Acta Veterinaria-Beograd 2026, 76 (2), 159-172
UDK: 636.09:[616.98-07:579.882
DOI: 10.2478/acve-2026-0011

Research article

EXPLORATION OF NOVEL TARGET VIA GENOME MINING AND ESTABLISHMENT OF PROOFMAN-LMTIA ASSAY FOR THE RAPID DETECTION OF *CHLAMYDIA ABORTUS*

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(Received 05 January, Accepted 14 May 2026)

Chlamydia abortus is an obligate intracellular Gram-negative pathogenic bacterium widely distributed globally. This pathogen primarily infects humans and various domestic animals, causing abortions in pregnant animals. It poses a significant threat to public health and the animal husbandry industry, leading to substantial economic losses. In this study, specific primers and a Proofman fluorescent probe for LMTIA (Ladder Melting Temperature Isothermal Amplification) were designed based on the genome of the *C. abortus* S26/3 strain through sequence alignment and screening of specific target sequences. These primers and probes were used to develop a rapid Proofman-LMTIA assay for detecting *C. abortus*. The reaction system and temperature were optimized, and the specificity, sensitivity, and reproducibility of the assay were evaluated. The optimal amplification temperature for Proofman-LMTIA was 59.5 °C, and the reaction time was less than 30 minutes. The established assay had a minimum detection limit of 101 fg/μL per test, indicating a high analytical sensitivity. The assay also showed excellent stability, with coefficients of variation below 3% in inter-group and intra-group reproducibility tests. In summary, the rapid and effective Proofman-LMTIA method for detecting *C. abortus* developed in this study provides a novel technical tool for the clinical diagnosis and control of *C. abortus* infections.

Keywords: *Chlamydia abortus*, Proofman-LMTIA, detection method, diagnosis

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INTRODUCTION

Chlamydia organisms are non-motile obligate intracellular Gram-negative pathogenic bacteria belonging to the order Chlamydiales and the family Chlamydiaceae [1]. More than 15 Chlamydia species are pathogenic to humans or animals, including *Chlamydia abortus*, *Chlamydia avium*, *Chlamydia buteonis*, *Chlamydia caviae*, *Chlamydia crocodili*, *Chlamydia felis*, *Chlamydia gallinacea*, *Chlamydia muridarum*, *Chlamydia percorum*, *Chlamydia pneumoniae*, *Chlamydia poikilotherma*, *Chlamydia psittaci*, *Chlamydia suis*, *Chlamydia trachomatis*, and *Chlamydia crocodilias* as well as four Candidatus (Ca) species, namely *Ca. C. ibidis*, *Ca. C. corallus*, *Ca. C. sanzinia*, and *Ca. C. serpentis* [2,3]. These species cause various diseases. *C. abortus* is an important livestock pathogen with a significant economic impact and a primary cause of the enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA) worldwide, except in New Zealand and Australia [4,5]. The pathogen mainly causes enzootic abortion disease in sheep and goats. Moreover, it causes sporadic reproductive failure in several animal species, including cattle, pigs, deer, and horses [6]. Although poorly recognized as a human pathogen, *C. abortus* presents a significant zoonotic risk to breeders, veterinarians, and pregnant women by causing spontaneous abortions and potentially fatal consequences for pregnant women [7-9].

The detection and control of *C. abortus* are challenging due to the absence of specific clinical symptoms [10]. Infected animals typically lack any clinical symptoms prior to the abortion (in the last 2–3 weeks of gestation) or the delivery of very weak newborns [11]. Although a live vaccine (against *C. abortus* 1B strain) is used in Europe for the management of the disease in sheep, this vaccine has limitations such as poor safety, low stability, shedding of infectious organisms at parturition or during the protective immune response [11-14]. Therefore, early detection of *C. abortus* infection is crucial for controlling the disease spread. *C. abortus* infections are diagnosed using various detection methods, including direct identification, culture, antigen detection, serological tests, and nucleic acid amplification [5]. However, these methods have limitations such as prolonged processing time, high costs, intricate operations, and require high expertise, limiting the effective detection of *C. abortus* in clinical specimens [15].

The Ladder Melting Temperature Isothermal Amplification (LMTIA) is a new method for rapid nucleic acid detection developed by our research group, based on the isothermal amplification of a ladder-like melting curve [16]. The LMTIA technique effectively and rapidly amplifies target genes in an isothermal environment within a rapid timeframe (less than 30 minutes). Moreover, the nucleic acids bind with specific fluorescent probes (e.g., Proofman) to further improve the sensitivity and specificity of the amplification reactions. This technology is currently applied in food adulteration detection, traditional Chinese medicinal materials identification, and pathogen detection [17-25]. In this study, a rapid Proofman-LMTIA method was established for the detection of *C. abortus*. The findings will provide critical information on diagnosis and prevention of the spreading of the pathogen.

MATERIALS AND METHODS

Control DNA samples, clinical Samples, and DNA extraction

The *C. abortus* (GN6 strain) genomic DNA used in this study was a generous donation from the animal zoonosis team of Henan Agricultural University. The genomic DNAs of *Escherichia coli* (ATCC 25922), *Salmonella* spp. (ATCC 13076), *Listeria monocytogenes* (BNCC 185986), African swine fever virus, *Toxoplasma gondii*, *Cryptosporidium parvum*, *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis* were stored in the Key Laboratory of Biomarker-Based Rapid-Detection Technology for Food Safety in Henan Province.

In October and December 2025, field rectal swab samples were collected from 32 goats in Xuchang and 30 sheep Xinyang, Henan Province. The research protocol was reviewed and approved by the Ethics Review Committee of Xuchang University. These goats and sheep had been vaccinated against foot-and-mouth disease (Foot and Mouth Disease Bivalent Vaccine, Inactivated; TypeO, Strain OHM/02+TypeA, Strain AKT-III) and peste des petits ruminants (Peste Des Petits Ruminants and Goat Pox Vaccine, Live; Strain Clone9 + Strain AV41), and tested negative for Brucella infection using the Rose Bengal Test (RBT). DNA was extracted from rectal swab samples using the TlANamp Genomic DNA Kit (TIANGEN, #DP304-03) according to the manufacturer's instructions. Each DNA sample was eluted to obtain a 50 μL final volume. The DNA quality was evaluated using a spectrophotometer (Nanodrop OneC, Thermo Fisher Scientific, Wilmington, DE, USA), and was stored at -20°C for subsequent analyses.

Target sequence selection and Proofman-LMTIA primer design

In this study, the genome of the *C. abortus* S26/3 strain was employed as the target sequence. Utilizing the Blastn local alignment software, pairwise comparisons were conducted between the whole-genome nucleic acid sequences of *C. abortus* published in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and those of other animal chlamydiae, such as *C. pecorum*, *C. suis*, *C. pneumoniae*, *C. trachomatis*, *C. caviae*, *C. felis*, *C. psittaci* and *C. muridarum*. By taking the genome of the *C. abortus* S26/3 strain as the reference, homologous sequence regions shared between the aforementioned other chlamydiae and the *C. abortus* S26/3 strain were identified and recorded one by one, then marked on the S26/3 genome. The unmarked regions on the S26/3 genome could potentially serve as specific diagnostic targets for *C. abortus*. Subsequently, the MEGA7 (https://www.megasoftware.net/) was used to analyze the conserved sequences within the unmarked regions of different *C. abortus* strains (e.g., S26/3, GIMC 2006:CabB577, GN6, 1H, CAAB7, MRI-10/19, etc.) in GenBank.

The Oligo7 software (https://www.oligo.net/) was utilized to identify sequences within the conserved sequences of different *C. abortus* strains that exhibited a ladder-like melting temperature profile, and diagnostic target sequences

(emb|CR848038.1|:698177-698377) specific for detecting *C. abortus* using the LMTIA method were carefully screened out (as illustrated in Figure 1).

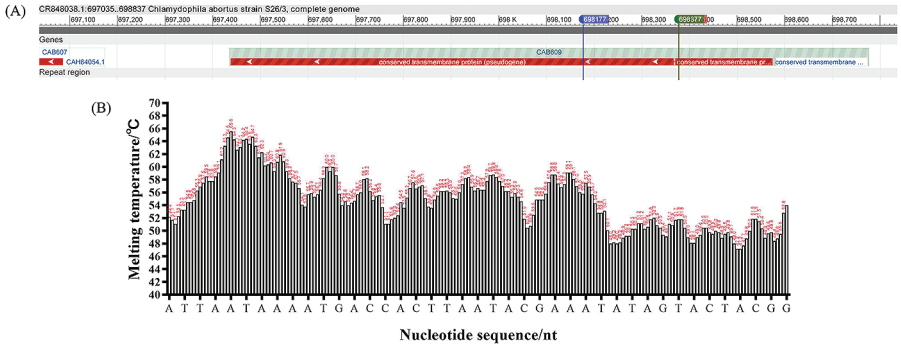

Figure 1. The melting temperature curve of the target sequence for detecting *C. abortus* using the Proofman-LMTIA method. **(A)** The target sequences specific for detecting *C. abortus* on the S26/3 genome. **(B)** The melting temperature curve of the target sequence.

The Primer3Plus tool (https://www.primer3plus.com/) was employed to design the primers for the LMTIA assay. The sequences of the primers and probes were synthesized by General Biological Systems (Anhui) Co., Ltd. The primers and probes utilized for the Proofman-LMTIA assay are presented in Table 1.

Table 1. Primers and probes for the Proofman-LMTIA.

Primer	Sequence (5'-3')
C. abortus-F (Forward primer)	TTCGGGGAGTTTGGATATGTTTTTAGCAATAGAGTTTCCAAATCAG
C. abortus-B (Reverse primer)	ACTCCCCGAAGACAAACATTTTCTATCCTTGAGTTATTTGTGA
C. abortus-LF (Loop primer forward)	GGAGAGAGTAACAGCCTTGGA
C. abortus-LB (Loop primer reverse)	GTGAGTAGATTGTCAAAAACGA
C. abortus-Proofman probe	BHQ2-AGAGTAACAGCCTTA-FAM
Target sequence	

AGCAATAGAGTTTCCAAATCAGGGATCTGCACTCCACGGCAAGCAAGTTGTACACGCTCAAAATCCAAGGCTG
TTACTCTCTCCTTACAATCCTCAGGCAACATATCCAAACTCCCCGAAGACAAACATAAGAGTAGCGCTCGCAATT
CTTTAAGAGTGAGTAGATTGTCAAAAACGAAATCACAAATAACTCAAGGATAG

Optimization of the Proofman-LMTIA reaction temperature

The Proofman-LMTIA reaction temperature was optimized with a reaction system comprising 10 μL (0.16 μL of *C. abortus*-F/B primer (100 μM), 0.04 μL of *C. abortus*-LF/LB primer (100 μM), 0.3 μL *C. abortus*-Proofman probe (10 μM), 5 μL of 2 ×

premix, 0.25 μL of Bst DNA Polymerase (Merit Biotech Shandong Co., Ltd., Heze, China), 2 μL of genomic DNA template, and 2.05 μL double distilled water). The reaction reagents (excluding the template DNA, 8 μL in each tube) were added to 16 tubes using a 96-well PCR plate. Among them, 2 μL ddH_2O was added to 8 tubes as a negative control, and 2 μL of *C. abortus* genomic DNA was added to the other 8 tubes. Subsequently, 20 μL of liquid paraffin was added to the 16 tubes to prevent aerosol contamination. The Gentier 96E fully automatic medical PCR analysis system was used for analysis. The temperatures were set at gradients of 59.5 °C, 60 °C, 60.5 °C, and 61 °C. Fluorescence signals were recorded every 45 seconds to obtain a total of 40 fluorescence signals.

Sensitivity and specificity evaluation

The extracted *C. abortus* genomic DNA was diluted to 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, 10 fg/μL, 1 fg/μL, and 0.1 fg/μL using a gradient dilution method to evaluate the detection limits of the Proofman-LMTIA assay. The diluted samples were added to the optimized Proofman-LMTIA reaction system. Double distilled water was used as a negative control, and each sample was evaluated in duplicates. Fluorescence signals were recorded every 45 s over 40 cycles.

Escherichia coli, *Salmonella* spp., *Listeria monocytogenes*, African swine fever virus, *Toxoplasma gondii*, *Cryptosporidium parvum*, *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis* genomic DNAs were used to determine the specificity of the Proofman-LMTIA assay. Double distilled water was used as a blank control, and *C. abortus* genomic DNA was used as a positive control. Each experiment was performed in duplicate. Fluorescence signals were recorded every 45 s over 40 cycles.

Reproducibility of the Proofman-LMTIA reaction

The reproducibility of the Proofman-LMTIA assay was evaluated by testing 10-fold dilutions of three samples of *C. abortus* genomic DNA, with the concentration ranging from 10^2-10^4 pg/μL per reaction. Each reaction was conducted in triplicate on three different days. Subsequently, the coefficient of variation (CV) of the cycle threshold values was determined. A CV value between 0% to 15% indicated good reproducibility.

Clinical sample analysis

The DNA samples extracted from 32 goats and 30 sheep rectal swab samples were tested using both the Proofman-LMTIA method and the PCR diagnostic method proposed in the agricultural industry standard NY/T 562-2015, which was issued by the Ministry of Agriculture of the People's Republic of China for the diagnosis of animal chlamydiosis. The PCR diagnostic method was performed using the following genus-specific primers targeting the OmpA gene: MP1: 5'-ATGAAAAAACTCTTGAAATCGG-3'; MP2:5'-TTAGAATCTGAATTGAGC

ATTCAT-3'; MP3:5'-CAGGATACTACGGAGATTATGTTT-3'; MP4:5'-GA TTAGATTGAGCGTATTGGAA-3'. PCR amplification was performed under the following conditions: denaturation at 95 °C for 5 min, 35 cycles of amplification at 95 °C for 1 min, 53 °C (1st)/52 °C (2nd) for 45 s, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Double distilled water was used as a negative control, and two samples of *C. abortus* genomic DNA were used as the positive control. All secondary polymerase chain reaction (PCR) products underwent bidirectional sequencing at Sangon Biotech Company (Zhengzhou, China). The sequence assembly and editing tasks were accomplished with the aid of DNAstar Lasergene Editseq 7.1.0 software. Multiple sequence alignment analysis was carried out using ClustalX 2.1 software on the obtained sequences and the reference sequences in the GenBank database to determine the chlamydial species.

RESULTS

Optimization of the Proofman-LMTIA reaction temperature

The optimal temperature range for the Proofman–LMTIA assay in detecting *C. abortus* is between 59.5 °C to 61 °C. The highest number of products was obtained at 59.5 °C, as detected by the fluorescence signal (Fig. 2). The temperature change did not significantly affect the primers and amplification began at around 10 cycles. The negative control (comprising double distilled water) showed no amplification. Consequently, 59.5 °C was chosen as the optimal reaction temperature for detecting *C. abortus* using the Proofman-LMTIA assay.

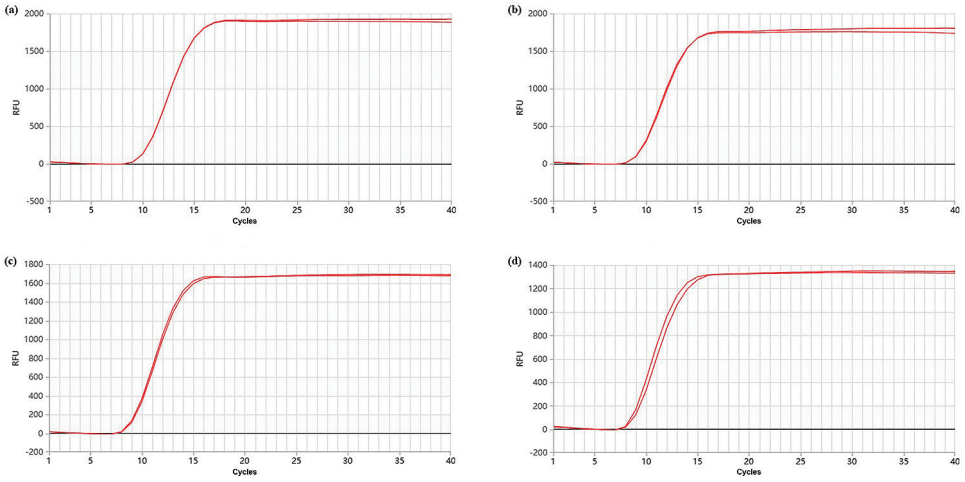

Figure 2. Proofman−LMTIA reaction for detecting *C. abortus* at different temperatures. **(a-d)**: Proofman−LMTIA reaction for detecting *C. abortus* at 59.5 °C, 60 °C, 60.5 °C and 61 °C. The red curve represents *C. abortus* genomic DNA, and the black curve represents double distilled water.

Specificity determination of the Proofman-LMTIA assay

The specificity of the Proofman−LMTIA assay was evaluated using *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, African swine fever virus, *Toxoplasma gondii*, *Cryptosporidium parvum*, *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis* DNA samples (Fig. 3). Proofman−LMTIA assay showed high specificity for detecting *C. abortus*, and there was no cross-reaction with other pathogens.

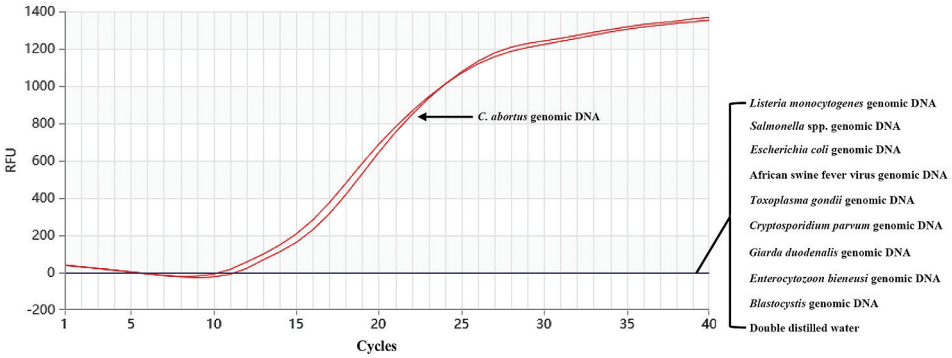

Figure 3. Specificity of the Proofman-LMTIA assay with different DNA types.

Sensitivity of the Proofman-LMTIA assay

The sensitivity and detection limits of the Proofman−LMTIA assay were evaluated by amplifying 10-fold serial dilutions (10^{-1}-10^{6} fg/μL) of *C. abortus* genomic DNA (Figure 4). The lower limit of detection for *C. abortus* genomic DNA was10^{1} fg/μL, indicating high analytical sensitivity.

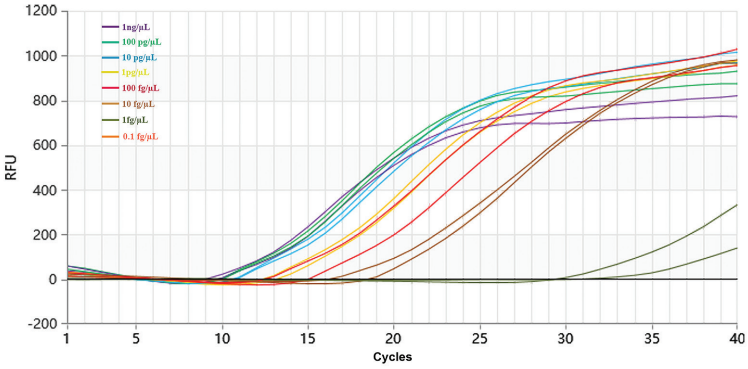

Figure 4. Sensitivity of the Proofman−LMTIA assay in detecting *C. abortus* genomic DNA.

The Proofman-LMTIA assay exhibits high repeatability and stability

Repeatability analysis revealed that the intragroup coefficients of variation were 2.60%, 1.88%, and 2.43%, and the intergroup coefficients of variation were 2.15%, 1.40%, and 0.72%. These values were less than 3%, implying that the method has a high repeatability and stability (Figure 5 and Table 2).

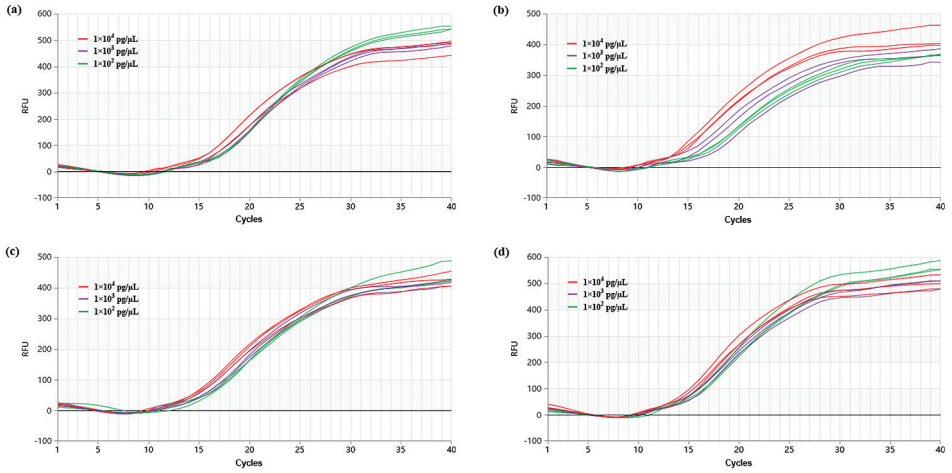

Figure 5. Reproducibility of the Proofman-LMTIA assay.

Table 2. Intra-assay and inter-assay variation of the Proofman-LMTIA assay.

Template DNA (pg/µL)	Replicates (n)	Intra-assay			Inter-assay		
		Cycles (AVG)	S.D	CV (%)	Cycles (AVG)	S.D	CV (%)
1×10^4	3	14.99	0.39	2.60	16.31	0.35	2.15
1×10^3	3	15.93	0.30	1.88	17.86	0.25	1.40
1×10^2	3	17.28	0.42	2.43	18.18	0.13	0.72

*__AVG__ – Average; __S.D__ – Standard Deviation; __CV__ – Coefficient of Variation.

Performances of Proofman-LMTIA and PCR assays for detection of *C. abortus* in field samples

The presence of *C. abortus* in 32 goat and 30 sheep rectal swab samples was detected using the Proofman-LMTIA assay, and the results were compared with the PCR diagnostic method. Proofman-LMTIA assay and PCR diagnostic method demonstrated that 2 goat and 4 sheep rectal swab samples were positive for *C. abortus*, a conclusion that was

further substantiated by sequencing analysis. More samples will need to be tested in the future to fully validate the sensitivity and reliability of the Proofman-LMTIA assay.

DISCUSSION

Abortion caused by *C. abortus* is prevalent in ruminants in various countries, including Europe, North America, Africa, and Asia [26]. Infected animals can shed large amounts of *C. abortus* in abortion products, such as fluids, placentas, dead/aborted lambs, fleeces, and stillborn/infected lambs, which can lead to the spread of the disease to other animals and humans [8,27]. Challenges in diagnosing the bacterium and low specificity of the tests available for detecting *C. abortus* result in underestimating the precise prevalence of animal chlamydiosis [5,28]. Although infections caused by *C. abortus* can be controlled through vaccination, detecting infection during pregnancy is vital in ensuring effective therapeutic intervention and controlling the spread of the disease [14].

The modified Ziehl–Neelsen (MZN) approach is the most reliable method for direct identification of *C. abortus*, as reported in the WOAH (World Organisation for Animal Health) Terrestrial Manual [29]. A Fluorescent antibody test (FAT) is commonly used to differentiate *Coxiella burnetii* from *C. abortus* due to the similar staining characteristics. Isolating pathogens from chicken embryos or cell cultures was previously considered the "gold standard" for chlamydia detection. However, this method is time-consuming and labor-intensive, requiring personnel to possess professional knowledge and expertise in operating specific equipment [26]. The complement fixation test (CFT) and indirect hemagglutination assay (IHA) test are the standard serological tests widely used for OEA diagnosis, especially in China [26,30-31]. Several commercial enzyme-linked immunosorbent assays (ELISA) kits have been developed for detecting *C. abortus* based on the MOMP, POMP and LPS antigens. ELISA based on these antigens is more sensitive and specific than CFT and IHA techniques but is associated with a high cost [26,32-35].

With advances in molecular techniques, PCR assays, Real-Time PCR, and DNA microarrays are widely used for detecting *C. abortus* strains or evaluating the evolutionary relationship of strains from different hosts or regions based on different target genes (such as ompA, 23S rRNA, 16S rRNA, pomp90–3, pomp90–4, and 16S–23S spacer region) [15,36]. In the present study, *C. abortus* target sequences were identified through BLAST comparison analysis of the *C. abortus* S26/3 genome and the genomes of other chlamydia species deposited in the GenBank database to validate the efficacy for Chlamydia detection. The *C. abortus* (emb|CR848038.1|:696891-703724) nucleic acid sequences were used to design primers for the Proofman-LMTIA assay. Clinical identification of EAE is often hindered by concurrent infections with other abortive pathogens such as *Campylobacter* sp., *Toxoplasma* sp., *Listeria* sp., *Brucella* sp., and *Salmonella* sp. [5]. In the present study, *Salmonella* spp., *Listeria monocytogenes*, and *Toxoplasma gondii* genomic DNA samples were used to determine the specificity of

the Proofman-LMTIA assay. The established Proofman−LMTIA assay showed high specificity in detecting *C. abortus*.

In several studies, isothermal nucleic acid amplification technology (the loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA)) has been used for the detection of *C. pecorum* and *C. trachomatis* [37-39]. These assays are highly sensitive and specific to the target pathogen. However, studies have not explored the application of isothermal amplification technology to detect *C. abortus*. The LMTIA assay is a new isothermal nucleic acid amplification technique, effective in diagnosing *Listeria monocytogenes* and African swine fever virus [16-17,19]. LMTIA is a simple, robust, and low-cost method that can be used as a simple screening assay in the field or at the point of care by clinicians. In the present study, we described the development and evaluation of a simple, sensitive, and specific Proofman-LMTIA assay for detecting *C. abortus*. The optimal amplification temperature for the Proofman−LMTIA assay was 59.5 °C, and the reaction time was less than 30 minutes. The established assay had a minimum detection limit of 10 fg/μL per test, indicating a high analytical sensitivity. Research should be conducted to explore the application of this technology in detecting *C. abortus* in many clinical samples to validate the effectiveness of the established Proofman−LMTIA assay.

CONCLUSION

In summary, a simple, sensitive, and specific Proofman-LMTIA assay was developed in this study to detect *C. abortus*. Further research should be conducted to explore the application of this assay in detecting other pathogens.

Acknowledgements

We would like to thank MogoEdit (https://www.mogoedit.com) for English editing during the preparation of this manuscript.

Author contributions

ZHC and DGW conceived and drafted the study. ZHC, YHH, XYH and WDZ performed the experiments. YNZ and JYW analyzed the data. ZHC drafted the manuscript. ZHC and JQL performed critical revisions of the manuscript. All authors have approved the final draft of the manuscript.

Declaration of conflicting interests

The authors declare no conflict of interest.

Funding

This research was funded by Natural Science Foundation of Henan (242300421590), Key Research Projects of Higher Education Institutions in Henan Province (24A230017), Key Research Projects of Xuchang University (2024ZD013).

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ISTRAŽIVANJE NOVIH CILJEVA KOMPJUTERIZOVANIM ISPITIVANJEM GENOMA „GENOME MINING" I USPOSTAVLJANJE PROOFMAN-LMTIA METODE ZA BRZU DETEKCIJU *CHLAMIDIA ABORTUS*

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Hlamidija abortus je obligatna intracelularna gram-negativna patogena bakterija široko rasprostranjena širom sveta. Ovaj patogen prvenstveno inficira ljude i razne domaće životinje, uzrokujući pobačaje kod gravidnih životinja. Predstavlja značajnu pretnju

po javno zdravlje i stočarsku industriju, što dovodi do značajnih ekonomskih gubitaka. U ovoj studiji, specifični prajmeri i Proofman fluorescentna sonda za LMTIA (Loop-mediated multiple cross displacement amplification) su dizajnirani na osnovu genoma soja *C. abortus* S26/3 putem poravnanja sekvenci i skrininga specifičnih ciljnih sekvenci. Ovi prajmeri i sonde su korišćeni za razvoj brzog Proofman-LMTIA testa za detekciju *C. abortus*. Reakcioni sistem i temperatura su optimizovani, procenjene su specifičnost, osetljivost i reproducibilnost testa. Optimalna temperatura amplifikacije za Proofman-LMTIA bila je 59,5 °C, a vreme reakcije je bilo manje od 30 minuta. Uspostavljeni test je imao minimalnu granicu detekcije od 101 fg/mL po testu, što ukazuje na visoku analitičku osetljivost. Test je takođe pokazao odličnu stabilnost, sa koeficijentima varijacije ispod 3% u intergrupnim i intragrupnim testovima reproducibilnosti. Ukratko, brza i efikasna Proofman-LMTIA metoda za detekciju *C. abortus* razvijena u ovoj studiji pruža novi tehnički alat za kliničku dijagnozu i kontrolu infekcija *C. abortus*.