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Posted Date: 2 October 2024

doi: 10.20944/preprints202410.0166.v1

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Article

Antimicrobial Susceptibility and Resistance Patterns of *Staphylococcus aureus* Isolated from Mastitic Cows in Hawassa City and Its Suburbs, South Ethiopia

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Abstract: Staphylococcus aureus is the primary cause of mastitis in dairy cattle globally and frequently exhibits multidrug resistance due to excessive antibiotic use on farms. This study aimed to isolate *S. aureus* from mastitis affected cows and evaluate its antimicrobial susceptibility. A total of 172 milk samples from confirmed mastitic cows were cultured using standard bacteriological methods. Forty-four *S. aureus* isolates were tested against 11 common antimicrobials using the Kirby–Bauer disk diffusion method. *S. aureus* was isolated from 51.2% of samples, with significant resistance observed against ampicillin (84.1%), penicillin (81.8%), tetracycline (36.4%), and amoxicillin-clavulanic acid (34.1%). MDR was noted in 43.2% of isolates. In contrast, *S. aureus* demonstrated complete susceptibility to ceftriaxone and gentamicin (100%), and high susceptibility to streptomycin (88.6%), erythromycin (88.6%), nitrofurantoin (72.7%), and cefotaxime (72.7%). The significant isolation rate of *S. aureus* and its MDR underscore the urgent need for enhanced veterinary practices and public health strategies. A comprehensive approach that includes improved management, ongoing education for veterinarians and dairy farmers, responsible antimicrobial usage, and regular monitoring of resistance is essential to tackle the escalating threat of antimicrobial resistance in bovine mastitis.

Keywords: antimicrobial resistance; bovine; culture; Ethiopia; mastitis; *S. aureus*

1. Introduction

Bovine mastitis, the inflammation of the mammary gland, is a major concern in dairy herds globally due to its economic repercussions, including reduced production and increased culling rates [1]. In Ethiopia, which has the largest cattle population in Africa, bovine mastitis significantly hampers dairy production, particularly in urban and peri-urban commercial farms. Prevalence rates vary from 3.9% to 73.7% across different regions, with an overall pooled prevalence of 43.6% [2].

Mastitis is primarily caused by various bacteria, with *Staphylococcus aureus* accounting for up to 76% of cases [3]. This pathogen reduces milk production and poses serious health risks to cattle [4]. Farmers often use antibiotics to treat mastitis [5], but overusing them can lead to antimicrobial-resistant (AMR) strains, financial losses, and diminished mastitis management benefits [6]. *S. aureus* has shown resistance to multiple antibiotics, including penicillin, amoxicillin, tetracycline, amikacin, gentamicin, and erythromycin, and concerns about drug residues are increasing [1].

The ability of *S. aureus* to develop antibiotic resistance complicates treatment and increases culling rates in affected herds. This scenario not only heightens the economic burden on dairy farmers through rising veterinary costs and reduced milk production but also raises consumer concerns about dairy product safety. AMR in *S. aureus* related to bovine mastitis is a complex issue, involving the production of β-lactamase enzymes [5], multiple resistance genes [7], virulence factors [8], biofilm production [9], horizontal gene transfer [8], and the misuse and overuse of antibiotics in dairy farming [10].

 doi:10.20944/preprints202410.0166.v1

In Ethiopia, *S. aureus* is the leading cause of bovine mastitis, accounting for up to 48.2% of cases [2]. However, research on the AMR profile of this bacterium in bovine mastitis is limited. Recent studies indicate resistance to oxacillin, amoxicillin, oxytetracycline, tetracycline, and sulfa [11–13]. There have been no recent investigations into the AMR profile of *S. aureus* in dairy farms around Hawassa, a key dairy production area in south Ethiopia. Regular AMR testing is vital for identifying resistant strains, guiding effective treatments, minimizing treatment failures, and preventing the spread of resistant bacteria within herds and to humans. Such testing enhances herd health management and promotes food safety and public health through responsible antibiotic use. An antibiotic sensitivity test is crucial for developing a careful and rational approach to antimicrobial treatment of mastitis in animals [5]. Thus, this study aims to isolate *S. aureus* from bovine mastitis cases and assess the antimicrobial susceptibility of the isolates.

2. Materials and Methods

2.1. Milk Sample Collection

A total of 172 milk samples were collected from 95 dairy farms in Hawassa City and its suburbs in southern Ethiopia, all from cows diagnosed with mastitis. Of these, 18 samples were from cows with clinical mastitis, while 154 were from those with sub-clinical mastitis. Aseptic procedures for sample collection, as outlined by National Mastitis Council [14], were strictly followed. Samples were collected before milking; udders and teats were cleaned and dried, and each teat end was scrubbed with a cotton pledge soaked in 70% ethyl alcohol. To prevent recontamination, the teats on the far side of the udder were scrubbed first, using a separate pledge for each teat. The first few streams of milk were discarded, and approximately 10 ml of milk was collected into a sterile universal sample bottle held horizontally. After labeling, each sample was placed in an icebox and transported to the microbiology laboratory at the Faculty of Veterinary Medicine, Hawassa University, where they were either cultured immediately or stored at 4 °C for up to 24 hours before culturing on standard bacteriological media.

2.2. Isolation and Identification of Staphylococcus aureus

S. aureus isolation and identification was performed according to National Mastitis Council (NMC) guidelines [14]. In refrigerated milk samples, bacteria can concentrate in the cream layer and form clumps with fat globules. To disperse the fat and bacteria, the samples were warmed to 25 °C for 15 minutes and shaken before plating on standard bacteriological media. A standard loop (0.01 ml) of each milk sample was streaked onto 7% sheep blood agar (Oxoid, Hampshire, England) using the quadrant streaking method. The plates were incubated aerobically at 37 °C for 24 to 48 hours. If no growth was observed after this period, the milk sample was reinoculated into enriched tryptone soya broth (Oxoid, Hampshire, England) to enhance bacterial growth. After incubation, plates were examined for morphological characteristics such as colony size, shape, color, and hemolytic properties. Presumptive colonies were selected, *sub cultured* on nutrient agar (Oxoid, Hampshire, England), and incubated aerobically at 37 °C for 24–48 hours to obtain pure cultures. Colonies were identified by their Gram reaction, cellular morphology (coccus or rod), arrangement, and the catalase test. The Gram and catalase-positive cocci were further characterized for mannitol fermentation on mannitol salt agar (Oxoid, Hampshire, England) before conducting a tube coagulase test. A sample was considered positive for *S. aureus* if at least one colony was identified as *S. aureus*.

2.3. Antimicrobial Susceptibility Test

An antimicrobial susceptibility test was performed on 44 randomly selected *S. aureus* isolates from a total of 88. The isolates were assessed against 11 antimicrobials commonly used to treat bovine mastitis in Ethiopia, employing the Kirby–Bauer disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. The identified *S. aureus* isolates were inoculated onto blood agar (Oxoid, Hampshire, England) and incubated at 37°C for 24 hours. The colonies were then transferred to 4-5 ml of tryptone soya broth (Oxoid, Hampshire, England) and

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incubated at 35-37°C until slight turbidity, typically within 2-8 hours, was observed. The turbidity was adjusted to the McFarland standard. A 100 µl suspension was spread on Mueller Hinton agar (HIMEDIA, India) using a swab, after which antimicrobial disks were placed aseptically on the agar. Incubation continued at 35°C for 24 hours, with S. aureus ATCC 25923 serving as a control. The following antimicrobial disks (HIMEDIA, India) and concentrations were tested: Amoxicillin-Clavulanic acid (30 µg), Ampicillin (10 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Erythromycin (15 µg), Gentamicin (10 µg), Kanamycin (5 µg), Nitrofurantoin (100 µg), Penicillin (10 µg), Streptomycin (10 µg), and Tetracycline (10 µg). The sizes of the zones of inhibition were interpreted as R (resistant), I (intermediate), and S (susceptible), taking into account the breakpoints reported by "Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals" [15].

3. Results

3.1. Bacterial Isolation

Of the 172 milk samples cultured, *S. aureus* growth was confirmed in 51.2%. Specifically, it was detected in 22.2% (4/18) of clinical mastitis samples and 54.5% (84/154) of subclinical samples. At the herd level, the isolation rate was 52.6% (50/95) (Table 1).

Table 1. Isolation rate of *Staphylococcus aureus* from clinical and sub-clinical mastitis.

Form of Mastitis	Number of milk samples cultured	Number of positive samples	Isolation rate (%)
Clinical	18	4	22.2
Subclinical	154	84	54.5
Total	172	88	51.2

3.2. Antimicrobial Susceptibility Test

Of the 88 *S. aureus* isolates from clinical and subclinical mastitis, antimicrobial susceptibility tests were conducted on 44 (50%) isolates against 11 antimicrobials. The results indicated that all the isolates exhibited resistance to one or more of nine antimicrobials tested. *S. aureus* was completely susceptible to ceftriaxone and gentamicin (100% each), and highly susceptible to erythromycin (88.6%), streptomycin (88.6%), cefotaxime (72.7%) and nitrofurantoin (72.7%). Conversely, the isolates showed high resistance to ampicillin (84.1%) and penicillin (81.8%), along with tetracycline (36.4%) and amoxicillin-clavulanic acid (34.1%) (Table 2).

Table 2. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis (n = 44).

Antimicrobial	Disc Content	Disk diffusion inhibition zone diameters (mm)		
		Resistant (%)	Intermediate (%)	Susceptible (%)
Ampicillin	(10 µg)	84.1	–	15.9
Amoxicillin-Clavulanic acid	(30 µg)	34.1	–	65.9
Cefotaxime	(30 µg)	0	27.3	72.7
Ceftriaxone	(30 µg)	0	0	100
Erythromycin	(15 µg)	2.3	9.1	88.6
Gentamicin	(10 µg)	0	0	100
Kanamycin	(5 µg)	9.1	36.4	54.5
Nitrofurantoin	(100 µg)	0	27.3	72.7
Penicillin	(10 µg)	81.8	–	18.2
Streptomycin	(10 µg)	0	11.4	88.6
Tetracycline	(10 µg)	36.4	13.6	50

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Mean	28.2	11.2	60.6

A total of 18 distinct patterns of antimicrobial resistance were identified. Out of the 44 isolates of *S. aureus*, 19 (43.2%) showed MDR, which involved three or more different classes of antimicrobials. The MDR patterns observed were penicillin-streptomycin-tetracycline, ampicillin-penicillin-streptomycin-tetracycline, ampicillin-kanamycin-penicillin-streptomycin, ampicillin-amoxicillin-streptomycin-tetracycline, ampicillin-amoxicillin-streptomycin-kanamycin-penicillin,ampicillin-erythromycin-penicillin-streptomycin-tetracycline, and ampicillin-amoxicillin-penicillin-streptomycin-tetracycline (Table 3).

Table 3. Percentage and frequency of antimicrobials resistance pattern of *S. aureus* (n=44) for selected antimicrobials agents.

No. of Antimicrobials	Name of Antimicrobials	Frequency	Percentage
1	Strep	2	4.55
2	Amp, Strep	2	4.55
	Amp, Amox	1	2.27
	Amp, Strep	1	2.27
	Amp, Pen	1	2.27
	Pen, Strep	1	2.27
3	Amp, Pen, Strep	6	13.64
	Pen, Strep, Tetra	4	9.09
	Amp, Amox, Pen	2	4.55
	Amp, Pen, Tetra	1	2.27
	Amp, Amox, Strep	1	2.27
4	Amp, Amox, Pen, Strep	7	15.91
	Amp, Pen, Strep, Tetra	8	18.18
	Amp, Kan, Pen, Strep	2	4.55
	Amp, Amox, Strep, Tetra	1	2.27
5	Amp, Amox, Kan, Pen, Strep	2	4.55
	Amp, Ery, Pen, Strep, Tetra	1	2.27
	Amp, Amox, pen, Strep, Tetra	1	2.27

Amp= Ampicillin; **Amox-cla**=Amoxicillin-Clavulanic acid; **Ery**=Erythromycin; **Kan**=Kanamycin; **Pen**=Penicillin; **Strep**=Streptomycin; **Tetra**=Tetracycline.

4. Discussion

Global studies consistently identify *S. aureus* as a common cause of bovine mastitis. In this study, *S. aureus* was isolated from over half (51.2%) of the cultured milk samples, including both clinical and subclinical cases. This rate exceeds those in previous studies: 30.6% [11], 33.05% [12], 15.52% [16], 46.5%[17], 5.5% [18], and 28.1% [19]. It also surpasses figures from other countries, such as 50% in Italy [9], 30.32% in Pakistan [10], 28% in India [20], and 11.3% in China [21]. However, a higher isolation rate of 60% and 76% were reported in Mexico [22] and south Africa [3], respectively. The variation in *S. aureus* isolation rates between the present study and previous ones can be attributed to various factors, including differences in cow breed and parity, lactation stage, udder and teat hygiene, herd size, milking practices, contamination of bedding materials used [23,24], as well as the presence of antimicrobial resistant genes [8] and virulence factors [7] that contribute to the persistence of the bacteria in the udder. Additionally, *S. aureus* is known for its robustness in different environmental conditions, enabling it to survive extreme temperatures and moisture levels, which greatly contributes to its persistence in dairy farm environments [7].

The study revealed significant variability in the antimicrobial susceptibility of *S. aureus* isolates from bovine mastitis. The isolates showed varying resistance to nine of the 11 antimicrobials tested. However, all of the *S. aureus* isolates were found to be 100% susceptible to gentamicin and ceftriaxone. The high rate of susceptibility to gentamicin is in quite agreement with studies in Ethiopia [12,18]

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that reported 100% susceptibility of *S. aureus* to gentamicin. However, other studies from elsewhere have reported a varying level of resistance to this drug from 36% to as high as 85.8% [9,25].

Ceftriaxone, a third-generation cephalosporin antibiotic, is effective against a wide range of bacterial infections in humans. It can target both Gram-positive and Gram-negative bacteria [26]. Although rarely used in veterinary medicine for bovine mastitis, this study discovered that all *S. aureus* isolates were susceptible to ceftriaxone. This suggests that ceftriaxone could be a potential treatment for bovine mastitis. However, further research is required to confirm its effectiveness and any potential complications.

In this study, erythromycin and streptomycin were the second most effective drugs, eliminating 88.6% of the tested *S. aureus* isolates. This finding is consistent with a previous study [18] that reported 100% susceptibility to erythromycin. However, it contradicts an Italian study [9] that found a high resistance rate of 98.7% for erythromycin. The susceptibility of *S. aureus* isolated from bovine mastitis to streptomycin varies. The high susceptibility rate observed in this study aligns with a previous Ethiopian study [13], which reported that 71.8% of the isolates were susceptible to streptomycin.

While some studies report *S. aureus* resistance to cefotaxime ranging from 58.8% to 100% [13,22], we found it to be the third most effective antimicrobial, with 72.7% of isolates susceptible. Cefotaxime, like ceftriaxone, is a third-generation broad-spectrum cephalosporin commonly used in veterinary medicine for treating bacterial infections in animals, particularly dogs and cats. The high rate of intermediate to full susceptibility indicates its potential as an antibiotic for treating bovine mastitis, especially given the prevalence of MDR bacteria, even though it is not routinely used for such infections in large animals [27].

Nitrofurantoin, which ranked as the third most effective antimicrobial, demonstrated the same susceptibility to *S. aureus* as cefotaxime. This synthetic chemotherapeutic agent is commonly used in veterinary medicine to treat urinary tract infections caused by bacteria such as *S. aureus* in small animals like dogs and cats [28]. However, its effectiveness in treating bovine mastitis has not yet been established. Nevertheless, the rarity of clinical resistance to Nitrofurantoin [28], coupled with the observation that *S. aureus* isolated from mastitic milk samples in this study exhibited a high rate of sensitivity, suggests that it may hold promise as an antimicrobial for bovine mastitis treatment.

Our investigation revealed a significant resistance of *S. aureus* to key beta-lactam antibiotics, with 84.1% of isolates resistant to ampicillin and 81.8% to penicillin. These findings align with prior studies in Ethiopia, which reported 97.4% to 100% resistance to penicillin [12,13], and in Mexico, showing 100% resistance to both penicillin and ampicillin [22]. Our results are also consistent with global systematic reviews of antimicrobial resistance in *S. aureus* from bovine mastitis, highlighting widespread resistance to penicillin [29]. Factors contributing to this high resistance include the production of β-lactamase (BlaZ) enzymes that inactivate these antibiotics, genetic mutations in the bacterial strains that affect antibiotic susceptibility [30], and the selective pressure from the extensive use of these antibiotics in veterinary practices. Additionally, the biofilm formation by *S. aureus* contributes to its persistence and resistance in the mammary gland tissue [31].

This study found that 34.1% of the *S. aureus* isolates developed resistance to amoxicillin-clavulanic acid, lower than the 75% reported in Ethiopia [11], and 42.5% & 64% in Pakistan [10,25] for amoxicillin alone. *S. aureus* from bovine mastitis often shows significant amoxicillin resistance, mainly due to β-lactamase production, an enzyme that degrades β-lactam antibiotics. This enzyme is usually encoded by transferable plasmids among bacteria, promoting the spread of resistance [32]. The lower resistance in this study is likely due to combining amoxicillin with clavulanic acid, a β-lactamase inhibitor that enhances the efficacy of the drug [30]. Supporting this, another Ethiopian study reported 100% susceptibility of *S. aureus* isolates to amoxicillin combined with clavulanic acid [18].

In this study, 36.4% of *S. aureus* isolates showed resistance to tetracycline, lower than the 66.7% [11] and 69.2% [13] reported in Ethiopia. Global research has identified varying resistance levels in *S. aureus* from bovine mastitis, with some regions reporting rates as high as 40-86.5% [9,22,33], all exceeding the current finding. The emergence of resistance genes and the misuse of antibiotics in veterinary medicine significantly contribute to this issue. Conversely, a very high susceptibility rate of 100% to tetracyclines has been noted in *S. aureus* from bovine mastitis [18]. This significant variation in tetracycline susceptibility rates across studies is likely attributed to the differing frequency of the drug's use in treating bovine mastitis in various regions.

Multidrug resistance (MDR) is defined as an isolate that is not susceptible to at least one agent in at least three antimicrobial classes [34]. Based on this definition, MDR was observed in 43.2% *S. aureus* in the present study that is alarming. This finding is considerably higher from the 11.6% finding in China [21], although it is somewhat lower than the 50% [10] and 52% [25] figures reported from Pakistan. In contrast, a study from Ethiopia reported that none of the *S. aureus* isolates tested were MDR [18].

MDR in *S. aureus* causing bovine mastitis poses a significant issue in veterinary medicine. Various factors contribute to this antibiotic resistance. Research shows that *S. aureus* isolates often harbor multiple resistance genes, including *mecA*, *tetK*, *blaZ*, and *aacA-aphD*, leading to significant antimicrobial resistance [7,8,10,12]. Additionally, *S. aureus* produces several virulence factors, such as hemolysins, leukocidins, enterotoxins, and superantigens, enabling it to evade the host's immune system and establish infections [8]. Notably, its strong biofilm formation further protects it from antibiotics and immune responses [9,20]. Furthermore, the misuse and overuse of antibiotics in dairy farming create selection pressure that fosters MDR strains [10]. *S. aureus* can also acquire resistance genes from other bacteria via horizontal gene transfer methods like conjugation, transformation, and transduction [8]. The present study found that a significant proportion of *S. aureus* isolates exhibited MDR. However, the use of culture methods and the absence of advanced techniques like molecular assays, due to financial constraints, hindered the detection of resistance genes and biofilm-producing strains. Addressing these limitations is crucial for future research on *S. aureus* antimicrobial susceptibility.

5. Conclusions

This study highlights that *S. aureus* is a significant cause of mastitis in dairy farms, isolating it from over half of mastitis-positive milk samples analyzed. The high isolation rate raises public health concerns regarding the consumption of raw milk and its products. Among the isolates tested, 39.4% showed varying resistance to nine out of eleven antimicrobials, with multidrug resistance (MDR) observed in 43.2% of *S. aureus* isolates. The highest resistance was noted against ampicillin and penicillin, while *S. aureus* was susceptible to gentamicin and ceftriaxone, followed by erythromycin and streptomycin. The significant isolation rate of *S. aureus*, coupled with considerable MDR to commonly used antimicrobials, underscores critical implications for veterinary practices and public health. Thus, a comprehensive approach integrating improved management, ongoing education for veterinarians and dairy farmers, prudent antimicrobial usage, and regular resistance monitoring is essential to combat the growing threat of antimicrobial resistance posed by *S. aureus* and other bacteria in bovine mastitis.

Author Contributions : Conceptualization, R.A. and H.H.; Data curation, H.H.; Formal analysis, R.A.; Funding acquisition, R.A. and H.H.; Investigation, H.H.; Methodology, R.A. and H.H.; Project administration, R.A.; Resources, R.A. and H.H.; Software, R.A.; Supervision, R.A.; Validation, H.H.; Visualization, R.A.; Writing—original draft, H.H.; Writing—review and editing, R.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: The authors are grateful to the owners of all dairy farms who participated in the study for their willingness and cooperation during sample collection.

Conflicts of Interest: The authors declare no conflicts of interest.

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