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Establishment of loop-mediated isothermal amplification (LAMP) for rapid detection of *Brucella* spp. and application to milk and blood samples

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ABSTRACT

Brucella spp. are facultative intracellular bacteria that infect humans and animals. In this study, the loop-mediated isothermal amplification (LAMP) was used to detect the *Brucella*-specific gene *omp*25. Reaction conditions were optimized as temperature 65 °C, reaction time 60 min, Mg^{2+} concentration 8.0 mmol/L, polymerase content Bst DNA, 0.5 μL, deoxyribonucleotide concentration 1.6 mmol/L, and inner/outer primer ratio 1:8. The LAMP method was evaluated with 4 *Brucella* species and 29 non-*Brucella* bacteria species. Positive reactions were observed on all the 4 *Brucella* species but not on any non-*Brucella* species. The limit of detection of the LAMP method was 3.81 CFU *Brucella* spp. Using the LAMP method, 7 of 110 raw milk samples and 5 of 59 sheep blood samples were detected positive of *Brucella* spp. Results indicated that LAMP is a fast, specific, sensitive, inexpensive, and suitable method for diagnosis of *Brucella* spp. infection.

1. Introduction

Brucella spp. infects humans and animals and cause disease worldwide (Matyas and Fujikura, 1984). Livestock that are most vulnerable to brucellosis include cattle, goats, and pigs (Radostits et al., 2000; Ettinger and Feldman, 1995). Infections often cause abortion in female animals and subsequently serious economic losses to the livestock industry. Currently, more than 60 animal species, including livestock, domestic fowl, other domesticated animals, and wildlife are known as reservoir hosts for *Brucella* spp. Transmission from animals to humans can occur easily via exposure to infected animals or ingestion of unsterilized meat or dairy products from infected animals (Wilkinson, 1993). Brucellosis, the disease caused by *Brucella* spp. infection has been reported in more than 170 countries and regions in the world, and about 1/5–1/6 of the world's population is under the threat of this disease. Each year, the total economic loss caused by brucellosis can be as high as several billions of U.S. dollars (Haerry and Gehring, 1996; Qiu, 2004). Exposure to feed or water contaminated with *Brucella* spp. can cause brucellosis in healthy livestock. Even spin-drift or dust contaminated by *Brucella* spp. can lead to respiratory tract infections. In addition, the occurrence and spreading of brucellosis is not restrained by geographic conditions. Although, in general, the infection rate is higher in pasture regions, lower in urban area and intermediate in non-pasture rural areas, the incidence of brucellosis is also relatively high in towns near pastures or in middle-sized and large cities that are heavily involved in the fur, dairy, and meat industries. The consumption of dairy and meat products from infected animals can also cause brucellosis in urban residents. The severe damage caused by brucellosis influence individuals, families, and society as a whole. Therefore, a fast, specific, highly sensitive, inexpensive method for diagnosing of *Brucella* would be of great importance to both human health and the world's economy. In this study, we first designed primers targeting the *Brucella*-specific gene *omp*25 using online LAMP primer design software (https://Primerexplorer.jpamp3.0/index). Then we optimized reaction conditions for *Brucella* detection, examined the specificity and limit of detection of the method and implemented the method to milk and blood samples. Our results demonstrate the feasibility of this LAMP approach for rapid detection of *Brucella* spp.

2. Materials and methods

2.1. Bacteria strains and PCR Primers

Four major *Brucella* species, *B. abortus*, *B. ovis*, *B. melitensis*, and *B. suis* and 29 non-*Brucella* species bacteria were selected for this study (Table 1). *Brucella* bacteria were retrieved from stock by plating onto Trypticase soy agar (TSA) and incubating at 37 °C for 48 h in the presence of 5% CO_2. Single colony from TSA plate was picked and inoculated into

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Table 1
Brucella and non-*Brucella* bacteria species used for LAMP amplification.

Bacteria	No.	Species	Strain no.	Source[a] of strains
Brucella species	1	*Brucella abortus*	CVCC12	CIVDC
	2	*Brucella ovis*	16M	China CDC
	3	*Brucella melitensis*	544A	China CDC
	4	*Brucella suis*	1330S	China CDC
Non-*Brucella* species	1	*Pseudomonas aeruginosa*	ATCC 27853	CGMCC
	2	*Staphylococcus aureus*	ATCC 6538	CGMCC
	3	*Staphylococcus aureus*	ATCC 25923	CGMCC
	4	*Salmonella typhimurium*	ATCC 14028	CGMCC
	5	*Salmonella enteritis*	ATCC 50041	CGMCC
	6	*Salmonella schottmuelleri*	CMCC50001	CGMCC
	7	*Proteus mirabilis*	ATCC 12453	CMCC
	8	*Francisella.philomiragia*	ATCC 25015	CGMCC
	9	*Francisella.guangzhou*	CGMCC 1.10236	CGMCC
	10	*Bordetella. bronchiseptica*	ATCC 4617T	CGMCC
	11	*Blastomyces albicans*	ATCC 10211	CGMCC
	12	*Shigella boydii*	ATCC 51522	CGMCC
	13	*Shigella flexneri*	ATCC 12022	CGMCC
	14	*Shigella flexneri*	ATCC 51537	CGMCC
	15	*Shigella sonnei*	ATCC 9290	CGMCC
	16	*Escherichia coli* O157	ATCC 85933	CGMCC
	17	*Escherichia coli*	ATCC 25922	CGMCC
	18	Enteroinvasive *E. coli*	ATCC 270311	CGMCC
	19	Dysentery bacilli	ATCC 48097	CGMCC
	20	*Bacillus subtilis*	ATCC 9372	CGMCC
	21	*Klebsiella pneumoniae*	ATCC 700603	CGMCC
	22	*Legionella*	ATCC 35303	CGMCC
	23	*Legionella*	ATCC 33152	CGMCC
	24	*Clostridium perfringens*	CMCC64609	CMCC
	25	*Enterobacter sakazakii*	CMCC45401	CMCC
	26	*Yersinia entercolitica*	CMCC 52218	CMCC
	27	*Listeria monocytogenes*	CMCC 54002	CMCC
	28	*Bacillus cereus*	ATCC11778	CGMCC
	29	*Bacillus cereus*	CMCC63302	CMCC

[a] Note: ATCC: American Type Culture Collection; CIVDC: China Institute of Veterinary Drugs Control; CGMCC: China General Microbiological Culture Collection Center; CMCC: National Center for Medical Culture Collections; CVCC: China Veterinary Culture Collection Center; China CDC: Chinese Center for Disease Control and Prevention.

Trypticase soy broth (TSB) for enriching at 37 °C for 24 h. We designed the LAMP primers targeting the *omp*25 gene in *Brucella abortus* (*B. abortus omp*25 gene with access number X79284.1 in Genbank). The outer primers were named F3/B3, and the inner primers were named FIP/BIP. All primers were synthesized by Shanghai Yingyun Biotech Co., Ltd.

2.2. Preparation of DNA template

DNA was extracted by exposure bacteria to freeze–thaw cycles. One mL of *B. abortus* TSB enrichment solution was centrifuged at 3000 *g* for 1 min, supernatants were discarded and 100 μL of sterile water was added to pellets and mixed thorough. The mixture was incubated in boiling water bath for 10 min, centrifuged at 1000 *g* for 1 min, and the supernatant contains DNA was collected and stored at −20 °C.

2.3. Optimization of LAMP reaction conditions

We first adopted LAMP conditions reported by Notomi et al. (2000) as default reaction conditions. Then we optimized reaction conditions by evaluating of reaction time (15, 30, 45, and 60 min), temperature (58, 60, 63, and 65 °C), Mg^{2+} concentration (0.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mmol/L), Bst DNA polymerase (New England Biolab, U.S.) concentration (0.1, 0.3, 0.5, 0.7, 0.9, and 1.0 μL), dNTP concentration (0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 mmol/L), and inner/outer primer ratio (1:4, 1:5, 1:6, 1:7, 1:8, and 1:10).

2.4. Specificity of LAMP

The specificity of the LAMP method was tested with the optimized LAMP reaction conditions. Four major *Brucella* species (*B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis*) and 29 non-*Brucella* bacteria strains (Table 1) were used for the testing. Conventional PCR was also used to examine the specificity of LAMP primers. The two LAMP outer primers, F3 and B3 were used to perform conventional PCR (95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min and extension at 72 °C for10min) on the genomic DNA of *B. abortus*. The PCR products were sequenced by Shanghai Yingjun Biotech Co., Ltd. and then compared to the corresponding sequences in GenBank.

2.5. Limit of detection of LAMP

The limit of detection of the LAMP method was examined by implementation of optimized LAMP procedure to *B. abortus* TSB enrichment solutions with different concentrations generated by serial dilution. *B. abortus* TSB enrichment solution was 10-fold serial diluted and 1 ml of each diluted solution was used for DNA extraction the same way as above. Concentrations of diluted solutions were determined by enumeration of colonies on TSA plates. In addition, after completion of LAMP reactions, fluorescent dye SYBR Green I (Beijing

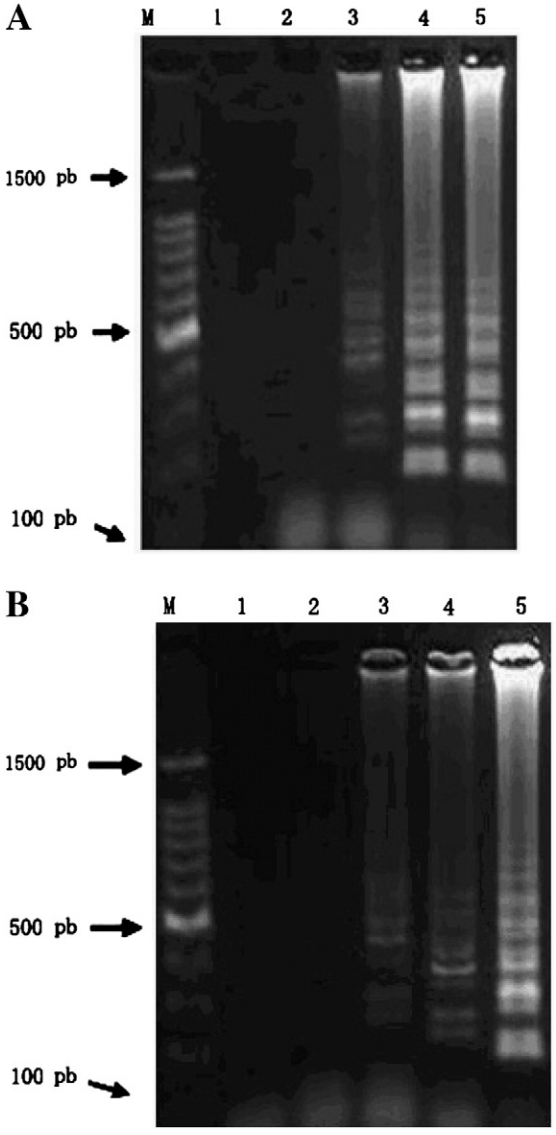

Fig. 1. Effects of temperature and reaction time on LAMP reaction. A. Effects of temperature. 1: negative control, 2: 58 °C, 3: 60 °C, 4: 63 °C, 5: 65 °C. B. Effects of reaction time. 1: negative control; 2: 15 min; 3: 30 min; 4: 45 min; 5: 60 min. In both A and B, M is the 100 bp ladder marker.