Detection of Aleutian disease virus by loop-mediated isothermal amplification

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Abstract In this study, a loop-mediated isothermal amplification (LAMP) assay was developed and optimized for the detection of Aleutian disease virus (ADV) in minks. The amplification could be completed within 45 min under isothermal condition by employing a set of six ADV genome-specific primers. The amplification results could be visualized directly with the naked eye by using fluorescent dye. Comparative experiments showed that the LAMP assay is superior to conventional polymerase chain reaction for the detection of both experimental and field samples. Results of current study indicated that the LAMP assay is a rapid and reliable technique for routine diagnosis of ADV infection in minks.

Keywords Aleutian disease virus · Diagnosis · Loop-mediated isothermal amplification

Aleutian disease virus (ADV), a naturally occurring autonomous parvovirus, is the causative agent of Aleutian disease of minks. Adult minks infected with virulent strains develop chronic and often fatal immune complex-mediated diseases, including glomerulonephritis and arteritis [4, 10]. Atypical interstitial pneumonia with respiratory distress can be observed in some virus-infected newborn minks [3].

In addition, depending on the virus strain and host factors, the animals may show progressive weight loss, reduced reproduction, poor pelt, lethargy, anorexia, polydipsia, polyuria, anemia, melaena and neurological symptoms [7, 10]. This disease is common in mink farms all over the world, causing considerable economic losses. Currently, there are no commercial vaccines available, screening of virus-infected animals followed by elimination is often applied to control the disease.

Counter-immunoelectrophoresis (CIEP) test based on in vitro grown ADV antigen is considered as the gold standard to screen the virus-infected animals. However, this method is laborious to perform. Other serological diagnostic methods, including modified CIEP [1, 2, 8, 19], rocket line immunoelectrophoresis [5], and enzyme-linked immunosorbent assays [6, 9, 12, 13, 20] have been reported. But these methods may show a slowdown in the process of disease diagnosis because preparation of the antigen used in these methods is both labor and time-consuming. Viral nucleic acid detection methods, such as polymerase chain reaction (PCR) and real-time PCR were developed for laboratory diagnosis [11, 14, 17, 18]. Even though they are sensitive and less time-consuming, they are not suitable for large scale application since specialized equipment and technical expertise are required, which are always not available in the field.

Loop-mediated isothermal amplification (LAMP) for nucleic acid has advantages of simplicity, specificity, rapidity and cost effectiveness, thus it is considered a promising nucleic acid amplification technique for pathogens [15]. In this paper, we report the development of a LAMP assay for ADV detection.

A mink ADV strain was first isolated by inoculating animal-derived homogenized organ samples on Crandell feline kidney (CrFK) cells according to previously reported

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Fig. 1 Oligonucleotide sequences of LAMP primers

procedure [16]. To prepare the template for LAMP assay, viral DNA was directly extracted from both tissue samples and virus-containing cell culture using commercially available DNA extraction kit (Qiagen, Hilden, Germany). A set of six oligonucleotides recognizing eight distinct regions in *vp2* gene of ADV, including forward primer F3 and backward primer B3 (outer primers), forward inner primer FIP and backward inner primer BIP (inner primers), and forward loop primer LF and backward loop primer LB (loop primers), were designed by the use of online program Primer Explorer version 4 (<http://primerexplorer.jp/elamp4.0.0/index.html>). All the primers were synthesized and high-performance liquid chromatography-purified by a commercial service (Invitrogen, Beijing, China) after their specificities were confirmed by BLAST searches against GenBank (Fig. 1).

The LAMP assays were performed in a 25- μ l reaction system with a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan) according to manufacturer's protocol. Briefly, reaction mixtures containing 40 pmol each of the primers FIP and BIP, 20 pmol each of the primers loop primer F and B, 5 pmol each of the primers F3 and B3, 12.5 μ l of 2 $\times$ reaction buffer (40 mM Tris-HCl [pH 8.8], 20 mM KCl, 16 mM MgSO₄, 20 mM (NH₄)SO₄, 1.6 M betaine, 0.2 % Tween 20, 2.8 mM each dNTP), 2 μ l of DNA sample and 1 μ l of *Bst* DNA polymerase enzyme were made. The LAMP reactions were carried out in regular laboratory water bath with temperature of 65 °C. To optimize the LAMP reaction conditions, incubating time were set from 15 to 60 min. All the reactions were terminated by incubation at 80 °C for 5 min, and LAMP products were analyzed by 2 % agarose gel electrophoresis. Results indicated that the primers amplified the template successfully at 65 °C. When the reaction mixtures were incubated for 15, 30, 45 and 60 min, respectively, under the predetermined reaction temperature, it was found that LAMP characteristic ladder-like DNA bands first appeared at 30 min, and then reached a maximum at 45 min. There was no obvious difference in the amount of LAMP product between 45 and 60 min

Fig. 2 LAMP products generated at different time intervals. Samples were removed one by one at 15 min interval to test the amplification of template DNA. NC negative control

amplification, therefore 45 min was set as optimal reacting time (Fig. 2a).

Under field conditions, it is highly desirable to determine the positive reaction directly without other equipments. Therefore we tested the usefulness of fluorescent detection reagent (FDR, Eiken Chemical Co. Ltd., Tokyo, Japan) in LAMP assays. With the presence of 1 μ l FDR in

Fig. 3 Comparative sensitivity of LAMP and PCR for the detection of ADV genome. Template DNA was 10 times serially diluted (from 3.3×10^6 to 3.3×10^0 copies). LAMP and PCR results were determined through agarose gel analysis (a and c). The positive LAMP reactions can also be inspected directly by naked eye (b). NC negative control

each tube, positive amplifications can be indicated by color change (from orange to green) of the reaction system, making the results can be visualized directly by naked eye. In concordance with the agarose gel analysis, the color changes were observed at the point of 30 min, suggesting that the LAMP assay is a rapid method for ADV genome detection (Fig. 2b).

To determine the analytical sensitivity of the LAMP assay, a pair of previously published primers was used to amplify ADV specific genome through regular PCR [18]. The PCR amplicon were purified using a gel extraction kit

and cloned into pMD18-T vector (Takara Bio Inc., Dalian, China). Then nucleotide sequencing-confirmed recombinant construct (namely T-ADV) was linearized using a vector-specific restriction enzyme (*SacI*), and quantified by measuring the concentration after purification. The template copy numbers were determined by using the following formula: copies/ μ l = concentration of linearized plasmid (g/ μ l)/(plasmid length $\times$ 660) $\times$ 6.022×10^{23} . A series of templates were created by 10 times dilution for linearized plasmids in DNase-free water. Conventional PCR was also performed on each template with F3 and B3 as forward and reverse primers, respectively. PCR was conducted in a 20 μ l reaction volume containing 10 μ l PCR master mix (Promega, Promega, Madison, WI, USA), 2 μ l (5 pmol/ μ l) each primers F3 and B3, 1 μ l DNA template, and 5 μ l deionized water, with a thermal cycler (Eppendorf, Hamburg, Germany) for 35 cycles. Each cycle consisted of 95 $^{\circ}$ C for 30 s, 50 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 40 s. A negative control (no template) was included. Results revealed that the detection limit for LAMP was approximately 3.3×10^2 copies of template DNA while the detection limit for conventional PCR was 3.3×10^4 copies, therefore LAMP assay was 100 times more sensitive than conventional PCR in this study (Fig. 3).

To assess the applicability of the LAMP assay with field samples, nine mink tissue samples (four spleen, three liver and two kidney samples) were collected from the animals which had been shown serological positive by CIEP test during the period of the establishment of this LAMP assay. Of nine suspected samples, LAMP and regular PCR showed equal sensitivities, i.e., all the samples were shown to be positive by the two methods. Then 26 field samples (one brain, three intestine, six spleen, ten liver and 13 kidney samples) collected from different mink farms during the year of 2013–2014 were subjected to the LAMP assay. Results indicated that all the samples that were shown to be positive by PCR were also positive by LAMP, and all the samples that were shown to be negative by LAMP were also negative by PCR. But one intestine, two spleens and two kidney samples were found to be positive by LAMP while negative by PCR. LAMP product amplified from PCR-negative sample was further confirmed by

Table 1 Comparison between the ability of LAMP and PCR to detect ADV from clinical specimens

Specimens	Detection positive rate for assay		Agreement ^a
	LAMP	PCR	
Suspected samples	9/9 ^b	9/9	30/35 (85.7 %)
Field samples	12/26	7/26	

^a Agreement indicates the percentage of the samples that gave the identical (including both positive and negative) result with both assays

^b The denominators indicate the total number of samples tested by assays, the numerators indicate the number of positive samples shown by assays

nucleotide sequencing. The LAMP showed 85.7 % agreement with PCR (Table 1).

Taken together, results of current study indicate that the LAMP assay can be carried out in a single tube with a simple visual method, only 45 min are required for completing the assay with high sensitivity. In addition, the LAMP assay does not require costly reagents and high technical expertise, suggesting that the LAMP developed in this study has great potential as a diagnostic tool for ADV infection, especially under field conditions. The applicability of the LAMP assay can be further evaluated by using the samples from living animals, such as blood.

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