

Development and Evaluation of a Loop-Mediated Isothermal Amplification Method for Rapid Detection of *Anaplasma ovis*[∇]

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***Anaplasma ovis* is an intraerythrocytic rickettsial pathogen of small ruminants. Loop-mediated isothermal amplification (LAMP) is a nucleic acid detection method in which the target DNA can be efficiently amplified with high specificity and sensitivity under isothermal conditions. In this study, a LAMP method was developed for the specific detection of *A. ovis*, using LAMP primers designed on the basis of the major surface protein 4 gene. LAMP was performed at 65°C for 30 min. Its specificity was confirmed by successful amplification of several *A. ovis* isolates and through EcoRI restriction analysis of LAMP products. No cross-reactivity with the *A. marginale* Lushi isolate, *Mycoplasma mycoides* subsp. *capri*, *Chlamydomphila psittaci*, *Theileria ovis*, *T. luwenshuni*, *T. uilenbergi*, or the *Babesia* sp. Xinjiang isolate was observed. Detection using the LAMP method was compared with that using conventional PCR in 227 field samples; LAMP demonstrated a sensitivity of 95.45%. In summary, LAMP is a specific, sensitive, and rapid test for the diagnosis of *A. ovis* infection, with the potential to be standardized as a detection method for *A. ovis* in areas of endemicity.**

Anaplasma ovis is an intraerythrocytic rickettsial pathogen of sheep, goats, and some wild ruminants (2, 11, 24). It is classified in the genus *Anaplasma* (Rickettsiales: Anaplasmataceae), along with *A. marginale*, *A. phagocytophilum*, and *A. bovis*, which also infect ruminants, and *A. platys*, which infects dogs (2, 5, 6). In China, *A. ovis* was first found in sheep in the Xinjiang Uygur Autonomous Region (17) and in goats in Liaoning Province (4). A later investigation revealed that *A. ovis* was widely distributed in sheep and goats in northwest China, including Inner Mongolia, Gansu, Shaanxi, Ningxia, and Qinghai Provinces (16). Experimental transmission studies showed that *Dermacentor nuttalli*, *Hyalomma asiaticum kozlovi*, and *Rhipicephalus pumilio* ticks act as biological vectors of *A. ovis* in China, transmitting *A. ovis* by transient infestation when they move from one host to another (16, 22). A recent study demonstrated that ruminant-infective *Anaplasma* species may also be widely present in the southwest of China (25).

The most frequently used method for diagnosing *A. ovis* infection involves microscopic detection of the *A. ovis* pathogen in blood following Giemsa staining. Serologically, the complement fixation test has also been used for field sample investigations in China (16); however, this test is difficult to perform and has fallen into disuse. A competitive enzyme-linked immunosorbent assay has also been used for diagnosis of *A. ovis* infections (21). Nucleic acid-based molecular tools, such as

PCR using the 16S rRNA gene (15) and major surface protein 4 (MSP4) gene (2) and the reverse line blotting method (1), have been shown to be of great diagnostic value in the identification of *A. ovis* infections. However, these methods are time-consuming and require expensive experimental devices and a high degree of laboratory experience in molecular biology. The present study aimed to develop a loop-mediated isothermal amplification (LAMP) method (20) for the rapid diagnosis of *A. ovis* infection and to evaluate its applicability by testing field samples and comparison with PCR.

MATERIALS AND METHODS

Preparation of template DNA. Genomic DNA (g-DNA) of *Anaplasma* species was prepared using a commercial kit, as described previously (15), and stored at –70°C until use. *A. ovis* DNA samples from the Yuzhong, Zhangjiachuan, and Yongjing isolates were used as positive samples for the establishment of the LAMP assay, while DNA samples from the *A. marginale* Lushi isolate, *Mycoplasma mycoides* subsp. *capri*, *Chlamydomphila psittaci*, *Theileria ovis*, *T. luwenshuni*, *T. uilenbergi*, and the *Babesia* sp. Xinjiang isolate and g-DNA of sheep were used as controls. Among the control DNA samples, *Mycoplasma* and *Chlamydia* DNAs were gifts from J. Zhou of the zoonotic disease group at our institute, and the others were reference samples collected by us (as members of the Vector and Vector-Borne Diseases Group). The concentrations of the DNA samples were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, China) and then diluted to 50 mg/ml.

Design of primer set for LAMP assay. The primer set was designed on the basis of the MSP4 gene of *A. ovis* (Table 1). The MSP4 genes of *A. ovis* from the Yongjing, Yuzhong, Zhangjiachuan, and Chengde isolates were amplified by PCR, as described previously (2). PCR products were cloned into a pGEM-T Easy vector (Promega, Beijing, China) and subjected to sequencing (Sangon Biotech, Shanghai, China). The forward outer primer (F3), backward outer primer (B3), forward inner primer (FIP), and backward inner primer (BIP) were designed on the basis of the sequences of these MSP4 genes using the Primer Explorer (version 4) program (<http://primerexplorer.jp/elamp4.0.0/index.html>), while the loop primers (LF and LB) were manually designed. To confirm the specificity of the LAMP reaction, two EcoRI restriction enzyme cleavage sites were created in the F1 complementary and F2 primers and the B1 complemen-

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TABLE 1. Nucleotide sequences of LAMP primers for detection of *A. ovis*

Primer	Type	Sequence (5'-3') ^a
MSP4F3	Forward outer primer	GTGTTGCACACAGATTGCC
MSP4B3	Backward outer primer	AGGCTTTTGTCTTCTCCGG
MSP4FIP	Forward inner primer (F1c + F2)	GCCCCTGTAGGCTAGCTTTGTG <i>gaattc</i> CCCATATGTGTGTGCCGG
MSP4BIP	Backward inner primer (B1c + B2)	TGGTGGTAGGTGGGTTCTACC <i>Agaattc</i> ATGTGCGGGGTATGTCCTTG
MSP4LF	Loop primer F	TGTCGACAAAGCTAGCACC
MSP4LB	Loop primer B	CGGACTCTTTGACGAGTCTT

^a Lowercase italics in FIP and BIP primer sequences indicate EcoRI restriction sites.

tary and B2 (Table 1) primers for subsequent restriction analysis of LAMP products. The primers were synthesized by TaKaRa (Dalian, China).

Establishment, specificity, and sensitivity of LAMP. LAMP was performed in a final volume of 25 μ l, containing 12.5 μ l 2 \times LAMP reaction buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄, and 0.2% Tween 20], 125 μ M each deoxynucleoside triphosphate (dNTP), 0.8 M betaine (Sigma-Aldrich), 8 U of *Bst* DNA polymerase large fragment (New England BioLabs, United Kingdom), 40 pmol each FIP and BIP primers, 20 pmol LF and LB primers, 5 pmol each F3 and B3 primers, and 2 μ l of target DNA. The reaction mixture was incubated at 65°C for 30 min using a conventional heating block (Eppendorf, Germany) and then heated at 80°C for 5 min to terminate the reaction. The specificity of LAMP was examined by testing 2 μ l of g-DNA of *A. ovis* Yongjing, *A. ovis* Yuzhong, *A. ovis* Zhangjiachuan, the *A. marginale* Lushi isolate, *M. mycoides* subsp. *capri*, *C. psittaci*, *T. ovis*, *T. luwenshuni*, *T. uilenbergi*, and the *Babesia* sp. Xinjiang isolate, as well as sheep g-DNA and a water control. The sensitivity of the reaction was evaluated by measuring the concentration of plasmid DNA containing the MSP4 gene of *A. ovis* Yuzhong using a Nanodrop 2000 spectrophotometer (Thermo Scientific), and the corresponding copy number was calculated using the method described by Lee et al. (12). The plasmid was diluted to contain 500 copies/ μ l and then serially diluted 10-fold. Two microliters was used in each reaction mixture when the sensitivity of the assay was evaluated. The LAMP products were subjected to electrophoresis on a 2% agarose gel containing 0.5 μ g/ml ethidium bromide, followed by visualization under UV light.

EcoRI restriction digestion of LAMP products. The LAMP products were purified using a PCR purification kit (TaKaRa, China) to remove the oligonucleotide residues. The purified LAMP products were digested with EcoRI (New England BioLabs, United Kingdom) at 37°C for 1 h. Digested products were analyzed by a 2.0% agarose gel electrophoresis, as described above.

Analysis of field samples using LAMP and PCR methods. A total of 227 field blood samples were collected from sheep in Yongjing County of Gansu Province. DNA extraction and preparation were performed using a DNeasy blood and tissue kit (Qiagen, China), following the manufacturer's instructions. These DNA samples were analyzed using both LAMP and a PCR method (2). The PCR primers were forward primer MSP4-5 (5'-GGGAGCTCCTATGAATTACAGAGAATTTGTTTAC-3') and reverse primer MSP4-3 (5'-CCGGATCCTTAGCTG AACAGGAATCTTGC-3'). The reactions took place in a final volume of 50 μ l, which contained 1.0 mM each primer, 5 μ l PCR buffer, 4 μ l dNTPs, 0.25 μ l *Taq* (5 U/ml; TaKaRa, China), and 1 μ l of DNA sample. Reactions were performed in an automated DNA C1000 thermal cycler (Bio-Rad, Beijing, China) for 35 cycles. After an initial denaturation step of 30 s at 94°C, each cycle consisted of a denaturing step of 30 s at 94°C, an annealing step of 30 s at 60°C, and an extension step of 1 min at 68°C. Reaction mixtures included g-DNA of *A. ovis* Yongjing as a positive control, and sheep g-DNA and distilled water were used as negative controls. The LAMP and PCR products were subjected to electro-

phoresis on agarose gels containing 0.5 μ g/ml ethidium bromide, followed by visualization under UV light.

Nucleotide sequence accession numbers. The sequences obtained were submitted to GenBank under accession numbers HQ456347 to HQ456350.

RESULTS

Optimum reaction conditions, specificity, and sensitivity of LAMP. The optimum incubation temperature for LAMP with the *A. ovis* primer set was established using a range of temperatures from 61 to 65°C. All temperatures gave positive results (data not shown), but 65°C was chosen as the reaction temperature for all applications. Time periods for the reactions ranging from 15 to 45 min were then tested at this reaction temperature. Analysis of the results indicated that incubation for 30 min was sufficient to allow the reaction to occur (Fig. 1). Under these conditions, six LAMP primers produced LAMP amplicons from g-DNA of *A. ovis* isolates (Yongjing, Yuzhong, and Zhangjiachuan) in a ladder pattern, while there were no products from DNA of other related pathogens, uninfected sheep DNA, or the water control (Fig. 2). To confirm that LAMP products correspond to the correct target sequence, the amplified products were digested with EcoRI enzyme (there are no EcoRI restriction sites in the target MSP4 gene sequence) and were introduced by use of FIP and BIP primers. LAMP products before and after digestion were analyzed by agarose gel electrophoresis (Fig. 3). The sensitivity of LAMP was evaluated by testing 10-fold serial dilutions of plasmid (starting from 1,000 copies) containing the MSP4 gene of *A. ovis*. The LAMP assay had a DNA detection limit of 1 copy of plasmid DNA (Fig. 4).

Detection of field samples using LAMP assay and PCR. Detection of field samples collected from 227 sheep in

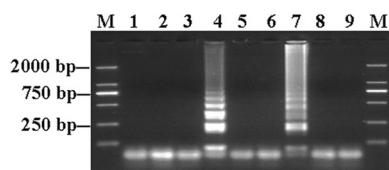


FIG. 1. Establishment of optimal incubation time for LAMP at 65°C. Lanes: M, DNA marker; 1, 4, and 7, DNA of *A. ovis* Yongjing; 2, 5, and 8, sheep g-DNA; 3, 6, and 9, water control; 1 to 3, reactions for 15 min; 4 to 6, reactions for 30 min; and 7 to 9, reactions for 45 min.

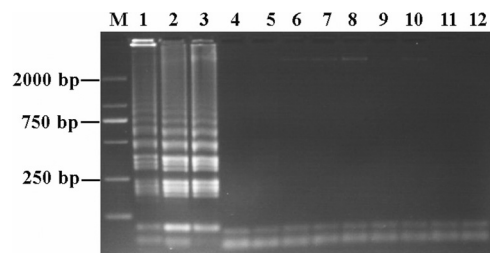


FIG. 2. Specificity of LAMP primers for *A. ovis*. g-DNA from the indicated species was incubated for 30 min at 65°C. Lanes: M, DNA marker (2 kb); 1, *A. ovis* Yuzhong isolate; 2, *A. ovis* Yongjing isolate; 3, plasmid containing MSP4 gene; 4, *M. mycoides*; 5, *C. psittaci*; 6, *A. marginale* Lushi isolate; 7, *T. ovis*; 8, *T. luwenshuni*; 9, *T. uilenbergi*; 10, *Babesia* sp. Xinjiang isolate; 11, sheep g-DNA; 12, water control.

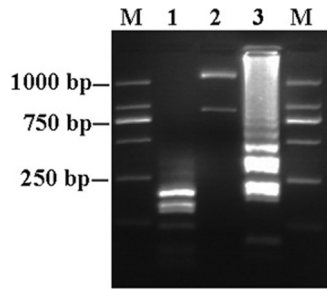


FIG. 3. Restriction digestion of LAMP products from *A. ovis*. EcoRI restriction sites were introduced in FIP and BIP primers, allowing restriction fragment analysis of the specificity of the LAMP reaction. Lanes: M, DNA marker; 1, EcoRI digest of *A. ovis* LAMP product; 2, EcoRI enzyme activity control by digesting reconstructed plasmid DNA in pGEM-T Easy vector; 3, LAMP products from g-DNA of *A. ovis*.

Yongjing County of Gansu Province was conducted using both the LAMP and the PCR methods. A comparison of the results is presented in Table 2, where LAMP detected *A. ovis* DNA from 157 samples (69.16%) and PCR detected DNA from 66 samples (29.07%). However, 94 samples found to be positive by LAMP were negative when tested by PCR. Likewise, 3 samples found to be negative by LAMP were positive when tested by PCR. Moreover, results for 63 positive samples and 67 negative samples were in agreement with both techniques. On the basis of the data in Table 2, using PCR as the reference method, the sensitivity and specificity of LAMP are 95.45% and 41.61%, respectively.

DISCUSSION

LAMP is a nucleic acid amplification method that has demonstrated the advantages of being rapid, easy to use, and less expensive than other nucleic acid-based tests for the diagnosis of infectious diseases (18, 20). It therefore has the potential for use in resource-limited veterinary laboratories in developing countries, such as China, where many endemic diseases exist. *A. ovis* has been found to be distributed over 44 counties in Gansu Province, with infection rates of 30.1 to 60.8% (16). Ovine *Theileria* and *Babesia* infections have also been found in regions of Gansu Province in recent years (13, 23). It is difficult to differentiate between *Piroplasma* and *Anaplasma* pathogens on the basis of clinical signs and microscopic examination, and a rapid diagnostic method, LAMP, was therefore developed in the present study. LAMP primers were designed on the basis of the MSP4 gene sequence of *A. ovis*, which is reportedly

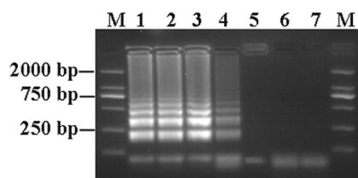


FIG. 4. Sensitivity of LAMP primers for *A. ovis* using 10-fold serial dilutions of plasmid DNA containing the MSP4 gene of *A. ovis*. Lanes: M, DNA marker; 1, 1,000 copies; 2, 100 copies; 3, 10 copies; 4, 1 copy; 5, 0.1 copies; 6, 0.01 copies; 7, 0.001 copies.

TABLE 2. Comparative evaluation of LAMP and PCR methods for detection of field samples

LAMP result	No. (%) of specimens with the following PCR result:		
	Positive	Negative	Total
Positive	63	94	157 (69.16)
Negative	3	67	70
Total	66 (29.07)	161	227

highly conserved among *A. ovis* isolates (2). Alignment of the MSP4 sequences from four *A. ovis* isolates from Yongjing, Jintai, Zhangjiachuan, and Chengde confirmed 99.8% sequence identity. Using such a conserved gene as a target gene for LAMP is important, because for the LAMP reaction to occur, all eight primers must specifically bind to the target sequences (19). The primers used in this study allowed the LAMP reaction to be completed within half an hour. The high level of efficiency was probably attributable to the introduction of the loop primers (LP and LB), which may have reduced the time taken for amplification by half (19).

We demonstrated that the LAMP primers specifically amplified g-DNA of *A. ovis* isolates but not *A. marginale*, confirming the high specificity of LAMP for the diagnosis of *A. ovis* infection. The ability of LAMP to amplify different isolates of *A. ovis* is critical to its usefulness for detecting the organism in a wide range of areas of endemicity. LAMP did not react with g-DNA of the *A. marginale* Lushi isolate, probably because of the low level of similarity (91.5 to 91.6%) of the MSP4 gene between *A. ovis* isolates and *A. marginale* Lushi (MSP4 gene GenBank accession number HM640938). *A. phagocytophilum* was not included in the current study, but alignment of the MSP4 gene sequences from *A. ovis* isolates and *A. phagocytophilum* strains (3) showed a maximum similarity of 64.4%, suggesting that there is unlikely to be any cross-reaction between these species; however, this should be confirmed experimentally. Digestion of the LAMP products by EcoRI further confirmed the specificity of the assay.

The sensitivity of LAMP using 10-fold serial dilutions of plasmid DNA containing the MSP4 gene indicated that it was able to detect levels of plasmid DNA as low as 1 copy. However, further studies are required to confirm the sensitivity of LAMP for the detection of *A. ovis* parasitemia. In a comparative evaluation of 227 field samples, the LAMP assay detected more positive samples (69.2%) than PCR (29.1%), implying a higher sensitivity. The higher sensitivity of LAMP than conventional PCR has been demonstrated in several studies (8, 14, 20), and the sensitivity of LAMP has been shown to be less affected by components of the clinical samples than PCR (7, 9). Nevertheless, three samples identified as negative by LAMP produced weak bands with conventional PCR, possibly reflecting the fact that PCR primers amplify g-DNA from *A. marginale*, as well as *A. ovis*. *A. marginale* is known to infect other ruminants (10). Attempts to sequence these three positive PCR products have not yet been successful. However, the MSP4 gene sequence obtained from an *Anaplasma* sp.-infected sheep in Jingtai County of Gansu Province (GenBank accession number HM195103) showed 98% similarity with the MSP

gene sequence of *A. marginale*, suggesting possible *A. marginale* infection in sheep in Gansu Province.

In summary, the LAMP method developed in this study shows great potential as a test for the easy diagnosis of ovine anaplasmosis in regions of endemicity in China. It could provide an accurate, sensitive, affordable, and easy-to-use method and a practical alternative to PCR for the routine diagnosis of anaplasmosis caused by *A. ovis*.

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