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Research Article

Prevalence and antibiotic resistance profiling of *Staphylococcus aureus* from cases of bovine mastitis in and around Warangal region of Telangana

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Abstract

Mastitis is an important disease of dairy animals incurring severe economic losses to the dairy industry worldwide. A wide range of pathogens are known to be causative agents of mastitis in animals. The prevalence of mastitis in dairy animals in the study region due to *Staphylococcal* spp. was found to be 71.42%, and due to *S. aureus* was 66.07%. The prevalence of other pathogens causing mastitis in the study region was found to be in the order *E. coli* (16%), *Streptococcus* spp. (7.14%), *Bacillus* spp. (3.57%), and *Proteus* spp. (1.78%). The antibiogram study of all 74 *S. aureus* isolates revealed that the organisms were found sensitive to antibiotics such as third generation fluroquinolone, levofloxacin (87.83%), azithromycin (79.8%), chloramphenicol (71.63%), gentamicin (62.5%), erythromycin (62.16%), and co-trimoxazole (50%). The high resistance was observed to commonly used antibiotics such as methicillin (73.0%), followed by the antibiotics in the order trimethoprim (70.28%), tetracyclines (70%), oxacillin (68%), doxycycline (66.21%), ciprofloxacin (63.52%), and co-trimoxazole (40.55%). The present findings warrant the danger of the injudicious use of antibiotics while treating mastitis cases in the Telangana region.

Keywords: Antibiogram, Mastitis, Methicillin, *Staphylococcus aureus*, Telangana

Highlights

- Resistance to methicillin was observed among 73.0% of the isolates.
- The isolates were resistant to commonly used antibiotics such as tetracyclines (70%), oxacillin (68%), doxycycline (66.21%), and ciprofloxacin (63.52%).
- The isolates were found sensitive to the antibiotics, levofloxacin (87.83%) followed by azithromycin (79.8%), chloramphenicol (71.63%), gentamicin (62.5%), erythromycin (62.16%), and co-trimoxazole (50%).
- The prevalence of multidrug-resistant *S. aureus* as a major etiological agent in clinical cases of mastitis revealed the need for isolation and testing for antimicrobial susceptibility of the etiological agent as an effective therapeutic measure to prevent the indiscriminate use of antibiotics.

INTRODUCTION

Mastitis is characterized as the inflammation of the udder involving the pathophysiological changes in the parenchyma of the udder and physical, chemical and microbiological changes in the milk, causing severe economic loss to the dairy farmers. It is one of the most prevalent and complex diseases in the dairy industry with severe economic losses due to decreased milk production, abnormal milk composition and high treatment costs leading to early culling of the animals (Thompson-Crispi *et al.*, 2014; Hoque *et al*., 2018; Hoque *et al*., 2022). It is estimated that the daily losses due to mastitis during the first 2 weeks of lactation range from 1–2.5 kg of milk per day, resulting in an estimated loss of 110–552 kg/lactation. This loss of milk production is attributable to huge economic losses of about Rs. 575 million annually and a reduction in milk production by 21% (Rajala-Schultz *et al*., 1999; Bardhan, 2013; Kour *et al*., 2023). Although, mastitis is a multi-complex disease condition, it can be classified as clinical and subclinical based on the clinical manifestation of the disease. The clinical mastitis can be detected by changes in udder parenchyma, abnormal milk production like changes in color, consistency and reduced milk production (Feyissa *et al*., 2023). The sub-clinical form of mastitis is more prevalent than the clinical form, reduces milk production, and adversely affects milk quality (Seegers *et al.*, 2003; Jyothi *et al*., 2018). A wide variety of microorganisms are responsible for causing mastitis, of which bacteria are the most frequently

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isolated pathogens. The bacterial agents causing mastitis can be contagious or environmental pathogens. The major contagious bacterial pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis*, and the major environmental pathogens include *Escherichia coli* and *Streptococcus uberis* (Bhat *et al*., 2017). In India, it has been reported that *Staphylococcus* is the major etiological agent, followed by *Streptococcus* and *E. coli* causing subclinical mastitis in cows (Jyothi *et al*., 2018). Apart from huge economic losses in terms of treatment costs and reduced milk production, the indiscriminate use of antibiotics may result in the emergence of multidrug-resistant bacteria, which pose a potential threat to public health (Bhat *et al*., 2017). Also, indiscriminate use of antibiotics without the selection of an accurate therapeutic drug may lead to treatment failure. For effective treatment of mastitis in dairy cows, proper identification of the etiological agents and proper selection of antimicrobials are prerequisites. This information guides in the successful treatment and control of mastitis and also addresses public health concerns (Dhakal *et al*., 2007; Jyothi *et al*., 2018). Rapidly-evolving multi-drug resistant *Staphylococcus aureus* is a global concern to animal and human health (Cosgrove *et al*., 2003). The present study was carried out to study the antibiogram of the major etiological agent, *Staphylococcus aureus,* from clinical cases of bovine mastitis in the study region.

MATERIALS AND METHODS

Clinical samples: A total of 99 milk samples were collected from 82 animals (more than one teat affected in 9 animals) with clinical cases of bovine mastitis presented to the veterinary hospitals, veterinary polyclinics, and from small, medium and large dairy farms in and around Warangal region of Telangana state for a period of 18 months from January 2021 to June 2022 as detailed in table 1.

Collection of milk samples: Milk samples from individual quarters from the animals presented to the veterinary hospitals were taken after washing the udder for visible debris, and teat ends were scrubbed with cotton soaked in spirit. From each affected quarter, 5 mL of milk was collected in sterile vials (HiMedia, Mumbai) after discarding the first few streaks of milk for culture and antibiotic sensitivity testing.

Isolation and identification of bacteria: The bacteriological isolation was done as per the method described (Bhat *et al*., 2017). The collected milk samples were plated with a sterile platinum loop on sheep blood agar (BA) plates (HiMedia, Mumbai), Mannitol salt agar (MSA) (HiMedia, Mumbai), MacConkey agar (MCA) and the inoculated plates were incubated at 37°C for 24 h. The bacterial colonies were identified initially by colony characteristics on BA, MCA and MSA. The morphological confirmation was done by gram staining and biochemical characterization by catalase, oxidase, and slide coagulase tests.

Extraction of bacterial DNA: For confirmation of *Staphylococcus aureus*, the bacterial genomic DNA was extracted using HiPurA® Genomic DNA Purification Kit (HiMedia, Mumbai) according to the manufacturer's instructions.

Confirmation of *Staphylococcus aureus* by Polymerase chain reaction: All the coagulase positive isolates were confirmed in Polymerase chain reaction (PCR) using the primers specific for virulent gene, *Spa* (*Staphylococcus aureus* protein A) which amplifies a 920 bp region in the genome (Aher *et al*., 2012).

Antibiogram study of the isolates: Bauer-Kirby (Bauer *et al*., 1966) described a disc diffusion method to conduct an antibiotic susceptibility test using antibiotic test discs. The turbidity of the cultures of *Staphylococcus aureus* in BHI broth was adjusted to McFarland standard tube 0.5 before charging on Mueller Hinton Agar (MHA). The culture was spread evenly with the help of a sterile cotton swab over the entire surface of MHA. Antibiotic discs were kept aseptically by using sterile forceps. The plates were then incubated at 37°C for 16-24 h. The zone of growth inhibition around the antibiotic discs

Table 1. Details of clinical samples collected

Place	Number of animals with clinical mastitis	No of milk samples collected
Veterinary hospitals, Hanamkonda and Warangal region	26	30
Small dairy farms (< 20 animals) in and around Warangal	23	32
Large dairy farms (> 20 animals) in and around Warangal	33	39
Total	82*	99

* Among 82 animals, 16 animals were below 3 years of age and 66 animals were more than 3 years of age

was measured to the nearest millimetre. The zone of growth inhibition diameter of each drug was interpreted using the interpretative criteria provided by the manufacturer (HiMedia, Mumbai). The results were qualitatively interpreted as susceptible, intermediate and resistant as per the CLSI guidelines (2020) and categorized as resistant, intermediate and sensitive. The antibiotics oxacillin (30 µg), gentamicin (10 µg), azithromycin (15 µg), erythromycin (15 µg), tetracycline (30 µg), doxycycline (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), co-trimoxazole (25 µg), trimethoprim (5 µg), chloramphenicol (30 µg) and methicillin (10 µg) were used in the present study.

RESULTS

In the present study, a total of 112 pure cultures of the bacteria were isolated from 99 clinical samples. Based on morphology, cultural characteristics on bacteriological media and biochemical tests, the bacterial genera *Staphylococcus*, *Streptococcus*, *E. coli*, *Proteus* and *Bacillus* spp. were tentatively identified. Among the isolates, 80 (71.42%) were found to be of *Staphylococcus* spp., 18 (16%) *E. coli*, 8 (7.14%) *Streptococcus* spp., 4 (3.57%) *Bacillus* spp., and 2 (1.78%) are *Proteus* spp. (Fig. 1).

In the present study, the majority of the bacteria were of *Staphylococcus* spp. which were morphologically identified in gram's staining as gram-positive cocci arranged in irregular clusters resembling bunches of grapes and on MSA, golden yellow colored colonies were noticed (Fig. 2). Among the 80 *Staphylococcus* spp. 74 isolates were found positive by slide coagulase test and the *Spa* gene coding for virulence factor, protein A was specifically amplified in PCR with an amplicon of 920 bp, confirmed the *Staphylococcus aureus* (Fig. 3). The prevalence of mastitis due to

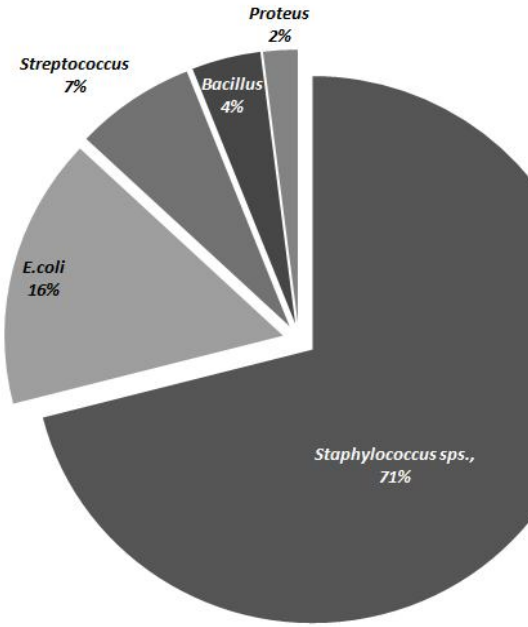

Fig. 1. Pie diagram showing prevalence of various bacterial agents associated with bovine mastitis in the study region

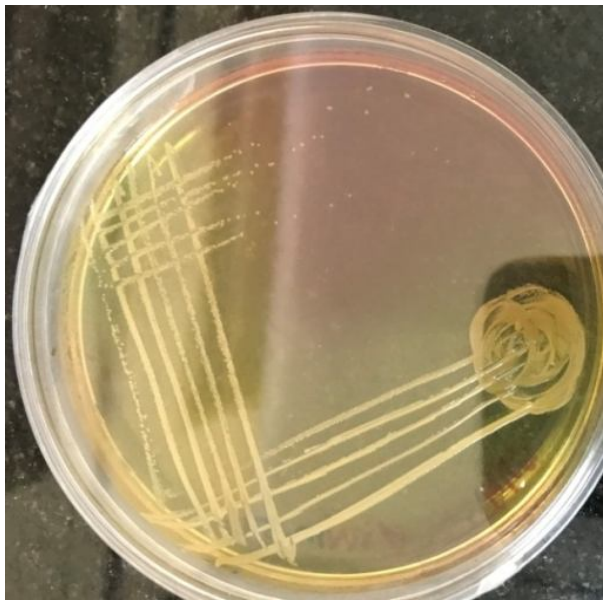

Fig. 2 Golden yellow colored colonies of *Staphylococcus aureus* on mannitol salt agar

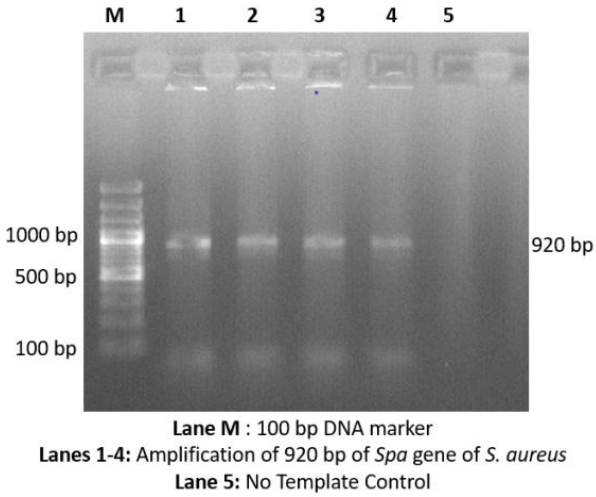

Fig. 3. Amplified 920 bp amplicon of *Spa* gene of *Staphylococcus aureus* in PCR

Staphylococcus spp. alone in clinical mastitis was identified as 71.42% (80/112) among the clinical mastitis cases in dairy cows in and around Warangal region. The prevalence of *Staphylococcus aureus* was identified as

66.07% (74/112) in clinical mastitis cases. Further, the presence of *Spa* gene in the isolates indicates the pathogenic nature of the isolates. The prevalence of other bacterial agents in clinical mastitis cases in the region was in the order 16% (*E. coli*), 7.14% (*Streptococcus* spp.), 3.57% (*Bacillus* spp.), and 1.78% (*Proteus* spp.). In the present study, the prevalence of mastitis was higher in adult animals than in young animals.

The antibiogram study of all 74 *S. aureus* isolates revealed the following result. The organisms were found sensitive to the antibiotics in the order of levofloxacin (87.83%), azithromycin (79.8%), chloramphenicol (71.63%), gentamicin (62.5%), erythromycin (62.16%), co-trimoxazole (50%), ciprofloxacin (36.48%), oxacillin (32%), tetracycline (30%), trimethoprim (29.72%), methicillin (27.0%), doxycycline (21.62%). The organisms were found intermediate sensitive to the antibiotics in the order azithromycin (17.5%), gentamicin (12.5%), doxycycline (12.16%), chloramphenicol (10.8%) and co-trimoxazole (9.45%). Highest resistance was observed against the antibiotic methicillin (73.0%), followed by the antibiotics in the order trimethoprim (70.28%), tetracyclines (70%), oxacillin (68%), doxycycline (66.21%), ciprofloxacin (63.52%), co-trimoxazole (40.55%), erythromycin (37.84%), gentamicin (25%), chloramphenicol (17.57 %), levofloxacin (12.17%) and azithromycin (2.7%). The antibiogram profile of the isolates is presented in Table 2 and depicted in Fig. 5.

Fig. 4. Antibiotic sensitivity testing of *Staphylococcus aureus* isolates on Mueller Hinton agar by disc diffusion method

DISCUSSION

Mastitis is a serious problem in dairy animals, causing huge economic losses in terms of reduced milk production by quantity and quality, as well as frequent treatment failures. Among the infectious causes of mastitis, *Staphylococcus* is the most prevalent pathogen causing mastitis in dairy animals worldwide, including India. In the present study, 5 different types of bacteria were identified from a total of 112 pure cultures of the bacteria isolated from 99 clinical samples. The *Staphylococcus* spp. were found to be predominant with 71.42% (80 isolates), followed by *E. coli* 16%, *Streptococcus* spp. 7.14%, *Bacillus* spp. 3.57%, *Proteus* spp. 1.78%. The present findings were in line with the findings of several other workers in India and abroad. *Staphylococcus aureus* was found to be the predominant bacterial pathogen (60%) causing subclinical mastitis in cattle in Madhya Pradesh

Table 2. Antibiotic sensitivity/resistance pattern observed among 74 *Staphylococcus aureus* isolates

Sl. No.	Antibiotic	Sensitive		Intermediate		Resistant	
		No.	(%)	No.	(%)	No.	(%)
1	Oxacillin (30 µg)	24	32.00	0	-	50	68.00
2	Gentamicin (10 µg)	46	62.50	9	12.50	19	25.00
3	Azithromycin (15 µg)	59	79.80	13	17.50	2	2.70
4	Erythromycin (15 µg)	46	62.16	-	-	28	37.84
5	Tetracycline (30 µg)	22	30.00	-	-	52	70.00
6	Doxycycline (30 µg)	16	21.62	9	12.16	49	66.21
7	Ciprofloxacin (5 µg)	27	36.48	-	-	47	63.52
8	Levofloxacin (5 µg)	65	87.83	-	-	9	12.17
9	Co-Trimoxazole (25 µg)	37	50.00	7	9.45	30	40.55
10	Trimethoprim (5 µg)	22	29.72	-	-	52	70.28
11	Chloramphenicol (30 µg)	53	71.63	8	10.80	13	17.57
12	Methicillin (10 µg)	20	27.00	-	-	54	73.00

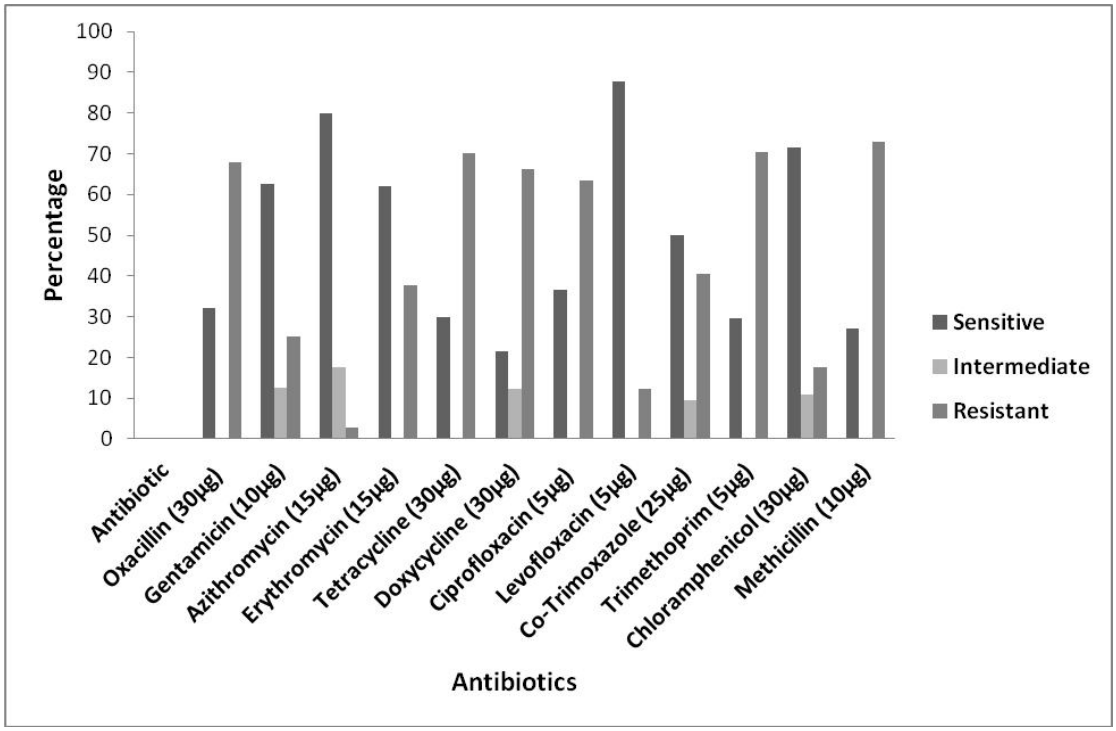

Fig. 5. Graphical representation of antibiogram pattern observed among 74 *Staphylococcus aureus* isolates

(Chhabra and Arora, 2006). Similar to our findings, *S. aureus* was found to be a major pathogen, while *Streptococcus* and *E. coli* were found as minor pathogens causing bovine clinical mastitis in Jammu region (Mohanty *et al*., 2013), West Bengal (Paul *et al*., 2013), Tamil Nadu (Jeykumar *et al*., 2013), Gujarat (Singh *et al*., 2017), Hyderabad (Jyothi *et al*., 2018), Chattisgarh (Hazari *et al*., 2018) and other parts of the world (Hoque *et al*., 2022; Feyissa *et al*., 2023).

Further, the *Staphylococcal* isolates were identified as *Staphylococcus aureus* in PCR with primers that specifically amplified the *Spa* gene coding for Protein A and produced an expected amplicon of 920 bp suggestive of *Staphylococcus aureus*. The presence of *Spa* gene in *Staphylococcus aureus* is indicative of its pathogenic nature. This is in accordance with the findings of other workers (Gorre *et al*., 2022). The prevalence of mastitis due to *Staphylococcus* spp. alone in clinical mastitis was found to be 71.42%, and the prevalence of *Staphylococcus aureus* was found to be 66.07% in dairy cows in and around Warangal region of Telangana state.

The antibiogram study of all the 74 isolates of *Staphylococcus aureus* revealed that the organisms were found to be sensitive to levofloxacin (87.83%) followed by azithromycin (79.8%), chloramphenicol (71.63%), gentamicin (62.5%), erythromycin (62.16%), co-trimoxazole (50%), ciprofloxacin (36.48%), oxacillin (32%), tetracycline (30%), trimethoprim (29.72%), methicillin (27.0%), doxycycline (21.62%). The organisms were found to be least sensitive to commonly used antibiotics against gram-positive infections in the present study. Similar to our findings, *S. aureus* was found to be sensitive to antibiotics like levofloxacin (Mohanty *et al*., 2013; Salauddin *et al*., 2020). Cloxacillin and co-trimoxazole were found to be effective in treating mastitis due to *S. aureus* in cattle in Madhya Pradesh (Chhabra and Arora, 2006). Ciprofloxacin and chloramphenicol were found to be effective against *S. aureus* from milk origin (Jeykumar *et al*., 2013; Opko *et al*., 2016). Intermediate sensitivity was observed for the antibiotics azithromycin (17.5%), followed by gentamicin (12.5%), doxycycline (12.16%), chloramphenicol (10.8%) and co-trimoxazole (9.45%). In the present study, the isolates were seen as resistant to penicillin group of antibiotics with highest resistance to methicillin (73.0%), followed by trimethoprim (70.28%) and tetracyclines (70%), oxacillin (68%), doxycycline (66.21%), ciprofloxacin (63.52%), co-trimoxazole (40.55%), erythromycin (37.84%), gentamicin (25%), chloramphenicol (17.57%), levofloxacin (12.17%) and azithromycin (2.7%). The resistance to penicillin G was observed and could be due to frequent use of these antibiotics, the organisms

could have gained resistance to these group of antibiotics (Chhabra and Arora, 2006). The *S. aureus* from milk origin shown highest resistance to tetracyclines and erythromycin (Okpo *et al*., 2016), tetracyclines and penicillins (Jeykumar *et al*., 2013; Paul *et al*., 2013; Bhat *et al*., 2017; Hazari *et al*., 2018; Salauddin *et al*., 2020; Hoque *et al*., 2022; Feyissa *et al*., 2023; Talaat *et al*., 2023). Also, the *S. aureus* in China were found resistant to β-lactams (50.68%), followed by quinolones (36.23%), macrolides (34.08%), sulfonamides (32.25%), tetracyclines (27.83%), aminoglycosides (26.44%), lincosamides (23.39%), and chloramphenicol (10.33%) (Wang *et al*., 2023). The differences in the resistance pattern of isolates could be due to different geographical locations and previous exposure to those antibiotics, since antibiotic resistance can be transferred by horizontal gene transfer among the bacteria. Hence, proper testing of the isolates against a variety of antibiotics is essential to initiate early and appropriate antibiotic therapy.

In Conclusion, the present study revealed that *S. aureus* was found to be the most common cause of clinical mastitis in dairy animals in the study region. The prevalence of methicillin-resistant *S. aureus* was found to be 73.0%. The organisms were found resistant to commonly used antibacterials to treat mastitis in animals. The isolates were found sensitive to antibiotics such as levofloxacin, azithromycin and chloramphenicol. Moreover, indiscriminate use of antibiotics may result in the emergence of multi-drug resistant strains of pathogens and wherever possible, antimicrobial sensitivity testing needs to be done before initiating the antimicrobial treatment to animals to prevent treatment failures. If the facility is not available, antibiotics such as levofloxacin along with azithromycin and gentamicin could be used to treat mastitis against *S. aureus* in the study region.

Conflict of interest: The authors declare that they have no conflict of interest.

Author's contributions: VKS: Isolated the bacteria, carried out the antimicrobial sensitivity part and drafted the manuscript; JSJ, GV: Participated in designing the study and assisted in research and drafting the manuscript; PA, RKL: Participated in sample collection from mastitis animals and checking the manuscript.

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