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Investigation of Antimicrobial Resistance and Virulence Genes among Methicillin-Resistant *Staphylococcus aureus* from Subclinical Mastitic Cows

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Abstract | Subclinical mastitis is one of the most common forms of mastitis at dairy farms. In addition to their economic importance, there is serious zoonotic potential due to the spread of bacteria and toxins through milk. The present study aims to determine Methicillin-Resistant *Staphylococcus aureus* (MRSA) antimicrobial resistance and virulence genes isolated from cases of cattle mastitis. A total of 260 milk samples were collected from apparently normal cows, and analysis indicated that 120 (46%) were positive in the California Mastitis Test (CMT). Specifically, we further aimed to apply various techniques, including conventional microbiological tests and molecular methods (amplification by *nuc* gene using polymerase chain reaction) to detect the presence of *Staphylococcus aureus*. Application of these techniques indicated that 41% The results of these techniques indicated that 41% were *S. aureus*. All isolates were subjected to cefoxitin, a surrogate marker for detecting the *mecA* gene. Phenotypically, all isolates were resistant to the cefoxitin disk, while genotypically, 30 (73%) carried the *mecA* gene and was considered MRSA. A total of 80% of isolates were resistant to Vancomycin, whereas 83% susceptible to sulfamethoxazole and Chloramphenicol. All 30 isolates were verified for the presence of virulence genes, including *hla*, *hlb*, and *eta*. The presence of *hla*, *hlb*, and *eta* was identified with a rate of 63.3%, 70 %, and 100%, respectively. Taken together, these findings revealed the MRSA-carrying bacteria in mastitis cases in cows, which could potentially transfer to humans and may pose antibiotic resistance or intolerance.

Keywords | Subclinical mastitis, Cows, MRSA, CMT, *mecA* gene, Antibiotic susceptibility tests

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INTRODUCTION

Bovine mastitis continues to be the most frequent and costly disease affecting dairy cattle due to its detrimental impact on health, welfare, and productivity (Naranjo-Lucena and Slowey, 2023).

Two forms of mastitis, including subclinical and clinical, are frequently reported; however, overt subclinical mastitis (SCM) signs are rarely seen in the milk or udder. Mastitis led to decreased milk output and an increase in somatic cells counts Williamson *et al.* (2022). On the other hand, clinical mastitis (CM) is characterized by quick onset, changed milk content, reduced milk production, and the emergence of basic, immediately identifiable signs of inflammation in the infected udder (Bude and Mengesha, 2021). One of the primary bacteria that causes mastitis in dairy cattle is *S. aureus* (Ali *et al.*, 2021). *S. aureus* is a facultative anaerobic,

gram-positive bacteria that is coagulase-positive, catalase-positive, and oxidase-negative. It is a spherical, non-spore-forming, non-motile bacteia with a diameter of 0.5 to 1.5 µm. *S. aureus* is a significant infectious agent affecting the population and the health field (Bitrus *et al*., 2018). Methicillin-resistant *Staphylococcus aureus* (MRSA), also referred to as resistant staph or superbug, is regarded as one of the primary bacteria that infect patients in hospitals and the general public.

The World Health Organization (WHO) has designated MRSA as a high-priority bacterium for additional study and management (World Health Organization, 2017).

Methicillin-resistant *Staphylococcus aureus* (MRSA) appeared, probably due to acquiring the *mecA* gene (Sangappa and Thiagarajan, 2012). However, *S. aureus* pathogenicity involves the production of virulence factors that help the bacterium evade host defenses, allowing the microorganism to colonize the mammary glands of ruminants (Turner *et al*., 2019). The prevalence of (MRSA) is the most significant issue, making *S. aureus* infections especially problematic (Abril *et al*., 2020). Cell wall-associated adhesins and toxins are just two of many virulence factors that Staphylococci have that help the bacteria evade the immune system and make infections more severe. However, most of these factors were originally identified in *S. aureus* (González-Martín, *et al*., 2020). Hemolysins are among the several exoproteins produced by *S. aureus*, facilitating the organism to proliferate and cause disease in its mammalian hosts (Dinges *et al*., 2000). While most strains identified from intramammary infection in bovine express β-hemolysin, sphingomyelinase, and α-hemolysin is cytotoxic, both toxins are known to promote *S. aureus* adherence to mammary gland epithelial cells (Zhang *et al*., 2018; Algammal *et al*., 2020).

Two exfoliative toxins (ETs) with different immunological profiles were found to create some strains of *S. aureus,* namely *eta* and *etb*. Additionally, they have been linked to a group of impetiginous staphylococcal infections known as staphylococcal scalded skin syndrome. Many molecular epidemiological studies have already been conducted on enterotoxigenic *S. aureus* isolated from sheep milk and food (Scherrer *et al*., 2004).

The class of serine proteases that includes exfoliative toxins exhibits remarkable substrate selectivity. This hydrolysis causes keratinocytes to dissociate in human and animal skin (Nishifuji *et al*., 2008). This study aims to identify the nature and genetics of *S. aureus* in mastitis cases and to underpin the genes driving the antibiotic resistance. The finding would be valuable information for both animal handlers and milk consumers.

MATERIALS AND METHODS

Samples collection

California Mastitis Test (CMT) was used to screen all 260 raw milk cows in Basrah province for subclinical mastitis from 21 November 2023 to 28 February. The milk was evaluated for *Staphylococcus aureus* using conventional and molecular methods.

Microbiological techniques

Bacterial isolation and identification

All samples were primarily submitted to the (CMT). The test relies on the reagent's interaction with the DNA of somatic cells found in milk (Philpot and Nickerson, 1991). Briefly, a tiny sample of milk was extracted from each udder quadrant using the white plastic paddle and gently mixed with 2 mL of the scam reagent. Depending on the degree of gel, within 20 seconds, based on how much gel formation has occurred, the test is concluded positive. The positive CMT samples were transported immediately to the Central Research Unit utilizing an icebox.

Upon laboratory arrival, the milk samples were inoculated in brain heart infusion broth and incubated overnight at 37°C.

Following preincubation, the samples were subcultured on mannitol salt agar (Himedia, India) and Chromogenic media (Pioneer/France). The plates were incubated aerobically for 24 hours at 37°C. Gram's staining was used to identify the suspected colonies on chromogenic agar.

Molecular techniques

The suspected isolates were confirmed by amplifying the *nuc* gene using PCR. By the instructions provided by the bacterial extraction kit manufacturer (Genaid, Korea), bacterial DNA was extracted. The primers were procured from Bioneer, Korea, and were specific to the *nuc* gene, which encodes a particular thermostable nuclease, Table 1.

Table 1: Sequence of primer for *nuc* gene with their manufacture.

Gene	Primer sequences (5→3)	Length	Product size	Source	Manufacturer
nuc	F: 5´- GCTTGCTATGATTGTGGTAGCC 3'	22	423 bp	(Wongboot *et al*., 2013)	Microgen/ Korea
	R: 5´- TCTCTAGCAAGTCCCTTTTCCA 3'	22			

Table 2: Sequence of primer for *mecA* gene with their manufacturer.

Gene	Primer sequences (5→3)	Length	Product size	Source	Manufacturer
mecA	F:5'-TCCAGATTACAACTTCACCAGG-3'	22	162bp	Stegger *et al.* (2012)	Microgen/ Korea
	R:5'-CCACTTCATATCTTGTAACG-3	20			

Table 3: Sequence of primers for *hla,hlb, eta* genes and their expected products.

Gene	Oligonucleotide sequences (5→3)	Length	Amplicon size (bp)	References
hla	F:5- CTGATTACTATCCAAGAAATTCGATTG-3'	27	209bp	Khodabux *et al.* (2023)
	R: 5-CTTTCCAGCCTACTTTTTTATCAGT-3	26		
hlb	F: 5- GTGCACTTACTGACAATAGTGC-3	22	309 bp	
	R: 5-GTTGATGAGTAGCTACCTTCAGT-3	23		
eta	F5-CGCTGCGGACATTCCAACATGG-3	20	676bp	
	R-5-TACATGCCCGCCACTTGCTTGT-3	20		

For the *nuc* gene, a total of 20µl of the PCR reaction mixture was prepared. Ten microliters of Green Master Mix, one microliter of each oligonucleotide primer, one microliter of DNA template, and seven microliters of nuclease-free water was used .PCR program was applied with 35 cycles of PCR conducted at 94˚C for 7 minutes and 58˚C for 30 seconds ,72˚C for 45 seconds and 72˚C for 7 minutes).

Phenotypic and genotypic detection of MRSA

To identify the MRSA phenotype, all identified *S. aureus* isolates were evaluated for antimicrobial sensitivity to cefoxitin (10µg) (NCCLS, 2023). The *mecA* gene was then found to molecularly validate MRSA isolates, Table 2.

Molecular detection of Methicillin-resistant *staphylococcus aureus* (MRSA) by (*mecA*) gene

Oligonucleotide primers for PCR amplification

By identifying the *mecA* gene using primer sequences shown in Table 2, MRSA isolates were molecularly confirmed.

The PCR reaction mixture for the *mecA* gene was prepared in a total amount of 20µl. One microliter of each oligonucleotide primer, one µl of DNA template, ten microliters of Green master mix, and seven microliters of nuclease-free water were combined. The thermocycling includes: 94˚C for 5 minutes and 30 cycles of (94˚C for 1 minute, 59˚C for 1 minute, 72 ˚C for 1 minute, and final 72 ˚C for 7 minutes).

Antimicrobial-susceptibility testing

Eight antibiotics were chosen for the antibiotic sensitivity assays based on their common use in the field. This test was conducted according to the method described earlier. (Bauer *et al.*, 1966). The antibiotic discs were provided by (Bioanalyse/ Turkey), including Erythromycin (30 µg), Gentamycin (30 µg), Ciprofloxacin (5 µg), Penicillin (10 µg), Tetracycline (10 µg), Vancomycin (10 µg), Sulfamethoxazole (10 µg), and Chloramphenicol(10 µg).

Molecular detection of virulence genes among MRSA isolates

Primers were employed to identify the virulence genes (including *hla, hlb*, and *eta*) in MRSA-positive isolates (Table 3).

A total of 20µl of the PCR reaction mixture for the *hla* and *hlb* genes was produced. With each of the oligonucleotide primers (0.5 µl), DNA template (2 µl), Green master mix (10 µl), and nuclease-free water (6 µl).

For the *hla, hlb* gene, a total of 20µl of the PCR reaction mixture was prepared, carrying 2 µl of DNA template, 0.5 µl of each oligonucleotide primer, 10 µl of Green Master Mix, and 6 µl of nuclease-free water.

The reaction mixture of (*eta*) consisted of 1 microliter of DNA template, 0.5 microliter of each oligonucleotide primer, 10 microliters of Green master mix, and 8 microliters of nuclease-free water.

The *hla* and *hlb* genes were amplified under the following conditions: 94˚C for 5 minutes, 35 cycles (94˚C for 30 seconds, 56˚C for 30 seconds, 72˚C for 30 seconds, and 72˚C for 5 minutes). Whereas, the (*eta*) gene was subjected to 30 cycles of PCR conditions, which included 95 ˚C for 5 minutes, 54 ˚C for 30 seconds, 72 ˚C for 30 seconds, and 72 ˚C for 5 minutes.

RESULTS

Subclinical mastitis detection

Based on CMT, a total of 120 (46.15%) samples showed positive results (Table 4; Figure 1).

Table 4: Percentage of subclinical mastitis according to CMT.

No. of samples	Positive results (%)	Trace (%)	Weak (%)	Distinct (%)	Strong (%)
260	120 (46.15)	8 (6.7%)	39(32.5%)	50(41.7%)	23 (19.1%)

Chi-square: 33.80, P-value < 0.001. Not all categories have the same distribution.

Table 5: Illustrate the number of *S. aureus* isolates detected by molecular detection, traditional microbiological methods, and cultural methods.

Total number of milk samples	Number of suspected isolates of *Staphylococcus aureus*				Confirmed isolates by using Molecular detection of *nuc* gene	
	Mannitol salts agar		CHROMagar™ Staph aureus			
No	No	%	No	%	No	%
260	110	53	80	31	41	16

Chi-square: 57.17, P < 0.001

Figure 1: Results of California mastitis test based on the degree of gel formation.

Staphylococcus aureus identification using PCR and standard microbiological methods

The percentage of suspected *S. aureus* isolates was confirmed using mannitol salts agar and CHROMagar™ *Staph aureus* was positive in 140 (53%) and 80 (31%), respectively.

Table 3 all suspected isolates were subjected to PCR targeting the *nuc* gene (Table 5, Figures 2 and 3).The molecular technique-based assay characterized *S. aureus* in 41 (16%) of samples.

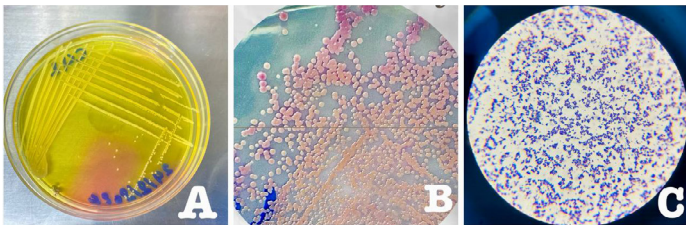

Figure 2: A: *Staphylococcus aureus* appeared as Golden yellow colonies on mannitol salts agar, B: Chromogenic agar: colonies seemed mauve (pink), C: Gram-positive cocci.

Methicillin-resistant *Staphylococcus aureus* can be identified phenotypically utilizing cefoxitin antimicrobial disk. All verified *S. aureus* isolates were resistant to a cefoxitin disk 41/41 (100 %) (Figure 4).

Figure 3: Electropherogram of *nuc* gene amplification. The PCR product was run on 1 % agarose gel, stained with ethidium bromide. L: mean Ladder and *nuc* gene product size approximately (423 bp).

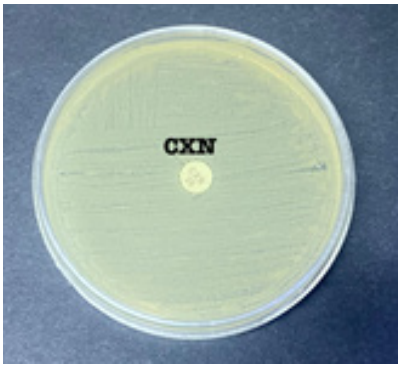

Figure 4: Cefoxitin disk test on muller Hinton medium.

Methicillin-resistant *staphylococcus aureus* (MRSA) isolates were detected using the *mecA* gene

All *S. aureus* isolates were submitted to molecular detection for the (*mecA*) gene using gene specific primers *of* 41 *S.aureus* isolates, only 30 isolates were *mecA* positive. The results are shown in Table 6 and Figure 5.

Table 6: Percentage of (*mec A*) gene in *S. aureus* isolates among subclinical mastitis.

No. of *S. aureus*	No. of *mec A* positive	
No	No	%
41	30	73

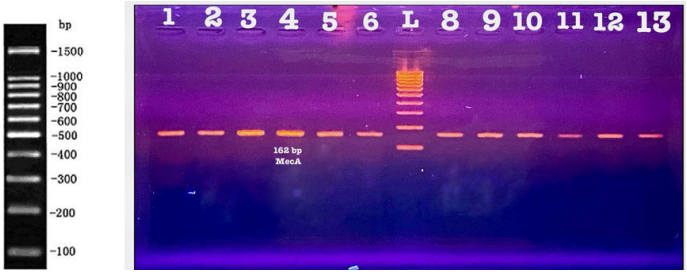

Figure 5: Electropherogram of amplification of *mecA* gene. The PCR products were run on a 1 % agarose gel stained with ethidium bromide. L: mean Ladder (Solarbio100 bp), *mecA* approximately (162 bp).

Antimicrobial-susceptibility testing

Eight antimicrobials were used to assess antibiotic sensitivity of *S. aureus* isolates, as shown in Table 7 and Figure 6. The isolates exhibited resistant to Vancomycin, Gentamycin, Penicillin, Erythromycin, and Tetracycline with percentages of (80%, 63.3%, 37%, 20 %, and 17%), respectively. In contrast, the isolates were vulnerable to *Sulfamethoxazole, Chloramphenicol, Tetracycline, Penicillin, Ciprofloxacin,* and *Gentamycin,* with a ratio of 83%, 83%, 67%, 60%, 47%, and 27%, respectively.

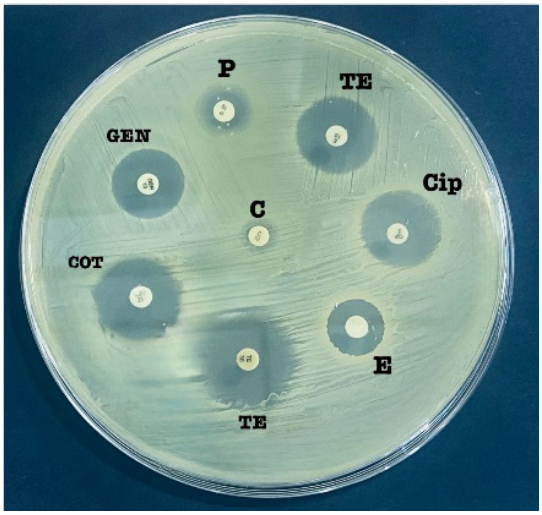

Figure 6: A: Antimicrobial susceptibility test on Muller Hinton medium.

Molecular detection of virulence genes (*hla, hlb* and *eta*)

Molecular detection of hemolysin alpha (*hla*), beta toxin (*hlb*) and Exfoliative toxin (*eta*)

The majority of prevalent virulence gene found in this investigation was *hlb* 70% (21/30), which was followed by *hla* 63.3% (19/30), as shown in Figures 7 and 8 the *eta* gene percentage in this investigation was (30/30) 100%.

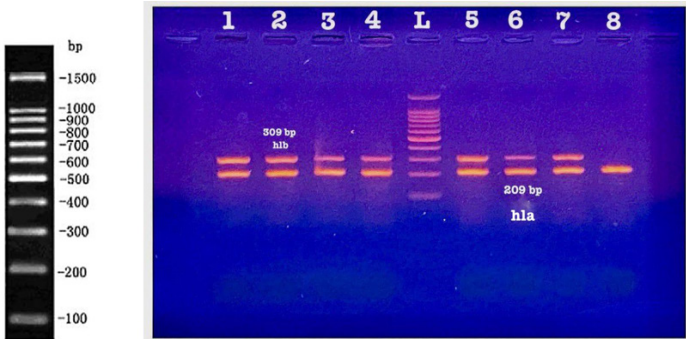

Figure 7: Electropherogram of amplification of *hla and hlb* genes. The PCR products were run on a 1 % agarose gel stained with ethidium bromide. L: mean Ladder (Solarbio100 bp), (*hla 209 bp, hlb*:309 bp approximately).

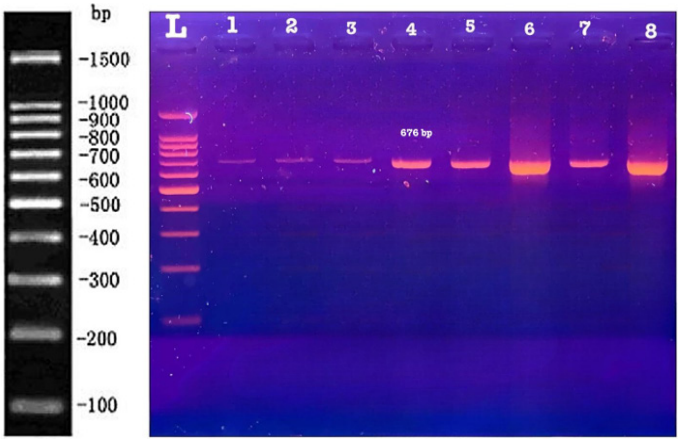

Figure 8: Electropherogram of amplified *eta* gene. The PCR products were run on a 1 % agarose gel stained with ethidium bromide. L: mean Ladder (Solarbio100 bp), (*eta:* 676 bp) approximately.

Table 7: Antibiogram of *S. aureus* isolates (n=30) against (8 antimicrobial agents).

Antimicrobial class	Antimicrobial agent	Code	Quality (µg)	*S. aureus* antibiotic resistance pattern		
				Resistant	Intermediate	Sensitive
Aminoglycosides	Gentamycin	GEN	30µg	19 (63.3%)	3 (10%)	8 (27%)
Amphenicol	Chloramphenicol	C	10µg	3(10%)	2 (7%)	25 (83.3%)
Fluoroquinolones	Ciprofloxacin	CIP	5µg	1 (3.3%)	15 (50%)	14 (47%)
Glycopeptides	Vancomycin	VA	10 µg	24 (80%)	4 (13.3%)	2 (7%)
Macrolides	Erythromycin	E	30µg	6 (20 %)	18 (60%)	6 (20%)
Sulfonamides	Sulfamethoxazole	COT	10µg	2 (7%)	3 (10%)	25(83.3%)
Tetracyclines	Tetracycline	TE	10µg	5 (17%)	5 (17%)	20 (67%)
β–lactam	Penicillin	P	10µg	11 (37%)	1 (3.3)	18 (60%)

DISCUSSION

The infection of the mammary gland, known as bovine mastitis, is one of the most serious diseases affecting dairy herds globally because of its financial implications. It causes considerable losses in decreased production and culling rates (Azooz *et al*., 2020; Sharun *et al*., 2021). Based on clinical features, clinical and subclinical mastitis can be differentiated. The former is characterized by watery discharges, clots, or flakes in milk, and the affected areas are usually swollen, hot, and unpleasant.

It is more difficult to diagnose subclinical mastitis because there are no outward signs in animals or milk. Its primary signs are decreased milk supply and an increased somatic cell count. Subclinical duration lasts longer than clinical, and infection allows the spread of pathogens within the herd (Cobirka *et al*., 2020). In this study, the California mastitis test results revealed that 140 (41.3%) samples tested positive for CMT.

In Iraq, studies including (Al-Iedani and Ghazi, 2016) recorded that the incidence of subclinical mastitis among cattle was 38.89% in Al-Sulaimaniyah Province. Compared with other studies conducted in Basrah, the detection rate of SCM using CMT ranged from 38% to 56.6% (Al-Iedani, 2016; Boss *et al*., 2016). The traditional microbiological methods used for *S. aureus* detection rely on enriching the milk sample in Brain heart infusion broth (BHI), differentiating it on Mannitol salts agar, and subculturing on Chromogenic agar. According to several studies, the pathogens most frequently present in mastitis cases are *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Escherichia coli, and Klebsiella pneumonia* (Morales-Ubaldo *et al*., 2023). The *S. aureus* isolation rate in this study from SCM was 16%. This rate was in line with a previous study in Nepal, which reported that 15.2% of CMT-positive milk was *S. aureus* (Király *et al*., 2024). *S. aureus* in our study is higher than in a study conducted by (Dive *et al*., 2013), 6(2.8%) cows with SCM were positive for *S. aureus*. Variations in the sample's attributes (size, season, type), isolation technique, and geographic locations may account for the discrepancy in *S. aureus* prevalence between this study and earlier research.

MRSA strains have emerged as formidable nosocomial pathogens and have disseminated globally due to their ability to develop antimicrobial chemotherapy resistance (Shittu *et al*., 2011). Although using antibiotics in modern medicine to treat infections caused by bacteria has changed the field, the indiscriminate, improper, and frequently abusive use of antibiotics has led bacteria to develop drug resistance (Chakraborty *et al*., 2022). Most of the world is plagued with bacteria and superbugs resistant to many drugs. Pathogens with antibiotic resistance dramatically increase the rates of morbidity and death.

Antimicrobial resistance is growing quickly and threatens to outpace the introduction of new antimicrobials (Christaki *et al*., 2020). Cefoxitin is phenotypically an alternative marker and potentially more sensitive for detecting methicillin resistance, as recorded in (Mohammed and Al-Iedani, 2020).

Consequently, (100%) of *S. aureus* isolates in the recent study were resistant to the cefoxitin disk. This result is higher than reported previously. Mohammed and Al-Iedani (2019), who *clarified that* the detection rate of MRSA using the cefoxitin disc diffusion method was 66.66%.

When it comes to MRSA isolation, one of the best methods is the detection of *mecA*. The *mecA* encodes the lowest β-lactam potential alters protein (PBP2a) found on SCCmec-resistant genomes (Jani *et al*., 2017). In the current study, the MRSA ratio according to the *mecA* detected by PCR was 73%. This rate agrees with a previous study (Ibrahim *et al*., 2023), where it is reported that the rate of MRSA according to the *mecA* detected by PCR was 86.66%.

In addition, Hammadi and Yousif (2013) have reported that 88% of *S. aureus* cases were MRSA; in contrast, Al-Jebouri and Mdish (2013) found only 10% of *S. aureus* cases to be MRSA.

By applying the disc diffusion technique, 30 *S. aureus* isolates were submitted to antimicrobial susceptibility tests against eight antimicrobial agents. According to the findings of the current study. *S. aureus* isolates were resistant to Vancomycin (80%) and 83% susceptible to Sulfamethoxazole and Chloramphenicol. These results are higher than reported earlier (Tassew *et al*., 2017), where it was reported that *S. aureus* isolates were resistant to Vancomycin in percentage (56.8%). Our findings are in agreement with another study Hoque *et al*. (2023), where it is reported that *S. aureus* isolates were susceptible to sulfamethoxazole in a higher percentage (77%).

Furthermore, this study found resistance against Gentamycin, Penicillin, and Erythromycin with decreasing rates of (63.3%, 37%, and 20%), respectively. These results are lower than those reported before Tassew *et al*. (2017), who have found resistance to penicillin (95.6 %), and in line with gentamycin (59.4%), On the other hand, the isolates were susceptible to Sulfamethoxazole, Chloramphenicol, Tetracycline, Penicillin, Ciprofloxacin and Gentamycin, with a ratio of 83%, 83%, 67%, 60%, and 47% and 27%, respectively.

These results are higher than those reported earlier (Shrestha *et al*., 2021), who found that Tetracycline was sensitive (48.3%).

In contrast, it has been found that maximum susceptibility to Ciprofloxacin is higher than in this study (92%) and similar to the percentage of Gentamycin in this study (69.23%) (Tassew *et al*., 2017). The source of isolates may cause minor variations in resistance, and usage of these antibiotics may be the cause of the ongoing rise in resistance.

Few studies investigated hemolysin genes in *S. aureus* strains isolated from mastitis milk of dairy species. (Aslantas *et al*., 2022). In the current study, *hla* (70.3%) and *hlb* (78%) genes in *S. aureus* isolates are lower than reported before (Acosta *et al*., 2018), with a higher percentage of *hla* (95.93%) and *hlb* 93.50%. In contrast, another study. Moraveji *et al*. (2014) reported the presence of *hlb* only in one of 20 isolates of *S. aureus* from bovine mastitis milk. Moreover, the percentage of exfoliative toxin (*eta*) in this study was 100%. This result disagrees with a previous study Yang *et al*. (2020), where it is clarified that none of the MRSA isolates carried the *eta* gene.

CONCLUSION

Our findings emphasize the critical presence of *S. aureus* on a wider scale and propose the importance of monitoring antibiotic resistance profiles before the application of antibiotics in animals.

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NOVELTY STATEMENT

The nature and genetics of *S. aureus* in mastitis cases, as well as the presence of virulence genes, underpin the driving of antibiotic resistance. The finding would be valuable information for both animal handlers and milk consumers.

AUTHOR'S CONTRIBUTION

NNJ, RAF: Supervision of writing review and editing.
ZAF: Data collection, formal analysis, writing original draft.
All authors contributed significantly to the research, discussed the results, and approved the final version of the manuscript.

Generative AI and AI-assisted technology statement

The authors have carefully reviewed all AI-assisted outputs to ensure accuracy, integrity, and compliance with the ethical standards of research and publication. Responsibility for the content of this manuscript remains entirely with the authors.

Conflict of interest

The author declares that there are no conflicts of interest related to this research. The study was conducted independently, without any financial, commercial, or personal relationships that could be construed as potential sources of bias. All experimental design, data collection, analysis, and interpretation were carried out solely by the author and collaborators within the academic framework.

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