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Molecular identification, antimicrobial resistance and virulence gene profiling of *Staphylococcus* spp. associated with bovine sub-clinical mastitis in Bangladesh

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ABSTRACT

This study was conducted to investigate the diversity and antimicrobial resistance profiling of *Staphylococcus* species causing sub-clinical mastitis (SCM) in dairy herds in Bangladesh as well as putative risk factors associated with the infections. Individual quarter milk samples were collected from a total of 284 lactating cows from 30 dairy farms were screened by means of California mastitis test; 178 (62.7%) of them had at least of quarter affected by SCM. After conventional microbiological isolation procedures, PCR tests were used for *Staphylococcus* species identification and detection of antimicrobial resistance and virulence genes. *S. chromogenes* (65.7%) was the most predominant species followed by, *S. epidermidis* (20.2%), *S. haemolyticus* (19.1%), *S. aureus* (15.7%), and *S. sciuri* (5.6%). High levels of antimicrobial resistance to ampicillin and amoxicillin/clavulanic acid were observed in *S. aureus* (82.1% and 75%) and *S. sciuri* (80% and 70%), while resistance to cefepime was markedly higher in *S. chromogenes* (95.7%), *S. haemolyticus* (94.1%), and *S. epidermidis* (97.2%). Multidrug resistance isolates were identified in all five species. The *mec*A gene was detected in *S. aureus* (32.1%) and *S. chromogenes* (5.98%). In addition, 20% *S. sciuri* and 17.7% *S. haemolyticus* carried the cytotoxin (*pvl*) gene, while 14.3% *S. aureus* harbored the toxic shock syndrome toxin (*tst*) gene. Multivariable logistic regression analysis identified "Old aged" (OR [CI]: 3.5 [1–12.4]); "Early stage of lactation" (OR [CI]: 3.4 [1.2–9.7]) and, "Firm udder condition" (OR [CI]: 4.2 [1.2–14.6]) as risk factors associated with SCM caused by *S. aureus, S. chromogenes*, and *S. haemolyticus*, respectively. Moreover, "Use of antimicrobials" (OR [CI]: 10.4 [3.4–32.1] and "History of previous clinical mastitis" (OR [CI]: 4.9 [1.2–19.7] for the carriage of methicillin-resistant *Staphylococcus* spp.

1. Introduction

Sub-clinical mastitis (SCM) is the most frequent diseases in dairy farming across the world causing serious economic burden (Hoque et al., 2018). Although there is usually a significant decrease in milk production due to damage of the mammary gland, there are no clinical signs of mastitis compromising diagnosis (Simojoki et al., 2009 and De Buck et al., 2021). Although staphylococci are considered as major mastitis-causing agents responsible for clinical and SCM (De Visscher et al., 2017), it is worth nothing that the contagious *Staphylococcus aureus* and others species of coagulase-negative staphylococci (CoNS) such as *Staphylococcus haemolyticus, Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus sciuri,* and *Staphylococcus simulans,* are frequently isolated from bovine SCM cases (De Visscher et al., 2017; De Buck et al., 2021). Nonetheless, differentiation between species of mastitis-associated CoNS by conventional microbiological methods is laborious and time consuming (Capurro et al., 2009) due to the close phenotypic resemblance within the CoNS species. CoNS species can colonize the mammary glands tissue for prolonged periods of time or even throughout the lactation period being continuously shed into milk

Abbreviations: SCM, Sub-clinical mastitis.

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(Taponen et al., 2007; Taponen & Pyörälä, 2009). Furthermore, CoNS species can be highly transmittable (Ster et al., 2013and Schabauer et al., 2018) and rapidly spread within the dairy farm through the milkers as well as by milking vehicles like milking machine or instruments. In addition, most of the organisms encode important virulence factors, as well as multiple antimicrobial resistance genes (Hosseinzadeh et al., 2014; Frey et al., 2013, Zhou et al., 2017 and González-Martín et al., 2020) and cause persistent infection in mammary gland (Cervinkova et al., 2013 and Mahato et, al.,2017). Some CoNS strains exhibit poor response to a wide range of antimicrobials leading to low cure and high culling rates in the dairy farms (Hoque et al., 2018 and Schnitt et al., 2021). Consequently, the emergence and spread of antimicrobial resistant CoNS species, particularly multidrug resistant (MDR) strains is of utmost concern for both mastitis treatment, (Dabele et al., 2021) and public health (Dhaouadi et al., 2020). Therefore, the rapid identification of *Staphylococcus* species associated with udder colonization in dairy herds is key importance for the control of the infection (Piessens et al., 2011). In this regard, rapid DNA-based molecular diagnostic techniques can be highly reliable, accurate, and reproducible (Zadoks & Watts, 2009) towards the identification of staphylococci at the species level as well as virulence and antimicrobial resistance determinants. Although, there are several studies on the damaging effect and pathogenic significance of *S. aureus* (Hoque etal., 2018; Priyantha et al., 2021 and Rana et al., 2022a), but there is few information on the species-specific significance, virulence and antimicrobial resistance profile of CoNS associated with bovine SCM in developing region including Bangladesh. Therefore, the aim of the present study was to characterize *S. aureus* and CoNS and identify risk factors associated with their occurrence in dairy herds in Bangladesh.

2. Materials and methods

2.1. Ethical approval

Both field (SCM screening and sample collection) and laboratory procedures of the current study were performed with the permission of the Chattogram Veterinary and Animal Sciences University's (CVASU) Ethical Approval Committee [Approval no. CVASU/Dir (R&E) EC/2020/165 (1)].

2.2. Study design

Across-sectional study was conducted in the Cumilla district of Bangladesh (N 23.4607°, E 91.1809°) from January 2020 to September 2022. The majority of them used crossbred cows (Holstein-Friesian × Indigenous), Jerseys, and non-descriptive breeds. Only dairy farms having more than 10 lactating cows and a minimum average milk production of 50 liters per day were selected for this study.

2.3. Data collection

Pre-tested questionnaires (supplementary file-1) were used for the collection of all farm and animal related data including husbandry management, udder health information, all hygienic practices and antimicrobials used for the treatment of bovine mastitis. Finally, individual data from a SCM positive cow were gathered to determine further association of various risk factors with identified *Staphylococcus* spp and antimicrobial resistance.

2.4. Screening and diagnosis of SCM

California Mastitis Test (CMT) was performed for all the lactating cows on each dairy farm for the initial screening of SCM. The test and interpretation were performed according to the protocol described by Abebe et al. (2016). In brief, 2 mL of milk from each quarter was collected in CMT paddles, and an equal volume of CMT reagent was mixed for 60 s in a gentle circular motion. The CMT results were scored and interpreted as 0 (negative [healthy quarter], somatic cell count [SCC] ≤100,000 cells/ml milk), 1+ (weak positive, SCC >100,000–500,000 cells/ml milk), 2+ (distinct positive, SCC >500,000–1000,000 cells/ml milk), and 3+; (strong positive, SCC ≥1000,000 cells/ml milk). Finally, udder quarters showing a CMT score of 1+ were considered as positive for SCM. Furthermore, if any of the tested cows showed mixed CMT results (different scores) within the same udder from different quarters, we considered the maximum CMT score for grading SCM in cows.

2.5. Sample collection and transportation

After confirmation of SCM, approximately 10 mL of milk was collected from each CMT positive quarter by following the procedures of the National Mastitis Council (NMC), 2004. Before milk collection the teat of the infected quarter was cleaned using 70% ethanol, then fore milk has been discarded. After that, the samples were taken aseptically into the individual sterile plastic tubes for microbiological analysis. Finally, all milk samples were transferred under refrigeration to the Department of Microbiology and Veterinary Public Health, CVASU. Samplings were performed weekly by a trained veterinarian.

2.6. Isolation and identification of Staphylococcus spp

Microbiological analysis was performed within 12 h of sampling. Initially, all milk samples were directly incubated at 37°C for 8 h which enhanced the growth rate of mastitic organisms. Then, 20 μL of milk was streaked on 5% bovine blood agar (Oxoid Ltd., Cambridge, UK) and the agar plates were incubated at 37°C for 48 h. All agar plates were examined in every 24 h to check the optimum growth of desired organisms. Suspicious staphylococci colonies were initially identified based on appearance (round, smooth, raised, pigmented or non-pigmented, yellow or creamy white, presence or absence of hemolysis) and Gram staining. In addition, selected staphylococci colonies were further subcultured on Mannitol Salt Agar (MSA) (Oxoid Ltd., Basingstoke, UK), and incubated at 37 °C for 48 h. Subsequently, a catalase test was performed to differentiate the catalase-positive *Staphylococcus* spp. from the catalase-negative *Streptococcus* spp. and tube coagulase test was performed to distinguish coagulase-positive staphylococci (CoPS) from coagulase-negative staphylococci (CoNS) or non-*aureus* staphylococci (NAS) (Quinn et al., 1998).

2.7. Extraction of bacterial chromosomal DNA and multiplex PCR program

In the current study, we used a multiplex- polymerase chain reaction (m-PCR) assay for the species-specific confirmation of investigated *S. aureus, S. haemolyticus, S. chromogenes, S. epidermidis, S. sciuri,* and *S. simulans*. The chromosomal DNA of *Staphylococcus* spp. was extracted from freshly grown cultures by the crude boiling lysis method described by Hoque et al. (2018) with some modifications. All staphylococcal genomic DNA were investigated for the confirmation of species level by m-PCR targeting different species specific oligonucleotide primers (Table 1) as described previously by Shome et al. (2011). A total of 50 μL of PCR reaction mixture was thermally cycled once for denaturation at 94°C for 5 min, followed by 30 times at 94°C for 30 s; 60°C for 30 s,72°C for 45 s and then once for final extension at 72°C for 10 min. Bacterial species was confirmed based on PCR-amplified product size, and the amplicons were measured using a 1 kb plus DNA ladder on a 1.5% agarose gel (Promega Corporation, Madison, WI, USA) containing ethidium bromide. Previously confirmed *S. aureus* was used as positive control, while *S. pseudintermedius* (Rana et al., 2020b) was used as negative control for every m-PCR reaction. Finally, all genomic DNA and PCR confirmed pure isolates were cultured on brain heart infusion broth (BHIB) (Oxoid Ltd., Basingstoke, UK) and stocked at –80°C for further

Table 1
Primer sequences used for the amplification of different *Staphylococcus* spp.

Organism	Gene primer	Primer designation	Primer sequence 5′–3′	Amplicon size (bp)	References
S. aureus	23S rRNA	SAS2F	AGCGAGTCTGAATAGGGCGTTT	894	Shome et al., 2011
		SAS2R	CCCATCACAGCTCAGCCTTAAC		
S. haemolyticus	*sodA*	SHS1F	CAAATTAAATTCTGCAGTTGAGG	214	
		SHS1R	AGAGCCCCATTGTTCTTTGA		
S. chromogenes	*sodA*	SCHS1F	GCGTACCAGAAGATAAACAAACTC	222	
		SCHS1R	CATTATTTACAACGAGCCATGC		
S. epidermidis	*rdr*	SERF	AAGAGCGTGGAGAAAAGTATCAAG	130	
		SERR	TCGATACCATCAAAAAGTTGG		
S. sciuri	*gap*	SSCGF	GATTCCGCGTAAACGGTAGAG	306	
		SSCGR	CATCATTTAATACTTTAGCCATTG		
S. simulans	*gap*	SSMF	AGCTTCGTTTACTTCTTCGATTGT	472	
		SSMR	AAAAGCACAAGCTCACATTGAC		

analysis.

2.8. Antimicrobial resistance profile of diverse Staphylococcus spp

After PCR confirmation, individual staphylococcal isolates were subjected to susceptibility testing by means of standard disk diffusion method using eleven different antimicrobials (Oxoid Ltd., Basingstoke, UK) from eight different antimicrobial classes: amoxycillin/clavulanic acid (AMC) 30 μg, ampicillin (AMP) 10 μg, ceftriaxone (CRO) 30 μg, cefoxitin (FOX) 10 μg, cefepime (FEP) 30 μg, erythromycin (E) 15 μg, gentamicin (CN) 10 μg, tetracycline (TE) 10 μg, ciprofloxacin (CIP) 5 μg, trimethoprim/sulfamethoxazole (SXT) 25 μg and imipenem (IMP) 10 μg. The zone of inhibition of individual disks was measured and interpreted as susceptible (S), intermediate (I), or resistant (R) by following the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2018) for veterinary pathogens. Any bacterial isolates exhibiting resistance against $\geq$3 different classes of antimicrobial was classified as MDR (Magiorakos et al., 2012).

2.9. Detection of resistance genes

Any staphylococcal isolates showing resistance to cefoxitin was further screened for the detection of the *mec*A gene by PCR, as described earlier by Larsen et al., 2008 (Table 2). A previously confirmed MRSA strain (Rana et al., 2020b) and nuclease-free water were used as positive and negative controls, respectively in PCR reactions.

2.10. Detection of virulence genes

All PCR confirmed *Staphylococcus* species were further screened for the detection of the following virulence genes: enterotoxins (*sea, seb* and *sec*), cytotoxin (*pvl*), exfoliative toxins (*eta* and *etb*) and a toxic shock syndrome toxin (*tst*) previously described by Zhou et al., 2017 (Table 2). In addition, all *S. aureus* isolates displaying positive results for the tube coagulase test were further confirmed for the presence of the *nuc* gene (Sasaki et al., 2010).

2.11. Data management and analysis

All the data obtained from the dairy farm during sample collection and laboratory analyses was entered into spreadsheets using commercial software (Microsoft Excel 2010). The descriptive statistics were performed to calculate the prevalence of the current study. The prevalence of SCM was calculated by dividing the number of SCM affected cows (numerator) by the total number of tested cows (the denominator). All putative risk factors for SCM were analyzed considering six outcomes in mastitic milk: the presence of *S. aureus, S. haemolyticus, S. chromogenes, S. epidermidis, S. sciuri*, and methicillin-resistant *Staphylococcus* spp. However, none of the milk samples tested positive for *S. simulans*. Shortly, univariable logistic regression analysis was performed to identify possible risk factors for the six mentioned outcomes. Any variables showing a p value of $\leq$0.20 were selected for the multivariable logistic regression model. Stepwise forward selection approach was followed to construct the absolute model. Variables having p value of $\leq$0.05 were considered significant for the final model. All statistical analysis was performed using STATA® 13.0 software.

3. Results

3.1. Prevalence of SCM

A total of 284 lactating cows (1136 quarters) were screened for SCM

Table 2
Primer sequences used for *nuc, mec*A and virulence genes amplification.

Organism	Gene Primer	Primer designation	Primer sequence 5′–3′	Amplicon size (bp)	References
Staphylococcus spp.	*nuc*	*nuc*F	TCGCTTGCTATGATTGTGG	359	Sasaki et al., 2010
		*nuc*R	GCCAATGTTCTACCATAGC		
	*mec*A	*Mec*F	TCCAGATTACAACTTCACCAGG	162	Larsen et al., 2008
		*Mec*R	CCACTTCATATCTTGTAACG		
	sea	SF	GGTTATCAATGTGCGGGTGG	102	Zhou et al., 2017
		SR	CGGCACTTTTTTCTCTTCGG		
	seb	SF	GTATGGTGGTGTAACTGAGC	164	
		SR	CCAAATAGTGACGAGTTAGG		
	sec	SF	AGATGAAGTAGTTGATGTGTATGG	451	
		SR	CACACTTTTAGAATCAACCG		
	tst	SF	ACCCCTGTTCCCTTATCATC	326	
		SR	TTTTCAGTATTTGTAACGCC		
	eta	SF	ATATCAACGTGAGGGCTCTAGTAC	1155	
		SR	ATGCAGTCAGCTTCTTACTGCTA		
	etb	SF	CACACATTACGGATAATGCAAG	604	
		SR	TCAACCGAATAGAGTGAACTTATCT		
	pvl	SF	GTGCCAGACAATGAATTACCC	255	
		SR	TTCATGAGTTTTCCAGTTCACTTC		

on 30 selected dairy farms. Out of them, 178 [62.7%, 95% Confidence Interval (CI): 56.9 to 68.1] cows were positive for the CMT and confirmed as having SCM (Table 3). Among the SCM affected cows, 71 (39.9%, 95% CI: 33 to 47.2) cases were associated with a single quarter, 58 (32.6%, 95% CI: 26.1 to 39.8) with two quarters, 27 (15.2%, 95% CI: 10.6 to 21.2) with three quarters, and 22 (12.4%, 95% CI: 8.2 to 18) with four quarters (Fig. 1). Moreover, a total of 356 (31.3%, 95% CI: 28.7 to 34.1) quarters were found positive for SCM through the CMT test. All the dairy farms (100%, 95% CI: 86.5 to 100) were positive for SCM (Fig. 2).

3.2. Detection rate of SCM based on CMT score

Considering all 284 lactating cows, 106 (37.3%, 95% CI: 31.9 to 43.1) had negative CMT results. Moreover, out of 178 SCM positive cows, 97 (55%, 95% CI: 47.2 to 61.6) were diagnosed as weak positive (1+). In addition, 71 (40%, 95% CI: 33 to 47.2) and 10 (6%, 95% CI: 3 to 10.2) cows were identified as distinct (2+) and strong positive (3+) for SCM, respectively.

3.3. Frequency of different Staphylococcus spp. on SCM affected cows

The frequency calculation was performed based on the different PCR confirmed *Staphylococcus* spp. out of 356 cultured milk samples (Fig. 3a). Among *Staphylococcus* spp., *S. chromogenes* was the most frequent pathogen in SCM-affected cows (117/178; 65.7%, 95% CI: 58.5 to 72.3) at the cow level (Table 3). *S. epidermidis, S. haemolyticus,* and *S. aureus* were identified in 36 (20.2%, 95% CI: 15 to 26.8), 34 (19.1%, 95% CI: 14 to 25.5) and 28 (15.7%, 95% CI: 12 to 21.8) cows, respectively (Table 3). On the contrary, *S. sciuri* was detected in ten cows only (5.6%, 95% CI: 3 to 11) and none of the mastitic milk samples were positive for *S. simulans*.

3.4. Mixed infection of diverse Staphylococcus spp. on udder of SCM affected cows

Across the 178 SCM-affected cows, 58 (32.6%) were co-infected by *Staphylococcus* spp. (Fig. 3). Among them, 4 cows harbored three different *Staphylococcus* species in same udder quarters, while 54 SCM-cows showed quarters with various two *Staphylococcus* species combinations (Fig. 3).

3.5. Antimicrobial use on the dairy herds for SCM treatment

From the antimicrobial therapeutic history of dairy farms, 19 (63.3%) and 9 (30%) herds used gentamicin and streptomycin (aminoglycosides) respectively for treatment of SCM. Furthermore, 15 (50%), 14 (47%), and 9 (30%) farms used β-lactam antibiotics such as ceftriaxone, amoxicillin, and penicillin (Fig. 2). Oxytetracycline 1 (3.3%) was the least used drug for the treatment of bovine SCM (Fig. 2).

Table 3
Prevalence of different *Staphylococcus* spp. associated with SCM affected lactating cows.

Total number of dairy farm	Total number of lactating cows	SCM affected cows	PCR confirmed bacterial species	Prevalence
30	284	178 (62.7%)	*S. aureus*	28 (15.7%)
			S. chromogenes	117 (65.7%)
			S. haemolyticus	34 (19.1%)
			S. epidermidis	36 (20.2%)
			S. sciuri	10 (5.6%)

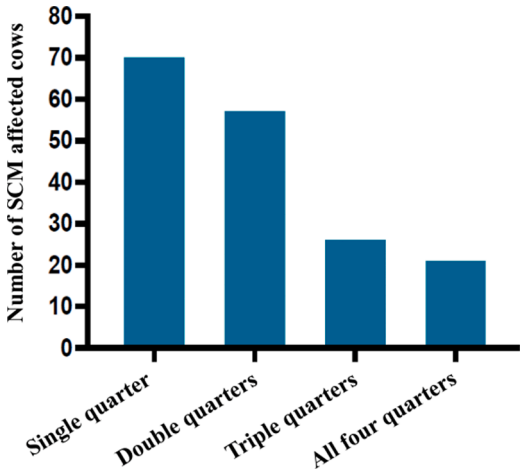

Fig. 1. Frequency of SCM in different quarters of CMT- positive cows.

3.6. Antimicrobial resistance profiles of Staphylococcus spp

Antimicrobial susceptibility profiles of *Staphylococcus* spp. are shown in Table-4. Overall, *S. aureus* isolates exhibited the highest resistance levels against ampicillin (82. 1%) and amoxycillin/clavulanic acid (75%) (Table 4). In addition, most of the *S. chromogenes* isolates were susceptible to majority of the tested antimicrobials, and the highest resistance levels were observed against cefepime, tetracycline, which were 95.7% and 54.7%, respectively. On the other hand, all *S. haemolyticus* were susceptible to ampicillin, amoxycillin/clavulanic acid, imipenem, and gentamicin (Table-4). Cefepime (97.2%), erythromicin (80.6%) and cefoxitin (77.8%) exhibit resistant against *S. epidermidis*, while 70% of *S. sciuri* isolates were susceptible to ceftriaxone and ciprofloxacin. All isolates were susceptible to imipenem (Table 4).

3.7. Detection rate of MDR in Staphylococcus spp

MDR isolates were identified among all five *Staphylococcus* spp. Among all MDR isolates, 19/28 (67.9%) *S. aureus* and 68/117 (58.1%) *S. chromogenes* showed resistance up to seven classes of antimicrobials. A lower frequency of MDR isolates was detected among *S. epidermidis* (16/36; 44.4%), *S. haemolyticus* (12/34; 35.3%) and *S. sciuri* (3/10; 30%).

3.8. Distribution of resistance genes among diverse Staphylococcus spp

The *mec*A gene conferring methicillin resistance was screened in all cefoxitin resistant staphylococci isolates. It was detected in 9 (32.1%) *S. aureus* (Fig. 3c) that were classified as methicillin-resistant *S. aureus* (MRSA). Isolates belonging to *S. chromogenes* 7 (6%), *S. epidermidis* 2 (5.6%), and *S. sciuri* 1 (10%), also carried the *mec*A gene (Table 5), and therefore were designated as MRSC, MRSE, and MRSS.

3.9. Distribution of virulence genes among diverse Staphylococcus spp

The enterotoxin-encoding genes *sea* was detected in *S. aureus* (17.9%) and *S. chromogenes* (1.7%), (Fig. 3d), while the *seb* gene was only detected for *S. aureus* (7.1%) (Table 5). The cytotoxin (*pvl*) gene was detected in *S. sciuri* (20%) and *S. haemolyticus* (17.7%) (Fig. 3e). The toxic shock syndrome toxin-encoding gene (*tst*) was detected in *S. aureus* (14.3%) (Fig. 3f).The exfoliative toxins-encoding gene (*eta*) was detected in *S. aureus* (7.1%) and *S. haemolyticus* (5.9%). None of the isolates carried *sec* or *etb* genes (Table 5).

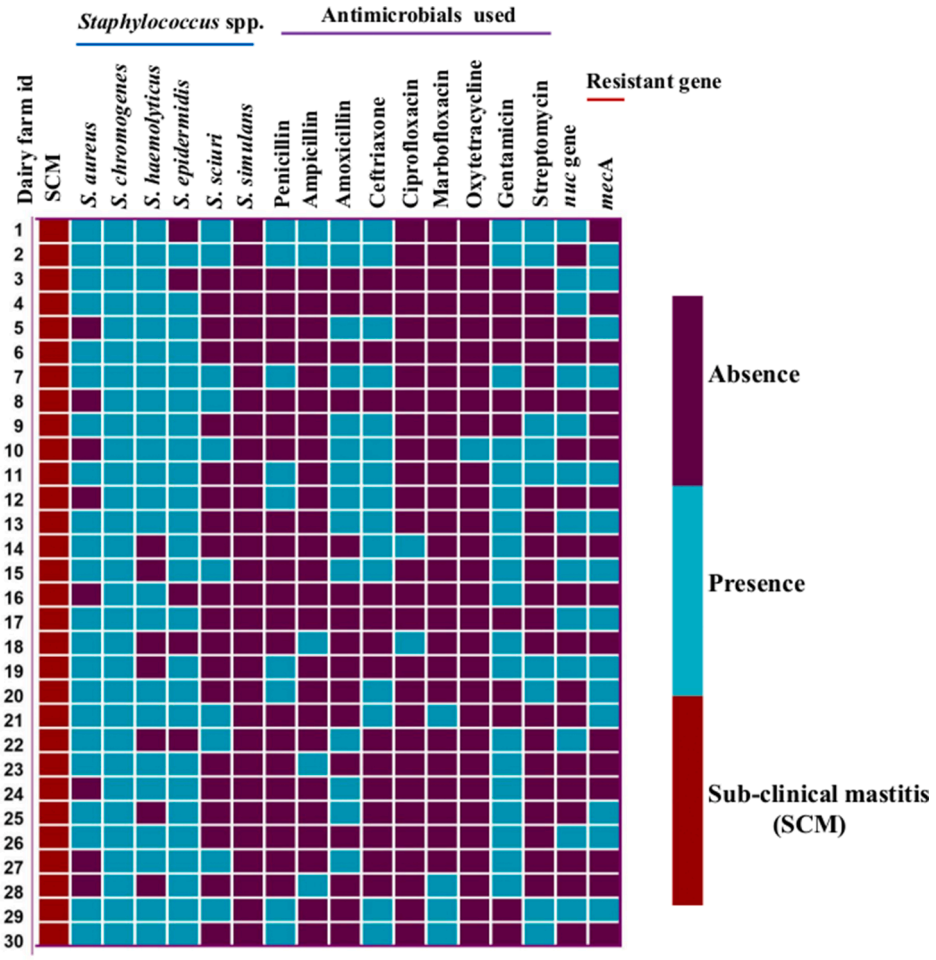

Fig. 2. The heat map represents the distribution of diverse *Staphylococcus* spp. isolated from SCM-affected cows and different classes of antimicrobials used in dairy farms, as well as the presence of the *nuc* gene and the methicillin-resistant gene (*mec*A). Each row represents an individual dairy farm.

3.10. Risk factors associated with Staphylococcus spp. carriage in SCM-affected lactating cows

The univariable logistic regression analysis identified five potential different risk factors ($p \leq 0.20$) associated with the carriage of *S. aureus* and *S. chromogenes* in SCM affected cows (Supplementary Tables 1 and 2). In the subsequent multivariable logistic regression analysis, two different factors were found to be significant risk factors associated with the carriage of both *S. aureus* and *S. chromogenes*. The significantly associated risk factors were "Old aged" (OR: 3.5, 95% CI: 1–12.4, $p \leq 0.05$) and "Front left side quarter" (OR: 2.5, 95% CI: 1.1–5.7, $p \leq 0.03$) for *S. aureus* (Table 6). In case of *S. chromogenes,* two factors, "Early stage of lactation" (OR: 3.4, 95% CI: 1.2–9.7, $p \leq 0.02$) and "Hind left side quarter" (OR: 2.1, 95% CI: 1.1–4.0, $p \leq 0.02$), were significantly associated with SCM (Table 6). Six potential risk factors ($p \leq 0.20$) were particularly associated with the presence of *S. haemolyticus* (supplementary Table 3). However, "Firm udder condition" (OR: 4.2, 95% CI: 1.2–14.6, $p \leq 0.02$) and "Front right side quarter" (OR: 2.8, 95% CI: 1.1–7, $p \leq 0.03$) were significantly associated with presence of *S. haemolyticus* in SCM affected cows. A total of four and two different factors ($p \leq 0.20$) were initially associated with *S. epidermidis* and *S. sciuri*, respectively (supplementary Table 4 and 5). In multivariable logistic regression model, none of the factors were significantly associated with SCM in cows. Fig. 4

3.11. Risk factors associated with carriage of methicillin-resistant Staphylococcus spp. in SCM affected lactating cows

Twelve out of the thirty factors were primarily identified in the univariable analysis as the potential risk factors for the carriage of methicillin-resistant *Staphylococcus* spp. in SCM affected lactating cows **(**supplementary Table 6**).** Two variables "Use of antimicrobials" (OR: 10.4, 95% CI: 3.4–32.1, $p \leq 0.00$) and "History of previous clinical mastitis" (OR: 4.9, 95% CI: 1.2–19.7, $p \leq 0.02$) were found significant in the final model (Table 6).

4. Discussion

Staphylococcal mastitis is an ongoing challenge for successful management and operation of dairy industry throughout the world, including Bangladesh. As per our knowledge, this is the first comprehensive molecular study for identification of diverse CoNS species which enabled us to representing the species-specific prevalence of SCM in affected cows in Bangladesh context. This study estimated overall prevalence of SCM on different dairy farms as 62.7% at cow level and 31.3% at quarter level. This result is higher than the previous findings of, Ramírez et al., 2014; Rana et al., 2022a and Zeryehun & Abera, 2017 in Colombia, Bangladesh, and Eastern Ethiopia, which were 37.2%, 38.4%, and 51.8%, respectively. The variation in prevalence may have occurred due to individual cow and farm factors like breed, age, stages of lactation, parity, udder immunity, farm ecology, management, hygiene, and milking practices in dairy farms (Rana et al., 2022a; Ashraf et al., 2018).

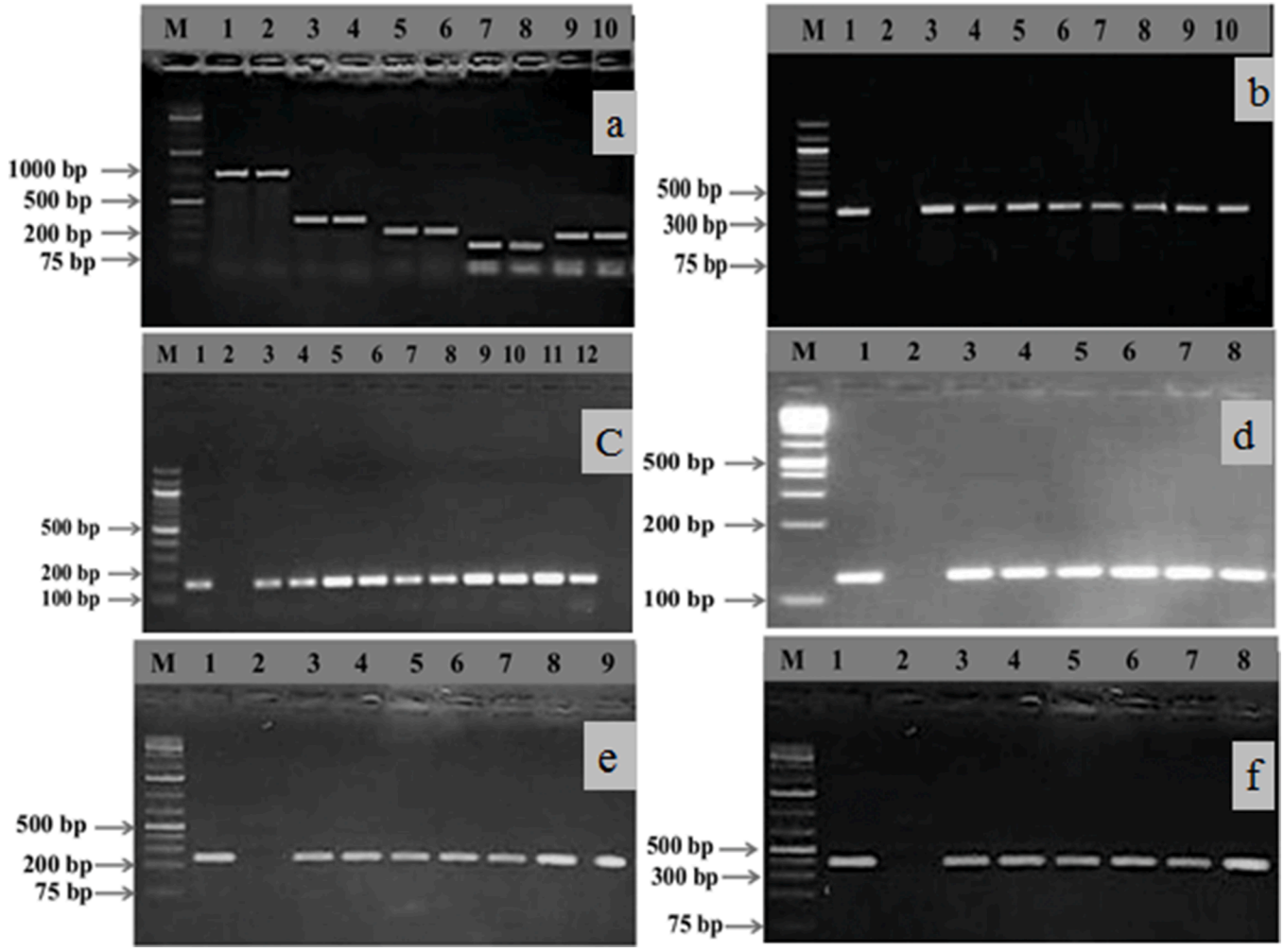

Fig. 3. Gel electrophoresis image (3a) of PCR products of *Staphylococcus* spp. showing specific amplified bands on 1.5% agarose gel, where lanes 1 and 2 = *S. aureus* (894 bp), 3 and 4 = *S. sciuri* (306 bp), 5 and 6 = *S. chromogenes* (222 bp), 7 and 8 = *S. epidermidis* (130 bp), and 9 and 10 = *S. haemolyticus* (214 bp). Image-3b and 3c display the specific amplification of the *nuc* (359 bp) and *mec*A (162 bp) genes, respectively. In addition, images 3d, 3e, and 3f exhibit the amplification of virulence genes like *sea* (102 bp), *pvl* (255 bp), and *tst* (326 bp), respectively. In all gel electrophoresis systems, we used a 1 kb plus DNA marker except for images 3b and 3c, where we used 100 bp DNA markers for confirmation of amplification of specific genes. Moreover, lanes 1 and 2 were used as positive and negative controls for the 3b, 3c, 3d, 3e, and 3f images, respectively.

Table 4
Antimicrobial sensitivity profiles of different *Staphylococcus* spp. causing SCM in lactating cows.

Organism	Antimicrobial susceptibility	AM	AMC	CRO	FOX	FEP	IMP	CIP	E	CN	SXT	TE
S. aureus (28)	Sensitive (%)	17.9	25	67.9	71.4	46.4	100	64.3	39.3	42.9	35.7	53.6
	Resistant (%)	82.1	75	32.1	28.6	53.6	0	35.7	60.7	57.1	64.3	46.4
S. chromogenes (117)	Sensitive (%)	58.1	71.8	63.3	73.5	4.3	100	67.5	66.7	76.1	74.4	45.3
	Resistant (%)	41.9	28.2	36.8	26.5	95.7	0	32.5	33.3	23.9	25.6	54.7
S. haemolyticus (34)	Sensitive (%)	100	100	64.7	73.5	5.9	100	91.2	61.8	100	76.5	88.2
	Resistant (%)	0	0	35.3	26.5	94.1	0	8.8	38.2	0	26	11.8
S. epidermidis (36)	Sensitive (%)	36.1	80.6	33.3	22.2	2.8	100	94.4	19.4	86.1	36.1	33.3
	Resistant (%)	63.9	19.4	66.7	77.8	97.2	0	5.6	80.6	13.9	63.9	66.7
S. sciuri (10)	Sensitive (%)	20	30	70	60	40	100	70	30	60	30	40
	Resistant (%)	80	70	30	40	60	0	30	70	40	70	60

AM = ampicillin, AMC = amoxycillin/clavulanic acid, CRO = ceftriaxone, FOX = cefoxitin, FEP = cefepime, IPM = imipenem, CIP = ciprofloxacin, *E* = erythromicin, CN = gentamicin, SXT = trimethoprim/sulfamethoxazole and TE = tetracycline.

Both CoPS and CoNS are the most prevalent pathogens involved in bovine SCM (Taponen & Pyörälä, 2009; Pyörälä & Taponen, 2009; Schabauer et al., 2018; Rana et al., 2022a), even if the species-specific bacterial prevalence and distribution mightily vary among different dairy herds. The isolation rate of *S. aureus* in the current study is in line with previous studies by Kasa et al., 2020, and Rana et al., 2022a. However, the secretion of the coagulase enzyme is one of the key virulence strategies of *S. aureus* that encourages the initiation of mammary gland damage and the pathogenesis of bovine mastitis (Bonar et al., 2018). Several studies have described that the presence of *S. aureus* is significantly involved in clinical and SCM and causes pronounced inflammatory responses in cow udder (De Buck et al., 2021).

Table 5
Distribution of antimicrobial resistance and virulence genes among different *Staphylococcus* spp. isolates from SCM affected lactating cows.

Organism	Gene	Number of isolates	Detection rate (%)
S. aureus (28)	*mec*A	9	32.1
	nuc	13	46.4
	sea	5	17.9
	seb	2	7.1
	sec	0	0
	pvl	3	10.7
	eta	2	7.1
	etb	0	0
	tst	4	14.3
S. chromogenes (117)	*mecA*	7	6
	sea	2	1.7
S. haemolyticus (34)	*pvl*	6	17.7
	eta	2	5.9
	tst	3	8.8
S. epidermidis (36)	*mecA*	2	5.6
S. sciuri (10)	*mecA*	1	10
	pvl	2	20

Table 6
Multivariable logistic regression model for assessing the risk factors independently associated with the *S. aureus, S. haemolyticus, S. chromogenes* and methicillin-resistant (MR) *Staphylococcus* spp. from SCM affected lactating cows.

Outcome variable	Explanatory variable	Description	OR (95% CI)	*p*-value
S. aureus	Age	Adult	1	Reference
		Young adult	1.8 (0.7–4.9)	0.21
		Old	3.5 (1–12.4)	0.05*
	Front left side quarter	Yes	2.5 (1.1–5.7)	0.03*
		No	1	Reference
S. haemolyticus	Udder condition	Firm	4.2 (1.2–14.6)	0.02*
		Normal	1	Reference
	Front right side quarter	Yes	2.8 (1.1–7)	0.03*
		No	1	Reference
S. chromogenes	Stage of lactation	Early	3.4 (1.2–9.7)	0.02*
		Middle	1.1 (0.5–2.4)	0.78
		Last	1	Reference
	Hind left side quarter	Yes	2.1 (1.1–4.0)	0.02*
		No	1	Reference
MR *Staphylococcus* spp.	Use of antimicrobials	Yes	10.4 (3.4–32.1)	0.00*
		No	1	Reference
	History of previous clinical mastitis	Yes	4.9 (1.2–19.7)	0.02*
		No	1	Reference

Abbreviation: OR= odds ratio; CI=confidence interval; significance *(≤0.05).

In addition, we revealed that *S. chromogenes* (65.73%) *S. epidermidis* (20.2%) and *S. haemolyticus* (19.1%) were the most prevalent CoNS species isolated from SCM affected cows. The isolation rate of these CoNS species was agreed upon in the previous study of the USA, Canada, and Argentina, which was reported by Hasan et al., 2022; Jenkins et al., 2019; De Visscher et al., 2016; Raspanti et al., 2016. As bovine skin and farm bedding materials are the fundamental sources of *S. chromogenes, S. epidermidis,* and *S. haemolyticus* (Taponen & Pyörälä, 2009; Schabauer et al., 2018) such reports give evidence to the origin of bovine mastitis. The internal udder environment or intrinsic factors of the mammary gland also favor the colonization of diverse groups of organisms, especially bacterial species (Rana et al., 2020a). Moreover, the CoNS species, especially *S. chromogenes, S. haemolyticus,* and *S. sciuri,* are responsible for persistent SCM and markedly increased somatic cell count (SCC) in lactating cows without visible clinical signs (De Buck et al., 2021; Simojoki et al., 2009). These observations were kept up in our study, and the majority of CoNS isolates have been recovered from CMT-positive milk from cows with usual udder conditions. As a result, the pathogenic potentialities of CNS species, ranging from commensal organisms to actual mastitis pathogens, remain uncertain (Isaac et al., 2017; Pyörälä & Taponen, 2009). The early detection of bovine SCM and its causal agent is crucial for making treatment and management decisions (Schabauer et al., 2018). Moreover, the exact identification and recording of bacterial species involved in mastitis is inevitable to ameliorate dairy herd health.

We identified 32.6% of SCM cows carrying mixed *Staphylococcus* spp. infection with multiple combinations, which was strongly supported by Ethiopian and Estonian dairy herd findings and described by Kasa et al., 2020 and Kalmus et al., 2011. The possible reasons for this mixed colonization of bacterial species in udder might be occurred due to coexistence of mastitis pathogens together in farm environments (Kalmus et al., 2011). Additionally, the high dominance of mixed *Staphylococcus* spp. infection might occur due to fugitive transmission of these pathogens during hand milking of dairy cows.

In this study, *S. aureus* isolates displayed a high level of resistance against ampicillin (82.1%), amoxicillin and clavulanic acid (75%), which were the frequently used antimicrobials in dairy farms. This high level of resistance is in agreed with other previous reports by Priyantha et al., 2021; Rana et al., 2022a. Furthermore, the results showed that a significant number of CoNS species were resistant to various β-lactam antimicrobials. β-lactams (ampicillin, amoxicillin, ceftriaxone and cefoxitin) are the most used antimicrobials in the majority of dairy herds for the treatment of bovine clinical and SCM. This could be one of the major causes of β -lactam resistance in *S. aureus* and the CoNS due to repeated exposure and selective antimicrobial pressure (Christaki et al., 2020; Rana et al., 2022a). Furthermore, the production of β-lactamase has been reported as a resistance mechanism for *Staphylococcus* spp., which is resistant to a variety of antimicrobials (De Buck et al., 2021). In addition, the current study, all the *Staphylococcus* spp. was highly susceptible to imipenem (100%). This is highly expected as imipenem has not been used in the field of veterinary practices in the context of Bangladesh, and this antibiotic is totally reserved for human health.

In the present study, all of the *S. aureus* and CoNS isolates displayed MDR (i.e. resistance to ≥ 3 antimicrobial classes), and these findings are supported the previous several studies conducted in dairy farms for mastitic cows (Rana et al., 2022a, Dabele et al., 2021). Overexposure and repetitive use of similar groups of antimicrobials and antimicrobials having similar mode of action may induce the development of MDR (Rana et al., 2022b, Das et al., 2022). On the other hand, bacterial intrinsic factors, biofilm formation, and the encoding of multiple resistance genes were also reported for MDR expression (Christaki et al., 2020).

Our study detected 9 (32.1%) *S. aureus* and certain percentages of CoNS species that encode the *mec*A gene that displayed resistance against cefoxitin, ampicillin, amoxicillin/clavulanic acid, cefepime, and ceftriaxone antibiotics. However, due to the encoding of the *mec*A gene, which modifies penicillin binding protein (PBP) 2a on the bacterial cell wall, these broad-spectrum -lactam antibiotics become resistant to staphylococci. And these methicillin-resistant phenomena have already been reported in various farm animals (Monecke et al., 2013; Rana et al., 2020a; Schabauer et al., 2018). Specifically, the methicillin-resistant *S. chromogenes, S. epidermidis,* and *S. sciuri* were already reported in different countries like China (73.2%), Switzerland (62%), the Netherlands (14.1%), United Kingdom (4.1%), the USA (2.4%) and Canada (0.9%) (Schnitt et al., 2021) but these findings are noble for Bangladesh. Moreover, it is a matter of great concern that methicillin-resistant CoNS species are pivotal reservoirs of AMR genes, which create a clinical and management challenge for successful control of bovine mastitis in dairy farms.

We identified both *S. aureus* and CoNS species harbored a multiple

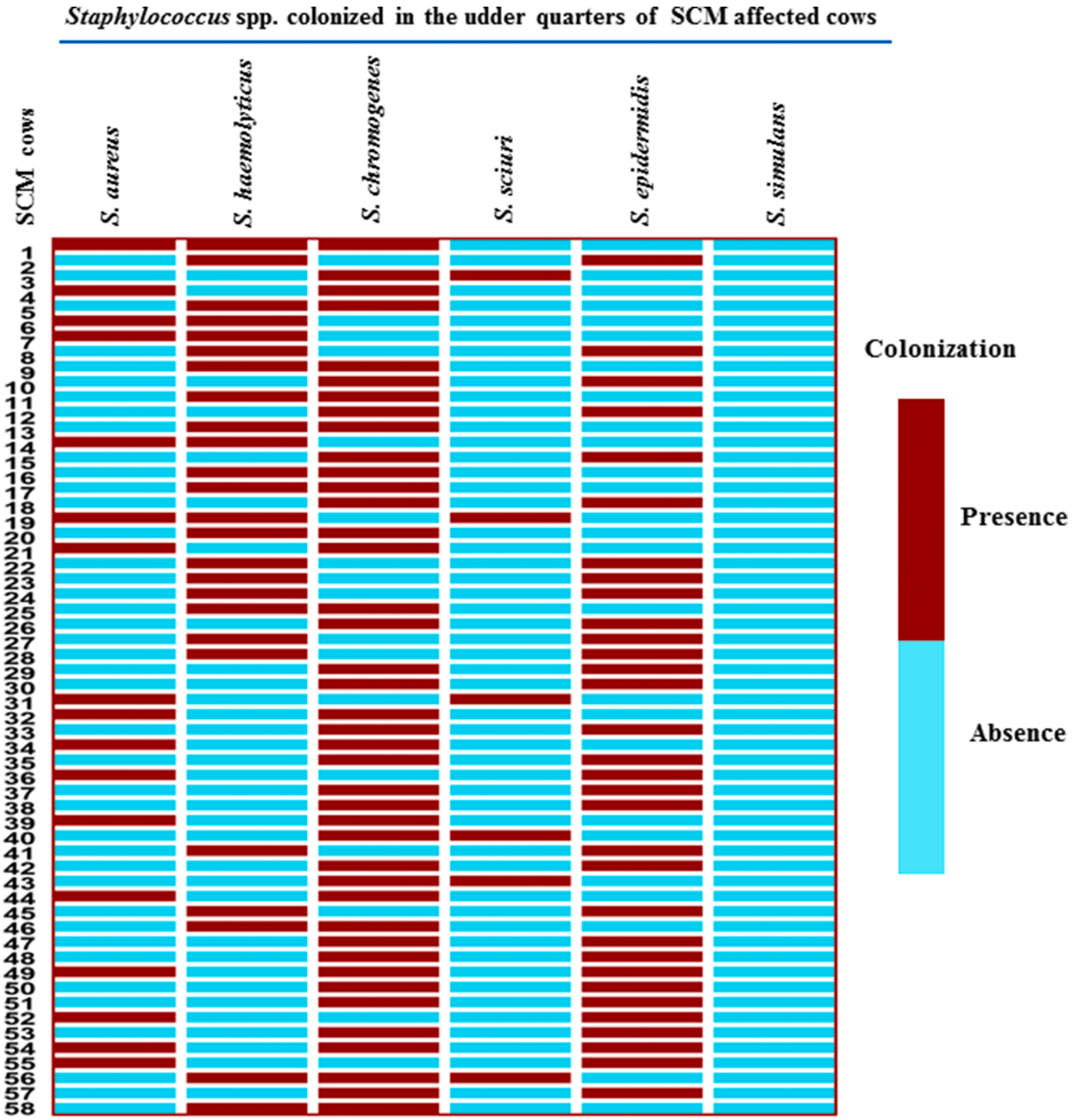

Fig. 4. The heat map depicts the mixed colonization of multiple *Staphylococcus* spp. on udder quarter of SCM affected cows.

number of virulence genes, including enterotoxins (*sea, seb* and *sec*), cytotoxin (*pvl*), exfoliative toxins (*eta, etb*) and a toxic shock syndrome toxin (*tst*). Interestingly, 17.9%, 14.3%, and 10.7% of *S. aureus* carried *sea, tst,* and *pvl* genes, respectively. These virulence determinants aligned with the previous reports of Mello et al., 2016, and Zhou et al., 2017. Moreover, alarming percentages of coagulase negative mastitis isolates like *S. haemolyticus, S. chromogenes,* and *S. sciuri* carried *sea, pvl, eta* and *tst* genes, and these findings are supported by the previous reports of González-Martín et al., 2020, Mahato et al., 2017. The presence of virulence genes and their known pathogenic potentialities are directly associated with bovine mastitis in farm environments. In addition, the information and evidence regarding the virulence factors of CoNS species and their gene regulations are much sparser in bovine mastitis.

The study also revealed that a significant statistical association between the prevalence of SCM and different risk factors like, age of cow, udder condition, stage of lactation, and position of the udder quarter. This finding is consistent with the findings of Lakew et al., 2019 who reported that the risk of SCM increases significantly with the advancement of lactation stage as well as the age of lactating cows. According to Radostitis et al. (2007) and Lakew et al. (2019) an older cow with more relaxed udder attachments (pendulous udder) and frequent floor touch of the cow's quarter allows contagious organisms to enter the cow's udder. In addition, previous history of clinical mastitis with prolonged mild infection dramatically increased SCC (McDougall et al., 2021) and initiated fibrosis of mammary gland tissue, changed the consistency of the udder to a firm texture, and, established SCM. Lactating cows' udder defense mechanism reduces the severity of the clinical nature of bovine mastitis (Lakew et al., 2019), which eventually conceals the clinical signs and cows perpetuate the disease's sub-clinical form.

We identified use of antimicrobials and history of previous clinical

mastitis as the risk factors associated with higher prevalence of methicillin resistant staphylococci in SCM cows and the similar factors were obtained by Rana et al., 2022a and Mahato et al., 2017.Concurrent use of antimicrobials for the clinical management of mastitis case might be a potent reason behind this association. In addition, antimicrobial therapy and its selection pressure is a well-documented factor for methicillin resistance in staphylococci (Gnanamani et al., 2017). However, the imprudent use of antimicrobials in the treatment of methicillin resistance staphylococci should be unsanctioned and the absolute mastitis treatment regimen should be based on the antimicrobial susceptibility testing.

However, due to resource constraints, we were unable to perform detailed genotypic characterization of *S. aureus* and CoNS species involved in bovine SCM in the current study. Moreover, it would be an appropriate task to explore the sequence types of all staphylococcal isolates that are circulating in bovine mastitis in the context of Bangladesh. Future research should overcome these limitations.

5. Conclusion

The SCM in the dairy herds was highly prevalent, and the exalted burden of CoNS, markedly *S. chromogenes, S. epidermidis,* and *S. haemolyticus* were associated with the SCM. The alarming percentages of isolates found were not only MDR, but a significant number encoded the *mec*A gene, which is noted for methicillin resistance. In addition to *S. aureus*, a particular number of CoNS species harbor multiple virulence genes and assert their pathogenic potentialities. Moreover, old aged, early stage of lactation, and firm udder condition were identified as potential risk factors that amalgamated with SCM, while previous use of antimicrobials and history of previous clinical mastitis seem to be positively related with the prevalence of methicillin resistant *Staphylococcus* spp. in SCM cows. Therefore, intensive monitoring of the MDR pathogen in SCM is pivotal since the transmission of *Staphylococcus* spp. is dynamic and involves farm animals, workers, consumers, and veterinarians.

Ethical approval

The field task (SCM screening and sample collection) and entire laboratory procedures of the current study were performed with the permission of Chattogram Veterinary and Animal Sciences University's (CVASU) Ethical Approval Committee [Approval no. CVASU/Dir (R&E) EC/2020/165 (1)].

CRediT authorship contribution statement

Md Abul Fazal: Conceptualization, Data curation, Methodology, Project administration, Resources, Validation, Formal analysis, Visualization, Writing – review & editing. **Eaftekhar Ahmed Rana:** Conceptualization, Funding acquisition, Data curation, Investigation, Methodology, Project administration, Resources, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Sazeda Akter:** Resources, Validation, Visualization, Writing – review & editing. **Mohammad Abdul Alim:** Visualization, Validation, Writing – review & editing. **Himel Barua:** Project administration, Supervision, Writing – review & editing. **Abdul Ahad:** Conceptualization, Funding acquisition, Project administration, Supervision.

Declaration of Competing Interest

We, the authors of this paper have no financial interest to declare or personal relations that could have influenced the findings presented in this manuscript.

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Supplementary materials

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