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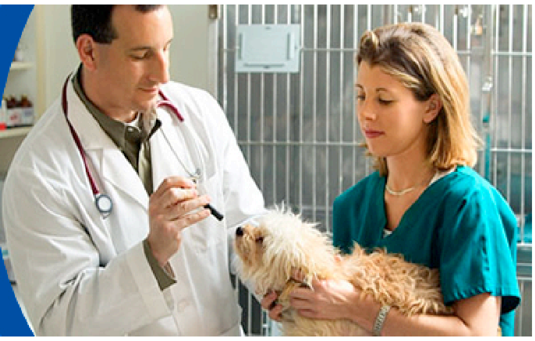

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Dr. T Ramasamy
M.V.Sc., Ph.D., Assistant Professor (Veterinary Pharmacology and Toxicology), Veterinary Clinical Complex, Veterinary College and Research Institute, Thalaivasal Koot Road, Salem, Tamil Nadu, India

Corresponding Author:
Dr. T Ramasamy
M.V.Sc., Ph.D., Assistant Professor (Veterinary Pharmacology and Toxicology), Veterinary Clinical Complex, Veterinary College and Research Institute, Thalaivasal Koot Road, Salem, Tamil Nadu, India

Antimicrobial resistance pattern of *Staphylococcus species* in mastitis affected dairy cows

T Ramasamy

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Abstract
Antimicrobial resistance (AMR) is a global health concern impacting both human and veterinary medicine. Mastitis, a major production disease in dairy cattle, is often caused by Staphylococcus spp., which increasingly shows resistance to multiple drugs. This study investigated the prevalence and antimicrobial resistance of Staphylococcus spp. isolated from mastitis-affected dairy cows in Tamil Nadu, India. Forty milk samples from clinical and subclinical cases (August 2024–March 2025) were screened by California Mastitis Test, cultured on mannitol salt agar, and tested for antimicrobial susceptibility against 12 antibiotics using CLSI guidelines. Staphylococcus spp. was isolated in 18 samples (45%), with high resistance to methicillin (77.78%) and penicillin G (72.22%), alongside notable resistance to enrofloxacin and amikacin (61.11% each). Amoxicillin–clavulanate (66.67%) and tetracycline (55.56%) retained the highest sensitivity. The high multidrug resistance highlights therapeutic challenges and zoonotic risks, emphasizing the need for culture-based therapy, antimicrobial stewardship, and a One Health approach.

Keywords: Mastitis, *Staphylococcus* spp., Anti-microbial resistance, Dairy cows, Antibiotic susceptibility

Introduction

Antimicrobial resistance (AMR) recognized as a major global public health threat, affecting healthcare systems not only in developing nations but also in high-income countries worldwide [1]. AMR arises when microorganisms—such as bacteria, viruses, fungi, and parasites—develop the ability to survive and proliferate despite exposure to antimicrobial agents that were previously effective in inhibiting or eliminating the organisms [2]. In light of the rising incidence of antibiotic resistance, the selection of antimicrobial agents for mastitis management should be guided by culture and sensitivity testing, rather than relying on empirical treatment approaches [3].

Mastitis is a global production disease affecting dairy cows and inflammation of the udder gland by the microbes that live inside the mammary gland [4]. The rearing environment and cattle production systems are linked to this multi-etiological disease. It is characterized by bacteriological, chemical, and physical alterations in the mammary gland [5].

It is recognized as a major health concern in dairy cattle and leads to significant economic losses for dairy farmers both globally and in India, primarily by impairing milk production and deterioration in milk quality [6]. Economic analyses estimate that subclinical and clinical mastitis collectively incur annual losses of approximately US$ 98.23 billion within the Indian dairy sector [7]. The mean daily treatment cost in Tamil Nadu was estimated at ₹417, with average illness duration of 4.19 days. Consequently, the total average treatment expenditure per illness episode amounted to ₹1,747.92 [8]. In the dairy sector, bovine mastitis is a major problem that both disturbs the health and welfare of the animals and results in significant financial losses [9].

A wide range of microorganisms have been implicated in the occurrence of mastitis. In comparison, the incidence of mastitis attributed to fungal and yeast infections were markedly lower than that caused by bacterial pathogens [10]. Among the bacteria, *Staphylococcus species* and *Escherichia coli* are contributing to the high occurrence of both clinical and subclinical mastitis [11, 12]. Antimicrobial treatment is currently essential to maintaining the equilibrium of bovine udder health, animal welfare, and economic considerations. Due to the diverse range of pathogens responsible for mastitis, antibiotic therapy remains the standard treatment approach and accounts for approximately 60% of the total antimicrobial usage in bovine veterinary practice [13]. However, the extensive use of antibiotics in mastitis management has contributed to the emergence of antimicrobial resistance and has raised significant concerns regarding the presence of antibiotic residues in milk, which may pose potential risks to public health [14]. Thus, this study was conducted to assess the prevalence of *Staphylococcus sp.* and its antimicrobial resistance pattern in bovine mastitis.

Materials and Methods

Study area

The present study was carried out in the department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai – 600 007. The samples were collected from the Large Animal Medicine Unit, Department of Clinics, Madras Veterinary College Hospital, TANUVAS, Chennai – 600 007. The study was conducted between August 2024 and March 2025.

Antimicrobial sensitivity test

Milk samples were obtained from cows diagnosed with mastitis under stringent aseptic conditions. The California Mastitis Test (CMT) was employed as a preliminary screening tool for the detection of mastitis. Antimicrobial susceptibility of the isolated pathogens was assessed using the disc diffusion method, following the standardized protocol recommended by the Clinical and Laboratory Standards Institute (CLSI).

Isolation and Identification of *Staphylococcus sp.*

Bacteriological examination of milk samples was initiated within 24 hours of collection to ensure sample integrity. Nutrient broth was prepared and dispensed into sterile test tubes in 10 mL volumes. The tubes were sealed, autoclaved for sterilization, cooled to room temperature, and stored after confirming sterility. For primary enrichment, sterile cotton swabs saturated with individual milk samples were aseptically introduced into the nutrient broth tubes, which were then closed with sterile cotton plugs and incubated at 37 °C for 18 hours to promote bacterial proliferation. Following incubation, the enriched cultures were streaked onto mannitol salt agar (MSA) plates and further incubated at 37 °C for 24 hours to facilitate the selective growth of *Staphylococcus* spp.

To standardize the inoculum density for antimicrobial susceptibility testing, a turbidity equivalent to a 0.5 McFarland standard was employed. The 0.5 McFarland standard was prepared by combining 50 µL of 1.17% barium chloride solution with 9550 µL of 1% sulfuric acid, yielding a suspension containing barium sulfate precipitate that simulates the optical density of approximately 1.5×10^8 CFU/mL.

Antimicrobial susceptibility testing was performed using the agar disc diffusion method on Mueller-Hinton agar, adhering to the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI). To ensure uniformity in inoculum density, bacterial suspensions were standardized to match the turbidity of a 0.5 McFarland standard. All isolates were evaluated for their susceptibility to twelve antibiotics routinely employed in veterinary practice. Commercial antibiotic discs (Himedia, Mumbai, India) were used, including enrofloxacin (10 µg), amoxicillin–clavulanate (15 µg), gentamicin (10 µg), ceftriaxone (30 µg), tetracycline (30 µg), penicillin G (10 U), methicillin, ampicillin, streptomycin, amikacin, cefotaxime, and clindamycin. The inhibition zone diameters were measured in millimeters and interpreted as susceptible, intermediate, or resistant, according to CLSI interpretive standards.

Results and Discussion

Forty bovine milk samples were aseptically collected from cases of clinical and subclinical mastitis. Among the forty samples, eighteen samples were shown positive for *Staphylococcus* spp. in MSA plates and the prevalence rate was 45% based of phenotypic examination (Figure 1). The current study revealed the prevalence of *Staphylococcus* spp. at 45%, which is in agreement with the findings of Neelam *et al.* (2022), who reported *Staphylococcus* infection in 10-44% of the mastitis cases [15].

Fig 1: Isolation of *Staphylococcus spp.* in mannitol salt agar.

The antibiotic sensitivity (ABST) pattern of *Staphylococcus* isolated from bovine mastitis milk samples revealed varied susceptibility profiles across the tested antibiotics. The results were shown in Table 1and Figure 2. The highest resistance was observed against Methicillin (77.78%) and Penicillin-G (72.22%), indicating a predominant resistance to beta-lactam antibiotics in this bacterial population.

Table 1: Resistance pattern of the isolated *Staphylococci Spp.*

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
Penicillin - G	16.67	11.11	72.22
Methicillin	11.11	11.11	77.78
Ampicillin	50.00	27.78	22.22
Amoxy-clav	66.67	22.22	11.11
Gentamicin	27.78	27.78	44.44
Enrofloxacin	22.22	16.67	61.11
Streptomycin	33.33	22.22	44.44
Amikacin	16.67	22.22	61.11
Ceftriaxone	44.44	11.11	44.44
Cefotaxime	27.78	11.11	61.11
Clindamycin	50.00	11.11	38.89
Tetracycline	55.56	16.67	27.78

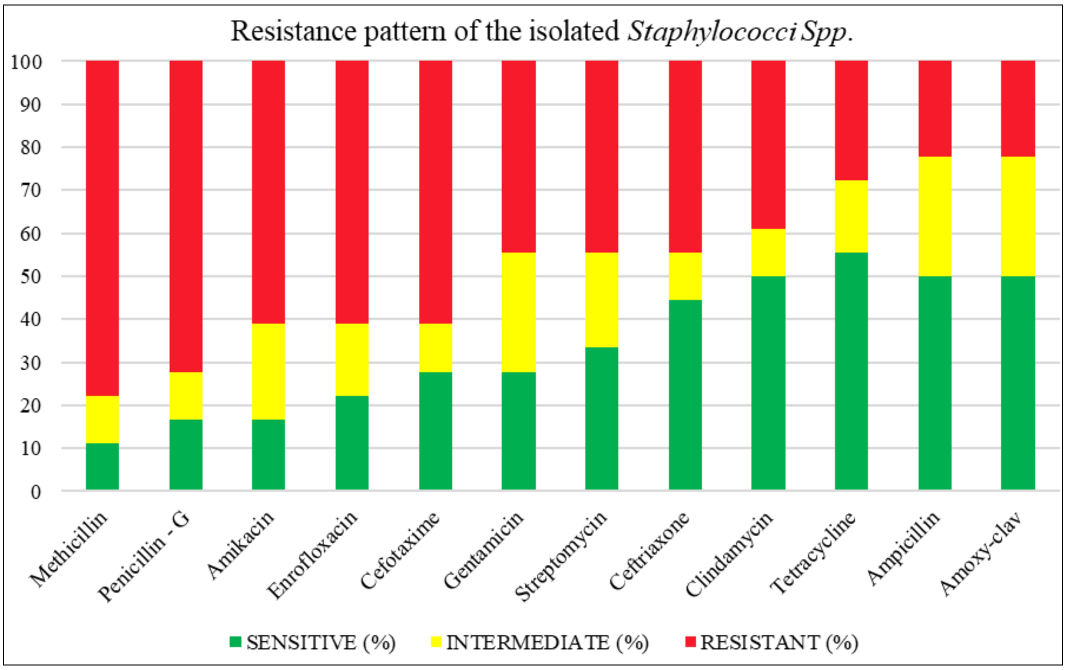

Fig 2: Resistance pattern of the isolated *Staphylococci Spp.*

The highest resistance was observed against Methicillin (77.78%) and Penicillin-G (72.22%), indicating a predominant resistance to beta-lactam antibiotics in this bacterial population. Ampicillin showed moderate sensitivity with 50.00% isolates sensitive and 22.22% resistant. Amoxicillin-clavulanic acid demonstrated the highest sensitivity among beta-lactams, with 66.67% of isolates sensitive and only 11.11% resistant. The particularly high methicillin resistance raises concerns about the potential presence of methicillin-resistant *Staphylococcus spp.* (MRSA), which have been increasingly reported in livestock-associated reservoirs [8]. Given that methicillin resistance is often linked to broader multidrug resistance, this poses significant challenges for both veterinary treatment and zoonotic transmission risks. Notably, amoxicillin-clavulanate (amoxy-clav) exhibited the highest efficacy (66.67% sensitivity), reinforcing the role of β-lactamase inhibitors in restoring susceptibility to β-lactam antibiotics. This aligns with studies demonstrating that clavulanate effectively counteracts staphylococcal β-lactamases, making combination therapies a viable option in resistant cases [16].

In Cephalosporins group, Ceftriaxone and Cefotaxime showed moderate to low sensitivity (44.44% and 27.78%, respectively) and relatively high resistance (44.44% and 61.11%, respectively). These findings are consistent with previous studies documenting widespread penicillin resistance in bovine mastitis isolates, likely due to the frequent use of β-lactams in dairy farming and the horizontal transfer of β-lactamase genes [11, 17, 18].

Among Aminoglycosides, Gentamicin and Streptomycin showed 27.78% and 33.33% sensitivity, respectively, with resistance rates of 44.44% for both. Amikacin exhibited a lower sensitivity (16.67%) and higher resistance (61.11%). Molineri *et al.* in 2021 reported that the continents of Africa, Asia, and Latin America exhibited elevated prevalence rates of antimicrobial resistance to penicillin G and gentamicin [19]. Signorini *et al.* in 2018 suggested that geographical heterogeneity in antimicrobial resistance profiles against gentamicin is likely shaped by divergent livestock production systems, regional variations in husbandry practices, and distinct regulatory frameworks governing antimicrobial usage in animal agriculture [20].

In the Fluoroquinolones, Enrofloxacin had a sensitivity of 22.22% and resistance of 61.11%. Fluoroquinolones (e.g., enrofloxacin, ciprofloxacin) are classified as Highest Priority Critically Important Antimicrobials (HPCIAs) by the WHO due to their significance in human medicine (WHO, 2019). Beuron *et al.* in 2014 reported 50% resistance in *Staphylococcus sp.* against enrofloxacin and also proposed that the utilization of enrofloxacin for dry cow treatment demonstrates a correlation with augmented *Staphylococcus aureus* resistance to it [21]. Clindamycin and Tetracycline displayed intermediate sensitivity, with 50.00% and 55.56% of isolates sensitive, respectively, and resistance rates of 38.89% and 27.78%. Overall, the data indicate a high prevalence of multidrug resistance among *Staphylococci* isolated from bovine mastitis cases, particularly against commonly used beta-lactams and aminoglycosides.

The Multiple Antibiotic Resistance (MAR) index is a valuable epidemiological tool used to assess the risk level of antibiotic resistance in bacterial isolates. MAR index greater than 0.2 generally indicates that the isolates originate from high-risk sources where antibiotics are frequently used or misused, such as in intensive farming or clinical settings [22]. In contrast, lower values suggest exposure to environments with minimal antibiotic pressure. Thus, the MAR index not only reflects the extent of resistance among isolates but also serves as an indicator of antibiotic usage practices in the environment from which they were recovered [23, 24]. Its application helps in understanding the epidemiology of antimicrobial resistance and in framing strategies for prudent antibiotic use.

The calculated MAR index among the 18 *Staphylococcus* isolates ranged from 0.00 to 0.75 (Table 2). The highest MAR index was observed in isolate 8 (0.75), followed by isolates 2, 3, and 7 (0.67), indicating that these isolates were resistant to more than two-thirds of the tested antibiotics. In contrast, isolates 12 and 14 exhibited a MAR index of 0.00, reflecting complete susceptibility to all antimicrobials tested.

Table 2: MAR index of *Staphylococcus spp.* Isolates

Isolate	Resistant Antibiotics	MAR Index
1	7	0.58
2	8	0.67
3	8	0.67
4	7	0.58
5	6	0.5
6	7	0.58
7	8	0.67
8	9	0.75
9	6	0.5
10	5	0.42
11	2	0.17
12	0	0
13	1	0.08
14	0	0
15	1	0.08
16	2	0.17
17	2	0.17
18	3	0.25

A MAR index value of >0.2 is generally considered an indicator of isolates originating from high-risk sources of contamination, where there is extensive antibiotic use.[22] In this study, 10 out of 18 isolates (55.6%) exhibited MAR indices above 0.2, suggesting a considerable degree of antibiotic pressure and potential misuse or overuse of antimicrobials in the farm environment.

The presence of isolates with high MAR indices demonstrates the emergence of multidrug-resistant *Staphylococcus* strains, which poses significant therapeutic challenges in mastitis management. On the other hand, the occurrence of isolates with MAR ≤ 0.2 indicates that not all bacterial populations are equally exposed to antimicrobial selection pressure, and some may still be sensitive to commonly used antibiotics.

Conclusion

This study highlights widespread resistance among *Staphylococcus spp.* from bovine mastitis, particularly to β-lactams, fluoroquinolones, and cephalosporins. While amoxicillin-clavulanate and tetracycline remain relatively effective, the high resistance to critical antibiotics necessitates urgent antimicrobial stewardship in dairy farming. A One Health approach, integrating veterinary and public health strategies, is essential to mitigate the spread of resistant staphylococci from livestock to humans. Future research should focus on longitudinal AMR surveillance and non-antibiotic interventions to combat mastitis sustainably.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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