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Phenotypic and Genotypic Characterization of *mecA* gene in Methicillin-Resistant *Staphylococcus aureus* with Detection of Homologous Sequences isolated from Hospital Acquired Infection Patients admitted at a Tertiary Care Hospital in Dhaka City of Bangladesh

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major concern for public health. Compared to infections caused by methicillin-susceptible *Staphylococcus aureus* (MSSA), MRSA causes severe infections with increased morbidity and mortality and also places a large economic pressure on our health care sector. **Objective:** The purpose of the present study was to determine the prevalence of MRSA in different clinical samples isolated from admitted patients in a tertiary care hospital, Bangladesh. **Methodology:** This cross-sectional study was carried out in the Department of Microbiology at the Microbiology Laboratory of Dhaka Medical College, Dhaka, Bangladesh from January 2019 to December 2019 for a period of one year. *Staphylococcus aureus* was isolated from pus, wound swab and blood samples by culture, microscopy and different biochemical tests. MRSA were isolated by oxacillin and cefoxitin disc diffusion method using Kirby-Bauer technique and MIC of oxacillin by agar dilutional method. PCR and sequencing of *mecA* gene was done. **Results:** Out of 208 samples 42.2% MRSA and 57.78% MSSA were detected. Sensitivity and specificity of oxacillin disc diffusion method were 100% and 96.15%, respectively and for cefoxitin disc diffusion method both were 100% in comparison with MIC of oxacillin. All the 19 MRSA strains were positive for *mecA* gene. **Conclusion:** In conclusion there is a high prevalence of MRSA. [*Bangladesh Journal of Infectious Diseases, December 2024;11(2):146-151*]

Keywords: Cefoxitin; *mecA* gene; MIC; MRSA; MSSA; Oxacillin; PCR; *Staphylococcus aureus*

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Introduction

Staphylococcus aureus is the most pathogenic member of the genus *Staphylococci* and the etiologic agent of a wide variety of diseases that ranges from superficial skin abscess, food poisoning and life-threatening diseases such bacteremia, necrotic pneumonia in children and endocarditis[1]. Resistance developments in these pathogens occur via drug target site alteration, antimicrobial agent

inactivation by enzyme, efflux pump and antimicrobial agent sequestration. Other resistance mechanisms have developed through acquisition of resistance determinants, position selection and spontaneous mutation[2-3].

MRSA is responsible for hospital, community and livestock acquired infection[4]. It is a leading cause of skin and soft tissue infections in both humans and animals[5-6]. It is also the second most common cause of blood stream infections in nosocomial associated outbreaks with high mortality and prolonged hospital stay[7]. *Staphylococcus aureus* acquired methicillin resistance through horizontal transfer of *mecA* which codes for a modified penicillin binding protein (PBP) with low affinity to beta-lactam antibiotics[8-9].

Antibiotic resistant *Staphylococcus aureus* has become a major threat in last few decades. Coordinated action is required to minimize emergence and spread of antibiotic resistance. The aim of this study was to detect the prevalence of MRSA from clinical isolates of hospital admitted patients.

Methodology

Study Settings and Population: This cross-sectional study was conducted from January 2019 to December 2019 in the Department of Microbiology at Dhaka Medical College, Dhaka, Bangladesh.

Samples Collection and Identification: Specimens were collected from clinical samples like pus, wound swab and blood of patients attending Dhaka Medical College Hospital and *Staphylococcus aureus* was identified by Gram staining, catalase test, coagulase test like slide and tube method, colony morphology, hemolytic property, pigment production and mannitol fermentation test in mannitol salt agar media as per standard procedures[10].

Antimicrobial Susceptibility Test: Antimicrobial susceptibility tests of the clinical isolates against different antimicrobials were performed in Muller-Hinton agar (MHA) using the standard disc diffusion technique like modified Kirby-Bauer method[11]. Zones of inhibition of cefoxitin (30µg) and oxacillin (1µg) were interpreted according to CLSI guidelines[12] and Khan et al[13] respectively.

Control Strain: *Staphylococcus aureus* ATCC 25923 was used as control strain to assess the performance of the method.

Agar dilutional method of Minimum Inhibitory Concentration (MIC)[14]: For each plate, 50 ml Mueller-Hinton medium was prepared and impregnated with 1 µl, 2 µl, 4 µl, 8 µl, 16 µl, 32 µl, 64 µl and 128 µl of oxacillin stock solution to achieve a concentration of 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml, 64 µg/ml, 128 µg/ml and 256 µg/ml per plate respectively. As 0.5 McFarland turbidity standard contains 1×10^8 CFU/ml, 10 times dilution of test inoculums was done to achieve 1×10^7 CFU/ml[15]. 1 µl of 10 times diluted test inoculum was placed on MHA plate and incubated aerobically at 37°C overnight. The lowest concentration of antibiotic impregnated into MHA showing no visible growth on agar medium was considered as MIC of that strain of bacteria.

Molecular Method[16,17]: Polymerase chain reaction (PCR) was done for the detection of *mecA* gene in *Staphylococcus aureus.*

Bacterial Pellet Formation: A loopfull of bacterial colonies from MHA media was inoculated into a microcentrifuse tube containing trypticase soya broth (TSB). After incubation overnight at 37°C, the tubes were centrifuged at 4000 g for 10 minutes at 4°C and the supernatant were discarded and then the tubes containing bacterial pellets were kept at -20°C as pellets until DNA extraction.

DNA Extraction: 300 µl distilled water was mixed with bacterial pellet and mixed well by vortex mixer. The mixture was kept in block heater (DAIHA Scientific, Seoul, Korea) for heating at 100°C for 10 minutes. After heating the tube was immediately kept on ice for further 5 minutes and then centrifuged at 14000 g at 4^0 C for 10 minutes. Supernatant was taken into another microcentrifuge tube. Extracted DNA was used as template DNA for PCR and was preserved at -20°C for future use.

Mixing of Mastermix and Primer with DNA Template: Tris-EDTA (TE) buffer was used for the dilution of primers according to manufacturer's instruction**.** PCR was performed in a final reaction volume of 25 µl in a PCR tube, containing 12.5 µl of master mix (mixture of dNTP, Taq polymerase, $MgCl_2$ and PCR buffer), 1 µl forward primer and 1 µl reverse primer (Promega Corporation, USA) 2 µl extracted DNA and 10.5 µl of nuclease free water. A brief vortex of the PCR tubes was done and were centrifuged in a microcentrifuge for few seconds.

Amplification through Thermal Cycler: PCR assays were performed in a DNA thermal cycler (Eppendorf AG, Master cycler gradient, Hamburg,

Germany). Each PCR run was comprised of preheat at 94^0 C for 10 minutes followed by 36 cycles of denaturation at 94^0 C for 1 minutes, annealing at 58^0 C for 45 seconds, extension at 72^0 C for 2 minutes with final extension at 72^0 C for 10 minutes.

Agarose Gel Electrophoresis: PCR products were detected by electrophoresis on 1.5% agarose gel, which was prepared with 1X TBE buffer (TrisBorate EDTA). For 1.5% agarose gel preparation, 0.18 gram of agarose powder (LE, Analytical grade, Promega, Madison, USA) was mixed with 12.5ml TBE buffer. Mixture was boiled for few minutes to dissolve and cooled to 60-70°C. A comb was placed in gel tray and poured the agarose gel. After solidification, comb was removed and 1µl loading dye was mixed with 6µl of amplicon on para film and then loaded into the well of agarose gel. 1µl loading dye was mixed with 2 µl of DNA ladder and was loaded into one well. Gel containing DNA ladder and amplicon were then placed on the electrophoresis tank having 1X TBE buffer at 100 volts for 35 minutes. Positive control and negative control were also loaded in separate well.

Staining and de-staining of the Gel: After electrophoresis, the gel was stained for 30 minutes with ethidium bromide (20 µl ethidium bromide in 200ml distilled water). It was then de-stained for 15 minutes with distilled water.

Visualization and Interpretation of Results: The gel was observed under UV Trans-illuminator (Gel Doc, Major science, Taiwan) for DNA bands. The DNA bands were identified according to their molecular size by comparing with the molecular weight marker (100 bp DNA ladder) loaded in a separate lane. Samples showing the presence of corresponding bp band were considered positive for the presence of that organism.

Primers used in this study[18]

Gene	Primer sequence (5'-3')	Size (bp)
mecA	Forward: AAAATCGATGGTAAAGGTTGGC	532
	Reverse: AGTTCTGCAGTACCGGATTT	

Procedure of DNA Sequencing: Purification of amplified PCR product was done by using DNA purification kit (FAVOGEN, Biotech Corp). Purified PCR products of *Staphylococcus aureus* were sent to 1st Base Laboratories, Malaysia for sequencing by capillary method (ABI PRISM 3500). BLAST analysis was performed for searching homologous sequences into the Gen Bank at www.ncbi.nlm.nih.gov.

Statistical Analysis: Statistical analysis was performed by Windows based software named as Statistical Package for Social Science (SPSS), versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Continuous data were expressed as mean, standard deviation, minimum and maximum. Categorical data were summarized in terms of frequency counts and percentages. Every effort was made to obtain missing data.

Ethical Clearance: All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional research ethics. Formal ethics approval was granted by the local ethics committee. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Results

Out of 275 samples, 208 bacteria were isolated among which *Staphylococcus aureus* were identified in 45 isolates. Out of 20 isolated MRSA (detected by oxacillin disc diffusion method), 1(5.0%) isolate had MIC of ≤2 µg/ml and was detected as MSSA (Table 1).

Table 1: MIC of Oxacillin among MRSA Detected by Oxacillin Disc Diffusion Method

MIC of Oxacillin	Frequency	Percent
256 µg/ml	15	75.0
128 µg/ml	2	10.0
64 µg/ml	0	0.0
32 µg/ml	0	0.0
16 µg/ml	1	5.0
8 µg/ml	1	5.0
4 µg/ml	0	0.0
2 µg/ml	1	5.0
Total	**20**	**100.00**

CLSI break point of MIC of oxacillin for *Staphylococcus* aureus: Sensitive ≤ 2 µg/ml, Resistant ≥ 4 µg/ml.

Among the three phenotypic methods of MRSA detection, oxacillin disc diffusion method identified highest number 20 (44.44%) of MRSA (Table 2).

Table 2: Detection of MRSA in Isolated *Staphylococcus aureus* by Different Methods

Methods	MRSA	MSSA
Oxacillin Disc	20(44.4%)	25(55.6%)
Cefoxitin Disc	19(42.2%)	26(57.8%)
MIC of oxacillin	19(42.2%)	26(57.8%)
PCR for *mecA* gene	19(42.2%)	26(57.8%)

Sensitivity of oxacillin disc diffusion method was 100.0% and specificity was 96.15% in comparison with MIC of oxacillin by agar dilution method (Table 3).

Table 3: Comparison between Oxacillin Disc Diffusion Method and MIC of Oxacillin by Agar Dilution Method in Detecting MRSA

Types	Oxacillin (1µg) DDM	MIC of Oxacillin	
		MRSA	MSSA
MRSA	20(44.4%)	19(100.0%)	1(3.8%)
MSSA	25(55.6%)	0(0.0%)	25(96.2%)
Total	**45(100.0%)**	**19(100.0%)**	**26(100.0%)**

DDM=Disc Diffusion Method

Sensitivity and specificity of cefoxitin disc diffusion method were 100% when compared with MIC of oxacillin (Table 4).

Table 4: Comparison between Cefoxitin Disc Diffusion Method and MIC of Oxacillin by Agar Dilution Method in Detecting MRSA

Types	Cefoxitin (30 µg) DDM	MIC of Oxacillin	
		MRSA	MSSA
MRSA	19(42.2%)	19(100.0%)	0(0.0%)
MSSA	26(57.8%)	0(0.0%)	26(100.0%)
Total	**45(100.0%)**	**19(100.0%)**	**26(100.0)**

DDM=Disc Diffusion Method

Highest MRSA were isolated from pus and wound swab than blood samples (Table 5).

Table 5: Distribution of MRSA & MSSA isolated from different samples (N = 45)

Samples	MRSA	MSSA
Pus & Wound Swab	18(94.7%)	23(88.5%)
Blood	1(5.3%)	3(11.5%)
Total	**19(100.0%)**	**26(100.0%)**

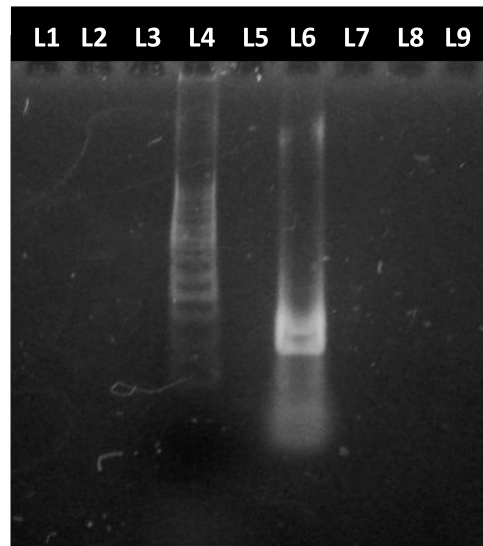

Figure I: Photograph of gel electrophoresis: negative control without DNA (Lan 1), negative control *Staphylococcus aureus* ATCC 25923 (Lan 2), hundred bp DNA ladder (Lan 4), amplified DNA of 532 bp for *mecA* gene (Lan 6), negative sample (Lan 8)

Discussion

The emergence of MRSA is an important threat that is resulting in the failure in treatment and control. MRSA developed new effective mechanisms to resist modern antibiotics, so it is considered as a life-threatening microorganism for both humans and animals[19,21]. The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by *Staphylococcus aureus*.

In the present study among 45 isolated *Staphylococcus aureus*, 20 strains were resistant to oxacillin and 19 strains were resistant to cefoxitin by disc diffusion method. For disparity of the result between these two methods, further confirmation was done by MIC method and detection of *mecA* gene by PCR and 19 (42.22%) strains were found as MRSA.

One MSSA strain which was labeled as MRSA by oxacillin disc diffusion method was probably due to their heterogenous nature of resistance or due to hyper production of β-lactamase and the result was also probably influenced by several factors like concentration of NaCl, temperature, inoculum size and test agent[22]. Cefoxitin is better for detection of MRSA because it is less affected by hyper production of β-lactamase and it induces in PBP2a production in vitro in undetectable low-level-MRSA (pre-MRSA) or in phenotypically MSSA[23].

In this current study, oxacillin disc diffusion method was less specific (96.15%) than cefoxitin disc diffusion method (100.0%) when compared with MIC of oxacillin. In this study, both sensitivity and specificity of cefoxitin disc diffusion method were 100% when compared with MIC of oxacillin by agar dilution method. This finding was almost similar to the previous study which reported that both sensitivity and specificity of cefoxitin disc diffusion method were 99.70 % for detection of MRSA[24].

In agreement with present study, a previous study in Bangladesh reported that 46% were MRSA in their study[25]. Another study in Pakistan reported that 45% were MRSA which is in accordance with present findings[26]. Geographic variations in the prevalence of MRSA and variations from one hospital to others may be due to efficacy of infection control practices in healthcare facilities and antibiotic usages that vary in different hospitals[27]. In this context Bangladesh is very vulnerable to develop MRSA due to irrational use of antibiotics[28].

Conclusion

The clinical isolates of *Staphylococcus aureus* of hospitalized patients exhibit a relatively high prevalence of MRSA which is alarming issues for our health system. Cefoxitin disc diffusion method is a reliable method and better alternative of oxacillin disc diffusion method for detection of MRSA.

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Conflict of Interest
There is no conflict of interest.

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Contribution to authors:
Noor-E-Jannat Tania conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. SM Shamsuzzaman contributed to the analysis of the data, interpretation of the results and critically reviewing the manuscript. Aminul Islam contributed to the data analysis and manuscript writing. Khadijatul Kubra and Maminur Rahman involved in the manuscript review and editing. All authors read and approved the final manuscript.

Data Availability
Any questions regarding the availability of the study's supportin g data should be addressed to the corresponding author, who can provide it upon justifiable request.

Ethics Approval and Consent to Participate
This study was approved by Research Review Committee (RRC) of Department of Microbiology and Ethical Review Committee (ERC) of Dhaka Medical College, Dhaka, Bangladesh (Reference number: MEU-DMC/ECC/2019/171).

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