NOTE *Virology*

Rapid detection of contagious ecthyma by loop-mediated isothermal amplification and epidemiology in Jilin Province China

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(Received 8 June 2015/Accepted 1 August 2015/Published online in J-STAGE 6 September 2015)

ABSTRACT. The aim of this experiment was to develop a loop-mediated isothermal amplification (LAMP) assay and to research the recent epidemiology of contagious ecthyma in Jilin Province, China, using the assay. A LAMP assay targeting a highly conserved region of the F1L gene was developed to detect contagious ecthyma virus (CEV). Three hundred and sixty-five cases from 64 flocks in 9 different areas of Jilin Province, China, from 2011 to 2014 were tested using the LAMP assay. The results showed that the sensitivity of the LAMP assay was 100 copies of the standard plasmid, which is 100-fold higher than the sensitivity of PCR. No cross-reactivity was observed with capripoxvirus, fowlpox virus, foot-and-mouth disease virus serotype O, foot-and-mouth disease virus serotype Asia I and bluetongue virus. The average positive rate was 19.73% (72/365), and the positive rate was highest in lambs aged 1–6 months. Our results demonstrated that CEV infection was very widespread in the flocks of Jilin Province and that the LAMP assay allows for easy, rapid, accurate and sensitive detection of CEV infection.

KEY WORDS: contagious ecthyma virus (CEV), epidemiology, loop-mediated isothermal amplification (LAMP)

doi: 10.1292/jvms.15-0340; *J. Vet. Med. Sci.* 78(1): 125–128, 2016

Contagious ecthyma (CE), also known as Orf, is a debilitating disease of sheep and goats caused by the contagious ecthyma virus (CEV). CEV is a dsDNA virus belonging to the Parapoxvirus genus of the Chordopoxvirinae subfamily and Poxviridae family. CEV is the cause of a papular dermatitis in sheep and goats known as CE and is zoonotic, affecting humans [10]. The disease is characterized by inflammatory, proliferative and scabby lesions on the lips, nostrils and muzzle, and its frequency of occurrence and severity are particularly high in lambs. CE has a worldwide distribution, and it affects the majority of small ruminants and some wild animals. In an outbreak, up to 10% mortality in lambs and 93% mortality in kids have been recorded [7]. This disease impacts the economic well-being of farmers and causes economic losses. Loop-mediated isothermal amplification (LAMP) is a highly specific, efficient and rapid nucleic acid amplification method that amplifies DNA under isothermal conditions [11]. Gene amplification products form a ladder-like pattern on an agarose gel and show a visual color change when the fluorescent dsDNA intercalating dye SYBR Green I is used. LAMP may be used more easily and rapidly than PCR in clinical medicine and has been successfully developed to diagnose many diseases [1, 6, 8, 9, 13, 17].

The outer primers (5′ GACCCCGAGCTCATGGT 3′ for F1L-F3 and 5′ GCCGCGTCTTCACCTGTA 3′ for F1L-B3), inner primers (5′ CCTCCTTGATGATCGC-GTCGTGACGTCTCGCTAGACGCCTA 3′ for F1L-FIP and 5′ GAGGTGTTCACGCTGGAGAAGCAGTACTC-GGGGTAGACCAC 3′ for F1L-BIP) and the loop primer (5′ GAGCTTCTTCATGCCGCC 3′) of the F1L gene were designed using the PrimerExplorer 4.0 software based on a conserved region of the F1L gene identified by sequence alignment. The primers were synthesized by Takara.

Viral genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A standard plasmid, pGEM-T-easy-F1L, was constructed by insertion of a F1L gene fragment generated using the F1L-F3 and F1L-B3 primers into the pGEM-T-easy plasmid (Promega, Madison, WI, U.S.A.). The sequence of the pGEM-T-easy-F1L plasmid was verified by sequencing at SinoGenoMax. The standard plasmid DNA was extracted using an AxyPrep Plasmid Miniprep Kit (Axygen BioScience, Union City, CA, U.S.A.). The concentration of plasmid was measured using an Epoch Multi-Volume Spectrophotometer System (BioTek, Winooski, VT, U.S.A.), and the copy number was then calculated.

The LAMP assay was performed in a reaction mixture of 25 μl containing 1×Bst buffer (NEB, Ipswich, MA, U.S.A.), 8.0 mM $MgSO_4$, 0.8 M betaine (Sigma-Aldrich Chemie GmbH, Munich, Germany), 1.4 mM dNTPs, 8 U Bst polymerase (NEB, Ipswich, MA, U.S.A.), 0.2 μM each of the F3 and B3 primers, 1.6 μM each of the FIP and BIP primers, 0.4 μM LoopF primer and 1 μl extracted DNA as the template. The reaction mixtures were incubated at 65°C for 45 min. The products were visualized with the naked eye after addition of SYBR Green I (Invitrogen, Thermo Fisher Scientific, Waltham, MA, U.S.A.). The products were also

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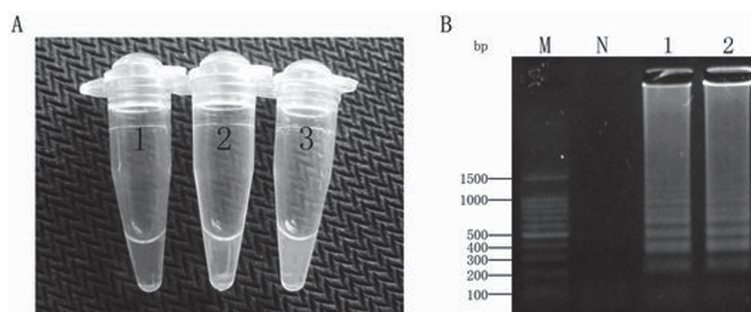

Fig. 1. Establishment of the LAMP reaction for orf virus. A: SYBR Green I staining. 1: Negative control. 2: ORFV genomic DNA. 3: Standard plasmid. B: Agarose gel analysis. M: 100 bp ladder marker. N: Negative control. 1: ORFV genomic DNA. 2: Standard plasmid.

resolved in 2% agarose gel and visualized on a UV transilluminator. An evaluation of the amplification temperature (57–69°C); effects of different concentrations of $MgSO_4$ (2–12 mM), the ratio of outer, loop and inner primers (1:2:1–1:2:14); and the reaction time (15–75 min) was performed to optimize the LAMP reaction.

The specificity of the LAMP assay was determined by applying the DNA from CEV, capripoxvirus (CPV), fowlpox virus (FPV), foot-and-mouth disease virus serotype O (FMDV-O), foot-and-mouth disease virus serotype Asia I (FMDV-Asia I) and bluetongue virus (BTV). These viruses were maintained in our laboratory. To evaluate the sensitivity of the LAMP assay, the CEV standard plasmid (1–10^8 copies) was used as template in PCR reactions. The PCRs were performed in a 25 μl reaction mixture containing 1×PCR buffer, 0.2 mM each dNTP, 0.2 μM F1L-F3 and F1L-B3 primers, 1 μl extracted DNA and 0.1 U Taq DNA polymerase (Takara, Otsu, Japan). The PCR reactions were performed using the following protocol: 95°C for 10 min; 35 cycles denaturation at 94°C for 30 sec, 30 sec of annealing at 55°C and 30 sec of primer extension at 72°C; and then a final extension at 72°C for 10 min. The LAMP products were visualized with the naked eye after the addition of SYBR Green I. Both the LAMP and PCR products were separated on 2% agarose gel and visualized on a UV transilluminator.

Positive results were obtained for the detection of CEV genome DNA and the standard plasmid with the LAMP method established in this study. After the LAMP reaction was completed, we observed a color change from orange to green with a positive LAMP reaction with the addition of SYBR Green I (1000×, 1 μl, Invitrogen, Thermo Fisher Scientific) to the reaction mixture (Fig. 1A). This color change was particularly significant against a black background. The positive LAMP reaction generated a laddering pattern with a set of different sized bands consisting of several inverted-repeat structures, as determined by 2% agarose gel electrophoresis (Fig. 1B).

Positive amplification was detected in 45 min with both genomic DNA and the standard plasmid when the reaction temperature was higher than 61°C. The gel electrophoresis bands were clearly visible when the reaction temperature was higher than 63°C, and no difference was observed when the reaction temperature varied from 63 to 69°C (Fig. 2A). The results indicate that the Mg^{2+} concentration must be at least 6 mM to yield a positive reaction, and no additional change was observed up to 14 mM Mg^{2+} (Fig. 2B). Positive reactions were obtained using ratios of outer, loop and inner primers ranging from 1:2:4 to 1:2:14, and no significant difference was observed (Fig. 2C and 2D). Positive amplification was detected within as little as 30 min, and product formation reached the maximal level after 45 min (Fig. 2E). The good conditions for the LAMP assay were determined to be 65°C for 45 min with 8.0 mM $MgSO_4$, 0.8 M betaine, 1.4 mM dNTPs, 0.2 μM each outer primer, 0.8 μM each inner primer, 0.4 μM loop primer and 8 U Bst polymerase.

All viruses were detected with this LAMP assay, and only DNA from the CEV was amplified; the results confirmed the specificity of this assay. Both SYBR Green I staining and agarose gel electrophoresis yielded the same result.

The detection limits for the LAMP assay and PCR were 10^2 and 10^4 copies of the standard plasmid, respectively. No amplified products were detected in the negative controls. Therefore, the sensitivity of the LAMP was 100-fold higher than that of PCR (Fig. 3). The fluorescent green and orange colored products were visualized after SYBR Green I staining, and the sensitivities were consistent with the gel electrophoresis results. The results showed that the LAMP assay has the potential to replace PCR because of its simplicity, rapidity, specificity, sensitivity and cost-effectiveness without the need for specialized equipment.

Jilin Province is the main sheep-breeding region in China, and CEV has been reported in the literature in China [3, 5, 18]. CE is an important zoonotic disease, and detailed and updated information on epidemiology in sheep would not only help in updating the knowledge of the scientific community but also would be useful for policy makers in formulating appropriate measures for control and eradication of the disease. Here, three hundred and 65 cases (including the skins of the back, udder, limbs and tail) from 64 flocks in 9 different areas of Jilin Province, China, from 2011 to 2014 were tested using the LAMP assay. The results showed that the average positive rate was 19.73% (72/365),

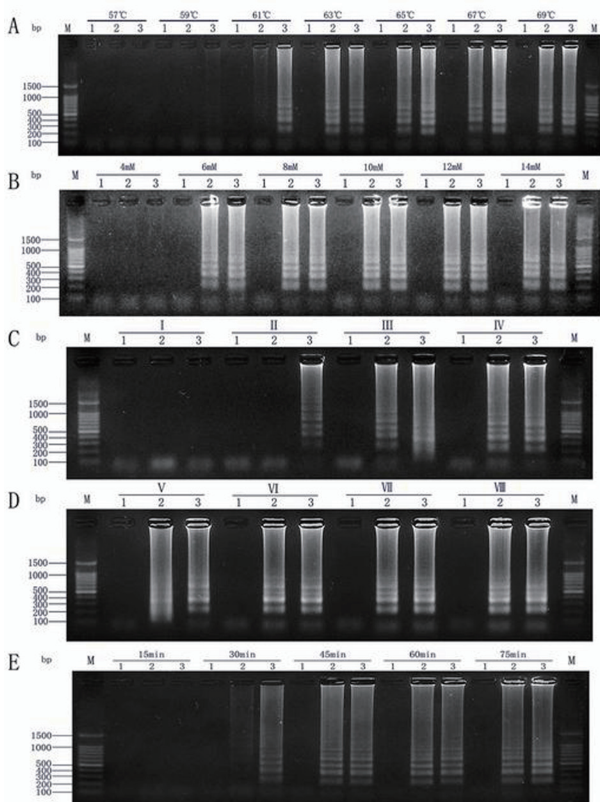

Fig. 2. Optimization of the LAMP reaction for orf virus. M: 100 bp ladder marker. 1: Negative control. 2: ORFV genomic DNA. 3: Standard plasmid. (A) The effect of temperature: lanes 1–7 (57, 59, 61, 63, 65, 67 and 69°C, respectively). (B) The effect of MgSO4: lanes 1–6 (4, 6, 8, 10, 12 and 14 mM, respectively). (C, D) The effect of the ratio of outer, loop and inner primers: 8 pairs see Table 2 (I–VIII). (E) The effect of reaction time: lanes 1–5 (15, 30, 45, 60 and 75 min, respectively).

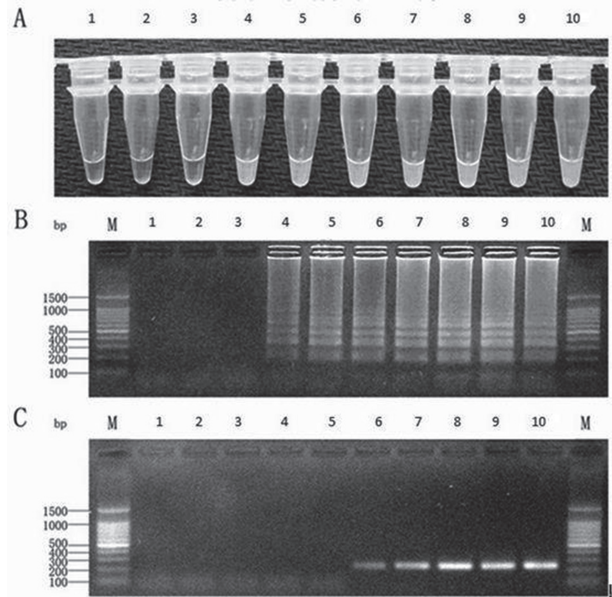

Fig. 3. The sensitivity of LAMP and PCR. The panels show the DNA templates used at 0–10^8 copies/reaction of the ORFV standard plasmid. M:100 bp ladder marker. A, B: The sensitivity of LAMP with SYBR Green I staining and agarose gel analysis. C: The sensitivity of PCR with agarose gel analysis.

Table 1. Detection rate comparative analysis of LAMP and PCR assays

Samples	No. of samples examined	No. (%) of samples detected by	
		LAMP	PCR
Lambs	154	52 (33.77)	52 (33.77)
Adult sheep	82	11 (13.41)	10 (12.2)
Ram	26	1 (3.85)	1 (3.85)
Ewe	103	8 (7.77)	7 (6.8)
Total	365	72 (19.73)	70 (19.18)

Table 2. The ratio of the primers and their concentration

Pairs	Primers ratio (F3:B3:LF:FIP:BIP)	Primers concentration (μM/l)
I	1: 1: 2: 1: 1	5: 5: 10: 5: 5
II	1: 1: 2: 2: 2	5: 5: 10: 10: 10
III	1: 1: 2: 4: 4	5: 5: 10: 20: 20
IV	1: 1: 2: 6: 6	5: 5: 10: 30: 30
V	1: 1: 2: 8: 8	5: 5: 10: 40: 40
VI	1: 1: 2: 10: 10	5: 5: 10: 50: 50
VII	1: 1: 2: 12: 12	5: 5: 10: 60: 60
VIII	1: 1: 2: 14: 14	5: 5: 10: 70: 70

and the positive rate was highest in lambs aged 1–6 months (Table 1). Conventional PCR assays were also used to evaluate the three hundred and 65 cases, and the positive rate for conventional PCR was 19.18% (70/365). The results showed that the LAMP assay is more sensitive than PCR for the detection of clinical samples. The CEV antibodies of 2104 serum samples collected from the flocks mentioned above were examined by indirect ELISA. The results showed that 812 serum samples were positive, and the average positive rate was 38.59% (812/2104). The epidemiology of CEV has not been reported in the literature in Jilin Province, China, and our results demonstrated that CEV infection was very widespread in the flocks of Jilin Province. According to the sites of pathological changes, the disease can be divided into the lip type, breast type, vulva type and mixed type, and among these types, the lip type was most common. None of the animals had been inoculated with a CEV vaccine, so the feeding density and differences in environment were considered to be the main causes of infection.

Currently, PCR is an important technique for disease diagnosis, A conventional PCR assay based on amplification of part of the CEV gene (B2L) has been developed [14]. Moreover, Real-time PCR has also proved to be accurate and effective in the quantification of Orf viral DNA [2, 4, 12], However, these assays require complex operations and high-precision instruments. LAMP is especially useful

in resource-limited situations. Reliable detection of CEV is fundamental for the exclusion of other rash-causing illnesses (e.g, capripox, foot-and-mouth disease, staphylococcal infection and bluetongue). To date, a LAMP assay based B2L for the detection of CEV has been reported [15]. In this study, the sensitivity of the LAMP assay was 100 copies, which was less sensitive than assays previously reported [14, 16]; however, the assay in this study can be completed within 45 min, which can save 15 min of time and the established LAMP assay in this study had good specificity.

ACKNOWLEDGMENTS. This work was supported by the China Natural Science Foundation (31372413) and the Key Projects of Jilin Provincial Science and Technology Department (No. 20110235).

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