

Cefoxitin Disc Test as a Marker for Detecting Methicillin Resistance in *Staphylococcus aureus* Isolates

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S. aureus is an important cause of nosocomial infections. It has overcome most of the therapeutic agents, of which the most important problem for the physicians and the microbiologists is the emergence of MRSA. In the present study, an effort has been made to detect and compare the efficacy of the cefoxitin disc diffusion test with other phenotypic methods like oxacillin disc diffusion, oxacillin agar screening test and determination of MIC and to the genotypic detection of *mec A* and *fem B* genes using multiplex PCR. Oxacillin disc diffusion test identified 85(32.44%) strains as methicillin resistant, from oxacillin agar screening 80(30.53%) were found to be methicillin resistant, whereas cefoxitin disc diffusion test detected 79(30.15%) strains to be methicillin resistant. On estimation of MIC 79 strains were found to be methicillin resistant with MIC value $\geq 4 \mu\text{g/ml}$. *mec A* and *fem B* genes were present in all the 79 strains detected as methicillin resistant by cefoxitin disc diffusion test and by the estimation of MIC. Cefoxitin disc diffusion test was found to be the most sensitive and specific phenotypic method of detection of methicillin resistance comparable to the detection of *mec A* and *fem B* genes.

Key words: Cefoxitin, *fem B*, *mec A*, MRSA.

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^{1,2} some studies have reported the prevalence of MRSA as high as 60%³⁻⁵.

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 heterogenous nature of methicillin resistance⁶. But still rapid and accurate detection of MRSA is very crucial for correct and effective treatment of the patients. So

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several methods for the detection of methicillin resistance in staphylococci have been evaluated like oxacillin disc diffusion⁷⁻⁹, oxacillin agar screening tests⁷, MIC determination by broth culture dilution¹⁰ and automated systems such as the Vitek FPS-SA card⁷. 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^{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 procedures¹⁵. 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 slabs and seeded with cork stoppers soaked with hot sterile paraffin until analysed¹⁵.

Oxacillin and cefoxitin disc diffusion test

All the isolates were subjected to oxacillin and cefoxitin disc diffusion test using oxacillin 1 µg disc and cefoxitin 30 µg disc. A 0.5

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 sensitive¹⁶.

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^{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 (≤2 µg)¹⁹ and resistant (≥4 µg)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 *femB* genes using the following oligonucleotides sequence. *mec A* 1-5' GTA GAA ATG ACT GAA CGT CCG ATA A-3', *mec A* 2-5' CCA ATT CCA CAT TGT TTC CGT CTA A-3', *fem B* 1-5' TTA CAG AGT TAA CTG TTA CC-3', *fem B* 2-5' 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 (0.5 µM), Taq DNA polymerase (0.25U), and MgCl₂ (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 out patients 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 ceftioxin 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

Table 1. Comparison of 3 different phenotypic methods for detection of methicillin resistance (n = 262)

Methicillin susceptibility	Test methods	No. of Strains
Sensitive by 3 tests	A+ Cn+ Ox+	171
Sensitive by 2 tests	A+ Cn- Ox+	2
Sensitive by 2 tests	A+ Cn+ Ox-	9
Resistant by 2 tests	A- Cn+ Ox-	3
Resistant by 2 tests	A- Cn- Ox+	4
Resistant by 3 tests	A- Cn- Ox-	73
Total		262

A= Oxacillin agar screen (6 µg) += sensitive
Cn= Cefoxitin disk (30µg) -= resistant

Table 2. Comparison of 3 phenotypic methods with pcr for detection of mrsa n=262

Test method	Detected as		Detected as		Sensitivity (%)	Specificity (%)
	MRSA	(%)	MSSA	(%)		
Oxacillin disc diffusion (1µg)	85	(32.44)	177	(67.56)	93.41	92.99
Oxacillin agar screen (6 µg)	80	(30.53)	180	(68.71)	97.57	98.33
Ceftioxin disc diffusion (6 µg)	79	(30.15)	183	(68.85)	100.00	100.00
PCR for <i>mec A</i> gene	79	(30.15)	183	(68.85)	100.00	100.00

≤ 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 ceftioxin 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.

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

**Fig. 1.** Multiplex PCR for Detection of *mec-A* (310 bp) & *fem-B* (651 bp) genes

1-3,5,7-10:*mec-A* & *fem-B* positive (MRSA)
4:*mec-A* negative & *fem-B* positive (MSSA)
6: DNA ladder

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 hetero-resistance 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 resistance²¹. 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.

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