Molecular Diversification and Frequency of mecA Gene in the MRSA Infections of Hospitalized Patients at Islamabad, Pakistan

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ABSTRACT

Objective: To determine the molecular diversification and frequency of mecA Gene in MRSA infections of hospitalised patients at Islamabad, Pakistan.

Study design: Cross-sectional study.

Place and Duration: Pakistan Institute of Medical Science, Islamabad, Pakistan, from Sep 2017 to Sep 2018.

Methodology: To explore the occurrence of pathogenic *S. aureus* in the hospitalised patients of Islamabad, 500 samples were collected. All the isolates were further characterised by gram staining, catalase test and DNAse media analysis validating the presence of *S.aureus*. The overall frequency of *S.aureus* was 19.5% in all the isolates. Six highly distinguished colonies were further evaluated through colony PCR by 16S r RNA analysis, and results revealed 99.9% homology of isolates with *S.aureus*. The degree of resistance was further evaluated for 12 isolates based on the previous MRSA resistance pattern. The extraction and concentration of DNA were done by the CTAB and Nanodrop methods. Then primers were designed for the presence of the mecA gene of S. aureus.

Results: Results showed 51 (52.7%) MSSA and 45 (47.4%) MRSA among all the isolates. Colony PCR by 16S r RNA analysis results revealed 97 (99.9%) homology of isolates with *S.aureus*. PCR of all the twelve extracted DNAs of isolates showed the frequency of the mecA gene in all the isolates.

Conclusion: The study is the first report the presence of resistance mecA gene in the hospitalised patients of Islamabad. It has depicted a resistant gene's presence and showed the rising trend of resistance in pathogenic S. aureus.

Keywords*:* Antimicrobial, Meca gene, MRSA, *S.aureus*, Susceptibility.

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INTRODUCTION

The ratio of patients dying due to Methicillin-resistant staphylococcus aureus (MRSA) is considerably greater than that for Methicillin-susceptible *S. aureus* (MSSA). Oxacillin resistance is generally linked to the mecA gene. MecA gene encodes the synthesis of additional penicillin-binding protein, PBP2a or 2′, with homogeneous or heterogeneous expression.[1] Transpeptidase activity, delivered by PBP2a, allows cell wall synthesis. PBP2a's little affinity to most β-lactams confers resistance to MRSA against several members of this class of antibiotics. Thus, PBP2 a indicates multiple targets for unique antibiotics designed to prevent MRSA risk by inhibiting bacterial cell wall biosynthesis.[2]

MRSA infections have amplified from 35.9% to 66.7% in Pakistan in the past ten years. The statistics of epidemiological typing of MRSA isolates from Pakistan are inadequate, and minimal studies have stated the dissemination of MRSA copies.[3]

Universal PCR primers, particularly for prokaryotes, are used to sequence the 16S rRNA gene for reviewing the configuration and to generate comprehensive taxonomic profiles of the microorganisms of these microbiotas.[4-6] In this study, we have also used this technology to study the phylogenetic profile of *S. aureus*.

Hence, the advanced detection of the highly infective and drug resistance genes in *S. aureus* is essential in tracking the MDR and hypervirulent strains related to high levels of morbidness/fatality.[7-9] Therefore, this study aimed to analyse the frequency of the mecA methicillin resistance gene and to determine antibiotic resistance patterns and phylogeneticity in *S. aureus* strains isolated from clinical specimens in patients hospitalised at PIMS Islamabad.

METHODOLOGY

This was a cross-sectional study, conducted at the Pakistan Institute of Medical Science, Islamabad, Pakistan, from September 2017 to September 2018. Different types of samples, including blood, urine,

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wound secretions, and nasal secretions, were collected from the patients at the Pakistan Institute of Medical Science (PIMS) Islamabad.

Non-probability consecutive sampling technique was used. The sample size was calculated through onlinecalculator.net, keeping the population proportion of 67%.[10] Ethical approval for the study was obtained from the Ethics Review Board Committee of the COMSATS Institute of Information Technology, Islamabad (CIIT/ Bio/ERB/17/25).

Inclusion Criteria: All the hospitalised patients of either gender, irrespective of age were included in the study.

Exclusion Criteria: Patients with any co-morbidity were excluded from the study.

Written consent was taken from the patients. The samples were taken through gel swabs or in blood values, and tests were conducted in the Applied Microbiology and Biotechnology Lab, COMSATS Institute of Information Technology, Islamabad, for 12-24 hours. Samples were cultured on the same day. The presence of *S. aureus* was checked through colonial morphology as they give a small grey-white colony of 1-2cm on agar. *Staphylococcus aureus* was further confirmed through gram staining, catalase and DNAse test as they are positive for all of these tests. After confirmation of staphylococcus aureus colonies were purified on nutrient agar.

The susceptibility of all positive cultures which yield *S. aureus* was checked on Mueller-Hinton agar. Oxacillin disc resistance results were used to confirm MRSA strains. Antibiotic susceptibility profiles of the isolates against nine commonly used antimicrobial agents, including Penicillin 10µg, Ciprofloxin 5µg, Kanamycin 30µg, Tetracyclin 30µg, Doxycycline 30µg, Erythromycin 15µg, Cefoxin 30µg, Clindamycin 10µg sulfamethoxazole 25µg, Oxazolidinone 30µg.

The CTAB method was used to isolate DNA from bacteria.[8] The isolated DNA was electrophoresed on 1% Agarose gel in 1 X TBE buffer (Annexure V). A gel doc system was used to visualise the gel. The samples were sent to Macrogen Inc. Korea to sequence the 16S RNA gene through the direct colony PCR method (Table-I). Slants were formed to transfer bacterial samples to Korea. 50 mL falcon tubes were used in which nutrient agar was poured and placed in a position to form slants. The falcon tubes were allowed to dry and agar to solidify. After some time, bacterial colonies were streaked in falcon tubes and incubated at 37 overnight. Samples were labelled and sealed with parafilm and sent to Korea for sequencing.

16S RNA sequences were further analyzed by using Megablast at NCBI and identified based on similarity with the kind of strain present in the EZTAXON database. The mecA gene from *S. aureus* was amplified, which is responsible for antibiotic resistance in *S. aureus*. Sequences of the gene of all the possible homologues were used in the design of primers. The available full length of sequences of strains was obtained and identified from EzTaxon for the mecA gene and retrieved from the NCBI database and Staphylococcus database (www.staphylococcus.com) and used to design primers.

Table-I: Standard service report for 16S rRNA gene.

Sample. No	Accession No	Description	Length	Start	End	Coverage	Bit	*e*-value	Match/Total	Pct. (%)
33507	CP0115261	S.aureus	2755072	503057	504546	0	2741	0.0	1488/1490	99
33214	CP0115261	S.aureus	2755072	503057	504553	0	2747	0.0	1494/1497	99
33358	CP0115261	S.aureus	2755072	503064	504552	0	2743	0.0	1488/1489	99
19879	CP0115261	S.aureus	2755072	503066	504546	0	2736	0.0	1481/1481	100
36494	CP0115261	S.aureus	2755072	503060	504553	0	2747	0.0	1492/1494	99
34699	CP0115261	S.aureus	2755072	503060	504553	0	2752	0.0	1493/1494	99

Retrieved gene sequences were aligned on CustalW as Multiple Sequence Alignment. The complementary sequences on the negative strand of DNA were obtained from www.justbio.com.[9] The retrieved sequences were placed on the given space and executed multiple alignments. Forward and reverse primers were designed by justbio "hosted tools and "Megablast" at the NCBI database.[10] The PCR profile, which was used in Thermocycler (Peq Lab, Germany), was shown in the Table-II.

Table-II: PCR profile used in thermocycler.

Initial Denaturation Temperature	950C	5 Minutes
Denaturation Temperature	950C	45 seconds
Annealing Temperature	Gradient	45 seconds
Extension Temperature	720C	1 min 30 sec
Final Extension Temperature	720C	10 minutes
Number of cycles	25	
Storage	40C	

The amplified gene sequence was purified and sent for sequencing. The PCR product was purified through PCR purification Kit (Thermo Scientific) using the manual guide provided in the kit.

Statistical Package for Social Sciences (SPSS) version 26.0 was used for the data analysis. Quantitative variables were summarized as mean ± SD and qualitative variables were summarized as fre-quency and percentages. Chi-square test was applied to find out the association. The *p*-value of ≤0.05 was considered statistically significant.

RESULTS

A total of 500 samples were collected from PIMS Hospital, Islamabad. Out of which 50 samples were from the blood (25 from females, 25 from the male patients). Out of 300 samples were from wound/pus in which, 100 samples were from pimples, 100 samples from accidental cuts, 100 samples from operative cuts, 50 samples were from urine and 100 samples were from a nasal fluid.

After Identifying *S. aureus* through gram staining, catalase test and DNase media test, all the *S. aureus* were grown on Mueller Hinton Agar to check their antibiotic disc resistance pattern. Out of 97 samples were positive for *S. aureus*. Penicillin was the first-ever antibiotic that was used against any bacteria. 97 (100%) samples of *S. aureus* were resistant to penicillin. Cipro-floxin is used against various bacteria causing skin infections. 6 (6%) *S.aureus* samples were susceptible, while 91 (94%) were resistant to Cipro-floxacin. Results against Kanamycin showed that 6 (6%) samples were intermediate, and 91 (94%) were resistant. Tetracycline was also used as an antibiotic against infectious bacteria. Its disc results showed that 35 (36%) of samples were susceptible, 6 (6%) were intermediate and 56 (58%) were resistant. Doxycycline was mostly used against the bacteria which caused acne and skin infections. 49 (51%) of samples were susceptible, 9 (9%) intermediate and 39 (40%) resistant against Doxycycline. 20 (21%) samples were intermediate, 3 (3%) were susceptible and 74 (76%) resistant against Erythromycin. Cefoxitin results showed that 36 (36%) of samples were resistant, 16 (13%) were intermediate and 48 (50%) were susceptible. 42 (43%) isolates were resistant, 6 (6%) were intermediate and 49 (51%) susceptible against Clindamycin. Sulfamethoxazole results showed that 46 (47%) samples were resistant, 5 (5%) were intermediate and 46 (47%) susceptible, while 53 (55%) samples were resistant, 17 (17%) were intermediate and 27 (28%) susceptible against Oxazolidinone.

Slants were prepared to send to Korea for sequencing. Samples were selected based on antibiotic resistance. The samples showing a maximum number of antibiotic resistance and MRSA were selected and sent for sequencing.

According to 16S rRNA standard report, a phylogenetic tree was designed for *S. aureus* samples. Figure-1 and Figure-2 showed phylogenetic trees for sample numbers 34699 and 33214, respectively. 16S rRNA gene sequence was analysed on ClustalW and then trimmed for submission to the NCBI database.

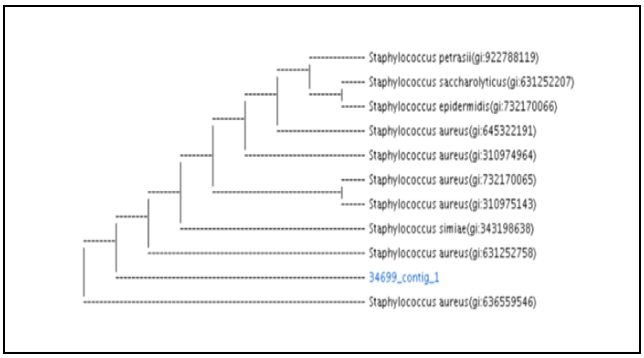

Figure-1: Phylogenetic tree of sample number 34699.

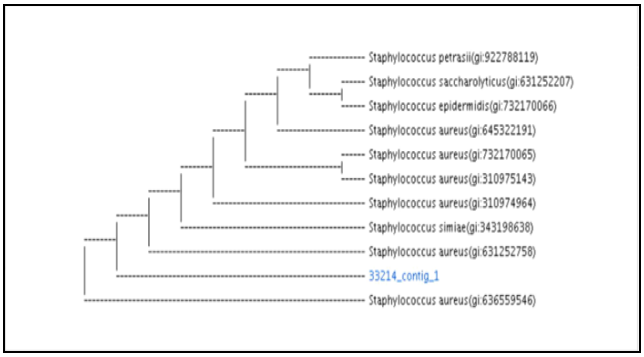

Figure-2: Phylogenetic tree of sample number 33214m.

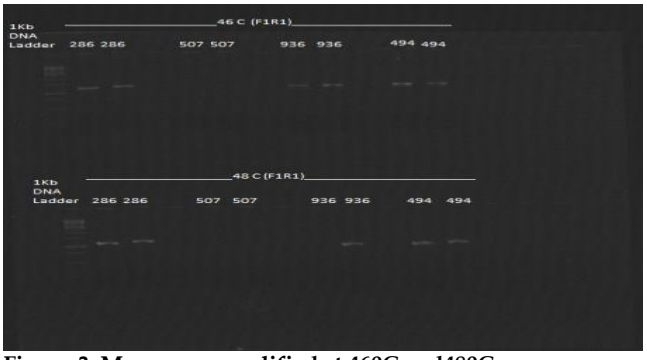

Figure-3: Meca gene amplified at 460C and480C.

MecA gene, responsible for MRSA and antibiotic resistance in *S. aureus*, was amplified. The gene was involved in MRSA as all the MRSA strains showed

positive results for the mecA gene. 50 uL PCR product was formed, and primers were used, specifically for the mecA gene. DNA was extracted from the isolate using the CTAB method for further gene amplification.

PCR was carried out using forward and reverse primer and given PCR conditions in methods. mecA gene was amplified, and the PCR product was run on the gel. Figure-3 showed the amplified mecA gene bands on the gel.

DISCUSSION

S. aureus is a chief source of bacteraemia in hospitals and is the most common cause of necrotizing pneumonia, skin, and soft-tissue infections in the community.[11-13] In our study, 48.2% of samples were MRSA, and 52% were MSSA.

16s rRNA analysis is an important method for the molecular identification of different strains of MRSA. The morphological identification features do not provide the exact information about the genetic diversity present among the various strains of MRSA, 1w2e3 therefore, 16s rRNA gene analysis has been performed through the colony PCR method. DNA extraction of the colony was done, and then metagenomics of the particular samples was perfor-med through universal primers. The data obtained were evaluated to determine the homology of strain at the species level. As identified from their characteriza-tion and response to a series of antibiotics, six extraordinary strains of MRSA were investigated for 16s rRNA analysis and determined their genetic diversity at the species level. Many sensitive and specified problems related to qPCR assay were overcome by sequencing. The molecular identification and genetic analyses of MRSA were, and many studies have shown various genes involved in the resistance of *S. aureus* against certain drugs and antibiotics.

The most common resistant gene is the mecA gene, which is considered a key player in developing the resistance of *S. aureus*. mecA gene has been reported in by many researchers, and much literature is available on this gene. The selected twelve isolates of MRSA isolated in this study were multidrug-resistant as evaluated from their initial antibiotic drug-resistant pattern. It was found that the mecA gene was positive in 100% of these isolates. Recent studies described that mecA containing multidrug-resistant *Staphylococcus aureus* strains has been common in hospital personnel, and the prevalence of MRSA was relatively high (44%).[14,15] In the current study, we have reported that the rate of MRSA is above 52%. Our results are pretty familiar to other researchers, such as reported by Boucher *et al*,[16] They revealed that methicillin-resistant *Staphylococcus aureus* (MRSA) infections had been unceasingly rising in hospital-acquired infections. The high prevalence of infection caused by MRSA led to the recommendation of glycopeptides for treatment.[17] The reports about the enhanced occurrence of VRSA amongst multidrug-resistance MRSA have been quoted about the current status and genetic adapta-bility of *S.aureus*.[18] The rise of antibiotic-resistant *Staphylococcus aureus* was also evaluated in the mecA and nuc gene in various resistant species of S. aureus.[19,20]

Among the twelve clinical samples, all were found to be mecA gene positive and showed 100% frequency for the mecA gene compared to other studies where 68% were found for mecA gene.[21] Our study could also be related to the research work performed by, who found 100% of MRSA isolated samples positive for mecA gene.[22]

Although our sample size was less than the entire population, at least it covers the periphery of PIMS hospital, and it could be evaluated that MRSA and MSSA are widespread in the PIMS hospital setting. At this moment, it was recommended that there should be regular periodic reviews of hospital-acquired infections comprising antimicrobial sensitivity examinations, and it would be pretty helpful in drawing antibiotic policy for infection control and alleviating the prevalence of MRSA.

STUDY LIMITATIONS

The current study was not without its limitations. The sample size was limited because of the overwhelming expenses of carrying out genetic studies in South Korea, resulting in financial constraints.

Conflict of interest: None.

Authors' Contribution

YR: Concept, design acquisition of data, RY: Writing original draft, AG: Review and editing, KI: Supervisor, AA: Data analysis, HB: Manuscript review.

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