STATUS AND DISTRIBUTION OF *MECA* GENE IN HOSPITALIZED PATIENT'S MRSA ISOLATES

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ABSTRACT

Detection of *mecA* gene, the gene encoding PBP2a, using PCR is considered to be the reference method. A total of 98 staphylococcal clinical isolates were used in this study. All the isolates were screened by a set of primers specific for mecA gene. In this study out of 98 isolates 78 isolates were classified as methicillin resistant based on disk diffusion method, while 20 were classified as MSSA. The mecA gene was found positive in 89 (MRSA & MSSA) isolates (87.22%), 20 out of 98 isolates which were categorized as MSSA by disk diffusion method 11 showed amplification of mecA gene by PCR. Complete concordance in our study between PCR results and those of disk diffusion method were found in 78 isolates. In terms of clinical implications, the discrepant finding among tests caution against the reliance on a single method for determining methicillin resistance in staphylococci as this can lead to erroneous results and patents receiving inappropriate medications. This study is first of its kind in a small city like Akola Maharashtra where Staphylococcus infection is increasing and becoming a menace which is resistant to methicillin.

Key Words: Clinical Samples, *Staphylococcus. aureus*, Methicillin resistance, Disk diffusion test, *mecA* PCR.

INTRODUCTION

Staphylococci are the commonest of all the clinical isolates responsible for several super active types of infections. They have a differential ability to spread and cause outbreak in hospitals (Barber M, 1961). However, treatment of these infections has become problematic due to development of methicillin resistance.

Methicillin resistance first appeared among nosocomial isolates of S. aureus in 1961(Barber, 1961). The incidence of methicillin resistance of S. aureus (MRSA) in India ranges from 30-70% (Verma, 2000). MRSA are implicated in serious infections and nosocomial outbreak. These strains shows resistance to wide range of antibiotics, thus limiting the treatment options to very few agents such as vencomycin and teicoplanin. The mechanism of methicillin resistance in heterogeneous and homogeneous population of MRSA is diverce. Primarily these include the production of a low affinity penicillin binding protein (PBP) which is an altered form of penicillin Binding Protein required for bacterial cell wall synthesis termed PBP2a in addition to usual PBPs and the production of β-Lactamase. The gene encoding the altered PBP is mecA.

It is clinically crucial to determine rapidly whether clinical isolates are methicillin resistant of not, as this is a paramount importance for both treatment and control. Diagnostic methods used to detect MRSA in clinical samples should be highly sensitive and specific and most important the result should be available within a short time. Accurate detection of methicillin resistance can be difficult due to presence of two subpopulations, one susceptible and the other resistant that may coexist within the culture of staphylococci. All cells in culture may carry the genetic information for resistance but only a small number may express resistance in vitro (Prere, 2006). This phenomenon is termed heteroresistance for distinction with homogenous resistance which is easily detectable, variations of growth condition influences PBP2a production. Classic disk diffusion methods with oxacillin disk or automated system allow only detection of high level PBP2a producing strains. Some strains show a heterogeneous resistance and more often strains producing low level of PBP2a are reported, these classes of strains escape classic detection and are misidentified as methicillin sensitive. The clinical consequences may be fatal (JAMA, 1999). Actually several phenotypic methods have been proposed to detect heterogeneous and low level resistance and to discriminate borderline resistance. Borderline resistance to oxacillin is attributed to the hyper production of normal staphylococcal β-lactamase but borderline strains remain sensitive to oxacilline (Prere, 1991). Japanese studies reported that cefoxitin induced greater production of PBP2a that oxacilline.

A recent fresh study compared different detection methods and proposed cefoxitin or moxalactam disk diffusion test as a good assay for detection of low level MRSA (Feltan, 2002).

However it appears that if the test is performed properly with respect to inoculum, temperature of incubation or NaCl concentration in medium, they fail to detect very low level of PBP2a production. On the other hand some strains grow slowly and may be misidentified as susceptible while they possess the mecA gene. Results using conventional phenotypic assay may be given after 48 hours or more starting from suspicious colonies on primary culture plate. In case of severe disease, the early detection of MRSA is essential. In addition to their high specificity, the main advantage of molecular genetic test is their fast executability. Thus detection of mecA gene using PCR is considered to be the reference method (Prere, 2006).

In the present study we have reported the presence of MRSA in different infection at different hospitals by mecA PCR in and around Akola, a central India city of Maharashtra.

MATERIALS AND METHODS

Bacterial isolates

A total of 98 staphylococcal clinical isolates collected between the periods 2007 to 2008 were used in this study (which includes reference strain of *S. aureus* ATCC 25923). All the strains were clinical MRSA isolates from different specimens such as pus, blood, sputum and other body fluids. Identification of staphylococcal isolates was done by morphology, Gram stain, standard biochemical characteristics and susceptibility testing. *S. aureus* was identified by using standard tube *coag*ulase test.

Disk diffusion test

The procedures routinely used in the clinical microbiology laboratories were employed for this study. The concentrations of oxacillin tested were 1µg/ml. Disk diffusion tests were performed with 1µg of oxacillin per disk placed on Mueller-Hinton agar with 4% NaCl supplementation. The zone of inhibition was determined after 24 hours of incubation at 37°C. Organisms giving an inhibition zone equal to or lesser than 10 mm were interpreted as resistant to oxacillin. Organisms with a zone equal to or greater than 12 mm were interpreted as susceptible while those with an inhibition zone of 11-12 mm were interpreted as intermediate. (Rallapalli, 2008)

Bacterial genomic DNA isolation

Bacterial culture was grown overnight in nutrient broth and 2 mL of the culture was transferred into a microcentrifuge tube and spun for 2 minutes. The pellet was resuspended in 567 µL of TE buffer to which 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K were added, mixed gently and incubated for 1 hour at 37°C. Following this, 100µL of 5M NaCl was added and mixed thoroughly. After addition of 80µL of 10% CTAB-0.7M NaCl solution and the tubes were incubated for 10 minutes at 65°C. Equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed well and centrifuged at 10,000 RPM for 10 minutes. The upper aqueous phase was transferred to a new tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and centrifuged at 10,000 RPM for 10 minutes. The upper aqueous phase was transferred to a new tube and 0.8 volumes of isopropanol was added, mixed gently until the DNA was precipitated. The DNA was washed with 70% ethanol and resuspended in 50 µL TE buffer (Rallapalli, 2008).

PCR protocol

Primers were purchased from MWG; PCR was performed with two complementary primers as follows

mecA-F AAA ATC GAT GGT AAA GGT TGG C
mecA-R AGT TCT GCA GTA CCG GAT TTG C

Bacterial genomic DNA (aliquot of 1µL containing 50 ng of genomic DNA) was added to PCR mixture consisting tenfold concentrated reaction buffer (500mM KCl, 100mM Tris-HCl, pH 8·3), with final concentrations of 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 0.1 µM of each *mecA* primer. This mixture was supplemented with 2U of Taq DNA polymerase. The final reaction volume for PCR was 20 µL. DNA amplification was carried out in an automated thermocycler (MJ Research PTC-200). After an initial denaturation step for 5 minutes at 95°C, 40 cycles of amplification were performed as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and DNA extension at 72°C for 90 seconds, followed by an additional cycle of 5 minutes at 72°C to complete partial polymerizations. Amplified products were analyzed using horizontal 1·5% agarose gel electrophoresis.

RESULTS AND DISCUSSION

The colonized 98 isolates of S. aureus on plate showed smooth, slightly domed colonies with 1-2mm diameter. All isolates were positive to catalase, manitol fermentation, tube coagulase and DNase test.

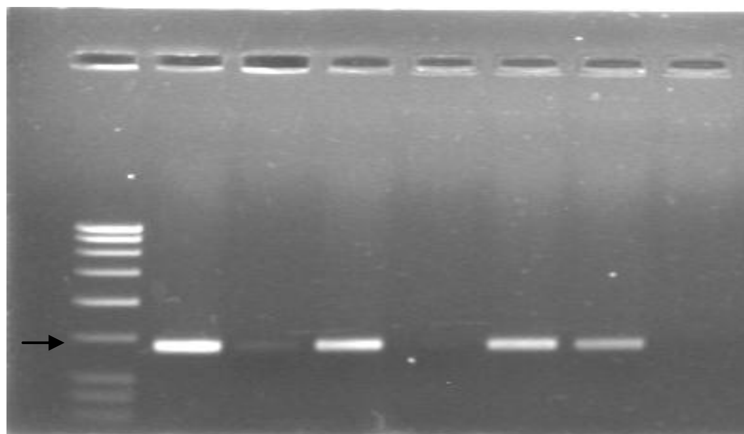
Detection of Methicillin resistance by disk diffusion test

The disk diffusion method was applied for detection of MRSA. Disk diffusion tests were performed with 1µg of oxacillin per disk placed on of Mueller-Hinton agar with 4% NaCl supplementation. Of the 98 isolates from different hospitals, 78 strains (76.44%) were identified as Methicillin resistant staphylococcus aureus and 20 (19.60%) as Methicillin susceptible staphylococcus aureus. Refer (Table I)

Table 1: MRSA MSSA isolates from different body fluid

Isolates	MRSA	MSSA	Total Isolates
Burn wound pus	31	5	36
Wound pus	18	1	19
Blood	13	6	19
Sputum	9	2	11
Throat swab	0	3	3
Urine	7	3	10

Fig:1 mecA PCR of MRSA MSSA isolates



mecA PCR of all the isolates

All the isolates were screened by a set of primers specific for mecA gene. An amplicon of 533bp was observed in all the 78 isolates, these isolates were confirmed by disk diffusion method as MRSA, remaining 20 isolates which were considered as MSSA by disk diffusion method were also screened for mecA gene PCR. Out of 20 MSSA isolates 11 isolates showed presence of mecA gene by PCR, remaining 9 isolates did not showed any amplification.

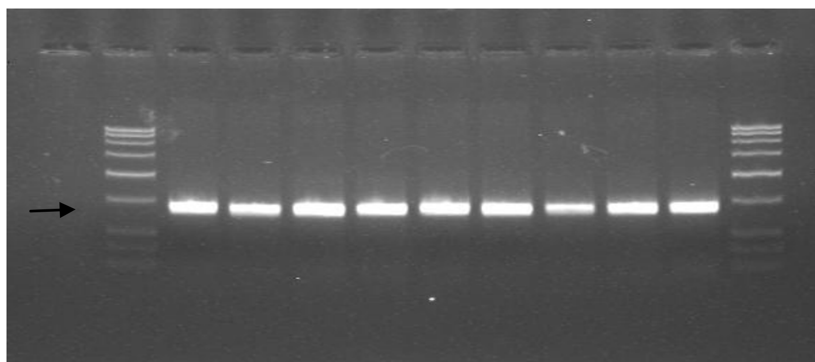
Gel picture of mecA PCR amplification product, column 1 is Low range DNA ruler (GeNei banglore) arrow in the picture is showing 600bp band.

Column 2, 4, 6 and 7 are 533bp mecA PCR product while the isolates of column 3, 5 and 8 did not show any amplification which were already considered as MSSA by phenotypic methods.

mecA amplicon (533bp) cloning and sequencing

A 533bp (JF778650 NCBI Nucleotide Database) amplification product of PCR from one of the isolate was cloned in to pGEM-T easy vector (Promega USA). Positive clones were confirmed by Blue white screening (As per instruction of Kit) and colony PCR. Positive clones were sequenced using ABI prism 377 sequencer. NCBI blast search confirmed the cloned amplicon as fragment of mecA gene.

Fig 2: Gel Picture of typical mecA amplification, arrow showing 600bp fragment of ladder



In this study out of 98 isolates 78 isolates were classified as methicillin resistant based on disk diffusion method, while 20 were classified as MSSA. The mecA gene was found positive in 89 (MRSA & MSSA) isolates (87.22%), 20 out of 98 isolates which were categorized as MSSA by disk diffusion method 11 showed amplification of mecA gene by PCR. Complete concordance in our study between PCR results and those of disk diffusion method were found in 78 isolates. The discrepant findings in our study cannot be attributed to technical problem related, for example, to colony selection, inoculum size, or incubation time, as repeat testing yielded the same results in each of the test. However an explanation of these discrepancies can be completed from other reports in the literature (Araj, 1999).

Discrepant results among conventional assays for detection of methicillin resistance were reported to be mainly due to the heterogeneous expression of resistance (Kocazog, 1997; Unal Hoskin, 1992; de Lencastre,1991). For example, Unal etal. (Unal, 1994) Reported that by using micro dilution testing, 186 of 1450 clinical isolates of S. aureus were preliminarily classified as oxacillin resistant; 15 of these isolates gave conflicting results by alternative methods and were classified further. Only two of these were mecA positive. The investigator concluded that significant number of S. aureus strains classified as resistant with an oxacillin may prove susceptible by other methods. 11 isolates in our study gave result that supported this statement. Other investigators also reported that the conventional susceptibility testing method including disk diffusion , broth of agar dilution tests are not always reliable in detecting methicillin resistance in staphylococci (Kocazog, 1997). The reliability of these tests have been ranked as follows agar screen greater than microdilution greater than disc diffusion method (de Lencastre, 1991, Chambers, 1997, Mackenzie AMR, 1995). Thus search for more reliable tests, based on genotypic marker were sought.

Since majority of the methicillin reisitant staphylococci carry *mecA* gene, test based on *mecA* detection, such as PCR based amplification or DNA hybridization have been expected to identify correctly even the most hetrogenous strains and were considered to be the best at detecting methicillin resistant staphylococcus. However several studies reported discrepancies, noting that some strains with negative mecA showed phynotypic resistance to methicillin while other with positive *mecA* showed phynotypic susceptibility to methicillin. York et al (York, 1996) reported that staphylococci strains with MIC < 2 µg/ml may be methicillin resistant and may be verified by oxacillin agar screen with incubation for 48hr. In addition, rare strains of mecA negative staphylococci, mostly coagulase negative, with higher level of methicillin or ocacillin resistance have been reported. Knapp *et al* (Knapp, 1996) noted that MRSA lacking the mecA gene could be classified as false resistant isolates by the oxacillin disk and plate method, and attributed this to hyper-production of β- lactamase. Discrepancies were also noted earlier by de Lancasre who compaired the oxacillin disk diffusion with dot bolt hybridization of a mecA DNA probe in detecting MRSA. They reported that of 49 strains said to be methicillin susceptible by disk diffusion, 11 were positive for mecA gene probe and of 59 strains determined MRSA by disk diffusion, ten showed no detectable hybridization by mecA probe. Thus, the discrepancies among the two methods ranged from 17% to 22% in both directions. The investigators suggested that the conflicting finding could be due to a variable and complex expression of resistance among the different MRSA sub-populations or strains. Besides, some MRSA

isolates may have incomplete regulator genes (mcl and or MECR1) and or high genomic diversity as was recently reported (Kobayashi, 1996). Moreover, not all strains that posse's mecA gene can express it (York, 1996). Such observations can help explain the discrepant findings among test in our study. It is worth nothing that standardized PCR based methodology can minimize discrepancies among different studies in the literature.

Although the expression *mecA* gene is considered an important mechanism of methicillin resistance in staphylococci, other mechanism alone or in combination have been detected in staphylococcus strains. For example methicillin resistance in *mecA* negative strains of S. aureus can arise because of hyperproduction of β-lactamase, production of normal PBP with altered binding capacity, and /or other as yet unidentified factors. Each of these mechanisms can contribute a certain degree of resistance against penicillinase resistant β- lactum antibiotics. In our study 76.44% of 98 isolates characterized by disk diffusion as MRSA were producers of β-lactamase. Unal et al (Unal S 1994) suggested that the prevalence of β-lactamase hyper producing strains of S. aureus in a specific institutional setting will influence the frequency of discordant results obtained by conventional and molecular-based susceptibility methods. Moreover, the mechanism of resistance was assumed to differ among isolates from different institutions as suggested by Kocayoz and Unal (Kocazog, 1997) who reported that in Turkey the mechanism of methicillin reisitance in staphylococci was ascribed to mecA in 94% and to β-lactamase in 6% of the isolates.

In terms of clinical implications, the discrepant finding among tests caution against the reliance on a single method for determining methicillin resistance in staphylococci as this can lead to erroneous results and patents receiving toxic or inappropriate medications. For example, Unal et al (Unal S 1994) when using the microbroth results alone would have withheld β-lactam therapy from 13 patients whose isolates were in fact mecA negative. Such false resistant strains accounted for 13 of 15 instances of discordant oxacillin susceptibility test results. On the other hand, disc diffusion did not identify two truly oxacillin resistant isolates.

In conclusion, the susceptibility testing of S. aureus and other staphylococcus should emphasize not only the accurate detection of oxacillin resistance but also the avoidance of false resistance to oxacillin. Moreover, as several resistance mechanisms are involved in mediating methicillin resistance in MRSA and since discrepancies are being observed among conventional and molecular tests used to detect MRSA.

This study is first of its kind in a small city like Akola Maharashtra where Staphylococcus infection is increasing and becoming a menace which is resistant to methicillin.

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