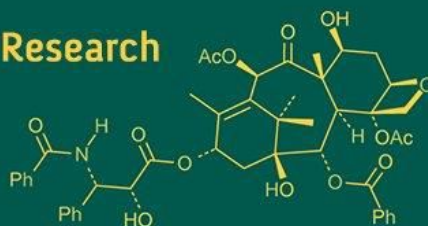


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High prevalence of culture-negative intramammary infections and diagnostic limitations of conventional microbiological culture in bovine mastitis of Bareilly region, India

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Abstract

This study evaluated the prevalence, bacteriological profile, and diagnostic limitations associated with conventional culture-based detection of mastitogens in bovine mastitis from Bareilly region of Uttar Pradesh, India. A total of 486 lactating cows comprising 1872 functional quarters were screened using clinical examination, California Mastitis Test (CMT), Somatic Cell Count (SCC), and Total Bacterial Count (TBC). Milk samples from CMT-positive quarters were subjected to microbiological culture, Gram staining, selective media isolation, and biochemical characterization. The prevalence of mastitis was 50.21% at cow level and 20.67% at quarter level. Staphylococci predominated (37.72%), followed by Streptococci (21.19%) and Coliform bacilli (12.66%). Notably, 19.37% of samples showed no growth and 5.68% were contaminated. Approximately 25.06% of samples failed to yield definitive microbiological results. The findings highlight significant diagnostic gaps in routine bacteriological culture and underscore the necessity of integrating molecular diagnostics for accurate detection of intramammary infections.

Keywords: Bovine mastitis, mastitogens, culture-negative mastitis, somatic cell count, intramammary infection

Introduction

Bovine mastitis remains one of the most economically consequential and pervasive diseases affecting dairy herds across the globe. As an inflammatory condition of the mammary gland, it not only compromises the physiological integrity of the udder but also disrupts the productivity and profitability of dairy enterprises at multiple levels. The disease manifests through localized inflammation of mammary tissue, often accompanied by systemic responses in severe cases, and leads to a marked decline in milk yield. In addition to quantitative losses, mastitis significantly alters milk composition, including changes in fat, protein, lactose, and mineral content, thereby diminishing its nutritional value and processing suitability. The economic burden extends beyond reduced production to encompass increased veterinary expenditures, labor costs, discarded milk during withdrawal periods, and premature culling of chronically affected animals. On a global scale, mastitis is responsible for substantial annual financial losses due to decreased milk output, compromised milk quality, therapeutic interventions, and herd replacement costs, as extensively documented in epidemiological and economic assessments ^[1, 2]. Consequently, mastitis is not merely a health disorder but a complex production disease with profound implications for animal welfare, farm sustainability, and the dairy supply chain.

Clinically, bovine mastitis presents in two principal forms: clinical and subclinical. Clinical mastitis is characterized by overt signs such as swelling, heat, pain, redness of the udder, and visible abnormalities in milk, including clots, flakes, or watery secretions. In contrast, subclinical mastitis, though lacking obvious external symptoms, is considerably more prevalent and economically detrimental. Its insidious nature allows it to persist undetected within herds, silently impairing milk production and quality over extended periods. Because affected animals appear outwardly healthy, subclinical cases often escape early detection and

timely intervention, facilitating ongoing transmission within the herd. Diagnosis of subclinical mastitis relies predominantly on indirect indicators of inflammation, including the California Mastitis Test (CMT) and somatic cell count (SCC) estimation. Elevated SCC reflects the influx of leukocytes into milk as part of the host immune response, serving as a widely accepted marker of intramammary infection and udder health status.

The etiological landscape of bovine mastitis is remarkably diverse. To date, more than 150 microbial species have been implicated in intramammary infections, underscoring the multifactorial and complex nature of the disease. These pathogens are broadly categorized into contagious and environmental groups based on their primary reservoirs and modes of transmission. Contagious pathogens, such as *Staphylococcus aureus* and *Streptococcus agalactiae*, are primarily transmitted during milking through contaminated equipment, hands of milkers, or direct cow-to-cow contact. These organisms are well adapted to the mammary environment and often establish persistent infections that are difficult to eradicate. In contrast, environmental pathogens, including *Escherichia coli* and environmental streptococci, originate from bedding, manure, soil, and water, infecting the udder opportunistically between milking sessions [3, 4].

Recent epidemiological investigations have highlighted a dynamic shift in the microbial profile of mastitis pathogens. Notably, coagulase-negative staphylococci (CNS), once considered minor or opportunistic pathogens, are increasingly recognized as significant contributors to both clinical and subclinical mastitis cases [5]. Their rising prevalence may reflect changes in herd management practices, antimicrobial usage patterns, and improved detection methodologies. Furthermore, several bacterial species implicated in mastitis possess virulence mechanisms that complicate disease control. Biofilm formation enhances bacterial survival within the mammary gland by protecting pathogens from host immune defenses and antimicrobial agents. Intracellular persistence within mammary epithelial cells enables certain organisms, particularly *Staphylococcus aureus*, to evade immune surveillance and establish chronic infections. Compounding these challenges is the growing concern of antimicrobial resistance, which limits therapeutic efficacy and necessitates judicious antibiotic stewardship [6]. Together, these factors underscore the evolving and multifaceted nature of mastitis pathogenesis.

Despite advancements in molecular diagnostics, conventional bacteriological culture continues to be regarded as the gold standard for mastitis diagnosis. Culture-based methods allow for pathogen identification and antimicrobial susceptibility testing, thereby guiding targeted therapy. However, a notable limitation of this approach is the occurrence of culture-negative mastitis. Multiple studies report that approximately 20-40% of milk samples obtained from mastitic quarters fail to yield bacterial growth under standard laboratory conditions [7, 8]. This phenomenon presents a significant diagnostic dilemma. Several explanations have been proposed, including low bacterial load below the detection threshold, intermittent shedding of pathogens, prior or ongoing antibiotic therapy suppressing bacterial growth, intracellular localization of organisms, and the presence of fastidious or slow-growing microbes requiring specialized media or extended incubation periods. Additionally, infections caused by viruses, fungi, or mycoplasma species may not be detected using routine

bacteriological techniques.

Culture-negative mastitis poses substantial challenges for veterinarians and dairy producers alike. The inability to identify a causative agent complicates therapeutic decision-making, often leading to empirical treatment strategies that may be ineffective or contribute to antimicrobial resistance. From a herd health perspective, undiagnosed or misdiagnosed infections hinder the implementation of appropriate control measures, thereby perpetuating economic losses. In the Indian context, particularly under diverse field conditions characterized by variations in management systems, climatic factors, and resource availability, data regarding the prevalence and epidemiology of culture-negative mastitis remain limited. This knowledge gap is especially evident in Northern regions of India, where systematic investigations into the proportion and underlying causes of culture-negative cases are scarce. Addressing this deficiency is essential for developing region-specific diagnostic protocols, optimizing treatment strategies, and strengthening mastitis control programs tailored to local dairy production systems.

Materials and Methods

A cross-sectional epidemiological investigation was undertaken in Bareilly district of Uttar Pradesh, India, to determine the prevalence and bacteriological profile of bovine mastitis under field conditions. Cross-sectional designs are widely employed in mastitis research to estimate herd-level and quarter-level prevalence at a defined point in time [9]. The study population comprised 486 lactating cows maintained under smallholder and organized dairy production systems. From these animals, 1,872 functional mammary quarters were examined, excluding blind or non-functional quarters to ensure uniform sampling. Animal-level data, including parity, stage of lactation, breed, and management conditions, were recorded to facilitate epidemiological interpretation [10].

Clinical Examination and Screening Procedures

Each cow underwent a systematic clinical examination of the udder prior to laboratory-based testing. Visual inspection and palpation were performed to detect classical signs of clinical mastitis, including swelling, increased local temperature, pain on palpation, fibrosis, or abnormalities in milk such as clots, flakes, or serous secretion [11]. Quarters exhibiting overt inflammatory signs were categorized as clinical mastitis cases.

Apparently normal quarters were screened for subclinical mastitis using the California Mastitis Test (CMT), a rapid cow-side assay that indirectly estimates somatic cell concentration through gel formation resulting from the reaction between detergent reagent and DNA of somatic cells [12]. Equal volumes of milk and CMT reagent were mixed in a four-compartment paddle and scored according to standard criteria. CMT remains a practical and widely accepted field diagnostic tool for subclinical mastitis detection [1].

Somatic Cell Count (SCC) was determined to quantitatively assess mammary inflammation. SCC is internationally recognized as a reliable indicator of intramammary infection, reflecting leukocyte influx into milk in response to pathogenic invasion [13, 14]. Quarters with SCC values exceeding established thresholds ($>2 \times 10^5$ cells/mL) were considered indicative of infection. Total Bacterial Count

(TBC) was also estimated to evaluate microbial load and hygienic quality of milk, following standard microbiological procedures [15].

Milk Sample Collection

Milk samples were collected aseptically from CMT-positive quarters following recommended guidelines of the National Mastitis Council [16]. Teat ends were cleaned and disinfected using 70% ethyl alcohol, and the first few streams of milk were discarded to minimize contamination from teat canal flora. Approximately 10-15 mL of milk was collected into sterile, labeled vials, ensuring no contact between the teat end and the container rim. Samples were transported to the laboratory in an insulated icebox at 4 °C and processed within 24 hours to preserve bacterial viability and prevent overgrowth of contaminants [17].

Bacteriological Examination

Bacteriological culture was performed following standard mastitis diagnostic protocols [16]. Milk samples were streaked onto 5% sheep blood agar plates and incubated aerobically at 37 °C for 24-48 hours. Blood agar supports the growth of a broad spectrum of mastitis pathogens and enables assessment of hemolytic patterns, which aid in preliminary identification [18]. Plates were examined at 24 hours and re-evaluated at 48 hours before classifying samples as culture-negative.

Preliminary identification of isolates was based on colony morphology, pigmentation, hemolysis, and Gram staining characteristics. Gram-stained smears were examined microscopically to determine Gram reaction, cellular morphology, and arrangement [18].

Selective and differential media were employed for further characterization:

- **Mannitol Salt Agar (MSA):** Used for isolation and differentiation of *Staphylococcus* species based on mannitol fermentation and salt tolerance [19].
- **Edward's medium:** Utilized for selective isolation of *Streptococcus* species, enabling differentiation through esculin hydrolysis and colony characteristics [20].
- **MacConkey agar and Eosin Methylene Blue (EMB) agar:** Applied for isolation and differentiation of coliform organisms based on lactose fermentation and characteristic colony morphology [21, 22].

The coagulase test was performed to differentiate coagulase-positive staphylococci (CPS) from coagulase-negative staphylococci (CNS), as coagulase production is a critical virulence determinant associated with pathogenicity [18]. Tube coagulase testing using rabbit plasma was conducted, and clot formation within 4-24 hours was interpreted as a positive reaction. Additional biochemical tests were carried out where necessary to confirm species-level identification.

Statistical Analysis

All observations were systematically recorded and entered into a spreadsheet prior to statistical analysis. Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 17. Descriptive statistics were calculated to determine prevalence rates at cow and quarter levels. Student's *t*-test was applied to compare mean SCC and TBC values between infected and non-infected groups. A *p*-value of less than 0.05 was considered statistically significant [23]. Statistical procedures were selected to ensure robust

inference and reliability of the findings.

Results

The present investigation revealed a substantial burden of bovine mastitis in dairy herds of Bareilly district, Uttar Pradesh. The overall prevalence was recorded at 50.21% (244/486) on a cow basis and 20.67% (387/1872) on a quarter basis, indicating that nearly half of the examined lactating cows harbored intramammary infection in at least one functional quarter. These findings underscore the endemic nature of mastitis in the region and reflect the continuing challenges associated with udder health management under field conditions. Comparable prevalence rates ranging between 40-60% have been documented across various Indian states, suggesting that mastitis remains one of the most prevalent production diseases in the country [2, 2].

Clinical and Laboratory Indicators

The mean California Mastitis Test (CMT) score, Somatic Cell Count (SCC), and Total Bacterial Count (TBC) differed markedly between healthy and mastitic quarters. In healthy quarters, the mean CMT score, SCC, and TBC were 0.18 ± 0.03 , $2.86 \pm 0.23 \times 10^5$ cells/mL, and 0.45 ± 0.18 cfu/mL, respectively. In contrast, mastitic quarters exhibited significantly elevated values of 2.87 ± 0.28 , $19.84 \pm 1.55 \times 10^5$ cells/mL, and 6.21 ± 0.78 cfu/mL, respectively. The increases corresponded to 1494.44%, 593.71%, and 1280.0% elevations compared to healthy quarters, and these differences were statistically significant ($p < 0.05$).

The nearly six-fold rise in SCC in mastitic quarters is consistent with internationally recognized diagnostic thresholds for intramammary infection [1, 13]. Elevated SCC reflects the influx of polymorphonuclear leukocytes into milk as part of the host's innate immune response to microbial invasion [13]. Similarly, the marked increase in TBC confirms the involvement of viable bacterial populations in inflamed quarters and highlights compromised hygienic quality of milk.

Bacteriological Findings

Out of 387 CMT-positive milk samples collected from mastitic quarters, 146 samples (37.72%) yielded *Staphylococcus* spp. isolates based on colony morphology, pigmentation, odor, and characteristic growth on Mannitol Salt Agar. Among these, 36 isolates (9.30%) were identified as coagulase-positive staphylococci (CPS), while 110 isolates (28.42%) were coagulase-negative staphylococci (CNS), as determined by the coagulase test. The predominance of CNS over CPS is noteworthy and suggests a shift in pathogen dynamics within the region.

Eighty-two isolates (21.19%) were identified as *Streptococcus* spp. based on growth characteristics on Edward's medium, including esculin hydrolysis and colony morphology. Additionally, 49 isolates (12.66%) were identified as Gram-negative coliform bacilli based on characteristic lactose fermentation patterns, including purple-green colonies with metallic sheen on Eosin Methylene Blue agar and pink colonies on MacConkey agar. Gram-positive organisms collectively predominated over Gram-negative bacteria, accounting for the majority of isolates recovered. This distribution pattern aligns with global epidemiological observations indicating that Gram-positive pathogens remain the principal etiological agents of

bovine mastitis [3].

Culture-Negative and Contaminated Samples

Among the 387 CMT-positive samples, 75 samples (19.37%) failed to yield bacterial growth on 5% sheep blood agar even after 48 hours of incubation. Furthermore, 22 samples (5.68%) were considered contaminated due to the presence of mixed bacterial growth without isolation of a predominant mastitis pathogen. When combined, approximately 25.06% of samples did not provide definitive microbiological identification.

The occurrence of culture-negative mastitis in nearly one-fifth of positive samples corroborates previous reports indicating that 20-40% of mastitic milk samples may remain culture-negative under conventional laboratory conditions [7, 8]. These findings emphasize inherent diagnostic limitations of routine bacteriological culture.

Discussion

The present study demonstrates a high prevalence of bovine mastitis in Bareilly district, reflecting significant challenges in udder health management under prevailing dairy production systems. The cow-level prevalence of 50.21% is consistent with earlier reports from India, where mastitis prevalence frequently exceeds 40% in both organized and unorganized sectors [2]. Such high prevalence may be attributed to suboptimal milking hygiene, inadequate teat sanitation, improper housing conditions, and limited routine screening for subclinical infections.

The marked elevation in SCC and TBC in mastitic quarters substantiates the inflammatory and microbial basis of the disease. SCC remains a globally accepted biomarker for intramammary infection, with thresholds above 2×10^5 cells/mL widely recognized as indicative of mastitis [1]. The observed six-fold increase in SCC aligns with the pathophysiological mechanism of leukocyte recruitment into the mammary gland in response to bacterial invasion [13]. Elevated TBC further confirms active bacterial proliferation and potential milk quality deterioration.

Shift Toward Coagulase-Negative Staphylococci

The predominance of *Staphylococcus* spp., particularly CNS, represents a significant epidemiological observation. Traditionally, CPS such as *Staphylococcus aureus* have been regarded as major contagious mastitis pathogens. However, increasing evidence suggests that CNS are emerging as important contributors to subclinical mastitis [5, 4]. The higher proportion of CNS (28.42%) compared to CPS (9.30%) in this study may indicate improved control of classical contagious pathogens alongside rising importance of opportunistic environmental or commensal organisms. Contrasting findings have been reported in buffalo populations from Haryana, where CPS predominated over CNS, highlighting regional and species-level differences in pathogen distribution [25]. Such variations may be influenced by management practices, breed susceptibility, and antimicrobial usage patterns.

Streptococci and Coliforms: Environmental Influence

The isolation of *Streptococcus* spp. (21.19%) and coliform bacilli (12.66%) reflects the contribution of environmental pathogens to mastitis in the region. Environmental streptococci and coliforms are often associated with poor bedding hygiene, fecal contamination, and inadequate housing sanitation [3]. Their presence underscores the importance of environmental management strategies, including clean bedding, dry housing conditions, and proper milking procedures.

The predominance of Gram-positive bacteria over Gram-negative organisms in this study aligns with global epidemiological trends [3]. Gram-positive pathogens, particularly staphylococci and streptococci, possess adaptive mechanisms such as biofilm formation and adhesion factors that enhance persistence within the mammary gland.

Culture-Negative Mastitis and Diagnostic Limitations

A notable finding was that 19.37% of CMT-positive samples were culture-negative. When combined with contaminated samples (5.68%), approximately one-quarter of samples failed to yield definitive microbiological results. Similar findings have been documented elsewhere, with culture-negative mastitis reported in 20-40% of cases [7, 8].

Several mechanisms may explain this phenomenon. Low bacterial load below detection thresholds, intermittent shedding of pathogens, and prior antimicrobial therapy can reduce recoverable viable bacteria [7]. Additionally, intracellular persistence of *Staphylococcus aureus* within mammary epithelial cells enables evasion of immune responses and hampers culture-based detection [6]. The ability of *S. aureus* to survive within phagocytes by interfering with phagolysosomal fusion further complicates microbiological recovery.

Fastidious organisms such as *Mycoplasma* spp. and *Prototheca* spp., which require specialized culture conditions, may remain undetected under routine protocols [8]. Furthermore, biofilm formation reduces bacterial shedding and viability in culture media.

Implications for Diagnosis and Control

The substantial proportion of inconclusive culture results highlights limitations of conventional microbiological methods. Advanced molecular diagnostic tools, including polymerase chain reaction (PCR), quantitative PCR, and next-generation sequencing, have demonstrated superior sensitivity and rapid pathogen detection compared to culture-based techniques [8, 4]. Integration of molecular diagnostics with routine culture could enhance detection accuracy, facilitate targeted antimicrobial therapy, and reduce indiscriminate antibiotic use.

Overall, the findings emphasize the evolving epidemiology of bovine mastitis in Bareilly region, characterized by high prevalence, predominance of Gram-positive organisms, emerging importance of CNS, and significant occurrence of culture-negative cases. Comprehensive mastitis control programs integrating improved hygiene, routine screening, and advanced diagnostics are essential to mitigate economic losses and enhance dairy productivity.

Table 1: Comparison of Milk Parameters in Healthy and Mastitic Quarters

Parameter	Healthy	Mastitis
CMT Score	0.18±0.03	3.17±0.28*
SCC (×10 ⁵ cells/ml)	2.86±0.23	19.84±1.55*
TBC (×10 ⁵ cfu/ml)	0.45±0.04	6.21±0.78*

*Significant at $p < 0.05$ **Table 2:** Distribution of Mastitogens in CMT-Positive Milk Samples

Microorganism	Prevalence (%)
Staphylococcus aureus (CPS)	9.30
Coagulase-negative Staphylococci	28.42
Streptococci spp.	21.19
Coliform bacilli	12.66
Others	3.36
Contaminated	5.68
No Growth	19.37

Conclusion

The present study provides compelling evidence of a notably high prevalence of bovine mastitis among dairy herds in the Bareilly region of Uttar Pradesh, underscoring its continuing significance as a major impediment to dairy productivity. The findings indicate a clear predominance of Gram-positive pathogens, particularly staphylococci and streptococci, suggesting that these organisms remain the primary contributors to both clinical and subclinical intramammary infections in this geographical area. The predominance of coagulase-negative staphylococci (CNS) over coagulase-positive staphylococci (CPS) further highlights the evolving epidemiology of bovine mastitis, reflecting a shift from traditionally recognized contagious pathogens to opportunistic and emerging mastitogens.

A salient observation from this investigation is that approximately one-quarter (25.06%) of all CMT-positive milk samples either failed to yield bacterial growth or were contaminated, thereby not producing definitive microbiological results. This significant proportion of culture-negative or inconclusive samples underscores the inherent limitations of conventional bacteriological techniques in accurately identifying mastitis pathogens. Factors such as low bacterial load, intermittent shedding, intracellular persistence of pathogens like *Staphylococcus aureus*, biofilm formation, and the presence of fastidious organisms likely contributed to these inconclusive outcomes. The study thereby emphasizes the need for a realistic understanding of the constraints of standard microbiological methods, acknowledging that more than 25% of samples may not yield a definitive result in field conditions, which aligns with findings reported in previous investigations globally.

Given these diagnostic gaps, the integration of advanced molecular approaches—such as polymerase chain reaction (PCR), quantitative PCR, and next-generation sequencing—alongside conventional culture methods is strongly recommended. Such combined strategies are likely to enhance pathogen detection accuracy, allow for earlier and more precise identification of both classical and emerging mastitis pathogens, and facilitate evidence-based decision-making in antimicrobial therapy. Adoption of these approaches can significantly strengthen mastitis control programs, optimize herd health management, and reduce economic losses associated with reduced milk yield,

discarded milk, and treatment costs. Ultimately, the findings of this study reinforce the need for comprehensive, multifaceted strategies that combine improved hygiene, routine monitoring, and advanced diagnostics to achieve sustainable mastitis control in dairy operations.

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