any of the authors.

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