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Isolation of bacteria causing mastitis from dairy cattle and its antimicrobial sensitivity in the South-Western region of Gujarat

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Abstract
Mastitis is one of the major conditions that can reduce livestock production, population, quality of milk and farmers' income. Many bacteria have been identified as etiological agents for mastitis. It has been frequently reported that many cases of mastitis are difficult to cure because of antimicrobial resistance. This study aimed to culturally identify various bacteria that cause mastitis in dairy cattle and their antimicrobial susceptibility testing. The study was conducted on 647 cattle milk samples received at the Veterinary Clinical Complex, Kamdhenu University, Junagadh with a history of clinical mastitis. Primary bacterial isolation was carried out by inoculating milk samples on the Brain Heart Infusion agar (BHI), morphological identification by Gram's staining, and biochemical tests like-catalase, oxidase, and KOH test. 590 (91.19%) bacterial and 4 (0.6%) yeast isolates were found. 76 (11.74%) milk samples were found negative for bacterial isolation. The prevalence was calculated based on the percentage of samples positive for specific bacteria or yeast as an etiological agent. The prevalence was observed during the study as *Staphylococcus* spp., (45.71%); Gram-negative *Bacilli*, (26.27%); *Bacillus* spp., (3.43%); *Streptococcus* spp., (3.88%); *Micrococcus* spp., (1.34%); *Corynebacterium* spp., (2.84%); *Pseudomonas* spp., (4.62%) and Yeast, (0.6%). Antibiotics sensitivity test revealed levofloxacin as highly effective against *Staphylococcus* spp. (71.57%), Gram-negative *bacilli* (67.61%) as well as *Bacilli* spp. (86.95%), While gentamicin and levofloxacin were highly sensitive against *Streptococcus* spp. (84.62%). Higher susceptibility of *Pseudomonas* spp. (90.32%) was observed against cefpodoxime, While *Corynebacterium* spp. (73.68%) was sensitive to gentamicin. *Micrococcus* spp. revealed equal susceptibility (77.77%) against chloramphenicol and gentamicin. Cefoperazone was highly resistant to all isolated bacteria.

Keywords: Mastitis, BHI, cultural isolation & antimicrobial susceptibility

Introduction
According to the Basic Animal Husbandry Statistics (BAHS) 2024-25, India ranks as the world's top milk producer, generating a total of 247.87 million tonnes, with Gujarat holding the fourth position and contributing 7.78% to the national output (DAHD, 2025) [1]. Despite this dominance, milk quality—especially its shelf life—often fails to meet global standards due to contamination, excessive antimicrobial use, and inconsistent hygiene protocols. The root cause of these challenges is predominantly mastitis. Mastitis represents an inflammatory condition of the mammary gland primarily triggered by microbial invasion, which can readily spread to healthy animals (Contreras *et al.*, 2007; Amri *et al.*, 2020) [2, 3]. While conventional mastitis control strategies have effectively lowered the occurrence of contagious pathogens, they have shown limited success against environmental ones (Hogan and Smith, 2003) [4]. The disease may also arise from physical trauma to the udder, allowing microbes to enter through damaged teat canals (Grohn *et al.*, 2004; Contreras *et al.*, 2007) [5, 2]. Common causative agents include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Klebsiella* spp., *Escherichia coli*, and *Enterobacter* spp. (Zadoks *et al.*, 2011; Artdita *et al.*, 2018; Suarnata *et al.*, 2018) [6, 7, 8]. These pathogens typically infect the udder locally or systemically, particularly during early lactation, and poor hygiene in housing or milking equipment further facilitates their transmission (Azis *et al.*, 2013; Zhao *et al.*, 2015) [9, 10].
Bacterial infections leading to mastitis are influenced by poor hygiene in housing, equipment, handling, and the surrounding environment (Windaria *et al.*, 2018; Nisa *et al.*, 2019) [11, 12].

(Windaria *et al.*, 2018; Nisa *et al.*, 2019) [11, 12]. Concave flooring in barns, as noted by Azis *et al.* (2013) [9], promotes water and dirt accumulation, increasing the risk of contamination. Mastitis often arises from inadequate farm management, resulting in reduced milk yield and quality, higher treatment expenses, and diminished animal value (Utami, 2012; Riyanto *et al.*, 2016) [13, 14]. Beyond quality impairment, it lowers overall productivity and causes substantial farm losses (Nisa *et al.*, 2019) [12]. Intramammary infections significantly impair milk quantity and quality more than factors like dry-off duration or lactation stage (Luengo *et al.*, 2004) [15].

Mastitis pathogens in ruminants include Gram-positive species such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* (Zadoks *et al.*, 2011) [6], alongside Gram-negative coliforms like *Escherichia coli* and *Klebsiella pneumoniae* (Artdita *et al.*, 2018) [7], plus *Klebsiella variicola*, *Klebsiella oxytoca*, and *Enterobacter aerogenes* (Hall and Rycroft, 2007; Zadoks *et al.*, 2011) [16, 6]. The disease generates considerable economic burdens through decreased production (Riyanto *et al.*, 2016; Nisa *et al.*, 2019) [14, 12], alongside expenses for therapy, reduced livestock marketability, medication, veterinary services, and occasional fatalities (Windaria *et al.*, 2018; Nisa *et al.*, 2019) [11, 12]. Prompt intervention is essential upon early symptom detection (Amri *et al.*, 2020) [3]. Antibiotic treatment incurs high costs, exacerbated by rising resistance (Maida and Lestari, 2019) [17].

Mastitis status in ruminants is commonly assessed via the California Mastitis Test (CMT), supplemented by Gram staining, catalase, mannitol, coagulase, and Voges-Proskauer tests (Azis *et al.*, 2013; Bulele *et al.*, 2019) [9, 18]. Gram-negative cases may require citrate, maltose, and lactose assays, while Gram-positive identification in subclinical milk benefits from standard biochemical methods (Zadoks *et al.*, 2011) [6]. Antibiotic resistance in mastitis pathogens stems from misuse and resistance gene proliferation. Given these challenges, investigating antimicrobial susceptibility testing (ABST) patterns in mastitis-affected cattle is crucial.

Methods and Materials

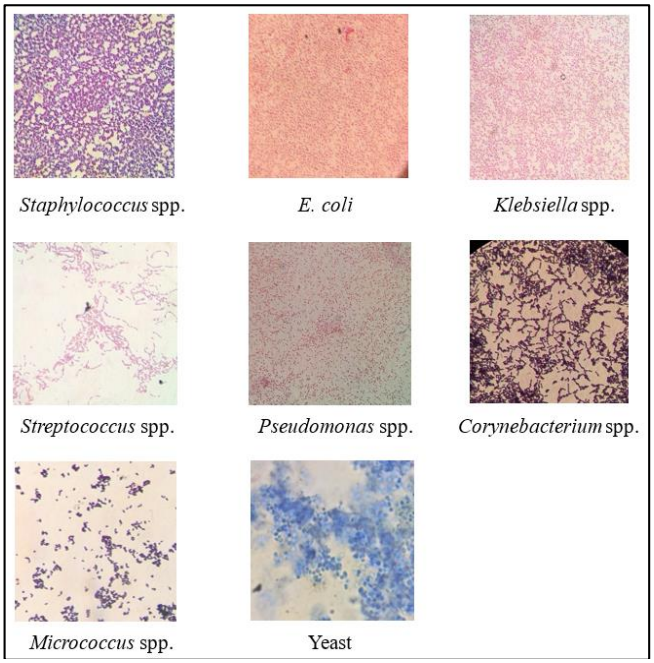
The present study was conducted in the Microbiology Clinical Research Laboratory, Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, spanning the period from 2023 to 2024. Milk samples were sourced from the Veterinary Clinical Complex (VCC), Junagadh, encompassing dairy farms within and surrounding Junagadh district. Subclinical mastitis was diagnosed using the California Mastitis Test (CMT). Bacterial isolation was performed by culturing the samples on Brain Heart Infusion (BHI) Agar, followed by identification through Gram staining. Milk samples were aseptically collected directly from cows exhibiting clinical signs indicative of mastitis. Each sample was placed in a sterile vial, securely capped, clearly labeled, and immediately transferred to a cooled transport box containing ice packs to maintain a temperature range of 5-10 °C. This ensured preservation of bacterial viability and prevented overgrowth during transit to the laboratory.

Bacterial Isolation

To isolate bacteria, a loopful of milk sample from cows confirmed positive for mastitis was streaked onto Brain Heart Infusion (BHI) agar plates. The inoculated plates were then incubated aerobically at 37 °C for 24 hours. After incubation, colonies displaying varied morphological features were observed and selected for further identification.

Gram Staining

Gram staining was performed to distinguish Gram-positive and Gram-negative bacteria. Smears were prepared on clean glass slides and heat-fixed by passing over a flame. Crystal violet was applied and allowed to act for 1 minute, followed by rinsing with running water. Slides were then treated with Lugol's iodine for 30 seconds and rinsed again. Decolorization was achieved with 96% ethanol until the runoff was colorless, immediately followed by a water rinse. Counterstaining with safranin was performed for 30-60 seconds, after which slides were rinsed, air-dried, and examined under a light microscope at 100× objective magnification with immersion oil. (Microscopic examination was found as follows.)



Antimicrobial Susceptibility Testing

In vitro antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller-Hinton Agar (MHA), following the protocol described by Bauer *et al.* (1966). Commercially available antibiotic disks (HiMedia Laboratories) containing the following eight agents and concentrations were employed: ampicillin/sulbactam (A/S-30/15 µg/disc), cefoperazone/sulbactam (CPZ-75/10 µg/disc), ceftizoxime (CZX-30 µg/disc), chloramphenicol (C-30 µg/disc), gentamicin (GEN-30 µg/disc), levofloxacin (LE-5 µg/disc), oxytetracycline (O-30 µg/disc), and cefpodoxime (CPD-10 µg/disc). The susceptibility profiles of the bacterial isolates were classified as susceptible, intermediate, or resistant according to the interpretive standards outlined by the Clinical and Laboratory Standards Institute (CLSI, 2018).

Data Analysis

This study employed descriptive statistical analysis. The data examined included the types of bacteria identified as causative agents of mastitis, along with their *in vitro* susceptibility patterns to the selected antimicrobial agents.

Results

Distribution of bacterial Isolates

In a comprehensive bacteriological analysis of 670 milk samples collected from cattle exhibiting signs of mastitis, *Staphylococcus* spp. emerged as the predominant isolate, accounting for 306 cases (45.71%), underscoring its role as a primary etiological agent in bovine intramammary infections, consistent with global reports where staphylococci frequently dominate mastitis pathology. Following closely were unspecified Gram-negative bacilli at 176 isolates (26.27%), likely encompassing environmental coliforms such as *Escherichia coli* or *Klebsiella* spp., while *Pseudomonas* spp. contributed 31 isolates (4.62%), *Streptococcus* spp. 26 (3.88%), *Bacillus* spp. 23 (3.43%), *Corynebacterium* spp. 19 (2.84%) and *Micrococcus* spp. 9 (1.34%). Minor findings included yeast in 4 samples (0.6%), with no bacterial growth observed in 76 samples (11.34%), a rate aligning with documented culture-negative outcomes in clinical mastitis that may reflect prior immune clearance, inhibited organisms or non-bacterial causes.

Table 1: Prevalence of bacteria isolated from mastitic milk samples

No.	Bacterial isolate	Cattle
1.	*Staphylococcus* spp.	306 (45.71%)
2.	G-ve Bacilli	176 (26.27%)
3.	*Pseudomonas*	31 (4.62%)
4.	*Streptococcus* spp.	26 (3.88%)
5.	*Bacillus* spp.	23 (3.43%)
6.	*Corynebacterium*	19 (2.84%)
7.	*Micrococcus*	9 (1.34%)
8.	Yeast	4 (0.6%)
9.	No growth	76 (11.34%)
	Total	670

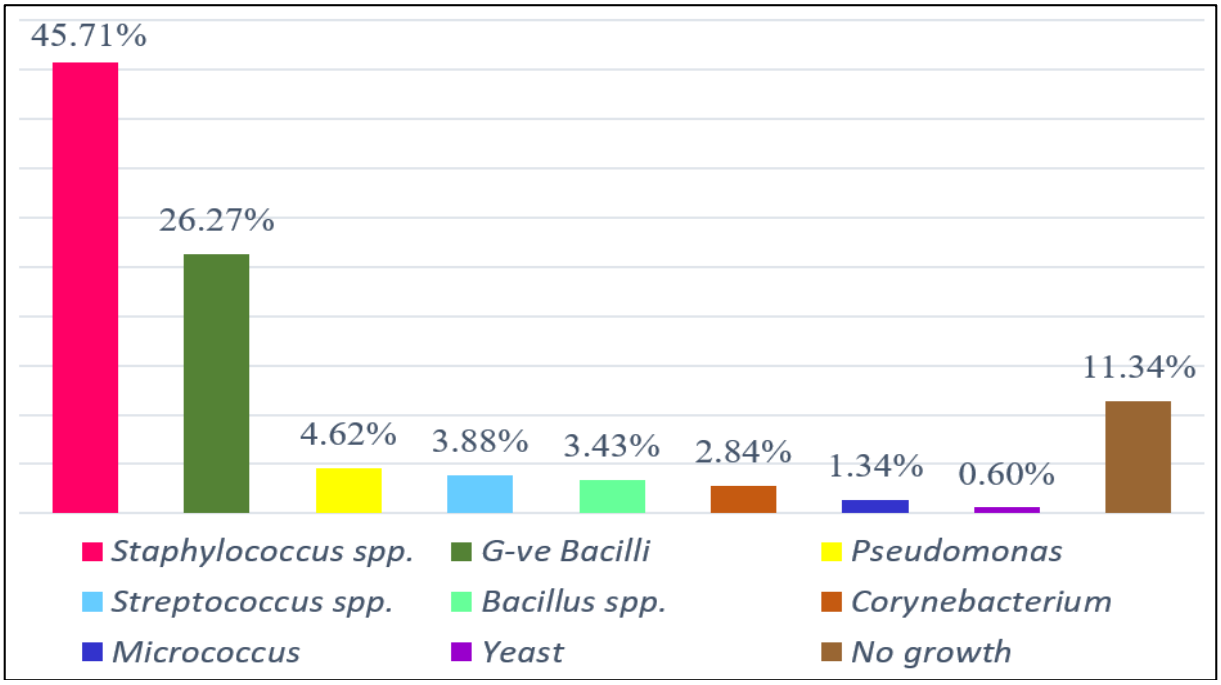


Chart 1: Distribution of bacterial isolates from milk samples of cows with mastitis

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility profiles of all bacterial isolates were assessed *in vitro* against selected antibiotics (as mentioned above) throughout the study duration. The susceptibility results for individual isolates are reported separately, with complete details summarized in Table 2.

Table 2: Antibacterial susceptibility of bacterial spp. isolated from mastitic milk

Bacterial isolate (n)	A/S	CPZ	CZX	C	GEN	LE	O	CPD
Staphylococcus spp. (306)	171 (55.88%)	62 (20.26%)	105 (34.31%)	172 (56.21%)	196 (64.05%)	219 (71.57%)	178 (58.17%)	183 (59.80%)
Gram-negative bacilli (176)	41 (23.30%)	17 (9.66%)	44 (25.00%)	78 (44.32%)	103 (58.22%)	119 (67.61%)	88 (50.00%)	92 (52.27%)
Pseudomonas spp. (31)	3 (9.68%)	4 (12.90%)	0 (0.00%)	6 (19.35%)	27 (87.09%)	26 (83.87%)	5 (16.12%)	28 (90.32%)
Streptococcus spp. (26)	15 (57.70%)	5 (19.23%)	7 (26.92%)	12 (46.15%)	22 (84.62%)	22 (84.62%)	12 (46.15%)	21 (80.77%)
Bacillus spp. (23)	14 (60.87%)	3 (13.04%)	5 (21.74%)	14 (60.87%)	16 (69.56%)	20 (86.95%)	13 (56.52%)	18 (78.26%)
Corynebacterium spp. (19)	6 (31.58%)	4 (21.05%)	5 (26.32%)	10 (52.63%)	14 (73.68%)	12 (63.16%)	10 (52.63%)	5 (26.32%)
Micrococcus spp. (9)	2 (22.22%)	0 (0.00%)	2 (22.22%)	7 (77.77%)	7 (77.77%)	6 (66.66%)	4 (44.44%)	4 (44.44%)

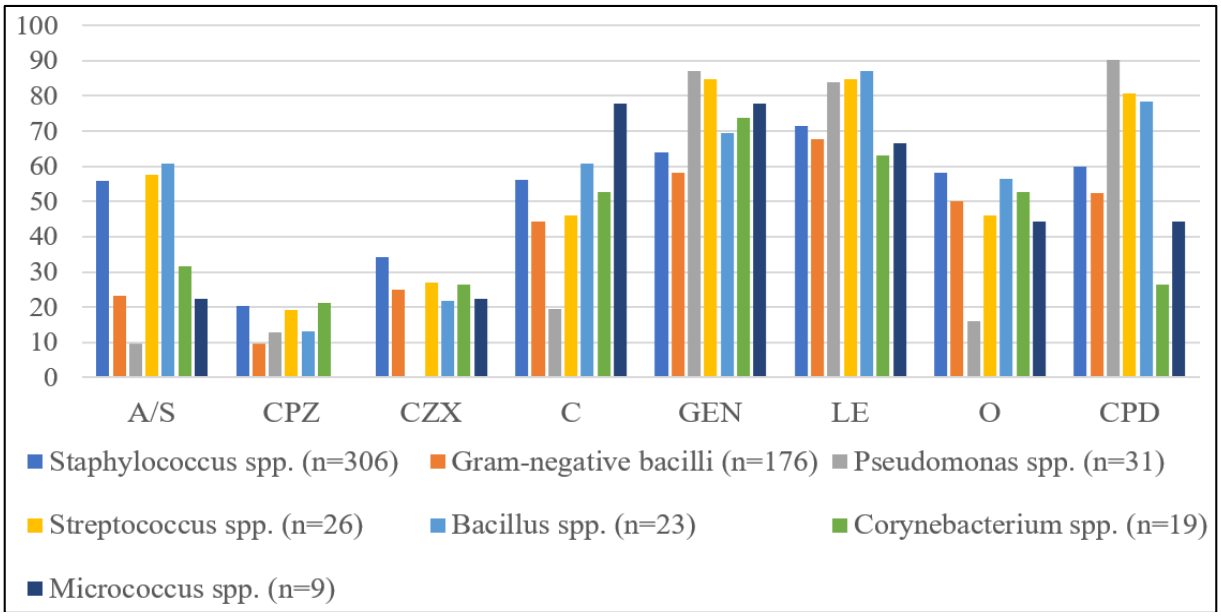


Chart 2: Antibiogram of bacterial isolates

Levofloxacin demonstrated significantly higher antibacterial activity against the majority of bacterial isolates, particularly *Staphylococcus* spp., Gram-negative bacilli, and *Bacillus* spp. Gentamicin also exhibited superior efficacy against *Pseudomonas* spp., *Streptococcus* spp., *Corynebacterium* spp., and *Micrococcus* spp. In contrast, β-lactam antibiotics, like cefoperazone and ceftizoxime, showed comparatively lower susceptibility rates across most of the bacterial groups evaluated.

Discussion

The dominance of staphylococci in bovine mastitis has been consistently reported in the literature. Asmaul *et al.* (2018) [19] and Hashemi *et al.* (2011) [20] reported prevalences of 55.55% and 32.10%, respectively. Similarly, Atyabi *et al.* (2006) [21] observed a prevalence of staphylococci of 30.27%, followed by Gram-negative bacilli (10.30%); however, these values were lower than those in the present study. In another investigation, Nabi *et al.* (2024) [22] documented a markedly higher prevalence of *Staphylococcus aureus* (73%), followed by *Escherichia coli* (24%). Likewise, Hosseinzadeh and Saei (2014) [23] and Rajkumar *et al.* (2024) [24] identified *Staphylococcus* spp. as the predominant pathogens in bovine mastitis, with prevalences of 71.5% and 66.21%, respectively, in culture-positive milk samples—both substantially higher than the rate observed here. Several earlier studies have also recognised *Staphylococcus* species as the primary bacterial agents of bovine mastitis (Harini and Sumathi, 2011; Birhanu *et al.*, 2017) [25, 26]. Furthermore, Kakati *et al.* (2024) [27] reported a higher prevalence of *Staphylococcus aureus* (66.5%), followed by *Escherichia coli* (10%) and *Streptococcus* spp. (6%), revealing a distribution pattern distinct from the findings of the present study.

In the present study, *Streptococcus* spp. isolates exhibited the highest resistance to cefoperazone and greatest susceptibility to levofloxacin, consistent with previous reports identifying levofloxacin as highly effective and ceftriaxone as less so (Gao *et al.*, 2012; Miranda *et al.*, 2018; Javia *et al.*, 2020; Parsana *et al.*, 2021) [28, 29, 30, 31]. For Gram-negative bacilli, 67.61% of isolates demonstrated sensitivity to levofloxacin, contrasting with higher reported sensitivity to chloramphenicol in other investigations (Sheela *et al.*, 2025) [32]. Among *Pseudomonas* spp., gentamicin displayed the strongest activity in this study, although lower efficacy has been noted elsewhere (Huang *et al.*, 2024) [33]. These discrepancies underscore regional and temporal variations in antimicrobial resistance patterns, emphasising the importance of continuous monitoring to inform targeted therapeutic approaches.

Conclusions

In conclusion, this study highlights *Staphylococcus* spp. as the predominant etiological agent of bovine mastitis in the southwestern region of Gujarat, accounting for 45.71%, followed by Gram-negative bacilli at 26.27%. Antimicrobial susceptibility testing revealed levofloxacin as highly effective against *Staphylococcus* spp. (71.57%), Gram-negative bacilli (67.61%), and *Bacillus* spp. (86.95%), while gentamicin showed superior activity against *Streptococcus* spp. (84.62%), *Corynebacterium* spp. (73.68%), and *Micrococcus* spp. (77.77%). These findings underscore the emergence of antimicrobial resistance, particularly to cefoperazone, emphasising the need for targeted antibiotic therapies based on local susceptibility patterns to mitigate economic losses in dairy farming. Future research should focus on molecular mechanisms of resistance and alternative control strategies to enhance mastitis management and milk quality.

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