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Detection of Brucellosis in Sika Deer (*Cervus nippon*) through Loop-mediated Isothermal Amplification (LAMP)

Qianhong Liu,[1,2,3,4] Jie Wei,[1,3] Qingsong Sun,[1] Ben Wang,[1] Yuting Wang,[1] Ying Hu,[1] and Wenrong Wu[1] [1]Jilin Agricultural Science and Technology University, No. 77, The Academician Road, Economic Development Zone, Jilin City, Jilin Province, 132101, People's Republic of China; [2]Key Lab of Preventive Veterinary Medicine in Jilin Provence, Jilin Agricultural Science and Technology University, No. 77, The Academician Road, Economic Development Zone, Jilin City, Jilin Province, 132101, People's Republic of China; [3]These authors contributed equally to this study; [4]Corresponding author (email: qianhongliu@126.com)

ABSTRACT: Brucellosis (*Brucella bovis*) in sika deer (*Cervus nippon*) can cause enormous losses to stag breeding, especially in areas in which stag breeding has become an important industry. It also poses a threat to humans because it is a zoonotic disease. Use of the loop-mediated isothermal amplification (LAMP) assay has been poorly described in the diagnosis of brucellosis in deer. We developed a LAMP assay targeting the *omp25* gene sequence to detect brucellosis in sika deer. The reaction can be completed in 60 min at 63 C and, with a detection limit of 17 pg, it was more sensitive than conventional PCR, with its detection limit of 1.7 ng. No cross-reactivity was observed with four bacteria: *Escherichia coli*, *Salmonella enterica* subsp. *enterica*, *Clostridium pasteurianum*, and *Pseudomonas aeruginosa*. We used 263 samples of blood to evaluate the reaction. The percentage of agreement between LAMP and PCR reached 91%; relative specificity reached 87%, and relative sensitivity reached 100%. The results indicate LAMP can be a simple and rapid diagnostic tool for detecting brucellosis in sika deer, particularly in the field, where it is essential to control brucellosis in deer with a rapid and accurate diagnosis for removal of positive animals.

Key words: *Brucella bovis*, brucellosis, LAMP, loop-mediated isothermal amplification, sika deer.

Brucellosis (*Brucella bovis*) is a severe zoonotic disease with a nearly worldwide distribution. It is also one of the major infectious diseases of deer and has seriously restricted further development of the deer breeding industry (Adams 1989; Myers et al. 2015). Sika deer (*Cervus nippon*) that are infected with *Brucella bovis* can serve as a bacterial reservoir and transmit the infection to humans (Li et al. 2009). In some areas of China, the prevalence of brucellosis among deer is 28%, which has seriously influenced the development of deer farming (Li et al. 2007). Therefore, to prevent the spread of brucellosis, a suitable, reliable, and rapid detection method is necessary.

At present, in China, the methods used to diagnose brucellosis in deer in the field rely mainly on plate agglutination and tube agglutination, but the detection rates of those methods are low, making it difficult to rely on them for control of brucellosis. Loop-mediated isothermal amplification (LAMP) is widely used in the diagnosis of many diseases because it is a rapid and simple procedure with high specificity and sensitivity and inexpensive instruments (Kurosaki et al. 2016; Venkatesan et al. 2016). This study evaluated the application of LAMP as a simple method for rapid detection of brucellosis in the field in deer.

The *Brucella melitensis* 16M, *Brucella abortus* 544A, and *Brucella suis* S1330 strains were inactivated through heating to 70 C for 2 h and preserved in our laboratory at –20 C. The S19 vaccine strain was obtained from the China Institute of Veterinary Drug Control (Beijing, China). *Clostridium pasteurianum* (no. 1.208), *Salmonella enterica* subsp. *enterica* (no. 1.10603), and *Pseudomonas aeruginosa* (no. 1.10712) were obtained from China General Microbiological Culture Collection Center (Beijing, China). *Escherichia coli* DH5α was isolated in our laboratory. Clinical samples were obtained from 263 sika deer from 17 deer farms in the Jilin Province, China, during the years 2011–13. Before fall breeding each year, all sika deer were anesthetized and a blood sample was taken from each deer for testing.

The DNA was extracted from the test bacteria and the blood samples using a DNA

extraction kit (Promega, Beijing, China) per the manufacturers' instructions. Both the quality and the quantity of the DNA molecules were evaluated on 1.0% agarose gel as well as by spectrophotometric analysis. Then, the DNA samples were stored at –20 C until use.

The PCR reaction was carried out in a 25-μL volume containing 2 μL of 10-fold dilution series DNA, 2.5 μL deoxyribose nucleoside triphosphate (dNTP; 2.5 mM), 1.5 μL Taq polymerase (1.25 U; Thermo Fisher Scientific, Beijing, China), 2.5 μL 10X Taq buffer, and 2.5 μL each of primers P1 and P2 (20 μM). The amplification was as follows: 3 min at 94 C, followed by 30 cycles of 94 C for 45 s, 65–55 C for 30 s, 72 C for 50 s, with a final elongation for 10 min at 72 C. In 30 cycles, there were two cycles each at an annealing temperature from 65 C to 55 C, then 10 cycles of 55 C. The PCR reaction yielded a product of about 500 base pairs. The LAMP primers were designed using an online LAMP primer design program (PrimerExplorer, Eiken Chemical, Tokyo, Japan; Eiken Chemical 2016) targeting the *omp25* gene of *Brucella* spp. All the primers are listed in the Supplementary Material and were synthesized by the Shenggong Company (Shanghai, China).

The LAMP reaction (New England Biolabs, Ipswich, Massachusetts, USA) was performed according to instructions from the manufacturer using a 25-μL mixture containing 2.5 μL of 10X Thermopol buffer (New England Biolabs; 20 mM Tris–HCl, 10 mM KCl, 10 mM $[NH_4]_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 1.5 μL $MgSO_4$ (10 mM), 3.5 μL dNTP mix (10 mM), 4 μL forward inner primers (10 μm/L), 4 μL backward inner primers (10 μm/L), 0.5 μL F3 primers (10 μm/L), 1.0 μL B3 primers (10 μm/L), 1.0 μL Bst DNA polymerase, large fragment (8,000 U/mL; New England Biolabs), 1.0 μL DNA sample, and 6 μL nuclease-free water. The amplification included incubation for 60 min from 57 C to 65 C to determine the optimal reaction temperature and was then terminated by heating to 80 C for 20 min. Meanwhile, the control group (without DNA as a template, with 7 μL nuclease-free water added) was established. The LAMP products were subjected to electrophoresis on a 2% agarose gel. Finally, the LAMP conditions were optimized for 60 min at 63 C.

To evaluate the sensitivity of the LAMP assay, a serial dilution of 16M DNA was tested as a template. The results indicate that the detection limit of the LAMP assay was 17 pg (Fig. 1A), whereas it was 1.7 ng using PCR (Fig. 1B). The sensitivity of LAMP was 100-fold better than that of PCR. Visual inspection was performed with the SYBR green I dye at the same time to validate the LAMP results. The samples changed to yellowish-green in positive reactions and remained reddish-orange in negative reactions (Supplementary Material Fig. S1). The results of both the gel electrophoresis and visual detection of color change were consistent, and those results indicated that visual detection could be correlated with the results from the gel electrophoresis.

The detection specificity of LAMP was determined by DNA from the *Brucella* spp. strains (16M, 544A, S1330, S19), and other potential cross-reacting pathogens (*Clostridium pasteurianum*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica*, and *Escherichia coli*) as described earlier, at 63 C for 60 min. Gel electrophoresis analysis indicated that the LAMP reaction was negative for the other four potential cross-reacting pathogens as templates. Results from the DNA templates of the *Brucella* spp. strains—16M, 544A, S1330, and S19—were positive (Fig. 2).

A total of 263 clinical samples from 17 deer farms were tested by LAMP and by PCR, and the DNA of the 16M strain was used as a positive control. Water was used as the blank control. The PCR operating conditions described above were used to determine that 75 samples (28.5%) were positive by both LAMP and PCR, and 164 samples (62.4%) were negative by both LAMP and PCR. There were 24 samples (9.1%) that were positive by LAMP but were negative by PCR, and no samples that were negative by LAMP but positive by PCR (Table 1). The sensitivity and specificity of LAMP compared with PCR were 100% and 87.2%, respectively.

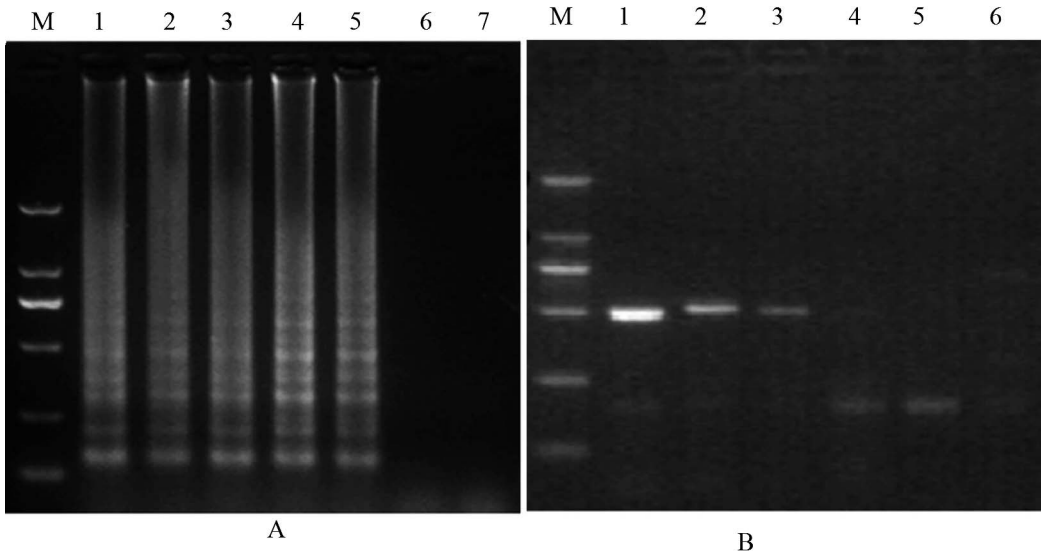

FIGURE 1. To evaluate the sensitivity of loop-mediated isothermal amplification (LAMP) and PCR, a serial dilution of *Brucella melitensis* 16M DNA was tested as a template. A represents the result of the LAMP assay. Lane M: DNA marker DL2000; Lanes 1–6: different 16M DNA concentrations subjected to LAMP (170 ng, 17 ng, 1.7 ng, 170 pg, 17 pg, 1.7 pg); and Lane 7: blank. B represents the results of the PCR assay. Lane M: DNA marker DL2000; Lanes 1–6: different DNA concentration subjected to PCR (170 ng, 17 ng, 1.7 ng, 170 pg, 17 pg); and Lane 6: blank.

Effective vaccines to control brucellosis in sika deer are not available. Commercially available vaccine strains used to control brucellosis in cattle have proven to be less effective in deer (Davis and Elzer 2002; Arenas-Gamboa et al. 2009a, b). Additional vaccination-related problems, including interference with diagnosis, resistance to antibiotics, and potential virulence for other animals and humans, have occurred. Population density of the sika deer is heavier in northeast China during the winter because of limited feeding conditions, and that increases the chance of infection. Because the disease appears to be highly correlated with feeding density, the heavier the feeding density, the greater the chance of exposure to infected animals (Etter and Drew 2006).

The detection rate of the methods (plate agglutination and tube agglutination tests) presently used in the field in China is so low that animals positive for the disease cannot be fully identified; therefore, infected animals cannot be eliminated from the herds. Rapid and accurate diagnosis and the removal of

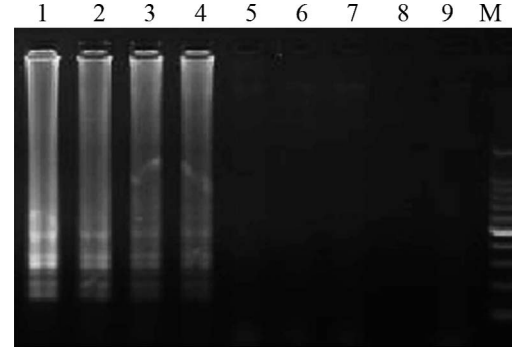

FIGURE 2. To detect the specificity of loop-mediated isothermal amplification (LAMP), the DNA from different *Brucella* spp. strains and other potential cross-reacting strains were used as templates. Lanes 1–4: LAMP products used the genome of *Brucella melitensis* 16M, *Brucella abortus* 544A, *Brucella suis* S1330, *Brucella melitensis* S19, respectively, as templates; Lanes 5–8: LAMP products used DNA from *Clostridium pasteurianum*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *Enterica*, and *Escherichia coli*, respectively; Lane 9: blank; and Lane M: DNA marker DL15000.

TABLE 1. Comparison of the specificity and sensitivity of loop-mediated isothermal amplification (LAMP) and PCR for the detection of the *Brucella bovis omp25* gene sequence in sika deer (*Cervus nippon*) from China. Percentage of agreement: (75+164)/263×100%=90.9%; relative specificity: 164/188×100%=87.2%; relative sensitivity: 75/75=100%.

	LAMP		
PCR	Positive	Negative	Total
Positive	75	0	75
Negative	24	164	188
Total	99	164	263

positive animals are the most effective way to control brucellosis. Using the LAMP technique, it is possible to diagnose disease without expensive training or equipment because the assay can be performed under isothermal conditions. In addition, the assay retains high specificity and sensitivity because of its use of four or more primers. These advantages mean that LAMP is especially suitable for use in the field.

Our results indicated that the sensitivity of LAMP was better than that of PCR. Of 263 samples, 99 (37.6%) were positive by LAMP, which is higher than what was reported by Li et al. (2007). The increased sensitivity of the test indicates that the prevalence of brucellosis in sika deer is very serious in northeast China. The increase in the scale of the breeding industry, the lack of effective detection methods, and the problems in isolating deer populations from domesticated cattle may be some of the reasons behind the increasing incidences of brucellosis. This study revealed that the LAMP assay was a potentially effective tool for detecting brucellosis in sika deer in the field because its rapidity, simplicity, high sensitivity and specificity, and the use of inexpensive equipment.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2016-05-105.

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