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Spa Typing and Virulence Attributes of Multidrug-Resistant *Staphylococcus aureus* in Goat: A Veterinary Hospital-Based Cross-Sectional Study

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Staphylococcus aureus (*S. aureus*) not only causes significant loss of livestock production but also has potential public health risks. This study was conducted with the objective of characterizing the antibiogram of *S. aureus* from goats attending a Teaching Veterinary Hospital in Bangladesh. We aimed to study the *spa* type, potential virulence factor(s), and the presence of methicillin and vancomycin resistance genes in the isolates. From a total of 200 goat nasal swab samples *S. aureus* was confirmed by PCR. Antimicrobial susceptibility testing was performed using the disc diffusion method followed by *mecA* and *vanA* gene PCR. Staphylococcal protein A (*spa*) typing was conducted for selected isolates. A set of major virulence determinants, including *sea*, *seb*, *pvl*, and *tst* were investigated by PCR. A total 11.5% (n=23) of *S. aureus* isolates were obtained. Ciprofloxacin resistance was the highest (100%; n=23) followed by cefoxitin (74%; n=17). About 91% of the isolates (n=21) were multidrug-resistant (resistant to ≥3 antimicrobial classes). One (6%) isolate was MRSA harboring *mecA*. Among 13 vancomycin-resistant isolates, three (23%) were *vanA*-positive. Of the virulence genes screened, *sea* was recorded in four (17%) isolates. The *spa* typing of two randomly selected strains revealed that one isolate belonged to *spa* type t5259, and the other seemed to be novel. Goats having cutaneous and mucosal lesions had higher *S. aureus* carriage (P=0.01) with a significant variation across the breeds (P= 0.02). This study provides insights into *S. aureus* strains circulating in the goat population in Bangladesh.

Key Words: *S. aureus*, MDR, PCR, *Spa* typing, Goat

INTRODUCTION

Antimicrobial-resistant *Staphylococcus aureus* (*S. aureus*) is a worldwide concern due to its ability to develop resistance to multiple antimicrobials within a short

period (1). *S. aureus* is a well-known opportunistic pathogen that colonizes the nasal cavity of humans and animals (2). Major virulence factors of pathogenic strains are hemolysin (α, β, ɣ, δ), leukocidin (Panton-Valentine leukocidin, PVL), enterotoxins (SEs), exfoliative toxins (ETA and ETB), and toxic shock syndrome toxin (TSST) (3). It infects both animals and humans causing infections ranging from superficial skin and soft tissue infections to life-threatening endocarditis, toxic shock syndrome and necrotizing pneumonia (4). Antimicrobials such as β-lactam, aminoglycoside, macrolides, tetracycline and quinolones are commonly used to treat staphylococcal infections (5, 6). Unfortunately, the level of resistance of these antimicrobials is increasing rapidly among staphylococci due to irrational use of antimicrobials in livestock production (5). Multidrug resistant (MDR) *S. aureus* is a major concern, which plays an important role in the transmission of resistance to different non- resistant bacteria through both mutation and/or the horizontal gene transfer (HGT) mechanism (7). Among β-lactam antimicrobials, penicillin, methicillin, cloxacillin, oxacillin, flucloxacillin, and dicloxacillin are the most useful antimicrobials to treat infections caused by *S. aureus* (7). Hence, methicillin-resistant *S. aureus* (MRSA) is a serious public health concern globally and is considered as one of the leading causes of hospital-acquired (nosocomial) infections in humans (8).In terms of mechanism, MRSA strains are resistant to all β-lactam antimicrobials by penicillin-binding protein (PBP_{2a}) which is encoded by the *mecA* gene (6). Despite host specificity, human infections with animal originated *S. aureus* strains have been reported frequently (9). Livestock-associated MRSA (LA-MRSA) has been described to be capable of colonizing and infecting humans such as veterinarians and farmers because of their close contact with animals (10, 11). Vancomycin is a recommended drug for the treatment of MRSA infection; however, currently, the emergence of vancomycin resistance is a major public health concern around the world (12). VRSA strains are characterized by the expression of 11 *van* genes among which *vanA* gene is very common (12).

In Bangladesh, goat rearing is generally regarded as subsistence, smallholder and small-scale commercial operations. The majority of the farmers (80.5%) follow a semi-intensive system but few farmers (12.2%) use the free-range system while only 7.3% maintain a confinement pattern for goat production (13). Studies show wide variation in the prevalence of *S. aureus* in goats ranging from 16.7% to 96.2% in different countries (14). Several studies have reported the transmission of drug-resistant *S. aureus* (particularly MRSA and VRSA) from animals, environments, and humans worldwide (10-12). Similarly, antimicrobial-resistant *S. aureus* has also been reported in Bangladesh among poultry, cattle, and humans (7, 15, 16). However, despite being one of the most important food animals, data on drug-resistant *S. aureus* colonization in goats is sparse compared to other animal species in Bangladesh. Emphasis needs to be put on the investigation of antimicrobial-resistant *S. aureus* in small ruminants in order to develop effective prevention and treatment guidelines for *S. aureus* infections. This study was conducted aiming to understand the carriage frequency of *S. aureus, spa* type(s), antimicrobial resistance patterns, and virulence characteristics of *S. aureus* isolated from goats in Bangladesh.

MATERIALS AND METHODS

Ethical approval

This study was approved by the ethical committee of Chattogram Veterinary and Animal Sciences University, Bangladesh with retrospective effect from the date of its commencement. The memo no. is CVASU/Dir (R&E) EC/2021/273 (1).

Study population and sampling

This study was conducted in Chattogram, the second-largest city in Bangladesh. Sahedul Alam Quaderi Teaching Veterinary Hospital (SAQTVH) of Chattogram Veterinary and Animal Sciences University (CVASU) was selected as the sampling site because of its wide service coverage across the city. We selected random dates to visit the hospital and, on each visit, we randomly selected 1 to 10 goats for sampling. The nasal swabs were collected from goats by inserting a sterile swab into the nasal cavity followed by gentle rubbing of the nasal mucosa. Samples were then placed in a sterile 15 ml Falcon tube

containing 5 ml Mueller Hinton broth (MHB) (Oxoid Ltd., UK) supplemented with 6.5% NaCl and transferred to the Microbiology and Veterinary Public Health Laboratory of CVASU for detailed investigation. The demographic and clinical data of animals were collected from the patient signalment sheets that were filled up by duty veterinarians.

Isolation and molecular identification of *S. aureus*

Swabs collected in MHB containing 6.5% NaCl were incubated at 37°C overnight for selective enrichment (17). After subsequent culture on 5% bovine blood agar, colonies showing hemolytic golden yellowish color were sub-cultured on Mannitol salt agar (Oxoid Ltd, UK). The phenotypic identification of presumptive staphylococci was done based on biochemical tests (18). Then, the primarily identified *S. aureus* was further confirmed by PCR targeting thermonuclease gene called *nuc* (19) which is species-specific and an indication of the pathogenic *S. aureus*. All PCR reactions were carried out in 25µl of a final volume containing 1µl DNA template (average concentration of 2.18ng/µl), forward and reverse primers of 1µl each (20 picomoles/µl), 12.5µl master mix (Thermo Fisher Scientific, Waltham, MA, USA) and 9.5µl nuclease-free water. MRSA ATCC 33591 strain and nuclease-free water were used as positive and negative controls, respectively.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the *S. aureus* isolates was performed by the disk diffusion method (20) with a panel of eight antimicrobials from six different groups. Frequently prescribed antimicrobials in clinical settings along with the literature-reviewed less common alternatives to treat *S. aureus* infections were considered to design the panel. The antimicrobials with their respective class were: amoxicillin-clavulanic acid (30µg), cefoxitin (30µg), cefepime (30µg), tigecycline (15µg), vancomycin (30µg), sulfamethoxazole-trimethoprim (25µg), ciprofloxacin (5µg) and gentamicin (10µg) (Oxoid, UK). For each isolate, the zone of inhibition around each disk was measured and interpreted as susceptible (S), intermediate (I) or resistant (R) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (21). *S. aureus* isolates showing resistance against at least three groups of antimicrobial agents (≥3) were defined as MDR (22).

Screening of MRSA and VRSA

Phenotypically, MRSA and VRSA were detected based on the CLSI guideline for cefoxitin (30µg) and vancomycin (30µg) disk diffusion with interpretive criteria of ≤ 21 and ≤ 16 mm inhibitory zone for methicillin resistance and vancomycin resistance, respectively (21). This phenotypic resistance was further investigated for the presence of the *mecA* and *vanA* gene by PCR as described earlier (23, 24). The primer sequences and PCR conditions are mentioned in Table 1. For *mecA* gene, positive and negative controls were used as mentioned earlier. For *vanA* gene, in-house isolated strain of vancomycin-resistant *Enterococcus faecium* and vancomycin-susceptible *Enterococcus faecalis* were used as positive and negative control, respectively.

Detection of virulence genes

All confirmed *S. aureus* isolates were screened by PCR assay for the identification of predominant virulence factors; Panton-Valentine Leukocidin (*pvl*), enterotoxin (*sea* and *seb*), and toxic shock syndrome toxin (*tst*). The primer sequences and PCR conditions of these virulence genes are stated in Table 1.

S. aureus spa typing and phylogenetic analysis

For *S. aureus* protein A (*spa*) typing, firstly amplification of the polymorphic X region of the *spa* gene of the isolates was performed by PCR. The primers and cycling conditions used to amplify the *spa* gene were according to the methodology

Table 1. Primer sequences of different genes used for the PCR in this study

Gene	Primer name	Primer sequences (5′- 3′)	Annealing Temp.	Amplicon size (bp)	Reference
nuc	nuc-F	GCGATTGATGGTGATACGGTT	55°C	270	Brakstad et al., 1992(19)
	nuc-R	ACGCAAGCCTTGACGAACTAAAGC			
spa	spa-1113F	TAAAGACGATCCTTCGGTGAGC	59°C	Variable	Kahl et al., 2005(25)
	spa-1514R	CAGCAGTAGTGCCGTTTGCTT			
mecA	mecA P4	TCCAGATTACAACTTCACCAGG	55°C	162	Larsen et al., 2008(23)
	mecA P7	CCACTTCATATCTTGTAACG			
PVL	Luk-PV-1	ATCATTAGGTAAAATGTCTGGACATGAT CCA	55°/50°C[a]	433	McClure et al., 2006(47)
	Luk-PV-2	GCATCAAGTGTATTGGATAGCAAAAGC			
sea	GSEAR-1	GGTTATCAATGTGCGGGTGG	57°C	102	Mehrotra et al., 2000(48)
	GSEAR-2	CGGCACTTTTTTCTCTTCGG			
seb	GSEBR-1	GTATGGTGGTGTAACTGAGC	57°C	164	Mehrotra et al., 2000(48)
	GSEBR-2	CCAAATAGTGACGAGTTAGG			
tst	GTSSTR-1	ACCCCTGTTCCCTTATCATC	57°C	326	Mehrotra et al., 2000(48)
	GTSSTR-2	TTTTCAGTATTTGTAACGCC			
vanA	vanA F	GGCAAGTCAGGTGAAGATG	55°C	713	Azimian et al., 2012(24)
	vanA R	ATCAAGCGGTCAATCAGTTC			

[a]First 10 cycles annealing temperature 55°C and for next 25 cycles the temperature is 50°C

described earlier (25) (Table 1). The PCR product of the *spa* gene was purified using a DNA purification kit (Favorgen Biotech Corp, Taiwan) and then sequenced with the support of a commercial service (Macrogen Inc., Seoul, South Korea). The *spa* type of the isolates was assigned using Ridom Staph Type 1.4.1 software (Ridom GmbH, Würzburg, Germany) (26). Numerical *spa* repeat and type were assigned. The *spa* gene sequences of this study along with 13 other *spa* sequences obtained from BLASTN search results from NCBI GenBank were aligned using clustalW in MegaX. The Maximum Likelihood method with a bootstrap value of 1000 replicates was applied to construct the phylogenetic tree.

Statistical analysis

All data were entered and analyzed using the "R" program (version 3.5.1). The univariate logistic regression analysis was performed to assess the relative association between the presence of *S. aureus* and different demographic factors and clinical manifestations. The heat-map depicting the distribution of antimicrobial resistance phenotype and genotype was prepared by using GraphPad Prism 7 (La Jolla, CA, USA). Variable(s) having *P*of ≤0.05 was considered as statistically significant.

RESULTS

S. aureus isolates

Out of the 200 animals investigated, 23 (11.5%; 95% CI 8-17%) tested positive for *S. aureus* based on the presence of the *nuc* gene (Fig. 1a).The univariate logistic regression analysis showed that the percentage of *S. aureus* was higher in goats with cutaneous and mucosal lesions than in goats without such lesions (*P*=0.01) and it varied significantly across the goat breeds (*P*= 0.02) (Table 2).

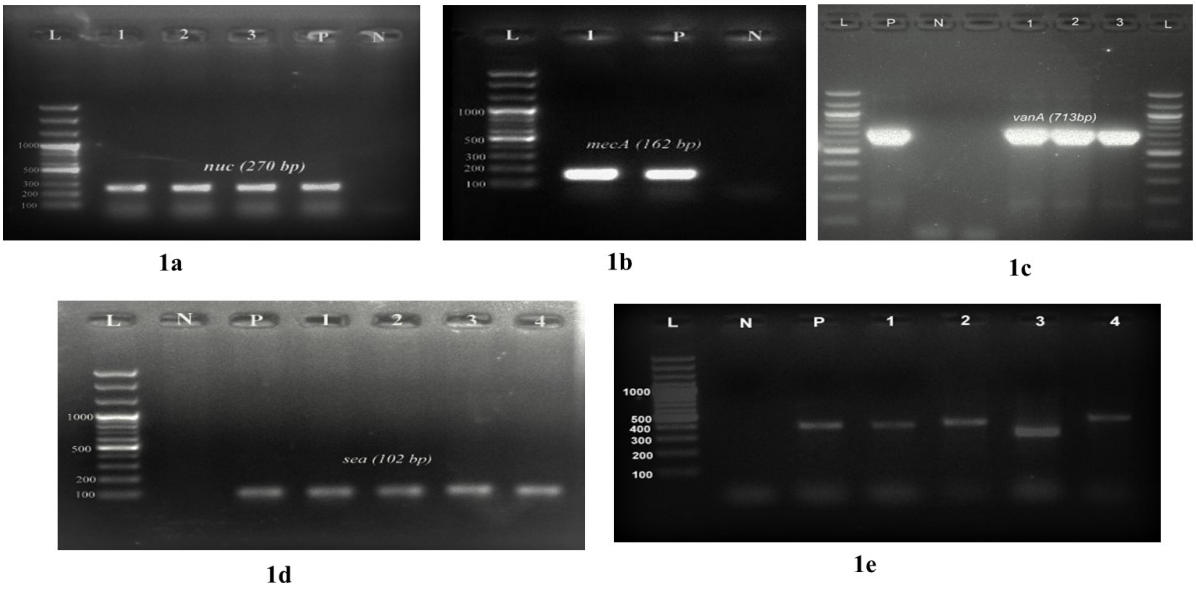

Fig. 1. Result of PCR assay for detection of *Staphylococcus aureus* (*nuc*, *spa*), antimicrobial resistance genes (*mecA*, *vanA*) and virulence gene (*sea*).

Table 2. Risk factors associated with prevalence of *S. aureus* in goats

Variables	Co-Variables	N	Percentage (%)	95% CI	*P*-value
Breed	Black Bengal	22	2(9)	1.34-29.0	0.02
	Cross	98	6(6)	2.58-12.98	
	Jamnapari	80	15(19)	11.59-28.77	
Sex	Female	120	15(12.5)	7.61-19.71	0.58
	Male	80	8(10)	4.92-18.75	
Age	< 1-year kid	80	9(11)	5.82-20.23	0.93
	Adult	120	14(11.7)	6.96-18.75	
Cutaneous and mucosal lesions	No	188	19(10.1)	6.49-15.32	0.01
	Yes	12	4(33)	13.55-61.20	

Antibiogram profiles of *S. aureus* isolates

The antimicrobial susceptibility pattern of 23 *S. aureus* isolates showed that all the isolates tested were resistant to ciprofloxacin (100%) followed by cefoxitin (74%), amoxicillin-clavulanic acid (65%), sulfamethoxazole-trimethoprim (65%), vancomycin (57%), cefepime (57%) and tigecycline (39%). On the contrary, the majority of the isolates showed sensitivity to gentamicin (78%) (Table 3). Individual antibiogram profiles of all isolates are illustrated in Fig. 2. Of all, 21 (91%) isolates were found as MDR. The MDR pattern of the isolates has been summarized in Table 3.

Detection of resistance genes

Among phenotypically 17 cefoxitin-resistant isolates, only one was found positive for the *mecA* gene to be considered as MRSA (6%; 95% CI 0-29%) (Fig. 1b). Therefore, the remaining 22 isolates were methicillin-susceptible (MSSA). In addition, among 13 phenotypically vancomycin-resistant *S. aureus* isolates, 3 (23%; 95% CI 8-51%) were positive for *vanA* gene detected in this study (Fig. 1c).

Table 3. Antimicrobial susceptibility profiles of *S. aureus* isolates (n= 23)

Antimicrobials	Susceptible (%)	Intermediate (%)	Resistant (%)
Amoxicillin-clavulanic acid	8(35)	-	15(65)
Vancomycin	10(43)	-	13(57)
sulfamethoxazole-trimethoprim	8(35)	-	15(65)
Ciprofloxacin	-	-	23(100)
Cefepime	9(39)	1(4)	13(57)
Gentamicin	18(78)	2(9)	3(13)
Cefoxitin	6(26)	-	17(74)
Tigecycline	4(17)	10(43)	9(39)

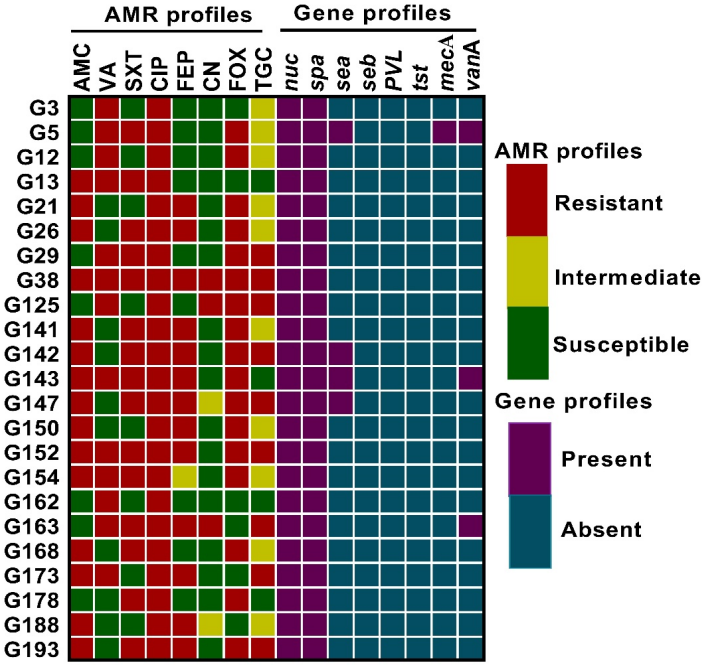

Fig. 2. Heat map showing the distribution of antimicrobial resistance phenotype and different virulence and resistance genes in *S. aureus* isolates.

Virulence attributes of *S. aureus*

Out of the virulence factors investigated in the 23 staphylococcal isolates, four (17%; 95% CI 6-38%) were positive for the *sea* gene (Fig. 1d) that encodes enterotoxin A, one of them was MRSA. No isolates tested positive in the PCR test for *seb, tst* and *PVL* gene.

spa types and phylogenetics

Amplification of polymorphic X region of the *spa* gene resulted in the formation of two different DNA bands with the sizes of ~ 300 to 400 bp (Fig. 1e), then we sequence one from each pattern as a representative. One of them was MRSA strain and another one was MSSA. The *spa* gene sequences were submitted to the NCBI GenBank (accession MT223771 and MT223772). The *spa* gene sequence (MT223771) of MSSA isolate was typed as t5259and another sequence (MT223772) of MRSA appeared to be novel, as it was not described yet in the *spa* Ridom Server used for analysis in this study. The

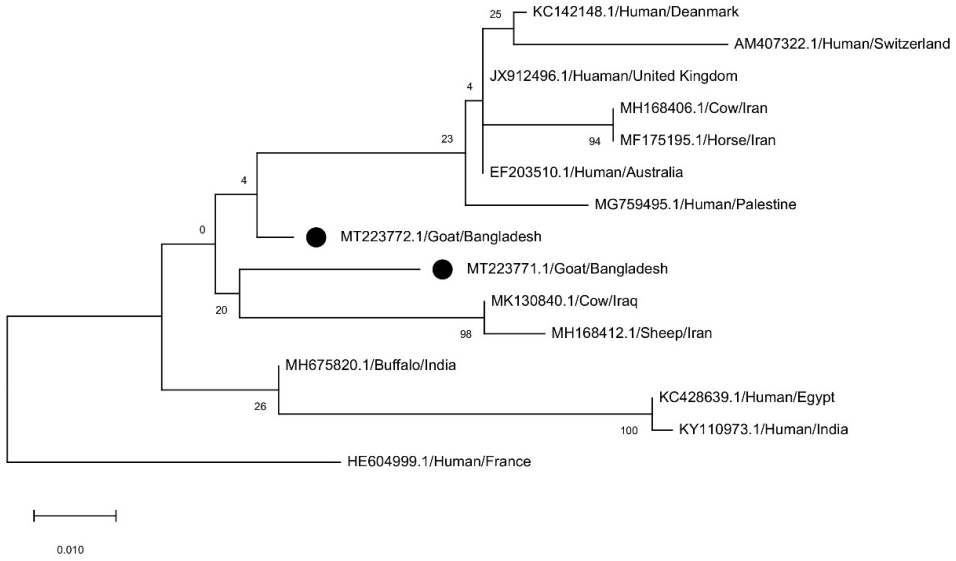

Fig. 3. Phylogenetic tree of partial sequences of *spa* genes.

repeat successions for these two sequences were 26-23-17-34-17-12-16 and 07-23-12-21-12-41-13-17-12-12-17 respectively. The Kreiswirth IDs of them were T1J1M1B1M1G1K1 and U1J1G1F1G1U2E1M1G1G1M1, respectively. The number of repeat units was 7 and 11, consecutively whereas the lengths of the entire VNTR were 168 and 264, respectively. For t5259, the repeat sequence started at 392 and for the untyped one, it started at 77. Phylogenetic analysis showed greater similarity between these two *spa* sequences (Fig. 3).

DISCUSSION

Studies have shown that goats with or without clinical condition(s) like respiratory illness can harbor *S. aureus* in their nares (4, 5). A wide variation in the prevalence estimate of *S. aureus* from goats has been reported which ranges from 5.6% to 43.2% or even more (27-29). Here, we reported the percentage of *S. aureus* as 11.5% in goats attending a teaching veterinary hospital, in Bangladesh.

S. aureus is widely resistant to the *β*-lactam group of antimicrobials which is evident in this study as well (1, 27). Almost 57% of isolates in this study were vancomycin-resistant (VRSA) as revealed by the disk diffusion method, which is the drug of choice for the treatment of MRSA infections though this antimicrobial is not commonly prescribed in goat practices in Bangladesh (30). Out of 13 phenotypically vancomycin-resistant isolates, 3(23%) were positive for the *vanA* gene which is lower than the previous study findings from other Asian countries (12). The remaining isolates might possess other *van* genes that were not tested in this study. However, heteroresistance might be responsible for seeing such a high rate of vancomycin resistance in *S. aureus* (31). Surprisingly, about 39% of isolates were resistant to the first glycylcycline antibiotic, tigecycline, which is regarded as an effective therapy against MSSA and MRSA without any co-resistance mechanisms yet to be known (32). Approximately, 91% of isolates were MDR. Selective pressure imposed by the use of antimicrobials as therapeutic or chemoprophylaxis might be the main cause of seeing such an unprecedented rate of multidrug resistance among *S. aureus* isolates (33). Isolation of MRSA circulating in the goat population has great public health significance since LA-MRSA can be transmitted to humans, especially to farmers, slaughter-house workers, and consumers of small-ruminant products in general (34) due to direct contact with animal, and can cause severe infection (9-11). In this study, 74% of isolates have shown resistance to cefoxitin, among which only one (6%) isolate was found to carry *mecA* gene confirming it as a methicillin-resistant isolate.

We acknowledge that due to lack of logistics, we studied only *mecA* gene for MRSA identification (which is considered as gold standard), we did not study *mecC* gene (a novel *mecA* homologue) as suggested for definitive identification of MRSA (35). However, marked variation in the prevalence of MRSA based on geography, host, environmental settings, etc. has already been described across Europe, Asia, and the USA (1, 27, 28, 32, 36). Among the virulence factors investigated, 4/23 strains were positive for the *sea* gene which is one of the classical enterotoxin producing genes. Although nine different genes are responsible for heat-stable staphylococcal enterotoxins (SEs), still the *sea* is considered the most prevalent one (37, 38) with occasional exceptions (3, 28). Epidemiological studies in humans suggested that the majority of *S. aureus*-associated infections and outbreaks have been caused by isolates with the *sea* type enterotoxin (39, 40). Notably, two strains were positive for both the *sea* gene and coagulase enzyme indicating high pathogenic potential.
A novel *spa* type from an MRSA isolate, not yet described in Ridom *spa* Server, was detected in this study. Another *spa* type, which is t5259, detected in this study was also rarely seen in the *S. aureus* isolates of small ruminants. A study in Japan reported the same *spa* type t5259 as MSSA from dairy cattle (41). The predominant *spa* types of *S. aureus* from goats in Europe are t1166 and t318 (42) and from sheep t002, t1534, t2678 and t3576 (43). There are few reports on the *spa* typing of *S. aureus* isolates in Bangladesh. One study involving *S. aureus* of ready-to-eat foods origin in Dhaka, Bangladesh identified seven different *spa* types with the predominance of t1198 and t315 (44). Notably, the novel *spa* type identified from this study was also an MRSA strain. Our study suggests that the carriage frequency of *S. aureus* was highest in goats with cutaneous and mucosal lesions such as dermatitis and oral lesions. Several previous studies indicated that animals with chronic infections such as dermatitis, and otitis require veterinary interventions and antimicrobial therapies more frequently which, in turn, increases the transmission burden of nosocomial pathogens like *S. aureus* and also their high antimicrobial resistance (45, 46).

In conclusion, this study has reported the high frequency of MDR *S. aureus* from goats in Bangladesh. Detection of *vanA* gene indicates the dissemination of vancomycin resistance among *S. aureus* limiting the treatment options. Since human transmission with a particular goat-originated MRSA strain is possible, a systematic study covering the whole country should be conducted coupled with an awareness campaign among livestock-associated personnel.

CONFLICT OF INTEREST

Authors have no potential conflict of interest

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