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Short communication

Establishment of reverse transcription loop-mediated isothermal amplification for rapid detection and differentiation of canine distemper virus infected and vaccinated animals

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ABSTRACT

Although widespread vaccination against canine distemper virus (CDV) has been conducted for many decades, several canine distemper outbreaks in vaccinated animals have been reported frequently. In order to detect and differentiate the wild-type and vaccine strains of the CDV from the vaccinated animals, a novel reverse transcription loop-mediated isothermal amplification (RT-LAMP) method was developed. A set of four primers—two internal and two external—were designed to target the H gene for the specific detection of wild-type CDV variants. The CDV-H RT-LAMP assay rapidly amplified the target gene, within 60 min, using a water bath held at a constant temperature of 65 °C. The assay was 100-fold more sensitive than conventional RT-PCR, with a detection limit of 10^{-1} $TCID_{50}$ ml^{-1}. The system showed a preference for wild-type CDV, and exhibited less sensitivity to canine parvovirus, canine adenovirus type 1 and type 2, canine coronavirus, and canine parainfluenza virus. The assay was validated using 102 clinical samples obtained from vaccinated dog farms, and the results were comparable to a multiplex nested RT-PCR assay. The specific CDV-H RT-LAMP assay provides a simple, rapid, and sensitive tool for the detection of canines infected with wild-type CDV from canines vaccinated with attenuated vaccine.

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1. Introduction

Canine distemper (CD) is a highly contagious and fatal disease of dogs and other terrestrial carnivores. CD is caused by the canine distemper virus (CDV), a single-stranded negative RNA virus of the genus *Morbillivirus*, family *Paramyxoviridae*, and is closely related to the viruses responsible for measles, rinderpest, peste des petits ruminants, phocine distemper, dolphin distemper, porpoise distemper, and equine morbillivirus (Fenner, 1976; Haas and Barrett, 1996; McCarthy et al., 2011; Soltan and Abd-Eldaim, 2014; Bellière et al., 2011).

It has been widely documented that CDV causes severe immunosuppression and persistent infection in the central nervous system in dogs (Müller et al., 1995; Wu et al., 2000; Meertens et al., 2003; Vandevelde and Zurbriggen, 2005). Moreover, the host spectrum of CDV is gradually increasing (Qiu et al., 2011). Currently, vaccination is the most effective means of controlling CD and one of the most frequently used vaccines is a modified live attenuated vaccine. Although widespread vaccination against CD has been conducted for many decades, this infection still represents an important disease for dogs (Elia et al., 2006). There is, therefore, a need for the development of assays that can distinguish dogs vaccinated with modified live attenuated vaccine from those infected with wild-type virus in herds.

Rapid and sensitive detection methods, including the reverse transcription polymerase chain reaction (RT-PCR) and multiplex PCR, are currently available for the diagnosis of CDV. However, the occurrence of false-positive PCR products for each of these methods hinders the identification of wild-type CDV strains (Ralston et al., 2007). Real-time quantitative PCR methods have

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also been reported to detect CDV. The advantages of real-time PCR, compared with conventional RT-PCR, include 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 (Coleman et al., 2014). Other methods such as serological assays (Indirect immunofluorescence assay (IFA), 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 (Gray et al., 2012; Gaedke et al., 1997; Rzezutka and Mizak, 2002; Frisk et al., 1999; von Messling et al., 1999).

Loop-mediated isothermal amplification (LAMP) is a novel method for the rapid, specific, and sensitive amplification of nucleic acids (Notomi et al., 2000). With its advantages, it has been applied widely in the detection of pathogenic microorganisms (Dinh et al., 2011; Li and Cai, 2011; Njiru et al., 2012) and genetically modified foods (Fukuta et al., 2004), analysis of shelf-life in melon (Fukuta et al., 2006) and chromosomal chimerism of bovine (Hirayama et al., 2007).

Rapid and cost-effective RT-LAMP assays for the pre-clinical detection of CDV have been described elsewhere (Cho and Park, 2005; Liu et al., 2010; Wen et al., 2012; Hu et al., 2012). As these assays target both wild-type CDV variants and the live attenuated vaccine strain, the interpretation of the results may be complicated in terms of eradication and control where a vaccination policy is practiced.

This study aimed to establish a RT-LAMP method which was specific for wild-type CDV, could differentiate from canines vaccinated with attenuated CDV vaccine, and which could be applied with relative ease in minimally-equipped laboratories and under field conditions.

2. Materials and methods

2.1. Viruses and field samples

CDV strains: wild-type CDV YB strain (Asia-1 subgroup), LDH (06) strain (Asia-1 subgroup), and ZH (05) strain (Asia-2 subgroup) were isolated by using Madin–Darby Canine Kidney expressed SLAM of dog (MDCK-SLAM) cells, and were identified and maintained at the Harbin Veterinary Research Institute (Harbin, China). Three locally supplied CDV vaccine products Vacc-A (Intervet, Netherlands: Onderstepoort strain), Vacc-B (Pfizer, USA: Snyder Hill strain) and Vacc-C (Merial, France: RECOMBITEK C4) were tested in the current study. All other non-CDV canine viruses: canine parvovirus (CPV), canine adenovirus type 1 and type 2 (CAV-1 and CAV-2), canine coronavirus (CCoV), and canine parainfluenza virus (CPIV) were provided by the Harbin Veterinary Research Institute (Harbin, China). Field samples ($n = 102$; including 37 rectal swabs, 20 conjunctival swabs, 15 urine samples, and 30 whole blood samples) (Table 1) were collected from different farms across several provinces of China where vaccination with live attenuated vaccine was in operation.

2.2. Ethical considerations

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. Our sampling processes were assisted by local authorities and veterinarians. All the owners of the dogs gave permission for their animals' samples to be used in this study.

Table 1
The information of the clinical samples collected from domestic dogs in 2012.

Location (Province)	Specimens	Numbers	Clinical signs
Heilongjiang	Rectal swabs	15	Fever, sneeze, depression, cough, diarrhea or bloody stool
	Conjunctival swabs	13	
	Urine	5	
	Whole blood	13	
Jilin	Rectal swabs	10	Spasm, fever, depression, sneeze and cough
	Urine	4	
	Whole blood	10	
Liaoning	Rectal swabs	12	Depression and diarrhea
	Whole blood	7	
Shandong	Conjunctival swabs	7	Sneeze, cough, spasm and fever
	Urine samples	6	

2.3. Primer design

Genomic sequences of N and H gene were aligned using the MegAlign program of DNAStar software (DNAStar, Madison, WI, USA). With online LAMP designing software PrimerExplorer V4 (http://primerexplorer.jp/e/), two sets primers (F3-N and B3-N, FIP-N and BIP-N) and (F3-H and B3-H, FIP-H and BIP-H) were designed based on the wild-type CDV YB strain (Table 2). The former was common for all types of CDV strains; the latter was conserved among wild-type CDV strains but not for the live attenuated CDV vaccine strains.

2.4. RNA extraction and cDNA synthesis

Viral RNA was extracted using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed in a final reaction volume of 20 μl containing: 10 μl RNA, 4 μl 5× RT Buffer, 1 μl dNTP (10 mM each), 50 pmol 9-Random Hexamers, 10 U M-MLV reverse transcriptase (Takara, Dalian, China), and 20 U RNase inhibitor (Takara, Dalian, China). The reaction was incubated at42 °C for 1 h followed by incubation at 70 °C for 10 min.

2.5. The universal CDV-N RT-LAMP assay for detection of CDV

The CDV-N RT-LAMP assay was performed in a total volume of 25 μl consisting of: 0.3 μM F3-N and B3-N primers, 2.4 μM FIP-N and BIP-N primers, 1.4 mM dNTPs, 2.5 μl 1× Thermo Pol buffer, 8 U*Bst* DNA polymerase (New England BioLabs, Herts, UK), and 2 μl cDNA. The reaction mixture was incubatedin a water bath for 45–50 min at 65 °C prior to incubation at 80 °C for 2 min. 6 μl of the LAMP products were subjected to electrophoresis on a 2%(w/v) agarose gel.

2.6. The specific CDV-H RT-LAMP assay for detection of wild-type CDV

The CDV-H RT-LAMP assay was performed in a total volume of 25 μl consisting of: 0.2 μM F3-H and B3-H primers, 1.2 μM FIP-H and BIP-H primers, 1.4 mM dNTPs, 2.5 μl 1× Thermo Pol buffer, 12 U *Bst* DNA polymerase (New England BioLabs, Herts, UK) and 2 μl cDNA. The reaction condition was same as above.

2.7. Evaluation of the specificity, sensitivity and reproducibility of CDV-H RT-LAMP assay

Specificity of the CDV-H RT-LAMP assay for wild-type CDV was assessed against CDV vaccine strains (Vacc-A, Vacc-B and Vacc-C),

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