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ORIGINAL ARTICLE

Evaluation of Accuracy of Phenotypic Methods in the Detection of Methicillin Resistant Staphylococcus aureus

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ABSTRACT

Objective: The current study was conducted to evaluate the accuracy of already in use phenotypic methods of identification of MRSA, keeping in view the gold standard method of mec-A gene detection using Polymerase chain reaction (PCR) and to compare the sensitivity and specificity of different phenotypic methods with the genotypic method (PCR).

Methodology: A descriptive cross-sectional study was carried out at Microbiology Lab, Pakistan Railway Hospital, Rawalpindi from October 2021 to September 2022, after approval from Institutional Ethical Review Committee (Riphah/IIMC/IRC/21/73), using non-probability sampling technique. A total of 222 samples of *Staphylococcus aureus* were isolated from all the clinical specimens received at Microbiology lab at Pakistan Railway Hospital. Among those isolates, 150(67.5%) were Methicillin sensitive *Staphylococcus aureus* (MSSA) and 72(32.4%) were Methicillin Resistant *Staphylococcus aureus* (MRSA). The results of Phenotypic methods were compared with the results of PCR by using Chi-square formula.

Results: The sensitivity and specificity of Cefoxitin Disc Diffusion method was 62.5% and 96.6%, Oxacillin Disc Diffusion method was 65.3% and 93.3%, Oxacillin Screen Agar technique was 81.9% and 96% while that of Oxacillin MIC by Agar Dilution was 91.6% and 89.3% respectively. Oxacillin screen agar demonstrates the better phenotypic technique for detection of MRSA in routine practice.

Key words: Cefoxitin Disc Diffusion, Oxacillin Disk Diffusion, Oxacillin Screen Agar, Oxacillin MIC, PCR.

Authors' Contribution:
[1,2]Conception; Literature research; manuscript design and drafting; [3,4] Critical analysis and manuscript review; [5,6] Data analysis; Manuscript Editing.

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Introduction

Staphylococcus aureus is one of the most common bacteria isolated in the clinical laboratory. It is a gram-positive bacterium and an important member of the normal flora.[1] It lives as a colonizer under normal circumstances but can cause several health issues when becomes invasive. In spite of hygienic improvements and usage of effective antimicrobials; it is an important contributor in both hospital and community infectious load.[2] *Staphylococcus aureus* produces a variety of enzymes and toxins making it a highly virulent organism.[3] It's invasiveness due to the surface proteins, endospore and biofilm formation, leads to recurrent infections.[4] Methicillin-resistant *Staphylococcus aureus* (MRSA); a clinical variant has raised serious health concerns because of rapid transmission and spread, causing outbreaks in the clinical setups. Multi drug resistance (MDR) has become a major concern mainly due to the empirical antimicrobial drugs

usage. Therefore administration of more expensive and tiresome management is required.[5] Methicillin/oxacillin-resistant *Staphylococcus aureus* are heterogeneous in their expression of resistance to β-lactam agents. Accurate identification of MRSA is very important for enhancing the reliability of the patient management.[6] β-lactams, are ineffective against MRSA strains except for some new cephalosporins e.g., Ceftaroline.[7] The mecA gene present at the staphylococcal cassette chromosome (SCC) is responsible for methicillin or oxacillin resistance and for resistance to nafcillin too; therefore, the resistance is independent of ß-lactamase production. There are 11 types based on the combination of mecA and ccr complexes. Hospital acquired-MRSA isolates have SCCmec types I, II, or III, while the routine community acquired-MRSA strains are SCC mec type IV or V, [8] mec A gene encodes an additional penicillin binding protein (PBP)-2a or PBP2' which has a low affinity for β-lactam antibiotics. The gold standard method for identification of mec-A gene is PCR, but this facility is neither available nor economical for routine purpose in most of the clinical laboratories.[9] Therefore, one of the available phenotypic methods is adopted in routine laboratory working. But it is not clear which of the phenotypic methods is most reliable in the detection of methicillin resistance. Although there are some studies available internationally which recommend using all the phenotypic methods along with the genotypic method. Most of the Pakistani studies have assessed the significance of Disc diffusion of Cefoxitin or Oxacillin for detection of Methicillin resistance.[10] But the efficacy of other phenotypic methods singularly or in combined form in the absence of genetic testing is not yet established. The current study was conducted to evaluate the accuracy of already in vogue phenotypic methods of identification of MRSA keeping in view the gold standard method of mec-A gene detection using Polymerase chain reaction (PCR) and to compare the sensitivity and specificity of different phenotypic methods (Cefoxitin Disc Diffusion, Oxacillin Disc Diffusion, Oxacillin Screen Agar, and MIC of Oxacillin by Agar Dilution Method) with the genotypic method (PCR).

Methodology

Our descriptive cross-sectional study was carried out at Microbiology Lab, Pakistan Railway Hospital, Rawalpindi from October 2021 to September 2022, after attaining the formal approval from Institutional Ethical Review Committee (Riphah/IIMC/IRC/21/73), using non-probability sampling technique. The expected sample size was calculated by using prevalence of 20% i.e., 0.20.[11]

A total of 222 *Staphylococcus aureus* isolates were obtained from blood, pus, HVS, sputum, urine, CSF, pleural and peritoneal fluid samples, regardless of age and gender of the patients. Duplicate samples from the same patients were excluded. The bacteria were identified by performing gram staining and standard biochemical tests (Catalase and Coagulase). At first the bijou bottles containing Brain Heart Infusion (BHI) Broth and *Staphylococcus aureus* were placed in the incubator at 37°C for 24 hours and then; when the growth of *Staphylococcus* was confirmed; the bottles were kept at -20°C for storage purpose. Bacteria were regrown on MacConkey agar plates[12] for performing all the following methods for the detection of mec-A-mediated resistance in *Staphylococcus aureus*. Kirby-Bauer disc diffusion technique was performed using 0.5 McFarland strength bacterial suspensions on Mueller-Hinton agar. Cefoxitin and Oxacillin impregnated antibiotic discs were applied. Cefoxitin applied plates were kept at 37°C while Oxacillin applied plates were kept at 35°C for 24 hours. The zones of inhibition were read using transmitted light. The recommended breakpoints of zone of inhibition for the 30µg Cefoxitin disc test were ≤21 mm and ≥22 mm for resistance and susceptibility respectively.[13] For Oxacillin, isolates showing an inhibition zone <13mm were considered resistant.[14] Muller-Hinton agar with Oxacillin powder, was

prepared as per directions of Oxoid to achieve a concentration of 6µg/ml of Oxacillin.[15] Direct colony suspensions of 0.5 McFarland strength of all the isolates were prepared.1µL of each bacterial suspension was applied on the plates. Plates were read after 24 hours of incubation at 20°C in the incubator for the presence or absence of growth for resistance and sensitivity against Oxacillin, respectively. Oxacillin agar plates were prepared for MIC of Oxacillin from 0.25 – 16.0 µg of Oxacillin per ml of MHA by making doubling dilutions of Oxacillin in Distill water: 320µg, 160µg, 80µg, 40µg, 20µg, 10µg and 5µg of Oxacillin powder each were added in 1ml of the MHA. The sterile plates were labeled from C to I; 1ml of each solution was poured in the petri dish accordingly along with the instillation of 19ml of MHA and was mixed thoroughly by gently shaking the plates. 1µL of each 0.5 McFarland strength bacterial suspension was applied on the plates. Plates were read after 24hours of incubation at 20°C. Presence of growth showed that organism was resistant to that strength of Oxacillin and absence of growth showed that organism was sensitive to that strength of Oxacillin. ≤2µg/ml was considered susceptible while ≥4µg/ml was considered resistant.

After obtaining bacterial growths, DNA extraction was done by boiling the thick McFarland solution at 100°C for one hour. Cuvettes were then arranged in the cuvette rack. 12.5µl of Master mix containing Taq polymerase, 3µl of extracted DNA, 1µl of Forward primer, 1µl of Reverse primer and 8.5µl of RNase free water were added in each cuvette and were placed in the Eppendorf for the amplification process. The amplification reactions were carried out by the setting of initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds and then annealing was done at 55°C for 30 seconds and for the purpose of extension it was performed at 72°C for 3 minutes and then the final extension was performed at 72°C for 2 minutes.[16] The sequence of Forward primer was (5'-ACT GCT ATC CAC CCT CAA AC-3') and the sequence of Reverse primer was (5'-CTG GTG AAG TTG TAA TCT GG-3'). The Amplicon size was 163 base pairs. After the completion of the amplification process. 1% Agarose gel was prepared using Tris base-Boric acid and EDTA (TBE-EDTA) buffer. 10µl of Positive control, Negative control, DNA ladder and amplified DNA samples each were added in the wells and 120V current was allowed to pass.[17] Bands produced were seen on Ultraviolet light illuminator and results were recorded.[18]

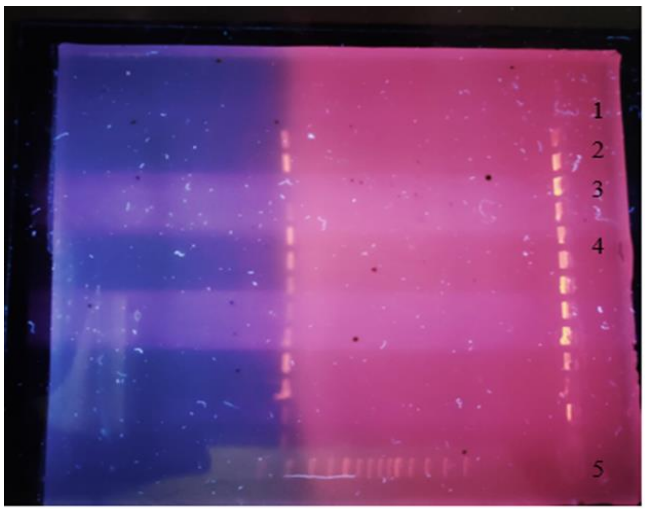

Figure 1. Bands of mecA, 1 Negative control, 2 Positive control, 3 MSSA (mec-A band absent), 4 MRSA (mec-A band present), 5 DNA Ladder

Data analysis was conducted using "Statistical Package for Social Sciences (SPSS) version 21.0". For each categorical variable, simple descriptive statistics (frequencies and percentages) were calculated. Pearson Chi-square was applied to calculate the sensitivity and specificity of each technique. p-value calculated was significant (<0.005).

Results

The analysis of 222 samples of *Staphylococcus aureus* was carried out. In this study using PCR for mec-A gene; MRSA were 32.4% (n=72) and MSSA were 67.56% (n=150), using Cefoxitin; MRSA were 22.5% (n=50) and MSSA were 77.4% (n=172), using Oxacillin; MRSA were 25.6% (n=57) and MSSA were

74.3% (n=165), using Oxacillin Screen agar (OSA); MRSA were 29.2% (n=65) and MSSA were 70.7% (n=157) and by using Oxacillin Agar Dilution; MRSA were 36.9% (n=82) and MSSA were 63.06% (n=140). Keeping in view mec-A gene as gold standard; Cefoxitin disc detected 45 True MRSA (20.2%), Oxacillin disc detected 47 True MRSA (21.17%), OSA detected 59 True MRSA (26.57%) and MIC of Oxacillin detected 66 True MRSA (29.72%) (refer to Table I: True & False Positive & Negative MRSA & **MSSA**). The results of Phenotypic methods were compared with the results of PCR by using Pearson Chi-square formula.

Table I: True & False Positive & Negative MRSA & MSSA

Method	True MRSA	False MRSA	True MSSA	False MSSA
PCR mecA detection	72		150	
Cefoxitin Disc	45	5	145	27
Oxacillin Disc	47	10	140	25
Oxacillin Screen Agar	59	6	144	13
MIC of Oxacillin	66	16	134	6

Table II Sensitivity and Specificity of phenotypic methods

Method	Sensitivity	Specificity
Cefoxitin Disc	62.5%	96.6%
Oxacillin Disc	65.3%	93.3%
Oxacillin Screen Agar	81.9%	96%
MIC of Oxacillin	91.6%	89.3%

The sensitivity and specificity of Cefoxitin Disc Diffusion (CDD) method was 62.5% and 96.6%, Oxacillin Disc Diffusion (ODD) method was 65.3% and 93.3%, Oxacillin Screen Agar (OSA) technique was 81.9% and 96% while that of Oxacillin Agar Diffusion (OAD) was 91.6% and 89.3% respectively (refer to Table II Sensitivity and Specificity of phenotypic methods).

Discussion

MRSA strains account for a sizable portion of infections in both hospital and community settings due to an increase in the drug resistance patterns.[19] MRSA must be accurately identified in order to provide proper treatment and preventive measures. The varied nature of methicillin resistance in *Staphylococcus aureus*, however, places limitations on the precision and dependability of phenotypic approaches for resistance detection. The mecA gene identification using PCR-based techniques by reference laboratories, are not feasible for routine application in diagnostic settings. Therefore, it is indicated to compare the PCR and phenotypic approaches for the identification of mec-A gene and methicillin resistance. International recommendations like CLSI took many initiatives over the past ten years to standardize and optimize phenotypic approaches for MRSA identification. Since no phenotypic test is 100% reliable for detecting methicillin resistance in *Staphylococcus aureus*, formation of an algorithm is recommended for accurate identification of MRSA in everyday practice.[20]

In this study, the evaluation of diagnostic ability of different phenotypic methods like CDD, ODD, OSA and MIC of Oxacillin by agar dilution and their comparison with PCR Assay, showed variable sensitivity and specificity from other international studies. The sensitivity of Cefoxitin disc diffusion is less from most of the studies already conducted that showed sensitivity of almost 100% but specificity is almost similar 100% [21], 100% [22] and 98.3% [23] respectively. The MICs of mec-C resistant organisms cannot be discovered by the tests that target mecA

or PBP2a, leading to an increase in percentage of MRSA strains among mec-A negative strains. Strains with mecC are often resistant to cefoxitin and susceptible to oxacillin (CLSI).

In the Oxacillin disc diffusion; results of previous studies [5,24]showed sensitivity and specificity of 100% and 73.6% respectively; is quite different from our study showing 65.3% and 93.3% sensitivity and specificity respectively. Similarly the study by [5,22] has higher sensitivity and lower specificity of 84.2% and 66.2% respectively. The reason for decreased sensitivity found in our study might be due to the excessive production of beta-lactamases resulting in phenotypic manifestation of resistance to oxacillin but sensitivity to cefoxitin in mecA gene negative strains, resulting in increase in the number of false positive (MRSA). The Oxacillin Screen Agar results were almost similar to the findings of the studies[5,21] which show sensitivity and specificity of 96% and 100% and sensitivity and specificity of 96.3% and 100% respectively. Likewise MIC of Oxacillin by Agar dilution method are close to the findings of [5], [25] which shows sensitivity and specificity of 97% and 100% respectively.

The results of our study favored the Oxacillin Screen Agar test for routine MRSA diagnosis and for further confirmation MIC of Oxacillin by Agar Dilution technique. MIC of oxacillin by agar dilution technique is a bit tricky but it detects the maximum number of true positive MRSA strains. Many other methods for MRSA detection are available. Latex agglutination method is a reliable technique having good sensitivity and specificity as shown in the study conducted.[26] Epsilometer-strip test (E-Test) is used for determining the MIC of different antibiotics. The results of the studies conducted showed that this test does not provide precise MIC as compared to Broth Microdilution Technique.[27] Mannitol-Salt Agar (MSA) detects MRSA by the appearance of yellow-colored colonies on the media after 48 hours, but this technique has a lower sensitivity and specificity as compared to other methods.[28]

Conclusion

Oxacillin screen agar was demonstrated as the better phenotypic technique for the detection of MRSA in routine practice.

Recommendations: The research can be extended by including these techniques too. Our study serves for the need of extensive research with large sample size from different areas of Pakistan to ascertain the status of different MRSA genes in this part and their comparison with other countries.

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