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Research Article

Molecular characterization of *Staphylococcus* spp. and *Escherichia coli* recovered from bovine mastitic milk in Palghar district, Maharashtra, India

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Abstract

Mastitis is an inflammatory response of the udder tissue to physical trauma or bacterial infections. The study was conducted to analyze bovine mastitic milk samples and environmental samples for the isolation and identification of *Staphylococcus* spp. and *Escherichia coli* using a cultural method and real-time PCR. A total of 214 samples comprising 162 pooled mastitic milk samples (one pooled sample per animal obtained by combining milk from all four quarters) (93 buffalo and 69 cattle) and 52 environmental samples (water, bedding material, feed, utensil swabs) from dairy farms in and around the Palghar region were collected and analysed for the detection of *Staphylococcus* spp. and *E. coli*. Out of 69 cattle and 93 buffalo milk samples screened by Modified California Mastitis Test and Somatic Cell Count (SCC), 47 (68.11%) and 22 (31.88%) cattle samples were reported as clinical and subclinical mastitic samples, repectively, whereas 36 (38.70%) and 57 (61.29%) buffalo samples were reported as clinical and subclinical mastitic samples, respectively. Among a total of 162 mastitic milk samples and 52 environmental samples analyzed, 117 and 38 samples were found to be positive for *Staphylococcus* spp., with an occurrence rate of 72.22 and 73.07%, respectively. Whereas, 78 and 28 samples were confirmed for *E. coli*, with an occurrence rate of 48.14 and 53.84%, respectively. The study concluded that the prevalence of *Staphylococcus* spp. and *E. coli* in mastitic milk and environmental sources emphasizes the need for strict implementation of biosecurity measures to reduce the occurrence of these pathogens, thereby safeguarding public health.

Keywords: Bovine mastitis, California Mastitis Test, *Escherichia coli*, Real-time PCR, *Staphylococcus* spp.

Highlights

- Clinical mastitis was more common in cattle, while buffaloes showed more subclinical cases.
- The study combined cultural methods and real-time PCR for pathogen detection.
- Both *Staphylococcus* spp. and *Escherichia coli* were frequently detected in milk and environmental samples.
- Improved hygiene and biosecurity practices are essential to control mastitis and protect public health.

INTRODUCTION

India has established itself as the global leader in milk production, contributing 230.58 million tonnes in 2022-23 (Press Information Bureau, 2023). Maintaining the quality and availability of milk is essential due to its significant nutritional and economic value. However, bovine mastitis remains a significant obstacle to dairy productivity, affecting animal health, milk yield, and quality (LeMaréchal et al., 2011). Due to the absence of prominent clinical signs, sub-clinical mastitis often remains undetected, which compromises milk safety and herd health. Thus, the prevalence of clinical and sub-clinical mastitis remains high across India (Krishnamoorthy et al., 2021a).

Staphylococcus spp. and *Escherichia coli* are among the most common pathogens associated with mastitis, presenting challenges in treatment and control due to poor antibiotic response (Rana et al., 2022). Drug resistance in *Staphylococcus* spp. has been a rising concern in the past few decades (Pal et al., 2021). Effective mastitis control involves accurate pathogen identification, segregation, dry cow therapy, and strategic culling (Hoque et al., 2018). Therefore, rapid detection methods are essential for timely intervention. However, phenotypic identification alone is insufficient for confirmation at the field level (Kaura et al., 2017).

Molecular characterization of pathogens using techniques such as real-time PCR (qPCR) provides

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sensitive, specific, and rapid pathogen detection, thereby improving disease management and epidemiological understanding (El-Sayed et al., 2017). Therefore, the current study focuses on the molecular detection and characterization of major mastitis-causing pathogens in dairy cattle using qPCR to enhance diagnostic accuracy and thus disseminate information about control strategies. The study aims to contribute to the development of region-specific approaches for mastitis surveillance and mitigation based on the geographic prevalence.

MATERIALS AND METHODS

Study area and sample collection

A total of 214 samples were collected, comprising 162 milk samples (one pooled sample per animal obtained by combining milk from all four quarters); i.e. 9, 10, 8, 10, 14, 11, 22, 22, 8, 12, 7, 13 and 16 from Farm A to M, respectively were collected aseptically from cattle (69) and buffalo (93) suspected for mastitis in and around Palghar region. Milk samples from affected quarters were collected according to the guidelines of the National Mastitis Council (2001). Fifty-two environmental samples (one each of water (50 mL), bedding material (50 mL), feed (100 g), respectively, and utensil swabs (50 mL) from 13 dairy farms around the Palghar region were collected using sterile swabs and maintained under chilled conditions for further analysis.

Screening of milk samples for mastitis using Modified California Mastitis Test (MCMT)

Approximately 3 to 5 mL of each milk sample was stripped from individual udder quarters of each cattle and buffalo into the CMT paddle cups, and an equal amount of modified CMT reagent was added and mixed for about 10-30 seconds. The results are interpreted as per the scores given by Abdulkhader et al. 2022.

Estimation of Somatic Cell Count (SCC)

Somatic cell count (SCC) of the samples was determined using DeLaval cassettes and a DeLaval Cell Counter (DCC). SCC is an established indicator of udder health and milk quality (Sharma et al., 2011) and was interpreted according to National Mastitis Council (2001) guidelines: <100,000 cells/mL (normal), ≥200,000 cells/mL (sub-clinical infection), and >200,000 cells/mL (clinical infection).

Microbiological analysis

Isolation and identification of *Staphylococcus* spp.: Isolation of *Staphylococcus* spp. was carried out as per IS 5887 (Part II): RA 2022 (Bureau of Indian Standards [BIS], 1976a). For mastitic milk samples, 0.1 mL of inoculum was plated on Baird Parker Agar (BPA) and incubated at 35-37°C for 24 hours, whereas for environmental samples, 10 mL each of water, bedding material and utensil swabs and 10 g of feed were pre-enriched in 90 mL of buffered peptone water (BPW) before plating onto BPA agar and incubated for 24 to 48 hrs at 37°C. Black colonies surrounded by an opaque halo are considered presumptive *Staphylococcus* spp. (Naas et al., 2019).

Isolation and identification of *Escherichia coli*: Isolation of *E. coli* was performed according to IS 5887 (Part I): RA 2022 (Bureau of Indian Standards [BIS], 1976b). For mastitic milk samples, 0.1 mL of inoculum was plated on Levin Eosin-Methylene Blue (L-EMB), and incubated at 35-37°C for 24 hours and for environmental samples, 10 mL each of water, bedding material and utensil swabs, along with 10 g of feed, were pre-enriched in 90 mL of buffered peptone water (BPW) and subsequently inoculated on L-EMB agar and incubated for 24 to 48 hrs at 37°C. The presence of *E. coli* was presumed by the presence of a metallic green sheen on the agar (Kabir, 2017), further confirmed by various biochemical tests.

Biochemical characterization of *Staphylococcus* spp. and *E. coli*: The presumptive isolates were subjected to various biochemical tests. The colonies were examined macroscopically for typical colony characteristics. Biochemical tests used for identifying *Staphylococcus* spp. included catalase, citrate, coagulase, indole, methyl red (MR) and nitrate reduction, while those for *E. coli* comprised catalase, oxidase, MR, Voges-Proskauer (VP), indole, citrate, nitrate reduction and hydrogen sulfide (H_2S) production.

Molecular characterization of *Staphylococcus* spp. and *Escherichia coli*

Extraction of DNA using the Qiagen Kit method: All confirmed isolates of *Staphylococcus* spp. and *E. coli* were subjected to DNA extraction using the QIAamp DNA Mini Kit, Qiagen. The obtained DNA was stored at -80°C until PCR processing. One representative isolate from each culture-positive sample was selected for molecular confirmation using qPCR.

Primers used in Real-Time PCR assay: Primers for molecular characterization of *Staphylococcus* spp. and *E. coli* were procured from M/s Eurofins Genomics India Pvt. Ltd., Bangalore. Specific primer details used for species detection are presented in Table 1.

Table 1. Details of primer sequences used for standardization of Real-Time PCR assay to detect 16S rRNA of *Staphylococcus* spp. and *Escherichia coli*

Sr. No.	Name of Organism	Target Gene	Primers	Nucleotide sequence
1	*Staphylococcus* spp.	16S rRNA	Forward primer	CGA AAG CGT GGGGAT CAA AC
			Reverse primer	CCC AGG CGG AGTGCT TAA TG
2	*E. coli*	16S rRNA	Forward primer	CAT TGA CGT TAC CCGCAG AA
			Reverse primer	CGC TTT ACG CCC AGTAAT TCC

Standardization of Real-time PCR: q-PCR targeting the 16S rRNA gene was performed by using *Staphylococcus* spp. (ATCC 12600) and *E. coli* (ATCC 25922) DNA as positive controls, while nuclease-free water was used as a negative control. qPCR for the detection of the 16S rRNA gene of *Staphylococcus* spp. and *E. coli* was standardized based on the protocols by Al-Shammari (2014) and Spano et al. (2005), respectively, with slight modifications. qPCR was performed using the Real-Time PCR Detection System (Applied Biosystems, Thermo Fisher Scientific, USA). The reaction mixture for *Staphylococcus* spp. (23.00 µL) and *E. coli* (20.00 µL) included 10.00 µL of 2X TaqMan Fast Advanced Master Mix, 1.50/1.50 µL and 1.00/1.00 µL of forward and reverse 16S rRNA primers, respectively, 3.00 µL and 2.00 µL of template DNA, and 7.00 µL and 6.00 µL of nuclease-free water.

qPCR Cycling Conditions: For *Staphylococcus* spp., qPCR was performed with an initial denaturation at 95°C for 10 min, 50 cycles of 95°C for 15 sec (denaturation) and 60°C for 60 sec (annealing and extension), whereas, for *E. coli*, the initial denaturation was set at 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 20 sec. A no-template negative control was included in each assay run. Samples with Ct values less than 40 were considered positive, whereas those above 40 were considered negative (Zhang et al., 2015).

RESULTS

Identification of clinical and subclinical mastitis

Out of the 162 mastitic milk samples analysed (69 from cattle and 93 from buffaloes), using the Modified California Mastitis Test (MCMT) and Somatic Cell Count (SCC), 47 (68.11%) of the cattle samples and 36 (38.70%) of the buffalo samples were positive for clinical mastitis (CM) while subclinical mastitis (SCM) was observed in 22 (31.88%) cattle samples and 57 (61.29%) buffalo samples. Overall, the occurrence rate of clinical and subclinical mastitis samples was 51.23% (83/162) and 48.76% (79/162), respectively (Table 2).

Table 2. Number of clinical and sub-clinical mastitic milk samples observed in different dairy farms using Modified CMT

Sr. No.	Source of samples	No. of milk samples collected			Samples positive for clinical mastitis			Samples positive for sub-clinical mastitis		
		Cattle	Buffalo	Total	Cattle	Buffalo	Total	Cattle	Buffalo	Total
1	FarmA	01	08	09	01	06	07	-	02	02
2	FarmB	01	09	10	01	04	05	-	05	05
3	FarmC	01	07	08	01	04	05	-	03	03
4	FarmD	01	09	10	01	03	04	-	06	06
5	FarmE	03	11	14	01	05	06	02	06	08
6	FarmF	01	10	11	01	06	07	-	04	04
7	FarmG	02	20	22	01	04	05	01	16	17
8	FarmH	03	19	22	01	04	05	02	15	17
9	FarmI	08	-	08	08	-	08	-	-	-
10	FarmJ	12	-	12	11	-	11	01	-	01
11	FarmK	07	-	07	03	-	03	04	-	04
12	FarmL	13	-	13	08	-	08	05	-	05
13	FarmM	16	-	16	09	-	09	07	-	07
	Total	69	93	162	47(68.11)	36(38.70)	83(51.23)	22(31.88)	57(61.29)	79(48.76)

Note: Figures in parenthesis indicates percent occurence of mastitis and sub-clinical mastitis in the milk samples analyzed

Table 3. Occurrence of *Staphylococcus* spp. and *Escherichia coli* in milk samples analyzed from various dairy farms

Sr. No	Source of sample	Number of milk samples analyzed	*Staphylococcus* spp.			*Escherichia coli*		
			Cattle	Buffalo	Total	Cattle	Buffalo	Total
1	FarmA	09	01	04	05 (55.55)	01	03	04 (44.44)
2	FarmB	10	01	04	05 (50.00)	01	04	05 (50.00)
3	FarmC	08	01	04	05 (62.50)	-	05	05 (62.50)
4	FarmD	10	-	06	06(60.00)	01	04	05 (50.00)
5	FarmE	14	02	06	08 (57.14)	01	07	08 (57.14)
6	FarmF	11	-	09	09 (81.81)	-	06	06 (54.54)
7	FarmG	22	01	16	17 (77.27)	01	12	13 (59.09)
8	FarmH	22	-	17	17 (77.27)	-	13	13 (59.09)
9	FarmI	08	08	-	08 (100.00)	03	-	03 (37.50)
10	FarmJ	12	10	-	10 (83.33)	02	-	02 (16.66)
11	FarmK	07	04	-	04 (57.14)	-	-	-(00.00)
12	FarmL	13	13	-	13 (100.00)	07	-	07 (53.84)
13	FarmM	16	10	-	10 (62.50)	07	-	07 (43.75)
	Total	162	51(31.48)	66(40.74)	117 (72.22)	24(14.81)	54 (33.33)	78 (48.14)

Note: Figures in parenthesis indicates percent occurence of organisms in the milk samples analyzed

Occurrence of *Staphylococcus* spp. in mastitic milk samples

Staphylococcus spp. were reported in 117/162 samples (72.22%), of which 51 (31.48%) belonged to cattle and 66 (40.74%) to buffaloes (Table 3).

In cattle samples, the highest number of isolates (n=13) was recorded from Farm L, while the lowest (n=1) was found in Farms A, B, C and G. In buffalo samples, the highest occurrence was observed in Farm H (17 samples), while Farms A, B and C had the lowest (4 samples each).

Occurrence of *Escherichia coli* in mastitic milk samples

Escherichia coli was reported in 78/162 samples, of which 24 (14.81%) were from cattle and 54 (33.33%) from buffalo milk samples (Table 3), with an overall occurrence of 48.14%. The highest positivity rate for *E. coli* in milk samples was observed in Farm C (62.50%), while no samples were found positive in Farm K. A single *E. coli*-positive isolate was isolated from Farms A, B, D, E and G.

Occurrence in environmental samples

Occurrence of *Staphylococcus* spp. in environmental samples: Out of 52 samples, 38 samples (73.07%) tested positive for *Staphylococcus* spp. The highest occurrence was reported in bedding material (13 samples; 25%), followed by water and utensil swabs (10 samples each; 19.23%) and feed (5 samples; 9.62%) (Table 4), similar to Woudstra et al. (2023), who reported the highest contamination rate in bedding material, followed by water and feed.

Occurrence of *Escherichia coli* in environmental samples: A total of 52 environmental samples analyzed, 28 samples (53.84%) showed the presence of *E. coli* (Table 4) with the highest contamination in water samples (10; 19.23%), followed by bedding material (9; 17.31%), utensil swabs (7; 13.46%) and feed (2; 3.85%).

Biochemical confirmation of isolates

All 155 isolates presumptively identified as *Staphylococcus* spp. exhibited typical jet-black, circular, smooth, and convex colonies on Baird-Parker agar. Biochemical analysis confirmed the isolates in agreement with the biochemical profile of *Staphylococcus* species.

Similarly, all 106 isolates of *Escherichia coli* presented typical metallic green sheen colonies with a dark center on Levine Eosin-Methylene Blue (L-EMB) agar, suggestive of lactose fermentation. Upon biochemical confirmation, the isolates exhibited characteristics as per the standard descriptions available for *E. coli* in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Molecular confirmation of *Staphylococcus spp.* and *E. coli* by Real-Time PCR

Assay sensitivity and specificity: The optimized thermal cycling conditions included an initial denaturation at 95°C and annealing temperatures ranging from 51°C to 55°C for *Staphylococcus* spp. and 55°C for *E. coli*. No amplification was observed in the negative controls.

Table 4. Occurrence of *Staphylococcus* spp. and *Escherichia coli* in environmental samples analyzed from different dairy farms

Sr. No	Source of sample	Number of Environmental samples analyzed	Number of environmental samples positive									
			Staphylococcus spp.					*Escherichia coli*				
			Water	Bedding material	Feed	Utensil swab	Number of positive samples	Water	Bedding material	Feed	Utensil swab	Number of positive samples
1	FarmA	04	01	01	01	01	04 (100)	01	01	01	01	04 (100)
2	FarmB	04	01	01	01	01	04 (100)	-	01	01	-	02 (50)
3	FarmC	04	01	01	-	01	03(75)	01	01	-	01	03 (75)
4	FarmD	04	01	01	-	01	03 (75)	01	01	-	01	03 (75)
5	FarmE	04	-	01	-	01	02 (50)	01	-	-	-	01 (25)
6	FarmF	04	01	01	01	01	04 (100)	01	01	-	01	03 (75)
7	FarmG	04	01	01	-	01	03 (75)	01	01	-	-	02 (50)
8	FarmH	04	01	01	-	01	03 (75)	-	01	-	-	01 (25)
9	FarmI	04	01	01	01	01	04(100)	01	01	-	01	03 (75)
10	FarmJ	04	01	01	01	-	03 (75)	01	01	-	-	02 (50)
11	FamK	04	01	01	-	-	02 (50)	-	-	-	-	-(00)
12	FamL	04	-	01	-	-	01 (25)	01	-	-	01	02 (50)
13	FarmM	04	-	01	-	01	02 (50)	01	-	-	01	02 (50)
	Total	52	10(19.23)	13(25)	05(9.62)	10(19.23)	38 (73.07)	10(19.23)	09(17.31)	02(3.85)	07(13.46)	28 (53.84)

Note: Figures in parenthesis indicates percent occurence of organisms in the environmental samples analyzed

Molecular confirmation of *Staphylococcus* spp. by qP*CR*: Out of 155 presumptive isolates subjected to qPCR targeting the 16S rRNA gene, 149 were confirmed by qPCR, comprising of 115 (70.98%) milk samples and 34 (65.38%) environmental samples. The molecular identity was confirmed with Ct values ranging from 16 to 18, resulting in an overall molecular confirmation rate of 96.12**%** for *Staphylococcus* spp. in the analyzed samples (Table 5, 6; Fig. 1).

Molecular confirmation of *E. coli* by qPCR: Out of the 106 presumptive isolates, 100 showed positive amplification in qPCR targeting the 16S rRNA gene, comprising 74 mastitic milk samples (45.67%) and 26 environmental samples (50%), with Ct values ranging from 10.5 to 22.0 (Table 5, 6; Fig. 2), thus confirming the overall molecular confirmation rate of 94.33% for *E. coli.*

Ct values

Samples with Ct values less than 40 were considered positive, whereas those above 40 were considered negative (Zhang et al., 2015). However, in the present study, the observed Ct values for analysis of milk and environmental samples for detection of *Staphylococcus* spp. and *E. coli* were 16 to 18 and 10.5 to 22, respectively. These observed values indicate a high concentration of DNA in mastitic milk samples.

DISCUSSION

Identification of clinical and subclinical mastitis: These findings of the current study agree with Kashyap et al. (2019), who reported an SCM prevalence of 68.33% in the Surajpur district of Chhattisgarh. However, in comparison, Sadashiv et al. (2013) reported lower incidences of SCM (48.21%) and CM (21.68%) in Karnataka. These differences may be due to possible variations, such as geographical location.

Occurrence of *Staphylococcus* spp. and *Escherichia coli* in samples: The overall isolation rate of *Staphylococcus* spp. was higher compared to previous studies by Krishnamoorthy et al. (2021b), who reported 67%. Studies conducted by Jena et al. (2015) and Verma et al. (2018) reported lower isolation rates of 46.3% and 42.55%, respectively. The overall detection rate of *E. coli* in this study was higher than earlier studies reported

Characterization of *Staphylococcus* and *Escherichia coli* from bovine mastitis

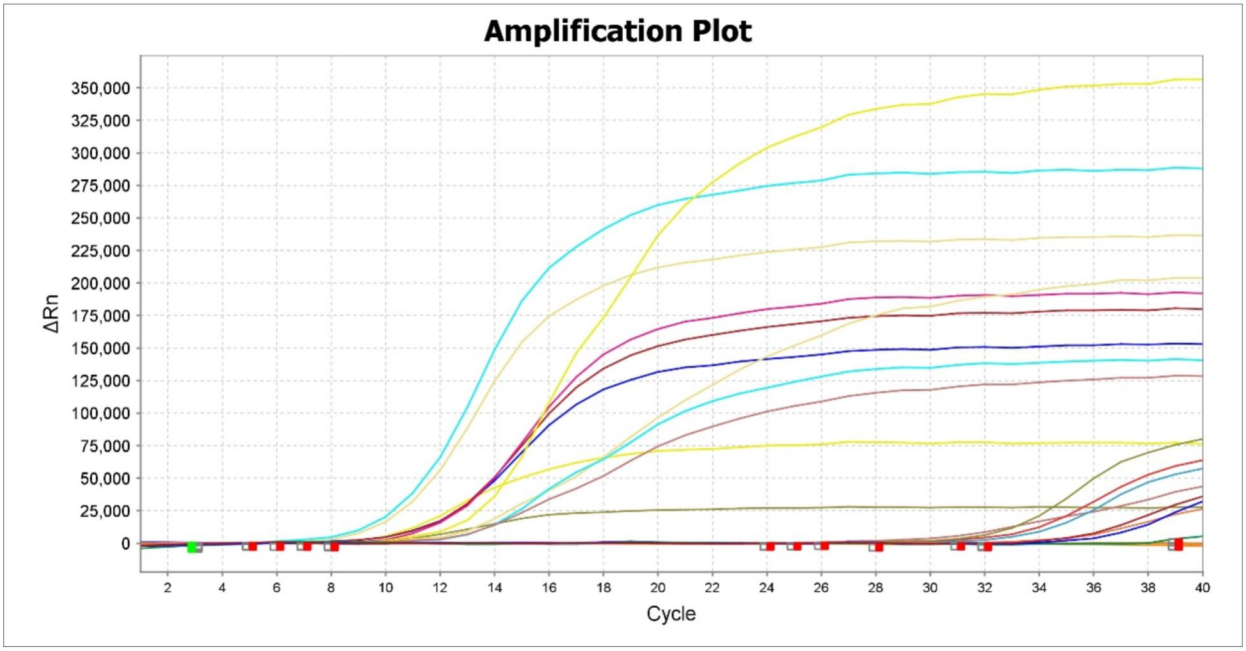


Fig. 1. Amplification plot for standard genomic DNA of *Staphylococcus* spp.

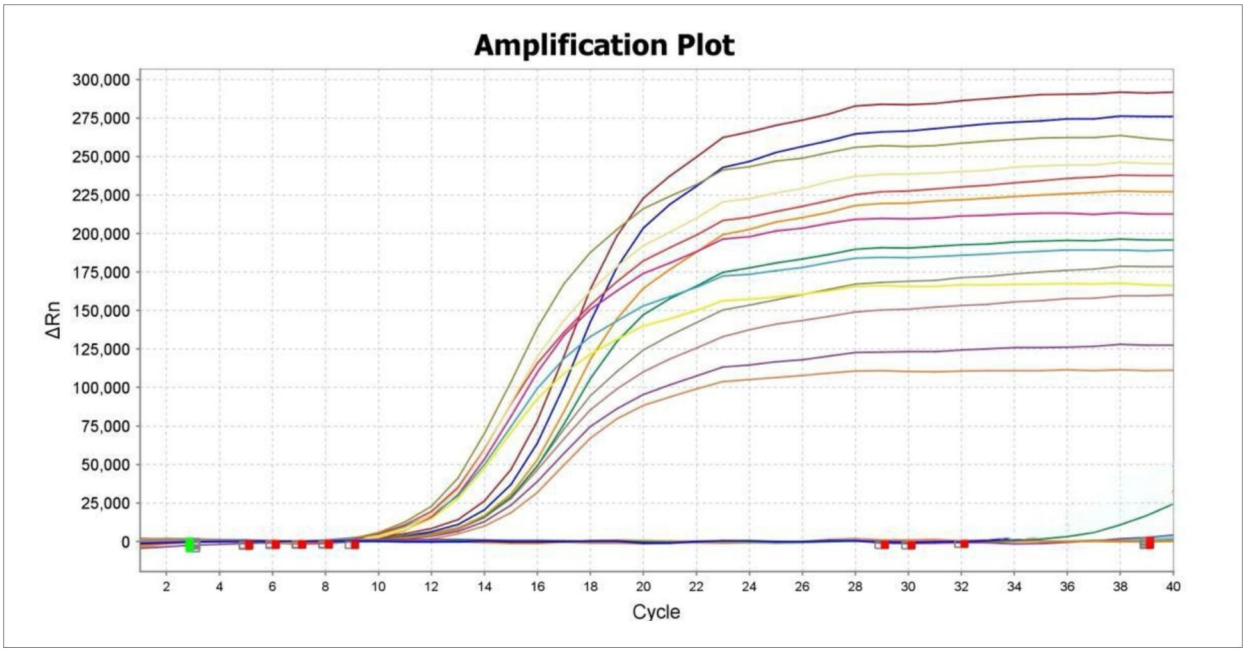


Fig. 2. Amplification plot for standard genomic DNA of *Escherichia coli*

Table 5. Occurrence of *Staphylococcus* spp. and *Escherichia coli* in milk samples analyzed by qPCR from various dairy farms using 16s rRNA

Sr. No.	Source of sample	*Staphylococcus* spp.		*Escherichia coli*	
		Number of culture positive samples	Number of positive samples by qPCR	Number of culture positive samples	Number of positive samples by qPCR
1	FarmA	05	05	04	04
2	FarmB	05	05	05	05
3	FarmC	05	05	05	05
4	FarmD	06	06	05	05
5	FarmE	08	08	08	08
6	FarmF	09	09	06	06
7	FarmG	17	17	13	11
8	FarmH	17	17	13	13
9	FarmI	08	08	03	03
10	FarmJ	10	10	02	02
11	FarmK	04	04	-	-
12	FarmL	13	11	07	05
13	FarmM	10	10	07	07
	Total	117	115 (70.98)	78	74 (45.67)

Note: Figures in parenthesis indicates percent occurence of organisms in the milk samples analysed

Table 6. Occurrence of *Staphylococcus* spp. and *Escherichia coli* in environmental samples analyzed by qPCR from different dairy farms using 16s rRNA

Sr. No.	Source of sample	*Staphylococcus* spp.					*Escherichia coli*				
		Number of environmental samples positive				Number of positive samples by qPCR	Number of environmental samples positive				Number of positive samples by qPCR
		Water	Bedding material	Feed	Utensil swab		Water	Bedding material	Feed	Utensil swab	
1	FarmA	01	01	01	01	04	01	01	01	01	04
2	FarmB	-	01	01	01	03	-	01	01	-	02
3	FarmC	01	01	-	01	03	-	01	-	01	02
4	FarmD	01	01	-	01	03	01	01	-	01	03
5	FarmE	-	01	-	01	02	01	-	-	-	01
6	FarmF	-	01	01	01	03	01	01	-	01	03
7	FarmG	01	01	-	01	03	01	01	-	-	02
8	FarmH	01	-	-	01	02	-	01	-	-	01
9	FarmI	-	01	01	01	03	01	01	-	01	03
10	FarmJ	01	01	01	-	03	01	01	-	-	02
11	FarmK	01	01	-	-	02	-	-	-	-	-
12	FarmL	-	01	-	-	01	-	-	-	01	01
13	FarmM	-	01	-	01	02	01	-	-	01	02
	Total	07	12	05	10	34 (65.38)	08	09	02	07	26 (50)

Note: Figures in parenthesis indicates percent occurence of organisms in the environmental samples analyzed

by Yadav et al. (2023), with a prevalence of 45%, Gangwal et al. (2017), and Verma et al. (2018), with the rates of 28% and 21.28%, respectively.

However, the rate of occurrence in *Staphylococcus* spp. in environmental samples was higher than that reported by Badawy et al. (2022), who observed 20% in bedding, 15% in water and 10% in feed samples. The occurrence rates of *E. coli* in environmental samples in this study were significantly higher than those reported by Aditya et al. (2023), who reported the occurrence as 9.09% in bedding material, 6.63% in water, and 7.14% in feed samples. The elevated levels are likely due to inadequate farm waste management and hygiene measures, such as overcrowded housing, infrequent replacement of bedding material, and limited access

to clean water, which may create favourable conditions for pathogen survival and dissemination within dairy farms.

Molecular confirmation of *Staphylococcus* spp. and *E. coli* by Real-Time PCR: The occurrence of *Staphylococcus* spp. in this study was lower (89.64%) than that reported by Chandrasekaran et al. (2014) but higher than that of Kouran et al. (2017) at 41.74%. The occurrence of *E. coli* in the present study is similar to Ayele et al. (2024) and slightly lower than that reported by Chandrasekaran et al. (2014) [50.64%], but notably higher than that observed by Kour et al. (2017) [25.24%]. These variations may reflect differences in herd health status and environmental hygiene.

In conclusion, the present study found that clinical mastitis was more prevalent in cattle, whereas subclinical mastitis was more prevalent in buffalo. A significant percentage of mastitic milk and environmental samples were positive for *Staphylococcus* spp. and *E. coli*, with regular occurrences across various farms and sources, including bedding and water. Molecular confirmation of the same using qPCR targeting the 16S rRNA gene showed agreement with conventional methods, confirming the reliability of both diagnostic approaches. The inclusion of environmental sampling provides valuable insights into possible infection sources and transmission pathways within dairy farms. These findings support the use of qPCR as a valuable tool for the early diagnosis and confirmatory detection of *Staphylococcus* spp. and *E. coli* in mastitic milk and environmental samples, facilitating timely management and intervention at the dairy farm level.

Conflict of interest: The authors declare no conflict of interest.

Author's contribution: SS: Performed the experiments; RZ, VV: Conceived and designed the study; BJ: Provided expert consultation and critical input; AN, NP: Drafted the manuscript and conducted proofreading; AS, BZ: Assisted in sample collection and related field activities. All authors contributed to the article and approved the submitted version.

Data availability statement: The data mentioned in the article will be available with the corresponding author.

Ethical approval- Not applicable as it is not under the purview of the IAEC Committee.

Use of artificial intelligence tools: During the preparation of this work, the author(s) used Grammarly *(Grammarly Inc., San Francisco, CA, USA)* for grammar, spelling and language editing. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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