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PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI* ISOLATED FROM BOVINE MASTITIS CASES IN SOUTHERN PUNJAB, PAKISTAN.

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ABSTRACT

Background: Mastitis in dairy cattle is a common health issue with major economic impacts across the global dairy industry. In Pakistan, limited regional data and the overuse of antibiotics have contributed to rising cases of antimicrobial-resistant pathogens in mastitic infections.
Objective: The objective of this research was to assess how frequently *Staphylococcus aureus* and *Escherichia coli* occur in mastitis-infected dairy cattle in the Muzaffargarh district, with particular emphasis on analyzing their resistance to antibiotics.
Methods: Milk samples (n = 100) were obtained from cows exhibiting clinical symptoms of mastitis across four tehsils in the Muzaffargarh district. The Surf Field Mastitis Test (SFMT) was used to screen these samples. Those testing positive were inoculated on selective culture media, and bacterial strains were identified through standard biochemical procedures. Antibiotic resistance was evaluated using the Kirby-Bauer disk diffusion technique by CLSI standards.
Results: Out of the 100 milk samples, 98% were positive for subclinical mastitis. *S. aureus* was the most frequently isolated pathogen (70.56%), followed by *E. coli* (23.52%). Alarmingly, 97.22% of *S. aureus* isolates were resistant to methicillin, while 83.33% of *E. coli* isolates exhibited resistance to gentamycin. Conversely, sulfonamides, tigecycline, chloramphenicol, and streptomycin showed higher efficacy.
Conclusion: The widespread occurrence of subclinical mastitis, along with the rise of multidrug-resistant *S. aureus* and *E. coli* strains, presents a significant threat to both animal health and public safety. These findings emphasize the importance of implementing targeted antibiotic management, routine microbial monitoring, and enhanced sanitation measures on local dairy farms.

INTRODUCTION

The dairy industry keeps merging into larger farms owing to people's demand for milk and milk derivatives; however, the sustainability of the dairy industry is threatened by various challenges. One such challenge is Bovine Mastitis (Morales-Ubaldo et al., 2023). Mastitis, an inflammatory condition of the mammary gland (Sharun et al., 2021), continues to impose substantial economic challenges on dairy production worldwide, including reduced milk yield and quality, elevated veterinary costs, and increased culling rates (Haq et al., 2024; Salman et al., 2023). Based on epidemiology, mastitis is divided into two groups: infectious mastitis and environmental mastitis. The primary cause is bacterial infections. The pathogenic microorganisms that cause the former include *Streptococcus agalactiae,* Mycoplasma spp., and *S. aureus*. These pathogens are commonly transmitted from infected to healthy cows during the milking process via contaminated hands, cloths, or milking apparatus. Conversely, the pathogens of environmental mastitis come through the environment, harboring the cow (Garcia, 2004). In Pakistan, particularly, the consequences are dire: nationwide studies have reported that approximately 57% of lactating cows suffer from subclinical mastitis, with *S. aureus* detected in about 30% of positive cases, and 50% of those isolates being multidrug- resistant (MDR), including methicillin-resistant strains (MRSA) (Ali et al., 2021). Detailed MRSA surveillance revealed that among subclinical mastitis samples, around 17% carried the mecA gene typical of MRSA, while resistance genes such as blaZ, tetK, and aacA-aphD were widespread, highlighting the alarming burden of antibiotic resistance in dairy herds (Haq et al., 2024). Similarly, a study found MRSA in 35.7% of mastitis bovines in Pakistan, with higher prevalence in older and multiparous cows and significant associations with poor milking hygiene and lack of teat dipping (prevalence of subclinical mastitis ~41%) (Haider et al., 2023)

Regional investigations confirm these trends: in the northwest of Pakistan, clinical mastitis affected approximately 17% of dairy cattle and buffaloes, while subclinical cases approached 57%, dominated by staphylococci (34%), *E. coli* (19.4%), and *Klebsiella* spp. (8%) (Ali et al., 2021). Buffalo-focused research in Lahore and Bhimber reported overall mastitis prevalence at ~49%, with clinical and subclinical cases around 10% and 39%, respectively, herd size being a significant risk factor (Hussain et al., 2020). Emerging work has begun to explore innovative mitigation strategies: for instance, a 2023 study evaluated sodium alginate-based antibiotic gels combined with MgO nanoparticles, finding notable inhibitory effects against *S. agalactiae* and *K. pneumoniae*, both implicated in mastitis, with improved efficacy compared to antibiotics alone (Manan et al., 2023).

Additionally, advanced diagnostics such as proteomics, immunoassays, and infrared thermography are gaining attention for early detection of mastitis, which is particularly critical in high-prevalence contexts to improve intervention outcomes (Said et al., 2022). Metagenomic profiling in Sahiwal cattle also revealed distinct shifts in microbial communities between healthy and mastitis milk, with overrepresentation of Proteobacteria and Firmicutes in infected samples, suggesting potential for microbiota-informed diagnostics (Salman et al., 2023). Despite these advances, localized data from key livestock-dense but underserved districts, like Muzaffargarh, remain scarce. Existing evidence links rising MDR pathogens largely to unregulated antibiotic use, self-medication, and insufficient adherence to withdrawal periods as noted in broader reviews of livestock antibiotic stewardship (Islam et al., 2025). Accordingly, the present study seeks to investigate the occurrence of *S. aureus* and *E. coli*. in bovine mastitis in selected regions, along with ascertaining their antimicrobial

resistance profiles. Understanding these patterns will provide us with valuable insights about targeted therapeutic strategies, advocating the appropriate use of antibiotics so as to accelerate the mastitis management before the onset of clinical mastitis.

Materials and Methods

Study Area and Sample Collection

The research was carried out in the Muzaffargarh District, situated in the southern part of Punjab, Pakistan, where livestock farming is a key economic activity. Four tehsils within the district were selected based on the density of dairy farms and ease of access. From June 2024 to May 2025, 100 milk samples were obtained from lactating cows showing signs indicative of mastitis. The selection of cows was based on clinical indicators such as inflammation of the udder, clotted or watery milk, or reduced milk yield. From each affected animal, approximately 15 to 25 milliliters of milk were collected aseptically from individual quarters using sterile polypropylene tubes. The samples were kept at 4°C during transportation to the Microbiology Laboratory at the University of Veterinary and Animal Sciences (UVAS), Lahore, and were analyzed within six hours of collection.

Mastitis Screening

The collected milk samples were initially tested for subclinical mastitis using SFMT. In this test, equal volumes of milk and a 3% solution of commercial detergent were mixed in a clean paddle. The appearance of visible gel formation was considered a positive result, with stronger gel consistency indicating a higher severity of infection. This simple, field-adaptable test was used to identify the clinical cases of mastitis prior to laboratory processing.

Isolation and Culturing of Pathogens

All samples that tested positive for mastitis were subjected to bacteriological analysis. Loopfuls of milk were streaked onto selective and differential media, including Mannitol Salt Agar (MSA), MacConkey Agar, EMB Agar, and Blood Agar. MSA was used specifically for the isolation of *S. aureus*, while MacConkey and EMB agars were employed to identify *E. coli* based on lactose fermentation and colony morphology. Blood Agar was used to observe haemolytic activity and support the growth of other fastidious pathogens. The culture plates were incubated under aerobic conditions at 37°C for 24 to 48 hours, and bacterial growth was assessed based on colony morphology, including size, shape, color, and hemolytic activity. Pure colonies were selected for further biochemical testing.

Biochemical Identification

Initial identification of bacterial strains was performed using conventional biochemical techniques. To distinguish between *Staphylococcus* and *Streptococcus* species, the catalase test was utilized. In this procedure, a small amount of 3% hydrogen peroxide was added to a bacterial smear on a sterile glass slide. The emergence of effervescence (bubble formation) indicated a catalase-positive reaction, confirming the presence of *Staphylococcus* spp., whereas no bubbling denoted *Streptococcus* spp. An oxidase test was also applied to determine the presence of cytochrome c oxidase, a distinguishing enzyme found in certain Gram-negative bacteria. The reaction was carried out using a specific oxidase reagent, with a dark purple coloration appearing within 30 seconds, indicating a positive result. For confirmation of *E. coli*, a series of IMViC biochemical tests, e.g., Indole, Methyl Red, Voges-Proskauer, and Citrate utilization, were conducted. *E. coli* was identified by its distinctive IMViC pattern: positive results for Indole and Methyl Red, and negative results for Voges-Proskauer and Citrate utilization. The detailed biochemical characteristics of *S. aureus* and *E. coli* are outlined in Table 1.

Table 1. Biochemical profile of *S. aureus* and *E. coli*

Biochemical Test	*S. aureus*	*E. coli*
Gram Staining	Gram-positive cocci	Gram-negative rods
Catalase Test	Positive (bubbling observed)	Negative
Oxidase Test	Negative	Negative
Indole Test	Negative	Positive (red ring formation)
Methyl Red Test	Variable (usually negative)	Positive (red color after adding MR)
Voges-Proskauer Test	Variable (usually positive)	Negative
Citrate Utilization Test	Negative	Negative
Coagulase Test	Positive	Not applicable

Gram Staining

Gram staining was utilized to observe the microscopic features of the bacterial isolates. Fresh colonies were used to prepare smears on grease-free glass slides, which were then heat-fixed and processed using a sequential staining method involving crystal violet, Gram's iodine, alcohol for decolorization, and a final counterstain with safranin. The stained slides were examined under a microscope using oil immersion at 1000x magnification. *S. aureus* was identified as Gram-positive cocci arranged in clusters, whereas *E. coli* appeared as Gram-negative rod-shaped cells.

Antibiotic Susceptibility Testing

The antimicrobial resistance profiles of the identified isolates were assessed using the Kirby-Bauer disk diffusion technique on Mueller-Hinton Agar, following the standards set by the Clinical and Laboratory Standards Institute (CLSI, 2022). For *S. aureus*, the antibiotics tested included methicillin (5 µg), sulfonamides (100 µg), tigecycline (15 µg), and amoxicillin (10 µg). In the case of *E. coli*, gentamycin (10 µg), chloramphenicol (30 µg), streptomycin (10 µg), and tetracycline (30 µg) were used. After incubation at 37°C for 18–24 hours, the diameters of the zones of inhibition were measured in millimeters using a standard ruler. Based on CLSI interpretive criteria, isolates were classified as sensitive, intermediate, or resistant to the respective antibiotics.

DATA ANALYSIS

Data from the study were compiled and analysed using Microsoft Excel. Descriptive statistics such as frequencies and percentages were used to conclude the prevalence of mastitis, distribution of bacterial isolates, and their resistance patterns.

RESULTS

Out of the 100 milk samples collected from clinically suspected mastitic cows, 98% tested positive for subclinical mastitis using the Surf Field Mastitis Test (SFMT). Bacteriological analysis confirmed the presence of *S. aureus* in 70.56% of positive cases, making it the most prevalent pathogen identified. *E. coli* was isolated from 23.52% of the samples. The remaining 5.92% of samples contained other or unidentified organisms based on colonial morphology. The prevalence of *S. aureus and E. coli* is shown in Table 2 and Figure 2.

Table 2. Prevalence of *S. aureus* and *E. coli in* Mastitis

Bacterial Species	Isolates (%)
S. aureus	**70.56**
E. coli	**23.52**
Others/Unidentified	**5.92**

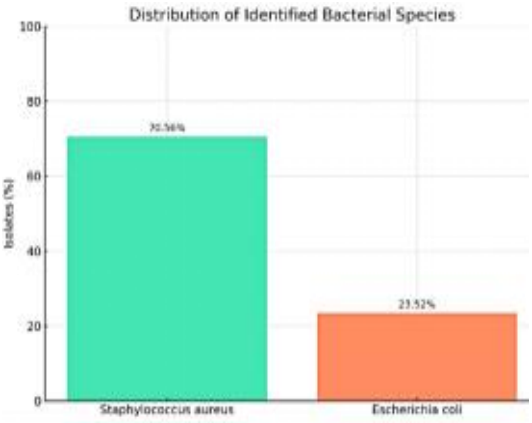

Figure 1. Prevalence of *S. aureus* and *E. coli* in Mastitis

Antibiotic Resistance in *S. aureus*

Resistance testing of *S. aureus* isolates revealed that 97.22% were resistant to methicillin, indicating a high prevalence of methicillin-resistant *S. aureus* (MRSA). Resistance to amoxicillin was also notable at 30.56%, while sulfonamides and Tigecycline showed better efficacy, with resistance rates of only 11.11% and 6.94%, respectively. The susceptibility of S. aureus is shown in Table 3 and Figure 2.

Antibiotic	Concentration Used (µg/disc)	Zone of Inhibition (mm)	Resistance (%)
Methicillin	5 µg	≤10 mm (resistant)	97.22
Sulfonamides	300 µg	≥17 mm (sensitive)	11.11
Tigecycline	15 µg	≥19 mm (sensitive)	6.94
Amoxicillin	10 µg	≤13 mm (resistant)	30.56

Table 3. Susceptibility pattern of *S. aureus*

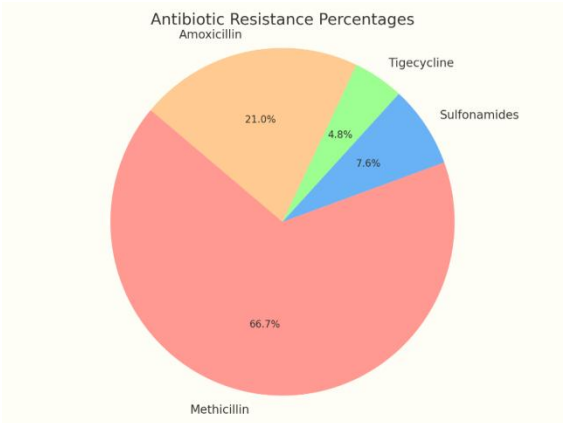

Figure 2: Susceptibility pattern of *S. aureus*

Antibiotic Resistance in *E. coli*

Among the *E. coli* isolates, 83.33% showed resistance to gentamycin, marking it as the least effective antibiotic against Gram-negative isolates in this study. Tetracycline resistance was recorded at 50%, while resistance to chloramphenicol and streptomycin remained comparatively lower at 12.5% and 16.66%, respectively. The susceptibility of *E. coli* is shown in Table 4 and Figure 3.

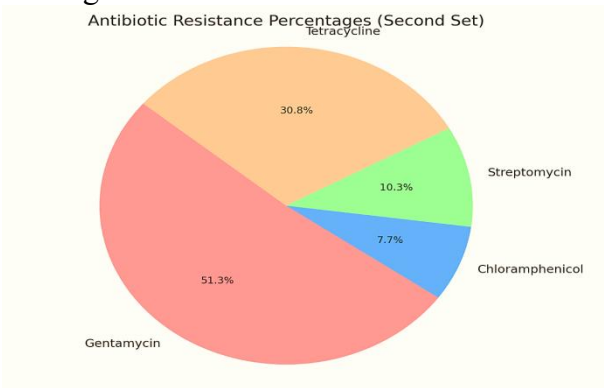

Figure 3: Susceptibility pattern of *E. coli*

Table 4. Susceptibility pattern of *E. coli*

Antibiotic	Concentration Used (µg/disc)	Zone of Inhibition (mm)	Resistance (%)
Gentamycin	10 µg	≤12 mm (resistant)	83.33
Chloramphenicol	30 µg	≥18 mm (sensitive)	12.50
Streptomycin	10 µg	≤11 mm (resistant)	16.66
Tetracycline	30 µg	≤14 mm (resistant)	50.00

DISCUSSION

The findings from this study reinforce that *S. aureus* and *E. coli* are the primary pathogens (El-Sayed and Kamel, 2021) responsible for subclinical mastitis in dairy cows in Muzaffargarh. With *S. aureus* detected in 70.6% of samples, this pathogen's prevalence is consistent with recent data from Pakistan and other regions prevalence rates often hover between 60% and 75% in Punjab and Sindh (Shahzad et al., 2024; Hussain et al., 2020) and align with global reports indicating its dominance in bovine mastitis (Shahzad et al., 2024). The organism's persistence is likely due to its robust biofilm formation, intracellular survival, and easy transmission through inadequate milking hygiene (Shahzad et al.,

2024). *E. coli*, detected in 23.5% of samples, is recognized as a primary environmental pathogen linked to acute mastitis, particularly where udder sanitation is poor (Murrad et al., 2020). Study outcomes in similar agro-climatic zones consistently report *E. coli* prevalence between 20% and 25%, primarily due to contaminated bedding and inadequate water hygiene (Ali et al., 2021).

Raising significant concern is the remarkably high methicillin resistance (97.2%) among *S. aureus* isolates. Comparable phenotypic resistance has been documented in studies from Lahore, with ~35% MRSA positivity (Selim et al., 2022), and Pothohar region findings reporting 21% mecA-positive rates with multiple virulence genes present. This high MRSA prevalence suggests unchecked beta-lactam antibiotic usage and represents a serious public health threat, given the zoonotic potential of MRSA (Schnitt et al., 2020). Conversely, lower resistance to tigecycline (6.9%) and sulphonamides (11.1%) suggests retained effectiveness, yet the presence of resistant strains to these advanced antibiotics calls for targeted antimicrobial surveillance (Shahzad et al., 2024). Amoxicillin resistance at 30.6%, echoing findings from Multan, further highlights the unregulated use of beta-lactams in dairy farming (Habib, 2023). A critical insight arose regarding *E. coli*: 83.3% gentamicin resistance sharply higher than prior regional reports of 30–50% indicates heavy aminoglycoside exposure in veterinary practice (Viana et al., 2025). Tetracycline resistance at 50% matches trends in Iran and Bangladesh, reflecting cumulative antibiotic selection pressure (Ahmad et al., 2021). Lower resistance to chloramphenicol (12.5%) and streptomycin (16.7%) aligns with global patterns, though their use remains complicated by potential toxicity (Ahmad et al., 2021). Alarmingly, regional studies also highlight metallo-β-lactamase (MBL)-producing *E. coli* strains and ESBL presence, carrying blaCTX-M, blaTEM, and blaNDM resistance genes further complicating treatment strategies (Maveke, 2023). Broad surveillance across livestock herds reveals widespread MDR patterns among both pathogens, including cefixime, fluoroquinolone, and beta-lactam resistance, largely linked to unrestricted antibiotic access and weak regulation (Toth et al., 2020; Liu et al., 2020; Shahzad et al., 2024) emphasize that MRSA persists even after standard cleaning and teat dips, underscoring the need for better farm biosecurity and treatment protocols. The consistent detection of mecA and virulence genes across multiple studies suggests that critical zoonotic strains are present in the region. Summing up, *S. aureus* remains a leading pathogen in Muzaffargarh dairy herds, now with alarming MRSA/MDR prevalence. *E. coli* continues as a significant environmental contributor, showing high resistance to gentamicin and tetracycline. These patterns underscore the urgent need for comprehensive antimicrobial stewardship, which should include routine culture-and-sensitivity testing, stricter antibiotic regulations, farmer education initiatives, adoption of teat dipping protocols, and regional antibiograms. Meeting these goals is essential to reduce antimicrobial resistance and enhance udder health in Pakistan's dairy sector.

CONCLUSION

This study reinforces the significant role of *S. aureus* and *E. coli* as the predominant pathogens causing bovine mastitis in Muzaffargarh, Pakistan. The exceptionally high prevalence of *S. aureus* (70.56%) and moderate presence of *E. coli* (23.52%) underline the urgent need to implement robust control measures targeting both contagious and environmental mastitis. Equally alarming is the extensive antibiotic resistance observed in both bacterial species. Nearly all *S. aureus* isolates were resistant to methicillin, highlighting the widespread presence of MRSA in the dairy sector. Similarly, *E. coli* exhibited substantial resistance to gentamycin and tetracycline, reducing the efficacy of commonly used antimicrobials. These findings reflect the

consequences of irrational antibiotic usage, the absence of routine sensitivity testing, and lack of awareness among dairy farmers. The results of this study emphasize the need for routine microbial surveillance, adherence to antibiotic stewardship programs, and implementation of strict hygienic practices at the farm level. Regional antibiograms must be developed and integrated into mastitis management protocols to ensure evidence-based therapeutic interventions. In addition, farmer education programs and regulatory frameworks must be strengthened to safeguard both animal health and public safety in the face of rising antimicrobial resistance.

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