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RESEARCH ARTICLE

Molecular detection, antibiogram, and risk factor analysis of *Staphylococcus aureus* from subclinical mastitis of goats in conventional and organized farms

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ABSTRACT

Objectives: The goal of this study was to isolate, molecularly identify, and antibiogram of *Staphylococcus aureus*, as well as to investigate risk factors for subclinical mastitis in the Jhenaidah and Chuadanga districts of Bangladesh.

Materials and Methods: A total of 100 milk samples were collected from apparently healthy goats at various farms in the Jhenaidah and Chuadanga districts. To detect samples with subclinical mastitis (SCM), the California Mastitis Test (CMT) was done. *S. aureus* was isolated and identified from milk samples that tested positive for CMT using a combination of standard bacteriological examinations, biochemical tests, and PCR confirmation. The disc diffusion test was used to determine the antibiogram status of the isolates. The risk factors for SCM in goats were analyzed using a semi-structured questionnaire with thirteen variables.

Results: Some characteristics were significantly associated with goat mastitis, including farm, breed, shed construction, floor surface condition, and farm adviser. The CMT identified 52 positive samples for SCM, with 73.07% (n = 38/52) suggesting the presence of the *nuc* gene. Antibiotic susceptibility testing revealed that the isolated *S. aureus* was totally resistant to the antibiotics Penicillin, Oxacillin, and Vancomycin (21%). On the other hand, Tetracycline, Gentamycin, Norfloxacin, and Levofloxacin were all susceptible to all isolates.

Conclusion: SCM is a severe problem in goats in Jhenaidah and Chuadanga districts. To minimize the risk of SCM infection in goats, hygiene precautions can be followed, and education among goat farm owners and farmers can be improved.

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Antibiogram; *nuc* gene; PCR; Subclinical mastitis; *S. aureus*.

Introduction

Bangladesh is a predominantly agricultural country with a large number of domestic animals. Goats contribute significantly to GDP by producing meat, milk, and skin, which account for approximately 27.0, 23.0, and 28.0% of total livestock production, respectively [1]. Around 45% of the population in Bangladesh lives below the poverty line [2], while 36% of all farm households in Bangladesh rear goats under scavenging conditions [2]. Goat farming is a significant source of cash-generating for poor farmers in Bangladesh. Mastitis is one of the most serious infections affecting mammals, including goats, throughout the world. Mastitis is a complex and infectious disease that affects the udders of dairy animals [3]. In Mymensingh and Joypurhat districts, the prevalence of SCM in goats was 44.59% using CMT screening and 5.273% with clinical diagnosis [4]. Rahman et al. [5] stated that mastitis in dairy goats was

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primarily caused by a lack of awareness of the owners. Goat rearing is done throughout Bangladesh, including the Jhenaidah and Chuadanga districts, due to excellent climatic conditions and the availability of feed resources. However, various diseases, including mastitis, are endemic in these two districts' goat populations.

Breed, age, parity, lactation stage, housing system, management techniques, season, and geographical location are all significant risk factors for the development of mastitis in goats [6-10]. Other factors such as the goats' nutritional health, concreted flooring, teat and host management, or environmental factors such as lactiferous sinuses and the milking system all contribute to the development of mastitis in goats [11]. Numerous bacteria are responsible for mastitis in goats, with *Staphylococcus* spp. being the most frequently reported [4,6,12-17]. *Staphylococcus aureus* was isolated and identified earlier using *nuc* gene amplification [18-23]. Martnez et al. [24], Lima et al. [25], Kabir et al. [26], and Islam et al. [27] all reported an antibiogram for *S. aureus* isolated from goats.

While goat farming is prevalent in Jhenaidah and Chuadanga districts, the frequency of mastitis and its associated causes remain unknown. Additionally, it is critical to understand the antimicrobial profile to treat and control mastitis in animals effectively. As a result, this study was conducted to reveal several factors linked with goat mastitis and perform molecular detection and characterize the antibiogram profile of *S. aureus* isolated from SCM samples.

Materials and Methods

Ethical statement

The research was conducted following established ethical norms and guidelines. Without injuring the goats, milk samples were obtained.

Survey

Between July and August 2018, a semi-structured questionnaire was used to collect all pertinent data, including age, breed, farm, shed structure, floor surface condition, responsible doctors, lactation period, shed washing interval, biosecurity fencing, disinfectant used, water resource, and other diseases in goat farms in the Jhenaidah (n = 25 farms) and Chuadanga (n = 29 farms) districts.

Sample collection

CMT was used to discriminate SCM samples. Milk samples were taken from the Jhenaidah Government Goat Farm (n = 35), the Wave Foundation goat farm (n = 13), and several local farms in the Jhenaidah and Chuadanga areas (n = 52). Using sterile test tubes, 10 ml of fresh milk was collected from each animal after dispensing a few droplets of milk beforehand. All samples were obtained according to established sample collection procedures without injuring or stressing any animals and immediately transported to Bangladesh Agricultural University's Microbiology Laboratory for bacteriological analysis using the cooling box.

Isolation of Staphylococcus aureus

To enrich microorganisms, primary culture was performed in nutrient broth (HiMedia, India). Pure cultures of *S. aureus* were obtained using the protocols described by Tanzin et al. [22]. These procedures included hemolysis on blood agar, Gram stain, morphological examination, biochemical features, catalase test, and coagulase test.

Molecular characterization by PCR

A pipette tip was used to extract the probable bacterial colony from the pure culture and dissolve it in 1 ml PBS (Phosphate-Buffered Saline) in the Eppendorf tube. To ensure appropriate mixing, the tube is placed in the vortex and centrifuged at 13,000 rpm for 2 min. After discarding the supernatant, 600 µl lysis solution is added and gently pipetted to mix. After incubation for 5 min at 80°C, 3 µl RNase solution is added. Pipette-mixed and incubated at 37°C for 45 min before cooling to room temperature. 200 µl of protein precipitation solution was added and mixed with a vortex. The mixture was then incubated on ice for 5 min before being centrifuged at 13,000 rpm for 5 min. As a result of the precipitation of protein, the pellet is discarded. The DNA-containing supernatant is collected. The supernatant is transferred to another Eppendorf tube containing 600 µl isopropanol and mixed by pipetting; the tube is centrifuged at 13,000 rpm for 2 min, the supernatant is discarded, and 600 µl 70% ethanol is added and mixed with the pellet; the tube is centrifuged at 13,000 rpm for 2 min as described by Mishra et al. [28].

The *nuc* gene (Table 1) was amplified using PCR with an initial denaturation step at 95°C for 1 min, followed by

Table 1. Primer sequence used in this study for detection of *S. aureus*

Primer name	Gene targeted	Primer sequence (5´-3´)	Amplicon size (bp)	Reference
nuc F	*nuc*	GCGATTGATGGTGATACGGT	279	Zhou et al. [13]
nuc R		AGCCAAGCCTTGACGAACTAAAGC		

Table 2. Prevalence of mastitis and *S. aureus*

Location	Farm Name	Total farm	Total sample	CMT positive No. (%)	*S. aureus* positive No. (%)
Chuadanga	Wave Foundation	1	13	8 (61.54)	8 (61.54)
	Local Farm	27	28	20 (71.42)	13 (46.42)
Jhenaidah	Jhenaidah Govt. Goat Farm	1	35	13 (37.14)	8 (22.86)
	Local Farm	23	24	11 (45.83)	9 (37.5)
Total	3 types	52	100	52 (52)	38 (38)

30 cycles of denaturation at 95°C for 5 min. The primers were annealed at 55°C for 45 sec and elongated at 72°C for 1 min. The final extension was carried out for ten min at 72°C. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and photographed using a Gel documentation system under ultraviolet light.

Antibiotic sensitivity test

The Clinical and Laboratory Standard Institute (CLSI) recommended an *in vitro* antibiotic sensitivity test [29]. This study utilized antibiotic discs impregnated with Levofloxacin (5 µg), Oxacillin (1 µg), Penicillin G (10 µg), Tetracycline (30 µg), Vancomycin (30 µg), Gentamycin (30 µg), and Norfloxacin (10 µg). The zone of inhibition was observed, and the results were reported as resistant and sensitive parameters.

Results and Discussion

Milk is an excellent food to consume. However, multiple experts have discovered that it is an optimal environment for bacterial growth. Diverse foodborne pathogens, such as *Escherichia coli*, *S. aureus*, and occasionally fungal, are confirmed in various food or food processing contexts [30-33]. The purpose of this study was to determine the risk factors associated with recognizing the common foodborne pathogen, particularly *S. aureus*. Within 100 milk samples 52 (52%) samples were positive by CMT in Jhenaidah and Chuadanga districts (Table 2).

The study discovered that five variables, including breed (n = 39, crossing), farm (n = 39/65 private farm samples), farm structure (Bambo shed), floor surface condition (n = 33/52), and responsible doctors (n = 29/54 farms in quak), had a significant effect on goat mastitis. Other variables such as age (out of 23 goats, n = 2/23 in 2 years and n = 21/23 in 3 years goats' sample) that was previously described by Yahia et al. [8], as supported by Gebrewahid et al. [9], and Razi et al. [10] (Table 3).

Among 100 samples, 38 (38%) included *S. aureus* that was Gram-positive, coagulase-positive, capable of fermenting sugar, and catalase-positive. Momin et al. [17] previously documented the same result of Grams staining

Table 3. Distribution of goat mastitis based on different risk factors of different areas of Bangladesh.

Factors	Variables	Significance	
		p-value	Level
Age	2 years		
	3 years	0.571	NS
Breed	Crossed	0.001	**
	Black Bengal Goat		
Location	Chuadanga		
	Jhenaidah	0.117	NS
Farm	Govt. Farm		
	Private Farm	0.001	**
Farm Structure	Concrete shed		
	Bambo shed	0.001	**
	Soil shed		
Disinfectant used	Yes	0.095	NS
	No		
Floor surface condition	Hygienic		
	Unhygienic	0.001	**
Water resource	Pond	0.516	NS
	Tube well		
	Others		
Washing interval of shed	<5 days		
	5-10 days	0.444	NS
	>15 days		
Biosecurity fencing	Yes	0.154	NS
	No		
Lactation Period	<93 days		
	93-95 days		
	95-97 days	0.208	NS
	>97 days		
Doctor	Quak		
	Registered Vet.	0.018	*
Correlation of other diseases	Fever, Cold and Mastitis	0.177	NS

Here, NS means not significant, * means .5% level of significance, and ** means 1% level of significance.

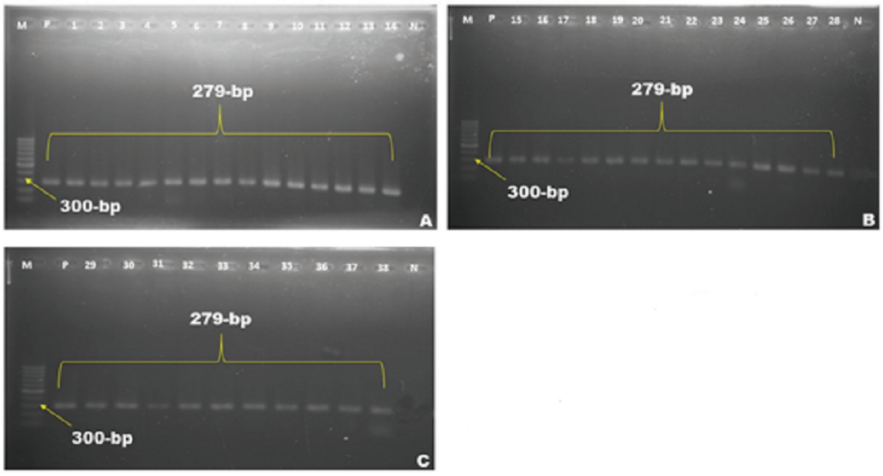

Figure 1. PCR amplification of nuc gene (279-bp) specific for the detection of *S. aureus*. Lane M = 100-bp ladder, P = Positive control, N = Negative control, Lane 1 to 14, 15 to 28, and 29 to 38 = amplified nuc gene.

Table 4. Antibiotic sensitivity profile of 38 coagulase positive *S. aureus*

Name of antibiotics	Resistant (%)	Sensitive (%)
Penicillin	100	0
Tetracycline	0	100
Gentamycin	0	100
Oxacillin	100	
Levofloxacin	0	100
Vancomycin	21.05	78.95
Norfloxacin	0	100

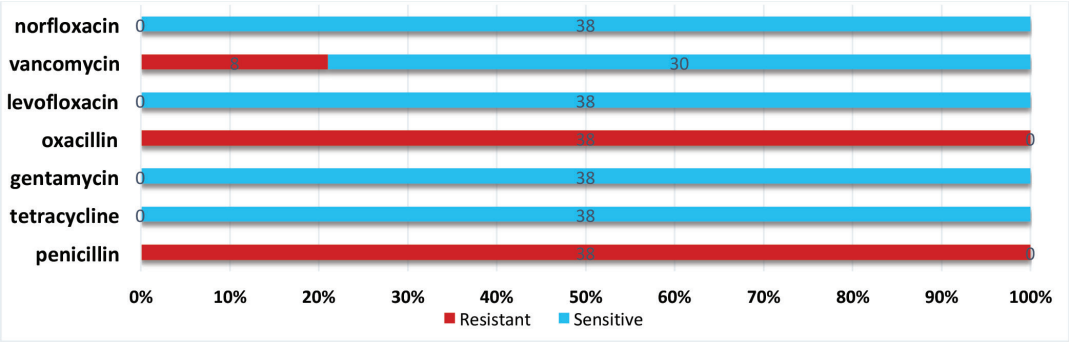

Figure 2. Results of antimicrobial susceptibility test of *S. aureus* (among 38 coagulase positive)

and biochemical testing for hemolysis on 5% sheep blood agar with the round, tiny, smooth elevated white colony. According to Islam et al. [27], 89.3% of *S. aureus* isolates from bovine origin were hemolytic. Gram staining revealed Gram-positive, violet-colored cocci that were shaped like grapes and clustered in grape-like clusters. The findings of this experiment indicated that 38 *S. aureus* had the *nuc* gene (amplicon size 279-bp, as shown in Fig. 1) but no *mecA* gene, as previously documented by Lopes et al. [18] and Yadav et al. [19].

Antibiograms of *S. aureus* revealed the most robust resistance to Penicillin and Oxacillin (Table 4, Fig. 2) [24,25]. Almost all isolates were susceptible to antibiotics such as Gentamycin, Vancomycin, Tetracycline, Levofloxacin, and

Norfloxacin, as reported by Momin et al. [17] previously reported. Although eradicating transboundary illnesses such as anthrax, rabies, and brucellosis is necessary, map-based correct immunization is required to eliminate mastitis, a multifactorial disease, and to maintain a sanitary environment [34,35,36]. Recently, BLRI developed a mastitis kit [37] that enables rapid diagnosis of the disease. However, producers are responsible for maintaining biosecurity and a hygienic environment to prevent their animals from mastitis.

Conclusion

The current research utilized two phages, namely survey and laboratory test. Among the risk variables, certain elements such as farm type, breed, shed floor, floor surface condition, and guidance from a subsequent person all have a direct positive significant effect on goat mastitis (p-value less than 0.05). Other variables like age, lactation period, washing interval, location, a serious disease, the shed's washing method, the disinfectant used to wash the shed, and biosecurity fencing are of no significance (p-value up to 0.05). *S. aureus* was detected in 38 (38%) of 100 CMT-positive mastitis samples from Jhenaidah and Chuadanga districts. Additionally, experimental observes that Penicillin and Oxacillin were highly resistant to the isolates. Gentamycin, Norfloxacin, and Levofloxacin had the best sensitivity pattern.

List of abbreviations

CMT, California Mastitis Test; SCM, Subclinical mastitis; *S. aureus*, *Staphylococcus aureus*; PCR, Polymerase Chain Reaction; GDP, Gross Domestic Product; UV light, Ultra-Violet light.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Authors' contributions

Conceptualization, KHMNHN and MLH; methodology, MAZ, AK and MMM; validation, KHMNHN and MLH; formal analysis, MAZ and MAHS; investigation, KHMNHN; resources, KHMNHN; data curation, MAHS; writing—original draft preparation, MSZ and MMM; writing—review and editing, MLH, and KHMNHN; visualization, KHMNHN; supervision, KHMNHN. All authors have read and agreed to the published version of the manuscript.

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