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Rapid detection of *Brucella* species by a loop-mediated isothermal amplification method targeting *BruAb2_0672* locus

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Abstract
Brucellosis is a concern in many regions, especially developing countries. The methods for detecting diseases based on nucleic acids, like LAMP, provide a rapid and user-friendly approach to disease identification. These approaches hold the promise of expediting early disease detection and significantly reducing disease transmission. In the current investigation, an Isothermal LAMP test was developed and validated as a specific and rapid method for *Brucella* spp. detection in clinical samples. The primers designed and custom synthesized, were tailored to target the genus-specific *BruAb2_0672* gene sequence of *Brucella*. The detection limit of *BruAb2_0672* LAMP was 28 fg of *Brucella* genomic DNA, whereas conventional PCR detected 2.8 pg of template extracted from pure culture. Out of 10 blood samples from cattle spiked with *Brucella abortus* S19, nine samples tested positive with both PCR and RT-PCR when using the *BruAb2_0672* LAMP outer primers. Additionally, all ten samples tested positive using LAMP. Furthermore, all ten negative samples were confirmed negative when subjected to PCR, RT-PCR, and LAMP.

Keywords: Brucellosis, LAMP assay, point-of-care, diagnostics, isothermal amplification

Introduction
Brucellosis, caused by various species within the genus *Brucella*, is a complex and severe zoonotic disease that presents a significant threat to global human and animal health. These gram-negative, facultative intracellular bacteria can manifest with various clinical symptoms, from fever and malaise to reproductive complications in livestock (Hayoun *et al*., 2020) [1]. The diversity of *Brucella* species, their varying host preferences, and the potential for co-infections highlight the need to accurately identify the causative agent (Khurana *et al.*, 2021) [2]. *Brucella* species, such as *B. melitensis*, *B. abortus*, *B. canis, and B. suis* display varying host preferences, but they have the capacity to infect a vast range of mammals, including livestock, wildlife, and humans. Additionally, the documented ability of multiple *Brucella* species to infect the same host simultaneously leads to complex clinical presentations and diagnostic challenges (OIE, 2022) [3]. The necessity for rapid brucellosis diagnostics is evident driven by several critical factors. Brucellosis, a zoonotic disease with severe health implications for humans and livestock, demands rapid and correct diagnosis to initiate timely treatment, preventing disease progression and chronic infections.

Brucellosis, a zoonotic disease with severe health implications for humans and livestock. Rapid and correct diagnosis is essential to initiate timely treatment, preventing disease progression and chronic infections. Early detection is instrumental in breaking the transmission chain, curbing the spread of brucellosis, and minimizing its economic impact on the livestock industry. Rapid diagnostics also enhance public health surveillance, enabling effective outbreak management and contact tracing contributing to overall disease control.

Brucellosis diagnosis currently relies on three primary methods. While isolating the bacterium from clinical samples is the gold standard, it is time-consuming. Additionally, this approach presents a substantial risk to lab personnel, given the organism's classification as a Biosafety Level III hazard (Liu *et al.*, 2022) [4]. Serological tests, while rapid, often yield unsatisfactory results due to their low sensitivity (Yagupsky *et al.*, 2019) [5].

Nucleic acid-based tests, such as PCR, are considered the gold standard, providing results within 1-2 hours. However, PCR requires an expensive and non-portable thermocycler,

rendering it unsuitable for resource-limited settings. Moreover, PCR requires intricate and time-consuming template preparation to ensure dependable results (Schrader *et al.*, 2012) [6].

Notomi *et al.* (2000) [7] introduced LAMP as a novel method which is highly robust, efficient, and suitable for simplified template preparations from various matrices. In recent years, LAMP has been widely used for detecting and identifying various microbial pathogens, including *Brucella* species. The specificity and sensitivity of LAMP assays depend on the selection of the specific gene capable of detecting organisms.

In the current investigation, a LAMP test targeting the *BruAb2_0672* locus was developed and validated. This region's suitability for the target detection was previously demonstrated in PCR assay development. The study aimed to establish and validate a rapid, specific, sensitive, and robust LAMP test for the accurate identification of *Brucella* spp in clinical samples, adaptable to the constraint resource-limited settings. To our understanding, this study represents the inaugural application of LAMP targeting the *BruAb2_0672* locus for *Brucella* detection in clinical samples.

2. Materials and Methods

2.1 Bacterial Isolates

In the current investigation, five reference *Brucella* strains including *Brucella abortus* S99, *Brucella abortus* S19, *B. melitensis* (16M), *B. suis* (1330) and *B. canis* were used for optimization of LAMP assay. Additionally, a set of 11 non-*Brucella* genera DNA including *Escherichia coli* (ATCC 43888), *Bacillus cereus* (ATCC 10876), *Salmonella typhi* (MTCC 3216), *Staphylococcus aureus* (ATCC 29213), *Actinobacillus lignieresii* (ATCC 33590), *Streptococcus agalactiae* (ATCC 27956), *Pasteurella multocida* B:2 (P52) vaccine strain, *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (MTCC 8165), *Streptococcus equisimilis* (ATCC 12394), and *Streptococcus uberis* (ATCC 700407), obtained from various laboratories of the institute our were also used.

2.2 DNA Extraction

Genomic DNA from reference species were extracted from the broth culture grown from a single pure colony using a commercial DNeasy Blood Mini Kit (Qiagen, Germany) following the instructions. The concentration and purity of the extracted DNA were determined by utilizing a UV spectrophotometer at wavelengths of 260 nm and 280 nm (Spectrophotometer ND100). The average concentration of genomic DNA obtained from *B. abortus* S19 was measured at 280 ng/µl, with a mean purity of ~ 1.8 as assessed using Nanodrop Spectrophotometer.

2.3 Primers used in the study

The primers designed for the specific detection of *Brucella* species were developed based on the conserved nucleotide sequence of the *BruAb2_0672* gene for *the Brucella* genus, accessible through the NCBI GenBank with accession number AE017224.1. This primer design process was executed using the Primer Explorer V5 Software (https://primerexplorer.jp/e/) (as indicated in Table 1). These primers were subjected to NCBI-BLAST to ensure their specificity. The primers were verified using the NCBI BLAST tool to ensure they specifically target the desired *BruAb2_0672* gene in *Brucella* species. The primers were commercially synthesized by IDT (IDT, USA) and subsequently utilized.

Table 1: Primers used for the specific detection by LAMP-assay

Assay	Primer Id	Sequence(5'-3')	Amplicon size	Reference
LAMP	F3	TGCCTGATCCTGTCACCG	200 bp	Genus specific LAMP (Designed)
	B3	CGCTGGTTTGCGACTGATG		
	FIP	GCGGCCAGTGGTACGTTTTCAG-AAGCTTGCGCGCTTCGT		
	BIP	TGTTCGCGATGCAGGAAGCAGG-GCGGCAAGCTTTGCATGAA		
	LF	CGTGCAGAAGGACG		
	LB	TGATGGCACCGCT		
PCR	*bcsp*31F	TGGCTCGGTTGCCAATATCAA	223 bp	(Baily *et al.*, 1992) [8]
	*bcsp*31R	CGCGCTTGCCTTTCAGGTCTG		
RT-PCR	F3	TGCCTGATCCTGTCACCG	200 bp	Designed
	B3	CGCTGGTTTGCGACTGATG		
	*bcsp*31F	TGGCTCGGTTGCCAATATCAA	223 bp	(Nyarku *et al.*, 2020) [9]
	*bcsp*31R	CGCGCTTGCCTTTCAGGTCTG		

2.4 Optimization of LAMP

In this study, a total of six primers were employed including F3 and B3 (outer primers) and FIP and BIP (inner primers), which were designed to the template. Additionally, loop primers were introduced to expedite the amplification process. Furthermore, various components and conditions were standardized to optimize the reaction. These modifications encompassed varying reaction times (ranging from 30 to 90 minutes), temperatures (between 63 and 69 °C), dNTP concentration (0.5 to 1.6 mM), *Bst* polymerase (4.0 to 10.0 units), and $MgSO_4$ concentration (2.0 to 8.0 mM).

The optimized reaction was conducted in a 25 µl reaction mixture that included the following components: 5 pmol/µl of F3 and B3, 20 pmol/µl of FIP and BIP, 10 pmol/µl of LF and LB, 0.5 mM/µl deoxynucleotide triphosphates, 2 mM/µl MgSO4, 4 units of *Bst* polymerase, 0.5M/µl betaine, and 2 µl template. Visualization of LAMP amplicons was achieved by adding 1.5 µl of SYBR dye (diluted at a 1:10 ratio) post-amplification. Amplicons that tested positive exhibited a distinct color change from orange to green upon the addition of dye, while negative amplicons retained their orange coloration. In addition to color change, amplicons were also subjected to 2.5% gel electrophoresis. Positive amplicons displayed a characteristic laddering pattern, whereas no such pattern in case of negative reactions.

2.5 PCR

The reaction was conducted using outer primers targeting *BruAb2_0672* gene, utilizing a gradient Thermal cycler (Biorad, United States). PCR was conducted in a 15 µl

reaction mixture consisting of 7.5 µl of 2x Dream Taq Master Mix (Thermo Scientific, USA), 5 pmol forward and reverse primers, 4.5 µl of nuclease free water and 2 µl of the template DNA. PCR was also done using published primer targeting *bcsp31* gene (Baily *et al.*, 1992) [8]. The resulting PCR amplicons, with an end product of 223 bp were visualized through 1.5% gel electrophoresis.

2.6 Real time PCR
The Real time PCR assay was conducted using LAMP outer primers targeting *BruAb2_0672* gene performed in a AriaMx Real-Time PCR System (Agilent Technologies, USA). RT-PCR was done in a reaction mixture of 10 µl, with a primer concentration of 5 pmol using 5 µl Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA), 3 µl template DNA and nuclease free water. Furthermore, the LAMP assay's specificity was evaluated by testing it against various bacterial species from different genera present in the laboratory.

2.7 Analytical Sensitivity and Specificity of LAMP
Ten-fold serial dilutions of *B. abortus* S19 DNA were prepared, starting from an initial concentration of 280 ng/µl to a final dilution of 2.8 fg/µl to determine the detection limit. The LoD is defined as the most dilute sample at which a positive reaction could be visually observed and detected on gel electrophoresis. This sensitivity was then compared with that of other nucleic acid-based tests, such as PCR and RT-PCR, using the same set of outer primers employed in the LAMP. The *BruAb2_0672* LAMP specificity was assessed by utilizing genomic DNA from both *Brucella* and non-*Brucella* strains as described in Section 2.1.

2.6 Evaluation of LAMP in spiked blood samples
The *BruAb2_0672* LAMP assay was further assessed for applicability using spiked samples. Ten-fold serial dilution of an overnight broth culture of *B. abortus* S19 was spiked in bovine blood samples. Twenty random blood samples collected for different experiments (other studies) were chosen for the spiking experiment. Before spiking, all blood samples underwent an initial screening using the RBPT (Rose Bengal Plate Test) and were subsequently subjected to ELISA, confirming their negative status. Ten blood samples were spiked with an initial bacterial count of 10^4cfu/ml 48h grown *B. abortus* S19. Additionally, ten unspiked samples served as negative samples.

3. Results
3.1 Conventional PCR
PCR was conducted on all five *Brucella* strains as detailed in the section 2.1. In each case, a distinct 200 bp band was observed, confirming the target *Brucella* spp (Fig. 1). These bands were clearly visible on a 1.5% gel. Conversely, no such bands were detected in any non-*Brucella* species. Under the specified conditions, the PCR detected 2.8 pg of genomic DNA (Fig. 2). Additionally, a conventional *bcsp31* PCR produced an amplicon size of 223 bp in all *Brucella* spp (Fig. 3). The PCR assay, under the same conditions exhibited a LoD of 28 pg (Fig. 4).

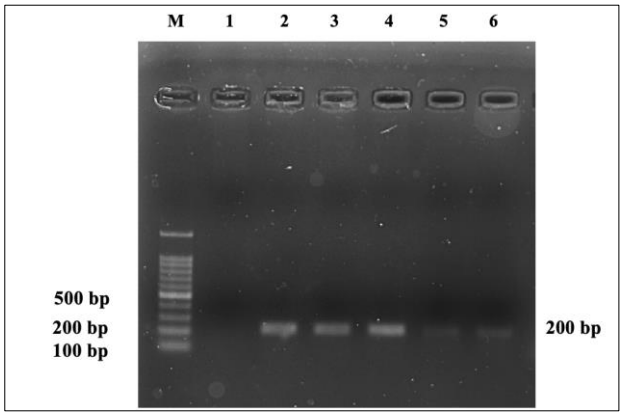

Fig 1: PCR amplification of genomic DNA by *Brucella* genus specific LAMP outer primer Lane M: 100 bp ladder, lane 1: Non-template control (NTC), lane 2: *B. abortus* S19, lane 3: *B. abortus* S99, lane 4: *B. melitensis*, lane 5: *B. suis*, lane 6: *B. canis*

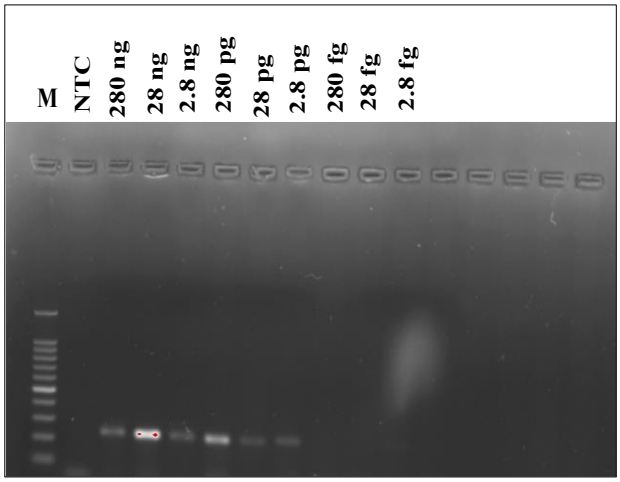

Fig 2: Limit of Detection (LOD) for PCR Using LAMP Outer Primers set at 2.8 pg. Lane M: 100 bp ladder, lane 1: NTC, lane 2-10: DNA extracted from serially diluted genomic DNA (280 ng – 2.8 fg)

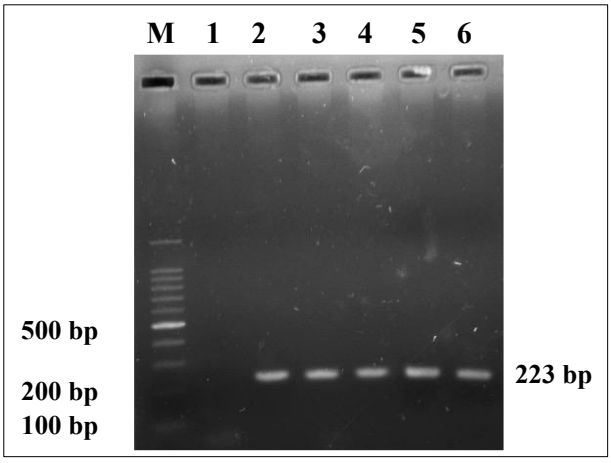

Fig 3: PCR amplification of *Brucella* spp using *bcsp31* (genus specific) PCR Lane M: 100 bp ladder, lane 1: Non-template control (NTC), lane 2: *B. abortus* S19, lane 3: *B. abortus* S99, lane 4: *B. melitensis*, lane 5: *B. suis*, lane 6: *B. canis*

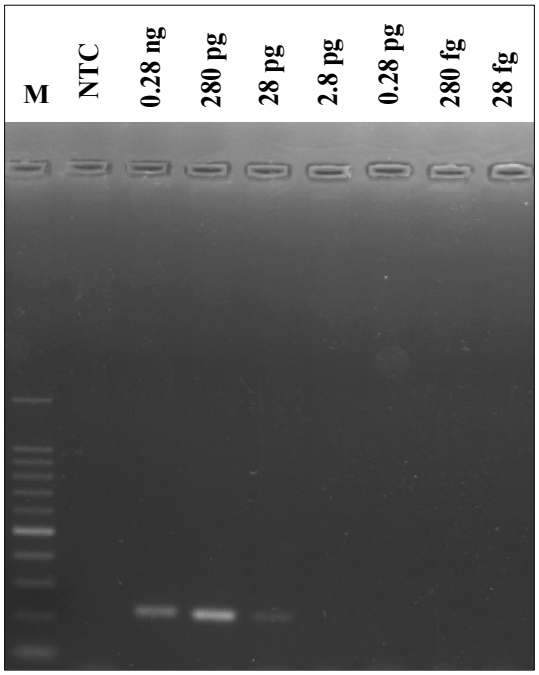

Fig.4: Limit of Detection (LOD) for PCR Using published primers (*bcsp31*) set at 28 pg. Lane M: 100 bp ladder, lane 1: NTC, lane 2-10: DNA extracted from serially diluted genomic DNA (0.28 ng – 28 fg)

3.2 Real time PCR

RT- PCR using *BruAb2_0672* LAMP outer primer (F3 & B3) was also compared with one of the reported RT-PCR assay (Nyarku *et al.*, 2020) [9]. The LoD of RT-PCR was 224 fg using LAMP outer primer (F3 & B3) primer and 280 fg using published primers. RT-PCR was conducted on all the five *Brucella* strains as detailed in section 2.1. All the 5 *Brucella* strains were found positive with RT-PCR whereas non-*Brucella* strains were found negative.

3.3 Establishment of *BruAb2_0672* LAMP

The LAMP assay effectively amplified the target gene (*BruAb2_0672*) at a temperature of 65 ºC within a 45-minute duration. A discernible ladder-like pattern was evident when analyzed using a 2.5% gel electrophoresis method (see Fig. 5). In addition, positive amplicons displayed a distinct color change from orange to green upon the introduction of SYBR dye, while negative amplicons retained their original orange hue.

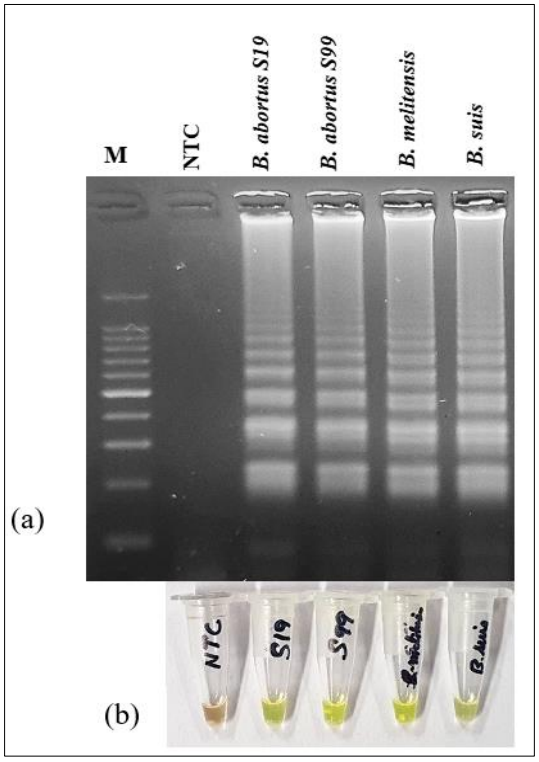

Fig 5: Optimized *Brucella* genus LAMP assay (a) Ladder pattern of LAMP amplicon. Lane M: 100 bp ladder, lane 1: Non-template control (NTC), lane 2: *B. abortus* S19, lane 3: *B. abortus* S99, lane 4: *B. melitensis*, lane 5: *B. suis* (b) Visualization of LAMP amplicon by SYBR Green I dye Green color indicates positive amplicon and orange indicate negative amplicon.

3.4 Analytical Sensitivity of LAMP

The lowest LoD of *BruAb2_0672* LAMP was determined to be 28.0 fg (Fig. 6). To assess the sensitivity, comparisons were made with conventional PCR and RT-PCR. Conventional PCR detected a LoD of 2.8 pg when employing LAMP outer primers, as illustrated in Fig. 2, and 28 pg when utilizing the published *bcsp31* primers (Fig. 4), using *B. abortus* S19 genomic DNA. Comparatively, the sensitivity of the RT-PCR method was determined to be one-tenth of the *BruAb2_0672* LAMP assay. Additionally, LAMP assay produced rapid results within just one hour, a marked contrast to the several days typically required for bacterial culture.

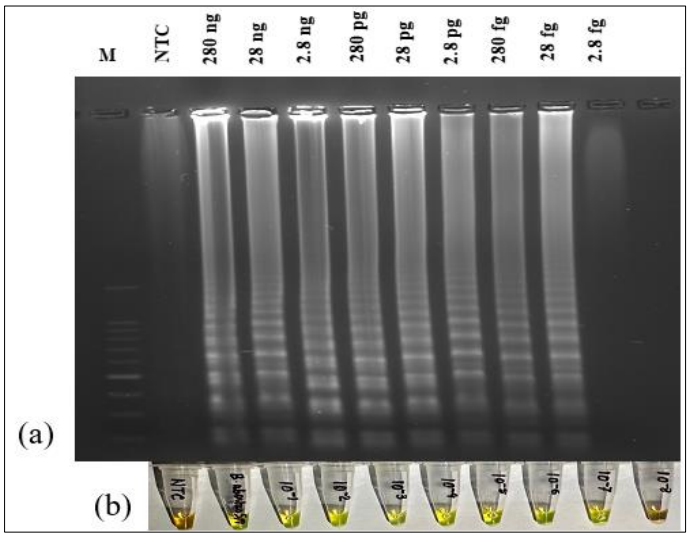

Fig. 6: Analytical sensitivity of LAMP assay displaying a detection limit of 28 fg (a) Ladder pattern of LAMP amplicon. Lane M: 100 bp ladder, lane 1: Non-template control (NTC), lane 2-10: DNA extracted from serially diluted genomic DNA (280 ng – 2.8 fg) (b) Visualization of LAMP amplicon by SYBR Green I dye Green color indicates positive amplicon and orange indicate negative amplicon.

3.5 Specificity of *BruAb2_0672* LAMP

The specificity of *BruAb2_0672* LAMP assay was assessed using 5 *Brucella* reference strains, *Brucella* vaccine strains, and other non-*Brucella* bacterial pathogen. The results indicated that *BruAb2_0672* LAMP assay exclusively identified all *Brucella* species and strains, while it couldn't identify any non-*Brucella* bacterial pathogens (Fig. 7).

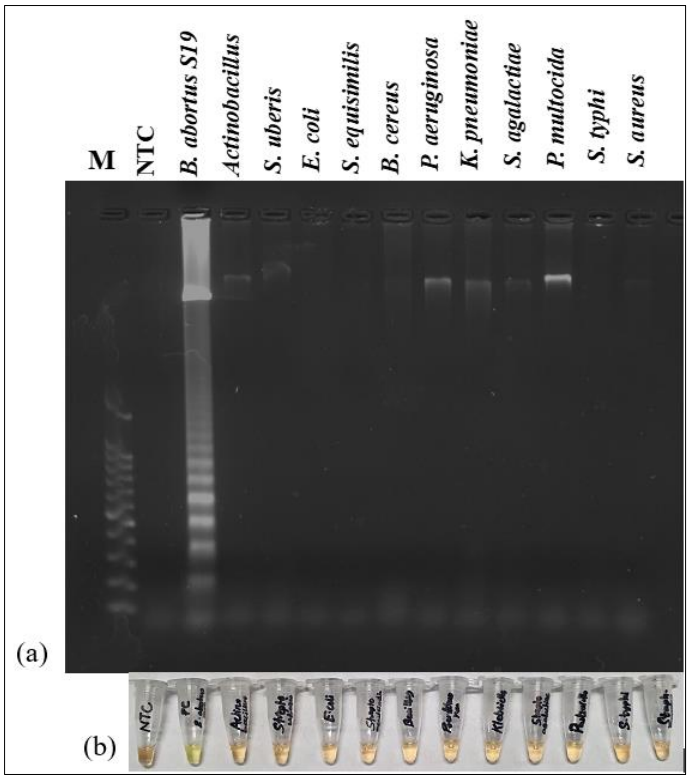

Fig 7: Specificity of LAMP assay with non-*Brucella* genera (a) Ladder pattern of LAMP amplicon. Lane M: 100 bp ladder, lane 1: Non-template control (NTC), lane 2: Positive control (*B. abortus* S19), lane 3: *Actinobacillus lignieresii*, lane 4: *Streptococcus uberis*, lane 5: *Escherichia coli*, lane 6: *Streptococcus equisimilis*, lane 7*: Bacillus cereus*, lane 8: *Pseudomonas aeruginosa*, lane 9: *Klebsiella pneumoniae*, lane 10: *Streptococcus agalactiae*, lane 11: *Pasteurella multocida* B:2, lane 12: *Salmonella typhi*, lane 13: *Staphylococcus aureus*. (b) Visualization of LAMP amplicon by SYBR Green I dye Green color indicates positive amplicon and orange indicate negative amplicon.

3.6 Evaluation of *BruAb2_0672* LAMP in spiked blood sample

The LAMP assay's applicability was evaluated using *Brucella* spiked blood samples. For this study, a random selection of 20 cattle blood samples was made. Among these, 10 samples were intentionally spiked with *B. abortus* S19 at a concentration of 10^4cfu/ml, while the remaining 10, which remained unspiked, served as negative controls. DNA was extracted from all 20 samples and subjected to testing with LAMP, PCR and RT-PCR for comparative purposes. Out of the ten spiked samples, all tested positive when assessed with the LAMP (Fig. 8), while only nine samples yielded positive results when analyzed with PCR and RT-PCR. In contrast, all unspiked samples consistently produced negative results across all three testing methods.

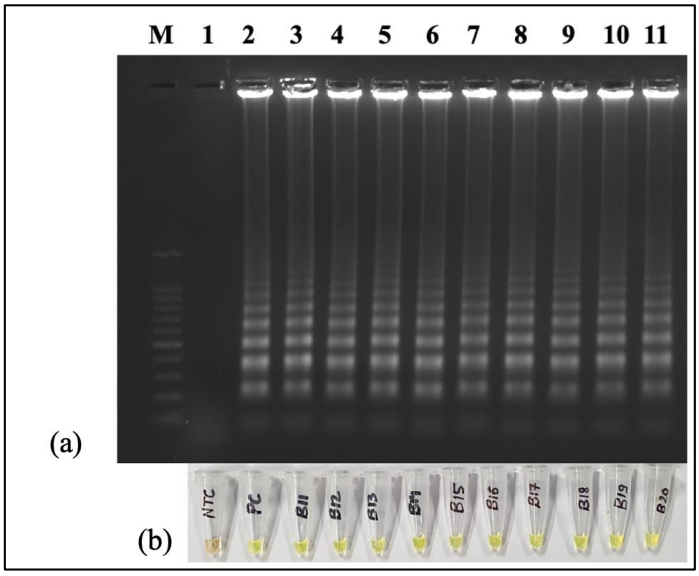

Fig. 8 LAMP for spiked blood samples (a) Ladder pattern of LAMP amplicon. Lane M: 100 bp ladder, lane 1: Non-template control (NTC), lane 2- positive control (*B. abortus* S19), lane 3-11: Clinical spiked blood samples showing positive laddering pattern LAMP (b) Visualization of LAMP amplicon by SYBR Green I dye: Green color indicates positive amplicon and orange indicate negative amplicon.

4. Discussion

Brucellosis is indeed categorized as a neglected zoonosis. Neglected zoonoses primarily impact animals but with the potential for human transmission, thereby presenting a substantial public health concern. However, they often receive insufficient attention and resources for research, prevention, and control. Given the significant prevalence, an early diagnosis and prompt medical intervention are crucial for preventing and managing this infectious disease (Xu *et al.*, 2020) [10].

Nonetheless, its effectiveness is constrained by the high occurrence of *Brucella*-specific antibody titres in countries with a substantial burden of brucellosis and its reduced sensitivity during the acute phase of the disease (Di Bari *et al.*, 2022; Moeini-Zanjani *et al.*, 2020) [11, 12]. The *Brucella* culture method is time-consuming, requiring days to weeks for results, which can lead to delayed diagnosis and treatment. Handling these biosafety level 3 (BSL-3) pathogens involves safety concerns and necessitates specialized facilities (Adone and Pasquali, 2013) [13]. Additionally, it may have limited sensitivity, especially in cases with low bacterial loads or early infection stages, potentially leading to false negatives (Perez-sanchao *et al.*, 2013 [14]. Consequently, culture is often supplemented with faster and more sensitive diagnostic methods in clinical practice. PCR as a point-of-care test can be limited by its complexity, bulky equipment, multi-step process, and sensitivity to sample quality and inhibitors, potentially causing delays and false-negative results (Demeke *et al.*, 2009) [15]. However, newer, more portable PCR devices address some of these challenges. LAMP excels as a point-of-care test. Its rapid results, simplicity, minimal equipment needs, robustness, high sensitivity, visual readout, and adaptability make it a cost-effective and efficient diagnostic tool for many healthcare scenarios, including resource-limited and remote areas (Craw *et al.*, 2012) [16]. The PCR procedure extends for approximately 90 minutes, amplifying 2.8 pg of *Brucella* DNA while demonstrating no non-specific reactions with negative controls.

In contrast, the LAMP assay efficiently amplified 28 fg target DNA in just 45 minutes, with its primers displaying no non-specific responses to negative controls comprised of bacterial DNA. The developed assay was also tested in spiked blood samples. Twenty cattle blood samples comprising ten artificially spiked and 10 unspiked samples were also tested with LAMP. All the spiked samples were detected positive with LAMP. In contrast, whereas only 09 samples gave positive results with PCR & RT-PCR. Comparing these two techniques, PCR required around 90-120 minutes, whereas *Brucella* LAMP achieved results in only 45 minutes. Interestingly, LAMP displayed a tenfold higher sensitivity than PCR, a finding consistent with the results reported by Moeini-Zanjani *et al.* in 2020 [12]. Regarding specificity, both methods demonstrated a high level of specificity, with no non-specific reactions observed in the negative controls.

Although various researchers have reported LAMP for the *Brucella* spp. detection using *bcsp31* gene (Trangoni *et al.*, 2015) [17], omp25 gene (Lin *et al.*, 2011; Pan *et al.*,2011; Song *et al.*, 2012; Soleimani *et al.*, 2013; Karthik *et al.*, 2016) [18, 19, 20, 21, 22] IS*711* gene (Pérez-Sancho *et al.*, 2015) [14], omp2a gene (Prusty *et al.*, 2016) [23], *BruAb2_0168* region of *B. abortus* (Karthik *et al.*, 2014) [24], *BMEI1661* gene (Ashmi *et al.*, 2023) [25], however, none of these assays have yet demonstrated practical applicability. This study marks the initial report of a *Brucella* LAMP assay targeting the

BruAb2_0672 gene.

The *Brucella* LAMP method, as assessed in this investigation, presents itself as a swift, exceptionally specific, and sensitive substitute for PCR assays. This technological advancement holds great value for the diagnosis of bovine brucellosis, particularly in areas where the disease is prevalent and within resource-constrained laboratory settings. The remarkable specificity of the LAMP primers minimizes the occurrence of false-positive results. Additionally, the low detection limit enables detecting minimal bacterial quantities, particularly during the initial phases of infection. Furthermore, the LAMP assay outperforms traditional methods in terms of speed and simplicity, reducing the diagnostic time and facilitating timely intervention. The introduction of this test for detecting *Brucella* species marks a ground-breaking leap in brucellosis diagnostics, holding significant implications for global health.

5. Conclusion

The applicability of a LAMP assay for detecting *Brucella* species offer a promising tool for quick and specific diagnosis of brucellosis. This assay can potentially revolutionize brucellosis diagnostics, particularly in resource-limited settings, by providing a cost-effective and user-friendly alternative to traditional methods. Further validation and field testing are essential to evaluate its performance in diverse epidemiological settings, but its potential impact on disease management and prevention is evident.

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