Journal of Virological Methods 260 (2018) 56–61

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Evaluation of an incubation instrument-free reverse transcription recombinase polymerase amplification assay for rapid and point-of-need detection of canine distemper virus

Jianchang Wang[a,c,1], Jinfeng Wang[a,c,1], Ruiwen Li[b], Ruihan Shi[a,c], Libing Liu[a,c], Wanzhe Yuan[b,*]

[a] Center of Inspection and Quarantine, Hebei Entry-Exit Inspection and Quarantine Bureau, Shijiazhuang 050051, China
[b] College of Veterinary Medicine, Agricultural University of Hebei, Baoding 071001, China
[c] Hebei Academy of Science and Technology for Inspection and Quarantine, Shijiazhuang 050051, China

ARTICLE INFO

Keywords:
CDV
N gene
LF probe
LFS
RT-RPA

ABSTRACT

Canine distemper, caused by Canine distemper virus (CDV), is a highly contagious and fatal systemic disease in free-living and captive carnivores worldwide. Accurate, rapid and simple detection of CDV is critical to improve disease management and prevent outbreaks. In this study, a visible and incubation instrument-free reverse-transcription recombinase polymerase amplification assay combined with lateral flow strip (LFS RT-RPA) was developed to detect CDV using primers and lateral flow (LF) probe specific for the nucleocapsid (N) protein gene. The CDV LFS RT-RPA assay was performed in a closed fist using body heat for 15 min, and the products were visible to the naked eyes on the LFS within 5 min. The assay could detect CDV, and there was no cross-reaction with the other viruses tested. Using the in vitro transcribed CDV RNA as template, the analytical sensitivity was 9.4×10^1 copies per reaction, which was the same result as that of a real-time RT-PCR. The assay performance was further evaluated by testing 32 nasal/oropharyngeal swab samples, and CDV RNA positive rate was 62.0% (20/32) by LFS RT-RPA, which was the same result as that of the real-time RT-PCR assay. The performance of the LFS RT-RPA was comparable to real-time RT-PCR, while the LFS RT-RPA assay was much faster and easier to perform. The novel CDV LFS RT-RPA assay provides an attractive and promising tool for rapid and reliable detection of CDV in the underequipped laboratory and point-of-need facility, which is of great significance in CD control in low resource settings.

1. Introduction

Canine distemper, caused by canine distemper virus (CDV), is a highly contagious and fatal systemic disease found worldwide not only in dogs and many other carnivores but also in some non-carnivores (Wilkes et al., 2014). CDV is a non-segmented, negative-stranded, enveloped RNA virus that belongs to the family *Paramyxoviridae* and the genus *Morbillivirus*, and is one of the most lethal infectious agents in both susceptible free-living and captive carnivores (Lednicky et al., 2004). CDV-infected dogs may develop respiratory, gastrointestinal, dermatologic, ophthalmic or neurological disorders that appear simultaneously or sequentially (Beineke et al., 2009; Decaro et al., 2004; Tan et al., 2011). The broad spectrum of clinical signs, not dissimilar from the signs observed in other respiratory and enteric diseases of dogs, hampers accurate and early clinical diagnosis of canine distemper (Seki et al., 2003). Therefore, rapid and accurate diagnosis of CDV infection would enable veterinarians to implement appropriate strategies in time to improve disease management and prevent outbreaks, particularly within a shelter environment (Elia et al., 2015).

A substantial number of assays based on viral nucleic acid detection have been described for CD diagnosis with a varying degree of sensitivity and specificity, such as reverse transcription polymerase chain reaction (RT-PCR) (Frisk et al., 1999), nested RT-PCR (Shin et al., 2004), real-time RT-PCR (Elia et al., 2006), reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Cho and Park, 2005) and reverse transcription insulated isothermal PCR (RT-iiPCR) (Wilkes et al., 2014). However, the RT-PCR assays are cold chain dependent and require relatively sophisticated equipment with experienced technicians, making these assays unsuitable for being used in underequipped laboratory and in field (Elia et al., 2006; Frisk et al., 1999; Shin et al., 2004). Compared to the current RT-PCR assays, the use of isothermal technologies reduces the need for high precision instrumentation and

* Corresponding author at: College of Veterinary Medicine, Agricultural University of Hebei, No.38 Lingyusi Street, Baoding, Hebei 071001, China.
E-mail address: yuanwanzhe2015@126.com (W. Yuan).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jviromet.2018.07.007
Received 7 February 2018; Received in revised form 12 July 2018; Accepted 12 July 2018

Table 1
Sequence of primers and probes for CDV RT-PCR, real-time RT-PCR and LFS RT-RPA assays used in the study.

Assay	Primers and probe	Sequence 5′-3′	Amplicon size (bp)	References
RT-PCR	H-F	TTAGGGCTCAGGTAGTCCA	1879	Zhao et al., 2010
	H-R	CTAAGKCCAATTGARATGTGT		
real-time RT-PCR	CDV-F	AGCTAGTTTCATCTTAACTATCAAATT	87	Elia et al., 2006
	CDV-R	TTAACTCTCCAGAAAACTCATGC		
	CDV-P	FAM-ACCCAAGAGCCGGATACATAGTTTCAATGC-BHQ1		
LFS RT-RPA	CDV-LF-F	GCTTACTTCAGACTCGGGCAAGAAATGGTTA	154	This study
	CDV-LF-R	Biotin-CAGTAGCTCGAATTGTCCGGTCCTCTGTTGT		
	CDV-LF-P	FAM-CTTGGCATCACCAAGGAGGAAGCTCAGCTGGT(THF) TCAGAAATAGCATCCA-C3-spacer		

consistent electrical power. The results are usually produced within 60 min for the above RT-LAMP and RT-iiPCR assays, and depend on the water bath or specialized instrument, POCKIT™ Nucleic Acid Analyzer (Cho and Park, 2005; Wilkes et al., 2014). A simple, rapid, accurate and user-friendly detection platform is still needed for reliable detection of CDV in the laboratories without access to real-time PCR instrumentation and at the point-of-need (PON) diagnosis.

Recombinase polymerase amplification (RPA) is an isothermal DNA amplification technique that has been demonstrated to be rapid, specific, sensitive, and cost-effective (Daher et al., 2016; Piepenburg et al., 2006). The RPA reaction uses enzymes called recombinases that form complexes with oligonucleotide primers and pair the primers with homologous sequences in DNA. A single-stranded DNA binding protein binds to the displaced DNA strand and stabilizes the resulting loop. The primer then initiates DNA amplification by a strand-displacing DNA polymerase (Piepenburg et al., 2006). Real-time detection of the RPA amplification mainly depends on the Exonuclease III and exo probe, while the direct visual detection of the RPA products depends on the Endonuclease IV, LF probe and the opposing amplification primer labeled at 5' end with a biotin. The LF probe oligonucleotide backbone includes a 5′-antigenic label (typically a FAM group), an internal abasic nucleotide analogue (a tetrahydrofuran residue or THF) and a 3′-polymerase extension blocking group (such as a C3-spacer). The amplicons are then detected by naked eyes in the 'sandwich' assay formats, such as a lateral flow strip (LFS) that contains anti-FAM gold conjugates and biotin-ligand molecules. Our laboratory had developed a real-time RT-RPA assay based on exo probe for real-time detection of CDV while the assay still depended on the specialized instrument, Genie III (OptiGene, West Sussex, UK) (Wang et al., 2017a). Series of LFS RPA assays had been developed for the detection of porcine parvovirus (PPV), peste des petits ruminants virus (PPRV) and bovine ephemeral fever virus (BEFV) (Hou et al., 2017; Yang et al., 2017, 2016).

In this study, we developed an incubation instrument-free RPA assay for rapid, specific and sensitive detection of CDV, which was combined with LFS (USTAR, Hangzhou, China) and performed by incubating the reaction tubes in a closed fist using body heat.

2. Material and methods

2.1. Virus strains and clinical samples

Canine distemper virus (CDV-FOX-TA strain, genotype: America-2), canine parvovirus type 2 (CPV-2, CPV-b114 strain), canine coronavirus (CCoV, ATCC VR-809 strain), canine parainfluenza virus (CPIV, CPIV/A-20/8 strain), and pseudorabies virus (PRV, Barth-K61 strain) were maintained in our laboratory. Thirty-two nasal/oropharyngeal swabs were collected from 20 dogs of both sexes (various breeds and ages) from the animal hospital of the Agricultural University of Hebei and 12 raccoon dogs from the farms in Hebei Province, China from 2014 to 2016 and snap-frozen for storage at −80 °C. All the dogs and raccoon dogs clinically were suspected of being CDV infected. Fifteen samples from the dogs and 5 samples from the raccoon dogs had been tested to be CDV positive with the Ct values ranging from 16.36 to 37.03, and the other 12 samples were CDV negative by a real-time RT-PCR (Elia et al., 2006).

2.2. DNA/RNA extraction

CDV, CCoV, and CPIV viral RNA was extracted using Trizol Reagent (Invitrogen, Waltham, USA), CPV-2 and PRV viral DNA was extracted using the TIANamp Virus DNA kit (Tiangen, Beijing, China), which were performed according to manufacturer's instructions, respectively. For viral RNA extraction from the nasal/oropharyngeal swabs, the swab was inoculated and vortexed in 1 mL sterile phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 10,000 rpm for 10 min at 4 °C. Two hundreds microliter of the supernatant was collected for viral RNA extraction using the Trizol Reagent, and the RNA was finally eluted in 20 μL of nuclease-free water. All RNA and DNA were quantified using ND-2000c and stored at −80 °C until use.

2.3. Generation of in vitro transcribed CDV RNA

The in vitro transcribed CDV RNA, which covers the nucleocapsid protein gene of CDV, was generated as described previously and diluted in ten-fold series to obtain RNA concentrations ranging from 9.4×10^4 to 9.4×10^{-1} copies/μL (Wang et al., 2017a).

2.4. RPA primers and LF probe

Nucleotide sequences of different CDV genotypes (accession numbers: AB490678, AF164967, AY386316, GU138403, HQ540292, KF856711, KF914669) available in GenBank were aligned to identify regions that are highly conserved in the N gene, and the primers and LF probe were designed, which were listed in Table 1 and synthesized by a commercial company (Sangon Biotech Co., Shanghai, China).

2.5. LFS RT-RPA

LFS RT-RPA reactions were performed in a 50 μL volume using a TwistAmp™ nfo kit (TwistDX, Cambridge, UK). Other components included 420 nM each RPA primer, 120 nM LF probe, 14 mM magnesium acetate, 200 U MMLV reverse transcriptase (Takara, Dalian, China), 40 U Recombinant RNase Inhibitor (Takara, Dalian, China) and 1 μL of viral or sample RNA. All reagents except for the viral template and magnesium acetate were prepared in a master mix, which was distributed into each 0.2 mL freeze-dried reaction tube containing a dried enzyme pellet. One microliter of viral RNA was added to the tubes. Subsequently, magnesium acetate was pipetted into the tube lids, which were closed carefully, and the magnesium acetate was centrifuged into the rehydrated material using a minispin centrifuge. The sample was vortexed briefly and spun down once again, and the reaction tubes were immediately incubated in the different technician's closed fist at room temperature. The RPA was performed using the body heat for 5, 10, 15 and 20 min, and an LFS was used to detect the amplicons that were dual-labeled with FAM and biotin. The LFS contains gold particle-conjugated anti-FAM antibody (rabbit polyclonal antibody) in the