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DETECTION OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS USING MEC A, RIBOTYPING AND ANTIBIOGRAM PROFILE OF PAKISTANI CLINICAL ISOLATES.

Adeela Fatima[1], Imran Sajid[2], Saba Riaz[3], Muhammad Saeed[4]

1. PhD Scholar
Department of Microbiology and Molecular Genetics,
University of the Punjab, Lahore, Pakistan.
2. PhD
Assistant Professor
Department of Microbiology and Molecular Genetics,
University of the Punjab, Lahore, Pakistan.
3. PhD
Assistant Professor
Department of Microbiology and Molecular Genetics,
University of the Punjab, Lahore, Pakistan.
Citi Lab and Research Center, Faisal Town Lahore, Pakistan.
4. M.Phil. (Microbiology)
Manager Pathology
Medical Lab Technology
DHQ Hospital Mandi Bahauddin.

Correspondence Address:
Dr. Saba Riaz
525A, Citi Lab and Research Center,
Faisal Town Lahore, Pakistan.
saba.mmg@pu.edu.pk

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ABSTRACT… The objective of this study was to determine the incidence of MRSA with their antibiotic susceptibility pattern and molecular characterization of these strains. **Study Design:** Cross sectional study. **Setting:** Microbiology section of Citilab and Research Centre, Lahore, Department of Microbiology annetiscd Molecular g. **Period:** March 2014 to June 2016. **Materials and Methods:** Bacterial isolates were retrieved from different specimens of pus/wound, blood and other body fluids. These were characterized using conventional (catalase, DNase, coagulase etc), phenotypic and molecular techniques (oxacillin and cefoxitin susceptibility, 16S rRNA gene sequencing and mec-A gene) methods of identification. Antibiotic sensitivity pattern was also detected by applying standard Kirby Bauer disc diffusion method. **Results:** Out of all the isolated strains, the frequency of MSSA (methicillin sensitive Staphylococcu saureus) was more than the MRSA and it was found that the male patients were more affected than the female patients. All of the isolates were resistant to cefoxitin and oxacillin while most of them showed positive band of mec-A gene. All of the MRSA isolates showed resistant to penicillin followed by azithromycin, erythromycin, co-trimoxazole and ciprofloxacin, while these strains were sensitive to linezolid and vancomycin, followed by teicoplanin, fosfomycin and fusidic acid. **Conclusion:** In conclusion, proper diagnosis of MRSA required conventional, phenotypic molecular techniques in our hospital diagnostic settings. This will help in choosing the effective antibiotics combat the infection.

Key words: Antibiotic Sensitivity Pattern, Cefoxitin, Kirby Bauer Method, Mec-A Gene, MRSA.

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INTRODUCTION

The Staphylococcu *S. aureus* is the most important pathogen that has developed resistance against most of the antibiotics, which are used as treatment option against its infection in the recent years. The contribution of penicillin in 1960's, mitigated the scenario of antibiotic resistance before the introduction of commercially available methicillin for the therapeutic purposes. The development of drug-resistant bacteria poses a great burden on the global health.[1] The emergence of methicillin resistant *Staphylococcus aureus* (MRSA) earlier in the hospitals and then in community worsened the situation specifically in young patients as skin and soft tissue infections. This multidrug resistant specimen cause severe infections with very high rate of mortality as its susceptibility is being reported frequently in community acquired infections to clindamycin, quinolones, macrolides, trimethoprim-sulfamethoxazole and tetracyclines.[2]

The choice of antimicrobial drugs against *S. aureus* is becoming narrowed down because of the development of resistance against most of the antibiotics. The majority of MRSA strains are resistant even to the routinely used anti-staphylococcal drugs such as aminoglycosides, fluoroquinolones, macrolide-lincosamides, tetracycline and trimethoprimsulfamethoxazole.[3] These are also become resistant to gentamycin, erythromycin, penicillin, ceftriaxone and ampicillin inTrinidad and Tobag.[4] The resistant is also being reported for oxacillin and penicillin in some Latin countries of America such as Mexico (penicillin and oxacillin), Brazil (penicillin), Chile(oxacillin and peni-

cillin).[5] For the treatment of these strains, vancomycin is considered to be a drug of last choice, though there are some issues in its use like toxicity, high cost and poor tissue penetration, especially in the lungs, bone and CNS. Moreover, the appearance of vancomycin intermediate and vancomycin resistant *S. aureus* (VISA&VRSA) strains globally, is a serious matter.[6]

For the detection of methicillin resistance various culture methods and assays are performed in routine bacteriology laboratory. These assays include the disk diffusion specially cefoxitin, E-test, broth microdilution, oxacillin agar medium, chromogenic agar medium, the detection of mecAgene by polymerase chain reaction (PCR), and latex agglutination assay.[7,8] The detection of mecAgene by PCR is considered as the gold standard, but most of the hospitals lacking the advanced laboratory services required for the molecular methods essential for fast and precise identification of methicillin-resistant isolates.[9] These days the cefoxitin disk diffusion test is commonly used in clinical microbiology setups. In the light of rules of the clinical and laboratory standards institute (CLSI), the cefoxitin disk diffusion assay should be used to confirm the mecA-mediated oxacillin resistance. The other methods that are commonly practiced are cefoxitin screen with an automated identification system like VITEK®-2 (bioMerieux SA, Marcy l'Etoile, France) or the BD Phoenix™ (BD Diagnostics, Franklin Lakes, NJ,USA), and the determination minimum inhibitory concentrations of oxacillin.[10,11]

The aim of this study was to estimate the incidence of MRSA with their antibiotic susceptibility pattern in the population of Lahore along with the molecular characterization of strains isolated from the samples of patients.

MATERIALS AND METHODS

Bacterial Isolates and Biochemical Identification

Clinical samples received at Microbiology section of Citilab and Research Centre, Lahore from March 2014 to June 2016 was included in the study. Clinical samples such as pus swab, wound swab, fluid were processed for Staphylococcus aureus identification following CLSI, 2014 criteria.[12] *S. aureus* were processed for bacterial identification using standard biochemical profiling, which includes Gram staining, Catalase, Coagulase, DNase along with culture characteristics and selective media mannitol salt agar.[13] Isolated strains were stored in 20 % glycerol at -80 °C. The current study was approved by Bio- ethics research committee (BREC) of the Citilab and Research Centre Lahore, Pakistan.

Detection of MRSA Using Phenotypic Methods

Confirmed *S. aureus* were further analyzed for detection of MRSA using phenotypic method. First of all, strains were tested against cefoxitin and oxacillin disc on Muller Hilton agar. Oxacillin was tested at 35°C for 24 hours, while cefoxitin was tested at 35°C for 24 hours. Bacterial growth aroundoxacillin and cefoxitin indicates positive phenotypic test for MRSA.[14]

Antibiotic Susceptibility Test

The antimicrobial susceptibility of these isolates was determined by Kirby Bauer disc diffusion method using different antibiotic discs (Bioanalyse) on Muller Hinton agar plates.[15] The CLSI standards of 2014 for antibiotic susceptibility were consulted to determine the sensitivity of all these isolates against a panel of antibiotics including: amoxicillin, penicillin, oxacillin, cefoxitin, ceftriaxone, cefaclor, teicoplanin, vancomycin, cephalexin, azithromycin, erythromycin, tetracyclin, ciprofloxacin, linezolid, clindamycin, co-trimoxazole, fosfomycin, fusidic acid, chloramphenicol.[12]

Detection of MRSA Using Molecular Method

All of the isolates were further confirmed as MRSA by sequencing a portion of mec-A gene (533bp) by using specific primers (mecA1F: AAA ATC GAT GGT AAA GGT TGG C, mecA1R: AGT TCT GCA GTA CCG GAT TTG C)[16] and by subsequent BLAST analysis (www.ncbi.nlm.nih.gov).

E-test MIC Determination of Vancomycin Against MRSA

The minimum inhibitory concentration (MIC) was carried out by E-test Method (Bioanalyse) and

interpreted according to manufacturer instruction following CLSI, 2014 guideline.

Ribotyping

The genetic characterization was performed by 16S rRNA gene sequencing after the extraction of genomic DNA by using the kit method (FavorPrep™, Cat# FATGK001-1). The PCR amplification of 16S rRNA gene of the isolates was carried out by using universal primers (27f: AGAGTTTGATCCTGGCTCAG) and (1522r: AAGGAGGTGATCCARCCGCA). The amplified products were purified by using gel purification kit (FavorPrep™, Cat# FAGPK001-1) and were sequenced. For the determination of the genetic similarity of these isolates with already reported data in gene bank, the sequenced data was analyzed through the BLAST search program at the NCBI website: http://www.ncbi.nlm.nih.gov/BLAST.[17] The nucleotide sequence data for each strain was deposited at the Gen Bank and get the accession numbers. After that the phylogenetic relationship was evaluated between these isolates by using MEGA6 software by applying neighbor joining method as alignment algorithm with bootstrap value of 1000 replicates.

RESULTS

Distribution of MRSA

Out of total 220 *S. aureus* isolates, the frequency of MRSA and MSSA (methicillin sensitive Staphylococcusaureus) was 32.7 % (n = 72) and 67.27 % (n = 148) respectively. Both phenotypic (Cefoxitin disc and Oxacillin) tests showed similar results. In molecular detection method 80.5 % (n= 58) strains are positive for mecA gene. Gender base analysis showed that samples from male patients were 58.3 % (n= 42) positive, while females patients suffered from infection caused by MRSA were 41.6 % (n= 30). In case of MSSA infection gender base analysis showed 58 .1% (n= 86) males and 41.9 % (n= 62) females were suffered. Specimen wise distribution showed that MRSA was most frequent in Pus/wound 75 % (n=54), 13.8 % (n= 10), 11.1 % (n= 8) samples following blood, fluids and others respectively. MRSA infection was more frequently present among the age group 40 to 60 years. MSSA infection was more frequently present among the age group 60 to 80 years.

Antibiotic Susceptibility Pattern of MRSA

The current study showed that all MRSA isolates were100 % resistant to penicillin and cefoxitin; however entirely (100 %) susceptible to linezolid and vancomycin followed by rifampicin (81.2 %), chloramphenicol (77.2 %), clindamycin (75.2 %), minocycline (67.3 %) and cotrimoxazole (65.3 %) (Figure-1, Figure-2A). MRSA showed high resistance (100 %) to penicillin followed by azithromycin (94.44 %), erythromycin (94.44 %), co-trimoxazole (94.44%) and ciprofloxacin (91.66%). In addition, MRSA isolates showed 100 % sensitivity to linezolid, 88.8% sensitivity to teicoplanin, 80.55% sensitivity to fosfomycin and 72.22% to fusidic acid (Figure-1).

MIC for Vancomycin

MIC for vancomycin is checked against MRSA, (n = 72) which showed that 50 isolates had MIC 2 µg/ ml followed by MIC 0.5 µg/ml (n = 10), 1µg/ml (n = 12) 2.5 µg/ml (n = 8), 4 µg/ml (n = 2) and 8 µg/ml (n = 2) (Figure-2B, 2C, 2D).

Molecular Identification of MRSA

Mec A gene

Out of 72 MRSA strains 63 showed positive single band, whereas 9 MRSA strains were negative. From the positive amplicons of MRSA, 5 were randomly selected for further amplicon sequencing for confirmation of MRSA including A1, A6, A7, A8, A9, which are submitted to Gen Bank for accession numbers (Table-I).

Ribotyping

In phylogenetic analysis, the MRSA strain A1, A2, A5, A5, A6, A7, A8, A9, A11, and A14 showed 100% similarity with Staphylococcus aureus strain NBRC 100910, N315, S33 R, MVF-7, ATCC 12600 and JH1 respectively. After obtaining the results of 16S rRNA gene similarity, the evolutionary history was inferred by using the neighbor joining method as alignment algorithm with bootstrap value of 1000 replicates.[17] The phylogenetic trees for 6 MRSA isolates have been shown in the Figure-3. The percentage of trees in which the associated taxa clustered together is

shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6.[18]

DISCUSSION

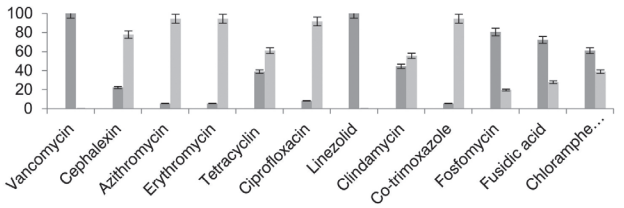

Figure-1. Antibiotic susceptibility pattern of all the MRSA isolates

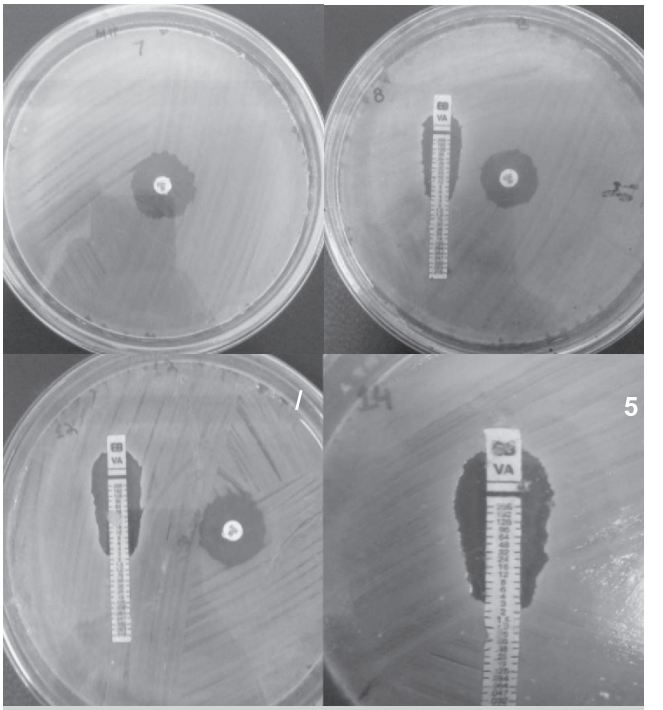

Figure-2. Sensitivity of MRSA isolates against cefoxitin disc (30mcg) (A) A7 (B, C, D) A8, A12 and A14 with EB strip for vancomycin resistance susceptibility

MRSA Strains	No. of Nucleotides Sequenced	% Similarity with	
A1	510	Staphylococcus aureus JCSC6945 mecA gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100%
A6	510	Staphylococcus aureus TN/CN/1/12 mecA gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100%
A7	510	Staphylococcus aureus JCSC6943 mecA gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100%
A8	500	Staphylococcus aureus TN/CN/1/12 mecA gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100%
A9	500	Staphylococcus aureus subsp. aureus N315 mecA gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100%

Table-I. Percentage similarity of mec-A gene of MRSA isolates with the genes already repoted in GenBank

Over the past 20 years the spread of methicillin-resistant Staphylococcus aureus (MRSA) has been proved to be a global public health issue.[19,20] The prevalence of MRSA is very high in different areas of the world like in Latin American countries it is calculated to be>80%, while it is increasing in other regions. The Australian region has experienced a rise in the prevalence of MRSA from 12% to 19% in the duration of 13 years from 2000-2013.[21,22] The situation in India is even more drastic as there is observed an increase in the infection rate with the proportions of 41±80% from 2008±2012.[21] However, the mean prevalence of MRSA is declining in European countries, in the United States and in Canada, while it is still high in most countries of the world, fluctuating from 15% to 45%.[21] In Scandinavian countries the successful surveillance strategies and infection control programs lessening the proportion of Staphylococcus aureus resistant to methicillin 1%.[23,24] The situation in Pakistan is worse because of the misuse of antibiotics and self-medication practices; the prevalence of MRSA has been reported with a varied range from 42% to 51%.[25]

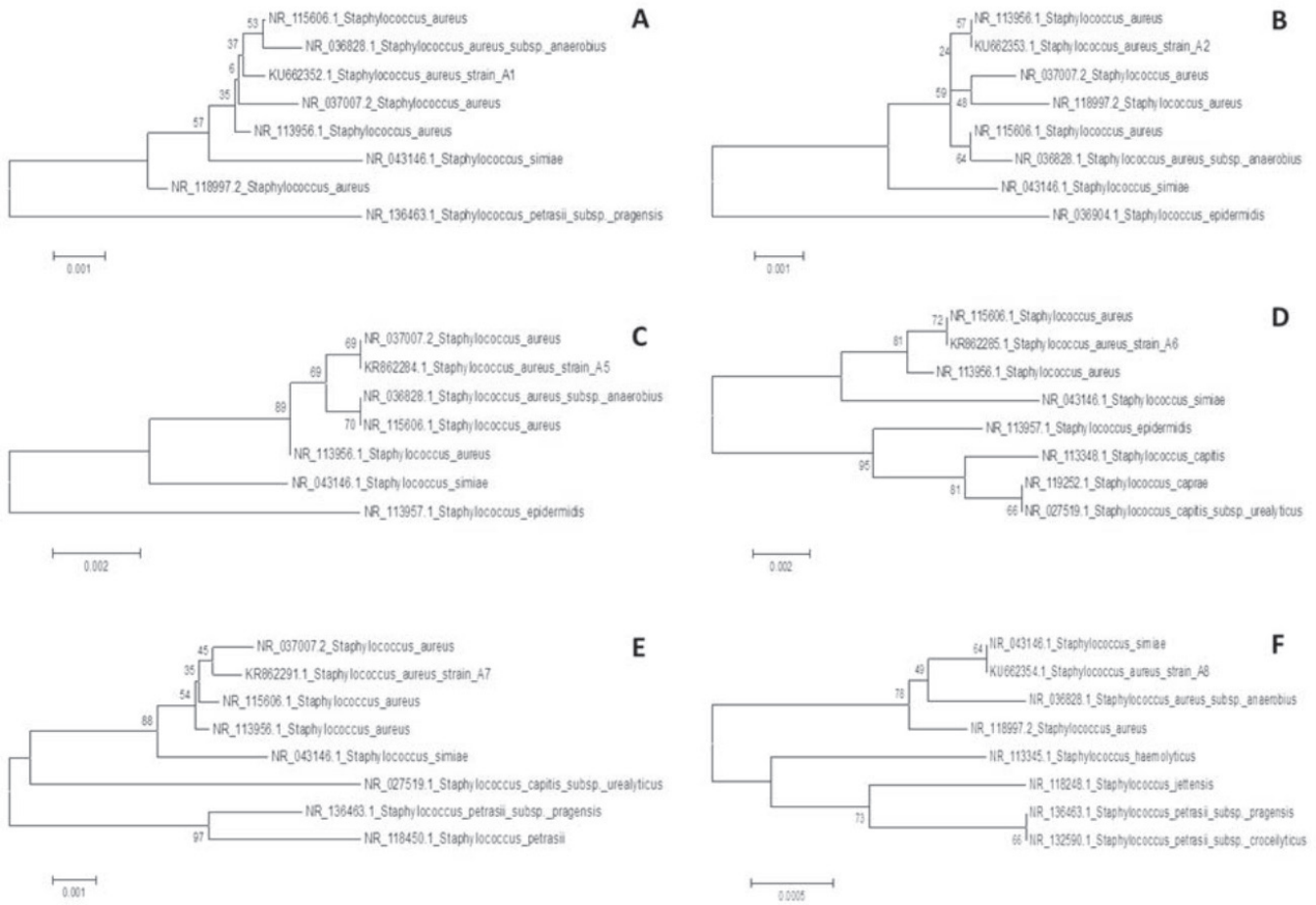

Figure-3. Neighbor joining phylogenetic trees of 16S rRNA gene sequence of MRSA isolatesStaphylococcus aureus A1 (A), A2 (B), A5 (C), A6 (D), A7 (E), A8 (F) along with their nearest homologues in NCBI database. Sequences were aligned and phylogenetic trees were made by using MEGA6, Bootstrap (1000 replicates) method

In our study, we isolated 220 *S. aureus* strains in which the frequency of MSSA was more than MRSA, as it was reported in an epidemiological study conducted at a tertiary care hospital in India.[26] On the basis of gender, it was revealed that the percentage of males infected with both of the two types, MSSA and MRSA was more than the females. This finding was comparable to a study conducted in a health care setup in Karachi, where the percentage of infection in males was reported to be 70%.[1] The laboratory diagnosis revealed that the frequency of MRSA in pus/wound samples was higher than other samples (75%), and it was in accordance with a study taken place in Hyderabad, Pakistan.[27]

The rapidly increasing problem of antibiotic resistance may only be addressed properly by improving the detection methods for the pathogens. These improved methods must be reliable and accurate enough, so these can help not in the early diagnosis of the infections but also in controlling the over whelming situation of resistance. The phenotypic method applied for the detection of MRSA in current scenario is by the use of oxacillin and cefoxitin disc susceptibility, which were given the same results in our case. These results were also comparable to the findings of a study conducted in India.[28] Another very important and rapid method to detect MRSA is by the typing of mec-A gene, which is now considered as a gold standard worldwide.[28] The MRSA isolates under study was also checked for the presence of this gene and most of them gave the positive result which was comparable to a study conducted in Denmark.[29]

The antibiotic sensitivity pattern of MRSA isolates was almost similar to that observed by Rashmi in a study conducted in a tertiary care hospital in India,[26] where all of the MRSA isolates showed resistant to penicillin, oxacillin, and cefoxitin, followed by azithromycin, erythromycin, co-trimoxazole and ciprofloxacin. All of the strains were sensitive to linezolid and vancomycin, followed by teicoplanin, fosfomycin and fusidic acid.[26] Genetic characterizations through 16S RNA gene sequencing has also been proved to be an important approach in identification of the MRSA. All of the strains were genetically characterized through 16S rRNA gene and then the phylogenetic relation was determined among them by using the neighbor-joining method based on Tamura-Nei model.[17]

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AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Adeela Fatima	Lab work, Data collection	
2	Imran Sajid	Principle investigator and supervisor.	
3	Saba Riaz	Manuscript writing and starin collection (MRSA).	
4	Muhammad Saeed	Manuscript writing and results interpretation.	