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A rapid and highly reliable field-based LAMP assay of canine parvovirus

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Summary. – Loop-mediated isothermal amplification (LAMP) is known as a rapid and reliable alternative to conventional single-step or nested PCR for detection of genomic DNA of various pathogens in clinical samples. In this study, LAMP assay was developed for canine parvovirus (CPV) and compared with single-step and nested PCR assays. Out of 50 fecal samples from dogs clinically suspected for CPV infections, 19 were found positive by single-step PCR, 22 by nested PCR and 26 by LAMP. LAMP products were subjected to restriction analysis and sequencing to check their specificity. LAMP assay turned out to be a rapid and fairly reproducible method, did not amplify other common canine pathogens and was more sensitive than nested PCR assay. Therefore, it can be regarded as a highly reliable method for routine field diagnosis of CPV infection.

Keywords: canine parvovirus; nested polymerase chain reaction; loop-mediated isothermal amplification; sensitivity; specificity

Introduction

CPV (the family *Parvoviridae*) was first identified in 1978. It has a single-stranded DNA genome with a length of about 5,200 nucleotides and expresses 2 structural (VP1 and VP2) and 2 non-structural (NS1 and NS2) proteins (Reed *et al.,* 1988). Clinical diagnosis of CPV infection is difficult since the main clinical signs of the disease, such as vomiting and diarrhea are common with other enteric diseases. Virus isolation, latex agglutination test (LAT), ELISA and PCR are the common diagnostic assays used for diagnosis of canine parvovirus infection (Hirasawa *et al.,* 1996). Some of the tests like LAT and ELISA lack specificity and sensitivity. Virus isolations are often time consuming and not suitable for routine diagnosis. Even though PCR is a highly sensitive and specific assay, it requires sophisticated equipment, which may not be available in most of the veterinary clinics. On the other hand, LAMP is a unique gene amplification method, in which DNA can be isothermally amplified using only one enzyme (Chen *et al.,* 2010). Since LAMP can amplify genes isothermally, the amplification reaction can be carried out with a simple heater. There is no need to use special devices like thermal cycler for PCR (Li *et al.,* 2010). Furthermore, it is applied in a wide range of fields, including single nucleotide polymorphism typing (Prompamorn *et al.,* 2011) and quantification of template DNA (Soliman and El-Matbouli, 2005). A large amount of DNA (10–30 μg/25 μl) can be synthesized in a short time (15–60 min), while maintaining high specificity of LAMP reactions (Zhang *et al.,* 2011).

In this study, a LAMP assay for CPV was developed and compared with single-step and nested PCR. The LAMP assay turned out to be a rapid and fairly reproducible method that did not amplify other common canine pathogens and was more sensitive than nested PCR. Therefore, it can be regarded as a highly reliable method for routine field diagnosis of CPV infections.

Materials and Methods

Samples. Fifty fecal samples were obtained from dogs suspected for CPV infection. Fecal samples were collected with a sterile swab and placed in a tube containing PBS.

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Abbreviations: CPV = canine parvovirus; LAMP = loop-mediated isothermal amplification; LAT = latex agglutination test; MDCK = Madin Darby Canine Kidney; NS1, NS2 = non-structural protein 1 and 2; VP1, VP2 = viral protein 1 and 2

LAMP assay. DNA was extracted from fecal samples using DNA extraction kit (NucleoSpin Tissue Kit, Marchery-Nagel, Germany) according to the manufacturer´s instructions. The isolated DNA was used as a template in the LAMP assay. For LAMP assay, four sets of primers (B3, F3, BIP, and FIP) recognizing a total of 6 distinct sequences (B1-B3 and F1-F3) on the VP2 gene of CPV, already described by Cho *et al*. (2006), were used in this study. The LAMP assay was performed in a 25 µl reaction mixture as reported by Cho *et al.* (2006). The reaction mixture was incubated at 60°C for 30 min and it was terminated by heating for 10 min at 80°C.

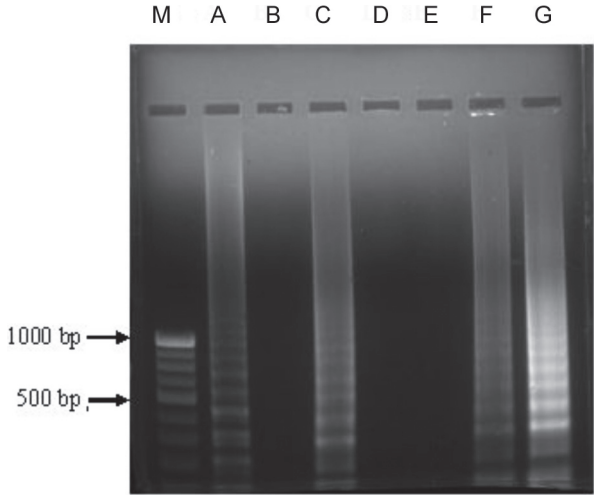

Fig. 1

Screening of CPV-suspected samples by the LAMP assay

100-bp DNA ladder (M), A = positive control, B = negative control, C–G = field samples.

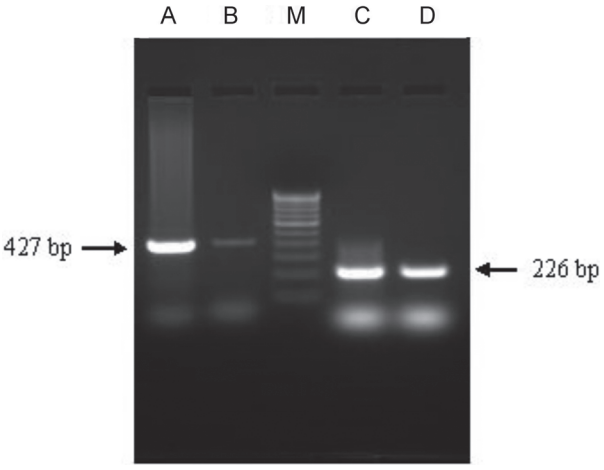

Fig. 2

Comparison of products of single-step and nested PCR assays of CPV

Agarose gel electrophoresis. Single-step PCR (A and B), 100-bp DNA ladder (M), nested PCR (C and D).

Single-step and nested PCR assay. Total DNA isolated from fecal samples was subjected to single-step PCR and nested PCR targeting the VP2 gene of CPV. The primer sequences and temperature profiles for single-step PCR and nested PCR described by Pereira *et al.* (2000) and Gupta *et al.* (2006), respectively, were used in this study. The total DNA from a vaccine strain of CPV grown in MDCK cells and uninfected MDCK cells were used as a positive control and negative control, respectively, for LAMP and nested PCR assays. Ten microlitres of the amplified products of the single-step PCR and nested PCR were analyzed in 1% agarose gel. Similarly, 10 µl of amplified LAMP products were analyzed in 1.5% agarose gel.

Restriction analysis and sequencing. The high molecular mass fraction of LAMP-amplified product was purified using PCR product purification kit (Qiagen, Germany). The purified product was subjected to four different sequencing reactions using B3, F3, BIP, and FIP LAMP primers. Sequencing was done in a Genetic analyzer (Applied Biosystems). LAMP amplicons were digested with *Nco*I restriction enzyme and analyzed by agarose gel electrophoresis.

Sensitivity of single-step PCR, nested PCR, and LAMP assay. Samples positive for CPV DNA were quantified using spectrophotometer. The DNA samples were serially diluted and subjected to single-step PCR, nested PCR, and LAMP assay to assess the sensitivity of each technique.

Specificity and reproducibility of LAMP assay. Total DNA from canine adenovirus, canine leptospira and cDNA from canine distemper virus were used to check the cross reactivity of LAMP primers. Positive and negative samples for LAMP reaction were tested thrice to check the reproducibility of the assay.

Results

A total of 50 fecal samples were screened using single-step PCR, nested PCR, and LAMP assay, and the results are summarized in Table 1. LAMP products of clinical samples are presented in Fig. 1. The single-step PCR (427 bp) and nested PCR (226 bp) amplicons are shown in Fig. 2. The sensitivity of nested PCR and LAMP assay was analyzed using serially diluted CPV-positive DNA samples (Fig. 3 and 4). The sequencing data of LAMP product showed 93% identity with other CPV isolates of Thailand (GenBank Acc. No. GV212791), Taiwan (GenBank Acc. No. FJ011097) and

Table 1. Comparison of various assays for the CPV positivity of fecal samples

Samples	Single-step PCR	Nested PCR	LAMP assay
Positive	19	22	26
Negative	31	28	24
Total	50	50	50

China (GenBank Acc. No. GQ169553) available in the GenBank. The restriction analysis of LAMP products is shown in Fig. 5. The LAMP product was also detected visually by adding SYBR Green I to the reaction tube, and the color change from orange to green was observed (Fig. 6).

Discussion

After the initial screening, few samples negative by single-step PCR were found positive by LAMP. Hence, in order to ascertain the higher sensitivity and specificity of LAMP technique, it was compared with nested PCR assay for the detection of CPV DNA in dog fecal samples. In comparison with nested PCR, the sensitivity of LAMP assay was higher. Some of the samples negative by single-step PCR and nested PCR were positive by LAMP assay. This clearly indicates that LAMP assay was more sensitive than nested PCR. The ladder like pattern of LAMP amplicons were obtained in this study as reported by Cho *et al.* (2006). Similarly, the reaction products of 427 bp (single-step PCR) and 226 bp (nested PCR) were obtained as reported by Pereira *et al.* (2000) and Gupta *et al.* (2006), respectively. Huang *et al.* (2010) reported that LAMP assay detected 100 fg of DNA, whereas single-step PCR detected 10 ng of DNA. In this study, using serially diluted DNA samples, we detected fg level of DNA in LAMP reaction, whereas single-step PCR and nested PCR detected 10 ng of DNA and 1pg of DNA, respectively. In order to ascertain the specificity of the LAMP reaction, LAMP-products were sequenced. When using all the four LAMP primers, only the FIP primer yielded the sequencing data. Prompamorn et al. (2011) used biotinylated LAMP amplicons of *Vibrio*

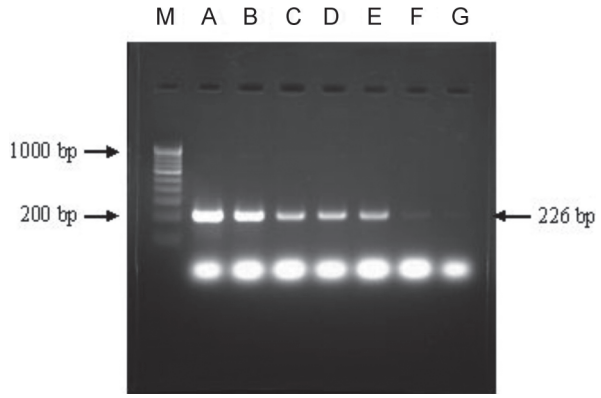

Fig. 3

Sensitivity of Nested PCR assay of CPV

100bp DNA Ladder (M), A = 100 ng, B = 10 ng, C = 1 ng, D = 100 pg, E = 10 pg, F = 1pg, G = 100 fg.

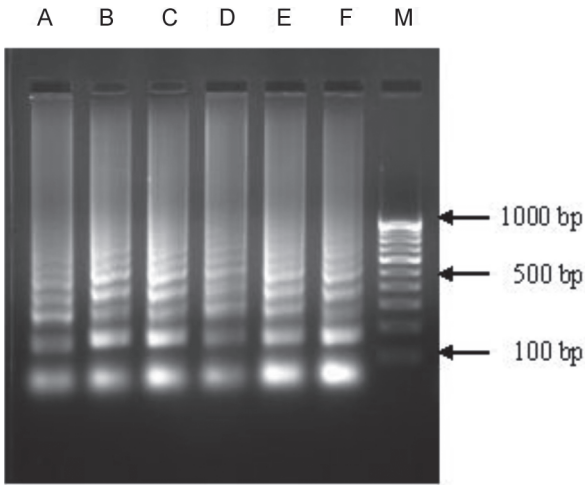

Fig. 4

Sensitivity of the LAMP assay of CPV

A = 10 ng, B = 1ng, C = 100 pg, D = 10 pg, E = 1pg, F = 100 fg, M = 100-bp DNA ladder.

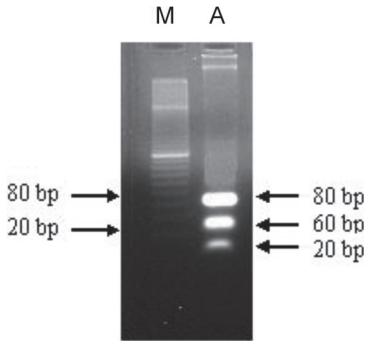

Fig. 5

Restriction analysis of CPV-positive LAMP products

Agarose gel electrophoresis of a 20-bp DNA ladder (M) and a *Nco*I-digested sample (A).

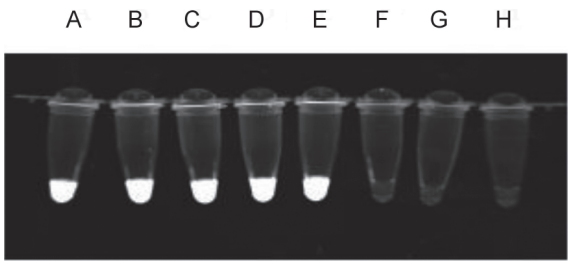

Fig. 6

Visual detection of LAMP products using SYBR Green I dye

UV-illuminated tubes with CPV-positive (A–E) and CPV-negative (F–H) samples.

parahaemolyticus thermolabile haemolysin gene and identified the product by hybridization probe. Liu *et al.* (2008) confirmed the specificity of the LAMP reaction of theileria parasite through *Eco*RI restriction analysis. Similarly, LAMP reaction of CPV was confirmed with *Nco*I restriction enzyme analysis. In our study, LAMP primers did not cross-react with other common canine pathogens such as canine adenovirus, canine leptospira and canine distemper virus, which clearly demonstrates the specificity of the primers. Chen *et al.* (2010) reported that the detection of *Haemophilus parasuis* using LAMP primers did not show any cross-reactivity with other non-*H. parasuis* strains. Moreover, the LAMP products were visualized after addition of SYBR Green I dye without using agarose gel electrophoresis, as reported by Soliman and El-Matbouli *et al.* (2005). Hence, LAMP is likely to be more suitable as a routine diagnostic tool than the single-step PCR or nested PCR, especially in clinics without sophisticated equipments such as thermal cycling machines and electrophoretic apparatus (Zhang *et al.,* 2011). In our study, LAMP assay amplified 6 distinct regions and multiple amplicons were obtained consistently. Hence, LAMP assay can be used as a reliable test in term of reproducibility.

LAMP assay can be used as an alternative method for very sensitive and specific nested PCR for early and rapid diagnosis of CPV infection in dogs, which helps in providing supportive treatment.

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