



Prevalence, Molecular Characterization and Antibiotic Resistance Profile of *Staphylococcus aureus* in Mastitis-Infected Dairy Cattle in Punjab, Pakistan

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Abstract

Mastitis is considered one of the most widespread bovine infections, affecting dairy herds with the prevalence varying globally. The following research was conducted to isolate and characterize *Staphylococcus aureus* from milk samples from mastitis-infected cows in major dairy-producing regions of Central Punjab, Pakistan. A total of 210 raw milk samples were aseptically collected from five regions: Sahiwal (n=48), Gujrat (n=40), Sargodha (n=42), Faisalabad (n=54), and Mandi Bahauddin (n=26). The isolates were recovered and identified by phenotypic, biochemical, and molecular tests. Antimicrobial susceptibility test (AST) was determined using the Kirby–Bauer disc diffusion method as per Clinical and Laboratory Standards Institute guidelines (CLSI). Among 210 milk samples, 38 (18.1%) were positive for *S. aureus*. The highest prevalence was recorded in Sahiwal (22.9%), followed by Sargodha (19.04%) and Gujrat (17.5%), while the lowest prevalence was found in Mandi Bahauddin (7.7%). AST showed high resistance to Penicillin G (79%), Azithromycin (47%), Erythromycin (45%), Ciprofloxacin (45%), and Trimethoprim/Sulfamethoxazole (39%). Conversely, Chloramphenicol (21%), Cefoxitin (26%), and Doxycycline (26%) showed lower resistance rates. In conclusion, this study highlights the presence of antibiotic-resistant *S. aureus* in raw milk samples from Punjab, Pakistan, and outlines the necessity of regular surveillance and conscious antibiotic use to manage this economically significant disease.

Introduction

Dairy products, especially milk, are among the most essential food sources for most of the world's population. Pakistan is the 4th largest milk producing country in the world. Approximately 81% milk is produced at a small scale in rural areas, which contributes 57 million tons of milk production (Sattar et al., 2022). Pakistan, with an estimated 57.5 million cows and 46.3 million buffalo, is the 4th largest milk producer in the world. Despite its significant production capacity, the dairy sector faces several challenges, with bovine mastitis being the most prominent (Ahmad et al., 2021).

Bovine mastitis (BM) is an inflammation of the tissues of the mammary gland, primarily caused by intramammary infection (IMI), and poses a significant problem to livestock health globally. The disease results in treatment failure, low yields, poor quality of milk, and significant economic losses in the dairy industry (Majumder et al., 2026). Mastitis is normally caused by bacteria. Bovine mastitis has been associated with more than 130 bacterial species. However, approximately 80 percent of cases of mastitis are caused by a few bacterial species, such as *S. aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*.

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(Kerro Dego & Vidlund, 2024; Zhang et al., 2024). Contagious pathogens are transmitted between cows at milking via contaminated equipment, the hands of milkers, or infected udders (Capurro et al., 2010; Touaitia et al., 2025).

One of the most significant bovine mastitis-causing pathogens in the world is *S. aureus*. It is a Gram-positive, catalase-positive, coagulase-positive, cluster-forming, non-motile, and non-spore-forming facultative anaerobic bacterium, primarily transmitted among cows during milking (Tong et al., 2025). The *S. aureus* may enter the milk via direct excretion of the udders with clinical or subclinical staphylococcal mastitis or by environmental contamination during the processing of raw milk (El-Ashker et al., 2015). The *S. aureus* can also be found in the milk with different concentrations when the udder is infected, reaching up to 10^8 CFU/mL (Grima et al., 2021).

Mastitis is closely related to subclinical mastitis due to its ability to induce persistent intra-mammary infections as it escapes the host immune defenses through intracellular persistence that leads to repetitive infections and long-term decreases in milk yield. The chronicity is also exacerbated by toxin-mediated tissue damage and biofilm formation (Tong et al., 2025). Some of the most recent observed key virulence factors include fibronectin-binding proteins (coded by *FnBPA* and *FnBPB*), collagen adhesion protein (coded by *Cna*), clumping factors (coded by *clfA*), and protein A (coded by *SpA*) α -hemolysin interacts with *ADAM10*, and β -hemolysin increases cell membrane permeability, which contributes to cell lysis. The superantigens (*SAGs*) are enterotoxins, toxic shock syndrome toxin-1 (*TSST-1*) (Tong et al., 2025). Due to its asymptomatic and chronic nature, recent developments in the diagnosis of mastitis have focused on the exploration of the host-derived biological molecules as sensitive factors of intramammary inflammation and infection (Dall'Olio et al., 2026).

Over the past few years, the incidence and epidemiology of bovine mastitis have changed because of the scale and management practices of dairy farming (Xu et al., 2022). Prevalence rates in Asia have been reported as 30.32% (94/310 subclinical mastitis samples) to 42.5% (85/200 milk samples), with MRSA in 11.7-21.0% of isolates. It may result in clinical mastitis, but the more frequent outcome is the subclinical and common chronic mastitis with an increased milk somatic cell count. In Narowal District, Pakistan, SCM was found to be more common (45.8%) compared to CM (37.5%), whereas Cameroonian MRSA isolates were mostly linked to clinical cases (72.5%) (Touaitia et al., 2025).

Mastitis is the most frequent reason for antimicrobial use in dairy cattle, and it is mostly given through intramammary and intramuscular routes. Nevertheless, the treatment is now

less effective with a cure rate of 10-30 percent only. Animal and human strains exhibit resistance to β -lactams, tetracycline, and macrolides, and variable resistance to methicillin. The occurrence of various resistance genes to antimicrobials such as *mecA*, *blaZ*, tetracycline, and macrolide resistance determinants, as well as efflux mechanisms, all play a role in causing multidrug-resistant *S. aureus* strains to resist treatment (Saeed et al., 2022). Antibiotic-resistant bacterial strains have become a worldwide issue of major concern in human and veterinary health (Javed et al., 2023). These pathogens can contaminate food products during processing or storage. This highlights the necessity of a One Health approach that integrates animal, human, and environmental health through improved antimicrobial stewardship, regulatory control, and surveillance systems (Singh et al., 2024).

The purpose of the current study was to determine the prevalence and isolation rate of *S. aureus* in raw milk samples collected from seven major dairy-producing districts of Punjab, Pakistan, with the help of the traditional biochemical tests and molecular methods based on the amplification of the 16S rRNA gene. Additionally, the antimicrobial susceptibility profiles of the identified *S. aureus* isolates were assessed using a panel of 10 commonly used antibiotics to analyze the magnitude and patterns of antimicrobial resistance.

Materials and Methods

Study Area and Sample Collection

A total of 210 raw milk samples from mastitis-infected cows were randomly aseptically collected from five main dairy-producing districts in Central Punjab, Pakistan: Sahiwal ($n=48$), Gujrat ($n=40$), Sargodha ($n=42$), Faisalabad ($n=54$), and Mandi Bahauddin ($n=26$). After disinfecting the cow teats with 0.5% iodine solution and drying with a clean paper towel, a 25-mL milk sample was collected into a sterile tube after discarding 4-5 streams of foremilk. All milk samples were transported to the laboratory within 2 hours of collection and stored at 4°C till further analysis.

Isolation and Identification of causative *S. aureus*

For enrichment, 25 mL of each raw milk sample was added to 225 mL of Tryptic Soy Broth (TSB). The broth was then incubated in a rotatory shaker at 150 rpm for 16 hours, and a loopful of broth was streaked over Mannitol Salt Agar (MSA) and 5% sheep blood agar. The purified presumptive *S. aureus* isolates were then identified by examining their hemolysis pattern and colony shape, which revealed salt-tolerant pure isolated *S. aureus* colonies. On MSA, *S. aureus* colonies were typically spherical, smooth, and golden-yellow. All colonies were sub-cultured on nutrient agar slants for 16 hours at 37°C before being identified

biochemically and stained with Gram’s stain. Under the microscope, *S. aureus* isolates appeared as Gram-positive cocci in cluster form. The isolates were negative for oxidase and indole, but positive for methyl red, Voges Proskauer (VP), Catalase, Urease, and Coagulase test.

Molecular identification of *S. aureus*

For DNA extraction, a loop of bacterial culture was suspended in 50 µL of sterile distilled water, heated for five minutes at 100°C, then centrifuged for five minutes at 12,000 rpm. 5 microliters of supernatant were taken for use in PCR. *S. aureus* isolates were confirmed using *nuc-F* and *nuc-R* primers targeting the 16S rRNA gene (Table 1). Molecular validation was accomplished by focusing on the 16S rRNA gene. The PCR reaction was carried out in a 20 µl volume, including 0.5 µl of each forward and reverse primer, 10 µl of 2× Master Mix, 2 µl of DNA template, and 7 µl of nuclease-free water. The reaction conditions were Denaturation at 95 °C for 3 minutes, followed by 30 cycles of 45 seconds at 94 °C, 45 seconds at 58 °C, 1 minute at 72 °C, and a final extension of 3 minutes at 72 °C.

Antimicrobial Susceptibility Testing

After PCR confirmation of the isolates, the antibiotic susceptibility test was performed using the Bauer et al. disk diffusion method (Bauer et al., 1966). The test was done to assess the antibiotic resistance of *S. aureus* against the antibiotics: Penicillin G (P, 10U), Cefoxitin (CFX, 30µg), Gentamicin (CN, 10µg), Ciprofloxacin (CIP, 5µg), Trimethoprim/Sulfamethoxazole (TMP/SMX, 5µg), Azithromycin (AZM, 5µg), Erythromycin (10µg), Chloramphenicol (30µg), Doxycycline (30µg), and Tetracycline (TE, 30µg). Tryptic Soy Broth (TSB) was used to refresh *S. aureus* cultures overnight at 37°C. The concentration was set at 0.5 McFarland turbidity. Antibiotic

discs were placed at a sufficient distance on the inoculated plates to prevent overlapping zones of inhibition in accordance with CLSI guidelines (Wayne, 2011).

Statistical analysis

The Chi-square test was used to evaluate the data in order to determine the prevalence of *S. aureus* across the various areas of Punjab, Pakistan. A *p*<0.05 was considered statistically significant.

Results

S. aureus Isolation from Raw Milk Samples

A total of 210 raw milk samples were randomly collected aseptically from five major dairy-producing areas in Punjab, Pakistan, from mastitis-infected cows. Out of which 38 *S. aureus* isolates were identified, with the highest isolation rate in Sahiwal (11/48, 22.9%) and the lowest in Mandi Bahauddin (2/26, 7.69 %). Other district-wise isolation rates were as follows: Gujrat (17.5%), Sargodha (19.04 %), and Faisalabad (18.51 %) as described in Table 2. *S. aureus* was first enriched in Tryptic Soy Broth (TSB), followed by streaking on Mannitol Salt Agar and 5% sheep blood agar and exhibited hemolytic growth on blood agar. MSA colonies were spherical and golden yellow.

Gram’s Staining and Biochemical Identification

Gram’s staining and biochemical identification were carried out on 38 verified *S. aureus* isolates, as shown in Table 5. After Gram staining under a light microscope, the isolates were revealed to be purple-colored cocci in clusters, consistent with the Gram-positive *S. aureus* morphology. Based on these characteristics, the isolates were presumed to be *S. aureus* and selected for further phenotypic and biochemical identification. The findings are presented in Table 3.

Table 1. Primer sequence targeting the 16S rRNA gene

Gene	Primer Sequence (5’ -3’)	Amplicon Size (bp)	Citation
<i>nuc-F</i>	TACAGGTGACTGCGGGCTTATC	270	(Tonu et al., 2012)
<i>nuc-R</i>	CTTACCGGGCAATACACTCACTA		

Table 2. Isolation rate of *S. aureus* out of 210 raw milk samples

District of Punjab	Total No of samples	Collected samples	Number of confirmed <i>S. aureus</i> isolates	<i>S. aureus</i> Isolation rate (%)
Sahiwal	n =210	48	11	22.9 %
Gujrat		40	07	17.5 %
Sargodha		42	08	19.04 %
Faisalabad		54	10	18.51 %
Mandi Bahauddin		26	02	7.69 %

Table 3. Biochemical tests for 38 confirmed *S. aureus* isolate from Raw Milk

Biochemical tests	Results
Catalase	+
Oxidase	-
Indole	-
Methyl red	+
Voges-Proskauer	+
Citrate	+

Molecular Confirmation via PCR

DNA extraction was performed for the isolates by the Simple Boiling method. 38 isolates of *S. aureus* were positive for genomic DNA. PCR amplification targeting the *16S rRNA* gene produced a 270bp specific band in all tested isolates, confirming their identity as *S. aureus*.

Susceptibility pattern in isolates

AST was performed on confirmed isolates using the method of Bauer et al. disk diffusion method (Bauer et al., 1966), according to the Clinical and Laboratory Standards Institute guidelines (Wayne et al., 2011). These samples were tested against ten antibiotics routinely used in human and veterinary medicine. The results were classed as sensitive (S), intermediate (I), or resistant (R) using the Clinical and Laboratory Standards Institute's zone diameter standards or antimicrobial disk manufacturer's guidelines. Table 4 summarizes the findings. High resistance rates were observed in Penicillin G (79%), Azithromycin (47%), Erythromycin (45%), Ciprofloxacin (45%), and Trimethoprim/Sulfamethoxazole (39%). Meanwhile, Chloramphenicol (21% resistance), Cefoxitin (26%), and Doxycycline (26%) showed lower resistance, indicating high susceptibility of the isolates to these antibiotics.

Table 4. Antimicrobial susceptibility profile of *S. aureus* isolates from raw milk

Total no of samples received	Positive for <i>S. aureus</i> with	Notified Antibiotic panel only	Resistant with %	Intermediate with %	Sensitive with %
210	n = 38 18.1%	Penicillin G	79%	0%	21%
		Cefoxitin	26%	0%	74%
		Gentamicin	34%	0%	66%
		Ciprofloxacin	45%	13%	42%
		Doxycycline	26%	5%	68%
		Trimethoprim/Sulfamethoxazole	39%	0%	61%
		Erythromycin	45%	34%	21%
		Azithromycin	47%	26%	26%
		Chloramphenicol	21%	11%	68%
		Tetracycline	37%	13%	50%

Statistical Analysis

The prevalence of *S. aureus* across the studied areas of Punjab is presented in Table 4. Statistically significant differences in prevalence were observed among the regions ($p < 0.05$). The highest prevalence was recorded in Sahiwal (22.9%), followed by Sargodha (19.04%) and Gujrat (17.5%), while the lowest prevalence was found in Mandi Bahauddin (7.7%).

Discussion

Mastitis is a major illness that poses a challenge to the dairy industry of the world, especially severe in Pakistan, because it has been a constraint to the growth of the dairy industry in the country. The studies have also revealed that 17% of the economic loss of animal disease is due to mastitis, and 80% of sub-clinical mastitis is *S. aureus* (Elsayed & Dawoud, 2015; Fareed et al., 2017). In the present study, 210 milk samples were collected from mastitis-infected cows from a selected region of Central Punjab, which were identified and categorized based on colony morphology and biochemical tests. *S. aureus* was 38/210 with an overall prevalence of 18.1 %. This work has shown a similar trend as determined by various earlier studies that found *S. aureus* to be a common etiological agent (Javed et al., 2022).

Among the studied districts, Sahiwal showed the highest prevalence (22.9%), followed by Sargodha (19.04%) and Gujrat (17.5%), indicating a comparatively higher prevalence of the pathogen in these areas. Such variations can be indicative of differences in herd size, milking, sanitation, and mastitis management awareness among dairy farmers. As *S. aureus* is mainly spread during milking using contaminated equipment, hands of milkers, or infected udders, an increase in prevalence can be an indicator of poor hygiene and inadequate control of contagious mastitis (Deddefo et al., 2023). On the other hand, a decreased

prevalence rate was observed in Mandi Bahauddin (7.7%), indicating better hand hygiene and biosecurity management. These observed variations can be due to differences in husbandry practices. All farms included in this study were privately owned and managed on a small scale, so there was poor management of cleanliness and hygiene practices. Pakistan's tropical climate also creates favorable conditions for the survival of pathogens (Javed et al., 2022).

However, the prevalence observed in this study is lower than various regional reports, which implies a considerable geographical variation. The highest prevalence rates have been observed in Muzaffargarh (70%), the Pothohar area of Pakistan (62.2%), and Khyber Pakhtunkhwa (57%), indicating a broader distribution of *S. aureus* in these areas (Akbar et al., 2025; Ali et al., 2021; Shahzad et al., 2024). Conversely, prevalence rates that are nearer to the current results are reported in Narowal (42.5%), indicating moderate endemicity of *S. aureus*-related mastitis in these regions (Shah et al., 2025).

Antimicrobial susceptibility test showed high resistance to Penicillin G (79%), Azithromycin (47%), Erythromycin (45%), Ciprofloxacin (45%), and Trimethoprim/Sulfamethoxazole (39%). Resistance to β -lactam antibiotics, particularly Penicillin G, is widely reported and is commonly associated with the production of β -lactamase enzymes and the presence of resistance genes such as *blaZ* and *mecA* (Lucas et al., 2021). This suggests that these isolates have developed mechanisms to resist the effects of these antibiotics. Moreover, resistance to macrolides and fluoroquinolones may be attributed to their frequent and often empirical use in mastitis treatment (Barlow et al., 2011). In contrast, relatively lower resistance was observed against Chloramphenicol (21%), Cefoxitin (26%), and Doxycycline (26%), indicating better in vitro activity however, their careful use is necessary to prevent the development of resistance. Higher sensitivity to these antibiotics may be due to their limited usage or less frequent exposure in dairy practice.

Overall, the identification of antimicrobial-resistant *S. aureus* in raw milk presents a serious threat to the health of animals, their dairy performance, and the health of people. These chronic intramammary infections due to resistant strains may result in failure in treatment, economic losses, and even the spread of the resistant bacteria through the food chain. This data indicates that regular laboratory-based diagnosis, antimicrobial susceptibility-based treatment, and high standards of hygiene are required during milking. Moreover, antimicrobial stewardship programs have to be adopted within a One Health framework to restrict the occurrence and transmission of resistance *S. aureus* strains for the management of bovine mastitis.

Conclusion

Staphylococcus aureus is a major cause of bovine mastitis in Punjab, Pakistan, with a significant proportion of isolates showing resistance to the commonly used antibiotics. The resistance to first-line drugs, particularly β -lactams and macrolides, is high, which means a long and frequent course of antibiotic treatment on the farm level lowers the efficacy of treatment. These results demonstrate the importance of regular laboratory diagnosis, antimicrobial susceptibility-based therapy, and enhanced hygiene practices on the farm. Antimicrobial stewardship must be adopted as a One Health approach to reduce the transmission of resistant *S. aureus* as well as ensure sustainable mastitis control and protection of animal and public health.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

Conceptualization, Writing-original draft preparation, Writing-review and editing; Summayah Shahid, Afra Ghufuran. The authors have read and agreed to the published version of the manuscript.

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