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Nanoparticle-based biosensors coupled with loop-mediated isothermal amplification for rapid, visual, and in-field identification of pathogenic *Leptospira*

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Abstract

Background Leptospirosis is a worldwide re-emerging zoonotic infectious disease caused by pathogenic spirochetes of the *Leptospira* genus. The clinical symptoms at leptospirosis onset are similar to those of other febrile illnesses, and early etiological identification of pathogenic *Leptospira* spp. is crucial to initiate immediate treatment and limit transmission. Here, we report a new molecular diagnostic assay termed loop-mediated isothermal amplification combined with a visual gold nanoparticle-based lateral flow biosensor (LAMP-AuNP-LFB) for rapid, specific, sensitive, and visual identification of pathogenic *Leptospira* strains.

Methods The AuNP-based LFB used here was successfully manufactured according to our device manual. The successful design of our unique *Leptospira*-LAMP primers was based on the *lipL41* gene from reference strains of 15 serovars representing 15 serogroups of pathogenic *Leptospira* spp. in China. The reaction conditions, including amplification temperature and time, were optimized, and the sensitivity and specificity of our assay were verified. The feasibility of our assay was tested through field mouse and leptospirosis clinical samples.

Results The AuNPs-based LFB used our study was successfully manufactured. The unique pathogenic *Leptospira* degenerate primers were successfully designed based on the *lipL41* gene. The optimal *Leptospira*-LAMP-AuNP-LFB detection process, including rapid nucleic acid isolation (less than 5 min), LAMP reaction (67 °C for 35 min), and visual AuNP-LFB readout (less than 2 min), could be accomplished within 45 min. Our assay detected target genes with as few as 5 copies per test and showed no cross-reactivity with other microbes in our study. The *Leptospira*-LAMP-AuNP-LFB assay results were consistent with culture-sequencing results in field mouse and leptospirosis clinical samples.

Conclusions The *Leptospira*-LAMP-AuNP-LFB assay is a valuable tool and has tremendous potential as a point-of-care (POC) testing approach for screening for pathogenic *Leptospira* infection, especially in resource-poor settings.

Keywords *Leptospira*, Loop-mediated isothermal amplification, Gold nanoparticle-based lateral flow assay, Point-of-care platform, Limit of detection

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Introduction

Leptospirosis is a serious global zoonotic infectious disease caused by pathogenic spirochetes of the *Leptospira* genus [1, 2]. Approximately one million new cases of leptospirosis occur globally, and 58,900 deaths have been reported annually [3]; of these cases, most occur in underdeveloped and developing regions, indicating that leptospirosis has become a reemerging zoonosis of global public health concern [1–4]. Leptospirosis is listed as a category B notifiable disease in China [5]. Worldwide, many animals, such as rodents and livestock, serve as important reservoirs of pathogenic *Leptospira* strains [6]. Pathogenic *Leptospira* species usually cause mild chronic or symptomless persistent infection in animals, and infected animals continuously discharge leptospires through their urine to contaminate soil and water, whereas humans can be infected through the mucosa or broken skin coming into contact with contaminated environment, or bitten by infected animal [7, 8].

The course of human leptospirosis varies from mild influenza-like manifestations to rapidly fatal forms, characterized by fever, myalgia, and severe symptoms such as renal failure, meningitis, and respiratory failure due to pulmonary diffuse hemorrhage [2, 9, 10]. Leptospirosis is frequently misdiagnosed because the clinical symptoms are not highly specific and resemble those of many other febrile diseases [11]. Early etiological diagnosis is critical for the prescription of more effective antibiotic treatments during the initial course of this disease. Hence, developing a rapid, accurate, visual point-of-care (POC) testing system for leptospirosis is crucial for treatment and limiting transmission.

Traditionally, the microscopic agglutination test (MAT) is most commonly used and is considered the "gold standard" diagnostic tool for leptospirosis identification [12]. However, this method does not permit early diagnosis because anti-*Leptospira* antibodies can be detected only in the later acute stage of infection [13]. Acquiring microbiological cultures of *Leptospira* is laborious and time consuming (approximately 6–8 weeks) [14]. In recent decades, nucleic acid amplification tests, such as polymerase chain reaction (PCR), real-time PCR, and multiplex PCR, have been used to identify *Leptospira* agents owing to their high sensitivity and specificity [15]. However, PCR-related methods are limited in terms of their application as rapid POC diagnostic techniques because expensive thermocyclers, trained technical personnel, and centralized laboratories are required to perform these methods. Moreover, PCR methods are time-consuming (approximately 2.5 h) [16].

To address the drawbacks of PCR-related approaches, many isothermal amplification tools have been developed and gradually used to accurately identify nucleic acids [17]. Gunasegar et al. (2021) demonstrated that LAMP has better diagnostic accuracy than PCR for *Leptospira* spp. in clinical samples [18], Ahmed et al. (2014) developed a recombinase polymerase amplification assay (RPA) for detection of pathogenic *Leptospira* based on TwistAmp chemistry [19], Jirawannaporn et al. designed an RPA-CRISPR/Cas12a-fluorescence assay for identifying the pathogenic *Leptospira* spp. isolates [20]. Loop-mediated isothermal amplification (LAMP) is a well-studied and promising isothermal amplification approach that has great potential for transforming POC testing platforms because of its high specificity and sensitivity, lack of need for specialized devices, and easy operation [21, 22], this method has been gradually used for the identification of many pathogens, such as *Mycobacterium tuberculosis*, *Brucella*, and human immunodeficiency virus (HIV) [23–25]. LAMP enables robust nucleic acid amplification with a *Bst* DNA/RNA polymerase for strand displacement activity and a set of specific primers (forward outer primer F3, backward outer primer B3, forward inner primer FIP, backward inner primer BIP, forward loop primer LF, and backward loop primer LB) for the recognition of eight fragments in the target sequence [21]. In addition, the DNA yield of LAMP is usually 100-fold greater than that of traditional PCR-based methods [26]. The gold nanoparticle-based lateral flow biosensor (AuNP-LFB) is a paper-based analytical device that has great potential as an ideal POC detection platform owing to its rapid detection, high sensitivity and specificity, on-the-spot detection, easy handling characteristics, and lack of need for specialized training [27, 28], and the AuNP-LFB has been widely used in the health diagnostic, environmental monitoring, and food quality control fields [27, 29, 30].

In the present study, for the first time, we combined loop-mediated isothermal amplification and a gold nanoparticle-based lateral flow biosensor (LAMP-AuNP-LFB) to develop a novel approach for accurate, rapid, sensitive, specific, on-the-spot, cost-saving, and visual identification of pathogenic *Leptospira* spp. by targeting the *lipL41* gene of these bacteria [31], which showed no homology to other microbe genomes in BLAST searches of the GenBank database. We verified the feasibility of our approach through field mouse kidney sample testing.

Materials and methods

Leptospira-LAMP primer design

The conserved *lipL41* gene sequences from 15 reference strains representing 15 serovars of 15 serogroups of pathogenic *Leptospira* spp. in China (GenBank accession nos. AY622673, AY622674, AY622675, AY622676, AY622677, AY622678, AY622679, AY622680, AY622681, AY622682, AY622683, AY622684, AY622685, AY622686, and AY622687) [32], were used for *Leptospira*-LAMP primer design through Primer Explorer v.5 (http://prim

erexplorer.jp/e/) and Primer Premier v.5.0 software. The set of 6 primers included two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB). For AuNP-LFB analysis, the 5′ ends of the FIP and LF primers were labeled with FAM and biotin, respectively. The specificity of the primer set was verified with the BLAST analysis tool. The primer locations are shown in Fig. 1, and the primer sequences and modifications are shown in Table 1. All primers were synthesized and purified via high-performance liquid chromatography (HPLC) at TsingKe Biotech Co., Ltd. (Beijing, China).

Leptospiral strains and genomic template preparation

The pathogenic *Leptospira* strains and other microbes used in the current study are shown in Table 2. The leptospires were cultured in Ellinghausen-McCullough-Johnson-Harris medium (EMJH) and stored in our laboratory. All microbial genome DNA templates were extracted rapidly with nucleic acid-releasing agents (GenDx Biotech Co., Ltd.; Suzhou, China; Cat No. NR201) according to the manufacturer's guidelines. In brief, 15 μl of bacterial suspension and 150 μl of nucleic acid-releasing agent were added to a 1.5 ml Eppendorf (EP) tube, and the mixture was subsequently incubated at 95 °C for 3 min to release nucleic acid. The genomic DNA was stored at −80 °C until use. *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai (56601) was used as a positive control. The genomic DNA concentrations were measured through a NanoDrop ND-2000 (Thermo Fisher Scientific, United States) at A260/280 nm, and the corresponding genome copy number was calculated from the weight of the *Leptospira* genome. One *Leptospira* genome was 5.22 fg (2.2×10^6 bp (genomic DNA length) × 665 Da/bp×1.67×10^{-24} g/Da).

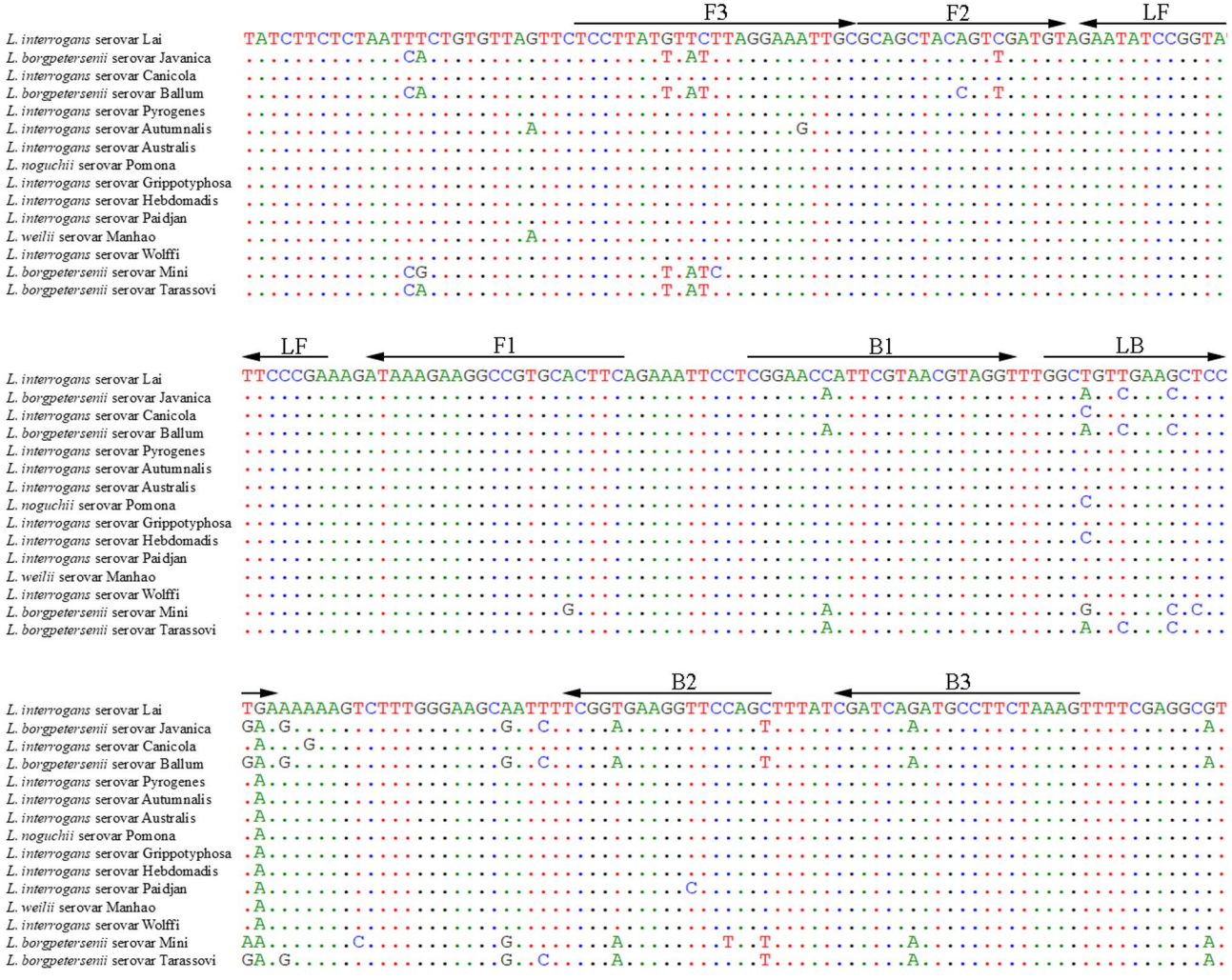

Fig. 1 Nucleotide sequences and location of the *lipL41* gene from 15 reference strains representing 15 serovars of 15 serogroups of pathogenic *Leptospiral* spp. in China, used to design the *Leptospira*-LAMP primers. The nucleotide sequence of the *lipL41* sense strand was shown. The sense and complementary sequences used were indicated by right and left arrows, respectively

Table 1 The *Leptospira*-LAMP-AuNP-LFB primers used in the current study

Primer name[a]	Sequence and modifications[b]	Length[c]	Gene
F3	5′-TCCTTATKTWYYTAGGAARTTGC-3′	23 nt	*lipL41*
B3	5′-CTTTAGAAGGCATYTGATCG-3′	20 nt	
FIP	5′-GAAGYGCACGGCCTTCTTTATGCAGCTACMGTYGATGT-3′	38 mer	
FIP*	5′-FAM-GAAGYGCACGGCCTTCTTTATGCAGCTACMGTYGATGT-3′	38 mer	
BIP	5′-CGGAACMATTCGTAACGTAGGTRCTRGARC	39 mer	
LF	CTTCWCCGA-3′	19 nt	
LF*	5′-TCGGGAATACCGGATATTC-3′	19 nt	
LB	5′-Biotin-TCGGGAATACCGGATATTC-3′ 5′-GGCNGTYGAASCYCCDRA-3′	18 nt	

FAM, 6-carboxy-fluorescein; nt, nucleotide; mer, monomeric unit

[a]FIP*, 5′-labeled with FAM, LF*,5′-labeled with biotin, when used for the AuNPs-LFB assay

[b]IUPAC code for mix bases: R, A/G; Y, C/T; M, A/C; K, G/T; S, C/G; W, A/T; H, A/C/T; B, C/G/T; V, A/C/G; D, A/G/T; N, A/C/G/T

[c]nt, nucleotide; mer, monomeric unit

AuNP-LFB preparation

The principle of the AuNP-LFB (60 × 4 mm) used in the current study is shown in Fig. 2B. In brief, the sample pad, conjugate pad, nitrocellulose (NC) membrane (detection region), and absorbent pad were laminated onto a plastic adhesive backing card. Crimson red dye streptavidin-coated gold nanoparticles (SA-AuNPs) (40 ± 5 nm, 10 mg/ml) were deposited on the conjugate pad. The NC membrane was fixed with the anti-FAM antibody (Ab) (0.2 mg/ml) and biotin-bovine serum albumin (BSA) (4 mg/ml) of the test line (TL) and control line (CL), respectively, and each Line was separated by 5 mm. Finally, the assembled biosensor was packaged on a plastic card with adhesive backing. The AuNPs-LFB used in the current study was manufactured by Tian-Jin HuiDeXin Biotech Co., Ltd. (Tianjin, China) based on our design specifications and stored at room temperature until use.

Standard Leptospira-LAMP reaction and detection

The LAMP reaction was performed in 25 μl of a mixture containing 1 μl of *Bst* 4.2 DNA/RNA polymerase (8 U) (HaiGene Biotech Co., Ltd. (Harbin, China), 1 μl of nucleic acid template, 0.1 μM of F3 and B3, 0.4 μM of FIP or FIP* (for AuNP-LFB only) and BIP, 0.2 μM of LF or LF* (for AuNP-LFB only) and LB, 2.5 μl of 10× *Bst* 4.2 buffer (Mg^{2+} free), 1.5 μl of 100 mM Mg^{2+}, 3 μl of dNTP mixture (10 mM), 1.5 μl of leuco-hydroxynaphthol blue (L-HNB, for colorimetry only), and double-distilled water to bring the volume to 25 μl.

Four analysis methods, visual biosensor AuNP-LFB, colorimetry (L-HNB), real-time turbidity, and agarose gel electrophoresis, were applied to monitor LAMP amplicons. For AuNP-LFB detection, two visible crimson lines (CL and TL) appeared simultaneously on the biosensor, indicating a positive *Leptospira*-LAMP result, and only a CL indicated a negative outcome. For L-HNB analysis, a mixture that turned from deep violet to light green indicated a positive *Leptospira*-LAMP result, and a mixture that remained deep violet throughout the reaction process indicated a negative result. For real-time turbidity assessment, a turbidity value of > 0.1 indicated positive *Leptospira*-LAMP. For 2% agarose gel electrophoresis assessment, an agarose gel presented ladder-like bands indicated a positive result, with no bands indicating a negative outcome.

Leptospira-LAMP-AuNP-LFB assay condition optimization

Reaction temperatures ranging from 63 to 70 °C (in 1 °C increments) were measured to determine the optimal amplification temperature at the stage of *Leptospira*-LAMP, and the reaction results were monitored through real-time turbidity. The optimal reaction time was confirmed by incubating the *Leptospira*-LAMP amplicons for 15 to 45 min (in 10-min increments) and determining their results with the AuNP-LFB and L-HNB visual reagents. Each test was performed in triplicate.

Leptospira-LAMP-AuNP-LFB assay sensitivity and specificity

The limit of detection (LoD) of the *Leptospira*-LAMP-AuNP-LFB assay was measured by evaluating the *Leptospira* genomic DNA with serial dilutions from 1.0×10^4 copies to 1 copy. The *Leptospira*-LAMP reactions were performed under optimal conditions, and the results were analyzed and compared with those of AuNP-LFB and a colorimetric indicator (L-HNB). Each test was performed at least six replicates. The specificity of the *Leptospira*-LAMP-AuNP-LFB assay was evaluated by comparing 15 reference strains of pathogenic *Leptospira* spp. in China with nucleic acids from various bacteria, and viruses (Table 2), and distilled water (DW) was used as a blank control. Each assay was confirmed in triplicate.

Verification of Leptospira-LAMP-AuNP-LFB feasibility through field mouse kidney and human blood samples testing

To verify the feasibility of our *Leptospira*-LAMP-AuNP-LFB assay, 56 suspected *Leptospira*-infected field mouse kidney samples and 26 clinical blood samples from patients with suspected *Leptospira* infection were collected between August 2023 and July 2024. The Ethics Committee of the Guizhou Provincial Center for Disease Control and Prevention approved the lawful and ethical collection and analyses. These samples were evaluated simultaneously with 16 S rRNA gene sequencing, real-time PCR, and our *Leptospira*-LAMP-AuNP-LFB

Table 2 Microbes used in this study

No.	Microbe	Serogroup[a]	Serovar[a]	Strain No. (source of strain)[b]	Leptospira-LAMP -AuNPs-LFB result[c]
1	*Leptospira interrogans*	Icterohemorrhagiae	Lai	Lai (56601)	P
2	*Leptospira borgpetersenii*	Javanica	Javanica	M10 (56602)	P
3	*Leptospira interrogans*	Canicola	Canicola	Lin (56603)	P
4	*Leptospira borgpetersenii*	Ballum	Ballum	Pishu (56604)	P
5	*Leptospira interrogans*	Pyrogenes	Pyrogenes	4 (56605)	P
6	*Leptospira interrogans*	Autumnalis	Autumnalis	Lin 4 (56606)	P
7	*Leptospira interrogans*	Australis	Australis	65 – 9 (56607)	P
8	*Leptospira noguchii*	Pomona	Pomona	Luo (56608)	P
9	*Leptospira interrogans*	Grippotyphosa	Linhai	Lin 6 (56609)	P
10	*Leptospira interrogans*	Hebdomadis	Hebdomadis	P7 (56610)	P
11	*Leptospira interrogans*	Bataviae	Paidjan	L37 (56612)	P
12	*Leptospira weilii*	Manhao	Cingshui	L105 (56615)	P
13	*Leptospira interrogans*	Sejroe	Wolffi	L183 (56635)	P
14	*Leptospira borgpetersenii*	Mini	Mini	Nan 10 (56655)	P
15	*Leptospira borgpetersenii*	Tarassovi	Tarassovi	(56671)	P
16	*Leptospira biflexa* (nonpathogenic *Leptospira* strain)	U	U	GZCDC	N
17	*Enterococcus faecalis*	U	U	ATCC 29,212	N
18	*Pseudomonas aeruginosa*	U	U	ATCC 27,853	N
19	*Shigella flexneri*	U	U	2nd GZUTCM	N
20	*Bacillus cereus*	U	U	GZCDC	N
21	*Streptococcus pneumoniae*	U	U	2nd GZUTCM	N
22	Invasive *Escherichia coli*	U	U	2nd GZUTCM	N
23	Enterohemorrhagic *Escherichia coli*	U	U	2nd GZUTCM	N
24	Enterotoxigenic *Escherichia coli*	U	U	2nd GZUTCM	N
25	Enteroaggregative *Escherichia coli*	U	U	2nd GZUTCM	N
26	*Mycobacterium tuberculosis*	U	U	GZCDC	N
27	*Staphylococcus aureus*	U	U	2nd GZUTCM	N
28	*Acinetobacter baumannii*	U	U	2nd GZUTCM	N
29	*Listeria monocytogenes*	U	U	2nd GZUTCM	N
30	*Klebsiella pneumoniae*	U	U	2nd GZUTCM	N
31	*Bordetella parapertussis*	U	U	2nd GZUTCM	N
32	*Haemophilus parainfluenzae*	U	U	2nd GZUTCM	N
33	*Vibrio parahaemolyticus*	U	U	GZCDC	N
34	*Acinetobacter lwoffii*	U	U	2nd GZUTCM	N
35	Hepatitis B virus	U	U	2nd GZUTCM	N
36	Hepatitis C virus	U	U	2nd GZUTCM	N
37	Human papillomavirus	U	U	2nd GZUTCM	N
38	Influenza B virus	U	U	GZCDC	N
39	Epstein-Barr virus	U	U	2nd GZUTCM	N

2nd GZUTCM, the Second Affiliated Hospital, Guizhou University of Traditional Chinese Medicine, *GZCDC* Guizhou Provincial Center for Disease Control and Prevention, *N* Negative

[a]U, unidentified serotype

[b]ATCC, American Type Culture Collection

[c]P, Positive

methods. For 16 S rRNA gene sequencing, the leptospires were cultured in EMJH-liquid medium and incubated for 3 weeks at 28 °C, and then the bacterial genomic DNA was extracted using Bacterial Genomic DNA Extraction Kit according to the manufacturer's instructions (Xi'an Tianlong Technology Co., Ltd.; Xi'an, China). The 16 S rRNA gene was amplified by the universal primers fD1 and rP2 [33], and then, the PCR products were sequenced by Sanger sequencing. The sequences were analyzed with basic local alignment search tool (BLAST) alignment (information available at www.ncbi.nlm.nih.gov/BLAST). *Leptospira* real-time PCR detection was performed with *Leptospira* Nucleic Acid Assay Kits (MABSKY Biological Co., Ltd., Shenzhen, China;

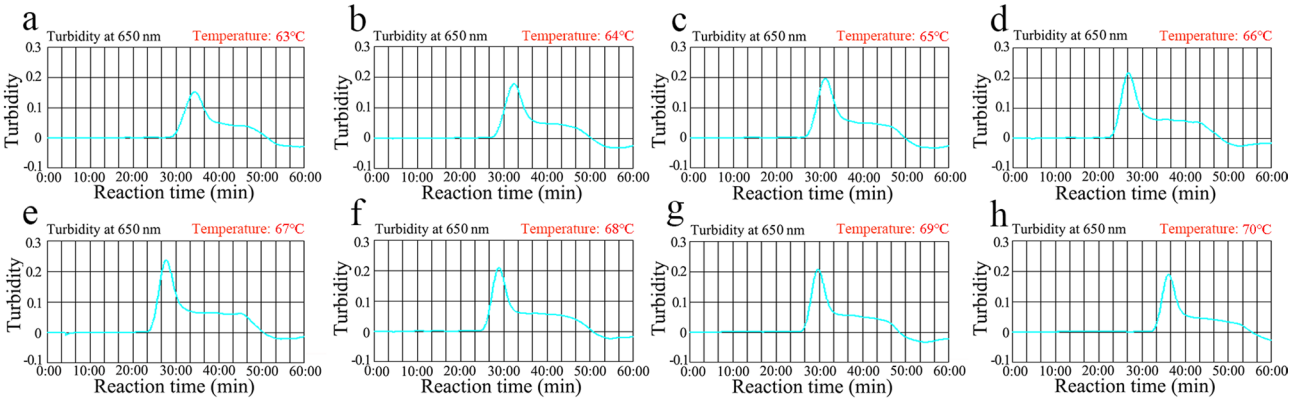

Fig. 2 Schematic diagram of *Leptospira*-LAMP-AuNP-LFB assay's principle (**A**) Workflow of the *Leptospira*-LAMP-AuNP-LFB assay. The whole detection process of each test can be accomplished within 45 min, including less than 5 min for rapid genomic DNA isolation (Step 1), 35 min for LAMP reaction (Step 2), and less than 2 min for AuNPs-LFB visual readout (Step 3) (**B**) Principle of AuNP-LFB for visual readout of *Leptospira*-LAMP products. **a** *Leptospira*-LAMP products (1.0 μl) and running buffer (100 μl; 100 mM phosphate-buffered saline with 1% Tween 20 [pH 7.4]) were added simultaneously on the sample pad. **b** The running buffer containing *Leptospira*-LAMP products moved forward to the conjugate pad and detection zone (NC membrane) through capillary action, the crimson dye streptavidin gold nanoparticle (SA-AuNP) was rehydrated with running buffer. **c** the SA-AuNP was integrated with FAM/biotin labeled *Leptospira*-LAMP amplicons, and then the FAM/biotin-*Leptospira*-LAMP-SA-AuNP complexes were captured by anti-FAM at the test line (TL), and the SA-AuNP were arrested by biotin-BSA at the control line (CL). **d** Interpretation of the *Leptospira*-LAMP-AuNP-LFB assay. CL and TL appeared simultaneously on the biosensor indicated a positive result. Only the CL was observed on the biosensor indicated a negative outcome. CL, control line; TL, test line

Cat. No. SKY-8645 F) on an Applied Biosystems™ 7500 Real-Time PCR System (Life Technologies; Singapore). A threshold cycle (Ct value) ≤ 36 was determined to indicate a positive result, and the sensitivity of this method was 500 copies/ml according to the manufacturer's guidelines. The *Leptospira*-LAMP-AuNP-LFB assay was performed at optimal reactions (67℃, 35 min). All of the identification investigations were performed at biosafety level 2 (BSL-2) based on the World Health Organization (WHO) Biosafety Manual (3rd edition). The outcomes of the real-time PCR and our *Leptospira*-LAMP-AuNP-LFB were compared with culture-sequencing, respectively. The statistical parameters were calculated through online tool from MedCalc (http://www.medcalc.org/calc/diagnostic_test.php) [34].

Results

Workflow and schematic mechanism of Leptospira-LAMP-AuNP-LFB assay

The workflow and schematic mechanism of our *Leptospira*-LAMP-AuNP-LFB approach are shown in Fig. 2. Briefly, genomic DNA templates were rapidly extracted with nucleic acid-releasing agents within 5 min (Fig. 2A, step 1). *Leptospira*-LAMP was carried out to specifically and rapidly amplify the target sequence for 35 min at a fixed temperature (67 °C). The *Leptospira*-specific *lipL41* gene was amplified with only a *Bst* 4.2 DNA/RNA polymerase with strand displacement activity and a set of 6 primers (two outer primers, F3 and B3; two inner primers, FIP and BIP; and two loop primers, LF and LB). For AuNP-LFB detection, the *Leptospira*-FIP* and *Leptospira*-LF* primers were labeled at the 5′ end with FAM and biotin, respectively. The *Leptospira*-LAMP products were then labeled simultaneously with the two signal molecules (Fig. 2A, step 2). The *Leptospira*-LAMP results were visually read through AuNP-LFB within 2 min (Fig. 2A, step 3). The whole detection process could be completed within 45 min.

The principle of the AuNP-LFB platform for the identification of *Leptospira*-LAMP amplicons is shown in Fig. 2B. In brief, *Leptospira*-LAMP products (1.0 μl) and running buffer (100 μl; 100 mM phosphate-buffered saline with 1% Tween 20 [pH 7.4]) were dripped simultaneously onto the sample pad of the biosensor (Fig. 2B-a). The running buffer-containing *Leptospira*-LAMP products moved to the conjugate pad and reaction region through capillary action. The SA-AuNPs were rehydrated and integrated with the FAM/biotin-labeled *Leptospira*-LAMP amplicons at the conjugation pad (Fig. 2B-b). In the detection region (NC membrane), anti-FAM was anchored at the TL and used to capture FAM/biotin-labeled *Leptospira*-LAMP amplicons, and biotin-BSA was fixed at the CL to arrest SA-AuNPs (Fig. 2B-c). The interpretation of *Leptospira*-LAMP results through the AuNP-LFB is shown in Fig. 2B-d. A positive result was indicated by both the CL and TL on the biosensor turning red, whereas a negative result was indicated by only the CL turning red.

Applicability of the Leptospira-LAMP-AuNP-LFB primer set

To validate the applicability of the LAMP primer set (Table 1) in the *Leptospira*-LAMP-AuNP-LFB reaction system, genomic DNA from 15 reference strains representing 15 serovars of 15 serogroups of pathogenic

Leptospira spp. in China, *Mycobacterium tuberculosis*, and *Brucella* was used as a template for the LAMP reaction at a constant temperature of 65 °C for 60 min, and DW was used as a blank control. 2% agarose gel electrophoresis, colorimetric indicator (L-HNB), and AuNP-LFB analysis were utilized to obtain the readout of the LAMP results. The templates from *Leptospira* spp. were amplified, and positive results were obtained with each analysis method (Fig. 3A-C). No amplification was observed for the negative controls (*M. tuberculosis* and *Brucella*) or the blank control (DW) (Fig. 3A-C). These findings confirmed that the designed LAMP primer set for the *lipL41* gene was appropriate for the development of the *Leptospira*-LAMP-AuNP-LFB assay.

Optimal Leptospira-LAMP temperature

An appropriate temperature is critical for high-efficiency LAMP. In the present study, *Leptospira*-LAMP reactions were performed at different temperatures (ranging from 63 to 70 °C at 1 °C intervals) with a genomic DNA template (1×10^4 copies per test) isolated from a purified culture (*L. interrogans* 56601). The LAMP protocol was carried out as described above, and the *Leptospira*-LAMP reactions were monitored via real-time turbidity measurement (Fig. 4a-h). The results showed that robust amplification for the *Leptospira*-LAMP assays occurred at 67 °C (Fig. 4e). Therefore, a reaction temperature of 67 °C was considered the appropriate temperature for the remaining *Leptospira*-LAMP-AuNP-LFB reactions in the present study.

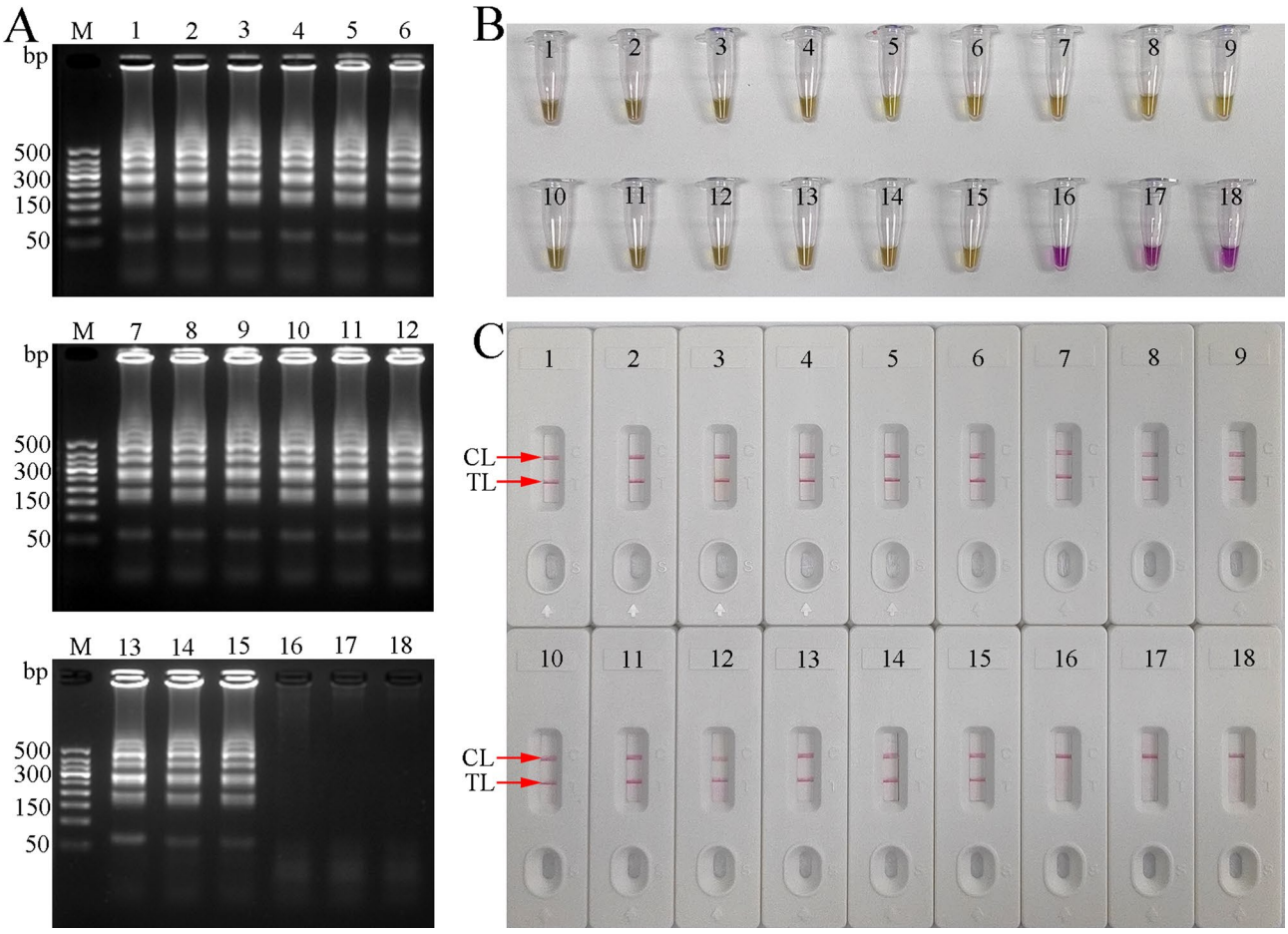

Fig. 3 Verification of *Leptospira*-LAMP products The *Leptospira*-LAMP products were tested simultaneously through (**A**) 2% agarose gel electrophoresis, (**B**) colorimetry (L-HNB), and (**C**) AuNPs-LFB. Templates of A1/B1/C1-A18/B18/C18 were *L. interrogans* serovar Lai (5601), *L. borgpetersenii* serovar Javanica (56602), *L. interrogans* serovar Canicola (56603), *L. borgpetersenii* serovar Ballum (56604), *L. interrogans* serovar Pyrogenes (56605), *L. interrogans* serovar Autumnalis (56606), *L. interrogans* serovar Australis (56607), *L. noguchii* serovar Pomona (56608), *L. interrogans* serovar Linhai (56609), *L. interrogans* serovar Hebdomadis (56610), *L. interrogans* serovar Paidjan (56612), *L. weilii* serovar Cingshui (56615), *L. interrogans* serovar Wolffi (56635), *L. borgpetersenii* serovar Mini (56655), *L. borgpetersenii* serovar Tarassovi (56671), *Mycobacterium tuberculosis*, *Brucella*, and distilled water (DW), respectively. CL, control line; TL, test line

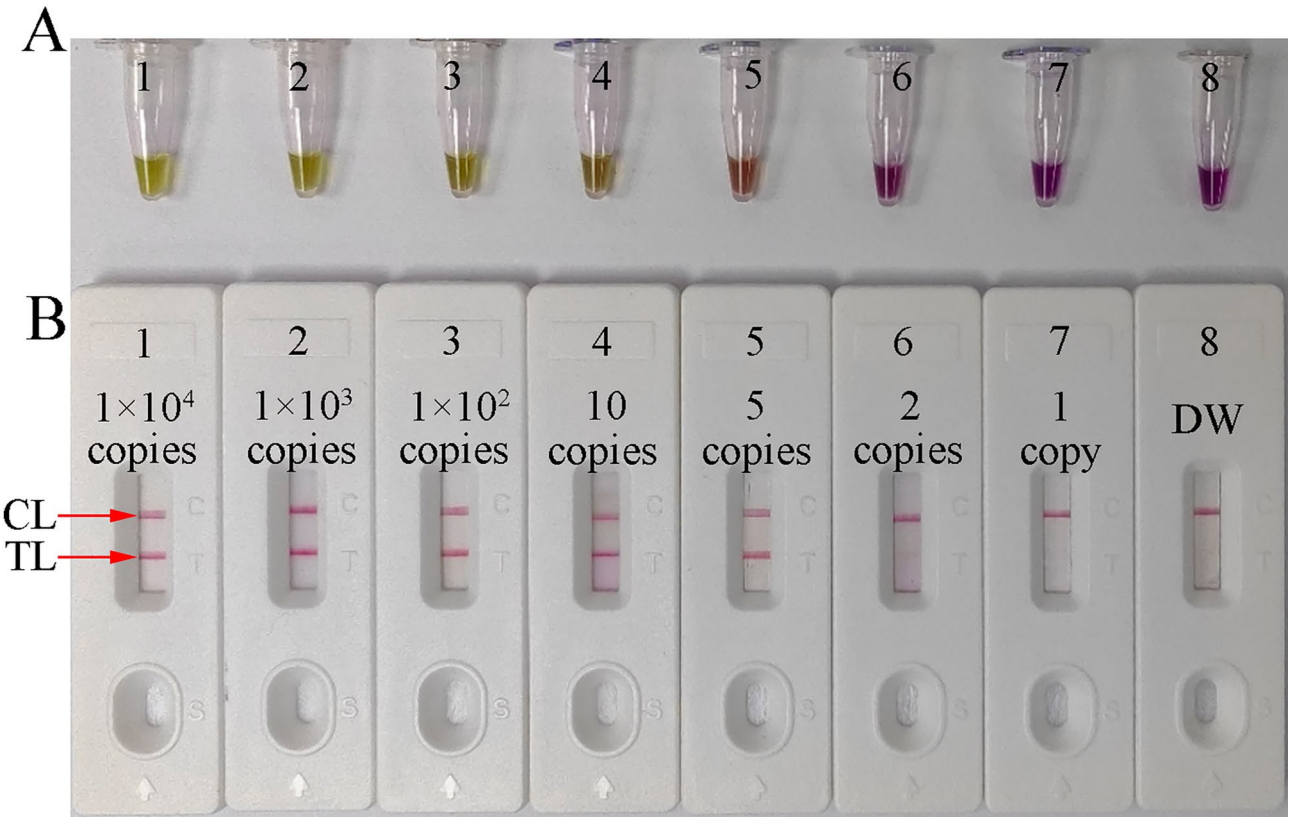

Fig. 4 Optimization of the temperature for *Leptospira*-LAMP reaction The standard LAMP reactions with target pathogen *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai (56601) DNA (1×10^4 copies per test) were monitored using real-time turbidimetry. Eight kinetic graphs (**a-h**) were generated at various temperatures ranging from 63–70℃ with 1℃ intervals. The threshold turbidity value was 0.1, and a turbidity > 0.1 indicated positive. The graphs from **f** (67℃) showed robust amplification

Sensitivity of the Leptospira-LAMP-AuNP-LFB assay

The sensitivity of our assay was evaluated with serially diluted genomic DNA from purified cultures (*L. interrogans* 56601) at concentrations ranging from 1.0×10^4 copies to 1 copy per test. *Leptospira*-LAMP reactions were performed at 67 °C for 60 min, and the LAMP products were analyzed through visual inspection with L-HNB reagents and AuNP-LFBs. The LoD of the *Leptospira*-LAMP-AuNP-LFB assay was 5 copies per reaction (Fig. 5B), which was the same as that obtained with the colorimetric indicator (L-HNB) (Fig. 5A).

Optimal Leptospira-LAMP-AuNP-LFB assay reaction time

To determine the optimal reaction time for our assay at the LAMP stage, four reaction times (15, 25, 35, and 45 min) were tested at the optimal reaction temperature (67 °C). The results were measured with an AuNP-LFB and colorimetric indicator (L-HNB). The results showed that the LoD of genomic DNA (5 copies per test) was detected when the amplification time was 35 min when an AuNP-LFB was used (Fig. 6C). However, LoD was not presented when the L-HNB method was used (Fig. 6A-D). Hence, the whole detection procedure for *Leptospira*-LAMP-AuNP-LFB detection, including rapid genomic DNA preparation (5 min), LAMP (35 min), and analysis of results using AuNP-LFB (< 2 min), could be completed within 45 min.

Specificity of the Leptospira-LAMP-AuNP-LFB assay

The specificity of our *Leptospira*-LAMP-AuNP-LFB assay was verified with 15 reference strains representing 15 serovars of 15 serogroups of pathogenic *Leptospira* spp. in China, nonpathogenic *Leptospira* spp. and non-*Leptospira* isolates (Table 2). Positive results were obtained for the nucleic acid isolated from all pathogenic *Leptospira* spp. strains, while negative results were obtained for the genomic DNA isolated from non-pathogenic *Leptospira* spp., all non-*Leptospira* strains and the blank control (Fig. 7). Therefore, these data indicated that our *Leptospira*-LAMP-AuNP-LFB assay could accurately identify pathogenic *Leptospira* spp. with no cross-reactivity with other strains.

Feasibility of Leptospira-LAMP-AuNP-LFB assay

To further confirm that the *Leptospira*-LAMP-AuNP-LFB assay is a valuable approach for the detection of *Leptospira* strains, 56 field mouse kidney samples and 26 clinical blood samples were collected and tested via

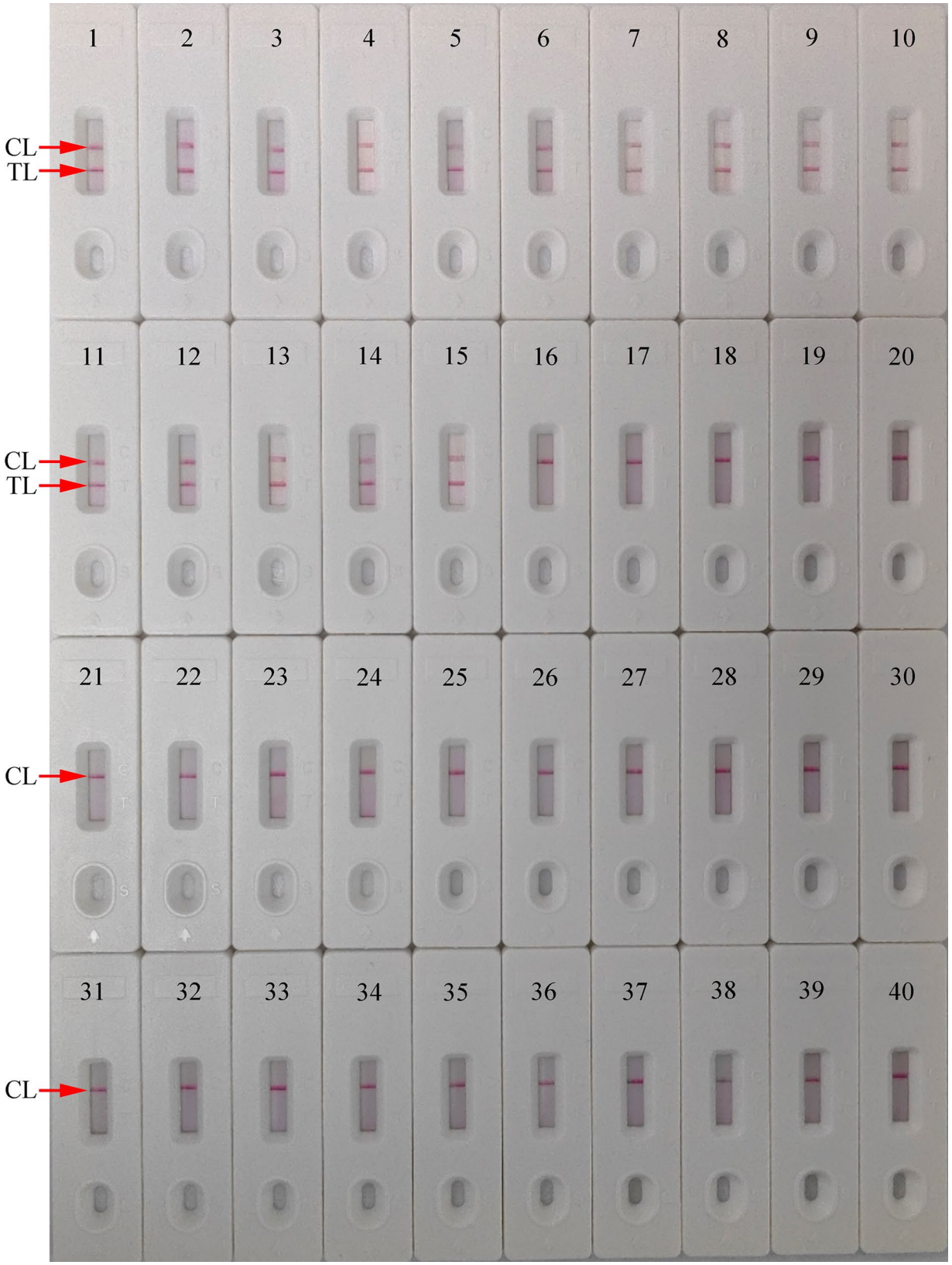

Fig. 5 Sensitivity analysis of *Leptospira*-LAMP assay Serial dilutions of target *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai (56601) DNA templates were subjected to standard LAMP reactions. The LAMP products were analyzed with visual reagent (L-HNB) and AuNP-LFB biosensor, respectively. Tubes/biosensors 1–8 (**A**, **B**) represented the genomic DNA levels of 1.0×10^4 copies, 1.0×10^3 copies, 1.0×10^2 copies, 10 copies, 5 copies, 2 copies,1 copy, and distilled water (DW), respectively. CL, control line; TL, test line

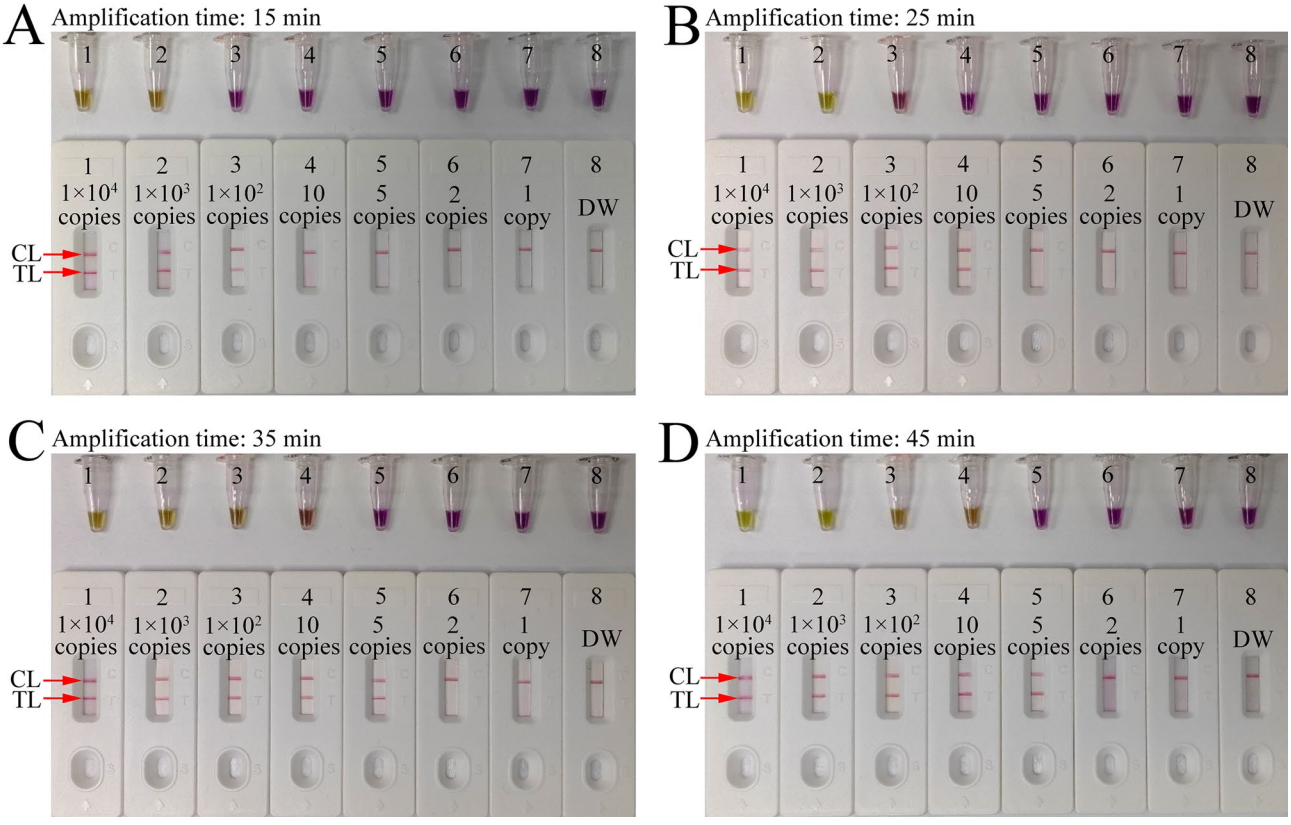

Fig. 6 Optimization of the reaction time for *Leptospira*-LAMP-AuNP-LFB assay Four different reaction times (**A**, 15 min; **B**, 25 min; **C**, 35 min; **D**, 55 min) were tested under optimal LAMP reaction temperature (67℃). The results were monitored simultaneously with colorimetric indicator (L-HNB) and AuNP-LFB biosensor. Tubes/biosensors 1–8 represented the *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai (56601) genomic DNA levels of 1.0×10^4 copies, 1.0×10^3 copies, 1.0×10^2 copies, 10 copies, 5 copies, 2 copies, 1 copy, and distilled water (DW), respectively. The signal of the LoD (5 copies per test) appeared with a 35 min reaction time through AuNPs-LFB biosensor (**C**). CL, control line; TL, test line

16 S rRNA gene sequencing after leptospiral cultivation and isolation, real-time PCR, and our *Leptospira*-LAMP-AuNP-LFB methods. The results revealed that 16 of the 82 samples tested as *Leptospira*-positive via the 16 S rRNA gene sequencing. The *Leptospira*-LAMP-AuNP-LFB assay results were consistent with sequencing results. Nevertheless, when real-time PCR was used, only 13 samples were confirmed as *Leptospira*-positive (Table 3 and Supplementary Materials TableS1). Compared with the cultivation-sequencing technology, the LAMP-AuNP-LFB sensitivity and specificity were 100.00% (95% CI: 79.41–100.00%) and 100% (95% CI: 94.56–100.00%), respectively (Table 3). Therefore, these data indicate that the *Leptospira*-LAMP-AuNP-LFB assay developed in this study can be used as valuable tool for the identification of *Leptospira* strains.

Discussion

In the present study, for the first time, we combined LAMP with an AuNPs-LFB to establish a novel *Leptospira*-LAMP-AuNP-LFB assay for rapid, visual, cost-saving, and in-field identification of pathogenic *Leptospira* strains. All the analytical data showed that the novel approach had a five-copy LoD and has no cross-reactivity with other microbes. The entire detection procedure could be completed within 45 min without the need for expensive facilities or trained personnel. The feasibility of our assay was tested through field mouse and leptospirosis clinical samples, and the results were compared with those of 16 S rRNA gene sequencing and real-time PCR methods.

Field mice serve as the main host of pathogenic *Leptospira* species [1–5], when mice come in contact with *Leptospira*-contaminated soil or water, the spirochetes rapidly enter the bloodstream to cause bacteremia, and then colonizes the epithelial cells of the kidneys. The pathogenic *Leptospira* spp. can continue to reproduce in the kidneys and be excreted through urine for a long time (even for life). At this point, field mouse becomes an asymptomatic carrier.

Traditionally, dark-field microscopy (DFM) is a rapid and simple diagnostic method for observing the presence of *Leptospira* in body fluids such as urine, serum, and blood [35]. However, DFM use is not recommended owing to its low sensitivity and specificity. A leptospiral concentration of 10^4 *Leptospira*/ml can be detected in

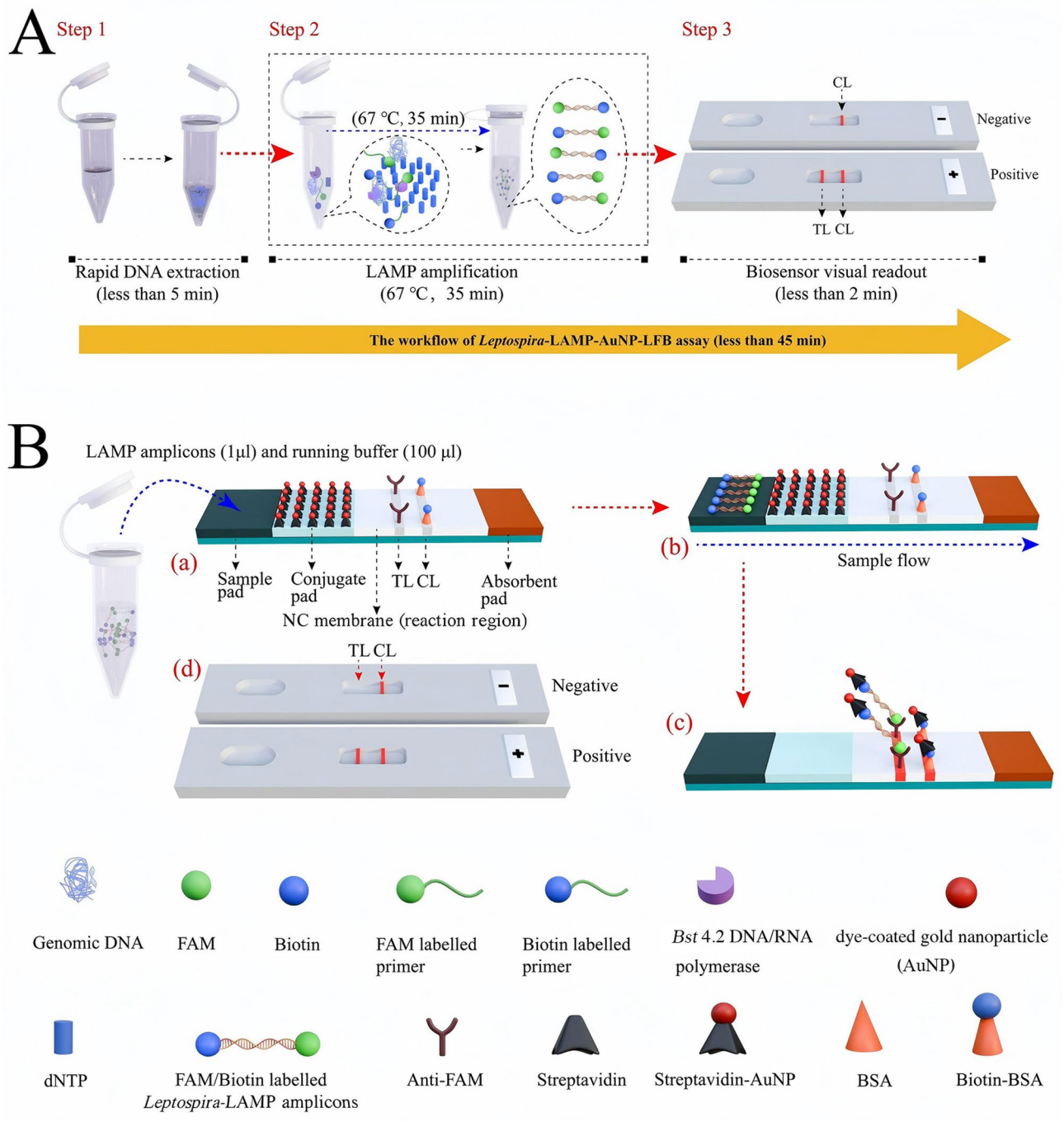

Fig. 7 Specificity analysis of *Leptospira*-LAMP-AuNPs-LFB assay with different microbes The specificity of our assay was evaluated using different nucleic acid templates. The *Leptospira*-LAMP were performed under optimal reaction conditions (67℃ for 35 min), and the amplification products were analyzed using AuNP-LFB. biosensors 1–15 used pathogenic *Leptospira* strains 56,601, 56,602, 56,603, 56,604, 56,605, 56,606, 56,607, 56,608, 56,609, 56,610, 56,612, 56,615, 56,635, 56,655, 56,671, respectively. biosensor 16 used nonpathogenic *Leptospira* strain *L. biflexa*. Biosensor 17–39 used *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Bacillus cereus*, *Streptococcus pneumoniae*, invasive *Escherichia coli*, enterohemorrhagic *Escherichia coli*, enterotoxigenic *Escherichia coli*, enteroaggregative *Escherichia coli*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Bordetella parapertussis*, *Haemophilus parainfluenzae*, *Vibrio parahaemolyticus*, *Acinetobacter lwoffii*, hepatitis B virus, hepatitis C virus, Human papillomavirus, influenza B virus, epstein-Barr virus, respectively. Biosensor 40, distilled water (Blank control). CL, control line; TL, test line

Table 3 Culture-sequencing, real-time PCR, and *Leptospira*-LAMP-AuNP-LFB comparisons for pathogenic *Leptospira* strains testing in in field mice kidney and human blood samples

Detection assay	Results	Standard method (culture-sequencing[a])		Sensitivity (%)	Specificity (%)
		+	-		
real-time PCR[b]	+	13 (field mice: 10; Human: 3)	0	81.25% (95% CI: 54.35–95.95%)	100% (95% CI: 94.56–100.00%)
	-	3 (field mice)	66 (field mice 43; Human: 23)		
Leptospira-LAMP-AuNP-LFB	+	16 (field mice 13; human:3)	0	100% (95% CI: 79.41–100.00%)	100% (95% CI: 94.56–100.00%)
	-	0	66 (field mice 43; Human: 23)		

[a]For culture-sequencing, the leptospires were cultured in EMJH-liquid medium and incubated for 3 weeks at 28°C, and then the bacterial genomic DNA was extracted using Bacterial Genomic DNA Extraction Kit according to the manufacturer's instructions (Xi'an Tianlong Technology Co., Ltd.; Xi'an, China). The 16S rRNA gene was amplified with PCR method and then were sequenced by Sanger sequencing. The sequences were analyzed with basic local alignment search tool (BLAST) alignment (information available at www.ncbi.nlm.nih.gov/BLAST)

[b]The real-time PCR diagnosis was carried out using commercial real-time TaqMan *Leptospira* Nucleic Acid Assay Kits (MABSKY Biological Co., Ltd., Shenzhen, China). The threshold cycle (Ct value) ≤ 36 was determined to indicate a positive result according to the manufacturer's guidelines

+, positive; -, negative

the body fluids of an infected individual. In addition, a sophisticated dark-field microscope and skilled personnel are required to perform DFM [35, 36]. Cultivation and isolation are considered standard techniques for the diagnosis of leptospirosis. As *Leptospira* is a slow-growing strain, 6–8 weeks are required for the cultivation of *Leptospira*, which is of low diagnostic value and does not permit early diagnosis [36, 37]. The MAT is regarded as the "gold standard" for leptospirosis, but it does not permit early diagnosis because the sensitivity of the MAT is low during the initial acute phase, and testing both acute- and convalescent-phase serum samples is required for accuracy [35–37]. In recent years, nucleic acid amplification technologies (NAATs) have been referred to as revolutionary techniques for the diagnosis of infectious diseases with specificity, sensitivity and a short turn-around time [35–38]. However, the use of NAATs in low- and middle-income regions is greatly limited owing to the high cost of equipment and need for trained experts. In this study, we used the isothermal amplification technique LAMP to amplify the target gene within a short time (35 min) at a fixed temperature (67°C), which can be achieved with simple devices, such as a heating block, a water bath, or a metal bath. More importantly, the *Leptospira*-LAMP products were objectively and visually analyzed with an AuNP-LFB.

The *Leptospira*-specific amplicons were generated through a LAMP system using only *Bst* 4.2 DNA/RNA polymerase with strand displacement activity and a set of six specific primers that span eight unique segments of the target sequence. The *Leptospira*-LAMP reaction conditions were optimized at 67 °C for 35 min. The specificity of our *Leptospira*-LAMP-AuNP-LFB assay was verified through pathogenic *Leptospira* strains and other microbes. As expected, the *Leptospira*-LAMP-AuNP-LFB technique specifically identified pathogenic *Leptospira* spp. strains but showed negative results for other isolates (Table 2; Fig. 7). In order to further confirm the specificity of our assay, more non-pathogenic *Leptospira* spp. or closely related species should be test in the next study. In the present study, a paper-based AuNPs-LFB was successfully manufactured to specifically recognize *Leptospira*-LAMP products. Owing to its cost-saving, high sensitivity and specificity, and ease of use, the AuNP-LFB platform is currently the most commonly used POC biosensor for medical diagnostic applications [27–29]. The integration of AuNPs as signal transducers has increased the analytical capabilities of these biosensors due to the unique physicochemical characteristics of these nanoparticles, such as a high surface-to-volume ratio, chemical stability, good biocompatibility, and low toxicity [39, 40]. In the present study, the colorimetric indicator L-HNB, agarose gel electrophoresis, and real-time turbidity were also applied to analyze *Leptospira*-LAMP amplicons. The L-HNB method is visual and equipment-free, but more reaction time is required for detection at the LoD (Figs. 5 and 6). Moreover, the visual result was ambiguous when the concentrations of the amplicon were low (Figs. 5 and 6). Specific and expensive facilities are required for the latter two methods, which are thus not suitable in resource-poor settings. In addition, the AuNP-LFB is easy to manufacture and cost-saving (approximately US $2.0 per test). Hence, the total cost of each assay, including genomic DNA isolation (~ US $0.5), LAMP (US $1.0), and AuNP-LFB detection (~ US $2.0), was US $3.5, which is lower than that of traditional real-time PCR testing (approximately US $7.0).

In previous studies, various isothermal amplification methods were used for the diagnosis of leptospirosis. Nurul Najian et al. (2016) reported a multiplex LAMP label-based AuNP lateral-flow dipstick biosensor approach targeting the *LipL32* gene for the detection of 13 representative pathogenic *Leptospira* species, and achieved an LoD of 3.95×10^{-1} genomic equivalent/ml [41]. The isothermal amplification process of their method takes 60 min, which is longer than that of our assay (45 min). Li et al. (2019) integrated multiple cross-displacement amplifications with a nanoparticle-based

Table 4 Comparison of the commonly used methods for pathogenic *Leptospira* detection

Technology	Limit of detection	Sensitivity (%)	Specificity (%)	Execution time	Advantages	Disadvantages	References
Microscopic agglutination test (MAT)	-	41% 1 st week; 82% 2nd–4th weeks; 96% beyond 4th weeks	94–97%	4–5 h	Widely available; Identifies serogroup	Specialized laboratory only; May be negative during the first 5–7 days; Cross-reactions may confuse interpretation	35–37
Dark field microscopy (DFM)	10^4 strains/ml	10^4*Leptospira*/ml can be detected	40%	1–5 min	Rapid detection	Low sensitivity and specificity	35, 36
Cultivation	-	48%	-	6–8 weeks	Directly demonstrates presence of the strains	Technically demanding and time-consuming; does not permit early diagnosis	36, 37
Real-time PCR	five copies per reaction	100%	75%	Approximately 2.5 h	Sensitivity and rapid results	Not serogroup specific	35–38
Label-based m-LAMP	3.95×10^{-1} genomic equivalent/ml	-	100%	60 min	High specificity and sensitivity	Just 13 pathogenic *Leptospira* specie can be detected.	41
MCDA-LFB	10 genomic equivalents per reaction	-	100%	70 min	High specificity and sensitivity	Just 13 pathogenic *Leptospira* specie can be detected; Need to open the reaction tube for results identification	31
LAMP combined with fluorescence detector	100 copies per test	-	100%	60 min	Easy to operation	Require expensive equipment	32
LAMP combined with visual reagent assay	10 leptospiral organisms per test	-	100%	60 min	High specificity; without opening the reaction tubes for results identification	The results are ambiguous when the LAMP product concentrations are low	42
LAMP-AuNP-LFB	5 copies per test	100% (95% CI: 79.41–100.00%)	100% (95% CI: 94.56–100.00%)	45 min	High sensitivity and specificity; accurate and visual interpretation; easy to operate and cost-saving	Need to open the reaction tube for results identification	Current study

LFB for the detection of 13 serovars representing 13 serogroups of *L. interrogans* in China and identified as few as 10 genomic equivalents per reaction of pure genomic DNA [31]. The isothermal amplification process of their method also takes 40-min. Lin et al. (2009) combined the LAMP reaction with a fluorescence detector for testing pathogenic *Leptospira* and achieved an LoD of 100 copies per test [32]. Ali et al. (2017) combined a LAMP assay with pre addition of dye for visual detection of *Leptospira*, and their results were ambiguous when the *Leptospira*-LAMP product concentrations were low [42]. In the present study, we designed a set of LAMP degenerate primers targeting the *LipL 41* gene from 15 reference strains representing 15 serovars of 15 serogroups of *Leptospira* spp. in China. The newly developed *Leptospira*-LAMP-AuNP-LFB assay was able to detect as few as 5 copies per test (Fig. 5), and the reference pathogenic strains of 15 serovars representing 15 serogroups were specifically covered in the scope of our *Leptospira*-LAMP-AuNP-LFB assay (Fig. 7). We summarized the commonly used methods for pathogenic *Leptospira* detection, their analytical properties and advantages/disadvantages were shown in Table 4. In addition, the crude nucleic acids were sufficient for LAMP because the *Bst* 4.2 DNA/RNA polymerase used is less readily inhibited than the *Taq* DNA polymerase used in traditional PCR-related methods [43]. In this study, the *Bst* 4.2 DNA/RNA polymerase contain hot-start ligand Aptamer, this ligand can block the enzyme activity with an efficiency of > 95% at < 30℃, and fully release the enzyme activity within 1 min at > 60℃. This characteristic is beneficial to establish the reaction system at room temperature and significantly reduce non-specific amplification under low temperature conditions. The whole detection process of our assay, including genomic DNA preparation (approximately 5 min), LAMP (35 min), and AuNP-LFB interpretation (less than 2 min), can be completed within 45 min, which is more time-saving than traditional techniques.

Our *Leptospira*-LAMP-AuNP-LFB detection platform also has some shortages. First, the degenerate LAMP

primers designed in this study specifically identify only 15 serovars of 15 serogroups of pathogenic *Leptospira* spp. prevalent in China. Owing to the high genetic heterogeneity of pathogenic *Leptospira* spp. and many serovars, the *Leptospira*-LAMP primers need to further refine for identifying many more serovars of pathogenic *Leptospira* species globally. Second, In this study, the *Leptospira*-LAMP reaction tube must be opened for AuNP-LFB interpretation, and will increase the risk of carry-over contamination. In our laboratory, spraying nucleic acid contamination scavenger soon after completing each AuNP-LFB test is an effective way to avoid nucleic acid contamination. For better adapt to clinical application, it is necessary to refine the *Leptospira*-LAMP-AuNP-LFB detection platform, and develop a sealed LAMP-AuNP-LFB device that avoids the tube opening procedure to prevent aerosol contamination. Third, owing to *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai is responsible for disease in over 60% of Chinese leptospirosis patients [44], this strain was used as a positive control for optimization of the *Leptospira*-LAMP-AuNP-LFB detection conditions (reaction temperature and time). However, The *Leptospira*-LAMP degenerate primers may introduce variability in amplification efficiency across serovars, the amplification efficiency metrics (e.g., sensitivity, reaction time) of each serovar could be further studied in the next study.

Conclusions

In the present study, we developed a new pathogenic leptospirosis molecular diagnostic method termed *Leptospira*-LAMP-AuNP-LFB, which is a combination of *Leptospira*-specific and rapid LAMP amplification with a visual and sensitive AuNP-LFB readout platform. Our assay can specifically detect the reference strains of 15 serovars representing 15 serogroups of pathogenic *Leptospira* spp. in China. The entire detection procedure can be completed within 45 min with no need for specific instruments. The newly developed *Leptospira*-LAMP-AuNP-LFB showed no cross-reactivity with other microbes and had a 5-copy LoD. Hence, our assay could be recognized as a valuable tool for rapid, visual, and in-field screening for pathogenic *Leptospira* infection.

Abbreviations

Spp	Species pluralis
LAMP	Loop-mediated isothermal amplification
AuNP	Gold Nanoparticle
LFB	Lateral flow biosensor
POC	Point-of-care
MAT	Microscopic agglutination test
PCR	Polymerase chain reaction
RPA	Recombinase polymerase amplification
CRISPR	Clustered regularly interspaced short palindromic repeats
HPLC	High-performance liquid chromatography
EMJH	Ellinghausen-McCullough-Johnson-Harris
No.	Number
NC	Nitrocellulose
SA-AuNPs	Streptavidin-coated gold nanoparticles
Ab	Antibody
BSA	Bovine serum albumin
TL	Test line
CL	Control line
LoD	Limit of Ddetection
DFM	Dark-field microscopy
NAATs	Nucleic acid amplification technologies

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-025-11645-4.

Supplementary Material 1. Table S1 Comparison of real-time PCR, Leptospira-LAMP-AuNP-LFB, and culture-sequencing assays for identification of pathogenic Leptospira strains in field mice kidney and human blood samples.

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Authors' contributions

XC and SL designed and conceived the experiments. XC, LX and SL designed and analyzed the LAMP primers. LX, YL, XY, and YC, performed the experiments. LX and YL collected and analyzed the data. XC and SL wrote and revised the manuscript. XC and SL revised the manuscript. All of the authors read and approved the final manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary materials

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Guizhou Provincial Center for Disease Control and Prevention (Approval No. G2019-01) and complied with the Declaration of Helsinki. Before our team obtained the clinical samples and conducted study, any personal identifiers of the suspected *Leptospira* infection had been removed by the monitoring stations. So the patient informed consents were waived by the Ethics Committee of the Guizhou Provincial Center for Disease Control and Prevention.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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