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## Short Communication

Department of Veterinary Pathology, College of Veterinary Medicine, Chonnam National University, Gwangju, Korea

# Detection of Canine Distemper Virus in Blood Samples by Reverse Transcription Loop-Mediated Isothermal Amplification

H. S. Cho[1] and N. Y. Park[1,2]

Address of authors: [1]Department of Veterinary Pathology, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea; [2]Corresponding author: Tel.: +82 62 530 2843; fax: +82 62 530 2847;
E-mail: nypark@chonnam.ac.kr

With 2 figures and 1 table

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### Summary

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) was used to detect canine distemper virus (CDV) genomic RNA. A set of four primers, two outer and two inner, were designed from CDV genomic RNA targeting the nucleocapsid protein gene. The optimal reaction time and temperature for LAMP were determined to be 60 min at 65°C. The relative sensitivity and specificity of RT-LAMP was found to be 100% and 93.3%, respectively, based on 50 canine blood samples and using RT-PCR as the gold standard. The detection limit of the RT-LAMP method was 100 times lower than with RT-PCR (10-1TCID50 $ml^{-1}$ versus 10TCID50 $ml^{-1}$). In addition to the advantage resulting from the visual detection of the end-product, the LAMP method is fast, requiring only 1 h to complete the assay. The LAMP method is a viable alternative to RT-PCR for diagnosing CDV infection in dogs. The LAMP method might be useful as an on site diagnostic assay for detecting CDV.

### Introduction

Canine distemper virus (CDV) is a member of the family Paramyxoviridae, genus *Morbillivirus,* and consists of a negative sense single-stranded RNA genome about 15 690 nucleotides in length encoding six structural proteins: nucleocapsid protein (NP), phosphoprotein, matrix protein, fusion protein, haemagglutinin protein, and large polymerase protein. The CDV causes canine distemper (CD), which is an important viral disease of dogs that has a high morbidity and mortality in unvaccinated populations. It is a highly contagious acute or subacute, febrile disease that has been known since 1760 (Murphy et al., 1999).

A clinical diagnosis of CD is difficult because the main clinical signs (nasal discharge and diarrhoea) are similar to other respiratory and enteric diseases (Jones et al., 1997). Currently, CD is a tentative diagnosis for dogs showing clinical signs of nervous system disease or chronic antibiotic-unresponsive respiratory disease. The more specific clinical signs of CD such as convulsions, lack of coordination and myoclonus occur more often in the later stages of infection after CDV infection has been established in the central nervous system (Moro et al., 2003). Furthermore, the variable clinical presentations of CD make it a difficult disease to diagnose. Therefore, an early diagnosis of the infection is important for quarantining infected dogs and providing an appropriate treatment. For this reason, a sensitive, specific and rapid method is needed to detect a small quantity of the virus early in an infection.

Several techniques have been used to detect CDV antigen or RNA (Potgieter and Ajidagba, 1989; von Mall et al., 1995; Gemma et al., 1996; Cho et al., 1999; Shin et al., 2004). However, the majority of these are laborious and time-consuming, can give false reactions or are unsuitable for antemortem diagnosis.

The reverse transcription-polymerase chain reaction (RT-PCR) has been widely applied for making a clinical CD diagnosis on account of its sensitivity and specificity (Shin et al., 2004). This assay is also rapid, requiring only 4 h to detect the viral nucleic acid. However, it can only be performed in a diagnostic or commercial laboratory with access to specialized equipment that is not generally available to veterinary clinics. A new technique, reverse transcription loop-mediated isothermal amplification (RT-LAMP), is an auto-cycling strand displacement DNA synthesis carried out using a reverse transcriptase and DNA polymerase with a high strand displacement activity and a set of specific primers that can recognize a total of six distinct sequences in the target cDNA (Notomi et al., 2000; Hong et al., 2004). This technique amplifies the target cDNA sequence with a high selectivity. The assay can also be carried out within 1 h, has a high sensitivity and produces an end-product that is visible to the naked eye (Notomi et al., 2000; Mori et al., 2001).

The aim of this study was to adapt the RT-LAMP technique for detecting CDV RNA in blood samples of dogs. A set of highly specific primers were designed and used to detect CDV using RT-LAMP, and these results were compared with those obtained using a conventional RT-PCR assay currently used to detect CDV nucleic acid in blood samples in clinically ill dogs.

### Materials and Methods

Blood samples were obtained from 50 dogs of both sexes (various breeds and ages) clinically suspected of being CDV-infected between 2003 and 2004. The blood was collected into

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an EDTA treated bottle and 400 $\mu$l was used to extract the viral RNA. The samples were stored at −70°C until used.

The RNA was extracted from the whole blood by TRIreagent® (Molecular Research Center, Cincinatti, OH, USA) according to the manufacturer's instruction. The RNA was pelleted by centrifugation, washed with 300 $\mu$l of 75% ethanol and dissolved in 10 $\mu$l of RNase-free water. As the control for RT-LAMP and RT-PCR, RNA was also extracted using the TRIreagent® from an attenuated live CDV vaccine strain (Cornell 780916/LP; ChoongAng Vaccine Lab, Co., Daejeon, Korea), canine coronavirus (CCV; American Type Culture Collection VR-809, Manassas, VA, USA) and blood samples from five adult healthy dogs.

The RT-LAMP reaction was performed as previously described (Notomi et al., 2000). One set of four primers (B3, F3, BIP and FIP), which recognized a total of six distinct sequences (B1 to B3 and F1 to F3) on the NP gene of the CDV RNA (GenBank accession No. AF014953), were designed using the PrimerExplorer V3 software (NetLaboratory; Fujitsu, Tokyo, Japan). Figure 1 shows the location and sequence of each primer. The RT-LAMP reaction was carried out in a total reaction mixture volume of 25 $\mu$l with 1 $\mu$l (40 pmol) of each of the primers CDV-BIP and -FIP, 1 $\mu$l (5 pmol) of the primers, CDV-F3 and CDV-B3, 12.5 $\mu$l of 2X reaction mixture [40 mM Tris-HCl, 20 mM KCl, 16 mM MgSO4, 20 mM (NH4)2SO4, 0.2% Tween 20, 1.6 M Betaine (Sigma-Aldrich, St Louis, MO, USA) and 2.8 mM dNTPs each], 1.0 $\mu$l of enzyme mixture [8 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA) and 5 U of AMV reverse transcriptase 0 (Takara Bio, Otsu, Japan)], 1 $\mu$l of template RNA and 7.5 $\mu$l of distilled water. In order to determine the optimal condition for the RT-LAMP assay, the primers and sample mixtures were incubated at 60°C, 63°C and 65°C for 15, 30, 45 and 60 min. At the end of each incubation period, the reaction was quenched by heating the sample at 80°C for 10 min. The reaction temperature was optimized at 65°C, and LAMP was carried out at a predetermined time (60 min). The optimal conditions were determined by separating the above LAMP products (5 $\mu$l) on a 1.5% agarose gels.

The sensitivity and specificity of the RT-LAMP assay were determined by carrying out RT-PCR using the primers designed in a previous study (Shin et al., 2004). RT and PCR were performed in the same tube using AccuPower RT/PCR PreMix (Bioneer, Seoul, Korea). After RT-PCR, 10 $\mu$l of the amplified PCR product were run in 1.5% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light.

A 10-fold diluted CDV-RNA (104TCID50 $ml^{-1}$ to 10–2TCID50 $ml^{-1}$) (Cornell 780916/LP) was used as a template for RT-LAMP and RT-PCR using predetermined conditions (as above). The experiment was performed three times on different days.

The sensitivity and specificity between the two assays in this study were compared. The agreement between the LAMP and the PCR was examined using kappa statistics (Altman, 1999).

## Results

The RT-LAMP and the RT-PCR were standardized with CDV RNA extracted from a tissue culture propagated CDV vaccine strain. The expected 297 bp fragment and ladder-like pattern of the NP gene was successfully amplified by both RT-PCR and RT-LAMP, respectively (Fig. 2). The specificity was confirmed by performing RT-LAMP and RT-PCR on RNA extracted from CCV and blood samples obtained from five adult healthy dogs. CDV-specific bands for RT-PCR and ladder like patterns were not detected.

The RT-LAMP and RT-PCR were carried out to determine the detection limit using 104TCID50 $ml^{-1}$ to 10–2TCID50 $ml^{-1}$ (diluted by 10-fold) of CDV-RNA as template. RT-LAMP could detect CDV-RNA at 10–1TCID50 $ml^{-1}$ (Figs 2a and c), whereas RT-PCR amplification detected CDV-RNA at 10TCID50 $ml^{-1}$ (Fig. 2b), which is 100 times lower than the detection limit of RT-LAMP.

With RT-PCR, 35 of 50 blood samples (70.0%) of clinically CDV-suspected dogs tested positive, whereas 39 of 50 blood samples (78.0%) tested positive by RT-LAMP (Table 1). Thirty-five blood samples (70.0%) tested positive by both RT-PCR and RT-LAMP. Four samples (8.0%) tested positive by RT-LAMP and negative by RT-PCR. No sample that

(a) Location and name of each target sequence as a primer in CDV NP gene

Nucleotide position

601 TATTGCTAGCTTCCATCTTGGCTCAAATTTGGATCCTGCTAGCTAAAGCGGTGACTGCTC
CDV-F3 CDV-F2

661 CTGATACTGCAGCCGACTCGGAGATGAGAAGGTGGATTAAGTATACCCAGCAAAGACGTG
CDV-F1

721 TGGTCGGAGAATTTAGAATGAACAAAATCTGGCTTGATATTGTTAGAAACAGGATTGCTG
CDV-B1 CDV-B2

781 AGGACCTATCTTTGAGGCGATTCATGGTGGCGCTCATCTTGGACATCAAACGATCCCCAG
CDV-B3

(b) Sequence of each primer

| Name of primers | Sequences |
|---|---|
| CDV-BIP | 5'-AAGACGTGTGGTCGGAGAATCCTCAGCAATCCTGTTTCT-3' (B1c-B2) |
| CDV-FIP | 5'-CTTCTCATCTCCGAGTCGGCGATCCTGCTAGCTAAAGCG-3' (F1c-F2) |
| CDV-B3 | 5'-CCATGAATCGCCTCAAAGA-5' |
| CDV-F3 | 5'-GCTTCCATCTTGGCTCAA-5' |

Fig. 1. The locations and names of the target sequences used as primers for CDV RT-LAMP within the NP gene (a). The name and sequence of each primer for CDV RT-LAMP is shown (b). B1c, sequence complementary to B1; F1c, sequence complementary to F1. The sequences of primers were obtained from GenBank A-F014953.

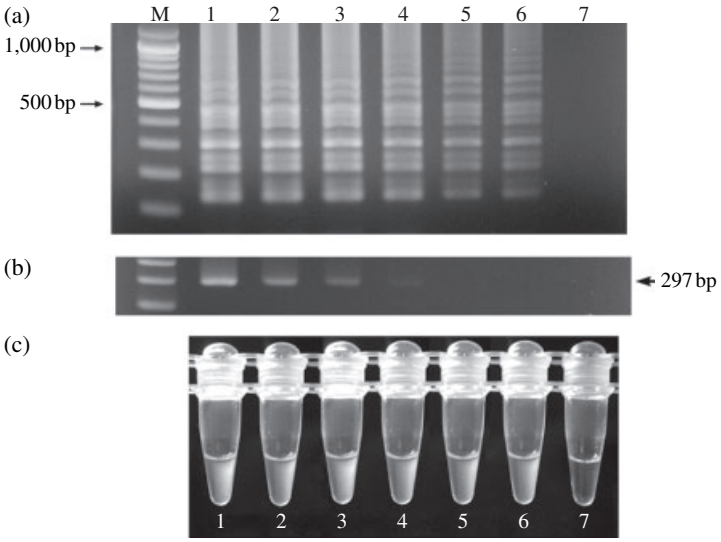


Fig. 2. Comparative sensitivities of RT-LAMP and RT-PCR for the detection of CDV RNA. The amplification by RT-LAMP (a) shows a ladder-like pattern, whereas the RT-PCR (b) shows a 297-bp amplification product, (c) RT-LAMP reaction tubes showing white precipitate of MgPPi when CDV RNA was present. Lanes: M, 100-bp DNA ladder (Bioneer); 1, 104TCID50 $ml^{-1}$; 2, 103TCID50 $ml^{-1}$; 3, 102TCID50 $ml^{-1}$; 4, 101TCID50 $ml^{-1}$; 5, 100TCID50 $ml^{-1}$; 6, 10-1TCID50 $ml^{-1}$; 7, 10-2TCID50 $ml^{-1}$; 8, negative control without target RNA.

Table 1. Comparative evaluation of the sensitivities and specificities of RT-LAMP and RT-PCR for the detection of CDV in blood samples

| | | RT-PCR | | |
|---|---|---|---|---|
| RT-LAMP | | + | − | Total |
| | + | 35 | 4 | 39 |
| | − | 0 | 11 | 11 |
| | Total | 35 | 15 | 50 |

Percent observed agreement = (35 + 11)/50 = 92.0%; sensitivity = 35/35 = 100%; specificity = 11/15 = 73.3%; Kappa = 0.78 (excellent).

tested positive by RT-PCR tested negative by RT-LAMP; and 11 (22.0%) tested negative by both tests. The agreement between the two tests was 92.0% (k = 0.78).

## Discussion

Various detection methods have been developed to diagnose CD (Potgieter and Ajidagba, 1989; von Mall et al., 1995; Gemma et al., 1996; Cho et al., 1999; Frisk et al., 1999; Shin et al., 2004). The sensitivity, specificity and rapidity of RT-PCR compared with conventional methods including electron microscopy, virus isolation, latex agglutination, haemagglutination and ELISA makes it the preferred diagnostic method. However, RT-PCR is technically demanding and requires 4–8 h for a complete diagnosis. In this study, a novel method called RT-LAMP was used to detect CDV-RNA in blood samples in dogs.

The RT-LAMP offers several advantages over PCR for the detection of CDV-RNA in blood of dogs infected with CDV. The whole assay requires only 2 h to complete, and it is a sensitive method that can amplify a few copies of DNA to a magnitude of 109 copies of target in $<1$ h under isothermal conditions (Notomi et al., 2000; Nagamine et al., 2002). The RT-LAMP method used for detecting CDV is highly sensitive, and it detects CDV-RNA template at concentrations as low as 10-1TCID50 $ml^{-1}$. A 100-fold increase in sensitivity was observed with RT-LAMP compared with RT-PCR, where the detection limit was 10TCID50 $ml^{-1}$. Therefore, it is likely that the sensitivity of RT-LAMP is also higher than RT-PCR, at least for cell culture-grown CDV.

The RT-LAMP was carried out using CDV-RNA as template in order to determine the optimal temperature and reaction time. The product was formed at both 63° and 65°C. However, 65°C was used as the optimal temperature because the higher temperature increases the specificity of the detection (data not shown). No amplification of product and precipitation were found in the reaction time of 15 and 30 min. However, at 45 and 60 min amplification at 65°C, precipitation and many bands of various sizes from approximately 200 bp to the loading well were produced (data not shown). Although well-formed bands could be detected at 45 min, the conditions were optimized for 60 min at 65°C. In RT-LAMP, reverse transcription was originally carried out prior to the PCR step (Fukuta et al., 2003) because AMV reverse transcriptase has a optimum activity near 40°C. However, the ability of AMV transcriptase to withstand higher temperatures with no adverse effects on its activity allows the RT-LAMP to be carried out at the higher temperature conditions.

In this study, the sensitivity and specificity of RT-LAMP was shown to be higher than that of RT-PCR. The correlation between RT-LAMP and RT-PCR results on blood samples of CDV-suspected dogs were considered to be good, showing 92.0% agreement and a kappa value of 0.78. This highlights the good sensitivity of RT-LAMP. We only used CCV for evaluating the specificity and it is recommended that more prescreened CDV negative field samples be further examined to prove the specificity for this test.

The RT-LAMP amplies a large amount of target RNA, and produces magnesium pyrophosphate as a by-product, which is visible as white turbidity (Notomi et al., 2000; Mori et al., 2001). In all cases, we could distinguish RT-LAMP-positive samples from negative samples simply by the turbidity of the reaction mixture (Fig. 2c).

Therefore, RT-LAMP-based CDV detection is highly sensitive and its speed of detection makes this a useful tool for diagnosing disease. In addition, the reaction does not require a special thermocycler system, meaning that the method can be used in field animal clinics.

## References


Altman, D. G., 1999: Practical Statistics for Medical Research. 1st edn. Chapman & Hall/CRC, Boca Raton/London/New York/Washington DC.

Cho, H., N. Y. Park, Y. H. Kim, K. O. Cho, H. S. Park, Y. S. Park, B. J. Lee, C. Y. Chung, and H. H. Im, 1999: Diagnosis of canine distemper by in situ hybridization. Korean J. Vet. Res. **39,** 583–592.

Frisk, A. L., M. Koenig, A. Moritz, and W. Baumgaertner, 1999: Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. J. Clin. Microbiol. **37,** 3634–3643.

Fukuta, S., T. Iida, Y. Mizukami, A. Ishida, J. Ueda, M. Kanbe, and Y. Ishimoto, 2003: Detection of Japanese yam mosaic virus by RT-LAMP. Arch. Virol. **148,** 1713–1720.

Gemma, T., K. Iwatsuki, Y. S. Shin, E. Yoshida, C. Kai, and T. Mikami, 1996: Serological analysis of canine distemper virus using an immunocapture ELISA. J. Vet. Med. Sci. **58,** 791–794.

Hong, T. C., Q. L. Mai, D. V. Cuong, M. Parida, H. Minekawa, T. Notomi, F. Hasebe, and K. Morita, 2004: Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. **42,** 1956–1961.

Jones, T. C., R. D. Hunt, and N. W. King, 1997: Canine distemper. In: Jones, T.C., R.D. Hunt, and N.W. King (eds), Veterinary Pathology. 6th edn. pp. 311–315. Williams & Wilkins, Baltimore.

von Mall, P., S. Alldinger, W. Baumgartner, and M. Adami, 1995: Distemper in wild carnivores: an epidemiological, histological and immunocytochemical study. Vet. Microbiol. **44,** 193–199.

Mori, Y., K. Nagamine, N. Tomita, and T. Notomi, 2001: Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. **289,** 150–154.

Moro, L., A. S. Martins, C. M. Alves, F. G. A. Santos, H. L. Del Puerto, and A. C. Vasconcelos, 2003: Apoptosis in the cerebellum of dogs with distemper. J. Vet. Med. B Infect. Dis. Vet. Public Health **50,** 221–225.

Murphy, F. A., E. P. J. Gibbs, M. C. Horzinek, and M. J. Studdert, 1999: Paramyxoviridae-canine distemper. In: Murphy, F.A., E.P.J. Gibbs, M.C. Horzinek, and M.J. Studdert (eds), Veterinary Virology. 3rd edn. pp. 411–426. Academic Press, San Diego.

Nagamine, K., T. Hase, and T. Notomi, 2002: Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes. **16,** 223–229.

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase, 2000: Loop-mediated isothermal amplification of DNA. Nucleic. Acids. Res. **28,** e63.

Potgieter, L. N., and P. A. Ajidagba, 1989: Quantitation of canine distemper virus and antibodies by enzyme-linked immunosorbent assays using protein A and monoclonal antibody capture. J. Vet. Diagn. Invest. **1,** 110–115.

Shin, Y. J., K. O. Cho, H. S. Cho, S. K. Kang, H. J. Kim, Y. H. Kim, H. S. Park, and N. Y. Park, 2004: Comparison of one-step RT-PCR and a nested PCR for the detection of canine distemper virus in clinical samples. Aust. Vet. J. **82,** 83–86.