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Original Article

Detection of multidrug-resistant methicillin-resistant *Staphylococcus aureus* from healthy black Bengal goat in Bangladesh

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Abstract

Introduction: The emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is a growing public health concern. The objective of this study was to determine the prevalence and multi-drug resistant (MDR) profiles of MRSA in goats in Bangladesh.

Methodology: A total of 150 samples from goats comprised of rectal swab (n = 50), nasal swab (n = 50), and milk (n = 50) were collected. Isolation of *S. aureus* from samples was conducted onto mannitol salt agar (MSA). Identification of *S. aureus* was performed by cultural characteristics, Gram staining, biochemical tests (catalase, coagulase, indole, methyl red, and Voges-Proskaur), and *nuc* gene-specific PCR assay. The MRSA was identified by cefoxitin disc diffusion test and *mecA* gene-specific PCR assay. The MDR profiles of MRSA were performed against ampicillin, amoxicillin, gentamicin, cefoxitin, vancomycin, azithromycin, cefotaxime, ciprofloxacin and nalidixic acid by disc diffusion method.

Results: The overall prevalence of *S. aureus* was 35.3% and MRSA was 7.3%. The prevalence of MRSA was 12% in rectal swabs, 8% in nasal swabs, and 2% in milk. The highest resistance of MRSA was against ampicillin (91%) followed by azithromycin (55%), amoxycillin (36%), nalidixic acid (27%), ciprofloxacin (18%) and cefotaxime (9%). Most MRSA isolates (90.9%) exhibited resistance to at least three classes of antibiotics and were MDR.

Conclusions: This study shows that goats may harbor MDR-MRSA, posing a risk to public health.

Key words: Methicillin-resistant *Staphylococcus aureus*; MRSA; healthy goat; public health.

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Introduction

A genetically different subgroup of *S. aureus* known as MRSA is also referred to as a superbug due to its resistance to widely used antibiotics. It is an emerging pathogen that poses significant risks to the public's health [1]. The use of antibiotics in the treatment of bacterial infectious diseases has considerably reduced both human and animal mortality. A major public health concern is the development of antimicrobial resistance (AMR), which is brought on by the misuse of antibiotics [2-4]. Long-term, indiscriminate antibiotic use is thought to have contributed to the development of MDR bacteria, including MRSA [6]. The penicillin-binding protein 2A (PBP2A), which is encoded by the *mecA* gene in the staphylococcal chromosomal cassette mec (SCCmec), is present in MRSA and prevents lactam antibiotics from inhibiting the formation of cell walls [6-8].

The first case of MRSA in livestock (LA-MRSA) was discovered in a pig in 2005 [9]. Although LA-MRSA colonization without symptoms is quite common, it can infect both humans and animals [10]. The MRSA infection in humans may spread through companion animals [11]. Domestic animals that were housed with MRSA-infected people were reported to be positive [12]. MRSA, which was initially identified in humans, is known to be present in pet animals, including dogs, cats, and horses [13-14]. Direct skin-to-skin contact and touching contaminated objects are the two main ways that MRSA is spread between people and animals. Animals mostly get skin infections as a result of it. Globally, the LA-MRSA is an emerging zoonotic pathogen [15]. Goats, poultry, dairy cattle, and beef cattle have all been documented to have MRSA [16-17]. MRSA has been found in cats and dogs in Bangladesh [18-19]. According to several studies, animals may contribute to the spread of MRSA from humans to animals [20]. People who continue to interact with animals are at greater risk of getting LA-MRSA. Animals colonized or infected with MRSA are easily able to spread the disease to farm workers [21]. The European Union's member states launched a scanning surveillance program to find LA-MRSA in retail beef, chicken, and pig meat because of the threat it poses to public health [16].

Ruminant-to-human MRSA zoonotic transmission has been documented [22]. In Bangladesh, goats have the biggest ruminant population, with a current population of over 26.94 million [23]. In Bangladesh's rural areas, raising goats is a financially successful endeavor due to the high demand for their meat and skin on both domestic and foreign markets. The rural Bangladeshi population occasionally consumes goat's milk. Most of the farmers in the village keep their goats inside their homes and have close relationships with them. Goats in Bangladesh have been documented to have MRSA and *S. aureus*-related pneumonia and mastitis [24-26]. A healthy animal can spread MRSA to people and other animals by serving as a reservoir for the bacteria [27]. The importance of goats as MRSA reservoirs, however, has not been investigated in Bangladesh. Therefore, the objectives of this investigation were to i) determine MRSA prevalence and ii) determine MDR profiles of MRSA in apparently healthy goats. The prevalence of MRSA in Bangladesh's healthy Black Bengal goats is reported for the first time in this study.

Methodology

Sample collection

Between July and November 2018, 150 samples were taken from black Bengal goats in five villages in the Mymensingh sadar Upazila (24.7500 °N, 90.4167 °E) and Tarakanda Upazila (24.8527541 °N, 90.313287 °E). These samples included 50 rectal swabs, 50 nasal swabs, and 50 milk samples. The black Bengal goats seemed to be in good health (Table 1). For each animal, only one sample—either a rectal swab, a nasal swab, or milk—was collected. Using sterile cotton swabs, rectal and nasal swabs were taken directly from the rectum and nasal passages. The swab was individually inserted into a test tube containing 5 mL of peptone broth. Goat milk (10 mL) was collected aseptically into a sterile test tube. All samples were delivered to the bacteriology laboratory in ice boxes for analysis.

Isolation and identification of S. aureus

By incubating the samples in trypticase soy broth for 24 hours at 37°C, the samples were enriched. A *Staphylococcus* spp.-selective medium, mannitol salt agar, was streaked with enrichment culture and incubated at 37 °C for 24 hours. Gram staining, biochemical tests (catalase, coagulase, indole, methyl red and Voges-Proskauer tests), and culture traits were used to identify *S. aureus.*

Extraction of bacterial genomic DNA

A procedure that had already been reported [28] was modified somewhat in order to prepare the template DNA from Staphylococcus species. A 1.5 mL microcentrifuge tube was used to produce a culture of *Staphylococcus* spp. at 37°C for 18–20 hours. Centrifugation was used to recover the bacteria for 10 minutes at 12000 rpm. The bacterial pellet underwent two sterile PBS washes before being resuspended in 100 µL of nuclease-free water. A 15-minute boil in a hot water bath was followed by a 20-minute snap cool on ice for a bacterial suspension in a 1.5 mL microcentrifuge tube. Following centrifugation of the bacterial suspension at 12000 rpm for 10 minutes at 4 °C, 50 µL of the supernatant fluid was used as a DNA template for PCR assays.

Molecular detection of S. aureus and MRSA by PCR assays

Two sets of synthetic oligonucleotide primers of 21 and 24 bases, respectively, were used in the PCR to amplify a sequence of the *nuc* gene, which encodes the thermostable nuclease of *S. aureus* [29]. Amplification of 279 bp fragment of the *nuc* gene was used to identify *S. aureus*. Briefly, the PCR mixture (20 µL) contained 5.5 µL of nuclease-free water, 12.5 µL of the 2PCR master mixture (Promega, USA), 1 µL of each of the forward and reverse primers (20 pmol), and 5 µL of genomic DNA. The PCR reaction was carried out in 30 cycles of initial denaturation at 95°C for 1 minute, annealing at 55°C for 45 sec, and extension at 72°C for 1 minute. The first denaturation took place at 95°C for 5 min. The last extension was carried out for 10 minutes at 72 °C. The positive and negative controls, respectively, were MRSA ATCC 33591 and nuclease-free water.

The amplification of a 533 bp fragment of the *mecA* gene was used to detect MRSA [30]. The PCR mixture was created as previously mentioned. Initial

Table 1. Detail information of sampling of goat.

Study area	Sampling site	Flock size	Samples collected		
			Rectal swab	Nasal swab	Milk
Mymensingh sadar Upazila	Village A	30	10	10	10
	Village B	30	10	10	10
	Village C	30	10	10	10
Tarakanda Upazila	Village D	30	10	10	10
	Village E	30	10	10	10

Table 2. Oligonucleotide primers used in PCR for detection of *nuc* and *mecA* genes of *S. aureus.*

List of Primers	Primer sequences	PCR product size (bp)	References
nuc F	5′-GCGATTGATGGTGATACGGTT-3′	**279**	[29]
nuc R	5′-AGCCAAGCCTTGACGAACTAA AGC-3′		
*mec*A F	5′-AAA ATC GAT GGT AAA GGT TGG C- 3′	**533**	[30]
*mec*A R	5′-AGT TCT GCA GTA CCG GAT TTG C -3′		

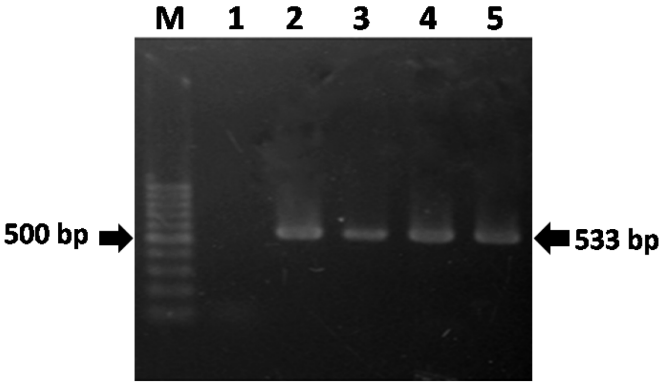
denaturation of the PCR was carried out at 95 °C for 5 minutes, followed by 30 cycles of annealing at 55 °C for 45 seconds, extension at 72 °C for 1.5 minutes, and final extension at 72 °C for 10 minutes. Used controls, both positive and negative, have already been described.

The PCR products were run on a 2% agarose gel at 100 volts for 30 minutes, stained with ethidium bromide, and captured using a gel documentation system (Biometra, Germany). Table 2 contains a list of primers.

Antibiotic susceptibility test

MRSA's antibiotic susceptibility pattern was determined using a cefoxitin disc diffusion test on a Muller Hilton agar plate in accordance with the Clinical and Laboratory Standards Institute's instructions [31]. Ampicillin (25 mcg), vancomycin (30 mcg), cefoxitin (30 mcg), gentamicin (10 mcg), cefotaxime (30 mcg), amoxicillin (30 mcg), azithromycin (30 mcg), nalidixic acid (30 mcg), and ciprofloxacin (5 mcg) were tested against MRSA for antibiotic susceptibility [32]. The guidelines of the Clinical and Laboratory Standard Institute were used to interpret the findings of the antibiotic susceptibility test [31]. As a control strain, MRSA ATCC 33591 was employed.

Figure 1. Detection of PCR amplicons (533 bp) of *mecA* gene in *S. aureus*.



Lane M: 100 bp size DNA ladder, Lane 1: Negative control, lanes 2- 4: PCR amplicons of *mecA* gene of *S. aureus* isolated from rectal swab, nasal swab and milk samples of goats and lane 5: positive control (*S. aureus* ATCC 33591).

Statistical analysis

The SPSS software was used to evaluate all the data, which was included in Excel sheets (MS-2010) (SPSS-24.0). Prevalence was calculated using descriptive analysis, and the level of significance was determined using the Chi-square test. Statistical significance was defined as a p value of 0.05 or below.

Results

Isolation and identification of Staphylococcus aureus

Colonies of *S. aureus* on MSA were small round and yellow. They produced β haemolysis on blood agar. They were arranged in grape-like cluster and were Gram positive. They were positive for catalase, coagulase, methyl red, and Voges-Proskaur tests and negative to indole test. They fermented five basic sugars: dextrose, maltose, lactose, sucrose and mannitol. The *nuc* gene was successfully amplified by PCR with the production of 279 bp PCR amplicons indicating that they were *S. aureus*.

Detection of MRSA

A total of 11 *S. aureus* isolated from rectal swab (n = 6), nasal swab (n = 4) and milk (n = 1) were found resistant to cefoxitin indicating that they were MRSA. PCR primers targeting *mecA* gene successfully amplified 533 bp fragments of DNA from *S. aureus* confirmed that they were MRSA (Figure 1).

Prevalence of S. aureus and MRSA

The overall prevalence of *S. aureus* was 35.3% (53 of 150). The prevalence of cefoxitin resistant *S. aureus* was 7.3% (11 of 150) .The highest prevalence of *S. aureus* was recorded in nasal swab (40%) followed by rectal swab (34%) and milk (32%) (Table 3). On the

Table 3. Prevalence of *S. aureus* and MRSA in rectal, nasal and milk samples of goat.

Type of specimens	No. of Sample tested	No. of samples positive for *S. aureus* (%)	*p*	No. of *S. aureus* isolates reststant to cefoxitin (%)	*p*
Rectal swab	50	17 (34)		6 (12)	
Nasal swab	50	20 (40)	0.684 NS	4 (8)	0.155 NS
Milk	50	16 (32)		1 (2)	

MRSA: Methicillin resistant *S. aureus;* NS: Not significant ($p > 0.05$).

Table 4. Prevalence of *S. aureus* and MRSA between flocks.

Flock location	No. of samples tested	No. of *S. aureus* positive samples (%)	*p*	No. of MRSA positive samples (%)	*p*
Village A	30	13 (43.3)		2 (6.7)	
Village B	30	8 (26.7)		4 (13.3)	
Village C	30	9 (30.0)	0.831 NS	0(0.0)	0.434 NS
Village D	30	13 (43.3)		3 (10.0)	
Village E	30	10 (33.3)		2 (6.7)	

MRSA: Methicillin resistant *S. aureus;* NS: Not significant ($p > 0.05$).

other hand, prevalence of MRSA in rectal swab, nasal swab, and milk was 12%, 8%, and 2%, respectively (Table 3). The flock-wise prevalence of *S. aureus* and MRSA in the study areas is shown in Table 4.

Antibiotic susceptibility profiles of MRSA

All MRSA were found susceptible to vancomycin and gentamycin. The highest resistance was observed against ampicillin (90.9%) followed by azithromycin (54.6%), amoxicillin (36.4%), nalidixic acid (27.3%), coprofloxacin (18.2%), and cefotaxime (9.0%) (Table 5). The 9.0% MRSA isolates were found resistant to two different classes of antibiotics, 36.4% MRSA isolates were resistant to three and four classes of antibiotics and 18.2% isolates showed resistance against five different classes of antibiotics (Table 6).

Discussion

MRSA is an important zoonotic pathogen responsible for a number of clinical illnesses in humans and animals which are very difficult to treat with antibiotics. Transmission of MRSA from goat to human was reported [33]. MRSA can be spread from infected animals to susceptible animals through direct contact, sharing the common house for living, and utensils used for feeding and drinking of animals. Animals living in crowded conditions are often contracted with MRSA. The reservoir roles of apparently healthy goats for MRSA have not been studied in Bangladesh. Humans may get infected with MRSA by direct contact with carrier animals [34]. MRSA got zoonotic importance when scientists suggested the possibility of goats as reservoirs for human MRSA infection. Therefore, the present research work was undertaken to determine the prevalence and MDR profiles of MRSA in goats reared by farmers in the rural areas of Bangladesh.

MRSA is known to colonize the intestinal tract of humans and animals. The LA-MRSA can cause health hazards to people engaged in livestock rearing and production practices [35]. Two human cases of MRSA

Table 5. Antibiotic susceptible pattern of MRSA against 10 antibiotics.

MRSA Isolates tested (n)	Antimicrobial agents	Resistant (%)	Susceptible (%)	Intermediate (%)
11	Ampicillin	10 (90.9)	1(9.0)	0 (0.0)
	Gentamicin	0 (0.0)	9 (81.8)	2 (18.2)
	Cefoxitin	11 (100.0)	0 (0.0)	0 (0.0)
	Vancomycin	0 (0.0)	11 (100.0)	0 (0.0)
	Amoxicillin	4 (36.4)	7 (63.6)	0 (0.0)
	Azithromycin	6 (54.6)	3 (27.3)	2 (18.2)
	Cefotaxime	1 (9.0)	7 (63.6)	3 (27.3)
	Ciprofloxacin	2 (18.2)	8 (72.7)	1 (9.0)
	Nalidixic acid	3 (27.3)	5 (45.5)	3 (27.3)

Table 6. Multidrug resistance profile of methicillin resistant *S. aureus.*

Isolates (n)	No. of Antibiotic (Class)	Multidrug Profile	No. of Isolates (%)	Prevalence of MDR %
MRSA (11)	1 (0)	Any one of the tested antibiotics	0 (0.0)	90.9
	2 (2)	AMP-CEF	1 (9.0)	
	3 (3)	AMP-CEF-CTX AMP-CEF-NAL AMP-CEF-AZM AMP-CEF-CIP	4 (36.4)	
	4 (4)	AMP-CEF-AZM-NAL AMP-CEF-AZM-NAL AMP-CEF-AZM-CIP AMP-CEF-NAL-CIP	4 (36.4)	
	5 (5)	AMP-CEF-AZM-CIP-NAL AMP-CEF-AZM-CIP-NAL	2 (18.2)	

AMP: Ampicillin; CEF: Cefoxitin; AZM: Azithromycin; CIP: Ciprofloxacin; NAL: Nalidixic Acid CTX: Cefotaxime; MDR: Multidrug resistance.

infections were linked with livestock reservoirs [11]. The LA-MRSA can infect veterinarians, livestock farmers, and abattoir workers as they frequently come in contact with animals [36-38]. Hospital staff involved in the direct care of an MRSA-infected dog were found MRSA positive. Transmission of this zoonotic pathogen can be possible either by fecal-oral route or through direct contact with animals [10]. MRSA were found to survive in feces for weeks which could be a source of infection for humans and animals. This study recorded a 12% prevalence of MRSA in rectal swabs of goats. A study conducted in Egypt found that 50% of *S. aureus* isolates were positive for MRSA in the fecal swabs of sheep [39].

The MRSA colonizes in the nasal cavity and is isolated from pneumonic goats [33]. This study recorded an 8% prevalence of MRSA in the nasal swabs. In Spain, 6% nasal carriage of MRSA in healthy dairy goats was found [40]. In Tunisia, MRSA prevalence was 3% in sheep nares and 0% in goat nares [41]. In Greece, a 76.9% prevalence of *S. aureus* was seen in the nasal swab of goats, and found no MRSA in nares [42]. In India, 76.15% prevalence of *S. aureus* was recorded in the nasal secretions of goats but none of the isolates were found positive for MRSA [43]. In Egypt 3.9% prevalence of MRSA was observed in goats [1]. The prevalence of MRSA was 2.6% in nasal swabs of animals that showed respiratory signs [17]. Higher prevalence of MRSA in the nasal swabs of apparently healthy goats recorded in the study might be due to the small number of samples screened. Animals can be infected with MRSA during sneezing and coughing of an infected animal. Sneezing and coughing of carrier animals may excrete MRSA through respiratory droplets which can be spread to humans.

MRSA was isolated from the milk of healthy goats as well as goats suffering from subclinical mastitis [26,44]. This study recorded a 2% prevalence of MRSA in milk. A study conducted in Italy reported a 1.23% prevalence of MRSA in bulk milk tanks [22]. In Greece, although 80% prevalence of *S. aureus* in caprine bulk tank milk was recorded but no MRSA was detected [42]. In Saudia Arabia, 9.2% prevalence of MRSA in mastitic milk was detected [17]. The presence of MRSA in milk is alarming from public health point of view. People may contract MRSA through drinking of contaminated milk. Humans may also get infected with MRSA during milking of carrier animals since MRSA was reported in teat skin samples of dairy goats [44].

Antibiotics are routinely used in food animal, production practices and as a growth promoter. Excessive use of antibiotics in veterinary medicine leads to the development of drug-resistant MRSA clones due to selection pressure [27]. Multidrug resistance is defined as the resistance of an isolate to three or more groups of antibiotics [45]. The MRSA isolates of goats in this study exhibited multidrug-resistant profiles (90.91%) since they were found resistant to at least three different classes of antibiotics. A study conducted in Saudi Arabia reported 100% MDR-MRSA in goat [17]. In Poland 92.9% of MDR strains of MRSA were reported in hospitalized patients [46].

A study conducted in Nigeria recorded 44% MDR strains of MRSA in fresh and fermented milk samples [47]. The MDR mastitis pathogens especially MRSA are considered as a potential threat to dairy goat production [22]. It also constitutes a significant public health problem worldwide [27].

MRSA are very difficult to treat because they are resistant to many classes of antibiotics. To treat MRSA infections, it is important to have adequate information and understanding the trends of antibiotic resistance patterns of these bacteria. In this study, MRSA exhibited resistance against ampicillin (90.9%), azithromycin (54.6%), amoxicillin (36.4%), nalidixic acid (27.3%), ciprofloxacin (18.2%) and cefotaxime (9.0%). Harrison *et al.* [11] stated that most of MRSA isolates are susceptible to penicillin when used in combination with β-lactamase inhibitors such as clavulanic acid. The present study is in agreement with the findings of other investigators [48] since we used only penicillin (amoxicillin) without a combination of β-lactamase inhibitors and recorded the resistant phenotype of MRSA against amoxicillin. These broad-spectrum antibiotics are used to treat bacterial infections in companion and food animals. The relatively high level to ampicillin and azithromycin resistance was observed in this study since these antibiotics are used as growth promoters and routine prophylaxis in livestock production practices in Bangladesh [49]. Penicillin, macrolide, quinolones, fluoroquinolones and third-generation cephalosporin resistance was previously reported in MRSA [19,32,50-51]. In this study, the MRSA strains (100%) were found susceptible to vancomycin which is the drug of last resort to treat MRSA. Similar findings were also reported by other investigators [19,52].

Conclusions

Data of this study suggest that goat harbors MRSA which might cause public health hazard if transmitted to human during close contact with carrier animals. This study will be helpful in creating awareness of

MRSA among goat raisers, veterinarians and goat meat consumers and motivate them to undertake bio-security and sanitary practices at farm. Regular surveillance and early detection of MRSA in goat are important to minimize its spread to other animals and humans.

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