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RESEARCH ARTICLE

Detecting *Mycoplasma bovis* by Sybr Green-based real-time quantitative PCR and Loop-Mediated Isothermal Amplification Methods

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ABSTRACT

Mycoplasma bovis (M. bovis) infection is causing substantial economic losses to the global dairy industry including Egypt through its role as a major mastitis pathogen in cattle. The present study was aimed to evaluate loop-mediated isothermal amplification methods (LAMP) and real-time quantitative PCR (rt-qPCR) assays for detecting *M. bovis* in milk samples from cows (n=30) and buffaloes (n=20) with mastitis across different governorates in Egypt between January and March 2023. Both assays were compared to traditional culture and counter-immunoelectrophoresis (CIEP) methods. The results of the culture methods were 16 (32%), CIEP was 10 (20%), PCR was 16 (32%), LAMP was 18 (36%), and rt-qPCR based on Syber Green was 19 (38%), respectively. The sensitivity of rt-qPCR has the highest sensitivity (100%), specificity (91.17%), and accuracy (100%) (kappa coefficient of 0.856). While LAMP has sensitivity 93.75%, specificity 91.17%, and accuracy 98% (kappa coefficient of 0.811). However, the lowest detection rate was found in culture methods and CIEP. Our research effectively utilized LAMP and Sybr Green-based rt-qPCR assays for the rapid detection of *M. bovis* in bovine milk samples. However, further validation is necessary with a larger sample size.

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INTRODUCTION

Mycoplasma is an important pathogen associated with mastitis in cattle and buffaloes (Ammar *et al.*, 2021). It is a member of the *Mycoplasmataceae* family within the Mollicutes class, characterized by the absence of cell walls and a smaller genome size compared to other bacteria (Behera *et al.,* 2018). It is a slow-growing bacteria, requiring a long incubation period, specific media, and special growth conditions, in addition to a false positive result (Saminathan *et al.,* 2016). *Mycoplasma bovis* is responsible for causing various bovine mycoplasmoses, such as mastitis, pneumonia, endocarditis, arthritis, and otitis, as reported by Ahmad *et al.* (2014). The considerable impact of these infections is evident in the high morbidity rates and substantial economic losses, particularly in cattle and buffaloes in developing countries (Abd El Tawab *et al.*, 2019). *Mycoplasma* mastitis is characterized by yellowish-brown, watery secretions with sandy flakes and purulent milk with a cottage-cheesy appearance (McDonald, 2012; Kapalamula *et al.,* 2021). Since *M. bovis* is largely incurable with chemotherapeutics and ineffective vaccination (Yilmaz *et al.,* 2016; Bernitz *et al.,* 2021), existing methods like culture are slow and inaccurate, while serological tests lack sensitivity and specificity (Ruijter *et al.,* 2021). On the other hand, molecular diagnosis like PCR is a very useful tool for detecting active infection in dairy animals (Gonçalves, 2022). Over the past decade, TaqMan probe-based rt-qPCR has emerged as a popular tool for detecting *M. bovis* in various animals (Adonu, 2017; Zeedan *et al.,* 2019; Zeedan *et al.,* 2020; Ammar *et al.,* 2022). While LAMP offers a promising alternative with its speed, simplicity, and isothermal nature (Zeedan *et al.,* 2022), it can be susceptible to false-positive results due to non-specific binding and primer interactions (Garg *et al.,* 2022). Numerous advancements have contributed to improving the efficiency of the LAMP assay over time (Wong *et al.,* 2018; Rolando *et al.,* 2020). However, the rt-qPCR assay utilizing a fluorescent probe and targeting the *16S rRNA* gene showed cross-reactivity with *M. agalactiae* (Rolando *et al.,* 2020). This study designed new diagnostic tools for detecting *Mycoplasma bovis* (*M. bovis*) in milk samples. These tools include LAMP and rt-qPCR assays, both targeting the *uvrC* gene.

MATERIALS AND METHODS

Samples collection: A total of 500 dairy animals (300 cows, 200 buffaloes) were studied in mastitis-prone regions of North-Upper Egypt (Jan-Mar 2023). Bloody/chocolate color milk and consistency changes, mainly in early lactations and post-calving, were observed. 50 mastitis samples (30 cows, 20 buffaloes) were analyzed for bacterial identification (serological & molecular).

***In-Vitro* isolation of *M. bovis*:** Milk samples were cultured in modified PPLO broth for *Mycoplasma bovis* (Darwish *et al.,* 2017). Colonies were identified using biochemical tests, CIEP, LAMP, PCR, and rt-qPCR.

Counter Immunoelectrophoretic Technique (CIEP): Milk samples were prepared & inoculated in broth medium (Abdeen and Mousa 2017). After incubation & centrifugation, the sediment was analyzed using CIEP with specific antisera. Precipitin line formation indicated positive results.

The prepared sample was considered as antigen and was put in a well in 1% agarose in sodium acetate buffer, while the references antisera were put in the wells as antibodies. Electrophoresis was carried out through constant electric current of 7m A per slide (2.5cm) for 45 minutes. Formation of precipitin line between the well of antisera and that of antigen indicates positive results. The slide was dried overnight with filter paper, then stained with 0.1% coomasie brilliant blue in 1-% acetic acid and examined using indirect light and stored at -20ºC for use.

DNA Extraction from milk samples

DNA extraction by QIA Amp® DNA mini kit: The *M. bovis*-DNA was extracted from broth by QIA Amp ® DNA Mini extraction Kit (Qiagen GmbH, Hilden, Germany) and extraction steps followed the manufacturer's instructions.

Extraction of DNA by boiling method: It was done according to methods described by Wen *et al.* (2019) with modifications; Specifically, one milliliter of Mycoplasma broth culture or 750µl of each milk sample was diluted 1:1 with ionized water that had been treated overnight with NTE buffer (pH 7.4), containing 0.5 SDS and 200µl Proteinase-K. The mixture was then centrifuged at 12,000rpm for 10 minutes, and the supernatant was discarded. The resulting pellet underwent two washes with 1x tris EDTA (TE) buffer, followed by centrifugation at 10,000rpm. After discarding the supernatant, 100µl of 1x TE buffer was added to the pellet, and the mixture was boiled in a heat block for 20 minutes. Subsequently, the solution was centrifuged at 12,000rpm for 10 minutes, and the supernatant was carefully collected into a new microcentrifuge tube.

PCR amplification: Specific oligonucleotide primer pairs targeting the UvrC gene of *M. bovis* were obtained from Microgen Company, South Korea (Table 1). PCR reactions for both *M. bovis* detection (*UvrC gene*) and Mycoplasma spp. identification (*16S rDNA*) were performed using the following parameters: Initial denaturation: 94°C for 2 minutes, 35 amplification cycles: Denaturation: 94°C for 30 seconds annealing: *UvrC* gene: 53°C for 30 seconds, *16S rDNA*: 60°C for 30 seconds, extension: 72°C for 2 minutes, final extension: 72°C for 5 minutes, analysis of amplified DNA: PCR products were mixed with 6X loading dye and electrophoresed on a 2% agarose gel. After staining with ethidium bromide, the gel was visualized under UV light. Band sizes were determined using a DNA molecular marker (Fig. 1 and 2).

Table 1: Primer's design of *M. bovis* used in PCR and rt-qPCR

Mycoplasma bovis	Sequence (5'-3')	Product size (bp)
*16S r*RNA gene *	AGA CTC CTA CGG GAG GCA GCA ACT AGC GAT TCC GAC TTC ATG	700- 1000 bp
Mbo gene for M. bovis , **	CCT TTT AGA TTG GGA TAGCGGATG CCGTCAAGGTAG CGT CAT TTCCTAC	360 bp
UvrC ***	AAGTTGAAGTTGACCGGTTTG TCCATATTTGGACCTAGTCCTTT	1000 to 1008 bp

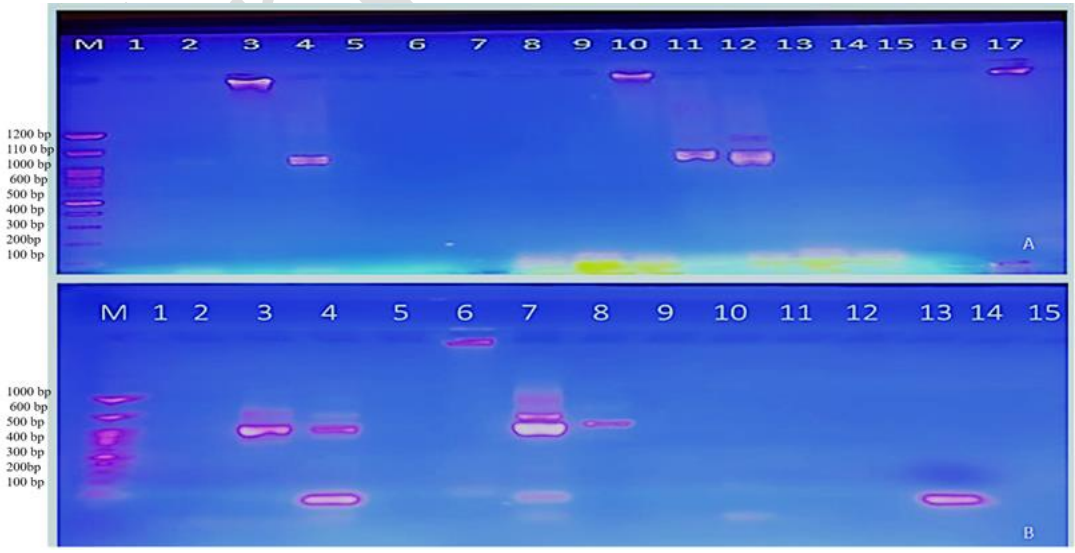

Fig. 1: A. Lane M: DNA 100 bp marker, Lane 1 negative control, Lanes 3, 10 negative samples. *Mycoplasma* other strain than *M. bovis*. Lanes 4, 11 and 12 PCR used *UvrC* primer specific for *M. bovis* 1000 to 1008 bp. Fig. 1: B. A. PCR based on *16S rRNA gene* sequences of *M. bovis* was designed for amplification of mycoplasma DNA at 700 to 1000 bp.

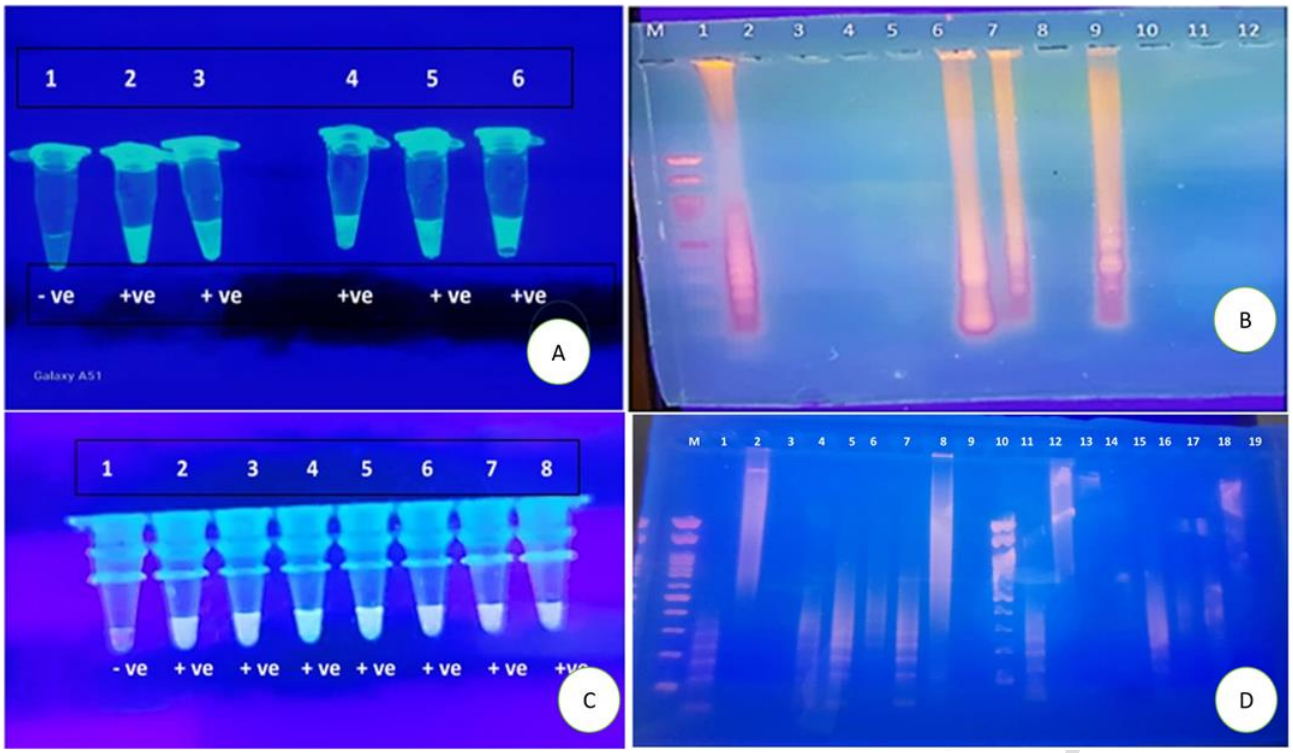

Fig. 2: A-C: Each panel shows positive (green) and negative (light/colorless) LAMP reactions under a UV transilluminator. Positive results indicate *M. bovis* detection. D: Lane M: DNA marker. Lanes 1, 6, 7, and 9 show positive bands for *M. bovis* samples. Lanes 2, 3, 4, 5, 8, 10, 11, and 12 represent negative samples. Fig. 2: C-D: LAMP products analyzed on 1.5% agarose gel show a characteristic ladder of multiple bands for positive samples (Lanes 1, 4, 5, 6, 7, 10, and 11), indicating *M. bovis* detection. Lanes 2, 8, 12, and 18 show bands from reactions not specific to *M. bovis*. Lanes 3, 9, 13, 14, and 19 represent negative control reactions.

Design of specific LAMP primers: *M. bovis*-specific primers targeted the *UvrC gene*, employing a loop-mediated isothermal amplification (LAMP) technique. This involved two sets of primers*: Outer primers: F3 and B3, Inner primers: FIP and BIP* (Wong *et al.*, 2018) as shown in Table 2.

LAMP reaction: The LAMP reaction mixture was assembled in a 25µl PCR tube, comprising 0.2µM F3 and B3 primers, 1.6µM FIP and BIP primers (details in Table 2), 1.4mM dNTPs mixture, 1mM betaine (Sigma Aldrich), 6mM MgSO4, 1x LAMP buffer (New England Biolabs, England), and 320U/ml *Bst DNA polymerase* (New England Biolabs, England). The reaction mixture was subjected to incubation within a temperature range of 59 to 60°C for 55 minutes using a dry heating block.

Detection of amplification products to detect LAMP products: The amplification products in LAMP reactions, Sybr Green (1:10 dilution of a 10,000× stock solution) was added to each reaction tube. The resulting color changes were observed under a UV lamp, where a green color indicated a positive result (Fig. 2A, B, and C). Additionally, the detection of LAMP amplification products was conducted through electrophoresis using a 1.5% agarose gel (Fig. 2 D).

Real-time PCR based on SYBER green assay: A real-time PCR assay was conducted using the identical primer set employed in conventional PCR, as described in previous protocols (Wen *et al.*, 2019). The total reaction volume was 20µl, consisting of 0.25µl each of forward and reverse primers (5 pmol each), 1µl template DNA, 5µl 2× Sybr Green-qPCR master mix, 3.3µl nuclease-free PCR-grade water and 0.2µl ROX (Low). The reaction conditions were optimized, including denaturation times ranging from 1 to 3 seconds.

Data analysis: Thirteen *M. bovis* cultures and 37 negative cultures from mastitic milk samples underwent molecular analyses (PCR, rt-qPCR, and LAMP assays). A Composite Reference Standard (CRS), defining *M. bovis* infection as positive when confirmed by either *M. bovis* presence or two or more positive results from molecular tests, served as the gold standard. Sensitivity, specificity, and percentage agreement (kappa coefficient) were computed using the MedCalc Diagnostic Test Evaluation Calculator available online (MedCalc's Diagnostic test evaluation calculator) and a trial version of MedCalc software. Real-time qPCR, LAMP, and PCR were carried out under reaction using same primers set and their sensitivities, specificity and accuracy evaluated as method described by Mabe *et al.* (2022).

RESULTS

***In-Vitro* isolation and identification of *M. bovis* in mastatic milk samples:** *M. bovis* was isolated on modified PPLO medium from mastitic milk showed fried-egg-shaped colonies and confirmed by biochemical identification. The total number of *M. bovis* isolates were 16 out of 50 (32%) in examined cow and buffalo milk (cows were 13 out of 30 (33.33%) and buffaloes 3 out of 20 (15%) as shown in Table 3.

Table 2: Primers used for LAMP assay

Method	Primer	Sequence (5'-3')
LAMP	B3	AAGCACCCTATTGATTTTTACTC
	F3	AGAAACAGACAAAAAATTAGTTCA
	FIP	GATTTTTGCATAGCTTTTAAAGTGATTTTGAAGGCAAACTAAGAAACATAAAAGG
	BIP	GACGCTTCAGTTGAAGAATTATCATTTTAATCCTTATTTTTAATGCTTTTGGC

Table 3: Comparison between CIEP, PCR, and LAMP for detection of *Mycoplasma bovis* in mastatic milk of cows and buffaloes.

Animal species	No. of animals	Culture methods				CIEP				PCR				LAMP			
		-Ve		+Ve		-Ve		+Ve		-Ve		+Ve		-Ve		+Ve	
		No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Cows	30	17	56.6	13	433	23	78.66	7	23.33	18	60	12	40	16	53.4	14	46.6
Buffaloes	20	17	85	3	15	17	85	3	15	16	80	4	20	16	80	4	20
Total	50	34	68	16	32	40	80	10	20	34	68	16	32	32	64	18	36

CIEP: Counter Current Immunoelectrophoresis PCR: Polymerase chain reaction LAMP: Loop-mediated isothermal amplification No: number of animals +ve: positive -ve : negative

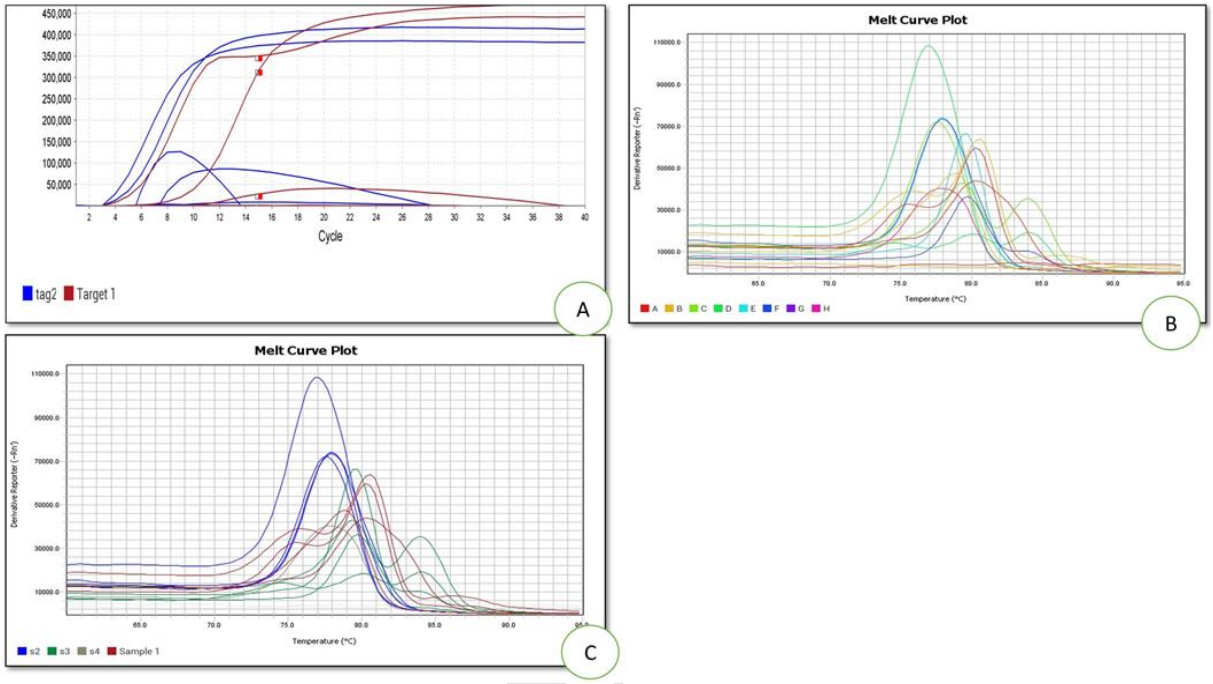

Fig. 3: A: Real-time PCR amplification plots show clear curves for both target 1 (*UvrC* gene, red) and Target 2 (mb gene, blue) in positive samples. Flat lines below the threshold (indicated as Ct ≤ threshold) represent negative controls. B and C: Melting curve analysis confirms specificity. Green and red lines with circle points (left panel) show melting peaks at specific temperatures (70°C, 75°C, 77°C, 78°C, and 85°C) for positive samples. Right panel shows melting peaks for a positive and negative control sample (blue and red lines, respectively.

Counter immunoelectrophoretic test: The results of *M. bovis* by CIEP were 7 out of 30 (23.33%) in examined cow's milk and 3 out of 20 (15%) in buffalo's milk as shown in Table 3.

Molecular detection and identification of *M. bovis*

PCR targeting specific primers of *M. bovis*: The total PCR positive results were 16 (32%) from examined cows and buffaloes milk samples (Table 3). PCR gave positive results of *M. bovis* that targeting *mb gene* at 340bp and slightly different from the size about 380bp, or PCR based *UvrC* primer specific for *M. bovis* 1000 to 1008 bp. While *16S rRNA* gene sequences of *M. bovis* were designed for amplification of mycoplasma DNA at 700 to 1000bp (Fig. 1 A and B).

LAMP assay: The LAMP assay exhibited a superior detection limit for DNA copies compared to PCR, as shown in Fig. 2 A, B, C and D. To evaluate sensitivity and specificity, 50 milk samples underwent analysis using the LAMP assay. The Sybr Green *uvr*C gene revealed that 18 samples (36%) were identified as *M. bovis* in mastitic milk (Table 3). LAMP detected at a similar rate (36%) to rt-qPCR's combined average (38%). However, rt-qPCR had higher detection in buffaloes (50%) compared to LAMP (36%) as shown in (Table 4). Furthermore, the LAMP products were subjected to 1.5% agarose gel electrophoresis, unveiling a characteristic ladder of multiple bands (Fig. 2 D). In the PCR results, *M. bovis* was detected in 14 samples (46.6%) out of the 30 examined cows and 4 samples (20%) out of the 20 examined buffaloes. The LAMP assay was further assessed for its ability to detect *M. bov*is in clinical samples, analyzing 50 mastitic milk samples from dairy cows and buffaloes (Table 3). The LAMP assay identified 16 positive cases of *M. bovis*, mirroring the same 16 positive cases detected by PCR in the corresponding mastitic milk samples.

Table 4: Comparison between LAMP and rt-qPCR for detection of *M. bovis* in milk samples

Sample	No. of samples	LAMP				rt-qPCR			
		-Ve		+Ve		-Ve		+Ve	
		No	%	No	%	No	%	No	%
Cow's milk	30	16	53.4	14	46.6	15	50	15	50
Buffaloes milk	20	16	80	4	20	16	80	4	20
Total animals	50	32	64	18	36	31	62	19	38

rt-qPCR: real time quantification polymerase chain reaction LAMP: Loop-mediated isothermal amplification No: number of animals +ve: positive -ve : negative

Table 5: Comparative between Sensitivity and Specificity results of rt-qPCR, LAMP, CIEP, and PCR assay

Assays	R	Culture [A]		Total	Sensitivity %	Specificity %	Kappa coefficient
		+	-				
PCR [B]	+	13	3	16	81.25	91.1	0.6131
	-	3	31	34			
CIEP test [B]	+	6	4	10	62.5	88.23	0.2131
	-	10	30	40			
LAMP [B]	+	15	3	18	93.75	91.17	0.8231
	-	1	31	32			
Rt-qPCR [B]	+	16	3	19	100	91.17	0.9767
	-	0	31	31			
Total		16	34	50			

Culture methods [A] +: Positive *M. bovis* isolates No *M. bovis* isolated −: negative results or bacteria other than mycoplasma.: PCR [B] +: PCR positive results −: PCR negative or no amplification

Detection of *M. bovis* in mastitic milk samples by syber green rt qPCR assay

Syber green rt qPCR assay conditions: Optimization was carried out with certain modifications, involving an initial step at 95°C for 3 minutes, succeeded by 40 cycles at 95°C for 3 seconds and 60°C for 20 seconds for annealing/extension, with a melting peak observed at 78.46°C. It's worth noting that the height of the melting peak may slightly vary higher or lower, attributed to the use of different *M. bovis* primers. The resulting amplification plot can be observed in Fig. 3 A, B and C.

Comparative between sensitivity and specificity between different assays: Conventional PCR, and CIEP assays detected *M. bovis* in non-culture milk samples directly without pre-culturing, and the sensitivity, specificity and accuracy of CIEP were 62.5, 88.23 and 88.6% (Kappa coefficient 0.2131), respectively. Real time- qPCR based on Sybr Green had higher sensitivity 100 and specificity 91.17% (Kappa coefficient 0.9767). While, LAMP had sensitivity, specificity and accuracy of 93.75 and 91.17% Kappa coefficient 0.8231), respectively (Table 5).

DISCUSSION

Mycoplasma bovis causes severe economic losses in dairy animals worldwide (Al-Farha *et al.,* 2017). Bovine mycoplasmosis is endemic in Egypt (Dudek *et al.,* 2020; Biesheuvel *et al.,* 2024). Chronically infected animals continue to shed *M. bovis*, which acts as a source of infection in dairy herds (Reis *et al.,* 2020; El-Demerdash *et al.,* 2023). The clinical signs observed in the examined animals included a severe drop in milk production, swelling, and severe congestion of the affected udders. Besides, there is a very poor response to mastitis therapy, and this result is in agreement with many recent studies conducted in Egypt (Ammar *et al.,* 2022; Algharib *et al.,* 2024). The absence of a detectable immune response in asymptomatic carrier animals before the onset of acute infection underscores the need for molecular assays for rapid diagnostic purposes (Abdalhamed *et al.,* 2021). In the current study, a new modified LAMP and rt-qPCR assay were developed targeting the *uvrC gene* of *M. bovis*, and these methods were compared to conventional detection methods. Our results indicated that the detection rates of *M. bovis* in mastitic milk samples were 16 samples (32%) by culture methods, 10 samples (20%) by CIEP, 16 samples (32%) by PCR, and 18 samples (36%) by LAMP, as shown in Table 3. These obtained results are in agreement with many authors, who indicated the high prevalence of mycoplasma-causing mastitis among dairy cows and buffaloes in Egypt (Zhao *et al.,* 2018; Waites *et al*., 2023). They reported that the prevalence of *M. bovis* in milk samples collected from bovines suffering from mastitis was 7.53% of the 53 *M. bovis* (7.48%). However, this result disagreed with Han *et al.* (2015), who reported higher percentages of *M. bovis* isolation from clinical mastitis milk (70.83%) in bovine. Traditional methods like culture and CIEP for *M. bovis* detection are slow and prone to errors. PCR methods targeting uvrC and 16S genes offer advantages of speed, sensitivity, and reduced labor (Table 3, Fig. 1A and 1B). Their sensitivity aligns with universal 16S PCR (Al-Farha *et al.,* 2017; Rifatbegović *et al.,* 2024). *16S rRNA* primers lack accuracy for closely related *Mycoplasma* like *M. bovis* and *M. agalactiae* due to identical *16S* sequences. While the *uvrC gene* primer offers good differentiation, our study confirms UvrC-based PCR superiority over CIEP for *M. bovis* detection, as shown in Table 3, and, as supported by Register *et al.* (2020), emerges as a suitable target for accurate *M. bovis* diagnosis.

The newly developed LAMP assay demonstrates high sensitivity in detecting *M. bovis* directly in milk samples, achieving a detection rate of 36%. This method presents several advantages over conventional detection techniques. Besides, the LAMP assay can be performed using a simple heating block, making it more accessible and practical for use in various settings, including field conditions where advanced laboratory equipment may not be available. One of the significant benefits of the LAMP assay is its straightforward visual readout. A positive result is indicated by a green color under UV light, as illustrated in Fig. 2. This eliminates the need for complex and costly equipment typically required for other molecular diagnostic methods, such as real-time PCR machines. The visual color change provides an easy-to-interpret result, facilitating rapid decision-making in the management of mastitis. In comparison to the conventional methods, such as culture and CIEP, which had detection rates of 32 and 20%, respectively, the LAMP assay shows superior sensitivity. The PCR method, which also detected 32% of the samples, requires more sophisticated equipment and technical expertise, making the LAMP assay a more viable option for widespread use. The high sensitivity and simplicity of the LAMP assay make it an excellent tool for early detection of *M. bovis* in mastitic milk. Early and accurate identification of this pathogen is crucial for effective disease management and control, reducing the

spread of infection and associated economic losses in the dairy industry. Moreover, the sensitivity of this method is comparable to that achieved with advanced TaqMan probes, as reported by Carrillo-Ávila *et al.* (2023). Our Sybr Green rt-qPCR method, targeting the uvrC gene for *M. bovis* detection, offers a rapid, sensitive, and less labor-intensive diagnostic approach compared to traditional methods. This method's high sensitivity surpasses conventional PCR, as demonstrated in Fig. 1A and B, matches the performance of advanced TaqMan probes, as reported by Tardy *et al.* (2020). Such efficiency holds promise for effective field applications. These holds promise for efficient field applications. In a direct comparison without pre-culturing, all three assays—CIEP, LAMP, and PCR were evaluated against 50 milk samples (Table 4). LAMP demonstrated the highest sensitivity for *M. bovis* detection, identifying 15 out of 16 culture-positive samples and 3 out of 30 culture-negative samples. LAMP was the most sensitive method for *M. bovis* detection in milk, correctly identifying 94% of positive samples and 3% of negative samples (kappa 0.812). Both LAMP and rt-qPCR showed higher sensitivity than culturing (Table 5). This suggests LAMP's potential as a reliable, field-ready tool for direct *M. bovis* detection. We agree with the assessment that both LAMP and rt-qPCR showed higher sensitivity than culturing, as detailed in Table 5. The superior performance of LAMP, particularly in detecting *M. bovis* directly from milk samples without the need for pre-culturing, highlights its potential as a reliable, field-ready tool, and these results were agreed upon (Villanueva *et al*., 2023). The LAMP assay's simplicity, requiring only a basic heating block and providing easy-to-interpret color change results under UV light, makes it especially suitable for use in field conditions and resource-limited settings. The findings suggest that LAMP, with its high sensitivity and practicality, could significantly enhance early and accurate detection of *M. bovis* in field conditions. This would allow for timely intervention and better management of mastitis in dairy herds, ultimately contributing to improved animal health and productivity, and these results were agreed upon (Zeineldin *et al.*, 2023).

The findings suggest that integrating advanced molecular techniques, especially LAMP, into diagnostic protocols could improve the early detection and management of mycoplasma mastitis. This would ultimately contribute to improved animal health and productivity in the dairy sector. Further studies and uses of these methods could solidify their role in combating mastitis caused by *M. bovis*.

Conclusion: This study highlights the significant roles of *M. bovis* on dairy animals worldwide, especially in mastitis. PCR and LAMP assays have demonstrated remarkable results for direct detection of *M. bovis* in milk samples, offering both sensitivity and speed. Furthermore, the Syber Green-based rt-qPCR based on the *uvrC* gene assay was able to detect low *uvrC* gene copies from mastitic milk samples. These findings suggest that both LAMP and Syber Green-based rt-qPCR may serve as valuable tools for *M. bovis* detection in clinical samples.

Ethical approval: The study was ethically approved by the Medical Research Ethical Committee, National Research Centre, and Egypt under registration number # 19-149 #.

Consent for publication: This work does not involve any personally identifiable information or data that necessitates obtaining consent for publication.

Availability of data and material: Data and materials are not applicable to this work as it does not involve experimental findings or require sharing of unique resources

Competing interests: The authors declare no conflict of interest regarding the publication of this work

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Authors contribution: AMA and GSGZ contributed to the research idea, planned the study design, performed data and sample collection, main laboratory worker, helped in sharing in the conception of the research idea, interpreting the data results, and manuscript preparation and work design. DAE provided different types of conventional primers for PCR and involved in sample collection. ESI helped in bacterial isolation and culture identification. All authors have read and agreed to the published version of the manuscript.

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