Infection and Drug Resistance

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METHODOLOGY

Lateral flow biosensor combined with loop-mediated isothermal amplification for simple, rapid, sensitive, and reliable detection of *Brucella* spp

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Abstract: *Brucella* species is responsible for brucellosis in human and animals, which is still of public health, veterinarian, and economic concern in many regions of the world. Here, a novel molecular diagnosis assay, termed loop-mediated isothermal amplification coupled with nanoparticles-based lateral flow biosensor (LAMP-LFB), was developed and validated for simply, rapidly, and reliably detecting all *Brucella* spp. strains. A set of six primers was designed based on the *Brucella*-specific gene *Bscp31*. The *Brucella*-LAMP results were visually reported by biosensor within 2 mins. A variety of bacterial strains representing several *Brucella* species, as well as several Gram-negative and Gram-positive bacterial species were used to determine the analytical sensitivity and specificity of the assay. Optimal LAMP conditions were 63°C for 40 mins, and the assay's sensitivity was found to be 100 fg of genomic DNA in the pure cultures. No cross-reactions to non-*Brucella* strains were obtained; thus, analytical specificity of LAMP-LFB assay is of 100%. Using the protocol, 20 mins for rapid DNA preparation followed by isothermal amplification (40 mins) combined with biosensor detection (2 mins) resulted in a total assay time of approximately 65 mins. In the case of 117 whole blood samples, 13 (11.11%) samples were *Brucella*-positive by LAMP-LFB, and the diagnostic accuracy was 100% when compared to the culture-biotechnical method. In conclusion, *Brucella*-LAMP-LFB technique developed in this study is a sensitive and specific method to rapidly identify all *Brucella* spp. strains, and can be applied as a potential diagnostic tool for brucellosis in basic, clinical, and field laboratories.

Keywords: *Brucella* spp., brucellosis, loop-mediated isothermal amplification, lateral flow biosensor, limit of detection

Introduction

Brucellosis, which is one of the worldwide zoonoses, is still of public health, veterinarian, and economic concern in many regions of the world.[1] Brucellosis is caused by a number of host adapted species of facultative intracellular bacteria of the genus *Brucella*.[2] *Brucella* spp. comprises six classical species (*B. melitensis, B. ovis, B. abortus, B. suis, B. neotomae, B. canis*), two marine species (*B. pennipedilis* and *B. ceti*), one human origin species (*B. innoponita*), and several newly described species (eg, *B. vulpis* and *B. microti*).[3,4] *Brucella* spp. is responsible for disease in both humans and animals.[5] Human brucellosis is considered as a life-threatening

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illness, and is the result of direct exposure to infected animals and their carcass, or animal products consumption.[6] Animal brucellosis is characterized by fetal death and spontaneous abortion in animals, and causes severe economic losses and restrictions on international animal movement and trade. In order to effectively prevent and control brucellosis, accurate early identification of *Brucella* spp. is very important.

Serology has been used in control and surveillance programs for brucellosis, while serological methods usually cause false-negative and false-positive results.[7] Isolation of the aetiological agent is the most specific diagnostic examination for the detection of *Brucella* spp. strains, but suffers from the disadvantages of low sensitivity, needing a long incubation period, especially in the chronic stage of the illness. Most importantly, the culture material must be carefully performed in bio-safety level laboratories, because several species (such as *B. melitensis* and *B. abortus*) of *Brucella* spp. are a class III organism and infects livestock and humans at a very low infectious dose.[8] Molecular techniques, such as traditional PCR and real-time PCR, have been proved to be efficient and rapid at different stages of the brucellosis, more sensitive than culture-based methods, more specific than serological diagnostics.[9] However, PCR-based techniques require the use of complex apparatus and skilled personnel, thus these assays are not readily available in all diagnostic laboratories in resource-poor settings and limits their use for wide-scale application at field level.[10]

To overcome the drawbacks posed by PCR-based assays, various other techniques, on the basis of isothermal amplification of nucleic acid, have been designed, which are able to rapidly synthesize DNA in large amounts without the use of any sophisticated apparatus.[11] Among these isothermal amplification methods, loop-mediated isothermal amplification (LAMP) is one of the most widely used techniques, which has been employed for rapid, sensitive, and specific detection of many bacterial pathogens including *Brucella* spp.[1,12–16] However, the results of *Brucella*-LAMP assays were displayed using agarose gel electrophoresis, color indicator (such as hydroxynaphthol blue, SYBR green I, calcein dye et al.), and real-time turbidity equipment. The use of gel electrophoresis to analyze *Brucella*-LAMP products is a complex procedure, and increases the risk of carryover contamination and degradation. Analysis of *Brucella*-LAMP products by color with the naked eyes is potentially subjective; thus, a practical sample may be somewhat ambiguous to the unaided eye when the concentration of *Brucella* genomic templates is very low. The analysis of *Brucella*-LAMP results by real-time turbidity requires a special optical instrument, and easily suffers from background interference.

The increasing application of LAMP assay has emphasized simplicity and speed as crucial criteria for adoption in "on-site" diagnosis, point-of-care (POC) testing, field detection and more. To well-suit for these applications, nanoparticles-based lateral flow biosensors (LFBs) have been designed, and increasingly employed as alternative tools for reporting LAMP results due to their simplicity, rapidness, and low cost.[17–19] Herein, we reported on the establishment of a visual, sensitive, and specific LAMP-based method, which successfully incorporated conventional LAMP assay with LFB (LAMP-LFB) for rapid and reliable detection of *Brucella* strains. The optimal conditions, analytical sensitivity, specificity, and feasibility of *Brucella*-LAMP-LFB method were validated using pure cultures and clinical samples.

Methods and materials

Reagents and instruments

Colorimetric indicator (Malachite Green, MG), universal isothermal amplification kits and biotin-14-dCTP were purchased from Bei-Jing HaiTaiZhengYuan. Co., Ltd. (Beijing, China). The LFB materials, including sample pad, nitrocellulose membrane (NC), conjugate pad, absorbent pad, and backing card were purchased from the Jie-Yi Biotechnology. Co., Ltd. (Shanghai, China). Dye (Crimson red) streptavidin-coated polymer nanoparticles (129 nm, 10 mg mL^{-1}, 100 mM borate, pH 8.5 with 0.1% BSA, 0.05% Tween 20 and 10 mM EDTA) were purchased from Bangs Laboratories, Inc.. (Indiana, USA). Anti-FITC (rabbit anti-fluorescein antibody) and biotin-BSA (biotinylated bovine serum albumin) were purchased from Abcam. Co., Ltd. (Shanghai, China). Genomic template extraction kits (QIAamp DNA minikits; Qiagen, Hilden, Germany) were purchased from Qiagen (Beijing, China).

Design of LAMP primer

A set of six primers, including two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB), was designed according to the *Bscp31* gene of the *Brucella* spp. published in GenBank in NCBI (Genbank accession no. M20404). *Bscp31* gene encodes a 31-kDa surface protein in all *Brucella* species and biovars.[20] According to the design principle of LAMP primer, Primers Explorer V4 (http://primerexplorer.jp/e/;

Eiken Chemical Co., Ltd., Tokyo, Japan) online primer design software was employed for screening out good LAMP primer sets. In addition, FITC (fluorescein isothiocyanate) was labeled at 5′ end of the FIP primer, and the new primer was named as FIP* and used for LAMP-LFB assay. The details, including primer sequences, modifications, and locations in the expression site of the *Bscp31* gene, are shown in Table 1 and Figure 1.

Bacterial strains and template preparation

A total of 42 *Brucella* strains (including *B. abortus, B. melitensis, B. ovis, B. canis, B. suis, B. neotomae*) and 19 non-*Brucella* isolates were employed at the current study (Table 2). Vaccine strain *B. suis* (GZ-CDC-S2) was used as a reference strain for optimizing the LAMP-LFB assay. According to the manufacture's instructions, the templates were prepared using DNA extraction Kits, and the extracted DNA were quantified using ultraviolet spectrophotometer (Nano drop ND-1000, Calibre, Beijing, China) at A260/280. The templates extracted from GZCDC-S2 were serially diluted ranging from 10 ng/μL to 100 aq/μL (10 ng/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, 10 fg/μL, 1 fg/μL, and 100 aq/μL), which was used for optimizing reaction temperature and testing assay's sensitivity. A volume of 1 μL of each dilution was used as a template for LAMP reactions.

Preparation of lateral flow biosensor

In this report, LFB was used for reporting LAMP results, and was constructed as previous publications.[17–19] In brief, LFB includes an absorbent pad, NC membrane, an immersion pad, a conjugate pad, and a backing pad. SA-PNPs (Dye streptavidin-coated polymer nanoparticles) were gathered in the conjugate pad. Then, anti-FITC and biotin-BSA were immobilized at test line (TL) and control line (CL), respectively.

Table 1 The primers used in the current report

Primers[a]	Sequences and modifications	Position within *Brucella* genome[b]	Length	Genes
F3	5′-TTACCCGGAAACGATCCAT-3′	1,189,462–1,189,480	19 nt	*Bscp31*
B3	5′-TCAGGTGTTCAGCCTTGA-3′	1,189,305–1,189,322	18 nt	
FIP	5′-CATCCAGCGAAACGCGCTTG-TGCGCGTAA GGATGCAAAC-3′	(1,189,392–1,189,411) (1,189,438–1,189,456)	39 mer	
FIP*	5′-FITC-CATCCAGCGAAACGCGCTTG-TGCGC GTAAGGATGCAAAC-3′	(1,189,392–1,189,411) (1,189,438–1,189,456)	39 mer	
BIP	5′-CGGGTTCTGGCACCATCGTC-TCTTCCGTG AGGCCGTAG-3′	(1,189,369–1,189,388) (1,189,328–1,189,345)	30 nt	
LF	5′-TCAGGTCTGCGACCGAT-3′	1,189,416–1,189,432	17 nt	
LB	5′-GCGCGTATCGTTCTTGA-3′	1,189,349–1,189,365	17 nt	

Notes: [a]FIP*, 5′-labeled with FITC when used in LAMP-LFB assay. [b]The primer position is based on the sequence *Brucella melitensis* strain BmWS93 with GenBank accession number CP034103.1.

Abbreviations: mer, monomeric unit; nt, nucleotide; FITC, fluorescein isothiocyanate.

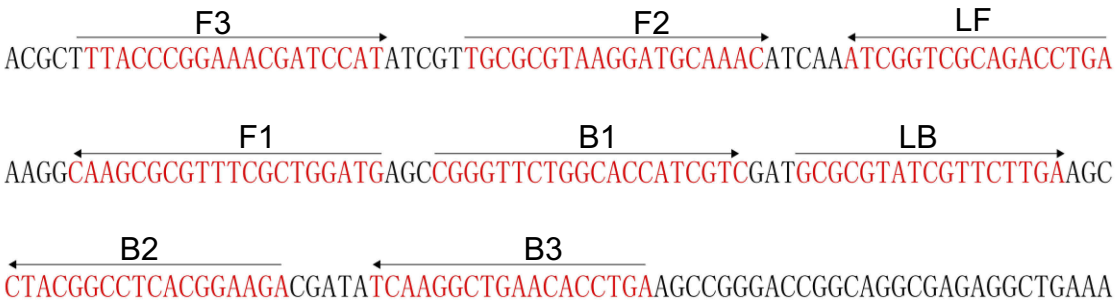

Figure 1 Sequence and location of *Bscp31* gene used to design loop-mediated isothermal amplification primers. The nucleotide sequences of the sense strand of *Bscp31* are listed. Right arrows and left arrows indicate sense and complementary sequences that are used.

Table 2 Bacterial strains used in the current study

Bacteria	Strain no. (source of strains)	No. of strains	LAMP-LFB result
Brucella species			
B. melitensis	Vaccine strain (GZ-CDC-M5)	1	*P*
B. melitensis	Isolated strains	32	*P*
B. suis	Vaccine strain (GZ-CDC-S2)	1	*P*
B. suis	Isolated strains	1	*P*
B. abortus	Vaccine strain (GZ-CDC-A19)	1	*P*
B. abortus	Isolated strains	3	*P*
B. neotoma	Isolated strains	1	*P*
B. ovi	Isolated strains	1	*P*
B. canis	Isolated strains	1	*P*
Non-Brucella species			
Leptospira interrogans	Isolated strains	1	N
Shigella dysenteriae	Isolated strains	1	N
Shigella boydii	Isolated strains	1	N
Shigella flexneria	Isolated strains	1	N
Shigella sonneri	Isolated strains	1	N
Enterococcus faecalis	ATCC35667	1	N
Enterococcus faecium	Isolated strains	1	N
Neisseria.meningitidis	Isolated strains	1	N
Bordetella pertussis	Isolated strains	1	N
Klebsiella pneumoniae	Isolated strains	1	N
Salmonella	Isolated strains	1	N
Bacillus cereus	Isolated strains	1	N
Enterotoxigenic E. coli	Isolated strains	1	N
Vibrio cholera	Isolated strains	1	N
Listeria monocytogenes	ATCC-EGD-e	1	N
Pseudomonas aeruginosa	Isolated strains	1	N
Vibrio parahemolyticus	Isolated strains	1	N
Streptococcus pneumonia	ATCC700674	1	N
Staphylococcus aureus	Isolated strains	1	N

Notes: Only *Brucella* species strains could be detected by the LAMP-LFB assay, demonstrating the high specificity of the target method.
Abbreviations: ATCC, American Type Culture Collection; GZ-CDC, Guizhou Center for Disease Control and Prevention; P, positive; N, negative.

Brucella-LAMP assay

Brucella-LAMP amplification was carried out in a one-step reaction in a 25-μL mixture containing 12.5 μL of supplied buffer (2 X), 0.4 μM each of outer primer F3 and B3, 0.8 μM each of loop primers LF and LB, 1.6 μM each of inner primers FIP and BIP, 0.4 mM biotin-14-dCTP, 1 μL (8 U) of *Bst* 2.0 DNA polymerase and 1 μL of template. Double distilled water (DW) was used as the template in the blank control (BC) sample, and non-*Brucella* genomic DNAs, including Staphylococcus *aureus* and *Salmonella* DNAs, were used as the templates in the negative control (NC) sample.

LAMP-LFB assay

A 1 μL aliquot of LAMP products, which were FITC and biotin-labeled LAMP products, was loaded into the sample zone. Then, a volume of 60 μL of running buffer was also loaded into the same region. As a result, the capillary flow can simultaneously transfer LAMP products and SA-PNPs from the conjugate region to TL and CL. Biotin-labeled LAMP products form complex with SA-PNPs via biotin-streptavidin-biotin interactions at the conjugated zone, and biotin/LAMP complexes were immobilized at the test band by interaction between hapten (FITC) and anti-FITC. SA-PNPs that did not construct complexes were captured at CL by interaction between streptavidin and biotin. Thus, SA-PNPs/LAMP/FITC complexes and non-complexed SA-PNPs were reported by visible line at TL and CL, respectively. Moreover, additional monitoring techniques, including real-time turbidity (LA-320C) and colorimetric indicator (Malachite Green, MG), were also used for reporting the *Brucella*-LAMP products.

Optimizing the reaction temperature of LAMP-LFB assay

The effect of different temperatures (from 60ºC to 67ºC, with 1ºC intervals) on LAMP amplification was determined. Amplification mixtures with 1 μL of template of *S. aureus* (isolated strain) and *Salmonella* (isolated strain) were used as NCs. Amplification mixtures with 1 μL of double DW were used as a BC.

Sensitivity of LAMP-LFB assay

Analytical sensitivity of LAMP-LFB assay was tested using serially diluted *B. suis* (GZ-CDC-S2) genomic DNA, and assay's sensitivity was verified as the last dilution of each positive test. For comparison, sensitivity of *Brucella*-LAMP assay using real-time turbidity and colorimetric indicator (MG) was also examined.

Optimizing the reaction time of LAMP-LFB assay

The effect of different times (from 30 mins to 60 mins, with 10 mins intervals) on LAMP amplification was evaluated, and the results were reported using LFB.

Specificity of LAMP-LFB assay

The specificity of LAMP-LFB was demonstrated using genomic DNA (at least 10 ng per microliters) from 42-*Brucella* strains and 19 non-*Brucella* strains (Table 2), and a 1 μL aliquot of genomic DNA was used as a template for LAMP reactions. All LAMP results were indicated using biosensor. All samples were repeated two times.

Evaluation of the feasibility of LAMP-LFB assay

A total number of 117 whole blood samples, which were suspected from human (21) and goat (96) brucellosis, were collected from different regions of Guizhou province, China (Ethic consideration: The National Health and Family Planning Commission of China determined that the collection of data from human cases of brucellosis was part of continuing public health surveillance of a notifiable infectious disease and was exempt from institutional review board assessment. All data were supplied and analyzed in an anonymous format, without access to personal identifying information).[21] These samples were used for *Brucella* spp. diagnosis using culture-based technique, PCR detection, and LAMP-LFB test. Traditional blood cultures were conducted with BACTEC FX system (Becton-Dickinson, Sparks, MD), incubated for six weeks and sub-cultured weekly.[22] In brief, about 3 mL fresh venous blood samples was aseptically inoculated into a two-phase culture flask (BIOVD, Zhengzhou, Henan, China) to cultivate and isolate *Brucella* strains. Post incubation at the conditions of 37ºC with 5% CO_2 for 3–5 days (or more 3–5 days cultivation for blind passage), the bacteria strain was streaked on blood agar plate and *Brucella* agar plate for pure cultivation. Subsequent methods including Gram stain, serum agglutination test, phage lysis test, were applied for the identification of the *Brucella* suspicious isolates. In addition, DNA templates from these practical samples (500 μL) were directly extracted using protocol of QIAamp for PCR and LAMP-LFB assays. PCR diagnosis was carried out using *Brucella* spp. specific primers (B4 and B5 primers) targeting *bscp31* gene having an amplicon size of 224 bp.[23] The examination results produced from LAMP-LFB method were compared with culture-based assay and PCR detection.

Results

Confirmation and demonstration of LAMP products

An appreciable LAMP reaction was observed when the assay was conducted at a fixed temperature of 63ºC for a duration time of 1 hr. The *Brucella*-LAMP amplified products exhibited light green under visible light, while the negative samples remained colorlessness (Figure 2A). Further validation of *Brucella*-LAMP amplification was visualized through the appearance of two crimson red bands (TL and CL) in biosensor, while the negatives samples only appeared a crimson red line (CL) in biosensor (Figure 2B). Our results confirmed that the LAMP primer set targeting *Bscp31* gene was appropriate candidate for establishment of LAMP-LFB assay for *Brucella* spp. detection.

Optimal temperature of LAMP primer set

Brucella-LAMP reaction condition was standardized to find out the optimum temperature. Eight amplification temperatures (ranging 60–67ºC, with 1ºC interval) were examined and compared under *Brucella*-LAMP protocol presented above. By real-time turbidity, all tested temperatures produced the kinetics graphs, and faster reactions were obtained for assay temperature of 62–64ºC (Figure 3). Assay temperature of 63ºC was employed for conducting the rest of LAMP reactions in the current study.

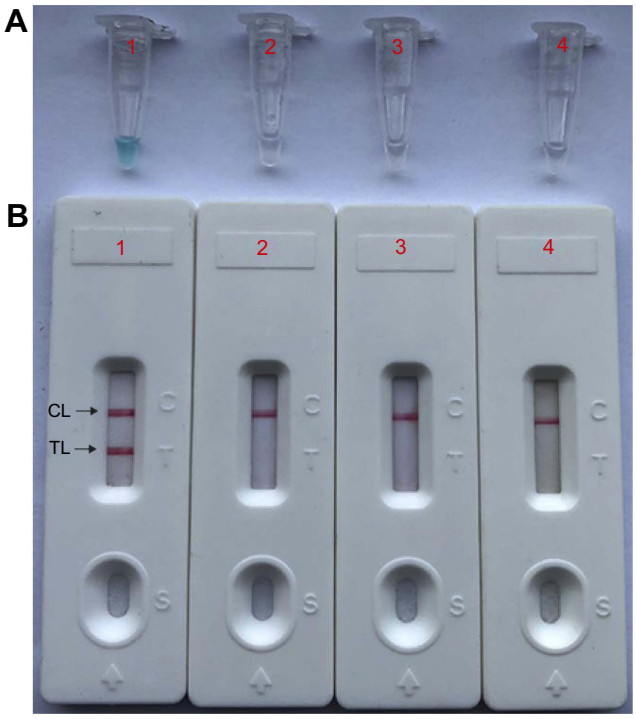

Figure 2 Validation of *Brucella*-LAMP products. (**A**), Color change of *Brucella*-LAMP tubes; (**B**), biosensor applied for visual detection of *Brucella*-LAMP products. Tube A1 (biosensor B1), positive amplification; tube A2 (biosensor B2), negative amplification (*Salmonella*), tube A3 (biosensor B3), negative amplification (*S. aureus*), tube A4 (biosensor B4), negative control (DW).

Sensitivity of *Brucella*-LAMP method

Analytical sensitivity of *Brucella*-LAMP assay was evaluated by limiting dilution of *Brucella* genomic DNA. The lowest limit of detection by *Brucella*-LAMP assay was found to be 100 fg per reaction. TL and CL were observed on the biosensor, displaying the positive LAMP results for *Bscp31* gene (Figure 4B). Moreover, the analytical sensitivity of *Brucella*-LAMP using biosensor was consistent with real-time turbidity detection (Figure 4C) and colorimetric indicator analysis (Figure 4A).

Optimal duration time of *Brucella*-LAMP assay

We evaluated the effect of different duration time (ranging from 20 mins to 50 mins with 10 mins interval) at optimal amplification temperature (63ºC), and the target DNA at the LoD level (100 fg) was detected when *Brucella*-LAMP reaction lasted only for 40-mins (Figure 5). Hence, an amplification time of 40-mins was used as the optimal LAMP time for conducting the rest LAMP amplifications performed at the current report. As a result, the whole procedure, including target DNA preparation (20 mins), *Brucella*-LAMP reaction (40 mins) and result indicating (2 mins), was completed within 65 mins.

Specificity of *Brucella*-LAMP method

The analytical specificity of *Brucella*-LAMP method was determined using *Brucella* vaccine strains, *Brucella* isolated strains, and non-*Brucella* bacterial pathogens. As shown in Figure 6 and Table 2, *Brucella*-LAMP assay specifically detected all *Brucella* species/strains, while non-*Brucella* bacterial pathogens were not detected. By biosensor, TL and CL simultaneously appeared at detection regions of LFB, suggesting the positive results for *Brucella* pathogens (Figure 6, **biosensor 1–10**). Only one crimson band (CL) appeared at the detection zone of LFB, reporting the negative results for non-*Brucella* strains and BC (DW) (Figure 6, **biosensor 11–30**).

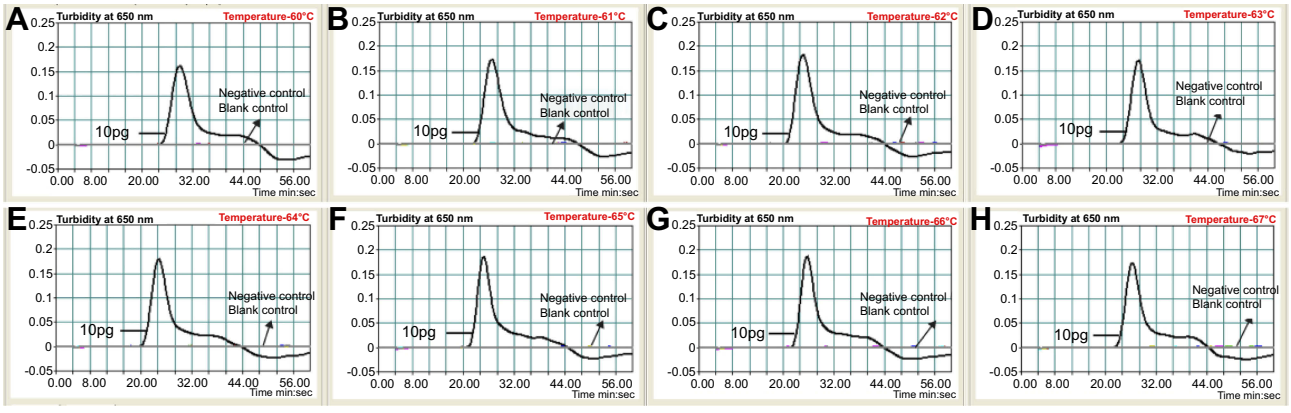

Figure 3 Optimal temperature for *Brucella*-LAMP primer set. The LAMP amplifications for detection of *Bscp31* sequence were examined by real-time measurement of turbidity. The corresponding curves of concentrations of templates were marked in the figures. Eight kinetic graphs (1–8) were generated at various temperatures (60–67°C, 1°C intervals) with target pathogens DNA at the level of 1 pg per tube (The threshold value was 0.1 and the turbidity of >0.1 was considered to be positive). The graphs from 3 (62°C) to 6 (64°C) showed robust reaction.

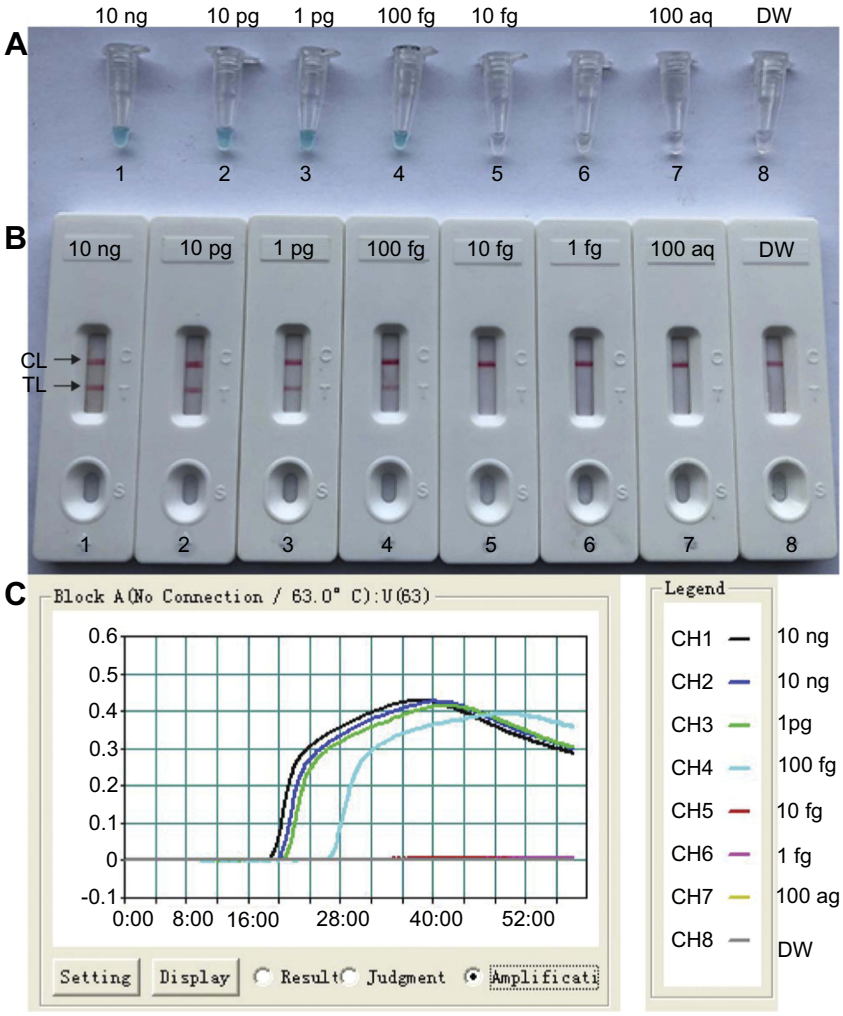

Figure 4 Sensitivity of *Brucella*-LAMP-LFB method using serially diluted genomic DNA with *B. suis* strain GZ-CDC-S2. Signals (**A**)/Tubes (**B**)/Biosensors (**C**) 1–8 represented the DNA levels of 10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 atto gram per reaction and blank control (DW). The genomic DNA levels of 10 ng to 100 fg per reaction yielded the positive reactions.

Demonstrating the feasibility of *Brucella*-LAMP-LFB assay by whole blood sample

We further determined the feasibility of *Brucella*-LAMP-LFB as a valuable tool for target pathogen detection. A total of 117 whole blood samples suspected from human and goat brucellosis were tested by LAMP-LFB assay, conventional PCR detection, and culture-biotechnical method. The results are summarized in Table 3. In the case of 117 biological samples, 13 (11.11%) and 13 (11.11%) samples were *Brucella*-positive by LAMP-LFB and traditional culture-biotechnical method, respectively. Thus, diagnostic accuracy obtained from LAMP-LFB assay was 100% when compared to the culture-biotechnical method. However, only 11 (9.40%) were *Brucella*-positive by conventional PCR technique. These results indicated that the *Brucella*-LAMP-LFB assay devised here was a valuable tool for target pathogen detection, and exhibited higher diagnostic ability when compared to the PCR method.

Discussion

Brucella spp. is responsible for brucellosis in both human and animals, and human brucellosis remains the world's most common bacterial zoonosis with more than 500,000 new cases annually.[24] Particularly, human brucellosis is related to substantial residual disability, and is a vital cause of travel-associated morbidity.[25] Diagnostic techniques, including microbiological isolation and identification of pathogens and nucleic acid amplification-based techniques, have been established for the detection of the target pathogens. However, traditional diagnostic assays for *Brucella* detection, such as culture-biotechnical methods

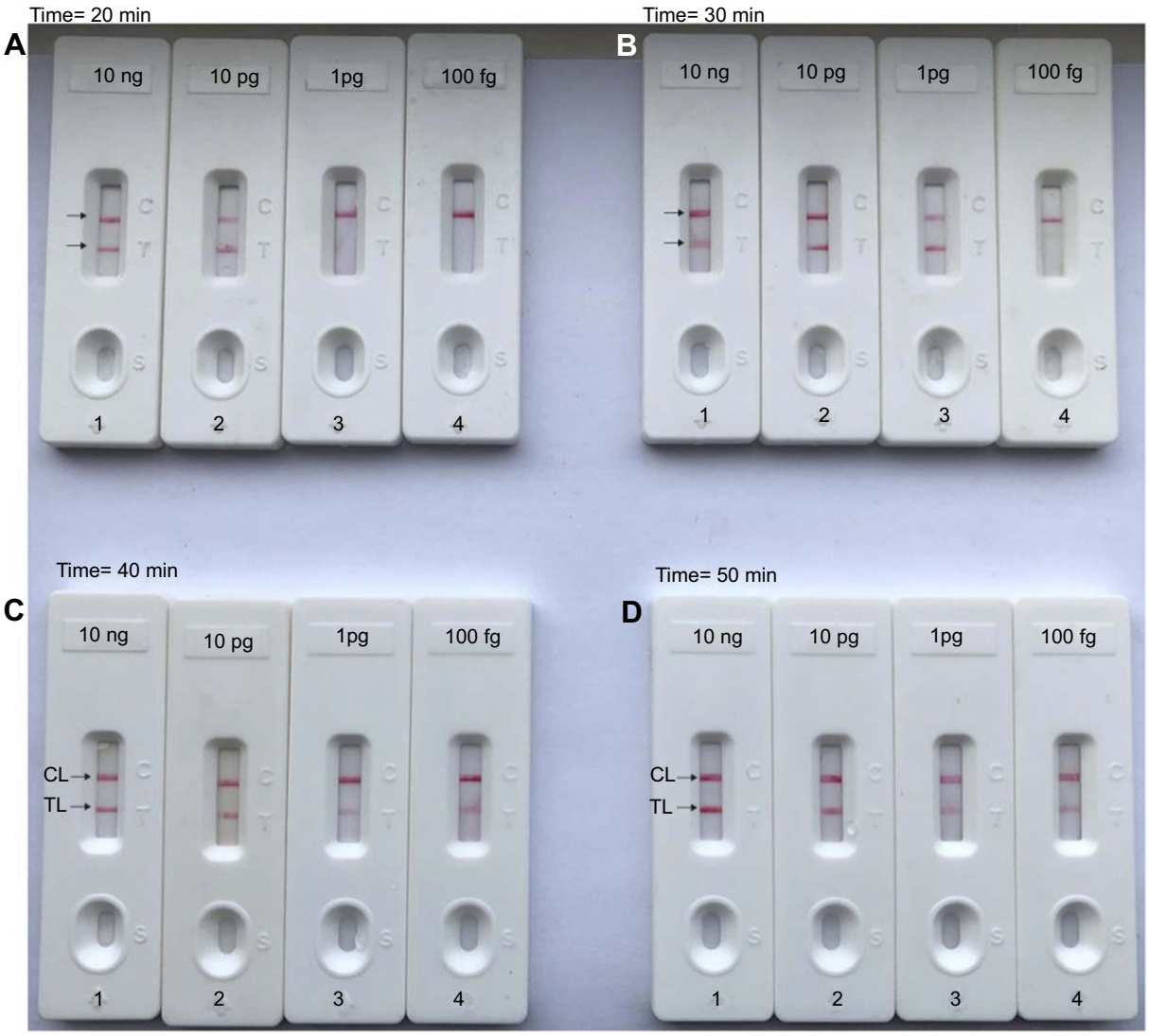

Figure 5 Optimal duration of time required for *Brucella*-LAMP-LFB assay. Four reaction times (**A**), 20 mins; (**B**), 30 mins; (**C**), 40 mins; and (**D**), 50 mins were tested and compared at optimal temperature (63°C). Biosensors 1, 2, 3, and 4 represent DNA levels of 10 ng μL^{-1}, 10 pg, 1 pg μL^{-1}, and 100 fg. The best sensitivity was obtained when the amplification lasted for 40 mins (**C**).

and PCR-based techniques, are time-consuming and laborious.[2] Hence, a newer diagnostic technique is required for rapid, simple, reliable detection of *Brucella* spp. strains in clinical, basic, and field laboratories.

To actualize more such effective detection tool, a LAMP-LFB assay (loop-mediated isothermal amplification coupled with nanoparticles-based LFB) has been developed for detection of *Brucella*spp. in this report. A set of LAMP primers (F3, B3, FIP, BIP, LF, and LB), which specifically recognized eight regions of the *Brucella*-specific gene (*Bscp31* gene), was designed on the basis of LAMP rules, thus providing a high degree of selectivity for target pathogen diagnostic (Figure 1).[26,27] To practically demonstrate the specificity of LAMP-LFB method, genomic DNAs extracted from *Brucella* spp. strains and non-*Brucella* bacterial strains were examined (Figure 6 and Table 2). *Brucella*-LAMP-LFB assay was able to detect all *Brucella* strains. All negative results were observed from the assay of non-*Brucella* spp. strains, and thus no cross-reactions to non-*Brucella* strains were obtained according to the specificity test. Herein, LAMP-LFB assay devised in the report detected these target pathogens with 100% specificity.

As an isothermal amplification technique, LAMP assay like multiple cross displacement amplification and cross-priming amplification only utilizes *Bst* polymerase enzyme which displays high resistance to known Taq polymerase inhibitors such as NaCl, hemoglobin, EDTA, N-acetyl

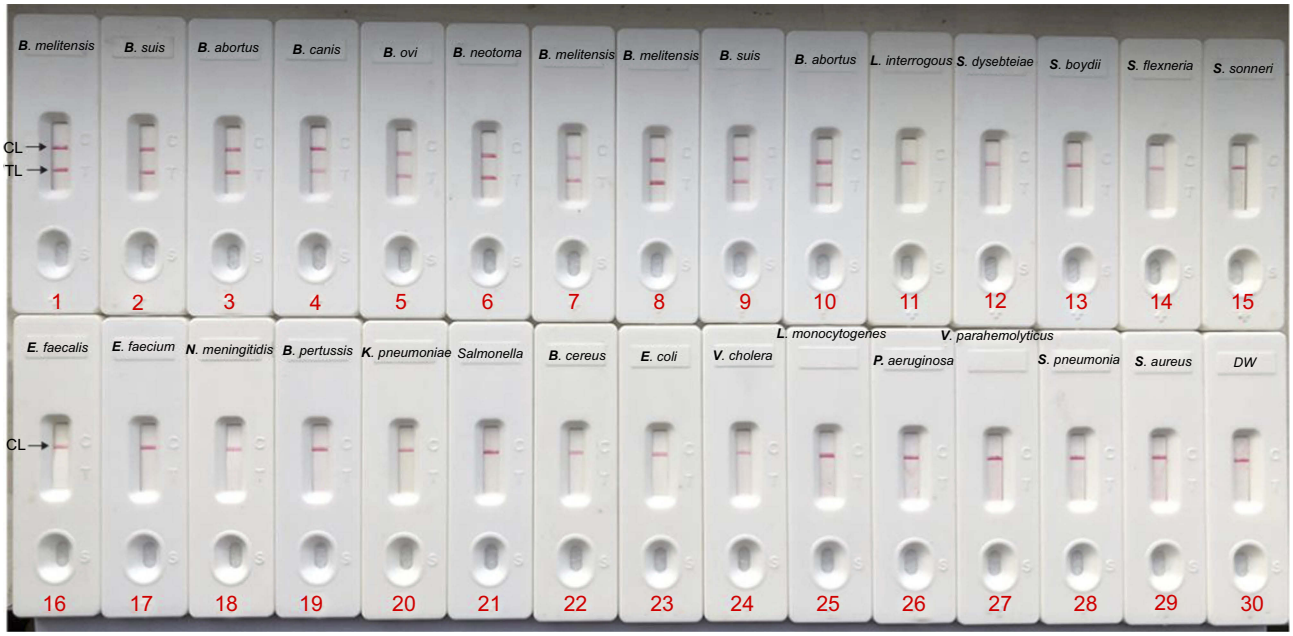

Figure 6 Specificity of *Brucella*-LAMP-LFB assay using different bacterial strains. The *Brucella*-LAMP-LFB was tested using different genomic DNAs. Biosensor 1, *B. melitensis* (GZ-CDC-M5); biosensor 2, *B. suis* (GZ-CDC-S2); biosensor 3, *B. abortus* (GZ-CDC-A19); biosensor 4, *B. canis* (isolated strain); biosensor 5, *B. ovi* (isolated strain); biosensor 6, *B. neotoma* (isolated strain); biosensor 7–8, *B. melitensis* (isolated strains); biosensor 9, *B. suis* (isolated strain); biosensor 10, *B. abortus* (isolated strain); Biosensor 11–29, *Leptospira interrogans; Shigella dysenteriae; Shigella boydii; Shigella flexneria; Shigella sonneri; Enterococcus faecalis; Enterococcus faecium; Neisseria meningitidis; Bordetella pertussis; Klebsiella pneumoniae; Salmonella; Bacillus cereus; Enterotoxigenic E. coli; Vibrio cholera; Listeria monocytogenes; Pseudomonas aeruginosa; Vibrio parahemolyticus; Streptococcus pneumonia; Staphylococcus aureus*; Biosensor 30, negative control (DW).

Table 3 Comparison of conventional LAMP-LFB, culture-biotechnical and PCR methods for the detection of *Brucella* in whole blood samples of human and goats

Detection methods	Whole blood samples (n=117)	
	Positive	Negative
LAMP-LFB	13	104
Culture	13	104
PCR	11	106

cysteine, and bile salts.[28,29] So far, LAMP assay has been used to detect a variety of pathogens, including *Brucella* spp.[14,30,31] Unfortunately, these *Brucella*-LAMP assays required the use of agarose gel electrophoresis, color indicator (such as hydroxynaphthol blue, SYBR green I, calcein dye et al) and real-time turbidity equipment for indicating the amplification results. As a result, these *Brucella*-LAMP methods relied on a complex procedure (gel electrophoresis), a special optical instrument (real-time turbidity instrument), or special indicator (hydroxynaphthol blue or SYBR green I), which hampered their wider application in field, "on-site" or POC laboratories.

Here, the first report, which employed LFB for analyzing LAMP products, was developed for rapid, visual, simple, and reliable detection of *Brucella* spp. strains (Figures 2–6). Comparing with the other monitoring techniques (ie, colorimetric indicator, gel electrophoresis, and turbidity) employed in previous publications, LFB showed its superiority on simple operation, rapid results, and ease of use in basic, clinical, and field laboratories. In particular, reporting *Brucella*-LAMP results by biosensor was able to avoid the use of special apparatus, reagent, and additional procedure, thus, *Brucella*-LAMP-LFB assay was more suitable than other *Brucella*-LAMP methods developed by previous studies for simple, visual, and rapid diagnostic of target pathogens.

The analytical sensitivity results showed that the *Brucella*-LAMP-LFB assay devised here was able to detect the target gene (*Bscp31* gene) even from 100 fg of the strain. The assay's sensitivity obtained from LFB detection was consistent with real-time turbidity detection and colorimetric indicator (MG) analysis (Figure 4). The whole procedure of LAMP-LFB detection, including genomic template preparation (20 mins), LAMP reaction (40 mins), and LFB analysis (2 mins), was competed within 65 mins. For further evaluating the practical availability of LAMP-LFB assay to target pathogens, this report evaluated 117 whole blood samples of goats and human using culture bio-technique method, PCR diagnostic and LAMP-LFB detection. The LAMP-LFB technique

exhibited high detection analysis for Brucella spp. Particularly, two biological samples were determined to be positive by culture-biotechnical assay and LAMP-LFB, but negative by traditional PCR detection. Lower diagnostic rate of PCR method may be due to the reasons that the copy numbers of target genomic templates were lower than the limit of detection or the presence of inhibitors specific to the conventional PCR technique affected the detection sensitivity. Moreover, comparing with culture-based assays and PCR method, *Brucella*-LAMP-LFB technique was conducted with only a simple instrument that provides a constant temperature of 63ºC, avoiding the long turnaround times and removing the use of expensive apparatus. But the LAMP-LFB assay has its own limitation as the LAMP results are indicated qualitatively as red colored bands.

In conclusion, a LAMP-LFB assay, which targeted the *Brucella*-specific Bscp31 gene, was successfully developed and validated for the detection of *Brucella* spp. The assay showed high analytical specificity for *Brucella* detection, and had the LoD of 100 fg per vessel with pure culture. The LAMP results were visually, rapidly, and indirectly reported using biosensor, which were objective, easy-to-use, and disposable. *Brucella*-LAMP-LFB assay established here was a simple, rapid, sensitive, and reliable method, which could be used as a potential diagnosis tool for target pathogens in basic, clinical, and field laboratory.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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