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ORIGINAL ARTICLE

Prevalence and antibiotic susceptibility profile of *Staphylococcus aureus* in clinical and subclinical mastitis milk samples

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Abstract

Background: *Staphylococcus aureus* is the most commonly isolated bacterial pathogen in clinical and subclinical mastitis among cows. This study aimed to investigate the prevalence of *Staphylococcus aureus*, including MRSA (Methicillin-resistant *Staphylococcus aureus*), in cows with clinical and subclinical mastitis in Cumilla and Chattogram regions. Additionally, antimicrobial resistance patterns were identified.

Methods: A total of 429 milk samples were collected from different teats of 125 cows with clinical and subclinical mastitis across 15 farms. Standard bacteriological methods were applied to isolate and identify *Staphylococcus aureus*. Confirmation of *Staphylococcus aureus* was achieved through PCR for the presence of the *nuc* gene. Antimicrobial susceptibility testing was conducted for 11 antimicrobials. Isolates showing resistance to cefoxitin and oxacillin underwent *mecA* gene screening to identify MRSA. Multivariable logistic regression models were used to identify risk factors associated with the presence of *Staphylococcus aureus*

Results: The study revealed that 13.54% (95% CI, 5.00-38.78%) of mastitis-infected cows tested positive for *Staphylococcus aureus*. *Staphylococcus aureus* isolates showed the highest resistance to penicillin, cloxacillin, and streptomycin (61.54%, 53.85%, and 53.85%, respectively), while manifesting the least resistance to ciprofloxacin, ceftriaxone, and sulfamethoxazole + trimethoprim (92%, 77%, and 77%, respectively), and gentamycin (69.23%). The prevalence of MRSA was 2.08% (95% CI, 1.55-38.34%).

Conclusion: This study represents the first report of MRSA in clinical and subclinical mastitis milk samples in Bangladesh. The findings emphasize the importance of ongoing monitoring of *Staphylococcus aureus* and MRSA in mastitis cases, considering their prevalence and patterns of antimicrobial resistance.

Key Words: Prevalence, MRSA, AMR, PCR, *nuc* gene, *mecA* gene

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Introduction

Mammary gland inflammation is referred to as mastitis, and it represents one of the most significant challenges faced by the dairy industry, resulting in substantial financial losses. Mastitis can permanently disrupt a cow's ability to produce milk regularly (Islam *et al*., 2010). In Bangladesh, subclinical mastitis alone leads to an annual financial loss of Tk. 122.6 million (USD 2.11 million) (Kader *et al*., 2003). Mastitis in animals can be caused by various types of bacteria, mycoplasma, fungi, and algae, with coagulase-positive *Staphylococcus aureus* and coagulase-negative *Staphylococcus chromogenes* being among the most common culprits. *Staphylococcus aureus*, in particular, can cause a range of clinical conditions in animals, from mild skin infections to life-threatening bacteremia. Particularly, it is also a significant contributor to nosocomial infections in humans, and it has been associated with an increase in community-associated illnesses (Baggett *et al*., 2003; Zinderman *et al*., 2004). The prevalence of clinical mastitis varies from 12% to 30% across different countries, imposing a high cost of treatment and negatively affecting milk composition (Kalinska and Slosarz 2016). Cows with clinical mastitis produce less milk for the remainder of the lactation period compared to healthy cows (Hagnestam *et al*., 2008).In a herd of 100 dairy cows, mastitis can cost an estimated EUR 4896 annually, while subclinical mastitis affects 45% of a herd and costs between USD 180 and 320 per case (Wilson *et al*., 1997). Clinical mastitis occurring within the first 100 days of the first lactation increases the likelihood of culling by 34% (Hertl *et al*., 2014). The pathogens most commonly isolated from cow milk samples are *Staphylococcus aureus, Streptococcus agalactiae*, and *Mycoplasma bovis*, as well as environmental pathogens like *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Corynebacterium bovis*, coagulase-negative *Staphylococcus* (CNS) species such as *Staphylococcus chromogenes* and *Staphylococcus epidermidis*. Staphylococci and streptococci, in particular, are frequently associated with mastitis (Lassa *et al*., 2013). The widespread use of antibiotics has contributed to the development of antibiotic-resistant Staphylococci, which poses a significant concern (Vandenesch *et al*., 2003). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains have been a concern since the early 1960s and Mary Barber at 1961 find out the prevalence and antimicrobial susceptibility pattern of invasive Methicillin-resistant *Staphylococcus aureus* isolates at a Japanese general hospital (Barber, 1961).

Materials and Methods

Study design

This cross-sectional study was conducted in Sadar South, Chandina and Daudkandi Upazilas of Cumilla District, as well as the Mirsharai Upazila of Chattogram district. These areas include a range of large, medium, and small-scale dairy farms. The study was conducted from January to June 2022.

Data collection

A pre-tested questionnaire was used to collect epidemiological and clinical data relevant to the study. The questionnaire contained general information about each dairy cow, their California Mastitis Test (CMT) score subclinical mastitis, history, antibiotic and drug usage.

Milk sample collection

After washing the udder, 1 ml milk from each quarter in the CMT tray was collected. CMT positive udder/udders milk 3-5 ml was also collected after disinfect the udder with 70% alcohol spray in falcon tube.

CMT Procedure

In the collected milk of CMT tray, 1 ml of CMT reagent was added and shaking well. In positive case viscosity develop. Very light viscid sample is CMT score 1, light viscid sample is CMT score 2, medium viscid sample is CMT score 3, deep viscid sample is CMT score 4 and clinical mastitis milk sample is CMT score 5.

S. aureus in clinical and subclinical mastitis milk samples

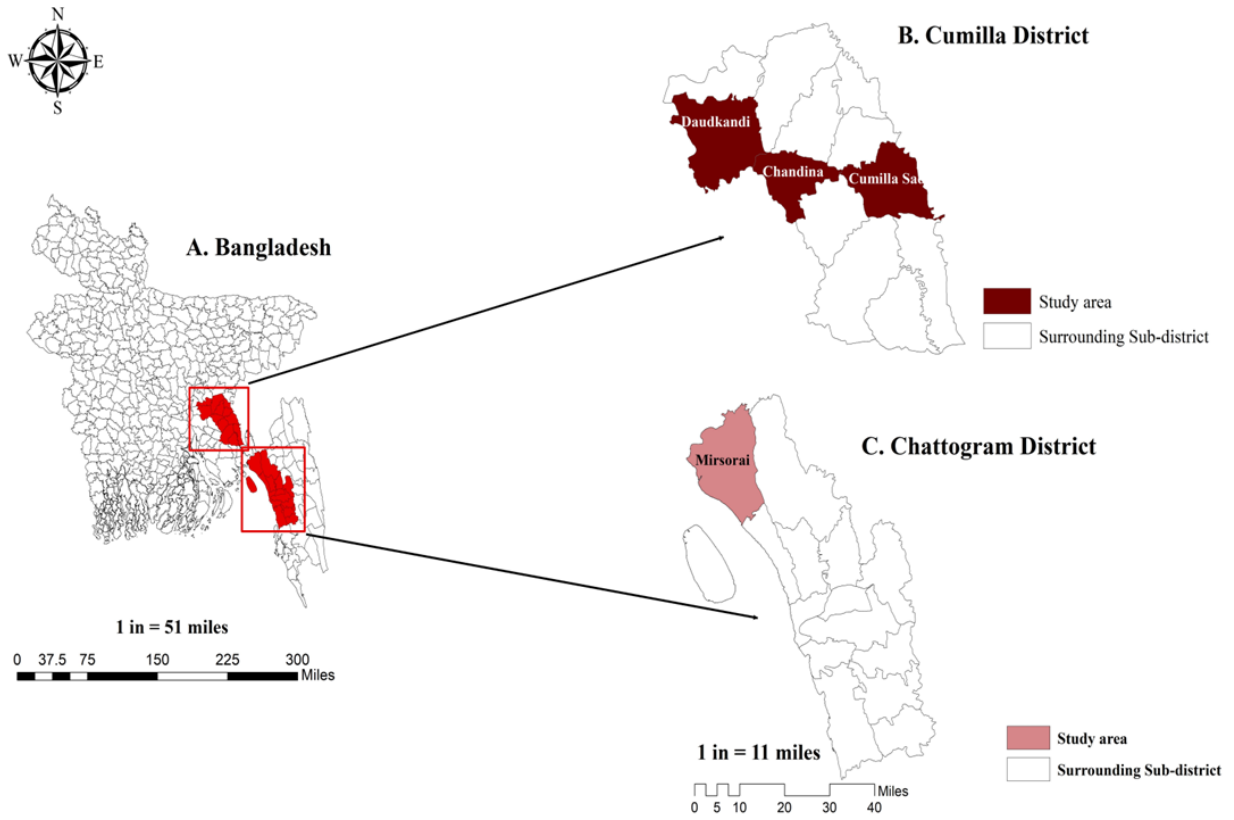

Figure 1: Map of Bangladesh showing study areas

Isolation and identification of *Staphylococcus aureus*

A total of 125 cows milk samples were collected from15 dairy farms in which 25(20%) cows are infected with clinical mastitis & 71(57%) cows tested positive with CMT scores ranging from 1 to 4. Subsequently, these samples were transported to the Department of Microbiology and Veterinary Public Health (DMVPH) laboratory at CVASU for further analysis. The milk samples were preserved in a deep fridge and incubated at 37°C for 6 hours before being streaked directly onto 5% bovine blood agar. They were then incubated for 24 hours at 37°C. Colonies displaying the characteristic appearance of Staphylococci on the blood agar plates were selected for initial phenotypic characterization. Any colonies that were catalase-positive and Gram-positive cocci were identified as Staphylococci. Suspected Staphylococci colonies were further cultured on Mannitol salt agar and incubated for 24 hours at 37°C, resulting in the production of bright yellow-colored colonies. These suspected positive colonies on Mannitol salt agar were then subculture onto blood agar and incubated at 37°C for 24 hours to assess their hemolytic properties.

Antimicrobial susceptibility test

The antimicrobial sensitivity testing of the obtained isolates was performed by Bauer-Kirby disc diffusion method with a panel of 11 antimicrobials (Bauer *et al*., 1966). The antimicrobials included amoxicillin, ceftriaxone, tetracycline, sulfamethaxozole + trimethoprim, ciprofloxacin, penicillin, cefotixin, oxacillin, maropenem, gentamycin and erythromycin. Muller-Hinton (MH) agar containing 2% NaCl was prepared according to the manufacturer's instructions (Oxoid). A bacterial turbidity equivalent to 0.5 McFarland standards was used as inoculum for each isolate evenly streaking on MH agar. The McFarland

standard was prepared by adding 0.5ml of 1% (1.75g/L) Barium Chloride to 99.5ml of 1% (0.36N) Sulfuric acid (Carter and Cole, 1990). After streaking antibiotic disc are placed and incubate as per protocol. For each isolate, the zone of inhibition around each disk was measured and interpreted as susceptible (S), intermediate (I) or resistant (R) according to CLSI document for veterinary pathogens (CLSI-2020). Methicillin resistance was determined by measuring zone diameter around oxacillin.

Table 1: CLSI 2020 guidelines for measurement of zone of inhibition during antimicrobial susceptibility test

Antimicrobial class	Antimicrobial agent	Disc content (µg)	Diameter of zone of inhibition (mm)		
			Susceptible	Intermediate	Resistant
β-lactam antibiotics	Penicillin	10 units	≥29	-	≤28
	Amoxicillin	10	≥29	-	≤28
	Oxacillin	1	≥18	-	≤17
	Cefoxitin	30	≥22	-	≤21
	Meropenem	10	≥22	-	≤21
Cephalosporin	Ceftriaxone	30	≥22	-	≤21
Tetracycline	Tetracycline	30	≥19	15-18	≤14
Aminoglycosides	Gentamicin	10	≥15	13-14	≤12
Quinolones	Ciprofloxacin	5	≥21	16-20	≤15
Macrolides	Erythromycin	15	≥23	14-22	≤13
Sulfonamides	Trimethoprim + sulfamethoxazole	1.25+23.75	≥16	11-15	≤10

Polymerase chain reaction (PCR)

Confirmation of *Staphylococcus aureus* was performed through PCR using an Applied Biosystem 2720 thermal cycler, following the protocol described by Sasaki *et al*. (2010). Bacterial DNA was extracted by boiling method. A loop full of fresh colonies (about 3/4) was picked from blood agar and transferred to 1.5 ml Eppendorf tube containing 200µl de-ionized ultrapure nuclease free water. The tubes were then vortexed to make a homogenous cell suspension. A ventilation hole was made on the top of each tube so that excess vapors were removed during boiling. Then the tubes were boiled at 99°C for 15 minutes in a hot water bath. Immediately after boiling, the tubes were placed into -20˚C for 5 minutes. After freezing, the tubes were again placed into a hot water bath at 99°C for 10 minutes and the boiled tubes were placed into -20˚C for 5 minutes. Thus allowed the cell wall to break down to release DNA from the bacterial cell. Finally, the tubes with the suspension were centrifuged at 10000 rpm for 10 minutes. Then 100 µl of supernatant containing bacterial DNA from each tube was collected and preserved at -20°C until used. Oligonucleotide primers *nuc*-F (GCGATTGATGGTGATACGGTT) & *nuc*-R (AGCCAAGCCTTGACGAACTAAAGC) used for detection of *nuc* gene (thermonuclease gene characteristics of *Staphylococcus aureus*). The annealing temperature was 56°C and bp size was 359. PCR reactions were conducted with a 25 µl reaction volume (Table 1). The cycling conditions are shown in Table 2. A total of 30 cycles were run.

S. aureus in clinical and subclinical mastitis milk samples

Table 2: PCR reaction mixture for the detection of *nuc* gene of *Staphylococcus aureus*.

SL No.	Contents	Volume
1	Master mix	12.5 µl
2	Forward primer	0.5 µl
3	Reverse primer	0.5 µl
4	Nuclease free water	9.5 µl
5	Extracted DNA	2 µl
6	Total	25 µl

Table 3: Cycling conditions used for PCR detection of *Staphylococcus aureus*.

SL No.	Steps	Temperature and time
1	Initial denaturation	95°C for 2 minutes
2	Final denaturation	95°C for 30 seconds
3	Annealing	56°C for 30 seconds
4	Initial extension	72°C for 30 seconds
5	Final extension	72°C for 2 minutes
6	Final holding	4°C for infinity

Visualization of amplified PCR products by agar gel electrophoresis

A gel tray was assembled with setting proper teeth sized gel comb in the tray. Then 1% agarose gel was prepared for electrophoresis of PCR- amplification products by mixing 0.5 gm of molecular grade agarose powder with 50 ml of 1X TAE buffer in a conical flask. Then the solution was boiled in a microwave oven for 2 minutes. The melted agarose was cooled at 40- 50°C in a water bath, having added with 5 µl ethidium bromide at a concentration of 5pg per ml. Finally, the melted agarose was poured into the gel tray and placed the comb and allowed about twenty minutes to stand for solidification of the gel. The gel was placed in an electrophoresis tank, already filled in with 50ml of lx TAE buffer. Then 5µl of each of the PCR products was loaded to gel-holes. One hole was loaded with DNA marker (Thermo Scientific O'Gene Ruler 1 kb plus) to compare the amplified size of the gene product. Negative and positive controls were used in each electrophoresis run. Electrophoresis was run at 100 volts and 80 mA for 35 minutes.

Data Analysis

The data from the field and laboratory were gathered and organized into the Microsoft Excel 2013 (USA) spreadsheet. The 95% confidence interval was estimated using binomial exact calculation. Following this, the data were analyzed for multivariate regression using SPSS v26 (IBM, USA). All the relevant independent variables associated with the dependent variable were chosen for univariable analysis. In the context of a univariable analysis using the Chi-square test, when more than 20% of the cells had an expected count of less than 5, the Fisher's exact test was employed instead of the conventional univariable Chi-square ($\chi 2$) test. The univariable logistic analysis of "risk factor" of herd level was performed initially. Significant variables ($p \leq 0.05$) from the univariable analysis were selected for a multivariate logistic regression analysis. After developing the final model, significant Likelihood ratio test statistic (LRTS) values were retained. The dependent (Outcome) variable was dichotomized, indicating PCR positive (1) and PCR negative (0). For the final regression analysis, backward stepwise regression was used to fit the model. Biologically plausible risk factors with significance ($p \leq 0.05$) were retained in the final step. Collinearity was assessed using Fisher's exact test, and variables with a significant value ($p \leq 0.05$) were considered collinear. To evaluate the "goodness-of-fit" of the final model, the sensitivity was assessed using the Hosmer-Lemeshow test.

Geo-spatial mapping and plot

ArcMap 10.7 was used to create a detailed study area map, providing important spatial context for this study. Subsequently, OriginPro software was used to create informative Venn diagrams and heat maps, which offered clear and concise visualizations of data, particularly with respect to specific attributes. To assess the relationships among isolated samples, dendrogram was constructed which enabling us to estimate Pearson's correlation, particularly in the context of antibiogram profiles. Additionally, genotype data was integrated into the Venn diagrams, specifically focusing on isolated *Staphylococcus aureus*, *mecA* and *nuc* genes.

Results

Descriptive statistics

Out of the 96 mastitis affected cows (25 cows with clinical mastitis and 71 cows with subclinical mastitis), 13 cows were found to carry *Staphylococcus aureus*, representing a prevalence of 13.5% (95% CI, 1.55-38.34%). Among the 25 cows with clinical mastitis, 2 cows tested positive for *Staphylococcus aureus*, resulting in a prevalence of 8% (95% CI, 1.55-38.34%). In contrast, among the 71 cows with subclinical mastitis which tested positive with the California Mastitis Test (CMT), 11 cows were found to carry *Staphylococcus aureus*, yielding a prevalence of 15.49%(95% CI, 55-90%).The prevalence of *Staphylococcus aureus* was observed to be highest in the left posterior quarter of the udder, with a prevalence of 17.39% (95% CI, 4.95-38.78%), followed by the front left quarter of the udder, where it was found in 12.5% (95% CI, 1.55-38.34%) of cases.

Antimicrobial susceptibility profiles of *Staphylococcus aureus*

All *Staphylococcus aureus* isolates (N=13) obtained from clinical and subclinical milk sample exhibited resistance to at least three different antimicrobials. The highest resistance was observed against Penicillin, Cloxacillin and Ampicillin (61.54%, %53.85 and 46.15%, respectively), as well as Cefotaxime and Tetracycline (38.46%). Conversely, the isolates showed maximum susceptibility to Ciprofloxacin, Ceftriaxone, and Sulfamethoxazole + Trimethoprim (92%, 77% and 77%, respectively) and Gentamycin (69.23%).

Prevalence of *mecA* gene in *Staphylococcus aureus*

The prevalence of *mecA* gene in *Staphylococcus aureus* is shown in Table 04.

Risk factors associated with the presence of *Staphylococcus aureus* in clinical and subclinical mastitis milk sample

Results of univariable logistic regression analysis to identify the risk factors associated with the frequency of *Staphylococcus aureus* in clinical and subclinical mastitis milk samples from different quarters of the udder in lactating cows are presented in Table 5.

S. aureus in clinical and subclinical mastitis milk samples

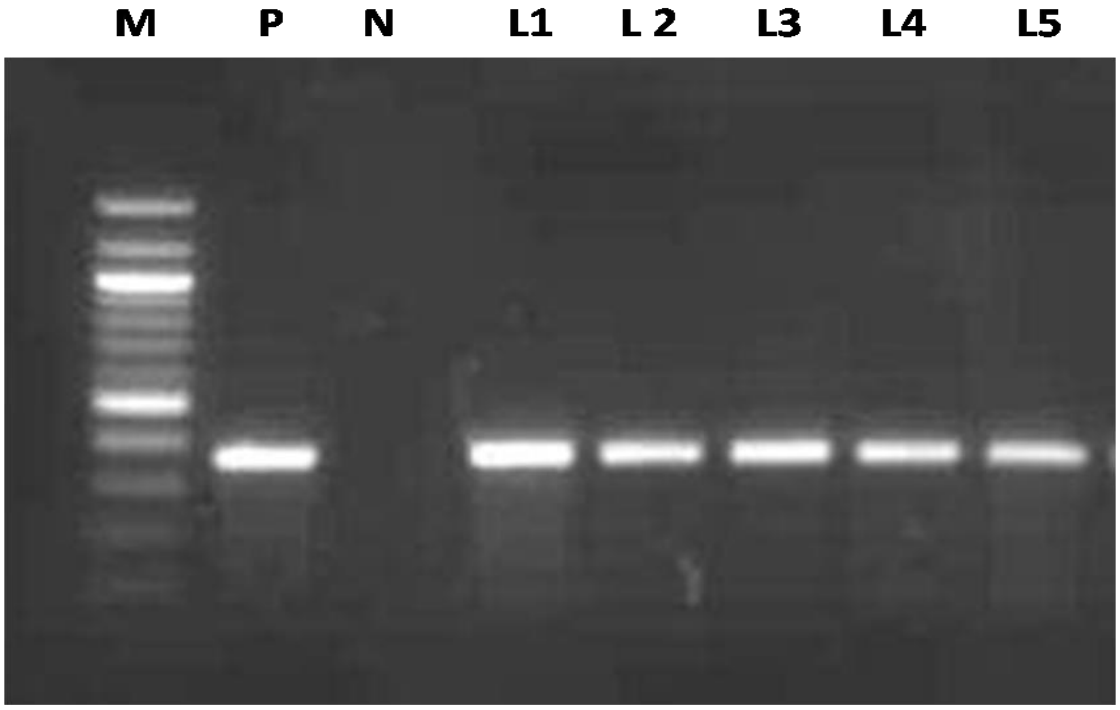

Figure 2: Electrophoresis on agarose gel showing the 359-bp PCR products after amplification with primers *nuc*F and *nuc*R. Amplifications were performed with chromosomal DNA from *Staphylococcus aureus* isolates. Lanes: M = 1 kb plus DNA Marker, P=Positive control, N=Negative control, L1-L5 = reaction specific for *Staphylococcus aureus*.

Table 4: Prevalence of *mecA* gene in methicillin-resistant *Staphylococcus aureus* isolates

Source of isolates	Number of *S. aureus* isolates	Oxacillin resistant isolates	*mecA* positive isolates	Prevalence (95% CI)
Clinical mastitis milk sample	2	1	0	0
Subclinical mastitis milk sample	11	6	2	18.18% (0.25-32)
Posterior Left (PL) Quarter	4	4	1	25% (0.16-30.23)
Front Left (FL) Quarter	2	2	1	50% (0.32-60.46)
Posterior Right (PR) Quarter	1	1	0	0
Front Right (FR) Quarter	0	0	0	0

CI: Confidence Interval

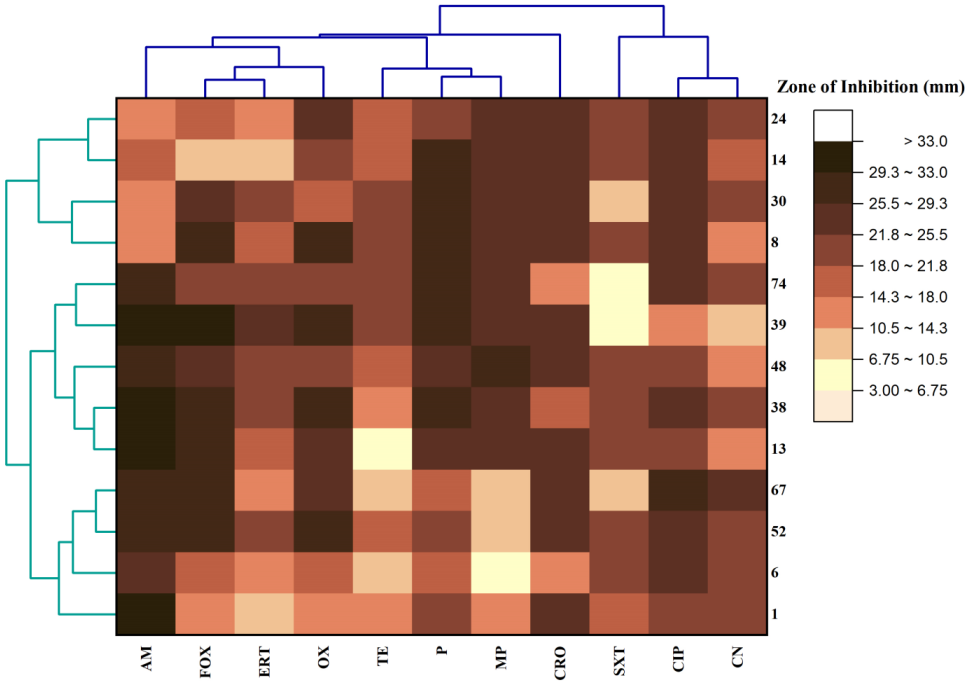

Figure 3: Heat map of antibiotic sensitivity test

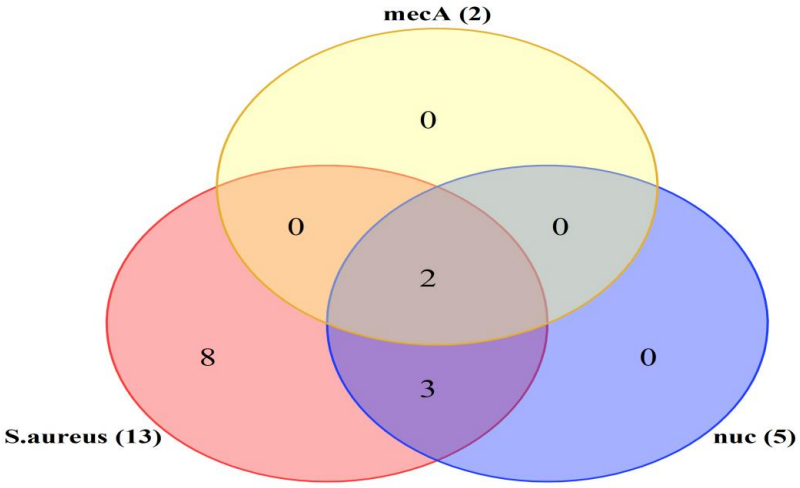

Figure 4: Inter relationship between *Staphylococcus aureus*, *nuc* gene and *mecA* gene

S. aureus in clinical and subclinical mastitis milk samples

Table 5: Univariable logistic regression analysis of risk factors for the presence of *Staphylococcus aureus* in both clinical and subclinical mastitis.

Variable	Categories	Tested	Positive (%) for *S. aureus*	Odds ratio (95% CI)	**P-value**
Bedding material	Yes	63	7 (11.11)	1	<0.01
	No	33	6 (18.18)	1.64 (0.59-4.47)	
Quarantine facility	Yes	34	3 (8.82)	1	<0.01
	No	62	10 (16.13)	1.83 (0.54-6.20)	
Isolation of sick animal	Yes	33	2 (6.06)	1	<0.01
	No	63	11 (17.46)	2.88 (0.69-12.24)	
Adequate drainage	Yes	78	10 (12.82)	1	0.028
	No	18	3 (16.67)	1.30 (3.98-4.25)	
Frequency of cleaning	Yes	72	7 (9.72)	1	0.081
	No	24	5 (20.83)	2.14 (0.75-6.13)	
Disinfectant use	Yes	28	1 (3.57)	1	<0.01
	No	68	12 (17.65)	4.94 (0.67-36.22)	
Foot bath	Yes	17	12 (17.65)	4.94 (0.67-36.22)	
	No	79	0 (0)	N/A	<0.01
Probiotic use	Yes	13	4 (30.77)	3.65 (0.93-14.32)	0.105
	No	83	9 (10.84)	1	
Vitamin and mineral supplement	Yes	38	4 (10.53)	1	<0.01
	No	58	9 (15.52)	1.47 (0.49-4.45)	

CI- Confidence interval

Discussion

The present study aimed to determine the prevalence and antimicrobial susceptibility profile of *Staphylococcus aureus* isolated from clinical and subclinical mastitis cases in the Cumilla and Chattogram regions.

In our current study, we observed a clinical mastitis frequency of 20%, which falls within the range of 12-30% reported in various countries by different authors (Kalinska and Slosarz 2016). Saeed *et al*. (2022) reported that an average of 16.5% of animals suffering from mastitis caused by *Staphylococcus aureus*, which was slightly higher than our findings of 13.54%. Our study also revealed that the left quarter is more susceptible to infection, with prevalence of 69.23%, while the right quarter showed a prevalence of 30.77%, which is consistent with these findings.

Regarding subclinical mastitis, our study reported a prevalence of *Staphylococcus aureus* is 15.49%, which was somewhat lower than previous studies and it was 31.4% (Saeed *et al*., 2022). Our findings align with those of other authors, demonstrating that *Staphylococcus aureus* infections are predominantly subclinical (15.49%), with a lower incidence in clinical mastitis (8%) (Janosi and Baltay 2004).

The prevalence of *Staphylococcus aureus* infections can vary based on factors such as breed, age, average milk production, and stage of lactation.

Some authors have noted that *Staphylococcus aureus* exhibits the greatest diversity of resistance, with all isolates in our present study displaying resistance to six antimicrobials, including Penicillin, Oxacillin, Cefoxitin, Tetracycline, Erythromycin, and Sulfamethoxazole. Our study also found that *Staphylococcus aureus* displayed high sensitivity to ciprofloxacin, consistent with the findings of Raviglione *et al*. (1990), who reported uniform susceptibility to ciprofloxacin (98.4%).

In our study, cefoxitin and oxacillin were employed as methicillin-resistant markers (Skov *et al*., 2006), revealing a prevalence of methicillin-resistant

Staphylococcus aureus (MRSA). Approximately 2.08% (2/96) of *Staphylococcus aureus* isolates tested positive for the *mecA* gene. This prevalence aligns closely with previous studies by Hendriksen *et al*. (2002) and Devriese *et al*. (1972), where the prevalence of MRSA was reported at 4.1%, demonstrating a similar trend in our current study. Variations in prevalence may be attributed to geographical factors and other risk factors, such as concurrent antibiotic use, chronic disease, and quarter variation, among others.

The management and treatment of MRSA infections pose a new challenge in veterinary medicine, particularly for dairy animals, given the limited therapeutic options available. Veterinarians should be aware of this zoonotic risk and take proper preventative measures to mitigate the transmission of MRSA from animals to humans, especially those handling dairy cows.

Conclusions

Our study revealed that 13.5% of the cows included in our research were carriers of *Staphylococcus aureus*. Interestingly, the prevalence of *Staphylococcus aureus* was notably higher among cows with subclinical mastitis compared to those with clinical mastitis. Moreover, when examining specific udder quarters, we found that the posterior left quarter showed the highest prevalence, closely followed by the front left quarter. We recommend implementing targeted management and intervention strategies aimed at minimizing the impact of *Staphylococcus aureus* infections in dairy cattle, thereby improving overall herd health and milk quality.

Acknowledgments

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Conflict of interest

The authors have declared no conflict of interest.

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