Development of a loop-mediated isothermal amplification for rapid detection of orf virus

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\textbf{A B S T R A C T}

A loop-mediated isothermal amplification (LAMP) assay using six primers targeting a highly conserved region of the B2L gene has been developed to diagnose orf virus. The assay produces a ladder-like pattern of products on an agarose gel that can be specifically digested with $BsrGI$ enzyme. The sensitivity of the LAMP assay, which was determined to be a single copy of the standard plasmid, was 100 fold and 10 fold higher than PCR and nested PCR, respectively; furthermore, no cross-reactivity was founded with the other tested viruses. By staining the products directly in the tube with PicoGreen or ethidium bromide, the products can be visualized with a similar sensitivity as by gel electrophoresis. Clinical samples were tested using PCR, nested PCR and LAMP assay and the positive rates were 60%, 70% and 70%, respectively. The LAMP assay allows easy, rapid, accurate and sensitive detection of infection with orf virus and is especially applicable in a resource-limited situation.

\section{1. Introduction}

The orf virus (ORFV) is a double-stranded DNA virus belonging to Parapoxvirus genus of Poxviridae family. The virus particle, with a size of 260 nm $\times$ 160 nm, has a cocoon shape with a regular spiral arrangement of surface tubules (Chan et al., 2007). There are 131 putative genes located within the virus genome, which is about 139 kbp in size. The genes are arranged in an orderly manner from the left to the right side and the conserved region, which includes essential genes such as the viral RNA polymerase, is located in the central region (Delhon et al., 2004). The orf virus has a world-wide distribution and causes an infectious skin disease known as contagious ecthyma in goats, sheep and other ruminants (Haig and McInnes, 2002). The disease lesions are characterized by a proliferative dermatitis in lips, nostrils, gums, tongues and teats (Murphy et al., 1999). Although it appears as a mild disease to lambs, mortality can reach 90% in young sheep because they are more predisposed to secondary infections in severe cases (Mondal et al., 2006). In addition orf is zoonotic and is known to cause nodular and papillomatous lesions in farmers, veterinarians and butchers who have had contact with infected livestock and their products (Torfason and Gunadottir, 2002). The routes of infection for humans include accidental abrasions, cutting, shearing, slaughtering and milking (Robinson and Mercer, 1995). The duration of the lesions ranges from 4 to 9 weeks, but normal healing may be prolonged due to complications (Torfason and Gunadottir, 2002). Generally, other than using electron microscopy at a high cost, PCR based detection of the B2L gene, which encodes a major envelope protein, has been the most widely used approach to diagnosis of orf and this method has shown good sensitivity and specificity (Inoshima et al., 2000; Mondal et al., 2006). However, this assay requires skilled technicians and specialized instrumentation. Loop-mediated isothermal amplification (LAMP) is a novel method that amplifies DNA with speedily and with high sensitivity under isothermal conditions using a specific set of primers and a DNA polymerase with strand displacement activity (Notomi et al., 2000). To date, it has been successfully developed to diagnose pseudorabies virus (PRV), avian influenza virus, human immunodeficiency virus (HIV), hepatitis B virus and foot-and-mouth disease virus (FMDV) (Curtis et al., 2008; Dukes et al., 2006; En et al., 2008; Imai et al., 2007; Li et al., 2005). It is especially useful in resource-limited situations. In this study, a LAMP assay was developed and compared with conventional PCR and nested PCR for the detection of orf virus. This approach was found to be an excellent tool with high sensitivity, high specificity and fast turn around time.

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Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Type</th>
<th>Sequences (5′-3′)</th>
<th>Length (bp)</th>
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<tr>
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<tr>
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<td>Reverse</td>
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</table>

<sup>a</sup> LAMP primers.

<sup>b</sup> PCR primers.

<sup>c</sup> Nested PCR primers.

2. Materials and methods

2.1. DNA extraction and construction of a standard plasmid

Samples were collected from the skin lesions around the muzzle and lips of goats during an outbreak of orf infection and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s protocol. Briefly, samples were homogenized in 0.1 M phosphate buffer saline, extracted with lysis buffer containing 1 mg/ml proteinase K and incubated at 56 °C overnight. The product was passed through the column and the DNA was eluted in 20 μl water. A standard plasmid, pT-Easy-B2L, was constructed by insertion of a B2L gene fragment generated using the OVB2LF1 and OVB2LR1 primers into the pT-Easy plasmid (Promega, USA). All primers used in this study are listed in Table 1. After verification by sequencing, the amount of plasmid present were measured spectrophotometrically using one OD<sub>260</sub> = 5.0 (copies/μl) and then the copy number was calculated by the formula: amount (copies/μl) = 6 × 10<sup>23</sup> (copies/mol) × concentration (g/μl)/MW (g/mol).

2.2. Designing the LAMP primers

The LAMP primers were designed using Primer Explorer V3 software based on a conserved region of the B2L gene identified by sequence alignment (Chan et al., 2007). The end stability of the candidate primers needs to follow the rule that the free energies of the 3′ ends of F2/B2, F3/B3, LF/LB and of the 5′ ends of F1c/B1c are less than −4 kcal/mol.

2.3. LAMP detection, PCR and nested PCR

The PCR reactions were performed in a 25 μl reaction mixture, which contained 2.5 μl of 10× buffer, 0.2 mM of each dNTP, 0.1 μM of OVB2LF1 and OVB2LR1 primers, 1 μl of extracted DNA and 0.5 μl of Taq polymerase (5 units/μl; NEB, USA). The PCR reactions were subjected to 94 °C for 10 min and then 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s and extension at 72 °C for 1 min with one final extension at 72 °C for 10 min. The second amplification of the nested PCR was performed with the same program except using 0.1 μM of the F3 and B3 primers.

Fig. 1. A region of the B2L gene of the orf virus was used to design the LAMP primers (A) and a schematic representation of inner primers (FIP and BIP), outer primers (F3 and B3) and loop primers (LF and LB), which are composed of the complementary sequences of the region between F1/F2 and B1/B2, respectively (B).
The LAMP reaction was carried out in a volume of 25 μl containing 1 x ThermoPol buffer (NEB, USA), 8 mM MgSO4, 0.4 M betaine (Sigma, USA), 1.2 mM dNTPs, 8U Bst polymerase (NEB, USA), 0.2 μM of each of the F3 and B3 primers, 1.6 μM of each of the FIP and BIP primers, 0.8 μM of each of the LoopF and LoopB primers and 1 μl of extracted DNA as template. The amplification was performed at 65 °C in a laboratory water bath for 1 h. A control containing no template was included in each test as the negative control. The products were visualized with the naked eye under a UV lamp after addition of an intercalating dye, PicoGreen (Invitrogen, USA) or ethidium bromide. They were also separated on a 2% agarose gel in 0.5× TBE buffer, followed by staining with ethidium bromide and visualization on a UV transilluminator. To further confirm the specificity of products, 2 μl of the reaction mixture was digested with BsrGI at 37 °C for 4 h.

2.4. Optimization of the LAMP assay conditions

An evaluation of the effects of different concentrations of MgSO4 (2–12 mM), of different concentrations of betaine (0–1.2 M), the amplification temperature (61–67 °C), the amount of dNTPs present (0–1.2 mM), the reaction time (15–75 min) and the ratio of outer and inner primers (1:1–1:12) were carried out to optimize the LAMP reaction. A hundred copies of the standard plasmid, pT-Easy-B2L, were used for each test (Fig. 1).

2.5. Sensitivity and specificity of the LAMP reaction

The sensitivity of the LAMP assay was tested and compared with PCR and nested PCR using the orf virus standard plasmid (1–10^5 copies) as template. Additionally, each product was visualized under a UV lamp after addition of an intercalating dye, PicoGreen (Invitrogen, USA) or ethidium bromide. The specificity of the LAMP assay was examined using DNA or cDNA from orf virus, FMDV, PRV, influenza virus, skin samples from normal goats and Madin-Darby ovine kidney (MDOK) cells. A control lacking template was included in each test as a negative control.

2.6. Evaluation of the LAMP assay using clinical samples

Twenty samples of the skin lesions around the muzzle and lips of affected goats were collected from seven outbreaks of orf disease in central Taiwan; the background information is listed in Table 2. Samples from normal goats were included as negative controls. All were tested by PCR, nested PCR and LAMP assay in parallel. The rate for the positive detection of orf virus in the samples and the sensitivity of the assay were calculated.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Geographic location</th>
<th>Date of sampling</th>
<th>Source</th>
<th>Host</th>
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<tr>
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<td>Nantou County</td>
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<td>Goat</td>
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<td>Hoping (HP)</td>
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</table>

Fig. 2. Optimization of the LAMP reaction for orf virus. (A) The effect of MgSO4: lane 1–6 (2, 4, 6, 8, 10 and 12 mM, respectively). (B) The effect of betaine: lane 1–7 (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 M, respectively). (C) The effect of temperature: lane 1–4 (61, 63, 65 and 67 °C, respectively). (D) The effect of dNTPs: lane 1–7 (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mM, respectively). (E) The effect of reaction time: 1–5 (15, 30, 45, 60 and 75 min, respectively). (F) The effect of the ratio of outer and inner primers: lane 1–5 (1:1, 1:2, 1:4, 1:8 and 1:12, respectively).
3. Results

3.1. Optimizing the LAMP assay conditions

All of the possible variables were tested. The results indicated that the Mg²⁺ concentration need to be at least 8 mM to give a positive reaction and no additional change was observed up to 12 mM (Fig. 2A). Slightly different yields were observed with the various amounts of betaine used and a concentration higher than 0.2 M was found to be appropriate (Fig. 2B). No difference was founded when the reaction temperature was varied from 61 to 67 °C (Fig. 2C). The size of the ladder increased as the concentration of dNTPs increased and a concentration greater 0.8 mM was found to be sufficient (Fig. 2D). A positive amplification could be detected as early as 30 min and product reached a maximal level after 60 min (Fig. 2E). Although positive reactions could be obtained using primer ratios ranging from 1:1 to 1:12, a more distinct pattern was shown when the ratio was above 1:8 (Fig. 2F). Taken together, the optimal conditions for the LAMP assay were determined to 65 °C for 1 h with 8.0 mM MgSO₄, 0.2 M betaine, 1.0 mM dNTPs, 0.2 µM each of outer primer, 1.6 µM each of inner primer, 0.4 µM each of loop primer and 8U Bst polymerase. The positive LAMP reaction generated a laddering pattern with a set of bands of different sizes consisting of several inverted-repeat structures. After the enzyme digestion, the specific pattern disappeared indicating a complete digestion of amplified products (Fig. 3A). Furthermore, a white precipitation composed of magnesium pyrophosphates was observed (Fig. 3B).

3.2. Sensitivity and specificity of the LAMP assay

The detection limits for the LAMP assay, the nested PCR assay and the standard PCR assay were 1, 10 and 100 copies of the standard plasmid, respectively. No amplified products were detected in the negative controls. Thus, the sensitivity of LAMP was 100 fold and 10 fold higher than those of the PCR and nested PCR assays, respectively (Fig. 4A). The fluorescent green and orange colored products could be visualized after PicoGreen and ethidium bromide staining, respectively, and their sensitivities were consistent with those obtained by gel electrophoresis (Fig. 4B). Across all viruses and cells tested using the orf LAMP assay, only DNA from the orf virus was amplified and this result confirmed the specificity of this assay (Fig. 5).

Fig. 3. Agarose gel electrophoresis (A) and visual inspection of the LAMP amplification products by turbidity (B). Lane M: DNA markers, lane 1: LAMP products, lane 2: LAMP products digested with BsrGI, lane 3: negative control, 4: LAMP products, and 5: negative control.

Fig. 4. The sensitivity of LAMP, nested PCR and PCR (A) and visual inspection with PicoGreen or ethidium bromide staining (B). Lane M: DNA markers, lane 1–6: 10⁵, 10⁴, 10³, 10², 10 and 1 copies/tube, respectively, and lane 7: negative control.

Fig. 5. The specificity of LAMP. Lane M: DNA markers, lane 1–6: orf virus, FMDV, PRV, influenza virus, normal goat tissues and MDOK cells, respectively, and lane 7: negative control.
in the samples did not affect the positive rate of detection as long as virus was present. A trace amount of virus beyond the limit of detection, inefficient amplifications and inhibitory substances seem to have contributed to a lower than optimum positive rate when using PCR. Even though this can be compensated for by the use of nested PCR, it is reasonable to predict a larger difference in the success rate for positive diagnosis may occur if more samples were included.

orf Occurs as an endemic infection in Taiwan. To date, a vaccination program has yet to implement. Although the mortality and economical losses are not very significant orf still can cause weight loss and poor performance because the disease prevents lambs from suckling. PCR and electron microscopy are routinely used for diagnosis of orf (Inoshima et al., 2000; Torfason and Gundadottir, 2002). Based on cost and simplicity, this assay can be used as a rapid, simple and economic alternative when undertaking large-scale screening and will greatly benefit world agriculture, as it will promote differentiation of orf from other diseases.

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References


