Staphylococcus aureus is one of the pathogens most frequently isolated from clinical specimens and is currently the most common cause of nosocomial infections. It has overcome most of the therapeutic agents that have been developed in the recent past and treatment of infections caused by S. aureus has become a problem. Currently the most important clinical challenge is Methicillin Resistant Staphylococcus aureus (MRSA). It is emerging as an important pathogen in the hospitals and in the community. The prevalence of MRSA varies from 30-35% in most of the settings but some studies have reported the prevalence of MRSA as high as 60%.

Methicillin resistance among Staphylococci is caused by expression of penicillin binding protein 2a (PBP2a), encoded by the mec A gene, which has low binding affinity to methicillin and to all the β-lactam antibiotics available in the clinical practice. However accurate detection of MRSA by routine susceptibility detection methods is very complicated due to the heterogeneous nature of methicillin resistance. But still rapid and accurate detection of MRSA is very crucial for correct and effective treatment of the patients. So...
several methods for the detection of methicillin resistance in staphylococci have been evaluated like oxacillin disc diffusion\textsuperscript{7-9}, oxacillin agar screening tests\textsuperscript{7}, MIC determination by broth culture dilution\textsuperscript{10} and automated systems such as the Vitek FPS-SA card\textsuperscript{7}. The phenotypic methods commonly used are time consuming and difficult as large number of factors may affect the expression of resistance and its detection. Therefore genotypic tests are considered more accurate than phenotypic tests.

However the routine use of the genotypic methods like mec A gene detection, which is considered the gold standard for the detection of methicillin resistance\textsuperscript{11,12} is beyond the scope of most of the microbiology laboratories especially in the developing world. Cefoxitin disc diffusion test is an upcoming phenotypic test the sensitivity and specificity of which is comparable to that of the genotypic methods. The main objective of this study was to compare the efficacy of cefoxitin disc diffusion test for the detection of methicillin resistance among the S.aureus strains with the phenotypic methods like oxacillin disc diffusion and oxacillin agar screening test as well as to that of the mec A and fem B gene detection by multiplex PCR.

**MATERIAL AND METHODS**

A total of 12001 samples were screened for S. aureus from in and outpatients of Jawaharlal Nehru Medical College Hospital, Aligarh, UP, India during a period from August 2005 to July 2007. The samples were cultured on 5-10% sheep blood agar, MacConkey agar, Mannitol salt agar and Robertson’s cooked meat broth. All the isolates suggestive of S.aureus were identified by the standard biochemical procedures\textsuperscript{15}. The methicillin susceptible strain ATCC 25923 was used as a control for the diagnostic procedures. All isolates were maintained in 0.5%-1% semisolid nutrient agar stabs and seeded with cork stoppers soaked with hot sterile paraffin until analysed\textsuperscript{15}.

**Oxacillin and cefoxitin disc diffusion test**

All the isolates were subjected to oxacillin and cefoxitin disc diffusion test using oxacillin 1µg disc and cefoxitin 30 µg disc. McFarland turbidity standard suspension of the isolate was made and lawn culture was done on Mueller-Hinton agar (MHA) plates containing 4% NaCl. Plates were incubated at 37°C for 18 hours and zone diameters were measured. An inhibition zone diameter of ≤10mm was reported as methicillin resistant and ≥13mm was taken as methicillin sensitive. For cefoxitin inhibition zone diameters of ≤19mm were reported as methicillin resistant and ≥20mm were taken as methicillin sensitive\textsuperscript{16}.

**Oxacillin screen agar**

Mueller-Hinton agar plates containing 4% NaCl and 6 µg oxacillin were prepared. Plates were incubated at 37°C for 24 hours. Plates were observed carefully in transmitted light for any growth. Any growth after 24 hours was considered oxacillin resistant\textsuperscript{17,18}.

**MIC determination**

MIC was determined by agar dilution test. 10 different dilutions of oxacillin were selected such that the concentrations that allowed determination of MIC breakpoints defining susceptible (≤2µg)\textsuperscript{19} and resistant(≥24µg)\textsuperscript{19} values were included. Lowest concentration at which the growth was inhibited by 80% or more was recorded as MIC.

**PCR amplification for mec A and fem B genes**

Multiplex PCR (20) was carried out on all the S.aureus strains found methicillin resistant on MIC determination. All the MRSA strains were for the mec A and fem B genes using the following oligonucleotides sequence: mec A1-5’ GTA GAA ATG ACT GAA CGT CCG A TA A-3’, mec A2-5’ CCA ATT CCA CAT TGT TTC CGT CTA A-3’, fem B1-5’ TTA CAG AGT TAA CTG TTA CC-3’, fem B2-5’ ATA CAA ATC CAG CAC GCT CT-3’. A 50 µl PCR reaction mixture consisted of 45 µl of mastermix containing PCR buffer (1X), dNTP mix (0.2mM of each), primer(0.5µM), Taq DNA polymerase (0.25U), and MgCl\textsubscript{2} (1.5mM) with 5 µl of template DNA. Cycling parameters were set to- hot start 94°C for 4 minutes followed by 35 cycles of melting at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute. Analysis of amplified products was done by gel electrophoresis. Amplicons of 310bp were consistent with mec A and of 651bp with fem B gene amplification. (Fig. 1).
RESULTS

Out of 12001 samples collected from various in and outpatients of JNMCH, a total of 262 *S.aureus* were isolated. On screening for methicillin resistance, 85 (32.44%) isolates were found to be methicillin resistant by oxacillin disc diffusion test. However by cefoxitin disc diffusion 79 (30.15%) isolates were found to be resistant to methicillin and by oxacillin agar screening 82 (31.29%) isolates were detected as methicillin resistant. All the *S.aureus* strains were subjected to MIC estimation against oxacillin using agar dilution method. *S.aureus* strains which had MIC ≤ 2 µg/ml were considered methicillin sensitive whereas those with MIC ≥ 4 µg/ml were considered methicillin resistant. It was found that oxacillin disc diffusion test identified 12 sensitive strains as resistant and 6 resistant strains as sensitive. Oxacillin agar screening gave only 2 false negative and 3 false positive results. However the sensitivity and specificity was highest for cefoxitin disc diffusion test with no false positive or negative. The results of the three phenotypic tests are shown in Table 1. The sensitivity and specificity of the three phenotypic tests as compared with the genotypic test are given in Table 2.

<table>
<thead>
<tr>
<th>Test method</th>
<th>Detected as</th>
<th></th>
<th>Detected as</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin disc diffusion (1µg)</td>
<td>85 (32.44)</td>
<td>177 (67.56)</td>
<td>93.41</td>
<td>92.99</td>
<td></td>
</tr>
<tr>
<td>Oxacillin agar screen (6 µg)</td>
<td>80 (30.53)</td>
<td>180 (68.71)</td>
<td>97.57</td>
<td>98.33</td>
<td></td>
</tr>
<tr>
<td>Cefoxitin disc diffusion (6 µg)</td>
<td>79 (30.15)</td>
<td>183 (68.85)</td>
<td>100.00</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>PCR for mec A gene</td>
<td>79 (30.15)</td>
<td>183 (68.85)</td>
<td>100.00</td>
<td>100.00</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by *S.aureus*. Although multiple methods of detection of this resistance have been developed, they are often too slow or not sufficiently sensitive or specific. Identification of the mec A gene is the gold standard for detecting the MRSA isolates, however not all laboratories can include molecular biology techniques in their routine clinical
practice. For this reason, it is essential that phenotypic techniques able to detect MRSA isolates rapidly and accurately should be introduced, to ensure correct antimicrobial therapy and to prevent the spread of MRSA isolates in the hospital and in the community.

Oxacillin disc diffusion test is the method most commonly used in the routine laboratories. However the sensitivity and specificity of this method is only 93.44% and 92.41% respectively. Whereas the sensitivity and specificity of cefoxitin disc diffusion test were 100%. Cefoxitin disc diffusion test correlates well with the PCR. It is considered as a better predictor than oxacillin for the detection of heteroresistance because it is a stronger inducer of PBP-2a. In addition it has high affinity for staphylococcal PBP4 and various experiments have shown a relationship between PBP2, PBP4 and methicillin resistance. The cefoxitin disc diffusion test should be preferred over oxacillin disc diffusion test for predicting methicillin resistance in S. aureus.

CONCLUSIONS

Among the phenotypic methods cefoxitin disc diffusion test is the best predictor of methicillin resistance in S. aureus. It is suggested that cefoxitin disc diffusion test should be used routinely in the microbiology laboratories for accurate detection of methicillin resistance.

REFERENCES

15. Colle JG, Miles RS. Test for identification of bacteria. In Mackie and Mc Cartney, Practical Medical Microbiology, eds Colle JG, Dugid JP.


