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Rapid and sensitive detection of elephant endotheliotropic herpesvirus 1 (EEHV1) in blood by loop-mediated isothermal amplification (LAMP)

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ABSTRACT. Elephant endotheliotropic herpesvirus type 1 (EEHV1) is the most important causative agent of an acute fatal hemorrhagic disease in Asian elephants (*Elephas maximus*). We employed loop-mediated isothermal amplification (LAMP) to develop a rapid and simple detection method for EEHV1 in blood. When used to test 21 clinical samples collected in Japan, the EEHV1 assay correctly identified one positive and 20 negative clinical samples. It was observed that when samples were spiked with synthetic DNA plasmids including EEHV1 polymerase gene, the detection limit of the LAMP assay was $10^{1.2}$ copies/*µl* and 100-fold higher than that of conventional PCR. These advantages of the LAMP assay for EEHV1 detection may facilitate better veterinary practices for treating elephants suffering from the acute disease.

KEY WORDS: acute hemorrhagic disease, Asian elephants (*Elephas maximus*), elephant endotheliotropic herpesvirus (EEHV), loop-mediated isothermal amplification (LAMP), polymerase chain reaction (PCR)

Elephant endotheliotropic herpesvirus (EEHV) was identified in 1999, and is recognized as the most common cause of acute fatal hemorrhagic disease (HD) prevalent in young Asian elephants (*Elephas maximus*) and African elephants (*Loxodonta* sp.) [12]. Seven types of EEHV (EEHV1 to 7) have been identified so far; the pathogenesis of each is distinct to elephant species and is different from each other [7]. EEHV1, which has subtypes 1A and 1B, was recognized as the most common type to cause fatal HD in Asian elephants [16, 21]. EEHV-associated HD is considered the most important disease resulting in death from shock caused by damage of the blood vessels and haemorrhaging, within 12–72 hr of the onset of clinical signs [19]. Clinical signs of the disease are easily distinguishable in the late stage, such as cyanosis of the tongue and a swollen head with edema. However, signs in the early stage are non-specific, and hence difficult to diagnose [1, 4]. Thus, accurate diagnostic tests are required for monitoring and detection of disease.

Antemortem diagnosis of EEHV viremia is currently performed by conventional polymerase chain reaction (PCR) or quantitative PCR (qPCR) mainly targeting the EEHV gene U38/DNA polymerase (POL) [6, 11]. However, the available molecular diagnostic assays are time-consuming and require a laboratory setup, hence are not commonly available near elephant herds (e.g., in zoos, elephant camps, and elephant conservation parks). An easy, rapid, and portable diagnosis method is needed, in order to respond quickly against the acute disease associated with EEHV. Loop-mediated isothermal amplification (LAMP) is a simple nucleic acid amplification technique, which is widely used for detecting bacterial and viral infections in the veterinary field [8, 9, 13]. LAMP assays have been shown to amplify, thus detect, nucleic acid with high speed, specificity and efficiency under isothermal conditions. In combination with a color change dye, amplicons can be observed with the naked eye. LAMP assay does not require complicated equipment and time-consuming post-reaction work for genetic confirmation, such as agarose-gel electrophoresis, making it ideal for field-deployment. In this study, we developed and evaluated a LAMP assay targeting the POL gene of EEHV1 for specific detection of EEHV1 in blood.

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Table 1. Primer sets designed for LAMP assay and PCR assay based on the EEHV1 specific POL gene

Purpose	Primer name	Nucleotide sequence (5′-3′)	Source
LAMP assay	FIP (F1c+F2)	CGACTTTCTAACATGCGGTTTTACGA-GAGGAGGATGTTACAATGGTCA	This study
	BIP (B1c+B2)	CTCTCGCAACTGTTAACGTCGTG-CATAAGGGGATCTTTACAATGCTTC	This study
	LF	ACCCTACCGGTGTTGGTGG	This study
	LB	CGGAACGCAAAGCTGTCAGG	This study
	F3	TGTACCCCAGTATCATTCAAGC	This study
	B3	GCACGTTAGTTTTAGAGCCAG	This study
PCR assay[a)]	6710	ACAAACACGCTGTCRGTRTCYCCRTA	[6]
	6711	GTATTTGATTTYGCNAGYYTGTAYCC	[6]
	6712	TGYAAYGCCGTNTAYGGATTYACCGG	[6]

a) First round PCR: 6710/6711, second round PCR: 6710/6712 [6].

A set of six primers for the LAMP assay, consisting of forward inner primer (FIP), backward inner primer (BIP), outer forward primer (F3), outer backward primer (B3), loop forward primer (LF), and loop backward primer (LB), were designed based on the published sequences of POL gene and terminase gene (TER) of EEHV1 (The GenBank accession numbers are HM568510, JF692761, and MF579090) by using the PrimerExplorer V5 software (Fujitsu Ltd., Tokyo, Japan). POL and TER gene were initially confirmed as highly conserved region in EEHV1 and have been used for targeting these regions in many PCR primers [12]. In this study, LAMP primer sets were designed targeting these regions because abundant sequence information was available on GenBank. The specificity of each primer was confirmed using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) by not showing the identity to nucleotide sequences of the other important pathogens in elephants, such as foot-and-mouth disease virus, rabies virus, and *Mycobacterium tuberculosis*-complex. The LAMP assay was carried out with a DNA amplification kit (Loopmap DNA Amplification Kit, Eiken Chemical Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's instructions. A 25 μl reaction mixture containing 12.5 μl of 2 × Reaction Mix, 1 μl each of FIP and BIP primers (40 μM of each), 1 μl each of F3 and B3 primers (5 μM of each), 1 μl each of LF and LB primers (20 μM of each), 1 μl *Bst* DNA polymerase, 1 μl Loopamp Fluorescent Detection Reagent (Eiken Chemical Co., Ltd.), 2.5 μl nuclease free distilled water and 2 μl DNA sample was prepared. The mixture was incubated using a heat block (MyBL-10C, ASONE, Osaka, Japan) at 60°C for 60 min, and the reaction was stopped at 95°C for 2 min. The reactions were visualized with the naked eye. Namely, the mixture turned green when the LAMP reaction amplified DNA in the presence of calcein−a fluorescence detection reagent, whereas the mixture remained orange when there was no amplification. The most sensitive primer set in a screening test using synthetic DNA plasmids was described in this paper (Table 1).

Conventional PCR was performed using a primer set (6710 and 6711) (Table 1) described previously, that targets the EEHV POL gene [6]. The PCR amplification for each round employed the following conditions: 94°C for 2 min, followed by 40 cycles at 98°C for 10 sec, 55°C for 30 sec, and 68°C for 30 sec, and a final extension at 68°C for 5 min, using KOD Fx Neo (TOYOBO Co., Ltd., Osaka, Japan), in accordance with the manufacturer's instructions. Amplicons were separated by agarose gel electrophoresis and visualized using ethidium bromide and a UV transilluminator. For the PCR screening test to confirm negative results, nested PCR was performed with the amplicons using an inner primer set (6710 and 6712) (Table 1) with the same conditions described above.

Twenty clinical whole blood samples were collected from twelve healthy Asian elephants (three males aged 3 months to 26 years and nine females aged 1 month to 51 years), in Ichihara Elephant Kingdom zoological park. All samples were confirmed EEHV negative by the PCR screening test. A positive clinical blood sample, which was collected from a female Asian elephant (aged 2 years) in Okinawa Zoo and Museum, was also used in this study. This specimen was collected in 2018, just before the elephant died, and was diagnosed with EEHV1A using the PCR assay. DNAs were extracted from whole blood samples by QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), in accordance with the manufacturer's instructions. The LAMP assay was performed with these samples to validate specificity. All assays were performed in duplicate. All 20 negative clinical blood samples were detected as EEHV1 negative and a positive clinical blood sample was detected as EEHV1 positive by the LAMP assay.

The sensitivity of the LAMP assay was assessed using two synthetic DNA plasmids carrying 558 bp target loci of EEHV1A and EEHV1B POL genes (HM568510 and JF692761, respectively). Ten-fold series dilutions (10^5–10^{-1} copies/μl) of each synthetic DNA were used as templates for the LAMP and PCR assay to confirm the detection limits. Salmon sperm DNA (BioDynamics Laboratory Inc., Tokyo, Japan) was added in dilutions as a carrier. The gene copy numbers of DNA were calculated by the concentration of synthetic DNA [20]. To imitate an actual infected blood sample, experimentally spiked blood samples (10^5–10^{-1} copies/μl in blood before DNA extraction) were prepared, and DNA was extracted using QIAamp DNA Blood Mini Kit. These assays were performed in duplicates with ten replicates to calculate detection limits. The detection limits of each assay, wherein 50% of samples were positive, were calculated using the Reed and Muench method [10]. Table 2 shows the results of sensitivity test of LAMP assay and the PCR assay using synthetic EEHV1A POL DNA plasmids. The 50% detection limits of LAMP assay and the PCR assay using ten-fold series dilutions were $10^{0.7}$ and $10^{3.3}$ respectively. The 50% detection limits of LAMP assay and the PCR assay using experimentally spiked samples were $10^{1.2}$ and $10^{3.5}$, respectively. Table 3 shows the results of sensitivity test using artificial synthetic EEHV1B POL DNA plasmids. The 50% detection limits of LAMP assay and the PCR assay using dilutions were $10^{0.5}$ and $10^{3.4}$, respectively, and using spiked samples were $10^{1.2}$ and $10^{3.5}$, respectively.

Table 2. Comparison of sensitivity between LAMP and PCR method using artificial synthetic EEHV 1A POL DNA plasmid

	Synthetic EEHV 1A POL DNA copies/μl in dilution or blood							Negative control or non-spiked samples	50% detection limit (copies/μl)
	10^5	10^4	10^3	10^2	10^1	10°	10^{-1}		
LAMP assay									
Dilution with water	20/20[a)]	20/20	20/20	20/20	10/20	6/20	0/20	0/20	$10^{0.7}$
Spiked blood samples	20/20	20/20	20/20	18/20	6/20	4/20	0/20	0/20	$10^{1.2}$
PCR assay									
Dilution with water	20/20	20/20	6/20	0/20	0/20	0/20	NT[b)]	0/20	$10^{3.3}$
Spiked blood samples	20/20	20/20	1/20	0/20	0/20	0/20	NT	0/20	$10^{3.5}$

a) Number of positive samples / number of examined samples. b) NT: Not Tested.

Table 3. Comparison of sensitivity between LAMP and PCR method using artificial synthetic EEHV 1B POL DNA plasmid

	Synthetic EEHV 1B POL DNA copies/μl in dilution or blood							Negative control or non-spiked samples	50% detection limit (copies/μl)
	10^5	10^4	10^3	10^2	10^1	10°	10^{-1}		
LAMP assay									
Dilution with water	20/20[a)]	20/20	20/20	20/20	14/20	5/20	0/20	0/20	$10^{0.5}$
Spiked blood samples	20/20	20/20	20/20	16/20	8/20	2/20	0/20	0/20	$10^{1.2}$
PCR assay									
Dilution with water	20/20	20/20	2/20	0/20	0/20	0/20	NT[b)]	0/20	$10^{3.4}$
Spiked blood samples	20/20	20/20	1/20	0/20	0/20	0/20	NT	0/20	$10^{3.5}$

a) Number of positive samples / number of examined samples. b) NT: Not Tested.

Generally, the LAMP assay has high specificity, which is directly attributed to the six sets of primers spanning eight distinct sequences of the target gene being amplified [9]. In this study, the LAMP assay did not amplify any whole blood samples of Asian elephants without EEHV1 (i.e., no false positive, meaning 100% specificity). BLAST search also showed high specificity of primers designed in the present study, and the LAMP assay indicated correct results in all clinical samples as expected. The detection limits of the LAMP assay for EEHV1A and 1B using either synthetic DNA plasmids or spiked blood samples were 100-fold higher than the conventional PCR assay. The slightly lower sensitivities of the LAMP assay and the conventional PCR assays in spiked blood samples than diluted samples may be due to the presence of many inhibitors in blood. The results demonstrated that the LAMP assay was more sensitive than the conventional PCR assays in the presence or absence of inhibitors.

While spiked blood samples may reflect the actual conditions of clinical positive samples, such as loss of viral DNA in blood, contamination by inhibitors in blood, and loss of DNA during extraction, the number of positive clinical samples used in this study was too small to discuss the sensitivity of the LAMP assay. To date, there is only one case diagnosed as EEHV-HD in Japan, and no other clinical positive elephants were reported [18]. Further studies are necessary to confirm higher sensitivity, portability, and reproducibility of this method in field, comparing with another diagnosis method such as qPCR, which was known have higher sensitivity than gel-based PCR using a number of positive samples under collaborations with researchers abroad.

Among recent case reports, viral load of EEHV in Asian elephant whole blood samples during the clinical period was more than 10^3 copies/μl [2, 3, 15, 17]. The detection limit of the LAMP assay using spiked blood samples was $10^{1.2}$ copies/μl and was enough to detect EEHV1 viremia during the clinical period. As the viral DNA can be detected several days prior to any clinical signs of EEHV-HD, routine blood DNA detection of at-risk Asian elephants for EEHV viremia has been suggested to prevent fatal disease or initiate treatment earlier [2, 4]. The portable LAMP assay for EEHV has the potential to alter current routine PCR monitoring performed in laboratories. Furthermore, Asian elephants are natural hosts of EEHVs and appear to carry latent EEHV with occasional asymptomatic reactivation [5, 11, 14]. Since the LAMP assay is highly sensitive compared with a conventional PCR, it may detect asymptomatic reactivation of EEHV more frequently, helping to understand the relationship of asymptomatic reactivation and fatal EEHV-HD. To establish clinical application in the future, it is necessary to define the minimum level of viral load needed for therapeutic approach and to modify the LAMP assay to be able to calculate the relative amount of DNA.

In this study, we developed a LAMP assay designed for detecting EEHV1 POL DNA in blood collected from Asian elephants. The LAMP assay is more sensitive than the conventional PCR assay. The results suggest that the LAMP assay is useful to diagnose EEHV1 viremia in Asian elephants as a potential substitute to the PCR assay. The LAMP assay takes less than 90 min from blood collection to analyzing the results and is much faster than the conventional PCR assay. For accurate and objective diagnostic purposes, amplified DNA should be measured using instruments such as real-time turbidimeter (LoopampEXIA, Eiken Chemical Co., Ltd.) and portable fluorimeters (Genie II, OptiGene, Horsham, U.K.). Also, the LAMP assay has the potential to use without DNA extraction because of more resistant to inhibitors than conventional PCR assay. Although additional studies with a large number of clinical samples are required, here we provide preliminary data to demonstrate how the LAMP assay can be useful for primary diagnosis of EEHV in the field.

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