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Original Research Article

Molecular Characterization and Antimicrobial Resistance Profile of Biofilm-Producing Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Caprine Mastitis

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ABSTRACT

The global rise of methicillin-resistant *Staphylococcus aureus* (MRSA) poses a significant threat to both human and animal health. This study aimed to detect MRSA in goat mastitis cases, identify resistance-associated genes, and assess biofilm-forming virulence. A total of 46 milk samples were collected from clinically mastitis-affected Black Bengal goats in Dinajpur Sadar and Chirirbandar. Conventional biochemical and cultural methods, along with PCR, were used for bacterial identification. The mecA gene, conferring methicillin resistance, and the nuc gene, a species-specific marker, were amplified via PCR. Statistical analysis was conducted using SPSS v25 and R v2024. The results showed an 82.6% prevalence of *S. aureus*, with MRSA identified in 13.16% (5/38) of isolates. Antibiotic susceptibility testing revealed 100% resistance to methicillin, oxacillin, vancomycin, and cefoxitin, followed by 85.71% resistance to ampicillin. The presence of biofilm-forming MRSA highlights its role in persistent infections and therapeutic failures. These findings underscore the need for effective, evidence-based control strategies to mitigate the spread of MRSA in livestock and safeguard public health.

1. Introduction

Methicillin-resistant *S. aureus* has recently become more prevalent, posing serious clinical and epidemiological problems around the world. Mastitis, also known as udder inflammation, lowers milk output and quality; it has a substantial negative economic impact on dairy farming (Najeeb et al., 2013). Impoverished rural residents are primarily affected by mastitis in goats as it lowers the revenue of people, and also, undermines the profitability of goat husbandry because it lowers milk output, and deprives youngsters of milk (Koop et al., 2016). Goat mastitis is mostly caused by *S. aureus*, Streptococcus agalactiae, and various environmental infections. *S. aureus* is the most prevalent pathogen that induces mastitis (Ribeiro et al., 2007). Aras et al. (2012) found that subclinical mastitis induced by *S. aureus* in goats had a prevalence of 5.6% to 37%. *S. aureus* can contaminate milk during subclinical mastitis without causing any visible changes in its appearance. Moreover, tainted milk and milk byproducts carry a risk of transmission to humans (Caruso et al., 2016). Methicillin-resistant *S. aureus* (MRSA), a bacterium that has surfaced on dairy farms, can colonize or infect farm workers. Additionally, MRSA can contaminate consumers by passing through the food chain (Papadopoulos et al., 2018). Livestock-Associated MRSA (LA-MRSA) serves as a reservoir of antibiotic-resistant bacteria and poses a significant zoonotic risk to humans, making it an alarming public health concern (Matuszewska et al., 2022). The first reports of MRSA in clinical mastitis affected goat milk in Bangladesh, highlighting the zoonotic potential and the need for effective control measures (Rana et al., 2020). In another research, Dutta et al. (2023) reported 6% mecA gene, which confirms MRSA, and only 11.5% were positive for *S. aureus*. MRSA is recognized globally as a significant public health concern due to its resistance to multiple antibiotics and its association with severe infections in humans and animals. While much attention has been focused on its impact in hospital and community settings, MRSA has also emerged as a critical pathogen in veterinary medicine. In particular, its role in causing mastitis in dairy goats highlights a concerning overlap between animal health and potential zoonotic transmission, underscoring the need to understand its prevalence and behavior in livestock populations.

Thus, the screening and genetic characterization of MRSA isolates are crucial for assessing the zoonotic potential of MRSA infections and for developing a successful prevention and control plan. The advantages of utilizing molecular techniques for MRSA characterization include speed, ease of use, enhanced sensitivity, specificity, and efficiency, as mentioned by Adzitey et al. (2013). These isolates may also be referred to as multidrug-resistant (MDR) isolates due to the fact that methicillin resistance makes them resistant to all beta-lactam antibiotics. MRSA acquires the mecA gene to confer resistance. Treatment costs are rising as a result of growing resistance, and handling or eating animal products carries an additional risk of MRSA transmission to humans. Because it can spread to people through contaminated animal products and occupational contact, livestock is a major source of *S. aureus*, and its zoonotic potential makes it a major threat (Smith et al., 2015). Surprisingly, there have been some reports of human infections with *S. aureus* that were associated with livestock. 21% One such instance involved the identification of MRSA in a six-month-old infant who worked in a pig farm in South Africa (Van Lochem, 2016). In addition to pathogenicity, a significant concern is *S. aureus*'s sharp increase in antibiotic resistance. Antibiotic resistance is one of the biggest risks to food security and global health, according to the World Health Organization (WHO), which highlights how urgent it is to address the problem of antibiotic resistance. A number of antibiotics, including β-lactam antibiotics (like penicillins, cephalosporins, and carbapenems) and non-β-lactam antibiotics

(like macrolides, azalides, and fluoroquinolones), are used to treat infections caused by *S. aureus* (Rayner et al., 2005). According to Van Boeckel et al. (2014), the global rise in antibiotic consumption is notable, with South Africa, Brazil, and China contributing to 76% of this increase; consequently, MRSA has been coined as a 'superbug'. Because of the complex host-pathogen interactions and extracellular hemolysin and toxin production associated with MRSA infections, these infections have become a major global public health concern. The consumption of milk contaminated with methicillin-resistant *S. aureus* might promote an outbreak of livestock-associated methicillin-resistant *S. aureus* in people, especially newborns. It is essential to assess the status of resistant strains of *S. aureus* within the research area. Understanding disease determinants or risk factors is necessary for both improved udder health and the proper prevention of mastitis (Koop et al., 2013). Recurrence of the infection may result from inadequate treatment or from failure to address the consequences of these risk factors (Koop et al., 2016). Compared to their healthier counterparts, animals in poorer health are five times more likely to develop mastitis. Furthermore, it is also vital to remember that animals are more susceptible to udder infections prior to parturition, during the early stages of udder and teat development. The findings indicate that MRSA strains present a significant risk to both veterinary and public health when unpasteurized goat milk is consumed. This study is highly relevant for addressing clinical problems in the study area or region in several ways including understanding the prevalence of MRSA in Goat Mastitis, or assessing the implications for public health and zoonotic transmission via direct contact, contaminated milk, environmental contamination, aerosol transmission, zoonotic transfer and also to evaluate the impact on livestock health and fairy industry. Very limited research was conducted about MRSA isolated from mastitis-affected goat milk in the northern part of Bangladesh. Because of the following issues, such as public health concern, biofilm formation increases the virulence of MRSA, antibiotic resistance threat, economic impact of the livestock industry, and lack of existing data in certain regions, this research is needed. This study will also be helpful as a guide for effective treatment strategies, improved hygiene protocols, vaccination development, and alternative treatments. By addressing these clinical challenges, the study plays a crucial role in improving animal health, public safety, and economic sustainability in the study region.

Therefore, this research aimed to isolate and identify methicillin-resistant *S. aureus* from mastitic goats, molecularly detect methicillin-resistant *S. aureus* using PCR for the mecA gene, and determine the biofilm formation status and virulence genes of MRSA.

2. Materials and Methods

2.1. Study area and Milk sampling

For this research, Clinically Mastitis-affected milk samples were obtained from goats in the Dinajpur District of Bangladesh. A total of 46 samples were collected from different upazillas (Sadar: 18, Parbatipur: 14, Chirirbondor: 6, and Birol: 8) of Dinajpur district. The 10 ml of milk samples from the goat were collected aseptically using sterile screw-cap tubes in accordance with the standard methods outlined by the National Mastitis Council Guidelines (NMC, 2017). Milk samples were collected at 8:00 AM. All labeled samples were transferred inside an ice box (4°C) to the microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh, for bacteriological analysis. The research work was conducted between July 2022 and June 2023.

2.2. Ethics approval and consent to participate

The Institutional Research Ethics Committee of Hajee Mohammad Danesh Science & Technology University, Dinajpur-5200, Bangladesh, approved the study. Notably, all animal experimentation was conducted in accordance with applicable laws, regulations, and guidelines, prioritizing animal welfare and minimizing any potential harm. Informed consent was obtained from all animal owners.

2.3. Selection and precise inclusion Criteria

The selection criteria for this study were established based on specific parameters, including farm location with origin (Roy Goat Farm: 18 samples (Sadar Dinajpur); Yeamin and Brothers Goat Farm: 14 samples (Parbatipur, Dinajpur); Nasir and Sons Agro Farm: 6 samples (Chirirbandar, Dinajpur) and Sarkar Goat farm: 8 samples (Birol, Dnajpur). The precise inclusion criteria, such as mastitis scoring, parity, day in milk (DIM), and milk yield details, were presented in supplementary Table S1. Mastitis scoring indicates score 1 (abnormal milk), score 2 (Abnormal milk + inflation of the udder), parity (Number of Parturitions), days in milk (DIM), and milk yields.

2.4. Phenotypic and biochemical characterization of isolates

According to Azam et al. (2023), bacterial isolates were primarily identified by cultural tests such as Nutrient Agar, Mannitol Salt Agar (MSA), Baird-Parker Agar (BPA) supplemented with egg yolk tellurite and mannitol salt agar, and Blood Agar media. 0.1 mL of milk samples were primarily streaked on Nutrient agar and incubated at 37°C for 24 hours. After incubation, bacterial colonies were taken with a loop and transferred into Mannitol salt agar using the same techniques. The colonies were incubated at 37°C for 24 hours. Similarly, bacterial colonies picked from Mannitol salt agar to Baird-Parker agar and Blood agar were aerobically incubated at 37 °C for 24 hours in a BioBase incubator (China). The tests were usually applied for the detection of pure isolates of suspected bacteria from goat milk by microscopic (gram staining) tests, and standard biochemical tests such as Methyl Red -Voges Proskauer (MR-VP broth), Indole (Tryptone broth), oxidase, Catalase, Triple Sugar Iron (TSI) (TSI agr), Motility Indole Urease (MIU), and Citrate utilization tests. After biochemical tests, all test tubes were incubated at 35- 37°C for 24-72 hours as per test requirements. The test reaction for TSI needs 18-24 hours of incubation. The test results were evaluated based on the test reaction observation. Under a microscope, *S. aureus* was observed as grape-like structures with a Gram-positive reaction. For this research, all microbiological media were procured from Difco in Bangladesh and Hi Media Private Ltd. in India.

2.5. Biofilm Formation Analysis (Congo Red Agar)

Biofilm formation was identified through the application of Congo Red Agar (CRA), which was modified with Brain Heart Infusion Agar (BHI) and Blood Agar Base described by Atshan and Shamsudin (2010); Freemn (1989). In BHI Agar, 0.8 grams of congo red dye and 36 grams of sucrose (both from Baker, UK) to make the final volume of the media, BHI Agar 52g (Oxoid, UK), water 1000 ml, whereas the final volume of media for Blood Agar base was measured with congo red dye 0.4 g, glucose 10 g, BAB-2 42 g (Oxoid, UK), and water 1000 ml. The bacterium was added to the medium after preparation, and after 48 hours of incubation at 37° C, it was left at room temperature for another two to four days. Biofilm production was determined based on the colony morphology and color: Strong biofilm producers: Black colonies with a dry, crystalline or rough texture, Moderate biofilm producers: Dark reddish-black colonies with a slightly rough surface and Non-biofilm producers: Red or pink colonies with a smooth and shiny appearance. This visual method allows for a rapid and effective screening of biofilm-forming capabilities among methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (Atshan and Shamsudin, 2010). Congo red agar was purchased from HI Media Pivate ltd., India.

2.6. Evaluating the Antibiotic Susceptibility

The sensitivity of isolates to antibiotics was determined using the disc diffusion method, in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (CLSI 2022). The isolates were carefully cultured in peptone water and incubated at a controlled temperature of 37°C for two hours. A petri dish containing Muller Hinton Agar (MHA) medium was carefully positioned in the incubator for 30 minutes to allow for drying. Subsequently, it was inoculated with 0.1 ml of the test culture using the spread plate technique, and then commercially available antibiotic discs were used. In order to examine the antibiotic sensitivity profile of various isolated bacteria, commercially available antibiotic discs such as ampicillin (25µg), methicillin (5µg), oxacillin (1µg), ciprofloxacin (5µg), gentamicin (10 µg), kanamycin (30 µg), tetracycline (30 µg), co-trimethoprim (25 µg), vancomycin (10 µg), cefoxitin (10 µg) and azithromycin (30 µg) were positioned on the surface of the inoculated medium with the use of sterile forceps. They were delicately pressed to ensure optimal contact with the medium's surface. The inoculated plates

were incubated at 37°C for 18–24 hours. Following the incubation period, the diameter of the zone of inhibition was measured in millimeters using a standard scale, in accordance with the manufacturer's specifications. Antibiotic discs used in the assay were obtained from Hi Media Private Ltd., India. All plating procedures were performed in triplicate to ensure accuracy and reproducibility.

2.7. MAR Index calculation

The Multiple Antibiotic Resistance (MAR) Index is used to assess the antibiotic resistance pattern of bacterial isolates. It helps identify the risk level of antibiotics. An Antibiotic susceptibility test (AST) using the Kirby-Bauer disk diffusion method was performed to determine resistance patterns. Standardized inoculum (0.5 McFarland turbidity standard) on Muller-Hinton agar was applied, and antibiotic discs on the agar were placed. Afterwards, the cultures were incubated at 37°C for 18–24 hours. Then the zone of inhibition was measured to interpret results as resistant (R), intermediate (I), or susceptible (S) based on CLSI (Clinical and Laboratory Standards Institute) or EUCAST guidelines (Azam et al., 2023; Tambekar et al., 2006; Krumperman, 1983). The MAR Index (M) is calculated using the formula:

$$M = a \backslash b$$

Where:

"a" = Number of antibiotics to which the isolate is resistant.

"b" = Total number of antibiotics tested.

2.7.1. DNA extraction of *S. aureus*

The DNA extraction of *S. aureus* was performed using the boiling-centrifugation method (recommended by Dashti et al., 2009). A single colony of *S. aureus* from Mannitol Salt Agar was inoculated into a microcentrifuge tube with 200 µL of DNAse-RNAse-free distilled water. It was then boiled at 100°C for 10 minutes and immediately chilled on ice for 5 minutes. The tube was subsequently centrifuged at 13,500 rpm for 10 minutes, and 5 µL of the supernatant was used as the template DNA in PCR. This DNA was preserved at 4°C for short-term applications and at -20°C for extended storage purposes. A total of 25 µL reaction mixture, including 5.5 µL of DNA template, 12.5 µL of 2X master mix (GoTaq Green master mix, Promega, Dane County, WI, USA), 1.0 µL of each forward and reverse primer, and 5.0 µL nuclease-free water, was adjusted for PCR assays. The primer details with references are presented in Tables 1 and 2, followed by the primer manufacturing protocols. For nuc gene of MRSA the thermocycler (Thermal Cycler Analytik Jena Biometra TOne 96G, Germany) reaction parameters are maintained as initial denaturation at 94°C for 1 min, denaturation at 94° C for 1 min, annealing 55°C for 30 sec, extension at 72° for 1.5 min and final extension at 72°C for 3.5 min with Nuc forward and reverse primers. For mecA gene of MRSA the thermocycler reaction parameters are maintained as initial denaturation at 95°C for 3 min, denaturation at 94° C for 1 min, annealing 53°C for 30 sec, extension at 72° for 1 min and final extension at 72°C for 6 min with mecA forward and reverse primers. (Tables 1 and 2). The purity of PCR-amplified DNA was evaluated using a Thermo Scientific NanoDrop 2000 spectrophotometer by measuring the A260/A280 ratio. Post-electrophoresis, the gel was carefully extracted from the chamber and initially visualized under a UV transilluminator (WUV-L50, Korea). Subsequent imaging and analysis were performed using a gel documentation system (UVD1-254). PCR product sizes were determined via 2% (w/v) agarose gel electrophoresis, stained with ethidium bromide (0.5 µg/mL), conducted at 70–100 V and 500 mA for 30–70 minutes (Aklilu et al., 2016). In this research, A 100 bp DNA ladder (Thermo Scientific, USA) was used as a size marker. The PCR band image was taken by a high-resolution camera using the Vtech software, a TV zoom lens, Japan.

2.8. Selection of primer for detecting *S. aureus*

Two types of primers were used in the current study.

i. Sau-234 (F) and Sau-1501 (R) primer: Band size 1267 bp in the agarose gel was indicative of the presence of *S. aureus* (Table 1).

ii. Sau-327 (F) and Sau-1645 (R) primer: Band size 1318 bp in the agarose gel was indicative of the presence of *S. aureus* (Table 1).

iii. MecA primer: The identification of the mecA gene is regarded as the definitive method for confirming MRSA. The band size of 533 bp in the agarose gel indicated the presence of *S. aureus* (Table 1).

iv. Nuc primer: For the detection of the virulence nuc gene. Band size 279 bp in the agarose gel was indicative of the presence of *S. aureus* (Table 1).

Table 1. Properties of Primers used in the present study

Primer name	Target gene	Primer sequence	Annealing temperature	Expected band size (bp)	Reference
Sau-234 (F) and Sau-1501 (R)	23S rRNA	F: 5'- CGA TTC CCT TAG TAG CGG CG -3' R: 5'- CCA ATC GCA CGC TTC GCC TA -3'	58 ºC	1267	Riffon et al., 2001
Sau-327 (F) and Sau-1645 (R)	23S rRNA	F: 5'- GGA CGA CAT TAG ACG AAT CA -3' R: 5'- CGG GCA CCT ATT TTC TAT CT - 3'	64 ºC	1318	Riffon et al., 2001
MecA (F) and MecA (R)	mecA	F: 5' - AAA ATC GAT GGT AAA GGT TGG C - 3' R: 5' – AGT TCT GGA GTA CCG GAT TTG C – 3'	53 ºC	533	Bühlmann et al., 2008
Nuc (F) and Nuc (R)	nuc	F: 5' – GCG ATT GAT GGT GAT ACG GTT – 3' R: 5' – AGC CAA GCC TTG ACG AAC TAA AGC – 3'	55 ºC	279	Wang et al.,1999

Table 2. The thermocycler parameters for PCR reaction (*S. aureus*)

Thermocycle	Sau-234 (F) and Sau-1501 (R)	Sau-327 (F) and Sau-1645 (R)	MecA primer	Nuc primer
Initial denaturation	94°C	94°C	95 °C	94°C
	2 min	2 min	3 min	1 min
Denaturation	94°C	94°C	94°C	94°C
	45 sec	45 sec	1 min	1 min
Annealing	58°C	64°C	53 °C	55 °C
	45 sec	1 min	30 sec	30 sec
Extension	72°C	72°C	72 °C	72 °C
	2 min	2 min	1 min	1.5 min
Final extension	72°C	72°C	72 °C	72 °C
	6 min	10 min	6 min	3.5 min
Cycle	35 x Step 2	35 x Step 2	35 x Step 2	35 x Step 2

2.9. Statistical analysis

We manually entered all data into a Microsoft Excel Spreadsheet 2016 and analyzed it using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). We analyzed the chi-square test for data comparison using R Studio version 2024.12.1+563. A P-value of 0.05> indicates statistical significance. The 95% Confidence interval was measured by R Studio version 2024.12.1+563 with the Wilson score interval method.

3. Results

3.1. Prevalence of bacterial isolate (*S. aureus*)

According to the study area, out of 46 samples, 38 (82.60%) positive isolates (*S. aureus*) were identified by several cultural and biochemical tests. *S. aureus* showed golden yellow colonies with a yellow zone on MSA (Figure 1A), black colonies on BPA (Figure 1B), strong biofilm formation with black colonies on CRA (Figure 1C), moderate biofilm-producing *S. aureus* in CRA with pink radish colonies (Figure 1D), and non-biofilm-producing *S. aureus* on CRA with pale color colonies (Figure 1E). *S. aureus* showed positive reactions for methyl red, catalase, and coagulase, whereas negative reactions for Voges-Proskauer, oxidase, and indole tests. The occurrence of these studies revealed that the highest number of positive cases was recorded in Chirirbandar, Dinaipur (100%), followed by Parbatipur, Dinajpur (85.71%), Sadar, Dinaipur (77.78%), whereas the lowest number of isolates (75%) was found in Birol, Dinajpur, (Figure 2). Table 3 represent the p-value of positive isolates from different location in Dinajpur including sadar, Dinajpur (p-value: 0.53); Parbatipur, Dinajpur (p-value: 0.60); birol, Dinajpur (p - value:0.47) and Chirirbandar, Dinajpur (p-value:0.47) and no p-value was statistically significant due to limited number of samples.

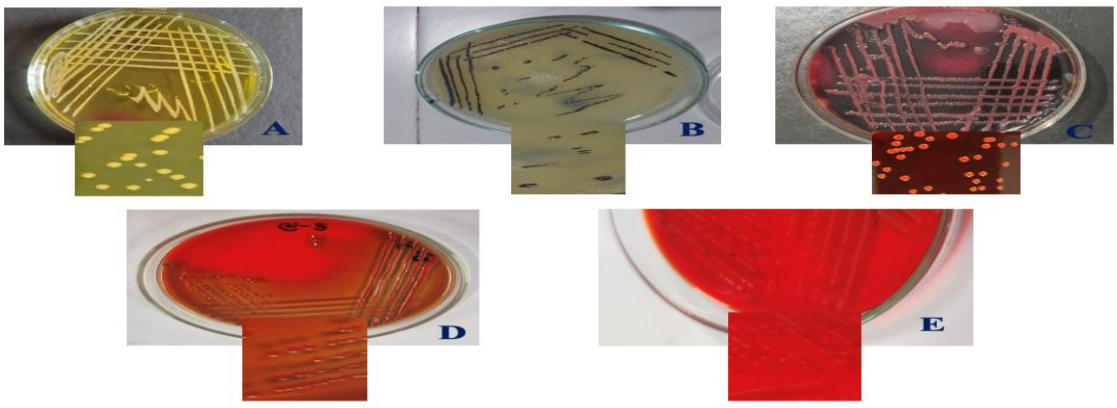

Figure 1: A: The culture of *S. aureus* on MSA shows a yellow colony. B: Culture of *S. aureus* in BPA forms a black colony. C: Biofilm production of *S. aureus* in CRA forms a black colony. D: Moderate biofilm production of *S. aureus* in CRA with pink radish colony. E: Non-biofilm-producing *S. aureus* on CRA.

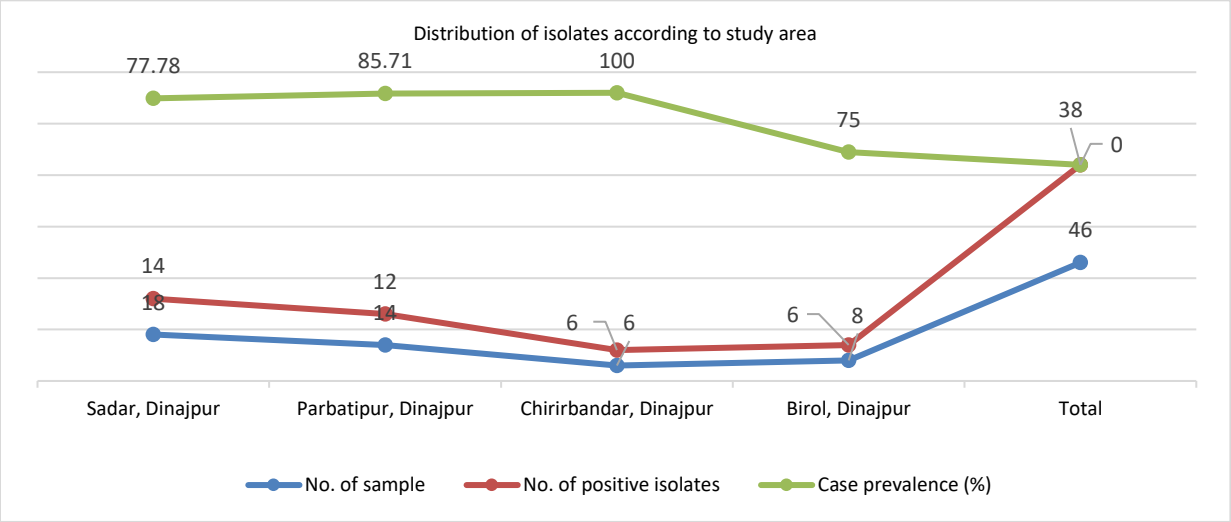

Figure 2: Distribution of isolates according to study area. In the graph blue color indicate number of samples isolated from mastitis affected goat milk from different regions of Dinajpur whereas yellow color highlights the positive isolates number and the top Ass color indicate percentage of isolates.

Table 3: Comparison of p-value between different study regions

Sampling area	No. of sample	No. of positive	Case prevalence (%)	95% CL		p-value
				Lower limit	Upper limit	
Sadar, Dinajpur	18	14	77.8	54.8	91	0.539
Parbatipur, Dinajpur	14	12	85.7	60.1	96	0.602
Chirirbandar, Dinajpur	6	6	100	61	100	0.472
Birol, Dinajpur	8	6	75	40.9	92.9	0.472

3.2. Biofilm formation status of *S. aureus*

According to the visual assessment of *S. aureus*'s biofilm formation status, 8 (42.10%) produced a high level of biofilm formation (+++) with black colonies on Congo Red Agar, whereas 6 (31.58%) of them produced moderate (++) biofilm formation with dark radish colonies, and 26.31% showed no (+) biofilm formation with pink colonies on Congo Red Agar media (Figure 3).

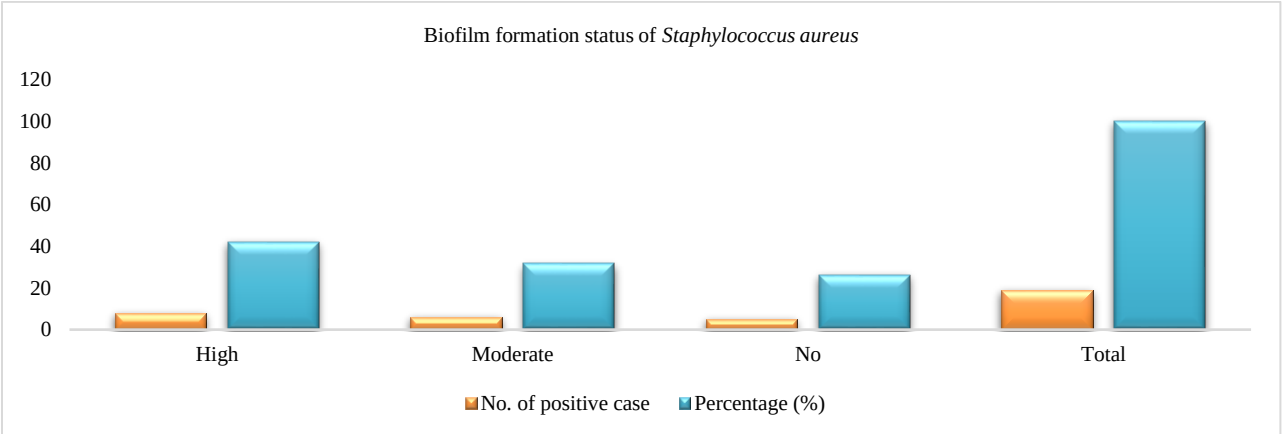

Figure 3: Biofilm formation status of *S. aureus*. Out of total 19 biofilm producing *S. aureus*, 8 positive *S. aureus* produce dark black colonies, 6 positive isolates produce dark radish colonies and only 5 positive isolates do not produce biofilms.

3.3. Correlation between Biofilm formation and Resistance genes

The correlation between biofilm formation and resistant genes was investigated in this research, and the overall biofilm formation was recorded as high, moderate, or no biofilm formation, correlated with resistant genes. In case of the mecA gene, the highest occurrence of biofilm formation was found to be 60%, whereas moderate biofilm formation was only 40%, and the non-biofilm formation 0% gene was detected. Consequently, in case of the nuc gene, the highest occurrence of biofilm formation was 42.86% of the total samples, followed by no biofilm formation at 42.86%, whereas 14.29% of the resistance gene was recorded as moderate biofilm formation. Furthermore, 44.44% of 23s rRNA resistant genes showed no biofilm formation, 33.33% showed moderate formation, whereas 22.22% produced high biofilm formation (Table 4). The data were annalyzed by Excel version 16 formulation. In this study, out of 38 positive isolates, 13.16% MRSA were detected, which produced biofilms in Congo Red Agar and were detected by visual assessment. Based on the mecA gene and nuc gene detection, we confirm biofilm formation of MRSA, out of 38 positive isolates, only 5 (13.16%) isolates associated with biofilm formation, which were positive for the mecA gene and nuc gene in the molecular method. Only when both the mecA gene and the nuc gene were positive with a PCR band did we call MRSA.

Table 4: Correlation between Biofilm formation and Resistance genes

Biofilm formation	Resistance Gene			
	mecA	nuc	23S rRNA	23S rRNA
High	3(60%)	3(42.86%)	2(22.22%)	3(42.86%)
Moderate	2(40%)	1(14.29%)	3(33.38%)	1(14.29%)
No	0(0.00%)	3(42.86%)	4(44.44%)	3(42.86%)
Total	5	7	9	7

3.4. Correlation between resistance genes and specific regions

In this study, out of 46 samples, we detected 38 positive isolates of *S. aureus*, and out of 38 positive isolates, we detected 5(13.16%) mecA gene, whereas 7(18.42%) nuc gene was detected by molecular techniques (PCR). We detected the mecA gene 3/14(21.42%) and the nuc gene 4/14(28.57%) in Farm 1, whereas in Farm 2 in Parbatipur, Dinajpur, 1/12(8.33%) of the mecA gene and 2/12(16.66%) of the nuc gene were found. Additionally, in farm 3 (Birol, Dinajpur), 1/16.66% mecA and nuc genes were recorded, whereas no resistance genes were detected in farm 4 (Chirirbandar, Dinajpur) (Table 5). According to location, the highest number of resistance genes of MRSA was identified in farm 1, and there was a correlation between farm location and resistance genes due to the environment and antibiotic use. The p-value was recorded as 0.711 for the mecA gene, whereas 0.67 was calculated for the nuc gene, and both p-values indicate this study is statistically non-significant.

Table 5: Correlation between resistance genes of MRSA and different locations

Farm and location	Isolates		mecA gene p-value	nuc gene p-value
	Methicillin-resistant *S. aureus*			
	mecA gene	nuc gene		
Farm 1 (Sadar Dinajpur)	3/14(21.42%)	4/14(28.57%)	0.7118233	0.6782905
Farm 2 (Parbatipur, Dinajpur)	1/12(8.33%)	2/12(16.66%)		
Farm 3 (Birol, Dinajpur)	1(16.66%)	1(16.66%)		
Farm 4 (Chirirbondor)	0/6(0%)	0/6(0%)		
Total	5/38(13.16%)	7/38(18.42%)		

Note: Farm 1: Roy Goat Farm: (Sadar Dinajpur); Farm 2: Yeamin and Brothers Goat Farm:(Parbatipur, Dinajpur); Farm 3: Sarkar Goat Farm: (Birol, Dinajpur); Farm 4: Nasir and Sons Agro Farm: (Chirirbandar, Dinajpur).

3.5. Antibiotic Sensitivity Tests

In the current research, 12 commercially available antibiotics were used against *S. aureus*. The highest resistance patterns were recorded for methicillin and cefoxitin, 38 (100%), followed by vancomycin, 36 (94.74%), 30(78.5%), and ampicillin, 20 (52.63%). Surprisingly, no resistant patterns were investigated for gentamycin and tetracycline against *S. aureus* (Figure 4). Out of 38 positive isolates of *S. aureus*, 100% resistance was found against Methicillin and Cefoxitin. *S. aureus* was found sensitive against Gentamicin, Tetracycline, and Co-Triethoprim. Furthermore, in Dinaipur Sadar, *S. aureus* was highly resistant to methicillin, cefoxitin, oxacillin, and vancomycin (100%), followed by ampicillin (85.71%), kanamycin, and azithromycin (28.57%). Additionally, in the case of Parbatipur, Dinaipur, *S. aureus* revealed that the highest resistance was found in the case of methicillin and cefoxitin. (100%), followed by vancomycin (83.33%), kanamycin, and oxacillin (50%). *S. aureus* samples were highly resistant in Chirirbandar, Dinajpur, toward methicillin, cefoxitin, oxacillin, and vancomycin (100%), followed by ampicillin and kanamycin (33.33%), compared to other antibiotics (Table 6). Notably, no resistant antibiotics

were measured, including ciprofloxacin, gentamycin, kanamycin, ceftriaxone, and azithromycin, whereas methicillin, cefoxitin, and vancomycin demonstrated 100% resistance in the Birol area of Dinajpur in this research

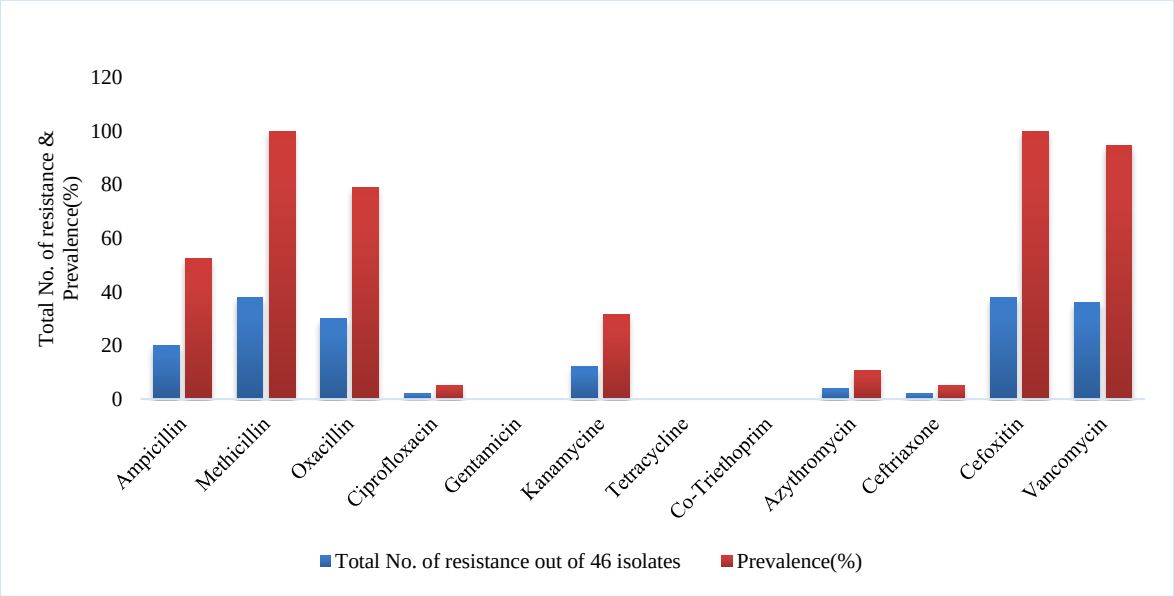

Figure 4. Overall antibiotic resistance profile of *S. aureus* isolated from mastitis affected goat milk

Table 6: Antibiotic resistance pattern of *S. aureus*

Name of Antibiotics	Location			
	Dinajpur Sadar 14 (R%)	Parbatipur 12 (R%)	Chirirbandar 6 (R%)	Birol 6 (R%)
Ampicillin	12 (85.71%)	2 (16.67%)	2 (33.33%)	4 (66.67%)
Methicillin	14 (100%)	12 (100%)	6 (100%)	6 (100%)
Oxacillin	14 (100)	6 (50%)	6 (100%)	4 (66.67%)
Ciprofloxacin	2 (14.28%)	0%	0%	0%
Gentamicin	0%	0%	0%	0%
Kanamicin	4 (28.57%)	6 (50%)	2 (33.33%)	0%
Tetracycline	0%	0%	0%	0%
Co-Trimethoprim	0%	0%	0%	0%
Azythromycin	4 (28.57%)	0%	0%	0%
Ceftriaxone	0%	2 (16.67%)	0%	0%
Cefoxitin	14 (100%)	12 (100%)	6 (100%)	6 (100%)
Vancomycin	14 (100%)	10 (83.33%)	6 (100%)	6 (100%)

3.6. MAR index calculation of *S. aureus*

Table 7 illustrates the calculation of the MAR index for isolated multidrug-resistant *S. aureus* from different locations such as Sadar, Parbatipur, Chirirbandar, and Birol in Dinajpur, whereas the MAR index ranged from 0.42 to 0.67 and the average MAR index was 0.54. According to our obtained result, the highest MAR index was measured in Dinaipur Sadar (0.67) and the lowest MAR index was calculated in Birol, Dinajpur (0.42) compared to other locations.

3.7. Molecular detection of MRSA

After phenotypic identification of MRSA by cultural and biochemical tests, all isolates were considered for genotypic identification with

several genes. After performing gel run electrophoresis for PCR band amplification, a 533 bp band was detected in the case of the mecA gene of *S. aureus*. In addition, the nuc gene with 279 bp, the 23S rRNA gene with 1318 bp, and the 23S rRNA gene with 1267 bp band were detected for *S. aureus*. The PCR bands of S. aureus for different genes are represented in Figure 5.

Table 7: Antibiotic Resistance Profile of Multidrug Resistance *S. aureus* from different locations with MAR index

SL. No	Resistance patterns	No. of antibiotics to which isolates were resistant (A)	No. of antibiotics to which isolates were exposed (B)	MAR index
1	AM, MET, OX, K, CEF, V, AZM, K	8	12	0.67
2	AM. MET, OX, K, CEF, V, k	7	12	0.58
3	AM, MET, OX, K, CEF, V	6	12	0.5
4	AM, MET, OX, K, CEF, V	6	12	0.5
5	AM. MET. OX. K, CEF, V	6	12	0.5
6	AM, MET, OX, K, CEF	5	12	0.42

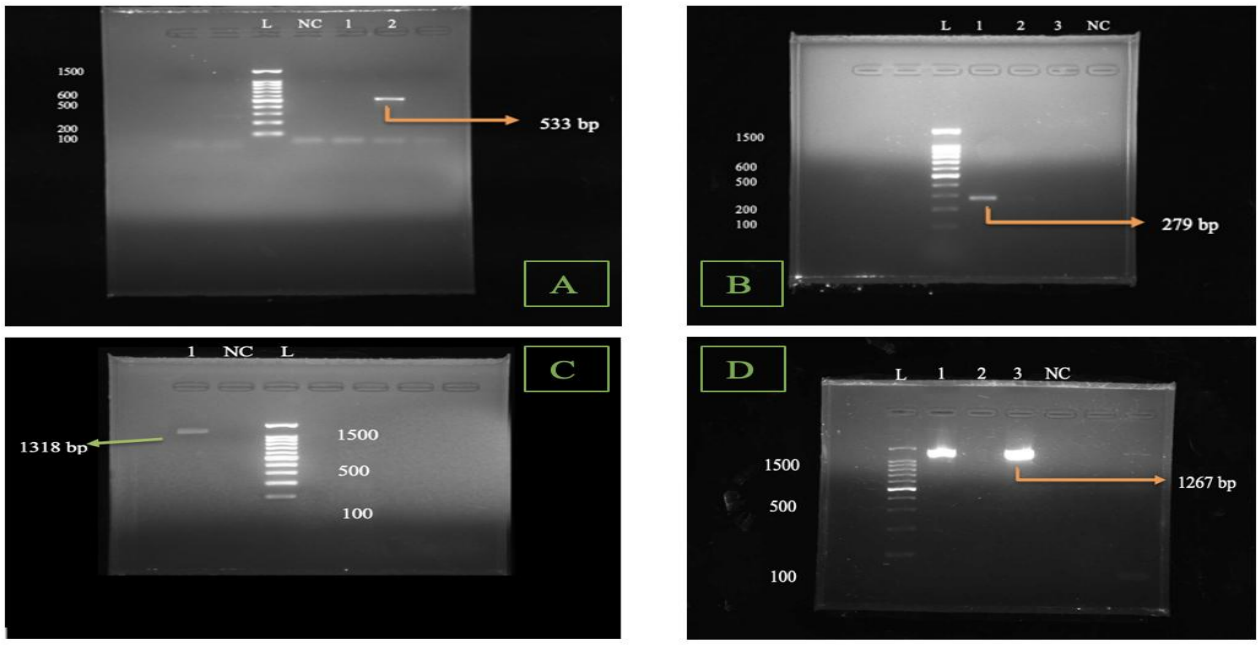

Figure 5: (A) mecA genes of *S. aureus* was detected by mecA primer design confirming 533 bp bands, L: Ladder, NC: Negative Control, Lanes 1-5 field sample, (B) nuc genes of *S. aureus* detected by nuc primer design confirming 279 bp bands, L: Ladder, NC: Negative Control, Lanes 1-3 field sample, (C) 23S rRNA genes of *S. aureus* detected by Sau- 327(F) and Sau- 1645 (R) primer design confirming 1318 bp bands, L: Ladder, NC: Negative Control, Lanes 1 field sample, (D) 23S rRNA genes of *S. aureus* detected by Sau- 234 (F) and Sau- 1501 (R) primer design confirming 1267 bp bands, L: Ladder, NC: Negative Control, Lanes 1-3 field sample.

3.8. Methicillin-resistance detection with oxacillin and cefoxitin disc diffusion tests

Table 8 represents the presence of the mecA gene of *S. aureus* on Muller-Hinton agar with oxacillin and cefoxitin disc concentrations. In this study, out of 19 isolates of *S. aureus*, 11 (57.89%) were detected as oxacillin resistant, whereas the presence of the mecA gene was found in only 5 (45.45%) isolates, and 13 (68.42%) were detected as cefoxitin resistant. In contrast, only 5 (38.46%) mecA genes were present.

Table 8: Oxacillin and cefoxitin disc diffusion tests and their comparison with the mecA gene (n=19)

Methods	Susceptibility		mecA gene	
	R%	S%	Present	Absent
Oxacillin disc diffusion test (1µg)	11 (57.89%)	8 (42.10%)	5 (45.45%)	6 (54.55%)
Cefoxitin disc diffusion test (30 µg)	13 (68.42%)	6 (31.57%)	5 (38.46%)	8 (61.54%)

R: Resistant; S: Sensitive

4. Discussion

Goat mastitis causes large financial losses because its treatment expenses are high, and it reduces milk yield. Moreover, there is a possibility of the infectious pathogen transmitting to humans, which may remain undetected (Caruso et al., 2016; Koop et al., 2016). The characterization of the causative agents and the risk factors that are linked with them is essential for the initial treatment, and also for implementing adequate control measures. The molecular characterization and isolation of MRSA from goat milk samples are the main objectives of this study.

Our study revealed that 38(82.60%) positive *S. aureus* were identified phenotypically by cultural and biochemical tests out of 46 milk samples from different goat farms, which agrees with the findings of Angelidis et al. (2020). In a previous study, in Greece, it was revealed that 50.2% of *S. aureus* were isolated and identified from mastitis-affected goat milk (Gelasakis et al., 2016), where our findings recorded 82.60%. Similarly, Saidani et al. (2018) in Indonesia also found in their study that 10.7% of the isolates were *S. aureus* in mastitis-affected milk. Our findings are also consistent with those of Jabbar et al. (2020) and Altaf et al. (2019), who mentioned *S. aureus* as the predominant bacteria for goat mastitis in Pakistan. Additionally, our study found positive MRSA 13.16%, which was related to findings about 8.96% MRSA in Bangladesh (Rana et al., 2020). In Greece another research recorded 5.6% MRSA isolated from mastitis affected goat milk (Gelasakis et al., 2016). In Pakistan, 35.92% MRSA was detected in mastitis-affected goat milk, which supported our findings (Javed et al., 2024). In another findings in Italy, they found only 2% MRSA in goat milk of mastitis affected goat milk (Cortimiglia et al., 2015). In the present study, it was observed that in nutrient agar the isolates produced a fairly large yellowish white colony; on Mannitol Salt Agar plates, it produced circular, 2-3 mm in diameter with smooth, shiny, glistening golden yellow colonies; and in blood agar media, it produced a smooth, shiny, round, and convex colony with beta hemolysis. This observation was supported by a number of scientists, Shareef et al. (2009) and Rashad et al. (2013).

According to our research, on average, methicillin and cefoxitin were found to have 100% resistance to MRSA. Vancomycin-intermediate *S. aureus* (VISA) strains develop a thickened cell wall that traps vancomycin molecules, preventing them from reaching their target sites (D-Ala-D-Ala residues in the peptidoglycan layer). This "trapping" effect leads to reduced efficacy (Cui et al., 2000). In another research, MRSA may develop mutations in genes regulating cell wall synthesis (e.g., walKR, graRS, or mprF) that change the structure or charge of the cell wall, reducing vancomycin access or binding (Howden et al., 2011). In contrast to previously published studies, we observed that 94.74% of MRSA showed resistance to vancomycin. In alignment with our finding parameters, a study conducted by Jabber et al. (2020) in Pakistan indicated that a percentage of *S. aureus* exhibited resistance to vancomycin. Furthermore, we mentioned in our study that 15 (78.95%) were resistant to oxacillin, which is in accordance with the findings of Jabbar et al. (2020), where they recorded that oxacillin resistance ranged from 93% to 100%. On the contrary, *S. aureus* was found highly sensitive to azithromycin 100% and ciprofloxacin 94.74% in our study, whereas a similar finding was reported in previously published studies with azithromycin 100% and ciprofloxacin 76.67% (Upadhyay & Kumar Kataria, 2009). In the course of the investigation, among the 19 isolated *S. aureus*, 11 (57.89%) exhibited resistance to oxacillin, with 5 identified as MRSA through polymerase chain reaction (PCR) testing. The mecA gene was not found in six isolates, which were resistant to oxacillin. It is plausible that these strains were hyperbeta-lactamase producers, which could explain their non-mecA-mediated oxacillin resistance or their borderline resistance (Broekema et al., 2009). Conversely, these 6 isolates may indicate false positive resistance to oxacillin, as discussed by Chowdhury et al. (2013). The current investigation revealed that 13 *S. aureus* strains, accounting for 68.42%, exhibited resistance to cefoxitin. Notably, mecA genes were identified in 5 of these strains, displaying a band of 533 bp on agar gel electrophoresis. An additional 8 resistant isolates were resistant to oxacillin and were found to be negative for the mecA gene, which is larger than that of Nicole et al. (2009). Similar to other genes, such as nuc, a PCR band of 278 bp was detected, which is in accordance with the findings of Chai et al. (2019) in Malaysia. Similarly, Chai et al. (2019) in Malaysia also found the mecA gene with a PCR band of 533 bp in *S. aureus*, which agrees with our findings.

Furthermore, our findings indicated that milk samples affected by mastitis may serve as a potential source for the propagation of multidrug-resistant *S. aureus* strains. This study indicates a significant presence of multidrug-resistant *S. aureus* in milk, drawing attention to the health implications for the goats and their kids who consume it. The study is similar to that of previous researchers, Altaf et al. (2019) and Asghar et al. (2019).

According to our study, the biofilm formation capacity of MRSA with the virulence gene was detected phenotypically on Congo red agar with black colonies with a crystalline, dry consistency that is formed by biofilm producers. Our findings agree with those of other authors who detected the virulence characteristic of *S. aureus* with the capacity to form biofilms, as mentioned in the golden standard methods (Zalewska-Piatek et al., 2009). Further investigation revealed that 57.90% of MRSA strongly produced biofilm, 36.84% produced moderate biofilm, whereas 52.6% recorded non-biofilm formation, which is in accordance with the findings of Angelidis et al. (2020). Antimicrobial agent treatment of bacterial diseases is hampered by biofilms, microbial communities that stick to one another and cause increased morbidity and persistent infections (Romling and Balsalobre, 2012; Sawhney and Berry, 2009). According to Chen et al. (2010), certain infections are linked to the formation of biofilms, which make it difficult to treat the infection because of its inherent resistance to high antibiotic dosages.

The emergence of high antibiotic resistance rates in bacterial pathogens, particularly methicillin-resistant *S. aureus* (MRSA), poses significant challenges in the management of caprine mastitis. The implications of this phenomenon extend beyond veterinary treatment difficulties to broader public health risks.

Antibiotic-resistant infections in goats result in reduced therapeutic efficacy, leading to: Persistent or chronic infections: Ineffective treatment may cause prolonged inflammation and damage to mammary tissues, impairing milk production. Increased economic losses: Farmers face higher costs due to

extended treatment periods, veterinary consultations, and decreased milk yield or culling of affected animals. Limited drug options: Resistance limits the availability of effective antimicrobials, especially in field conditions where broad-spectrum antibiotics are often used empirically.

The zoonotic potential of resistant pathogens like MRSA underscores a serious public health concern: Occupational exposure: Farmers, veterinarians, and dairy handlers are at risk of acquiring resistant strains through direct contact with infected animals or contaminated environments. Food safety concerns: Consumption of raw or unpasteurized goat milk or dairy products contaminated with resistant bacteria may serve as a transmission route to humans. Antibiotic resistance spread: Resistant genes can transfer between bacteria in the animal and human microbiota, contributing to the wider antimicrobial resistance crisis. 3. Broader Public Health and One Health Concerns: The presence of resistant pathogens in livestock reinforces the importance of antimicrobial stewardship across veterinary and human healthcare sectors.

To control Methicillin-resistant *S. aureus* (MRSA) in goat farming, a combination of preventive strategies is crucial. It highlights the need for routine surveillance, hygiene biosecurity practices, and alternative therapies including regular cleaning and disinfection of barns, equipment, and milking machines, hand hygiene for farm workers before and after handling animal, use of personal protective equipment (PPE) like gloves and aprons, footbaths and designated clothing/footwear for different farm zones to prevent cross-contamination. Another preventive measure, like antimicrobial stewardship, includes avoiding unnecessary antibiotic use, using culture and sensitivity testing before choosing antibiotics, following proper dosage and withdrawal times to prevent resistance development, educating farm staff on responsible antibiotic use, and using proper vaccination as per the veterinarian.

Therefore, the study found that milk yield and other relevant factors, like the care given by the milkers and hygienic practices during milking, are important determinants in this situation. Similarly, the increased prevalence of biofilm formation in MRSA isolates suggests the importance of incorporating biofilm detection in standard laboratory practices to effectively address antibiotic resistance and reduce the likelihood of treatment failure.

Due to resource and time limitations, we could not increase our sample size and geographic location, and we could not perform phylogenetic tree analysis or whole genome sequencing of identified biofilm-producing MRSA. These limitations should be acknowledged so that the findings can be interpreted cautiously. Addressing them in future studies—through multicenter designs, larger and more diverse samples, inclusion of longitudinal data, and advanced molecular techniques—would strengthen the evidence and improve the applicability of the results to both veterinary and public health sectors.

5. Conclusion

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a critical concern in both human and veterinary medicine. In dairy goats, the detection of MRSA in mastitis-affected milk with the mecA gene poses a significant public health risk due to its zoonotic potential transmission to humans through direct contact or consumption of contaminated milk. The emergence of MRSA strains resistant to all beta-lactam antibiotics in the Dinajpur district highlights a serious therapeutic challenge, as these antibiotics are widely used in both human and veterinary practices. Our findings suggested that for the treatment and control of mastitis in goats, identification of MRSA and antibiotic sensitivity tests is most important.

The study underscores the need for stringent biosecurity measures, routine MRSA surveillance, and the implementation of rational antibiotic use policies. Enhancing public awareness among farm workers, promoting hygienic milking practices, and adhering to good veterinary practices are essential to mitigate the spread of MRSA. The presence of multidrug-resistant MRSA not only compromises animal health and farm productivity but also poses a zoonotic threat, potentially facilitating cross-species transmission and limiting treatment options across sectors.

Declarations

Ethical consideration

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author's Contribution

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Supplementary material

The supplementary material associated with this work includes inclusion criteria of goat farm and uncropped gels of *Staphylococcus aureus* detected by the Nuc primer design. The supplementary material for this article can be found online : here .

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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