Letter to the Editor

Combination of Loop-Mediated Isothermal Amplification Assay and Nested PCR for Detection of *Borrelia burgdorferi sensu lato* in Human Serum Samples*

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A set of universal loop-mediated isothermal amplification (LAMP) primers targeting the *fla* gene was designed to detect *Borrelia burgdorferi sensu lato* (*B. burgdorferi* s.l.) in human samples. The sensitivity of LAMP was 20 copies/reaction, and the assay did not detect false positives among 11 other related bacteria. A positive LAMP result was obtained for 9 of the 24 confirmed cases and for 12 of 94 suspected cases. The positive rate of LAMP was the same as that of nested PCR. The LAMP is a useful diagnostic method that can be developed for rapid detection of *B. burgdorferi* s.l. in human sera. Combination of the LAMP and nested PCR was more sensitive for detecting *B. burgdorferi* s.l. in human serum samples.

Lyme disease is a global tick-borne disease caused by infection with *Borrelia burgdorferi sensu lato* (*B. burgdorferi* s.l.). The distribution of *B. burgdorferi* s.l. is diverse and wide in China[1]. The laboratory diagnosis of Lyme borreliosis depends mainly on serological and molecular biology methods. Serological methods are routine testing, including screening tests such as enzyme-linked immunosorbent assays (ELISA), indirect immunofluorescence assays (IFA), and confirmation tests such as Western blot[2-4]. Because of the distribution of the different genomic species, every country requires to build its own criteria for the standardized Western blot[2-4]. Molecular techniques such as conventional PCR, nested PCR, and real-time PCR are based on some specific gene detection, for example, *ospA*, *rrs*, *rrf-rrl* intergenic spacer, *groEL*, *recA*, *hbb*, *fla*, and so on. However, these techniques have intrinsic disadvantages: time-consuming, unideal sensitivity, easily contaminated, or requiring specific expensive instruments for the amplification. Because the incidence of *B. burgdorferi* s.l. infection is high in forest regions, particularly in rural areas in China, rapid, simple, cheap, and effective diagnostic methods are urgently required.

Notomi et al.[5] developed a novel approach to nucleic acid amplification, loop-mediated isothermal amplification (LAMP) assay, which has been applied for the molecular detection of various pathogens. A LAMP assay based on 16S rRNA gene was developed to detect *B. burgdorferi* s.l. in ticks[6]. However, LAMP assay was not reported to detect *B. burgdorferi* s.l. in human serum samples. In this study, we aimed to develop a simple, rapid, sensitive, and specific LAMP assay to detect *B. burgdorferi* s.l. in human serum samples for the clinical diagnosis, epidemiological investigation, and surveillance of Lyme disease.

We chose five strains of four species of *B. burgdorferi* s.l., including B31 (*Borrelia burgdorferi sensu strict*), PD91 and Fuji (*Borrelia garinii*), FP1 (*Borrelia afzelii*), and QX-S13 (*Borrelia valaisiana*), and collected the genomic DNA. A total of 11 strains of different species bacteria were used to determine the specificity of the LAMP assay. The genomic DNAs of 8 members of Rickettsiales including *Rickettsia conorii*, *Rickettsia slovaca*, *Rickettsia hone*, *Coxiella burnetii*, *Orientia tsutsugamushi*, *Bartonella Quintana*, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and 3 members of common clinical pathogens including *Escherichia coli*,

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Leptospira spp., and *Brucellosis bacteria*, were obtained from relevant departments of National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention (China CDC).

One hundred eighteen serum samples were collected from Mudanjiang Forestry Center Hospital in 2010. This study was approved by the Ethical Review Committee of National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention (China CDC). Participants also provided their written informed consent to participate in this study. The 118 suspected cases were confirmed by the serological diagnostic criteria of *B. burgdorferi* s.l., tested by indirect immunofluorescence assays (IFA) first, then by Western blot to detect IgM and IgG antibodies[2]. DNA was extracted from the remaining serums using the QIAamp DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) for *rrf-rrl* intergenic spacer nested PCR[7] and the LAMP assay described in this study. The primers of nested PCR were as follows: of the first step, the forward primer 5'-CGACCTTCTTCGCCTTAAAGC-3' and the reverse primer 5'-TAAGCTGACTAATACTAATTACCC-3'; of the second step, the forward primer 5'-TCCTAGGCATTCA CCATA-3' and the reverse primer 5'-GAGTTCGCGG GAGA-3'.

The LAMP primers specific for *B. burgdorferi* s.l. were designed on the basis of its *fla* gene. The primers of nested PCR were as follows: the forward outer primer (F3) 5'-GCTGTTGAGCTCCTTCTTG-3'; the reverse outer primer (B3) 5'-CACCAGCGTCACTTTC AG-3'; the forward inner primer (FIP) 5'-TGCAAAT CTATTCTCTGGCGAAGGTTGAGCACCTTCTTGAACA-3'; the reverse inner primer (BIP) 5'-ACAGCAATCGCTT CATCTTGATTCTCAAGCTTCTTGGACC-3'. To compare the sensitivities of the LAMP assay and conventional PCR, performed with the primers B3 and, a recombinant plasmid containing the target sequence of *B. burgdorferi* s.l. *fla* gene from the strain PD91 (*B. garinii*) was constructed. All LAMP reactions were performed with the Loopamp DNA amplification Kit (Eiken Chemical Co. Ltd., Tokyo, Japan) in 25 μL reaction system and were incubated in a real-time turbidimeter LA320C (Teramecs, Tokyo, Japan) at 63 °C for 60 min and then at 80 °C for 5 min to terminate the reaction.

Positive results from the two methods were counted to analyze the difference between the LAMP assay and nested PCR using the chi-square test by SPSS software. For the analysis, *P* value less than 0.05 was considered to indicate statistical significance.

The results showed that the five reference strains of *B. burgdorferi* s.l. were positive by LAMP assay, with ladder pattern observed appeared on agarose gel (Figure 1A), which is characteristic of the LAMP reaction and indicates successful amplification. In addition, successful amplification were confirmed by sequencing and then the sequences obtained were compared to reference sequences from GenBank. While the 11 control strains, were negative by LAMP assay (Figure 1B). This indicated that the LAMP assay was specific to *B. burgdorferi* s.l. and no false-positive amplification with these heterologous species. Therefore, because of its four primers that recognize six distinct regions on the target DNA, the LAMP assay was very specific to *B. burgdorferi* s.l. The high specificity of LAMP methods has also been reported for other pathogens[8].

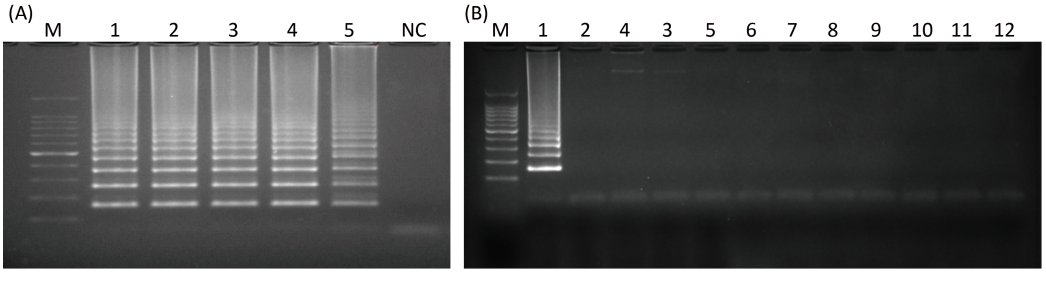

Figure 1. Specificity of the LAMP assay. 2% agarose gel electrophoresis analysis, Lanes M, 100 bp DNA ladder; NC, negative control. (A) Lanes 1 to 5, B31, PD91, Fuji, FP1, and QX-S13 strains, respectively; (B) Lanes 1, PD91; Lanes 2 to 12, the 11 control bacteria strains including *Rickettsia conorii*, *Rickettsia slovaca*, *Rickettsia hone*, *Coxiella burnetii*, *Orientia tsutsugamushi*, *Bartonella Quintana*, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *Escherichia coli*, *Leptospira* spp., *and Brucellosis bacteria*, respectively.

The limits of detection of LAMP and conventional PCR (with F3, B3 primers) for the *fla* gene were 10^1 copies/μL (20 copies per reaction) and 10^2 copies/μL (400 copies per reaction), respectively (Figure 2). This indicated that the LAMP assay was more sensitive than the conventional PCR for detecting *B. burgdorferi* s.l. DNA. Moreover, the LAMP assay was able to amplify the 10^6 copies/μL of recombinant plasmid in 18 min, indicating that LAMP achieved more rapid detection than conventional PCR. In the study by Yang[6], the sensitivity of 16S rRNA LAMP assay were 0.2 pg (10^2 copies/μL), 0.2 pg (10^2 copies/μL), 0.02 pg (10^1 copies/μL) for BO23, B31, PBi, respectively.

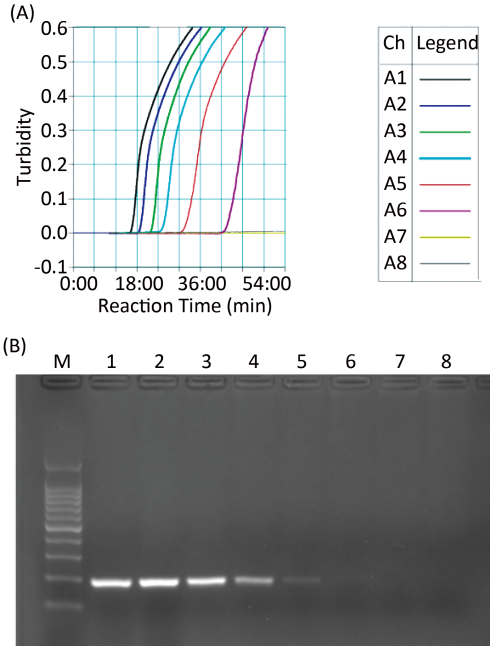

Figure 2. Comparative sensitivities of the LAMP assay and the conventional PCR for detection of the serially 10-fold diluted reference plasmid containing the target DNA. (A) The sensitivity of the LAMP assay was monitored by real-time measurement of turbidity (LA-320C, Teramecs). The abscissa was the reaction time and the vertical axis was the turbidity (400 nm). A1-A7: 10^6-10^0 copies/μL reference plasmid DNA template; A8: negative control. (B) The sensitivity of the conventional PCR was demonstrated by 1% agarose gel electrophoresis. Lane M, 100-bp DNA ladder; Lane 1 to 7: 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copies/μL, respectively; Lane 8: negative control.

A total of 118 samples were first tested by IFA and Western blot, 24 samples were serologically confirmed samples. 118 samples were then assayed by LAMP assay and nested PCR and the results were shown in Table 1. So, in the 24 confirmed samples, the positive rate of LAMP assay or nested PCR was 37.5% (9/24). In the 94 suspected samples, the positive rate of LAMP assay or nested PCR was 12.77% (12/94). This was similar to the result of the 16S rRNA LAMP assay in ticks[6]. In the total 118 samples, LAMP was in agreement with 5 (4 plus 1) positive and 81 (10 plus 71) negative nested PCR samples. However, discrepancies also occurred between LAMP and nested PCR: 16 (5 plus 11) positive LAMP samples were negative according to *rrf-rrl* nested PCR, 16 (5 plus 11) positive nested PCR samples were negative according to LAMP. This was probably because of the different sensitivity or the different target gene of the two methods. Although LAMP or nested PCR is sensitive, it could also miss positive samples, the reason might be an active infection with *Borrelia* does not necessarily need to be manifested as bacteria in the blood, it can be just bacteremia in local tissues or organs, not in blood. It was more sensitive (23/94, 24.47%) of detecting samples by both LAMP and nested PCR. In the 94 suspected samples, there were 12 (12.77%) positive by the LAMP and nested PCR, respectively. Therefore, serological methods could result in false-negative results, and molecular biology methods such as LAMP and nested PCR could compensate for the lack to some degree.

For LAMP amplification efficiency, the water bath is same as the real-time turbidimeter, but the latter could real-time monitor the result. The amplification

Table 1. Results of Loop-mediated Isothermal Amplification Versus Nested PCR in 24 Confirmed Serum Samples and 94 Suspected Serum Samples

Nested PCR	LAMP		Total
	Positive	Negative	
24 confirmed serum samples			
Positive	4	5	9
Negative	5	10	15
Total	9	15	24
94 suspected serum samples			
Positive	1	11	12
Negative	11	71	82
Total	12	82	94

efficiency of the LAMP method is extremely high due to continuous amplification under isothermal conditions, which results in the production of a large amount of target DNA. The LAMP assay with high amplification efficiency also produced a large amount of by-product magnesium pyrophosphate, a white-colored precipitate in the reaction mixture, which leaded to turbidity[9]. Therefore, the LAMP results were easily detected by naked-eye observation of increased turbidity or by real-time monitoring of the turbidity in an inexpensive photometer, which could reduce false-positive results and apply the technique in low-technology settings. For LAMP result reading, there were not any differences between agarose gel electrophoresis and turbidimeter. Therefore, the inexpensive photometer to monitor the turbidity of LAMP products is suitably applied in local clinics testing due to a lack of contamination resulting false-positive results.

We developed a simple, rapid, sensitive, and specific LAMP assay for detecting *B. burgdorferi* s.l. Combination of the LAMP and nested PCR was more sensitive for serum samples. Because the LAMP assay is fast (less than 1 h), cost effective, and sensitive for testing clinical samples just with water bath and simple operation, it may potentially be a valuable tool for rapid detection *B. burgdorferi* s.l. for the clinical diagnosis, epidemiological investigation, and surveillance of Lyme disease.

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