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Research article

Prevalence, risk factors, and antimicrobial resistance of *Staphylococcus aureus* in Holeta dairy cows, Oromia, Ethiopia

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Abstract

Staphylococcus aureus (S. aureus) is a major cause of bovine mastitis and a source of foodborne disease; its toxin-mediated virulence and antimicrobial resistance further complicate its control. In Ethiopia, widespread mastitis and the emergence of multidrug-resistant (MDR) *S. aureus* pose significant challenges to milk production, public health, and effective treatment strategies. A cross-sectional study was conducted in Holeta town, Oromia, Ethiopia, from February 2024 to April 2025, to estimate the prevalence of *S. aureus* in lactating cows, identify associated risk factors, and characterize antimicrobial resistance. A total of 224 cows from 36 randomly selected dairy farms were sampled. Milk samples were screened for mastitis using the California mastitis test (CMT) and cultured. Presumptive *S. aureus* was isolated from 55 of 224 cows (24.6%; 95% confidence interval (CI): 19.4–30.6). Due to resource limitations, particularly the availability of PCR reagents and laboratory capacity, only 32 of the 55 phenotypically positive isolates were selected for molecular analysis and tested for the *nuc* gene. Of these, 65.6% (21/32) were confirmed as *S. aureus*. The confirmed prevalence was therefore at least 9.4% (21/224); the true prevalence is likely below the presumptive estimate. Among the 21 confirmed isolates, resistance was highest to penicillin (19/21, 90.5%), streptomycin (17/21, 81.0%), tetracycline (12/21, 57.1%), and erythromycin (11/21, 52.4%), with 61.9% of isolates exhibiting a multidrug resistance profile. Prevalence of *S. aureus* was significantly associated with management system, cleaning frequency, breed, parity, lactation stage, and body condition score. The presence of MDR *S. aureus* underscores the need for improved mastitis control, including farm biosecurity, proper milking hygiene, targeted farmer training, and routine antimicrobial susceptibility testing. Confirmatory molecular testing of all isolates is needed for accurate prevalence estimation.

Keywords: Ethiopia, Holeta, Mastitis, Molecular detection, Risk factors, *Staphylococcus aureus*

Introduction

Foods of animal origin are frequently contaminated with bacterial pathogens such as *Staphylococcus aureus (S. aureus)*, *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp. *S. aureus* is commonly found in bovine-derived products, including milk, cheese, yogurt, and butter [1]. It is associated with toxin-mediated virulence and antimicrobial resistance [2] and produces heat-stable enzymes and enterotoxins that cause food poisoning characterized by vomiting, abdominal pain, and diarrhea [3, 4]. In dairy production, *S. aureus* is a major cause of bovine mastitis, a disease with substantial economic impact due to decreased milk production, mammary gland loss, premature culling, and occasional death of affected cows [5, 6, 7, 8].

The persistence of *S. aureus* in mastitis is linked to diverse virulence factors, including quorum sensing, immune evasion strategies, and the production of staphylokinase, lipase, and hemolysins [9, 10]. It readily acquires antibiotic resistance through mobile genetic elements. Methicillin-resistant *S. aureus* (MRSA) strains carry the staphylococcal cassette chromosome *mec* (SCC*mec*), which harbors the *mecA* gene encoding penicillin-binding protein 2a (PBP-2a) and confers resistance to methicillin and often to multiple other antibiotics [11, 12]. Resistance to penicillin may also arise from the *blaZ*-encoded penicillinase, which hydrolyzes the β-lactam ring of penicillin derivatives [13, 14, 15]. The thermostability of *S. aureus* is linked to the *nuc* gene, which encodes a heat-stable thermonuclease, allowing *nuc*-positive strains or their toxins to survive heat-processed foods, although not all isolates harbor this gene [16].

Previous studies in Holeta have isolated *S. aureus* from cow's milk and assessed its phenotypic antimicrobial susceptibility, while research in the wider West Shewa Zone has also examined the *nuc, mecA,* and *blaZ* genes [12]. Antibiotic residues in milk may trigger allergic reactions, disrupt the intestinal microbiota, and contribute to antimicrobial resistance [17]. In Ethiopia, *S. aureus* isolates from mastitic milk have shown resistance to several antibiotics, including ampicillin, penicillin, polymyxin B, and streptomycin [18].

The prevalence of multidrug-resistant (MDR) *S. aureus* in raw or inadequately processed milk is a serious public health concern, particularly in countries where raw milk is widely consumed. Scholars have reported that contaminated milk can transmit resistant bacteria and

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staphylococcal enterotoxins to consumers, causing foodborne illnesses and limiting treatment options [4, 19]. The persistence of *S. aureus* and its heat-stable toxins jeopardizes dairy safety [3], and the spread of MDR *S. aureus* throughout the dairy chain adds to the global antimicrobial resistance burden [20]. The zoonotic risk of resistant *S. aureus* via milk is especially alarming in countries with weak milk hygiene regulations, such as Ethiopia [21, 22].

Given the close contact between livestock and humans and weak milk hygiene regulations in Ethiopia, resistant *S. aureus* can be potentially transmitted through milk consumption [22]. MDR *S. aureus* complicates mastitis treatment and poses risks to other cattle and farm workers, including veterinarians, highlighting concerns about zoonotic transfer via the food chain [21, 23]. Therefore, this study aimed to isolate *S. aureus* from cows' milk, molecularly detect thermonuclease and resistance genes, estimate prevalence, assess associated risk factors, and determine the antibiogram profile of the isolates in the study area.

Materials and methods

Study area

The study was conducted in Holeta town, Oromia, Ethiopia (Figure 1). Holeta town is located in Sheger city, west of Finfinne, at a distance of 40 km from Finfinne. The 2007 national census reported that Holeta town's total population was 25,593, of whom 12,605 were men and 12,988 were women. The town has a latitude of 9°3'1" 0"-9° 5'0" N and a longitude of 38°28'3 0"- 38° 31'30" E, and an altitude of 2391 meters above sea level. The area has a mild subtropical highland climate, with minimum and maximum annual temperatures of 6.3°C and 22.1°C, respectively, and an average of 14.5°C. The area also has a bimodal rainfall pattern, with a long rainy season from June to September and a short rainy season from March to April. Annual rainfall ranges from 834 mm to 1300 mm [24].

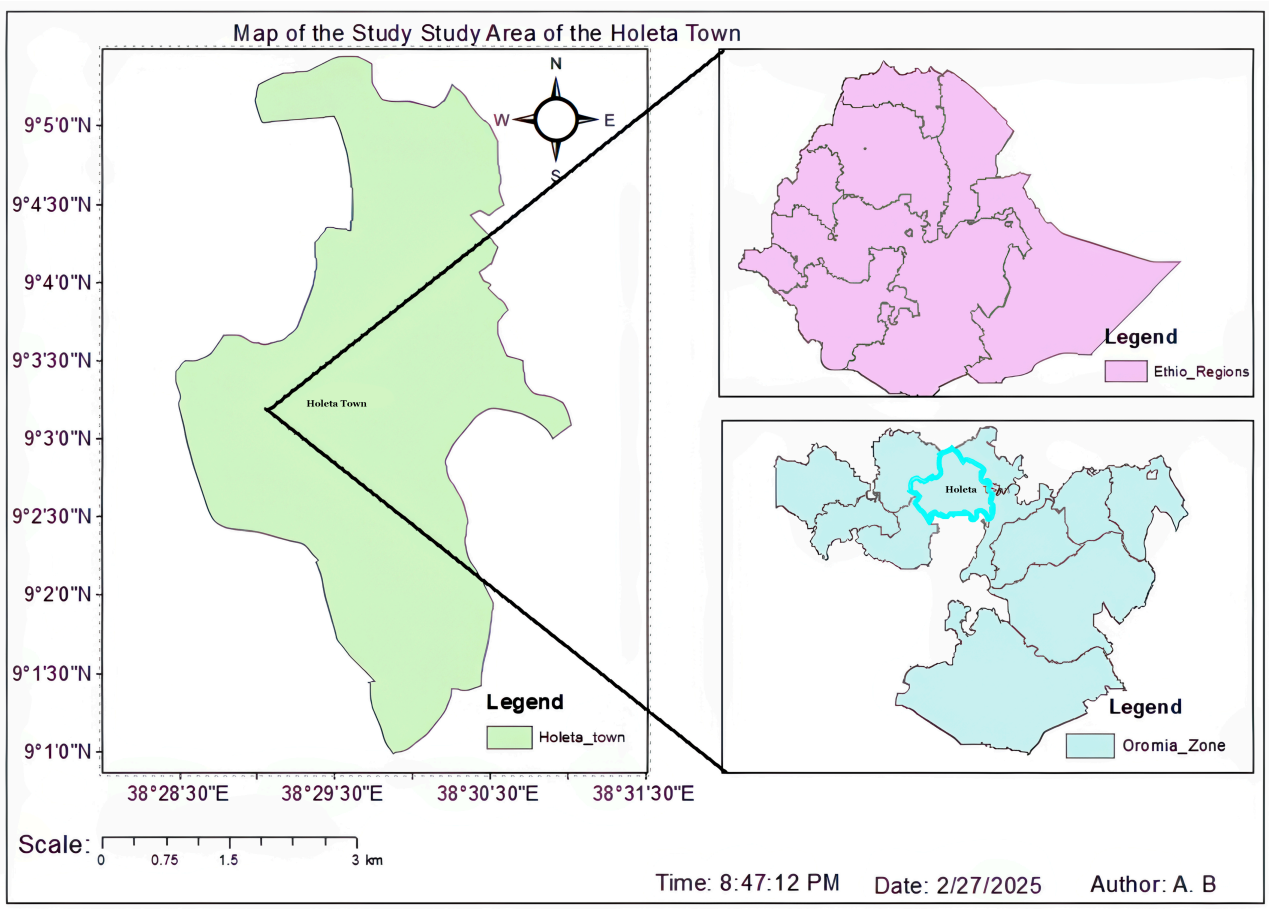

Figure 1: Map showing the geographical location of Holeta Town, the study area, within the Oromia Region, Ethiopia, and its surrounding administrative zones (ArcGIS).

Study animals

The study was conducted on lactating cows from dairy farms in Holeta town. The animals were maintained under both intensive and semi-intensive management systems, reflecting the common husbandry practices in the area. The study population included two main breeds: local indigenous cattle and crossbred cows, which are commonly kept for milk production. The cows varied in

age, parity, and lactation stage, representing the typical diversity of dairy herds in the region. These animals were selected to provide a representative sample for assessing the prevalence, antimicrobial resistance, and molecular characteristics of *S. aureus* in the local dairy sector.

Study design and study period

A cross-sectional study was conducted in Holeta town from February 2024 to April 2025. This design allowed data collection on the prevalence, antimicrobial resistance, and molecular characteristics of *S. aureus* from lactating cows at a single point in time across multiple dairy farms. The cross-sectional approach provided a snapshot of current infection and resistance patterns, facilitating identification of associated risk factors within the study population.

Sample size determination

The sample size for lactating cows was determined according to Thrusfield (2013) [25] using a 95% confidence interval, a 5% absolute level of precision, and an expected prevalence of 10.6%, as previously reported by Gebremedhin et al. (2022) in Holeta town [26]. Hence, the sample size calculated using the formula given below was 146.

$$n = \frac{\left(Z_{\frac{\alpha}{2}}\right)^2 P_{exp}(1-P_{exp})}{d^2} = \frac{1.96^2 * 0.1069(0.8931)}{0.05^2} = 146$$

Where n = required sample size; p_{exp} = expected prevalence; d = desired absolute precision (usually 0.05); $z_{\alpha/2}$ = the statistic for the confidence level (1.96). As Naing and Winn (2006) recommended, if the investigator suspects differences due to non-response or missing values, it is better to oversample the calculated sample size to achieve the desired precision and increase data representativeness [27]. Accordingly, about 53% of the calculated sample size was added to the 146 calculated samples, increasing the total sample size to 224 lactating cows.

Sampling method and sampling technique

To determine the total number of dairy farms and the number of lactating cows, a baseline survey was conducted. For this study and to facilitate data analysis, herd size was categorized as small (≤ 5 cows), medium (6-10 cows), and large (≥ 11 cows) [28]. This is because, in the Ethiopian context, farms with ≤ 5 cows are small-scale farms run as a side business.

According to data obtained from the Holeta Town Agriculture and Livestock Production Office, the town has 108 dairy farms, comprising 40 small, 38 medium, and 30 large farms. After stratifying farms by population size, this study included only one-third of the farms. Accordingly, a total of 224 lactating cows were randomly sampled from 36 randomly selected dairy farms. The 36 farms included 13 small, 13 medium, and 10 large farms. To determine the appropriate sample size, two, six, and twelve lactating cows were sampled from small, medium, and large farms, respectively. This means the study included 26 samples from small farms, 78 from medium farms, and 120 from large farms, each representing an individual lactating cow. The farms and individual lactating cows were selected using a simple random sampling method.

Inclusion criteria

Dairy farms were selected based on owners' willingness to participate and provision of informed consent. Within participating farms, 224 lactating cows were purposively selected based on eligibility criteria, including age, breed, lactation stage, and health status. Therefore, the cow-level sampling was non-random, which may limit the representativeness of the findings and their generalizability to the wider dairy-cow population.

Exclusion criteria

Cows that were not lactating at the time of the study were excluded because they do not contribute to milk production and are less relevant for assessing *S. aureus* prevalence in dairy products. Additionally, lactating cows that were not selected according to the study's sampling protocol were excluded to maintain the representativeness and consistency of the study population.

Questionnaire survey

A semi-structured questionnaire survey was prepared to collect information on farm attributes, cow attributes, and potential risk factors for mastitis in dairy cows. Data on animal identification, farm management, and other factors that enable identification of potential risk factors for the occurrence of the disease in dairy cows were collected through physical observation and interviews with farm owners or handlers.

Physical examination of the udder

The udders were carefully inspected and palpated for abnormalities. Clinical mastitis was identified by signs such as udder warmth, swelling, pain, and abnormal milk secretion, including clots, blood flakes, or watery discharge. Chronic mastitis was characterized by atrophy, hardness, or fibrosis of the udder quarters. Tick infestation and udder wounds were also recorded as potential risk factors for mastitis [29]. Subclinical mastitis was assessed using the California mastitis test (CMT).

Sample collection, transportation, and handling

An aseptic procedure was followed during milk sample collection to prevent contamination by microorganisms present on the skin, udder, and teats, on the hands of samplers, and in the barn environment. Milk samples were collected from each clinically and subclinically

mastitic non-blind quarter of the selected lactating cows for bacterial isolation. First, the dairy cow's udder and teat orifice were thoroughly washed, cleaned, and dried before milk samples were collected. In addition, dirt and other debris were removed from the udder and teat with a dry towel. Then, the teats were further scrubbed with cotton and soaked in 70% alcohol to prevent recontamination.

Teats on the far side of the udder were first scrubbed with alcohol and sampled, and those on the near side were sampled later. After milking out and discarding the first two drops, about 2 mL of milk from each quarter was tested on a CMT paddle, and about 20 mL of milk was aseptically collected from each mastitis-positive quarter using a sterile universal bottle [30]. Finally, all milk samples were labeled and transported within 2–3 h in an icebox to the Animal Biotechnology Research Laboratory of the National Agricultural Biotechnology Research Center (NABRC), then stored in a refrigerator at 4 °C for subsequent laboratory work.

California mastitis test (CMT)

The CMT was used to screen the collected milk samples. One squirt of milk from each udder quarter was placed in each cup of the CMT paddle, and each cup was thoroughly mixed with an equal volume of 3% CMT reagent. Based on gel formation, CMT results were classified as positive or negative, ranging from (+1) to (+3). CMT findings for subclinical cases were used to detect mastitis. Accordingly, the degree of coagulation was assessed as "-" (negative), "+" (slightly positive), "++" (moderately positive), and "+++" (very positive) [31].

Isolation and phenotypic identification of *S. aureus*

Bacteriological culture was performed using the standard microbiological technique recommended by Gebremedhin et al. [26]. A loopful of each infected quarter's milk sample was taken and inoculated separately onto blood agar base enriched with 5% ovine blood using the quadrant streaking method. All plates were incubated aerobically at 37 °C and examined after 24 hours for growth. Colonies were provisionally identified based on morphology (i.e., circular, black, convex, with or without a light halo on blood agar base), hemolytic pattern, and Gram stain reaction. Representative colonies were subcultured on blood agar base and nutrient slants and incubated at 37 °C [32].

The identification of *S. aureus* was carried out using the following tests: Catalase test, mannitol salt agar, and Coagulase test. Before further biochemical tests, the isolated bacteria on blood agar were subcultured onto nutrient agar. Each culture was subjected to Gram staining to determine its shape and Gram reaction. A catalase test using 3% Hydrogen peroxide (H_2O_2) was performed to identify catalase-positive and catalase-negative bacteria [33]. Mannitol salt agar with 1% maltose was used to differentiate *Staphylococcus* species; cultures were incubated at 37°C and examined after 24–48 h for mannitol and maltose fermentation, respectively [34].

Molecular identification of *S. aureus* by PCR

After a series of bacterial cultures, Gram stains, and biochemical tests, pure bacterial colonies were obtained, and the colony showing typical *S. aureus* characteristics was selected for molecular detection. The pure bacterial colonies were then inoculated into nutrient broth and stored at -40 °C. Finally, the sample was maintained under a cold chain for molecular detection.

Genomic DNA was extracted from 24-hour-grown S. aureus cultures in nutrient broth using a commercially available EZ-10 spin column genomic DNA Minipreps kit (Bio-Basic, Markham, Ontario, Canada), following the manufacturer's instructions [35].

A conventional PCR reaction was performed using the *S. aureus*-specific *nuc* gene (F 5´-CGCG ATT GAT GGT GAT ACG GTT-3´ and R 5´-ACG CAA GCC TTG ACG AAC TAA AGC-3´) for identification. Each 25 µl reaction mixture contained 3 µl genomic DNA, 12.5 µl 2X PCR Master Mix (Promega®, USA), 1 µl of each primer, and nuclease-free water to a final volume of 25 µl. Amplification was performed on a thermocycler (Master Cycler Personal® - Eppendorf®, Germany) with an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 45 seconds, and extension at 72°C for 1 min. The amplified PCR products were resolved by electrophoresis [36].

The PCR products were separated by 1.5% w/v agarose gel electrophoresis in 0.5× Tris-acetate-EDTA buffer stained with GelRed. In the first well, a DNA molecular weight marker was added to estimate the sizes of the amplified fragments. Five microliters of each PCR product were combined with six-fold loading buffer, loaded into separate wells of the prepared gel, and then run on the electrophoresis device for sixty minutes at 120 V. After staining with GelRed, the PCR products' band sizes were examined by viewing the appropriate DNA bands in the red-stained agarose gel under a UV transilluminator [37].

Antibiotic resistance test

Following molecular confirmation, antimicrobial susceptibility testing of *S. aureus* against eight antimicrobial disks was performed using the Kirby-Bauer disk diffusion method, following the standard procedure of the Clinical and Laboratory Standards Institute (CLSI). All identified *S. aureus* isolates were evaluated with the following antibiotics: erythromycin (E-15µg), streptomycin (S-10µg), gentamicin (G-10µg), tetracycline (TE-30µg), chloramphenicol (C-30µg), penicillin G (P-10µg), sulfamethoxazole (SXT-25µg), and kanamycin (K-

30µg) (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom).

Colonies isolated from pure culture were transferred into a 5 mL test tube of Brain Heart Infusion Broth. The turbidity of the incubated broth was adjusted with sterile saline or by adding more isolated colonies to match a 0.5 McFarland standard. A Mueller-Hinton agar (MHA) plate was prepared, and a sterile cotton swab was dipped into the suspension and swabbed across the MHA plate surface. Antibiotic discs were then placed on the agar plate using sterile forceps and gently pressed to ensure complete contact with the agar surface. Plates were read 24 h after incubation at 37 °C under aerobic conditions. Isolates were classified as susceptible, intermediate, or resistant for each antibiotic tested, following CLSI guidelines and the manufacturer's instructions, by measuring the zone of inhibition around the antibiotic disc [38].

Data management and analysis

Microsoft Excel was used for data entry, computation of descriptive statistics, and graphing. The associations among variables collected during this study were computed using various statistical tools in SPSS version 27. Descriptive statistics such as percentages and proportions were used to analyze some of the data. The Chi-Square (X^2) test was used to compare the prevalence of *S. aureus* with associated risk factors at a 5% significance level. Antimicrobial resistance patterns were evaluated by comparing the zone of inhibition for each drug with 0.5 McFarland turbidity.

Results

Of 224 cows examined, 55 (24.6%) were CMT-positive, indicating mastitis (Table 1, Panel A). CMT positivity was 3/29 (10.3%) among cows with clinical mastitis and 52/195 (26.7%) among cows with subclinical mastitis. PCR was performed on 32 CMT-positive samples available for molecular testing, of which 21 (65.6%) were confirmed positive for *S. aureus* (Table 1, Panel B): 2/11 (18.2%) among clinical cases and 19/21 (90.5%) among subclinical cases. Because PCR was performed only on CMT-positive samples, the two panels are not directly comparable, and no statistical comparison between CMT and PCR is presented. Agarose gel electrophoresis of PCR products showed amplification of the *nuc* gene in *S. aureus*, producing the expected ~274 bp amplicon.

Table 1: Screening of mastitis by CMT and confirmatory identification of *S. aureus* by PCR.

Panel A: CMT screening (all cows, n = 224).

Mastitis type	No. examined	CMT-positive n (%)
Clinical	29	3 (10.3)
Subclinical	195	52 (26.7)
Total	224	55 (24.6)

Panel B: PCR confirmation of *S. aureus* among CMT-positive cows (n = 32).

Mastitis type	No. CMT-positive tested by PCR	PCR-positive n (%)
Clinical	11	2 (18.2)
Subclinical	21	19 (90.5)
Total	32	21 (65.6)

CMT = California mastitis test. CMT is a non-specific screening test for mastitis and does not identify *S. aureus*; PCR was performed on CMT-positive samples to confirm *S. aureus*. Percentages in Panel A are of cows examined; percentages in Panel B are of CMT-positive cows tested by PCR. The two panels are not directly comparable.

Management and husbandry factors associated with *S. aureus* prevalence

Several herd- and management-related factors were associated with *S. aureus* prevalence (Table 2). Cows managed under semi-intensive systems (33.9%) had a higher prevalence of *S. aureus* positivity than those under intensive management (13%) ($p < 0.001$). Manure-cleaning frequency also showed a clear pattern, with prevalence rising from 10.6% in barns cleaned four times daily to 20.4% in barns cleaned three times daily and 38% in barns cleaned twice daily ($p = 0.001$). In contrast, floor type was not associated with *S. aureus* positivity (14.9% on good floors, 32.1% on bad floors, and 24.8% on muddy floors; $p = 0.128$), nor was farm hygiene (19.8% on low-hygiene vs 27.3% on high-hygiene farms; $p = 0.209$). The use of individual towels during milking was also not associated with *S. aureus* positivity (23.5% where towels were not used vs 25.0% where they were used; $p = 0.814$).

Cow-level factors

Several cow-level factors were associated with *S. aureus* prevalence (Table 3). Crossbred cows had a higher prevalence than local cows (36.1% vs 10.8%; $p < 0.001$). Prevalence increased with parity, from 12.2% in cows with 1–2 parities to 19.5% in cows with 3–5 parities and 36.4% in cows with more than six parities ($p = 0.003$). Lactation stage showed a similar pattern, with prevalence rising from 12.1% in early lactation to 20.0% in mid lactation and 35.4% in late lactation ($p = 0.003$). Body condition score was also associated with prevalence, with 15.1% in cows with poor condition, 24.1% in cows with medium condition, and 34.7% in cows with good condition ($p = 0.023$).

Antimicrobial susceptibility of *S. aureus* isolates

The antimicrobial susceptibility profile of the 21 PCR-confirmed *S. aureus* isolates showed that all isolates were completely susceptible to gentamicin, chloramphenicol, and sulfamethoxazole. High resistance levels were observed for penicillin G (90.5%), streptomycin (81.0%), tetracycline (57.1%), and erythromycin (52.4%). Kanamycin showed moderate resistance (14.3%). These results highlight antimicrobial resistance among *S. aureus* isolates, particularly to commonly used antibiotics (Table 4). Among the 21 *S. aureus* isolates tested for antimicrobial susceptibility, 13 (61.9%) exhibited multidrug-resistant profiles, defined as resistant to three or more antibiotic classes (Table 5). The isolates displayed eight distinct resistance patterns, with PEN–STR–ERY, PEN–STR–KAN, and PEN–TET–STR the most frequent. Although the patterns varied in frequency, a chi-square goodness-of-fit test indicated that these differences were not statistically significant ($\chi^2 = 1.57$, df = 7, $p = 0.97$). This suggests that no single resistance pattern predominates significantly over the others among the MDR *S. aureus* isolates (Figure 2).

Table 2: Association between herd and management-level factors and *S. aureus* positivity (univariable chi-square analysis, n = 224).

Variable	Category	No. tested	Positive n (%)	χ^2 (df)	*p*-value
Management system	Intensive	100	13 (13.0)	13.02 (1)	< 0.001
	Semi-intensive	124	42 (33.9)		
Floor type	Good	47	7 (14.9)	4.11 (2)	0.128
	Bad	56	18 (32.1)		
	Muddy	121	30 (24.8)		
Cleaning frequency	4× per day	47	5 (10.6)	13.50 (2)	0.001
	3× per day	98	20 (20.4)		
	2× per day	79	30 (38.0)		
Farm hygiene	Low	81	16 (19.8)	1.58 (1)	0.209
	High	143	39 (27.3)		
Individual towel use	No	68	16 (23.5)	0.06 (1)	0.814
	Yes	156	39 (25.0)		

n = number; % = percentage; χ^2 = Chi-square; df = degree of freedom

Table 3: Association between cow-level factors and *S. aureus* positivity (univariable chi-square analysis, n = 224).

Variable	Category	No. tested	Positive n (%)	χ^2 (df)	*p*-value
Breed	Local	102	11 (10.8)	19.17 (1)	< 0.001
	Crossbreed	122	44 (36.1)		
Parity	Few (1–2)	49	6 (12.2)	11.81 (2)	0.003
	Medium (3–5)	87	17 (19.5)		
	Many (> 6)	88	32 (36.4)		
Lactation stage	Early (1–3 months)	58	7 (12.1)	11.78 (2)	0.003
	Medium (4–6 months)	70	14 (20.0)		
	Late (> 7 months)	96	34 (35.4)		
Body condition score	Bad	73	11 (15.1)	7.58 (2)	0.023
	Medium	79	19 (24.1)		
	Good	72	25 (34.7)		

N = number; % = percentage; χ^2 = Chi-square; df = degree of freedom

Table 4: Antimicrobial susceptibility profiles of PCR-confirmed *S. aureus* isolates.

Antimicrobial class	Antimicrobial	Disc conc. (µg)	S%	I%	R%	95% CI for R%	*p*-value (R > 0)
Aminoglycoside	Gentamycin	10	21 (100)	0 (0)	0 (0)	0.0–16.1	1.0
	Kanamycin	30	16 (76.2)	2 (9.5)	3 (14.3)	3.1–36.3	0.013*
	Streptomycin	10	3 (14.3)	1 (4.8)	17 (81.0)	58.1–94.6	< 0.001**
Beta-lactam	Penicillin G	10	2 (9.5)	0 (0.0)	19 (90.5)	69.6–98.8	< 0.001**
Macrolide	Erythromycin	15	8 (38.1)	2 (9.5)	11 (52.4)	29.8–74.3	0.002**
Phenols	Chloramphenicol	30	21 (100)	0 (0.0)	0 (0.0)	0.0–16.1	1.0
Sulfonamides	Sulfamethoxazole	25	21 (100)	0 (0.0)	0 (0.0)	0.0–16.1	1.0
Tetracycline	Tetracycline	30	8 (38.1)	1 (4.8)	12 (57.1)	34.0–78.2	0.001**

S = susceptible; I = intermediate; R = resistant; CI = confidence interval; * $p < 0.05$; ** $p < 0.01$

Table 5: Multidrug resistance patterns of *S. aureus* isolates.

MDR	Resistance pattern	Isolate no	Percentage (%)
Three	PEN–STR–ERY	2	9.52
	PEN–STR–KAN	2	9.52
	PEN–STR–TET	1	4.76
	PEN–TET–KAN	1	4.76
	PEN–TET–STR	2	9.52
Four	PEN–TET–STR–ERY	1	4.76
	PEN–TET–STR–KAN	2	9.52
Five	PEN–TET–STR–KAN–ERY	2	9.52
Total MAR	–	13	61.9

MDR = Multidrug Resistance, PEN = Penicillin, STR = Streptomycin, ERY = Erythromycin, TET = Tetracycline, KAN = Kanamycin

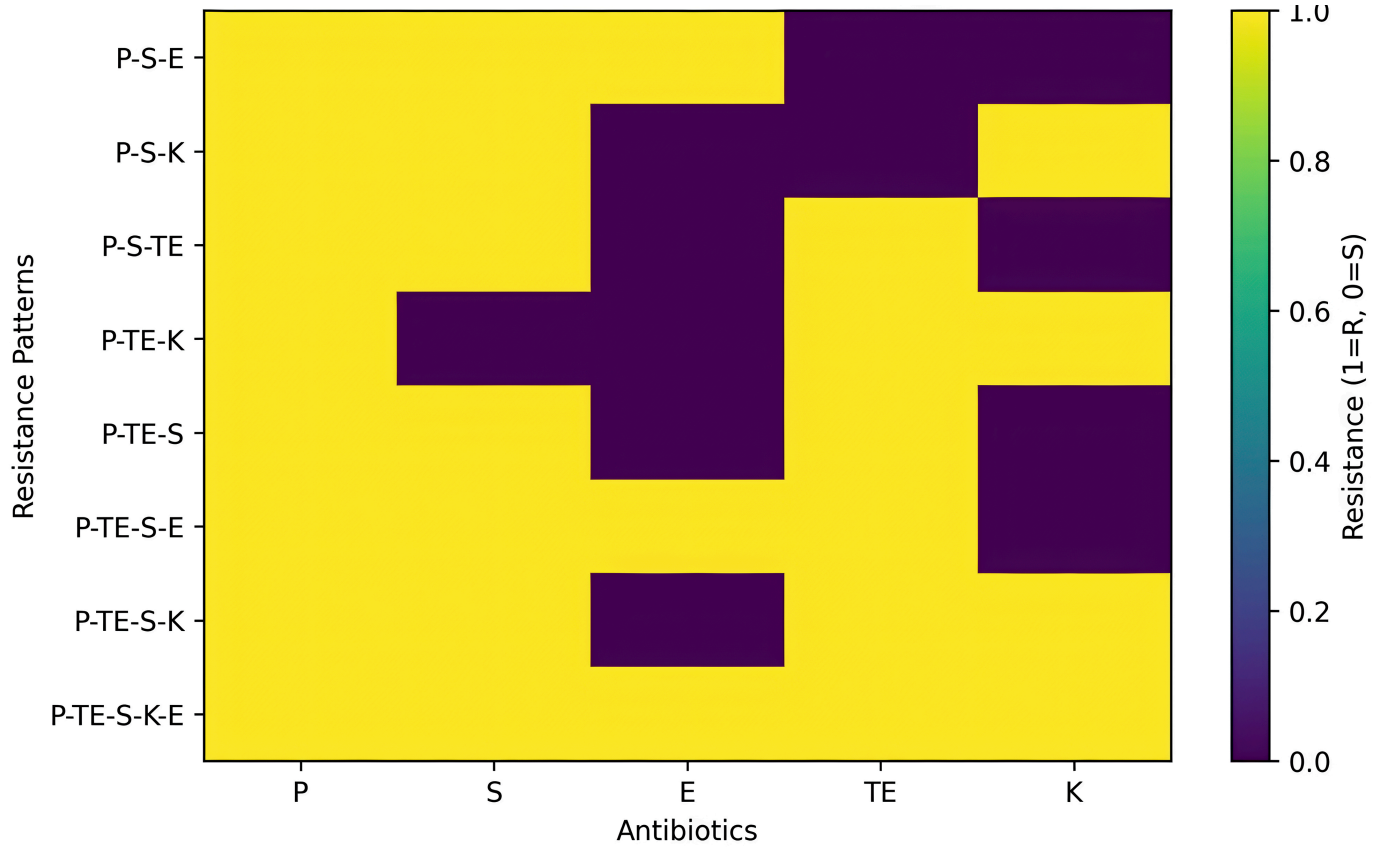

Figure 2: Heat map showing multidrug resistance (MDR) patterns of *S. aureus* isolates across tested antibiotics. Rows represent observed resistance combinations, columns indicate antibiotics, and shaded cells denote resistance presence (yellow) or absence (violet). P = Penicillin, S = Streptomycin, E = Erythromycin, TE = Tetracycline, K = Kanamycin.

Discussion

Bovine mastitis is one of the most prevalent and costly diseases affecting dairy herds, and *S. aureus* is among the pathogens most often responsible. The economic burden comes from decreased milk yield, treatment costs, and the early removal of affected cows from the herd. Beyond its impact on productivity, *S. aureus* mastitis also matters for public health, because the organism can enter the food chain through contaminated milk and dairy products [5, 7, 9]. The overall prevalence of *S. aureus* in lactating cows' milk in the study area was 24.6% (55/224; 95% CI: 19.38–30.59), consistent with previous reports from Debrezeit (29.1%), the sub-cities of Addis Ababa (27% and 24.6%) [39], and Hawassa, South Ethiopia (25%) [40]. Comparable prevalence rates were also documented in central Ethiopia (21%) [41] and Sebeta town (23.4%) [42]. Conversely, the current prevalence is higher than that reported from Mukaturi and Sululta (16.6%) [43], Holeta town (13.8%) [44], Malaysia (12.4%) [45], Asella (14.9%) [46], Italy (12.9%) [47], Gujarat, India (10.16%) [48], Iran (5.8%) [49], and China (8.2%) [50]. These variations may reflect differences in cow breed, parity, lactation stage, sample size, geographical location, milking practices, environmental contamination, and the use of screening tests such as the CMT, which improve detection of subclinical infections. Additionally, *S. aureus* persistence

is supported by its virulence factors, antimicrobial-resistant genes, and environmental resilience.

However, the present study's prevalence of *S. aureus* was lower than that reported in Shinshicho town, Southern Ethiopia (38.75%), Asella (39.1%), Adama (42.1%), Holeta town (47%), and Addis Ababa (48.3%) [51, 52, 53, 54, 55]. Internationally, higher prevalence has been reported in Morocco (40%) [56], Palestine (36.9%) [57], Bangladesh (75%) [58], and India (61%) [59], which may reflect epidemiological or methodological variation [59].

In this study, *S. aureus* was more frequently associated with subclinical mastitis than with clinical cases, supporting previous observations that it primarily causes long-lasting subclinical infections [60, 61]. This characteristic facilitates bacterial shedding through milk and transmission to healthy cows during milking.

Housing and management practices influenced prevalence. Semi-intensive management systems had a higher prevalence than intensive systems (33.9% vs 13.0%; $p < 0.001$), consistent with results from Sululta and Mukaturi [43]. This may reflect greater exposure to environmental pathogens and less controlled hygiene during grazing. In contrast, floor type was not associated with *S. aureus* positivity in this study (14.9% on good floors, 32.1% on bad floors, and 24.8% on muddy floors; $p = 0.128$). This differs from a finding in Adama, Ethiopia, where muddy floors were associated with higher infection [62] and may reflect differences in flooring management, drainage, or the smaller number of cows sampled per floor category in the present study.

The frequency of cleaning cow houses was associated with infection. Cows housed in facilities cleaned twice daily had a higher prevalence than those cleaned three or four times daily (38.0% vs 20.4% vs 10.6%; $p = 0.001$), highlighting the critical role of regular manure removal in limiting bacterial contamination. Farm hygiene ($p = 0.209$) and the use of individual towels ($p = 0.814$) were not associated with prevalence, in contrast to reports from Shinshicho town [51], which found larger herds to be at higher risk.

At the individual cow level, breed, parity, lactation stage, and body condition score were associated with infection. Crossbred cows were more likely to be positive than local breeds (36.1% vs 10.8%; $p < 0.001$), likely due to anatomical and physiological differences, genetic susceptibility, and higher milk yield [63]. Prevalence increased with parity, from 12.2% in cows with 1–2 parities to 36.4% in cows with more than six parities ($p = 0.003$), reflecting cumulative udder stress, prolonged pathogen exposure, and reduced antibiotic efficacy in heavily secretory mammary glands [53]. Infection was also more frequent in late lactation than in early or mid-lactation (35.4% vs 20.0% vs 12.1%; $p = 0.003$), consistent with studies from Adama and Hawassa [62]. Late-stage cows may be more susceptible due to repeated milking, reduced body condition, and nutritional deficits. Body condition score was also associated with prevalence (15.1% in bad, 24.1% in medium, and 34.7% in good condition; $p = 0.023$), which may reflect the relationship between body condition and overall metabolic and immune status during lactation.

PCR confirmation identified *S. aureus* in 65.6% (21/32; 95% CI: 46.8–81.4) of the phenotypically positive isolates tested, consistent with the 68% reported in previous studies from Adaberga, Ambo, and Bishoftu [64]. Antimicrobial susceptibility testing showed 100% susceptibility to chloramphenicol, sulfamethoxazole, and gentamicin, and high susceptibility to kanamycin (76.2%), consistent with previous studies [65]. High resistance was observed to penicillin G (90.5%), streptomycin (81.0%), tetracycline (57.1%), and erythromycin (52.4%), supporting prior findings from multiple regions of Ethiopia and abroad [18, 26, 38, 41, 63, 66, 67, 68, 69, 70, 71]. High resistance to β-lactams is attributed to β-lactamase production, while widespread tetracycline resistance reflects its common use in veterinary and human medicine.

MDR *S. aureus* was observed in 61.9% (13/21) of isolates, including resistance to three, four, or five antibiotic classes, consistent with reports from Holeta town (62.34%) and Babile district (67.34%) [26, 72]. This highlights the urgent need for strict regulations on antibiotic use in animal production to limit the emergence and dissemination of resistant *S. aureus* strains [73].

Conclusion

The study demonstrated that *S. aureus* is a significant pathogen in dairy cows in the study area, contributing substantially to both clinical and subclinical mastitis. Its prevalence was associated with multiple farm-level and host-related factors, including management system, manure-cleaning frequency, cow breed, parity, lactation stage, and body condition score. Molecular analysis confirmed the presence of the *nuc* gene in most isolates, supporting phenotypic identification, while antimicrobial susceptibility testing revealed high resistance to commonly used antibiotics such as penicillin G, streptomycin, tetracycline, and erythromycin. The high proportion of MDR *S. aureus* isolates (61.9%, 13/21) underscores the persistence and spread of resistant strains within farms, posing serious challenges for effective mastitis control, animal health, and milk production.

To reduce the prevalence and impact of *S. aureus* and MDR *S. aureus* on dairy farms, it is crucial to provide targeted training for farmers, farm staff, and veterinarians on mastitis prevention, proper milking hygiene, and farm biosecurity. Implementing appropriate milking practices to minimize teat injury and cross-contamination should

be prioritized, along with careful consideration of individual cow factors, such as breed, parity, lactation stage, and body condition, when introducing animals to herds. Awareness campaigns should inform stakeholders about the risks of multidrug-resistant pathogens, and routine antimicrobial susceptibility testing should be performed to guide prudent antibiotic use, limit resistance development, and ensure effective treatment strategies. In addition, routine monitoring and record-keeping of mastitis cases are essential to support judicious antibiotic use and early intervention.

Declarations and supplementary information

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