BMC Microbiology

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One-pot, uracil-DNA-glycosylase-aided, and multi-indicator nanobiosensor detection platform for identifying *Brucella melitensis* from the *Brucella* spp

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Abstract

Background Brucellosis, a zoonotic chronic contagious disease caused by *Brucella* spp., is primarily associated with species such as *Brucella melitensis* (*B. melitensis*) and *B. abortus*, among which *B. melitensis* is recognized as the most virulent in causing human brucellosis.

Methods Herein, we report a newly "one-pot" detection protocol for the identification of *B. melitensis* from the genus *Brucella* using the multiplex multiple cross-displacement amplification (MCDA) combined with dUTP-assisted uracil-DNA-glycosylase (UDGase) and gold nanoparticles lateral flow assay (AuNPs-LFA) biosensor (termed mMCUDA). Two sets of MCDA primers targeting the *bcsp31* and *BMEII0466* genes were designed. Various clinical isolates and whole blood samples were used to optimize and evaluate the mMCUDA assay.

Results The optimal condition for mMCUDA is 65℃ for 60 min. The limit of detection (LoD) of the mMCUDA assay was confirmed to be 7.5 fg/reaction. The mMCUDA assay can accurately identify *B. melitensis* from the *Brucella* spp. used in the study, and no cross-reaction with non-target pathogens was observed. The protocol has a powerful capability to eliminate carryover contamination (approximately 1.5×10^{-13} g/reaction).

Conclusions Taken-together, these investigative data demonstrated that the mMCUDA assay is a straightforward, one-pot, easy-to-obtain, friendly-to-use, and highly specific detection platform, which can be used as a useful potential screening tool in clinical applications.

Keywords *Brucella* spp., *Brucella melitensis*, Multiple cross-displacement amplification, Gold nanoparticles lateral flow assay, Uracil-DNA-glycosylase

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Background

Brucella causes brucellosis, a zoonotic chronic contagious disease that is widely prevalent worldwide [1, 2]. The main clinical characteristics of the disease are chronic and recurrent attacks, manifested as long-term fever (e.g., Malta fever, Mediterranean fever, Gibraltar fever, etc.), sweating, arthritis, and hepatosplenomegaly in infected humans [3–5]. Globally, there are approximately 5,00,000 new cases of brucellosis annually [1]. Currently, biological species identified within the genus *Brucella* (i.e., *Brucella* spp.) are categorized according to their pathogenicity and preferred host as *Brucella melitensis* (*B. melitensis*), *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, *B. ovis*, *B. ceti*, *B. microti*, etc [6, 7]. Among them, *B. melitensis*, initially isolated in Malta in 1887, is attributed to the vast majority of brucellosis cases worldwide and is recognized as the most virulent species in *Brucella* spp. to humans, primarily infecting domestic livestock and causing reproductive issues such as abortion and infertility, while also posing significant zoonotic risks [4, 8–11]. Other species like *B. abortus*, *B. suis*, and *B. canis* similarly target domestic animals and can infect humans, whereas *B. neotomae*, *B. ovis*, *B. ceti*, and *B. microti* mainly affect specific wildlife with limited but occasional zoonotic potential [4, 8–11]. Moreover, due to the diverse and non-specific clinical symptoms of brucellosis, it is easy to fail to recognize and misdiagnose, leading to long-term illness and inadequate treatment [12, 13]. In summary, it is particularly essential to early and accurately identify *B. melitensis* from the *Brucella* spp. to prevent and control brucellosis.

Isolation and cultivation of bacteria from whole blood specimens of suspected cases remain standard methods for diagnosing and identifying *Brucella* spp. in clinical laboratories [14]. However, the lengthy culture period (3–4 days) and more than two weeks required to confirm the results make it difficult to meet the early diagnosis of *Brucella* [15]. Based on this, a rapid detection protocol for the identification of *Brucella* spp. is urgently needed to meet the early diagnosis purposes. There are conventional serological tests, such as serum agglutination test (SAT), Rose Bengal test (RBT), complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA), etc [13, 16, 17]. Although serological tests are characterized by rapidity, they are limited by low sensitivity and selectivity, false positives, complicated workflow, and experienced trained personals [13, 14]. Meanwhile, none of them can be used alone to diagnose different stages of *Brucella* infection, but only in combination, resulting in the complexity of the diagnosis protocol [13]. A simplified, sensitive, specific, one-pot, and easy-to-obtain protocol for *Brucella* detection is urgently needed to meet early diagnosis requirement.

Nucleic acid amplification tests (NAATs) like polymerase chain reaction (PCR) and PCR-based techniques have improved the analytical sensitivity and specificity for target pathogen detection [15, 18, 19]. In particular, the classical real-time fluorescent quantitative PCR (real-time PCR) technique that was developed has dramatically improved the efficiency of nucleic acid tests and has played a vital role in controlling infectious diseases like leptospirosis, tuberculosis, etc [20–22]. Previously, a real-time PCR technique was also established here for the detection of *Brucella* in practical applications [15]. However, PCR-based assays have always been limited by the requirements for specialized thermocyclers and trained personnel, which has hindered their application in basic laboratories in resource-poor regions [14, 23, 24].

As mentioned above, to overcome these limitations, thermostatic-based NAATs techniques were devised, including recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), multiple cross-displacement amplification (MCDA), etc [25–29]. MCDA, is a novel NAATs technique, has a higher sensitivity than LAMP assay (~ 10-fold), and the amplification theory (only requires a single amplification enzyme), is uncomplicated than RPA assay [29]. The MCDA-based NAATs techniques have been used to detect various pathogens (e.g., bacteria, viruses, and other re-emerging infectious events) [29–31]. However, classical amplicons validation methods, like other thermostatic-based NAATs assays, can only identify single target, including visualization dyes (e.g., hydroxy naphthol blue and SYBR Green I), real-time turbidimeter, and agarose gel electrophoresis [32–34]. Meanwhile, these recognition methods are challenging to accurately determine thermostatic-based NAATs-specific and non-specific amplification. Thus, devising a novel and multi-indicator gold nanoparticles-based lateral flow assay (AuNPs-LFA) biosensor seems to be able to overcome the single-target defects mentioned earlier. Admittedly, thermostatic-based NAATs combined with an AuNPs-LFA biosensor require open-tube tests, which increase the risk of false positives caused by carryover contamination. In previous studies, NAATs techniques such as MCDA supplemented with dUTP (Deoxyuridine triphosphate)-assisted heat-labile uracil-DNA-glycosylase (UDGase) were confirmed to be helpful in preventing and eliminating carryover contamination certainly [35, 36]. In addition, many genes targeting *Brucella* spp. (e.g., *bcsp31*, *omp2*, *omp31*, *omp28*, 16 S rRNA, etc.) and *B. melitensis* (e.g., *BMEII0466*, *IS711*, etc.) have been proved to be effective molecular targets [14, 18]. For the detection of *Brucella* spp., *bcsp31* is commonly used, while for the identification of *B. melitensis*, *BMEII0466* is more frequently employed. Taken-together, multiplex MCDA (mMCDA) combined with dUTP-supplemented

UDGase enzymes and AuNPs-LFA biosensor targeting specific genes for the identification of *Brucella melitensis* from the *Brucella* spp. is an attractive protocol.

In this work, a novel multi-indicator AuNPs-LFA biosensor was designed according to the mechanism of mMCDA amplification (Fig. 1). Based on the capture principle of AuNPs-LFA biosensor, two sets of MCDA primers targeting conserved regions of the *bcsp31* and *BMEII0466* genes were devised and modified with Carboxyfluorescein (FAM) and Digoxigenin (Dig), respectively. Subsequently, the *m*ultiplex *M*CDA *C*ombined with dUTP-aided *U*DGase and *D*etector-based *A*uNPs-LFA biosensor (termed mMCUDA) was developed and applied to identify *B. melitensis* from the *Brucella* spp. Finally, various strains and clinical whole blood samples were used to evaluate the mMCUDA assay established in the current study.

Methods

Reagents and apparatus

Bst 6.0 DNA polymerase (stored in 10 mM Tris-HCl, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 50% Glycerol (pH 7.5)), 10 × reaction buffer (200 mM Tris-HCl, 500 mM KCl, 100 mM $(NH4)_2S0_4$, 20 mM $MgS0_4$, 1% Tween-20 (pH 8.8)), Uracil-DNA Glycosylase (Heat-labile, Bacterium), and 100 mM $MgSO_4$ (Beyotime Biotechnology Co., Ltd., Shanghai, China). Bacterial genomic DNA extraction kits and viral qEx-DNA/RNA extraction kits (Xi'an Tianlong Science &Technology Co., Ltd., Xi'an, China), dUTP (100 mM), dATP (100 mM), dCTP (100 mM), and dGTP (100 mM)

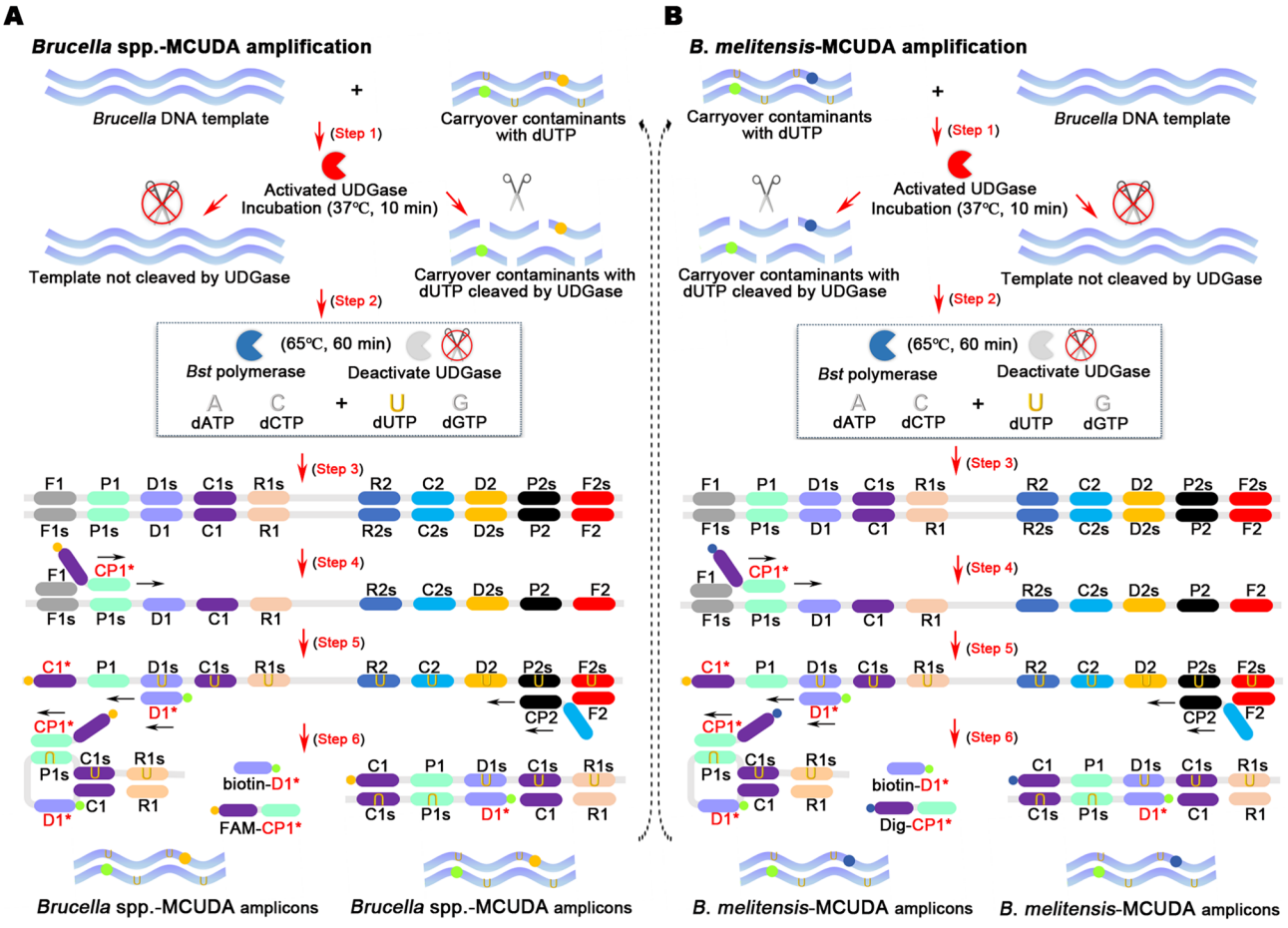

Fig. 1 Mechanism diagram of the mMCUDA protocol. The mMCUDA assay mainly includes UDGase-mediated cleavage action that followed dUTP-assisted MCDA amplification. Briefly, the UDGase-mediated cleavage for potential carryover contamination caused by mMCDA amplicons carrying dUTP was performed at 37 °C for 10 min after the premixed reaction mixtures (A and B, step 1). Then, the target fragments were exponentially amplified under the action of *Bst* DNA polymerase with strand displacement activity and ten MCDA primers capable of recognizing and binding to complementary regions on the target. Here, dTTPs in the dNTP mixtures are wholly replaced by dUTPs to ensure that produced amplicons carry uracil sites recognized by UDGase (A and B, steps 2–5). Finally, the FAM/Dig-CP1* and biotin-D1* primers were extended by binding to the target regions to yield abundant duplex FAM/Dig-amplicons-biotin complexes that can be captured by anti-FITC/anti-Dig antibodies (A and B, step 6). FAM, 6-carboxyfluorescein; mMCUDA, *m*ultiplex *M*CDA *C*ombined with dUTP-aided *U*DGase and *D*etector-based *A*uNPs-LFA biosensor; MCDA, multiple cross-displacement amplification; AuNPs-LFA, gold nanoparticles-based lateral flow assay

(Thermo Fisher Scientific, Beijing, China). These materials required for the preparation of multi-indicator LFA biosensors, including a backing card, sample pad, conjugate pad, nitrocellulose membrane (NC membrane), and absorbent pad (Jie-Yi Biotechnology. Co., Ltd., Shanghai, China). Biotin-BSA (Biotinylated bovine serum albumin), rabbit anti-FITC (Fluorescein isothiocyanate) antibody, and sheep anti-Dig antibody (Abcam. Co., Ltd., Shanghai, China). Crimson red Dye, SA-AuNPs (Streptavidin-coated gold nanoparticles) (Bangs Laboratories, INC., Indiana, USA). Real-time turbidimeter (*LA*–500) (Eiken chemical Co., Ltd., Japan) and ChemiDoc MP imaging system (Bio-Rad, USA).

Primers and plasmids design

A set of MCDA consists of 10 primers that can recognize ten different regions on the target, including two cross primers (CP1, C1 binding to P1, and CP2, C2 attaching to P2), two displacement primers (F1 and F2), and six amplification primers (C1, C2, R1, R2, D1, and D2) (Fig. S1 and Table S1). After sequence alignment and screening using the BLAST software (Basic Local Alignment Search Tool), two sets of MCDA primers, including *Brucella* spp.-MCDA primers targeting the *bcsp31* gene (GenBank accession no. M20404.1) and *B. melitensis*-MCDA primers targeting the *BMEII0466* gene (GenBank accession no. CP026337.1) were designed using Primer-Explorer V5 and Primer Premier software (5.0), respectively. According to the design principle of AuNPs-LFA biosensors, the CP1 and C1 primers were modified with FAM (*bcsp*-CP1*- and *bcsp*-C1*-FAM) and Dig (*BMEII*-CP1*- and *BMEII*-C1*-Dig), respectively. Four amplification primers were modified with biotin (*bcsp*-D1-, *BMEII*-D1-, *bcsp*-C1-, and *BMEII*-C1-biotin), respectively. According to the principle of MCDA reaction, the combination of labeled primers (CP1, C1, and D1) was selected in the MCDA mixed primers by randomly combining CP1 and C1 primers with C1 or D1 primers to avoid the cross-reactivity between labeled primers (Fig. S1, S2, and Table S1). All HPLC-purified grade primers used in the current study were synthesized and modified commercially (Tianyi-Huiyuan Biotech Co., Ltd., Beijing, China). Moreover, the *bcsp*- and *BMEII*-plasmids were prepared and quantified by synthesizing and/or attaching with the target fragments of *bcsp31* and *BMEII0466* genes, and subsequently cloning them into a pUC57 vector. The preparation procedure, including cloning and extraction, was performed by Tianyi-Huiyuan Biotech Co., Ltd. (Beijing, China). The original concentration of *bcsp*- (5 μg) and *BMEII*-plasmids (3 μg) was serially diluted (5×10^5, 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0, 5×10^{-1}, and 5×10^{-2} fg/μL).

Preparation of multi-indicator AuNPs-LFA biosensor

In this work, the multi-indicator AuNPs-LFA biosensor was devised according to the specific binding mechanism between antigens and antibodies. Firstly, the anti-FITC antibodies (0.15 mg/ml), anti-Dig antibodies (0.2 mg/ml), and biotin-BSA (2.5 mg/ml) were embedded on the NC membrane to form three lines, including test line 1 (TL1), test line 2 (TL2) and control line (CL). The TL1, TL2, and CL lines were separated by 5 mm intervals. Besides, the crimson red SA-AuNP 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 imbedded on a conjugate pad. And finally, the sampling pad, conjugate pad, NC membrane, and absorbent pad are successively fixed on the backing card utilizing plastic adhesive to form the sampling zone, conjugate zone, capture zone, and absorbent zone. The CL serves as a validation signal to confirm the operational integrity of the LFA sensor. The presence of an adequate amount of SA-AuNPs ensures the flow of unbound FAM-amplicons-biotin or primer-biotin-SA-AuNPs to the CL line. A red CL signal indicates proper LFA functioning and validates the result as reliable, whereas a colorless CL signifies malfunction and invalidates the result. Based on the above design, the multi-indicator AuNPs-LFA biosensor was assembled by TianJin HuiDeXin Biotech. Co., Ltd. (Tianjin, China). These assembled multi-indicator AuNPs-LFA were stored in a dark place at 4–30℃ for one year. Design principles of the multi-indicator AuNPs-LFA biosensor were displayed in Fig. 2.

Nucleic acid preparation

A total of 37 pathogens, including 17 *Brucella* and 20 non-*Brucella* strains, were used to evaluate the analytical specificity of the mMCUDA assay (Table S2). Here, these pathogens were prepared for the nucleic acid templates using the bacterial genomic DNA extraction kit or the viral qEx-DNA/RNA extraction kit. These extraction processes are performed using a fully automated nucleic acid extractor following the manufacturer's instructions. Moreover, 44 *Brucella* isolates and 54 whole blood samples from suspected patients with *Brucella* infection were used to evaluate the identification applicability of the mMCUDA assay in clinical tests. Isolates genomic DNAs were extracted using a rapid extraction protocol. Simply put, 200 μL of cultures (colonies scraped into the TE buffer solution) were placed in an enzyme-free EP tube, and then 100 μL of DNA extraction solution were added to it. Then, the EP tube was heated and boiled for 8 min, subsequently centrifuged at 12,000 rpm for 5 min, and the supernatant was used as an amplification template (stored at −20℃ until before use). The DNAs of whole blood samples were extracted using the QIAamp DNA

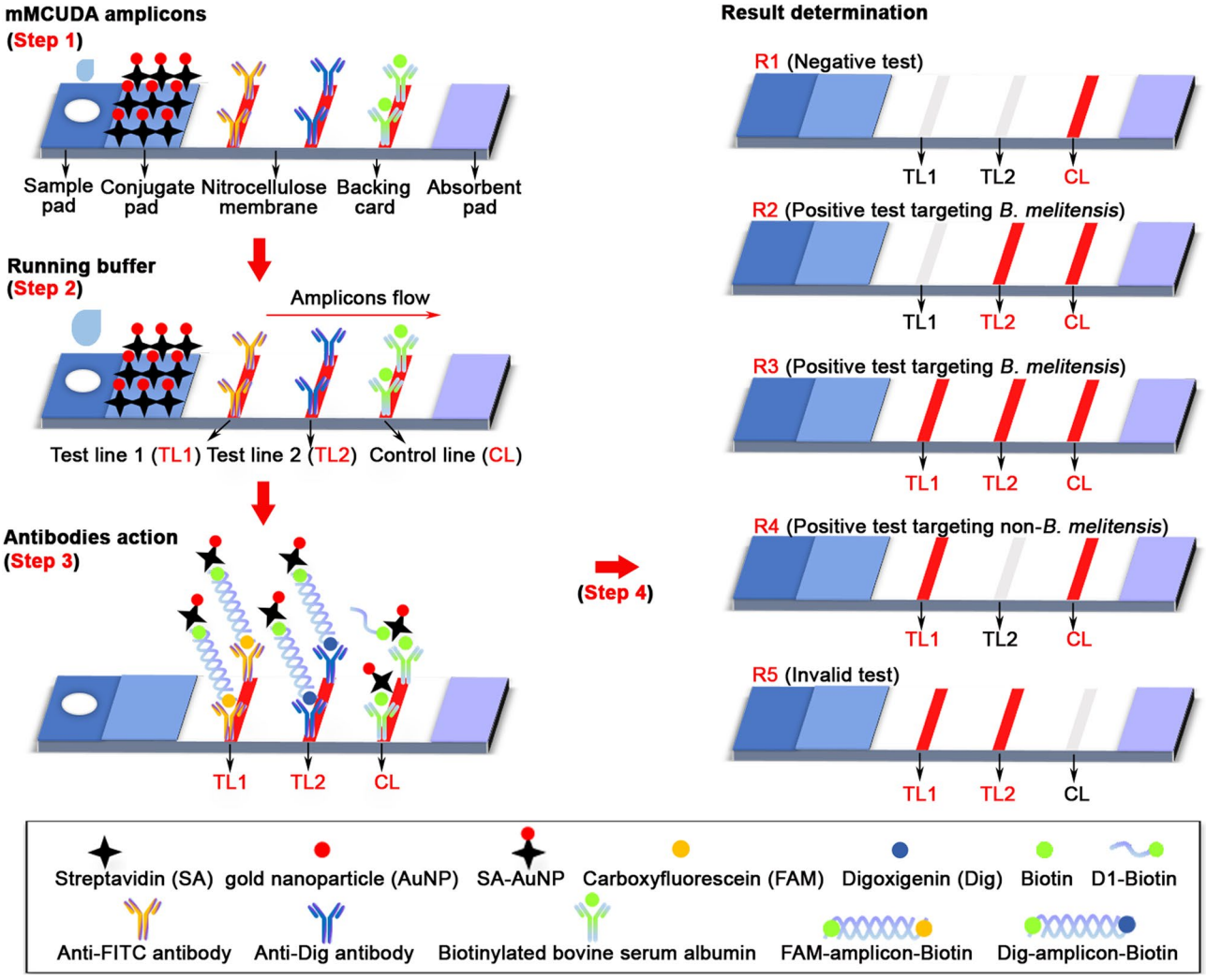

Fig. 2 Schematic diagram of the design principle of AuNPs-LFA biosensor. The AuNPs-LFA biosensor designed in the current study has four functional regions: sampling, conjugation, antibody capture (namely, NC membrane action zones), and absorption (step 1). These duplex FAM/Dig-amplicons-biotin complexes produced by MCUDA amplification were captured by anti-FITC and anti-Dig antibodies pre-embedded on the NC membrane to form test and control lines when they flowed the antibody capture zone under pushed by the running buffer (steps 1–3). Potential five results were displayed on the AuNPs-LFA biosensor (step 4), including a negative test (R1), single-positive test (R2, targeting *B. melitensis*), double-positive test (R3, targeting *B. melitensis*), single-positive test (R4, targeting other members of *Brucella* spp. excluding *B. melitensis*), and invalid test (R5). FAM, 6-carboxyfluorescein; mMCUDA, *m*ultiplex *M*CDA *C*ombined with dUTP-aided *U*DGase and *D*etector-based *A*uNPs-LFA biosensor; MCDA, multiple cross-displacement amplification; AuNPs-LFA, gold nanoparticles-based lateral flow assay

Blood Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Isolation and cultivation

Meanwhile, according to the routine operation procedures, 54 whole blood samples were isolated and cultivated for *Brucella*. Briefly, these whole blood samples were injected into a two-phase culture flask, followed by incubation at 37 °C and 5% CO_2 atmosphere for 3–5 days, respectively. All primary positive cultures were inoculated onto blood agar and *Brucella* agar plates for isolation and cultivation. Subsequently, frequently-used PCR assays targeting the B4 and B5 loci were performed to identify the genus *Brucella* by extracting genomic DNA. To confirm the presence of *Brucella* species, we employed AMOS PCR, a multiplex PCR method designed to differentiate between various *Brucella* species. The AMOS PCR reactions were performed in a 50 µL reaction volume containing 25 µL of 2 × Taq MasterMix, 2 µL of template DNA, and sterile water to a final volume of 50 µL. The primers used were *IS711*, A, M, O, and S, with concentrations and other details including primer sequences, expected amplicon sizes, and thermal cycling conditions as described in the original method by Bricker and Halling [37]. Moreover, the criteria for judging the cultivation results are that the culture flask will

be tilted and re-cultured for 30 days if no colony growth is found in the abovementioned cultivation condition. Once characteristic colony growth in the predetermined period is observed, it is determined as the primary culture-positive [38]. If no colony growth is still observed during the preset period, it will be considered as culture-negative [38].

The singlex MCUDA reaction mixtures

The singlex MCUDA (sMCUDA) reaction mixtures (*Brucella* spp.- and *B. melitensis*-MCDA) consist of 1 μL of *Bst* 6.0 DNA polymerase (40 U), 1.5 μL of $MgSO_4$ (100 mM), 2.5 μL *Bst* reaction buffer (10 ×), 1.4 mM dATP, 1.4 mM dCTP, 1.4 mM dGTP, 1.4 mM dUTP, 1 μL of heat-labile UDGase enzyme (1 U), 0.4 μM each of F1 and F2, 0.8 μM each of C1, C2, R1, R2, D1*, and D2, 1.6 μM each of CP1* and CP2, 1.5 μL of templates extracted from cultures, and DNase/RNase-free water were added to 25 μL. The sMCUDA reactions were digested by the UDGase enzyme at 37℃ for 10 min, followed by incubation at 65℃ for 60 min. Then, the sMCUDA amplicons were validated by AuNPs-LFA biosensors, real-time turbidimeter (*LA*-500), and 1.5% agarose gel electrophoresis.

Optimization of the primer ratio in the mMCUDA reaction

Typically, there is a specific competitive amplification between different primer sets in a multi-target reaction system, resulting in the cover of one or more with weak amplification efficiency. Based on this, the proportion of different single primer sets was optimized to explore the more balanced or directional amplification of *Brucella* spp.- and *B. melitensis*-MCDA sets in the mMCDA reaction system. Ten different blending schemes have been implemented for *Brucella* spp.- and *B. melitensis*-MCDA primer sets in the mMCDA mixed system (details are shown in Fig. S3). Finally, these mMCDA amplicons with different primer ratios were validated using a AuNPs-LFA biosensor by observing the color depth of TL1 and TL2 lines.

The mMCUDA reaction mixtures

The mMCUDA reaction mixtures consist of 1 μL of *Bst* 6.0 DNA polymerase (40 U), 1.5 μL of $MgSO_4$ (100 mM), 2.5 μL reaction buffer (10 ×), 1.4 mM dATP, 1.4 mM dCTP, 1.4 mM dGTP, 1.4 mM dUTP, 1 μL of heat-labile UDGase enzyme (1 U), 0.17 μM each of *bcsp*-F1 and *bcsp*-F2, 0.34 μM each of *bcsp*-C1, *bcsp*-C2, *bcsp*-R1, *bcsp*-R2, *bcsp*-D1*, and *bcsp*-D2, 0.68 μM each of *bcsp*-CP1* and *bcsp*-CP2, 0.23 μM each of *BMEII*-F1 and *BMEII*-F2, 0.46 μM each of *BMEII*-C1, *BMEII*-C2, *BMEII*-R1, *BMEII*-R2, *BMEII*-D1*, and *BMEII*-D2, 0.92 μM each of *BMEII*-CP1* and *BMEII*-CP2, 1.5 μL of templates extracted from cultures or 2 μL of templates extracted from whole blood samples, and DNase/RNase-free water were added to 25 μL. The mMCUDA reaction mixtures were digested by the UDGase enzyme at 37℃ for 10 min, followed by incubation at 65℃ for 60 min. Then, the mMCUDA amplicons were validated by AuNPs-LFA biosensors, real-time turbidimeter (*LA*-500), and 1.5% agarose gel electrophoresis. Moreover, to ensure test reliability, negative controls (1.5 μL of nucleic acid from non-target pathogens and 1.5 μL of environmental samples collected with sterile cotton swabs) and blank controls (1.5 μL of DNase/RNase-free water) were included in the study.

Assessment tests

The confirmation tests were conducted to validate the feasibility of the s- and m-MCUDA assays. 1.5 μL of plasmids was used as a positive control. 1.5 μL of DNA template extracted from *Listeria monocytogenes*, 1.5 μL of environmental sample in this test, and 1.5 μL of DNase/RNase-free water were used as amplification templates. The assessment test's reaction system and amplification conditions were performed as described above. Finally, the s- and m-MCUDA amplification products were verified using AuNPs-LFA biosensors, 1.5% agarose gel electrophoresis, and real-time turbidimeter (*LA*–500).

Temperature and time optimization tests

To achieve optimal detection efficiency, the incubation conditions for the s- and m-MCUDA assays were optimized by setting different temperatures and times. Here, a total of twelve different reaction temperatures (59–70℃, with 1℃ internal) were pre-screened according to the sMCUDA reaction systems using the plasmids (5×10^5 fg/μL). Subsequently, further validation was conducted within the pre-screened temperature range to determine the optimal incubation temperature using the diluents of the plasmids (5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0, 5×10^{-1}, and 5×10^{-2} fg/μL). The amplification result was reported using a AuNPs-LFA biosensor and real-time turbidimeter. In addition, the optimal incubation time of mMCUDA was also optimized by setting a serial of amplification times (40–70 min, ten internals). The serial dilution of plasmids (5×10^5, 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0, 5×10^{-1}, and 5×10^{-2} fg/μL) was used as a template, respectively. The reaction system of mMCUDA was described above. The results were validated using a AuNPs-LFA biosensor. Of note, the optimal reaction time is defined as the shortest time that can detect the lowest concentration of plasmids diluent, which is consistent with longer reaction time used in this study.

Confirmation of the capability to eliminate carryover contamination

The simulated tests were performed to evaluate the capability of the mMCUDA system to eliminate carryover contamination. There are two mMCUDA test groups: one is the UDGase-mediated group, and the other is the absence of the UDG enzyme. Simulated carryover contamination from mMCUDA amplicons was quantified and prepared into a series of dilutions ranging from 1×10^{-8} to 1×10^{-22} g/μL. 1.5 μl of the diluents above were used as amplification templates. All mMCUDA reactions, including UDGase and no-UDGase groups, were digested at 37 °C for 10 min, followed by immediate incubation at 65 °C for 60 min. Finally, the results of simulated carryover contamination were reported using AuNPs-LFA biosensors.

Analytical sensitivity and specificity

To verify the analytical sensitivity of the s- and m-MCUDA assays, the sensitivity test was performed according to its optimal reaction conditions (UDG enzyme digestion at 37℃ for 10 min, followed by incubation at 65℃ for 60 min). To investigate whether the self-owning activity intensity of MCDA reaction is limited by the presence of UDG enzymes in the MCUDA reaction, the sensitivity of the s- and m-MCDA assays without UDGase was also tested. 1.5 μl of series diluents of plasmids were used as amplification templates (5×10^{5}, 5×10^{4}, 5×10^{3}, 5×10^{2}, 5×10^{1}, 5×10^{0}, 5×10^{-1}, and 5×10^{-2} fg/μL). Besides, to validate the mMCUDA reaction's reliability for the detection of a single target, each set of dilutions of single *bcsp*-plasmids and *BMEII*-plasmids were utilized as templates to evaluate the analytical sensitivity of the mMCUDA assay, respectively. The amplicons were subsequently monitored using the AuNPs-LFA biosensors. Moreover, to confirm the mMCUDA assay's analytical specificity, 37 pathogens, including 17 target pathogens and 20 non-target pathogens, were used to evaluate its accuracy for identifying *B. melitensis* from the *Brucella* spp. (Table S2). The mMCUDA assay was carried out as previously described, and its amplicons were validated using AuNPs-LFA biosensors.

Applicability of mMCUDA for identifying isolates and whole blood samples

To evaluate the applicability of mMCUDA for detecting isolates and whole blood samples, 98 test objectives were detected, including 44 *Brucella* isolates and 54 whole blood samples from suspected infection patients with *Brucella* spp. These isolates were identified by mMCUDA assays. These whole blood samples were cultivated and immediately identified as described above. In addition, the DNA templates prepared from these samples were also directly detected using mMCUDA and real-time PCR assays. The mMCUDA assay was performed according to the optimal reaction conditions described earlier, and their amplicons were reported using AuNPs-LFA biosensors. Real-time PCR was implemented following the manufacturer's guidelines (Suzhou Youbosen Biotechnology Co., Ltd.). Finally, the applicability of the mMCUDA assay was evaluated by comparing test results with cultivation and real-time PCR assays.

Results

Overview of reaction principle of mMCUDA assay

The mMCUDA mixture developed in the current study mainly includes UDGase-mediated cleavage (Fig. 1A and B, step 1), dUTP-supplemented mMCDA amplification (Fig. 1A and B, steps 2–6), and AuNPs-LFA-mediated mMCDA amplicons validation (Fig. 2). Firstly, UDGase-mediated cleavage for potential carryover contamination caused by previous round of mMCDA amplicons carrying dUTP was performed at 37 °C for 10 min after the premixed reaction mixtures (Fig. 1A and B, step 1). Notably, the UDG enzyme is only active against the potential carryover contamination caused by dUTP-bearing amplicons and does not cleave the free dUTP in the mMCUDA reaction system (Fig. 1A and B, step 1). Subsequently, mMCUDA amplification was triggered with the aid of MCDA primers driven by the *Bst* DNA polymerase (Fig. 1A and B, step 2). In the mMCDA system, the free dTTPs were replaced by dUTPs to ensure the mMCDA amplicons carrying dUTPs assured that the UDG enzymes were able to recognize and cleave the potential carryover contamination. Meanwhile, CP1*-FAM or CP1*-Dig can bind to D1*-biotin in mMCDA reaction to form abundant FAM-amplicons-biotin and Dig-amplicons-biotin complexes (Fig. 1A and B, steps 3–6). These complexes were captured by anti-FITC and anti-Dig antibodies pre-embedded on the NC membrane when 0.8–1.2 μl of mMCDA amplicons were pushed by 100–150 μl of running buffer (Figs. 2 and 3B, steps 1–3). Finally, potential five results were generated on the AuNPs-LFA biosensor, including negative test (Fig. 2, R1), positive test (Fig. 2, R2, targeting *B. melitensis*), positive test (Fig. 2, R3, targeting *B. melitensis*), positive test (Fig. 2, R4, targeting other members of *Brucella* spp. excluding *B. melitensis*), and invalid test (Fig. 2, R5), when the AuNPs-LFA-mediated mMCDA amplicon validations were completed (Fig. 2, step 4). The whole workflow of MCUDA assay includes DNA preparation (Fig. 3A, step 1), UDGase-mediated digestion (Fig. 3A, step 2), MCDA amplification (Fig. 3A, step 3), and AuNPs-LFA verification (Fig. 3B, steps 1–3), as illustrated in Fig. 3.

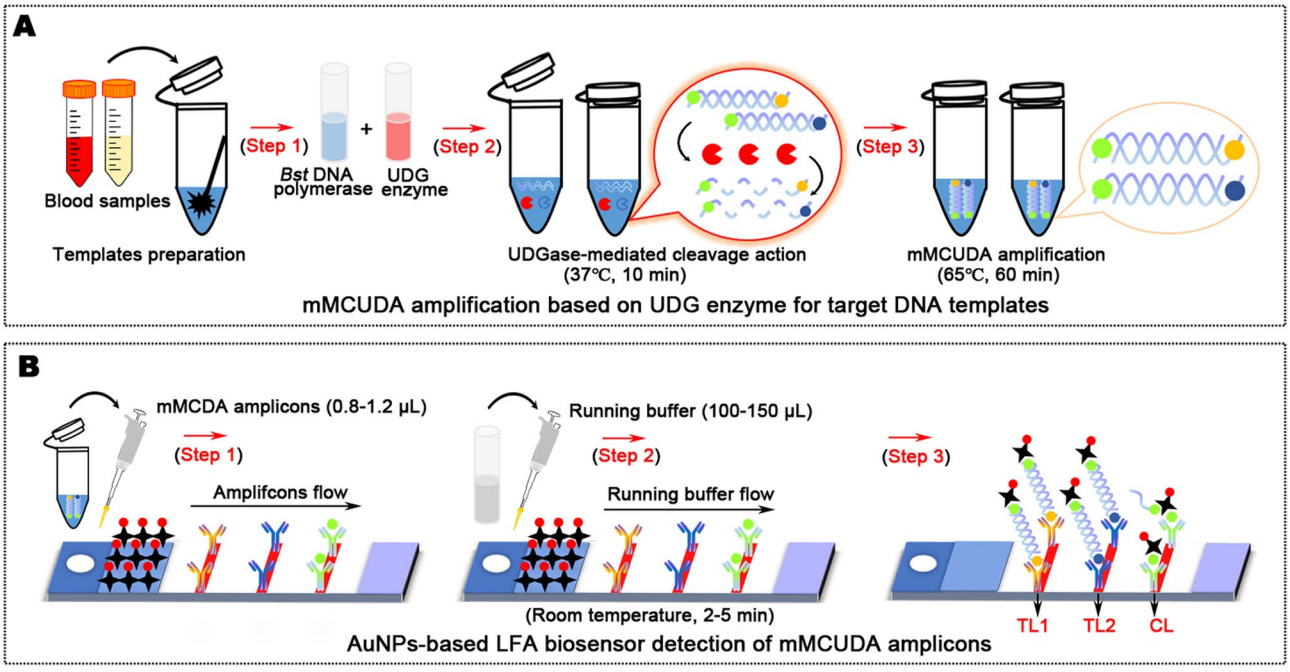

Fig. 3 Overview diagram of the whole workflow of mMCUDA assay. The whole workflow of MCUDA assay includes rapid DNA preparation (**A**, step 1), UDGase-mediated digestion (**A**, step 2), dUTP-assisted MCDA amplification (**A**, step 3), and AuNPs-LFA biosensor verification (**B**, steps 1–3). mMCUDA, *m*ultiplex *M*CDA *C*ombined with dUTP-aided *U*DGase and *D*etector-based *A*uNPs-LFA biosensor; MCDA, multiple cross-displacement amplification; AuNPs-LFA, gold nanoparticles-based lateral flow assay

Assessment tests of s- and m-MCUDA assay

In positive amplification, the CL, TL1 and/or TL2 lines appeared red on the AuNPs-LFA biosensor (Fig. S4, S5, and S6 A1-2); a turbidity value greater than 0.1 (the turbidity threshold was automatically set to 0.1) utilizing a real-time turbidimeter (Fig. S4, S5, and S6 B1-2); the characteristic gradient lanes were generated by 1.5% agarose gel electrophoresis (Fig. S4, S5, and S6 C1-2) in s- and m-MCUDA tests; and in negative amplifications, only the CL line displayed red (Fig. S4, S5, and S6 A3-5); the turbidity value was less than 0.1 (Fig. S4, S5, and S6 B3-5); and there were no characteristic gradient lanes in s- or m-MCUDA tests (Fig. S4, S5, and S6 C3-5).

Optimal reaction conditions of mMCUDA assays

As shown in Fig. S7 and S9, there was no difference in the amplification efficiency ranging from 59 to 70℃ measured by AuNPs-LFA biosensor in *bcsp*- and *BMEII*-MCUDA assays; and when the amplification process was monitored using a real-time turbidimeter, the *bcsp*-MCUDA assay exhibited robust kinetic curves with identifiable differences in the temperature range of 62–65℃ (Fig. S8, D-G), while the range of the *BMEII*-MCUDA test was 63–68℃ (Fig. S10, E-J). Interestingly, there is a phenomenon precisely opposite to the above sMCUDA experiments in the mMCUDA reactions, where its optimal pre-screening temperature range was 64–66℃ (Fig. S11, strips 6–8), that can only be achieved using AuNPs-LFA biosensors rather than real-time dynamic curves (Fig. S11 and S12). When the amplification temperature of the mMCUDA was 65℃ (Fig. S13, plot-B), it was steadier than other temperatures via further fine testing of the abovementioned pre-screening temperatures. Comprehensively, the optimal incubation temperature of the s- and the m-MCUDA test was 65℃ in the current study. Besides, the optimal incubation time of the mMCUDA assay was also obtained according to the definition in the Methods section. The lowest concentration of serial diluents of plasmids that can be detected by mMCUDA assay was 7.5 fg/reaction when amplification times were 60 and 70 min (Fig. S14). Thereby, the optimal amplification condition for the mMCUDA assay was 65℃ for 60 min (Fig. S14, C).

Applicability of eliminating carryover contamination

As shown in Fig. S15, the applicability of the mMCUDA assay for eliminating carryover contamination was evaluated by stimulated tests. In the current study, the maximum amount of carryover contamination caused by mMCDA amplicons that can be eliminated by mMCUDA assay was approximately 1.5×10^{-13} g/reaction (Fig. S15, strips 6–15). A threshold for the total mass of simulated carryover contaminants that cannot be prevented entirely by high-efficiency particulate air filters or fibrous pipette tip filters in biosafety cabinets is 1.0×10^{-18} g, which is approximately equivalent to an aerosol droplet with a

0.2 μm-diameter, according to previous publications [35, 39, 40]. This powerful capability of eliminating carryover contamination can significantly downgrade the possibility of false-positive amplification, and it is 10^2~10^5 folds higher than similar MCDA-based tests (approximately 1.0×10^{-15} or 1.0×10^{-18} g/reaction) [35, 36].

Sensitivity of s- and m-MCUDA assays

After repeated tests, the sMCUDA (UDGase present) and sMCDA assays (UDGase missing) can detect the lowest concentration of 7.5 fg/reaction for serial diluents of plasmids (Fig. S16 and S17). Similarly, the detection limit of the single-target mMCUDA assay was 7.5 fg/reaction (Fig. S18). Importantly, the analytical sensitivity of the multi-target mMCUDA assay developed in the study has been proven to be consistent with the mMCDA assay (UDGase missing) (Fig. 4 and S19), confirming the rationality and reliability of the mMCUDA protocol.

Specificity of mMCUDA assay

As expected, the mMCUDA assay developed can accurately detect all *Brucella* spp. strains (including *B. melitensis*, *B. abortus*, and *B. suis*) used in this study and identify *B. melitensis* from them (Fig. 5). No cross-reactions with non-*Brucella* strains were observed here, confirming its analytical specificity of 100%. Namely, there are four results generated using the AuNPs-LFA biosensor: (1) CL, TL1, and TL2 or only CL and TL2 lines appeared red were positive amplification targeting *B. melitensis*; (2) only the CL and TL1 lines appeared red were positive amplification targeting other members of *Brucella* spp.; (3) only CL lines appeared red were negative amplification.

Practicality of identifying isolates or whole blood samples

To verify the practicality of the mMCUDA assay for identifying clinical isolates or whole blood samples, 44 isolates and 54 whole blood samples were used for evaluation tests. Here, 42 clinical isolates were identified as *B. melitensis*, and the other 2 strains were non-*B. melitensis* in number of the genus *Brucella* by mMCUDA assay (agreement rate was 100% with initial results). Notably, the 4 whole blood samples from patients with suspected *Brucella* infection were identified as *B. melitensis*-positive by the mMCUDA assay (4/54), and 50 samples were negative (Table 1). The detectable rate was consistent with the canonical culture method (4/54) (Table 1). Meanwhile, 4 samples were identified as *Brucella* spp.-positive (4/54) and 50 as negative via real-time PCR assay (Table 1). Token together, these data confirmed that the mMCUDA assay devised in this study can be used as a useful identification tool for clinical isolates and whole blood samples.

Discussion

To date, brucellosis caused by *Brucella* spp. is still an infectious disease that cannot be ignored, especially in countries or regions where livestock farming is the primary income source [2, 41]. Among the members of the *Brucella* spp., *B. melitensis* is the most common and disease-producing species frequently associated with human brucellosis outbreaks globally [4, 15]. And yet other species, like *B. ovis* and *B. neotomae*, have not been reported to be associated with infection in humans [13]. Because of this, it is essential to identify members of the genus *Brucella*, especially highly pathogenic species such as *B. melitensis*. Moreover, the clinical symptoms of brucellosis are always nonspecific, associated with fever of unknown origin (FUO) [4, 42]. Until now, traditional diagnostic techniques such as culture and serology tests have not simultaneously possessed the characteristics of being fast, sensitive, specific, and easy-to-use, making them unsuitable as an ideal single-examination protocol for *Brucella* infection [13].

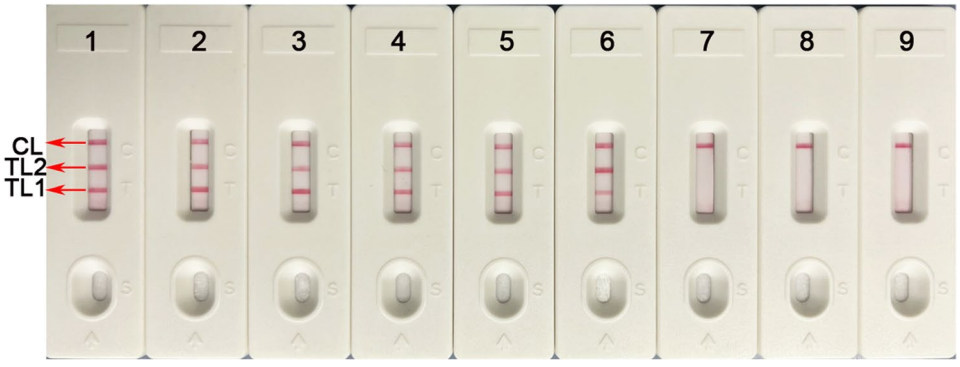

Fig. 4 Analytical sensitivity of mMCUDA assay using an AuNPs-LFA biosensor. The mMCUDA assay was performed using serial diluents of synthesized plasmids as templates (5×10^5 to 5×10^{-2} fg/μL). Biosensors 1–8 (A-C) correspond to 5×10^5, 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0, 5×10^{-1}, and 5×10^{-2} fg/μL. Strips 9 correspond to blank control (DNase/RNase-free water). As a result, the detection limit of the mMCUDA assay was 7.5 fg/reaction. mMCUDA, *m*ultiplex *M*CDA *C*ombined with dUTP-aided *U*DGase and *D*etector-based *A*uNPs-LFA biosensor; MCDA, multiple cross-displacement amplification; AuNPs-LFA, gold nanoparticles-based lateral flow assay; TL1, test line 1; TL2, test line 2; CL, control line

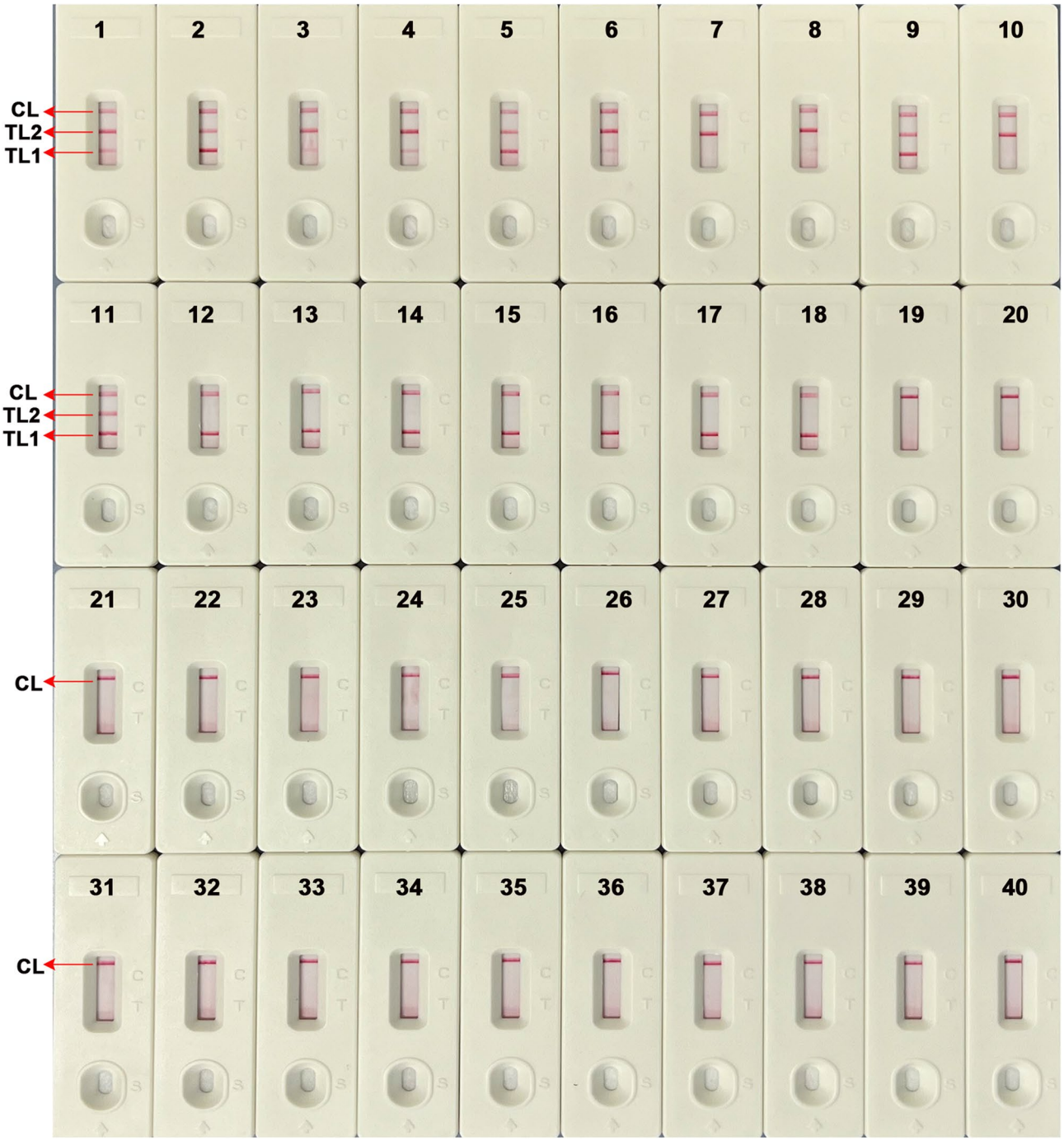

Fig. 5 Analytical specificity of mMCUDA assay using an AuNPs-LFA biosensor. The mMCUDA assay was implemented using nucleic acids extracted from various pathogens as templates. Strips 1–5 correspond to plasmids, *B. melitensis* (M5), *B. melitensis* (16 M, NCTC 10094), *B. melitensis* (Ether, NCTC 10509), and *B. melitensis* (standard material). Strips 6–11 correspond to *B. melitensis* (isolates). Strip 12–15 correspond to *B. abortus* (544, NCTC 10093), *B. abortus* (A19), *B. abortus* (isolate), and *B. abortus* (standard material). Strips 16–18 correspond to *B. suis* (1330, NCTC 10316), *B. suis* (S2), and *B. suis* (standard material). Strips 19–38 correspond to *M. tuberculosis* H37Rv (ATCC 27294), *M. tuberculosis* H37Ra (ATCC 25177), *M. tuberculosis* isolate (GZCDC), *M. malmoense* (ATCC 29571), *M. bovis* (ATCC19210), *Listeria monocytogenes* (GZCDC), *Staphylococcus aureus* (GZCDC), *Streptococcus pneumoniae* (GZCDC), *Escherichia coli* (GZCDC), *Klebsiella pneumoniae* (GZCDC), *Pseudomonas aeruginosa* (GZCDC), *Shigella* spp. (GZCDC), *Streptococcus suis* (GZCDC), *Orientia tsutsugamushi* (GZCDC), *M. leprae* (GZCDC), *Salmonella* spp. (GZCDC), *Bordetella pertussis* (GZCDC), *Neisseria meningitidis* (GZCDC), Human cytomegalovirus (BNCC), and Influenza virus (GZCDC). Strips 39-40 correspond to negative control (environmental samples in the experiment) and blank control (DNase/RNase-free water). NCTC, National Collection of Type Cultures; BNCC, BeNa Culture Collection; GZCDC, Guizhou Provincial Center for Disease Control and Prevention; ATCC, American Type Culture Collection; mMCUDA, *m*ultiplex *M*CDA *C*ombined with dUTP-aided *U*DGase and *D*etector-based *A*uNPs-LFA biosensor; MCDA, multiple cross-displacement amplification; AuNPs-LFA, gold nanoparticles-based lateral flow assay; TL1, test line 1; TL2, test line 2; CL, control line

Table 1 Comparison of detection results of whole blood samples

Methods[a]	Culture	
	Positive	Negative
mMCUDA		
Positive	4	0
Negative	0	50
Real-time PCR		
Positive	4	0
Negative	0	50

MCDA multiple cross-displacement amplification, *AuNPs-LFA* gold nanoparticles-based lateral flow assay, *PCR* polymerase chain reaction

[a]mMCUDA *m*ultiplex *M*CDA *C*ombined with dUTP-aided *U*DGase and *D*etector-based *A*uNPs-LFA biosensor

MCDA technique, as a novel NAAT method based on thermostatic incubation, which is more sensitive than LAMP and PCR assays, seems to be able to satisfy the above requirements. However, conventional MCDA amplicon validation patterns (e.g., visualization reagents, agarose gel electrophoresis, real-time turbidity meter, etc.) can only perform single-target detection and have low specificity [29]. A multi-indicator AuNPs-LFA biosensor designed in the current study was able to recognize double-target products of MCDA amplification. Furthermore, there is an additional CL line on the AuNPs-LFA biosensor to monitor the verification results, thus ensuring the reliability of the test results. Currently, the risk of carryover contamination caused by MCDA open-tube tests is also a concern that must be addressed. In previous studies, many dUTP-assisted UDGase-mediated assays that can finitely eliminate carryover contamination, such as LAMP-UDGase, MCDA-UDGase, etc., have been established and verified to be effective [35, 36, 43]. In general, many UDGase-mediated tests use a combination of dTTP and dUTP in reaction mixtures [35]. In our study, dTTP was utterly replaced by dUTP, which ensures that the synthesized mMCDA amplicon contains abundant Uracil sites that the UDG enzyme can recognize and cleave. As demonstrated in the simulation test, the mMCUDA has a more robust capability to prevent and eliminate contamination (approximately 1.5×10^{-13} g/reaction) than similar MCDA-based assays established previously (approximately 1.0×10^{-15} and 1.0×10^{-18} g/reaction) [35, 36], improving by about two orders of magnitude or more (Fig. S15). The ultra-strong clean-up performance has a crucial practical significance in ensuring the reliability of test results, especially for open-tube detection pathways based on isothermal NAAT techniques, like lateral flow assay, agarose gel electrophoresis, etc. Also, to avoid carryover contamination as much as possible, each step of the mMCUDA assay is performed in a different experimental zone despite introducing the UDGase-mediated eliminating carryover contamination in this study.

In fact, the selection of target genes is crucial for NAAT-based assays, like PCR, PCR-based, RPA, LAMP, and MCDA assays. Previous studies have established single LAMP and MCDA protocols targeting the *bcsp31* gene for detecting *Brucella* spp [36, 44]. Single hybridization assay targeting the *BMEII0466* gene has also been developed to identify *B. melitensis* species accurately [9]. Although these techniques have demonstrated a specific capability to detect the single-target, they cannot perform multi-target detection in a single reaction (namely, one-pot test). So, the UDGase-mediated multiplex dUTP-assisted MCDA amplification combined with a multi-indicator AuNPs-LFA biosensor (i.e., mMCUDA) was creatively designed and successfully established to detect simultaneously the member of the genus *Brucella* and identify *B. melitensis* from them in the current study. As expected, both sMCUDA and mMCUDA can effectively detect prescribed targets without non-specific amplification occurring in negative and blank controls, as corroborated by our confirmatory experiments (Fig. S4, S5, and S6). Importantly, in addition to the AuNPs-LFA biosensor verification manner, the real-time turbidity and canonical agarose gel electrophoresis were also applied to the product analysis of MCDA, which was entirely consistent with biosensor results and also confirmed the feasibility of the MCUDA assay.

Of note, since the species of the genus *Brucella* have a high degree of genomic homology, especially between *B. melitensis* and *B. abortus*, it is incredibly challenging to accurately identify them by conventional molecular methods [1]. A highly conserved region in the *BMEII0466* gene (approximately 80 bp) was found by sequence alignment when homologous analysis was performed with the whole genome sequence of *B. abortus* strains (notably, A19 vaccine strain). To ensure accurate differentiation between *B. melitensis* and *B. abortus*, the *BMEII*-CP1* primer located in the specific region is labeled with Dig. As a result, the *BMEII*-CP1* primer does not complement sequences in non-*B. melitensis* genomes, resulting in the purpose of precisely identifying *B. melitensis*. Besides, the specificity tests also confirmed that the mMCUDA assay targeting *bcsp31* and *BMEII0466* genes can detect all members of the genus *Brucella* used in the study and accurately identify *B. melitensis* from them (Fig. 5). Due to the limitation of the source, partial typical representative *Brucella* reference strains were used here for specificity validation (Table S2). Thereby, the mMCUDA assay being used as a laboratory diagnostic tool in the future may require further evaluation using other reference strains if necessary.

In this study, there was a competitive reaction between *bcsp*-MCDA and *BMEII*-MCDA in the mMCUDA reaction mixture, as shown in Fig. S11. This phenomenon is more pronounced when the ratio of the two primer sets

is more balanced or within an undesired temperature range, especially in the latter. Therefore, the pre-mixing ratio of two primer sets and amplification temperatures were optimized to obtain a more favorable result for the *BMEII*-MCDA reaction in the mMCUDA reaction to ensure the precise identification of *B. melitensis* from the members of the genus *Brucella*. This biased choice does not affect the detection efficiency when the single-target sensitivity of the mMCUDA was confirmed to be consistent with its double-target sensitivity (Fig. 4 and S18). As demonstrated by specificity experiments, the red color of the TL1 line was weaker than the TL2 line and even disappeared when *B. melitensis* isolates were detected by mMCUDA, while it showed the expected deep red in the identification of other members of the *Brucella* spp. (Fig. 5).

Moreover, the mMCUDA exhibits higher detection sensitivity compared to the canonical PCR assay [19], which holds practical significance for improving detection rates, especially in samples with low bacterial loads (Fig. 5). The sensitivity of the UDGase-assisted s- and m-MCUDA assay has been confirmed to be consistent with the s- and m-MCDA reaction without UDGase, allowing this study to provide the two different detection protocols mentioned above. However, the amplification time required for mMCUDA is longer than that of the previously established MCDA-based technique [36], possibly due to the limited availability of conserved regions in the *BMEII0466* gene of *B. melitensis* that can be used to design effective MCDA primers. As we mentioned earlier, real-time turbidity and classical agarose gel electrophoresis cannot recognize multi-target objectives in a one-pot test, as shown in assessment and optimization experiments (Fig. S4, S5, and S6). Together, the two suboptimal verification methods mentioned above attach with special instrument requirements (e.g., real-time turbidimeter, Imager, and so on) and increase detection complexity (namely, multi-step operation). Conversely, the biosensor designed in this study can reduce the requirements of the apparatus and simplify the operation procedure. For MCDA or LAMP methods, the risk of cross-reactivity between primers caused by multi-primer pairs requirements is higher than that of PCR or RPA methods triggered by dual-primers, and these shortcomings also need to be further improved. The validation test may be necessary to avoid non-specific reactions, especially for multi-primer modifications such as between CP1*, D1*, and C1* primers in the current study (Fig. S2).

In this report, the potential applicability of the mMCUDA assay was also analyzed. It was found that the assay can detect all isolates and exhibits consistent performance compared with canonical culture and real-time PCR methods for detecting whole blood samples in clinical settings (Table 1). Admittedly, the mMCUDA protocol cannot perform quantitative detection like real-time quantitative PCR and can only achieve qualitative detection, which is also a self-owned limitation and a critical concern that needs to be overcome in the future. Still, quantitative testing is not necessary, qualitative strategy is intended for most screening programs (yes or no), and culture is often the final diagnostic criterion. Additionally, the mMCDA amplification procedure can be completed in 60 min, which is shorter than the reaction time required for real-time PCR (approximately 1.5 h). Thereby, these research findings confirmed that the mMCUDA assay is a credible, easy-to-use, and highly accurate detection protocol for the identification of *B. melitensis* from the *Brucella* spp.

Conclusions

In summary, two detection targets, the *bcsp31* gene targeting *Brucella* spp. and *BMEII0466* gene targeting *B. melitensis*, were creatively integrated into a one-pot reaction to detect genus and species simultaneously. An easy-to-use multi-indicator AuNPs-LFA biosensor was designed, and a strategy utilizing the dUTP-aided UDGase enzyme to eliminate carryover contamination was intruded in the current report. Token-together, the mMCUDA assay established in this study is a straightforward, one-pot, friendly-to-use, and highly specific detection protocol for the identification of *B. melitensis* from the *Brucella* spp., which can be used as a competitive potential screening tool in clinical settings.

Abbreviations

mMCUDA	Multiplex MCDA Combined with dUTP-aided UDGase and Detector-based AuNPs-LFA biosensor
MCDA	Multiple cross-displacement amplification
AuNPs-LFA	Gold nanoparticles-based lateral flow assay
PCR	Polymerase chain reaction
LoD	Limit of detection
RPA	Recombinase polymerase amplification
LAMP	Loop-mediated isothermal amplification
UDGase	Uracil-DNA-glycosylase
sMCUDA	Singlex MCUDA

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04189-9.

Supplementary Material 1.

Acknowledgements

Not applicable.

Authors' contributions

XY was involved in conceptualisation, data curation, formal analysis, investigation, software, methodology, validation, and drafting and writing of the manuscript. SL was involved in conceptualisation, data curation, funding acquisition, investigation, methodology, project administration, resources, supervision, visualisation, validation, and writing and reviewing of the manuscript. WY was involved in investigation, resources, software, and methodology. SM, YL, FC, JH, XZ, and HJ were involved in methodology and

formal analysis. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Funding
This study was funded by grants from National Natural Science Foundation of China (82273758), Health Commission of Guizhou Province (gzwkj2024-055), and Project for Public Health Talent Cultivation of China (Grant No. Guo Jikong Zong Ren Han [2024]122).

Data availability
All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate
This study was approved by the Human Ethics Committee of the Guizhou Provincial Center for Disease Control and Prevention (Ethical approval no. S2022-14) and complied with the Declaration of Helsinki. All clinical samples obtained in this study were collected with informed consent from participants and were anonymized to exclude any personal identifying information.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 7 December 2024 / Accepted: 1 July 2025
Published online: 02 August 2025

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