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Rapid On-Site Detection of Major Mastitis Pathogens in Ruminants Using a Colorimetric Loop-Mediated Isothermal Amplification Assay

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ABSTRACT

Current diagnostic methods for clinical and subclinical mastitis typically involve a bacterial culture phase, which can be time-consuming and requires specialized laboratory facilities. To overcome these limitations, our study optimized a loop-mediated isothermal amplification (LAMP) assay for the detection of pathogens causing mastitis, including *Escherichia coli* (*E. coli*), *Salmonella species*, and *Staphylococcus aureus* (*S. aureus*), in milk samples collected from ruminants. Detection of *S. aureus*, *Strep. agalactiae*, and *E. coli* in milk samples by multiplex PCR was 97(22.6%), 54(12. 6%), and 69(16.1%), respectively. Results showed that the LAMP reaction was completed within 30min at 65°C. The positive reaction showed a green color, and the negative reaction remained unchanged under UV light. The sensitivity of multiplex PCR assay showed a high sensitivity for most pathogens, ranging from 86.9 to 98.1%, nearly similar to the LAMP assay, which ranged from 89.7 to 97.03%. The specificity of multiplex PCR and LAMP assays ranged from 92.7 to 98.7%. In conclusion, the LAMP assay is a promising method for fast on-site assays that are simple, high sensitivity, high specificity, and rapid for detecting mastitis-causing bacteria.

Keywords: Loop-mediated isothermal amplification, Mastitis, *E. coli*, Salmonella Species, *Staphylococcus aureus*, PCR.

INTRODUCTION

Subclinical mastitis is extremely dangerous in dairy animals because it has no clinical symptoms, occurs frequently, and is difficult to assess (Abdalhamed et al. 2022; Asfour et al. 2022). Treatment is very costly in the case of chronic mastitis and mostly results in the loss of the infected quarter (Abdalhamed et al. 2021a; Mengistie 2022). The conventional diagnostic tests for the detection of subclinical mastitis are the culture and diagnosis of milk, which are considered the gold standard for detecting mastitis based on microbial content and biochemical identification (Zeedan et al. 2023). However, this method is often complicated, time-consuming, and has limitations in clinical application (Abdalhamed et al. 2021b; Zeedan et al. 2023). Laboratory-based tests based on cultured pathogens for identification require expensive equipment, and complicated procedures that necessitate trained personnel to operate (Zeedan et al. 2014). Due to the limitations of conventional laboratory-based tests for diagnosing infectious animal diseases, a molecular diagnostic assay based on DNA amplification was developed that amplifies target genes under isothermal conditions with high efficiency, specificity, and sensitivity (Chakraborty et al. 2019; Zeedan et al. 2022). The assay called loop-mediated isothermal amplification (LAMP), requires simple reaction equipment such as a water bath and a heat block (Chakraborty et al. 2019).

LAMP has been widely used for the detection of various pathogens, including viral, bacterial, and parasitic agents. This technique has an advantage over PCR in that expensive equipment for temperature cycling and detection of results is not required, and the conditions for the extraction and purification of DNA are less demanding (Hansen, 2002; Noro et al. 2005). Several studies describe the ability of LAMP to detect pathogens in milk samples (Harrigan 1998). LAMP is a promising technology for detecting bacteria that overcomes the

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limitations of other diagnostic methods; it amplifies DNA at 65°C for 30 min, uses four to six DNA primers, achieves a limit of detection similar to that of conventional PCR, and requires only a simple heating element for assay operation as opposed to complex thermocyclers (Suriyasathaporn 2011; Middleton et al. 2014; El-Demerdash et al. 2023). Therefore, this study aimed to develop and evaluate LAMP assays to detect major mastitis-causing pathogens in milk samples collected from different animals.

MATERIALS AND METHODS

Ethical approval

All laboratory procedures were carried out at the National Research Center laboratories located in Giza, Egypt. approved by the Ethics Committee of the National Research Centre, Cairo, Egypt vide # 19-148/2022. The experiment procedures were approved by the Institutional Animal Ethics Committee to ensure the well-being of the animals and minimize any potential distress or harm, Samples were collected following established protocols. The mammary skin was cleaned with warm water and disinfected with 75% alcohol before collecting milk samples to ensure sterility and avoid contamination (Burvenich et al. 2003).

Samples collection

Udder secretions were collected from a number of lactating sheep (n=305), goats (n=175), camels (n=108), cattle (n=325), and buffaloes (n=265) with CM and normal or SCM at different governorates (Beni-Suef, Giza, Monofia, Alexandria, Sharika, and Marsa Matruh) from January 2022 to July 2022. Sterile plastic vials were used to collect approximately 10 mL of milk samples from animals. The quarter milk samples obtained from apparently healthy animals were subjected to the California mastitis test (CMT) by pouring about 2 mL of milk per quarter into the CMT paddle (Table 1) according to the methods of Cheng and Han (2020). The milk was visually assessed for consistency, color, and clots, and then mixed with an equal amount of CMT reagent and blended using a circular motion. Any positive reaction observed in the milk samples indicated subclinical mastitis that had received no medical treatment for 7 to 10 days, as shown in Table 1 (Garedew et al. 2012; Dhanashekar et al. 2012). At the time of sampling, milk, and swab samples were transferred within 2 to 4 h from the time of sampling to bacteriological analysis laboratories at the NRC at room temperature. 20 to 40µL of the milk were cultured on blood agar and Edwards's agar for identification based on morphology, the catalase test, potassium hydroxide (KOH) test, and Gram staining. Positive isolates were subcultured on blood agar, and pure cultures were inoculated on stab agar for 8–12 hrs at 37°C before storage at 4°C, according to the guidelines (Jaja et al. 2020). Further identification of the isolates was confirmed by PCR.

Bacterial Strains

Positive controls for the bacterial strains *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus agalactiae, and Salmonella spp.* were obtained from the previous work (Abdalhamed et al. 2021a).

Bacteriological examination

Milk samples were collected and subjected to microbiological analysis. Specifically, 20 to 40µL of each sample was streaked onto blood agar and MacConkey agar plates, which were then incubated at 37°C for 24-48 hours. After incubation, the plates were cultured on MacConkey and Methylene Blue agar and subjected to certain biochemical tests, including Indole, Methyl Red, Voges-Proskauer, and Citrate (IMViC) tests. Individual colonies were selected for further identification using the methods outlined. To identify specific bacterial species, present in the milk samples, a series of PCR assays were performed using E. coli, Salmonella, Staphylococcus, and Streptococcus. Each sample was tested with all primer sets listed in Table 2 in individual duplex or multiplex PCR assays, following the protocol outlined by Dhanashekar et al. (2012).

DNA extraction

Preparation of bacterial DNA for PCR

Bacteria were grown overnight in 20mL of TSB, and then 1.5mL of this culture was centrifuged at 5,000 xg for 10min as per the manufacturer's instructions and then stored at -20°C. For the milk samples, 500µL of suspended bacteria were used for DNA isolation. In the case of the bacterial isolates, 250µL of suspended bacteria were used. To isolate the template DNA, the bacterial suspensions and milk samples were incubated with Tris-EDTA buffer and protein for one hour at 37°C. After this, a buffer from the same kit was added, and the mixture

Table 1: Oligonucleotide primers for different bacterial pathogen identification for PCR

Primer	Target gene	Genes and Sequence (5´ to 3´)	bp	Annealing temp (°C)
S. aureus	*23S*	F 5-GGACGACATTAGACGAATCA-3 R 5-CGGGCACCTATTTTCTATCT-3	318	64
S. agalactiae	*16S*	F 5-CGTTGGTAGGAGTGGAAAAT-3 R 5-TGCTCCGAAGAGAAAGCCT-3	586	65
E. coli	*16S*	F 5-ATCAACCGAGATTCCCCCAGT-3 R 5--TCACTATCGGTCAGTCAGGAG-3	232	64
		F 5-AGCAGGTTTCCCAC CGGATCACCA-3, R 5-GTGCTCAGATTCTGGGTCTC-3	171	59
		F 5-TTTATTTCTGTATTGTCTTT-3, R 5-ATTACAACACAG TTCACA G-3	275	61
Salmonella spp.	*16S*	F 5 - CGA ATA TGC TCC ACA AGG TTA-3, R 5 - AGT GCC ATA CTT TTA ATG AC -3	316	63
		F 5 - ACT ATG TCG ATA CGG TGG G-3 R 5 - CCCGCTTACAGGTGGACTAC-3	432	65

Table 2: Oligonucleotide primers (Purification: salt-free) for amplification of major pathogens of mastitis in LAMP assay

Pathogens	Sequence Name	Sequence 5' - 3'	Scale (nmole DNA Oligo)
E. coli	F3	GCCATCTCCTGATGACGC	40
	B3	ATTTACCGCAGCCAGACG	40
	FIP	CTGGGGCGAGGTCGTGGTAT-TCCGACAAACACCACGAATT	100
	BIP	CATTTTGCAGCTGTACGCTCGC-AGCCCATCATGAATGTTGCT	100
Salmonella	*invE* F3	GTTACGAAATTGCGCCAGC	40
	invE B3	TGGCTCAACCTCCGGTAT	40
	invE FIP	AGGATTCGTCTCCAGGGGCGCCGCTGACATTTCGTCCG	40
	invE BIP	TGCGGCCTGTTGTATTTCCGCTTGTCCCGGCAGACATCT	40
	fliC F3	CTGTCAGTGGATACGATGA	40
	fliC B3	CTGCATCTGCAACTCCT	40
	STM4495 FIP	GCACTCCGGTTGTAAACCATTATTTGTACTGAGGCTAATTCACCAC	40
	STM4495 BIP	CGCAGCGTAAAGCAACTCATCCAAAAGGGTTTGTTTTTGATG	40
Staphylococcus aureus	F3–2	AACAGTATATAGTGCAACTTCAA	40
	B3–2	CTTTGTCAAACTCGACTTCAA	40
	FIP-2[3]	TGTCATTGGTTGACCTTTGTACATTAAAATTACATAAAGAACCTGCGA	100
	BIP-2[4]	GTTGATACACCTGAAACAAAGCATCATTTTTTTCGTAAATGCACTTGC	100
	Floop	AACMTATACCATCAATCGCTTTA	40
	Bloop	AAGGTGAGAGAAATATGGTCCT	40

was incubated for one hour at 56–60°C. A mixture of 400µL of ethanol and 3µL of HCl (25%) was added to the mix. Membranes were washed following the manufacturer's guidelines, and the isolated DNA was eluted using 50L of water, as outlined in (Jaja et al. 2020).

PCR reaction

PCR was performed using a standard protocol. Briefly, a reaction volume of 25µL was used containing 2.5µL 10x PCR buffer, 1.5µL magnesium (12.5mmol/L), 2µL dNTP (12.5mmol/L), 0.25µL Taq DNA polymerase (2.5IU), 1µL each primer (Table 2) and 1.5µL DNA extract. Sterilized deionized water was added.

Loop-mediated isothermal amplification

The LAMP reaction was performed based on methods described by Zeedan et al. (2023) with some modifications. Positive control was used at a concentration of 1ng/L (2µL), and 8µL of Warmstart Colorimetric LAMP 2 Master Mix (New England Biolabs, UK) was employed for the assay. Standard desalted oligonucleotide primers were used, synthesized at 40 to 100nmol, as shown in Table 3. Reactions were incubated at 65°C in a real-time PCR system (Simenies' USA). The negative control (sterile deionized water instead of DNA extract) was set in each LAMP reaction. This table provides information on the oligonucleotide primers used for the amplification of the major pathogens of mastitis in LAMP assays. The pathogens included in the table are E. coli, Salmonella, and Staphylococcus aureus. For each pathogen, four oligonucleotide primers are listed: two outer primers (F3 and B3), a forward inner primer (FIP), and a backward inner primer (BIP). Additionally, for Staphylococcus aureus, two loop primers (Floop and Bloop) were listed.

Statistical Analysis

Data expressed as percentages for the positive test rate (95% confidence interval (CI)) of bacterial-affected animals were calculated and expressed as percentages. The significance level was considered at $P<0.05$. Statistical analysis was carried out in SPSS version 26.

RESULTS

A clinically diseased animal with acute mastitis showed moderate swelling, visible signs of chunks of milk, firmness, clots in milk, and, in some cases, milk becoming viscous. Out of the 108 camels, eight exhibited symptoms of CM, representing a prevalence rate of 7.4%. Similarly, 17.5% of the 325 cattle (57 out of 325) and 15.1% of the 265 buffalo (40 out of 265) showed signs of acute mastitis, such as changes in milk, swelling, and pain. Subclinical mastitis, which is defined as the presence of mastitis without any clinical signs, was observed in 18.5, 21.2, 21.2, 20, and 21.5% of camels, cattle, buffaloes, sheep, and goats, respectively. Subclinical mastitis was found in 26% of the sampled quarters. In total, milk samples from 46% of the udder quarters were collected, as shown in Tables 1, 4, and Fig. 1. The prevalence of clinical mastitis (CM) among dairy animals varied among species. 17.7 and 12% from sheep and goats, respectively (Fig. 1). The results showed the number of positive samples and the percentage of different bacterium types identified by isolation and biochemical identification from different animals. In cattle with clinical mastitis (CM), there are *S. aureus*, *E. coli*, and *S. agalactiae*. The percentages of positive samples for these bacteria range from 10.5 to 22.8%, as shown in Table 4. In subclinical mastitis (SCM), S. *aureus*, *E. coli*, and *S. agalactiae* also remain the most prevalent bacteria, with percentages ranging from 18.8% to 26.08%. Buffaloes: Similar to cattle, *S. aureus*, *E. coli*, and *S. agalactiae* are the predominant bacteria in both CM and SCM cases in buffaloes. The percentage of positive samples for these bacteria ranges from 12.5 to 22.5% in CM and from 18.8 to 26.08% in SCM. Camels: The most common bacteria in goats with CM and SCM are S. aureus, E. coli, and S. agalactiae, with percentages of positive samples ranging from 11.6 to 28.5% in CM and from 6.9 to 21.5% in SCM.

Specificity of primers

The primer sequences were designed with the specific purpose of amplifying and identifying the main bacteria responsible for small and large ruminants'

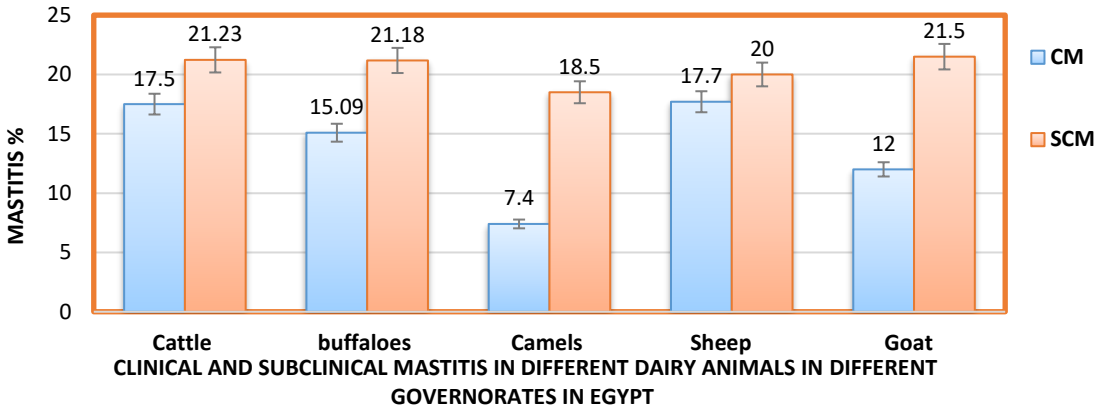

Fig. 1: Clinical mastitis (CM) and subclinical mastitis (SCM) in different dairy animals in different governorates in Egypt.

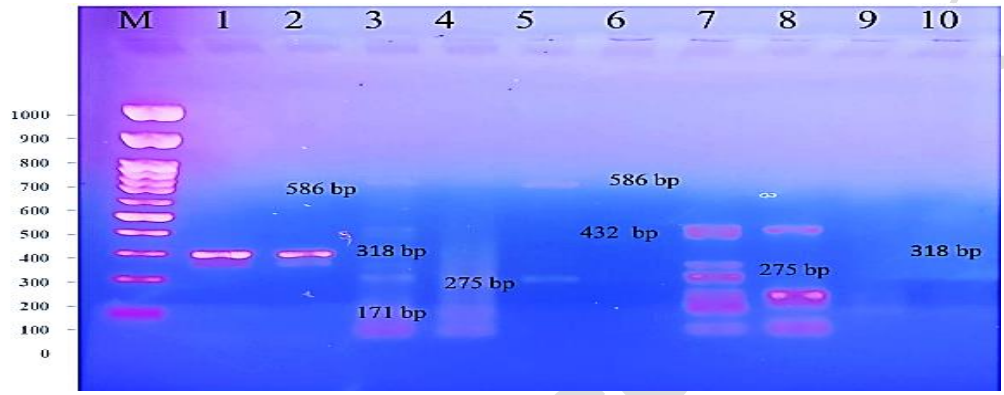

Fig. 2: Demonstrated the specificity of the molecular probes in the multiplex PCR assay with purified bacterial DNA from different species The resulting amplification products generated by different primer combinations were subsequently subjected to electrophoresis on a 1.5 % agarose gel. The gel lanes were labeled as follows: Lane A: 100-bp DNA ladder by analyzing the electrophoresis results showed DNA bands from the specific bacterial species, 1, 2, and 3: primers with *S. aureus* primers for *23S ribosome* gene 318 bp, *S. agalactiae for 16S ribosome gene 586 bp*, E. coli for *16S ribosome gene* 232 bp, 171 bp, 275 bp, and *Salmonella spp*. for 16S ribosome gene 316 bp and 432 bp.

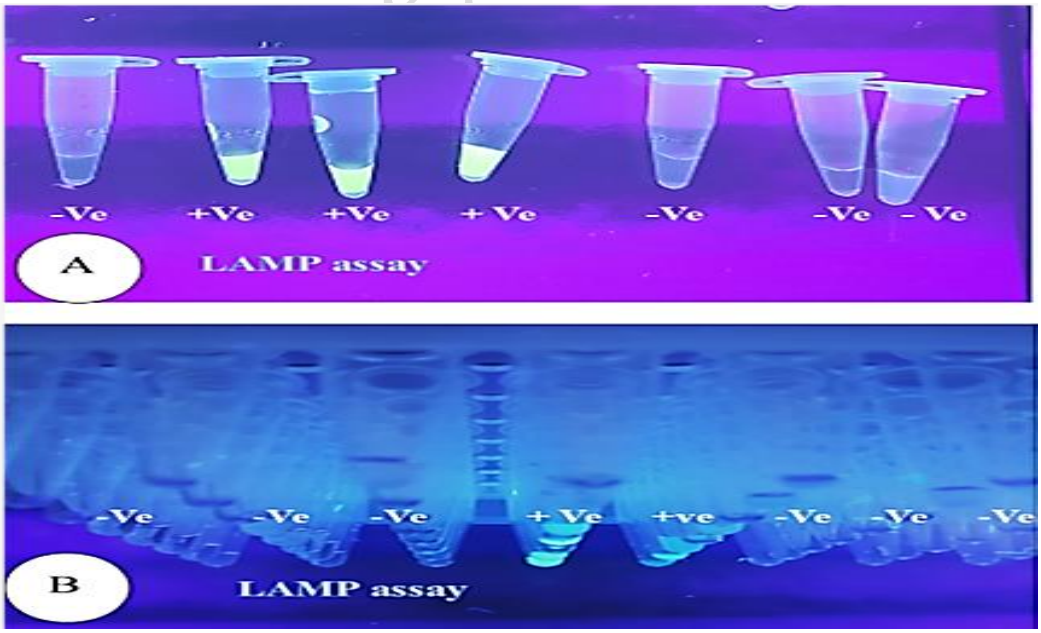

Fig. 3: (A and IB) **A):** Visualization of the LAMP reaction using UV light in 0.2mL PCR tubes 1 serves as the negative control, tube 2 is the positive (+) control, and tubes 3 to 5, 13 to 16, 18 to 21, 23 to 26, and 28 to 31 represent the tested positive samples. The LAMP reaction can be observed with the naked eye. Tube 1, when viewed under UV light, represents the negative control, while tubes 6 to 12, 17, 22, and 27 represent the tested negative samples. **B):** Visualization of the LAMP reaction through naked-eye observation in a 0.2 mL real-time PCR 96-well plate Columns 1 to 3 correspond to the negative samples; column 4, well 1, represents the positive control; and wells 2, 4, and 5 represent the tested positive samples.

Table 3: Culture method for the isolation and identification of bacteria causing mastitis in cattle and buffaloes

Bacteria types	Conventional method (Isolation and identification							
	Cattle (n= 126)				Buffaloes (n= 96)			
	CM		SCM		CM		SCM	
	+ve	%	+ve	%	+ve	%	+ve	%
S. aureus	13	22.8	18	26.1	6	15	9	16.1
E. coli	6	10.5	13	18.8	5	12.5	12	21.5
S. agalactiae	8	14.5	7	10.1	9	22.5	7	12.5
S. aureus + E. coli	7	12.5	3	4.3	3	7.5	3	5.3
S. agalactiae + S.aureus	6	10.5	4	5.8	6	15	3	5.3
E. coli + S. agalactiae	9	15.7	2	2.8	4	10	7	12.5
Salmonella spp	3	5.3	3	4.3	2	5	3	5.3
Mixed infection	4	7	6	8.6	3	7.5	5	8.9
Other causes	3	5.3	3	4.3	2	5	6	10.6
Percentage	17.5		21.2		15.1		21.2	

CM: Clinical mastitis, SCM: Subclinical mastitis, +Ve: Positive samples, -Ve: Negative samples, %: Percentage of positive or negative samples, *S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, S. agalactiae: Streptococcus agalactiae*.

Table 4: Conventional method for detection of bacteria causing mastitis in camels and small ruminants

Bacteria types	Conventional method (Isolation and identification)											
	Camels (n= 28)				Sheep (n= 115)				Goat (n= 64)			
	CM		SCM		CM		SCM		CM		SCM	
	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%
S. aureus	1	12.5	4	20	13	24	19	31.1	6	28.5	8	18.6
E. coli	2	25	3	15	12	22.2	11	18	3	14.2	5	11.6
S. agalactiae	1	12.5	4	20	7	12.9	6	9.8	2	9.5	3	6.9
S. aureus + E. coli	1	12.5	3	15	3	5.5	4	6.5	3	14.2	6	13.9
S. agalactiae + S. aureus	1	12.5	2	10	4	7.4	3	4.9	3	14.2	5	11.6
E. coli + S. agalactiae	-		3	15	6	11.1	7	11.4	2	9.5	6	13.9
Salmonella spp	1	12.5	-	-	2	3.7	2	3.2	1	4.7	3	6.9
Mixed infection	-		-	-	6	11.1	3	4.9	1	4.7	7	16.3
Other causes	1	12.5	-	-	5	9.2	6	9.8	-	-	-	
Percentage	7.4		18.5		17.7		20		12		21.5	

CM: Clinical mastitis, SCM: Subclinical mastitis, +Ve: Positive samples, -Ve: Negative samples, %: Percentage of positive or negative samples, *S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, S. agalactiae: Streptococcus agalactiae*.

mastitis (SLRM), as indicated in Table 2 and represented in Fig. 2. This further validated the specificity of the primer pairs and their ability to selectively amplify the target DNA regions.

Multiplex PCR

The results of the multiplex PCR assay for different bacterial species in the tested samples are shown in Table 5. The data reveal the presence of various bacterial species in different animal species. In cattle, *S. aureus* was detected in 22.8% of the samples, while *E. coli* was found in 10.5% of the samples. *S. agalactiae* and *Salmonella spp*., were detected in 14.3 and 5.2% of the samples, respectively. The occurrence of mixed infections was observed in 7.01% of the samples. Similarly, in buffaloes, *S. agalactiae* was the most prevalent species, detected in 22.5% of the samples. *S. aureus, E. coli*, and *Salmonella spp.* were found in 15, 12.5%, and 5% of the samples, respectively. Mixed infections were observed in 7.5% of the samples. Among camels, *E. coli* was the most frequently detected species, occurring in 25% of the samples. *S. aureus* and *S. agalactiae* were detected in 12.5% of the samples, respectively. Mixed infections were not detected in this animal species. In sheep, *S. aureus* was the most prevalent species, detected in 24.07% of the samples, followed by *S. agalactiae* in 22.2% of the samples. *E. coli, Salmonella spp.*, and mixed infections were observed in 12.9, 3.7, and 11.1% of the samples, respectively. Goats exhibited S. aureus as the most prevalent species, detected in 28.6% of the samples. *E. coli, S. agalactiae,* and *Salmonella spp*. were found in 14.2, 9.5, and 4.7% of the samples, respectively. Mixed infections occurred in 4.7% of the samples.

LAMP

The positive control tube and sample tubes turned green, indicating the presence of LAMP products (target DNA). Conversely, the negative control tube remained orange (Fig. 3). The developed LAMP method was used to detect *S. aureus, E. coli,* and *Salmonella*, respectively. The results demonstrated that the amplification product was positive only when the specific pathogen was present. Despite increasing the isothermal amplification time to 1 hour, DNA from other strains could not be detected, indicating the high specificity of the LAMP method for detecting pathogens that cause mastitis (Fig. 2). Ten-fold serial dilutions of DNA extracted from *S. aureus, E. coli,* and *Salmonella* were used as templates for LAMP and PCR amplification, respectively.

The results of the LAMP assay for different bacterial species in the tested samples are presented in the table. The data shows the detection of specific bacterial species in different animal species using the LAMP method. In cattle, *S. aureus* was detected in 24.5% of the samples, while *E. coli* was found in 12.28% of the samples. *Salmonella spp*. and mixed infections were detected in 7% of the samples. *S. agalactiae* was not tested in this animal species using the LAMP assay. Among buffaloes,

S. aureus and *E. coli* were detected in 15 and 12.5% of the samples, respectively. *Salmonella spp.* and mixed infections were not detected using the LAMP method. *S. agalactiae* was also not detected in buffaloes. Camels showed the presence of *S. aureus and E. coli* in 25% of the samples, respectively. *Salmonella spp*. In sheep: *S. aureus* was detected in 24.07% of the samples. *E. coli* and mixed infections were detected in 3.7 and 11.1% of the samples, respectively. *Salmonella spp*. was not detected using the LAMP method. Goats exhibited the detection of *S. aureus* in 28.5% of the samples and *E. coli* in 14.2% of the samples. *Salmonella spp. S. agalactiae* and mixed infections were not detected using the LAMP assay. The mixed infection category refers to samples that showed the presence of DNA from more than one type of bacteria. For instance, in the cattle CM group (n=57), 14 (24.5%) samples tested positive for *S. aureus* using the LAMP method, while only 13 (22.8%) samples tested positive using the multiplex PCR method. Similarly, for the sheep SCM group (n=61), 19 (31.1%) samples tested positive for *S. aureus* using the LAMP method, while 11 (18 %) samples tested positive using the multiplex PCR method as shown in Table 5.

Table 5 shows the sensitivity and specificity values for both multiplex PCR and LAMP methods for detecting different types of bacterial DNA in milk samples. Multiplex PCR and LAMP assays had a sensitivity of 97.03% and a specificity of 98.7%. Comparing the results of the multiplex PCR (MPCR) and LAMP assays for the detection of bacterial species in the tested samples, several observations can be made: Detection of Bacterial Species Both MPCR and LAMP assays successfully detected various bacterial species in different animal species, including S. aureus, E. coli, S. agalactiae, and Salmonella spp. In cattle, the MPCR assay detected higher percentages of S. aureus (22.8%) and S. agalactiae (14%) compared to the LAMP assay (24.5 and 7 %, respectively). On the other hand, the LAMP assay detected higher percentages of E. coli (12.3%) and mixed infections (7%) compared to the MPCR assay (10.5% and 7.01%, respectively). In some cases, certain bacterial species were detected by one assay but not the other, the MPCR assay detected Salmonella spp. in cattle (5.2% of samples) and buffaloes (5% of samples).

Comparison between sensitivity and specificity of Multiplex PCR and LAMP Assays

The results in Table 6 showed that both the multiplex PCR and LAMP assays exhibit high sensitivity and specificity for the detection of different bacterial species. Both assays perform equally well, with a sensitivity of 97.03% for all targeted species, including *S. aureus, E. coli, Salmonella spp*., and mixed infections. This indicates that both methods are effective in detecting the presence of these bacteria in the tested samples. Similarly, the specificity of both assays is also high, indicating their ability to accurately identify the absence of the targeted bacterial species. The specificity values range from 92.7 to 98.7% for both multiplex PCR and LAMP.

DISCUSSION

Currently, there is still no highly effective vaccine against small and large ruminants' mastitis (SLRM), and to control its spread, it is crucial to have sensitive, rapid, and specific tests that can identify the primary bacteria responsible for significant losses in the dairy industry (Ashraf and Imran 2018). Traditional methods used to identify pathogens are time-consuming and require substantial labor, while many commercially available identification systems are not specifically designed to detect and identify key veterinary pathogens associated with the disease (Yüksel Dolgun et al. 2022). PCR is less labor-intensive than bacterial culture and conventional identification methods, and eliminating the lysis step led to cost savings in reagents and time (Asmare and Kassa 2017). Fast, sensitive, on-site tests are preferred to target treatments in cases of clinical and subclinical mastitis. So, the present study aimed to develop and evaluate a LAMP assay for this purpose. The prevalence of clinical mastitis (CM) and subclinical mastitis (SCM) in different dairy animal species, with varied prevalence rates of CM and subclinical mastitis, was observed in 18.5% of camels, 21.18% of cattle, 21.23% of buffaloes, 20% of sheep, and 21.5% of goats. SCM percentages of 17.5% in cattle, 21.23% in buffaloes, 7.4% in camels, 17.7% in sheep, and 12% in goats (Tables 1, 4, and Fig. 1). The highest prevalence of both clinical and subclinical mastitis was found in cattle, followed by buffaloes and sheep. The

Table 5: Detection of bacterial DNA in milk samples by LAMP and multiplex PCR

Species		Multiplex PCR										LAMP									
		S.aureus		*E. coli*		*S.agalactiae*		*Salmonella spp*		Mixed infection		*S.aureus*		*E. coli*		*S.agalactiae*		*Salmonella spp*		Mixed infection	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Cattle	CM (n=57	13	22.8	6	10.5	8	14.03	3	5.2	4	7.1	14	24.5	7	12.3	-	-	4	7	4	7
	SCM (n=69)	18	26.1	13	18.8	7	10.14	3	4.3	6	8.6	20	28.9	13	18.8	-	-	3	4.3	6	8.6
Buffaloes	CM (n=40)	6	15	5	12.5	9	22.5	2	5	3	7.5	6	15	5	12.5	-	-	2	5	3	7.5
	SCM (n=56)	9	16	12	21.4	7	12.5	3	5.3	5	8.9	9	16.1	12	21.4	-	-	3	5.3	5	8.9
Camels	CM (n=8)	1	12.5	2	25	1	12.5	1	12.5	0	0	2	25	2	25	-	-	2	25	0	0
	SCM (n=20)	4	20	3	15	4	2	0	0	0	0	4	20	3	15	-	-	0	0	0	0
Sheep	CM (n=54	13	24	12	22.2	7	12.9	2	3.7	6	11.1	13	24.7	12	22.2	-	-	2	3.7	6	11.1
	SCM (n=61)	19	31.1	11	18.3	6	9.8	2	3.2	3	4.9	19	31.1	11	18	-	-	2	3.2	3	4.9
Goats	CM (n=21)	6	28.6	3	14.2	2	9.5	1	4.7	1	4.7	6	28.5	3	14.2	-	-	1	4.7	1	4.7
	SCM (n=43)	8	18.6	5	11.6	3	6.9	3	6.9	7	16.2	8	18.6	5	11.6	-	-	3	6.9	7	16.2
Total	429	97	22.6	69	16.1	54	12.6	19	4.4	35	8.2	101	23.5	73	17	-	-	22	5.1	31	7.2

CM: Clinical mastitis, SCM: Subclinical mastitis, +Ve: Positive samples, -Ve: Negative samples, %: Percentage of positive or negative samples, *S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, S. agalactiae: Streptococcus agalactiae*.

Table 6: Comparative between sensitivity and specificity of Multiplex PCR and LAMP Assays for Detection of different Bacterial Species

	Multiplex PCR								LAMP							
	S. aureus		*E. coli*		*Salmonella spp*		Mixed infection		*S. aureus*		*E. coli*		*Salmonella spp*		Mixed infection	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Total number of bacteria	97	22.6	69	16.1	19	4.4	35	8.2	101	23.5	73	17	22	5.12	31	7.2
Sensitivity	97		93.2		86.9		89.7		97		93.2		86.9		89.7	
Specificity	98.7		94.5		95.5		92.7		98.7		94.5		95.5		92.7	

N: Number of positive samples, %: Percentage of positive; *S. aureus: Staphylococcus aureus*, *E. coli*: *Escherichia coli*, *S. agalactiae*: *Streptococcus agalactiae*.

lowest prevalence was found in camels and goats, as previously reported by Ashraf and Imran (2018).

The prevalence of mastitis-causing bacteria varied among animal species, with *S. aureus, E. coli, and S. agalactiae* identified in cattle, buffaloes, camels, sheep, and goats. While mixed bacteria, including S. *aureus, E. coli, S. agalactiae,* and *Salmonella spp.*, were also observed, the findings of this study were consistent with the results reported by Field et al. (2003) and Devi et al. (2021). The advantages of the CMT are that it can be performed quickly and inexpensively on the animal's side (Cremonesi et al. 2009). However, this test may produce false-positive results in certain situations, such as non-infected quarters (Abed et al. 2021). The study found that mixed infections involving two or more bacterial species were not uncommon. The combinations of *S. aureus, E. coli,* and *S. agalactiae* were the most frequently identified mixed infections. These findings provide insights into the level of environmental microbial contamination. Ashraf and Imran (2018) suggest that *S. aureus* may increase the susceptibility of animals to infection by coliforms or other pathogens. While immunological methods may have limitations in terms of sensitivity and specificity, recent advances in molecular techniques have made rapid and sensitive detection of bacterial pathogens possible (Field et al. 2003; Cremonesi et al. 2009). In cattle, the multiplex PCR assay detected *S. aureus* in 22.8% of the samples, while *E. coli* was found in 10.5% of the samples. *S. agalactiae* and *Salmonella spp.* were present in 14.03 and 5.2% of the samples, respectively, with mixed infections observed in 7.01% of the cases. Conversely, the LAMP assay revealed *S. aureus* in 24.5% of the samples and *E. coli* in 12.28% of the samples. *Salmonella spp.* and mixed infections were also detected in 7.01% of the samples. *S. agalactiae* was not tested using the LAMP assay in cattle. In camels, the multiplex PCR assay detected *E. coli* in 25% of the samples, while *S. aureus* and *S. agalactiae* were present in 12.5% of the samples each. The LAMP assay provided similar results, identifying *S. aureus* and *E. coli* in 25% of the samples, respectively. The study found that the PCR assay could detect pathogens in bovine mastitic milk samples. While there is a detection limit for false-positive results, it can detect dead bacteria (Heikkilä et al. 2018; Abed et al. 2021). The study's results showed that 46% of the mastitic milk samples collected from subclinical cases of bovine mastitis were positive for *Staphylococcus species* (Notomi et al. 2000; Sahoo et al. 2016; Mohsin et al. 2022).

For sheep, both the multiplex PCR and LAMP assays consistently identified *S. aureus* as the most prevalent species, detected in 24.07% of the samples. *S. agalactiae, E. coli,* and mixed infections were found in 22.2, 12.9, and 11.1% of the samples, respectively. The multiplex PCR and LAMP assays successfully detected various bacterial species in different animal species, including *S. aureus, E. coli, S. agalactiae,* and *Salmonella spp.* However, there were variations in the occurrence percentages of these species between the two assays (Tie et al. 2012).

The comparison of sensitivity and specificity between the multiplex PCR and LAMP assays reveals that both methods are highly sensitive and specific for the detection of various bacterial species. The specificity values ranging from 92.7 to 98.7% for both methods suggest a low rate of false-positive results (Song et al. 2017; Zhang et al. 2020). The sensitivity and specificity values of multiplex PCR and LAMP indicate their suitability for the detection of bacterial DNA in milk samples (Deshmukh et al. 2016). Further studies with larger sample sizes and diverse populations of dairy animals would be beneficial to confirm and validate these findings (Srimongkol et al. 2020; Nnachi et al. 2022). Finally, LAMP assays can offer improved sensitivity and field settings. However, further research and validation are necessary to establish its effectiveness and reliability, taking into account various animal species and types of mastitis.

Conclusion

The LAMP assay has been successfully developed for the detection of major pathogens causing mastitis in milk samples from dairy animals. The high sensitivity and specificity of the LAMP assay make it a valuable tool for early detection and treatment of mastitis infections, which can improve milk quality and animal welfare in the dairy industry.

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Conflict of interest

There is no conflict of interest.

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