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Original Research Article

Evaluation of Phenotypic and Genotypic Test Methods to Detect Methicillin Resistant *Staphylococcus Aureus*

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ABSTRACT

Background and Objectives: Methicillin resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community acquired infections. Hence, accurately detection of MRSA is not only important for the control measures but also to control the endemicity of MRSA. The present study was undertaken to evaluate the efficacy of different phenotypic methods with *mecA* based PCR for the detection of MRSA.
Materials and Methods: A total of 1000 isolates of *S. aureus* were included in this study. Methicillin resistance was determined by oxacillin disc diffusion, oxacillin MIC, cefoxitin disc diffusion and the oxacillin screen agar test was compared with *mecA* based PCR.
Results: Out of 1000 isolates from our hospital, 265 (26.50%) and 301 (30.1%) were identified as MRSA based on Cefoxitin and Oxacillin susceptibility test respectively. In all phenotypic methods, Cefoxitin disc diffusion test better correlates with gold standard PCR method for detection of MRSA.
Conclusion: Our study revealed that cefoxitin disk diffusion method had a high sensitivity and specificity comparative to other phenotypic methods for detection MRSA. Result of prevalence of MRSA by cefoxitin disk diffusion test is in concordance with the PCR for *mecA* gene and it can be used as alternatives to PCR for the detection of MRSA.

Key words: MRSA, mecA, Cefoxitin, oxacillin MIC, PCR.

INTRODUCTION

Staphylococcus aureus is a prominent human pathogen that can cause a varied range of diseases ranging from minor skin infections to life-threatening infections such as endocarditis, pneumonia, and sepsis. Its impact is enhanced by the development of antibiotic resistance, most notably methicillin-resistant *S. aureus* (MRSA). MRSA is defined as a strain of *S. aureus* that is resistant to a large group of antibiotics called β-lactams that includes penicillin's and cephalosporins. [1]

Meticillin resistance in *S. aureus* is associated with production of an altered penicillin-binding protein, a 78 kDa protein termed PBP2a, which has a low affinity for β-lactam antibiotics. These strains show resistance to a wide range of antibiotics, thus limiting the treatment options to few agents, such as teicoplanin and vancomycin. Therefore, it is clinically essential to rapidly

determine whether *S. aureus* isolates are methicillin resistant or not because this determination is important to ensure correct antibiotic treatment in infected patients as well as control of MRSA isolates in hospital environments that is to avoid spreading of them.

Identification of the mecA gene, or its product, PBP2a, PCR method is the most reliable and gold standard method for detecting MRSA. however all laboratories do not have molecular biology techniques in their routine clinical practice mainly in developing countries and performing this test is costly. [2] Hence, it is essential to evaluate the phenotypic techniques which are able to detect MRSA isolates in a rapid and accurate manner, in order to ensure correct antibiotic treatment and to avoid the spread of MRSA isolates in the hospital environment.

Various conventional phenotypic methods include, oxacillin disc diffusion, oxacillin MIC and oxacillin screen agar, cefoxitin disc diffusion, latex agglutination have evolved for rapid detection of methicillin-resistant staphylococci, but the optimal method of detection remains controversial. Most of the methods require subculture on solid media, and many are unable to detect methicillin resistance and species at the same time.

There are many factors which make the detection of MRSA complicated. Discrepancies in detection have lead to an adverse effect on patient management, thereby highlighting the importance of accuracy in detection. Recently, the Clinical and Laboratory Standards Institute (CLSI) recommended the use of the cefoxitin disc diffusion method for MRSA detection. Cefoxitin is a cephamycin type antibiotic and best inducer of the PBP2a-encoding mecA gene.

The aim of our study was to evaluate the efficacy of different phenotypic test methods as marker for MRSA by comparing it with molecular detection of *mec a* gene by PCR, considered as Gold standard test.

MATERIAL AND METHODS

Strain

A total 1000 nonduplicate isolates of *S. aureus* from various clinical samples including blood, pus, surgical site, wounds, fracture sites, sputum, tracheal aspirates, and urine between Dec 2010 to Jun 2012 were used in this study at our institute Bharati Vidyapeeth university medical college and hospital, Sangli. The isolates were identified using conventional methods like Colony morphology, Gram staining, Catalase test, tube coagulate and slide coagulase test, mannitol fermentation and DNase test. In the present study all testing was done and results read according to the CLSI or the manufacturer's recommendations.

Phenotypic methods for detection of MRSA.

Cefoxitin Disc Diffusion Test

Cefoxitin disc diffusion test was carried out using a 30 μg disc of cefoxitin on Muller Hinton agar plate on all isolates of *S. aureus*. Lawn culture of the bacterial suspension standardised to 0.5 Mc Farland standards was done on the agar plates. The plates were incubated at 37°C for 18 to 24 hrs and zone diameters were measured. Zone diameters ≤19mm was reported as methicillin resistant and zone diameters ≥22mm was considered as methicillin sensitive. Colonies that grew within the zones were tested again and the zone of inhibition reported.

Oxacillin Disk Diffusion Test

Disk diffusion test was performed on all isolates of *S. aureus* with 1 ug of oxacillin per disk on Mueller‑Hinton agar with 4% NaCl. Incubated at 35ºC. The zone size was interpreted according to the CLSI that is susceptible ≥13 mm and resistant ≤ 10 mm. [3]

Oxacillin Screen Agar

Muller-Hinton agar plates containing 4% NaCl and 6 µg/ml of oxacillin were prepared. The Oxacillin screen agar (OSA) test was performed on the same isolates, following CLSI guidelines by using direct colony suspension and adjusted to match 0.5 McFarland turbidity standards. The suspension of the isolate was deposited as a spot on the agar surface inoculated on Oxacillin screen agar (OSA). Plates were incubated at 35°C. The plates were observed carefully in transmitted light for any growth. Growth of any number of colonies after 24 hours was interpreted as oxacillin resistance. Isolate with confluent visible growth on OSA was identified as homogenous MRSA and those with scanty growth after 24 hrs incubation that was transformed perfect visible growth was identified as heterogonous MRSA.

Minimum Inhibitory Concentration to Oxacillin by Agar Dilution

Minimum inhibitory concentration to Oxacillin was done using agar dilution method. The bacterial suspension was prepared by emulsifying portions of 4-5 discrete colonies into 4-5 ml of nutrient broth, opacity adjusted by McFarland standard 0.5. Gradient plates of Muller-Hinton agar (MHA) containing 4% NaCl were prepared with doubling dilutions from 0.25 to 256µg/ml of oxacillin. The plates was inoculated as spot of about 5-8mm in diameter using sterile cotton swab stick and incubated at 35^0 C for 24hours. MIC of oxacillin was $\leq 2\mu g$/ml indicated that strain was susceptible and MIC $\geq$ 4µg/ml indicates methicillin resistance (NCCLS 2003). NCCLS has not made recommendations for using Cefoxitin to define methicillin resistance using agar dilution tests.

Molecular detection of mecA gene by PCR was done using standard procedures on MRSA isolates as per result of different phenotypic methods.

Genotypic methods for detection of MRSA.

PCR for detection of *mecA* gene is done at molecular laboratory of I.C.M.R. Regional center, Belgaum by following technique.

Bacterial DNA was extracted from overnight cultures of *S. aureus* by CTAB- NaCl method. [4]

The quality and quantity of isolated DNA was determined using Nanodrop 1000 spectrophotometer (JH Biosciences, USA. Model: ND1000) at 260/280 nm as well as visually by horizontal gel electrophoresis in 1% agarose. PCR for the detection of *mecA* was carried out following the method of Unal *et al*. [5] Primer sequences used for mecA detection are *mecA* (F): 5'- GTA GAA ATG ACT GAA CGT CCG ATA A-3' and *mecA* R 5' CCA ATT CCA CAT TGT TTC GGT CTA A 3'

Briefly, 1µl of 60ng of the extracted DNA was added to 24 µl of PCR amplification mix consisting of 16 µl of doubled distilled autoclaved water, 2.5 µl of 10X Taq buffer, 1µl of 2.5mM dNTP mix (Merck, India), 0.5 µl of 3U/µl Taq polymerase (Merck, India), and 0.5mM of each primer. The *mecA* gene was amplified using the primers (Sigma, India) as described by Jonas *et al*., 1999. [11] Amplifications were carried out in a thermal cycler (iCycler, BioRad Inc., USA) with conditions that consisted of 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1 min with a final extension at 72°C for 2 min. Amplicons of 310 bp were consistent with *mecA* gene amplification. The PCR products were subjected to agarose gel electrophoresis using gel red dye and images were acquired using Alpha Imager gel documentation system (JH biosciences, USA. Model: D E 400).

S. aureus ATCC 25923 (*mecA* negative) and ATCC 43300 (*mecA* positive) were used as controls for all phenotypic tests

and genotypic test. The sensitivity and specificity of each test were calculated using the PCR results as a gold standard test.

Data Evaluation

In order to understand the overall performance of phenotypic methods in the identification of MRSA isolates, sensitivity, specificity, positive and negative predictivity values were calculated according to the *mecA* gene positivity of MRSA strains.

RESULT

Out of 1000 strains of *S. aureus* isolated in our hospital, 265 (26.50%) were identified as MRSA based on Cefoxitin disc diffusion method. By oxacillin disc diffusion method, oxacillin MIC method and oxacillin screen agar, 30.1%, 27.7% and 27% strains were identified as MRSA respectively. As is seen here, 36 isolates (3.6%) showed discrepancy between oxacillin and cefoxitin disc diffusion susceptibility results. These same 36 isolates which were susceptible to cefoxitin shown to be negative for *mec A* gene by PCR. There were no isolates that were sensitive to Oxacillin but resistant to Cefoxitin. Result of phenotypic methods and PCR method for *mec A* gene is given in table 1 below.

Table No. 1. Result of phenotypic Methods with genotypic method for detection of MRSA

Phenotypic methods	MRSA	MSSA
Mec A positive	239	761
Oxacillin disk diffusion test	281	719
Oxacillin MIC	247	753
Oxacillin Screen agar	241	759
Cefoxitin disk diffusion test	239	761
Total numbers of S. aureus :- 1000		

Out of 1000 *S. aureus* strains, 319 were subjected to detection of *mecA* by PCR. In all above phenotypic methods, Cefoxitin disc diffusion test better correlates with gold standard PCR method for detection of MRSA. Fig.1 shows PCR result.

Table No. 2:- Sensitivity and specificity of phenotypic methods and genotypic method for detection of MRSA.

Methods	Sensitivity	Specificity	PPV	NPV
Oxacillin Disc Diffusion	100%	95.10%	88.03%	100%
Cefoxitin Disc Diffusion	100%	100%	100%	100%
Oxacillin MIC	100%	98.36%	95.66%	100%
Oxacillin Screen Agar	100%	99.31%	98.14%	100%
PCR for mecA	100%	100%	100%	100%

MIC-minimum inhibitory concentration, PPV-Positive predictive value, NPV- Negative predictive value.

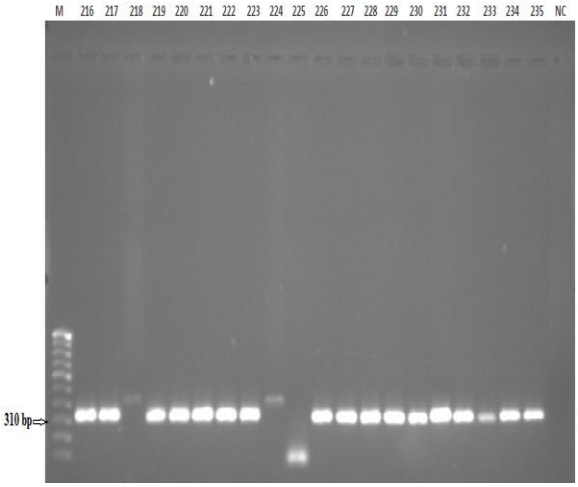

Fig No 1:- Agarose gel electrophoresis for *mecA* (310 bp) gene. Ladder No. 1 positive control, ladder No. 22 is negative control. Strain No. 218, 224, 225 negative for *mecA* gene

DISCUSSION

MRSA are being recognized as highly virulent and important human pathogens causing significant morbidity and mortality in hospitals as well as in community and are difficult to eradicate because they are becoming multidrug resistant. Rapid and accurate detection of MRSA is an important role of clinical microbiology laboratories to avoid treatment failure.

Expression of Methicillin resistance in *S. aureus* isolates possessing the *mecA* gene could be heterogeneous or homogenous in nature. Thus, heterogeneous strains are composed of two populations of cells, relatively susceptible cells and highly resistant cells. These strains appear phenotypically sensitive to methicillin. [6] In these strains methicillin MICs are at or just

above the susceptibility breakpoint e.g., oxacillin MICs of 4 to 8µg/ml. These are called BORSA strains. Borderline resistance strains do not contain the gene coding for methicillin resistance and resistance is not considered due to the production of PBP2a or *mecA* gene but as a result of modifications in the normal PBP genes, their overexpression or excessive production of staphylococcal β-lactamases. In in-vivo condition, when treatment with β-lactums, the PBP2a production may be induced and the cells which were susceptible to oxacillin in vitro become oxacillin resistant.

In vitro susceptibility studies, experimental data from animal studies and some clinical data show that treatment with β -lactam antibiotics is very effective for the infections caused by these *mecA* gene negative, non-PBP2a-producing BORSA. [7,8] Therefore it is essential to detect the presence of *mec*A gene to accurately identify the strains to be MRSA and thus PCR is a reference method in most clinical laboratories.

Correct identification of MRSA using conventional methods is complex, and some strains are difficult to classify, a strain can appear susceptible by one method and borderline or resistant by another. [9,10] For these reasons, several molecular methods have been developed to detect the mecA gene in MRSA clinical isolates. [11,12] However, genotypic tests involving *mecA* gene detection by PCR, which is considered to be the reference, are not practical for routine use in clinical laboratories.

The PCR technique has many added advantages over the conventional techniques. The time taken for diagnosing MRSA by conventional methods is 48-72 h, which is more as compared to PCR which takes 18-24 h. But the cost of PCR is high as compared to the conventional phenotypic methods. The critical parameters for success of a PCR based test are cost, reliability, speed, accuracy, and sensitivity. It is rapid with a high degree of sensitivity and specificity, but is expensive and all laboratories do not have PCR facilities.

Various phenotypic methods are available but the optimal method of detection remains controversial. In recent years there are multiple published report suggest the use of cefoxitin as surrogate marker for the detection of *mecA* gene mediated Methicillin - resistant Staphylococcus aureus. CLDSI also mentioned test for *mecA* gene or protein expressed by *mecA* gene that called PBP2' is most accurate test to identify MRSA. Isolates which harvest any one of this should be reported oxacillin resistant as very rare mechanism other than MecA cause oxacillin resistant. [13] Same time CLSI guidelines recommended cefoxitin to be used to identify MRSA. According to CLSI recommendation a 30 ug of cefoxitin disk is used and a zone of less than 19 mm or equal is considered as resistant strain. [14]

Currently surveillance data for MRSA are difficult to interpret, because there is no uniform testing method for detection of MRSA, and laboratories vary in their Standard operating procedure and interpretation of breakpoint values. [15] Discrepant results among conventional assays for detection of methicillin resistance were reported to be mainly due to the heterogeneous expression of resistance. [16] Other factors also influence the phenotypic expression of resistance such as addition of sodium chloride or sucrose to culture medium, incubation at 30°C or passage in the presence of b-lactam antibiotics enhances the expression of resistance (Hartman & Tomasz, 1986). These factors also necessitate the requirement for a simple, rapid, accurate and sensitive method for the detection of MRSA in routine diagnostic laboratories.

Disk diffusion method is an easy method for performance in microbiology, laboratories of MRSA. As already reported, the oxacillin disk diffusion test was the least reliable test for detection of MRSA. [17] The oxacillin screen agar test showed 100% sensitivity and 99.31% specificity for MRSA detection in our study. Swenson et al. (2001) noted that sensitivity decreased when heterogeneous resistant strains were tested and specificity decreased with strains having borderline MIC.

Several studies including the current one have reported that the results of the cefoxitin disk diffusion test correlate better with the presence of *mecA* compared with those of the oxacillin disk diffusion test. [18] Cefoxitin is a better inducer of *mecA* expression; this could explain why heterogeneous MRSA populations variably expressing the *mecA* are better detected by disk diffusion with cefoxitin than with oxacillin, which is a weak inducer of PBP2a production. This is considered to be the underlying mechanism for the higher sensitivity of cefoxitin than oxacillin. Anand *et al*. reported the sensitivity and specificity of the cefoxitin disk method to be 100%, which is correlate with our results. [19]

In addition, in that study, the sensitivity and specificity of the oxacillin disk was determined to be 100% and 95.10%, respectively. The lower specificity in the present study could be because of differences in the manufacturer's disk. In the study of Sakoulas *et al*., the sensitivity and specificity of the oxacillin MIC method was 99 and 98.10%, respectively, and the specificity finding was consistent with the results of the present study 100 and 98.36%, respectively. [20] Wallet *et al*, compared the MIC method with PCR and the sensitivity was 96%, which was slightly lower than our results 98.10%. [21] 36 strains which were resistant by oxacillin DD, but sensitive by cefoxitin DD had MIC values <8 mcg/ml. These strains probably are BORSA (Borderline resistant strains) that hyper produce beta lactamase and while they appear oxacillin resistant, they do not possess the usual genetic mechanism for such resistance. This was corroborated by the fact that all the isolates that were resistant to oxacillin but sensitive to cefoxitin were positive for mec A by PCR.

Regarding cefoxitin disk diffusion, Anand *et al. and* many other studied reported that the results of cefoxitin disk diffusion tests correlate better with the presence of *mecA* than do the results of disk diffusion tests using oxacillin. [22,23] The oxacillin disk diffusion method was found to be less sensitive for the detection of MRSA.

CONCLUSION

Our study revealed that cefoxitin disk diffusion method had a high sensitivity and specificity comparative to other routinely used methods for detection MRSA. Cefoxitin is a more potent inducer of the *mecA* regulatory system and an accurate surrogate marker for the detection of MRSA in the routine susceptibility testing. This method can be preferred in clinical microbiology laboratories because it is easy to perform, do not require special technique, incubation temperature, media preparation and more cost-effective in comparison to other methods.

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