

Role of Oxacillin Susceptibility Testing Strategy in Changing Scenario of *mecA* Positive *Staphylococcus aureus* Isolates (OS-MRSA) Detection

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Abstract

Staphylococcus aureus strains that are *mecA* and PBP2a positive but phenotypically susceptible to oxacillin are becoming more and more abundant, according to research from all around the world. The oxacillin susceptibility of *Staphylococcus aureus* (OS-MRSA) contributes to consequent treatment-failure due to misidentification by conventional susceptibility tests. Therefore, the objective of the current study was to ascertain the prevalence of OSMRSA in a tertiary care facility located in Mysore, South India. 395 MRSA isolates collected from diverse clinical samples were included in this lab-based prospective investigation. These isolates were tested using an oxacillin 1µg disc phenotypically by standard disc diffusion test, and simultaneously MIC to Oxacillin was determined from Vitek2 systems. Additionally, MRSA specific *mecA* gene detection was applied to these isolates in order to confirm their MRSA status genotypically. PCR findings demonstrate that 65% of the isolates were MRSA. The vitek2 system detected 4.06% OS-MRSA isolates with an oxacillin MIC of ≤2µg/ml. The disc diffusion method identified a total of 13.75% isolates as oxacillin sensitive and 10% isolates were oxacillin intermediately sensitive. Oxacillin sensitivity was shown for 1.87% of the *mecA*-positive MRSA isolates using the VITEK2 and disc diffusion techniques. This analysis found isolates with lower oxacillin MICs but relatively reduced OS-MRSA incidence. Using an oxacillin disc for routine laboratory MRSA detection might occasionally produce false negative results, which can result in improper antibiotic administration and treatment failure. In order to distinguish OS-MRSA from MRSA, it is crucial to combine phenotypic and genotypic techniques.

Keywords: Oxacillin Sensitive-MRSA (OS-MRSA), *mecA* Gene, PCR, MRSA, Vitek 2 System, MIC

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INTRODUCTION

S. aureus is the predominant origin of infections, in the community and in hospital environments. MRSA is also resistant to all other beta-lactam antibiotics, which exacerbates the issue. In October 1960, MRSA initially appeared in hospitals, and then reappeared in 1990 as a community-based illness. MRSA is a significant global health issue that is widespread in hospitals and healthcare facilities across many nations.¹

MRSA poses a serious health issue because of its infectiousness, antimicrobial resistance, and presence of virulence factors. The activation of *mecA* gene, which is encoded on the mobile genetic element staphylococcal cassette chromosome *mec* (SCC*mec*), is the main contributor to methicillin resistance in *S. aureus* (MRSA). The *mecA* gene encodes a modified penicillin binding protein (PBP2a) with a very low affinity for beta-lactam antibiotics, allowing *S. aureus* to survive beta-lactam antibiotic treatment. MRSA is defined as *Staphylococcus aureus* with either the *mecA* gene or a minimum inhibitory concentration (MIC) of oxacillin greater than 4 µg/ml or zone of inhibition ≤10mm by disc diffusion method. There have been reports of *S. aureus* that are *mecA* and PBP2a positive but phenotypically sensitive to oxacillin. There is general agreement that these *S. aureus* isolates should be classified as *mecA* positive, oxacillin sensitive/susceptible *S. aureus* (OS-MRSA).²

Furthermore, *mecA*-positive and oxacillin-sensitive isolates of *Staphylococcus epidermidis* and *Staphylococcus hominis* have also been reported. Because PBP2a is induced when these isolates are exposed to -lactams in-vitro, they are referred to as being "cryptically resistant". Due to their phenotypical vulnerability to oxacillin, they will be misidentified in the normal MRSA screening. These isolates can create a potential concern to patients who, once colonised with one, may soon find themselves colonised or infected with MRSA, for instance during an antimicrobial therapy cycle. The frequency of phenotypic resistance induction brought on by antibiotic exposure and the potential significance of dormant MRSA as a source of hospital acquired infections in both healthcare workers and in-patients.³ Clinical ramifications result from the

discovery of OS-MRSA because OSMRSA infections have been suggested in the report Although OS-MRSA is phenotypically responsive to oxacillin, the presence of *mecA* makes it potentially more susceptible in emergence of highly defiant MRSA under choice of antibacterial agents.²

MATERIALS AND METHODS

This study was conducted on 395 MRSA isolates collected in the department of microbiology from various clinical samples that included pus, blood, endotracheal aspirates, ear swabs, sputum, urine, and other sterile body fluids, from both in-and-out patients.

Ethical clearance was obtained from the IEC (Institutional Ethics Committee) (JSSMC/IEC/260822/38NCT/2022-23 dated 01-09-2022). All the *S. aureus* isolates from the clinical samples were processed and identified by standard microbiological methods in the hospital laboratory. Concisely, the clinical isolates were inoculated onto Blood agar (BA) and MacConkey agar for the isolation of the pathogens. Those samples yielded the growth of *Staphylococcus aureus* that were identified by standard procedures like catalase test, coagulase test and Vitek 2 ID were further included in the study. Resistance patterns of the isolates were documented from Vitek 2 system.

The disc diffusion method (Kirby Bauer) was used to detect methicillin resistance using a cefoxitin 30 µg disk. Oxacillin susceptibility was detected by using an oxacillin 1 µg disk. *S. aureus* isolates were lawn cultured onto Muller Hinton agar and the plates were left undisturbed at 37°C overnight after the application of both the antibiotic disk. Strain with the zone of inhibition ≤21mm on MHA around the cefoxitin 30 µg (HiMedia) disk and ≤10mm around the Oxacillin disk was considered as MRSA, as per CLSI guidelines 2021. Strain with the zone of inhibition 11-12mm on MHA around the Oxacillin disk was considered intermediate sensitive or borderline resistant, and a zone of inhibition ≥13mm was considered as oxacillin sensitive. (Figure 1)

MIC data of Oxacillin sensitivity was extracted from automated Vitek2 system. Strains with ≤2 µg/ml MIC are considered as oxacillin sensitive and ≥4 µg/ml are considered as oxacillin resistant.



Figure 1. Showing a strain with cefoxitin and oxacillin disk diffusion result on MHA media

Genotypically, MRSA was confirmed by uniplex PCR, a gold standard method, using specific primer sets targeting the *mecA* gene obtained from Oliveira and de Lencastre (2002), with forward primer *mecA* F¹- TCCAGATTACAACCTCACCAGG and reverse primer *mecA* R¹- CCACTTCATATCTTGTAACG (162bp).⁴

DNA Extraction and Amplification: DNA was previously extracted from each *S. aureus* isolate using the PCI (Phenol-Chloroform-Isoamyl Alcohol) method. The 500µl of lysis buffer (trisHCl+EDTA+NaCl+SDS) and 5µl of proteinase K were added to a 2ml micro-centrifuge tube containing 3 to 4 colonies of *S. aureus* from a fresh culture media plate and incubated at 56°C for three hours at 50rpm. After Incubation, 500µl of PCI (24:24:1) mixture was added, and centrifuged for 15 minutes at 5000rpm. Transferred the supernatant in 2ml tube and added 500 µl of a chloroform:isoamyl alcohol (24:1) mixture,

then centrifuged for 15 minutes at 5000 rpm. Supernatant was collected and mixed with 500µl of sodium-acetate:ethanol (1:9) mixture and kept at -20°C for an hour. After one hour of cold incubation, immediately follow that with 10 minutes of centrifugation at 10,000 rpm, at 4°C. The solution was discarded and 70% ethanol was added and centrifuged at 10000rpm for 10 minutes at 4°C. The tubes were dried at room temperature after the entire fluid was discarded. Elution buffer was added and spun the tubes before using a nano-drop instrument to measure DNA. All DNA samples that had been extracted were kept at -20°C until being processed.

30µl reaction mixture was prepared for the PCR consisting of the following, Template DNA 1µl, Primers 0.8µl/each (10pmol each primer), Master-mix 7.2µl and Nuclease free water 21.8µl. The PCR amplification was carried out in an automated thermal cycler (Biorad, T100). The cycling conditions were an initial denaturation at 95°Cx5 min, 34 cycles of 95°C x 30sec, 55.7°C x 30s and 72°C x 45sec, followed by a final extension at 72°C x 5 min. All the PCR amplified products were subjected to gel electrophoresis to confirm the presence of the *mecA* gene. The result was established by gel documentation image, captured by GenSys software.

RESULTS

Among 395 *S. aureus* isolates, a total of 62.27% (n=246) isolates were identified as MRSA phenotypically by the cefoxitin disk diffusion method. Isolates were collected from various clinical samples such as pus samples (86.17%), blood (3.65%), Et swabs (3.65%), ear swabs

Table 1. Showing total no. Of isolates and their Cefoxitin and oxacillin screening result by Kirby Bauer disk diffusion and Vitek2 system

Method	Cefoxitin screen (No. Of isolates)			Oxacillin Screen (No. Of isolates)		
	Sensitive	Intermediate Sensitive	Resistant	Sensitive	Intermediate Sensitive	Resistant
Disk diffusion method	149 (37.72%)	-	246 (62.27%)	36 (14.63%)	24 (9.75%)	186 (75.6%)
Vitek 2 system	-	-	395	10 (4.06%)	-	236 (95.9%)

(2.84%), sputum (1.6%), urine (0.81%), and other sterile body fluids (1.21%). Only phenotypically identified cefoxitin-resistant *S. aureus* isolates (MRSA) were considered for oxacillin disk diffusion test and PCR amplification for *mecA*-gene. A total of 75.6% (n=186) isolates were identified as Oxacillin resistant, 14.63% (n=36) isolates were oxacillin sensitive and 9.75% (n=24) isolates were intermediate sensitive, out of 246 methicillin-resistant *S. aureus* isolates. (Table 1)

The polymerase chain reaction technique confirmed a total of 65% (n=160) isolates as MRSA, of which 13.75% (n=22/36) isolates were identified as oxacillin sensitive and 10% (n=16/24)

isolates were intermediately sensitive by disk diffusion method. A total of ten (4.06%) isolates showed oxacillin MIC of $\leq 2\mu\text{g/ml}$ by the Vitek2 method, of which only six (3.75%) were identified as OS-MRSA by the PCR method. (Table 2) Only three (1.87%;n=3/6) *mecA* positive isolates were established as oxacillin sensitive by both disc diffusion and the VITEK2 method. (Table 3, Figure 2). Sensitivity and specificity of disk diffusion and Vitek was calculated using two table method and it was found that disk diffusion test to be 64.04% sensitive and 31.34% specific and Vitek showed 76.85% and 23.47%, respectively.

Table 2. Showing total number of isolates detected as *mecA* positive MRSA by PCR and their oxacillin susceptibility result

Oxacillin screen (Disk diffusion method)			Oxacillin MIC (Vitek 2 system)			<i>mecA</i> positive by PCR method
Sensitive	Intermediate Sensitive	Resistant	Sensitive	Intermediate Sensitive	Resistant	
22 (13.75%)	16 (10%)	122 (76.25%)	6 (3.75%)	-	154 (96.25%)	160 (65%)

Table 3. Showing isolates which are oxacillin sensitive-MRSA by both Vitek2 method and Kirby Bauer disk diffusion method

Oxacillin MIC (Vitek 2 system)	$\leq 0.25\mu\text{g/ml}$	$0.5\mu\text{g/ml}$	$2\mu\text{g/ml}$
Oxacillin screen (Disk diffusion method)	18mm	11mm	20mm
No. of isolates	1	1	1
Total no. Of OS-MRSA isolates	3 (1.87%)		

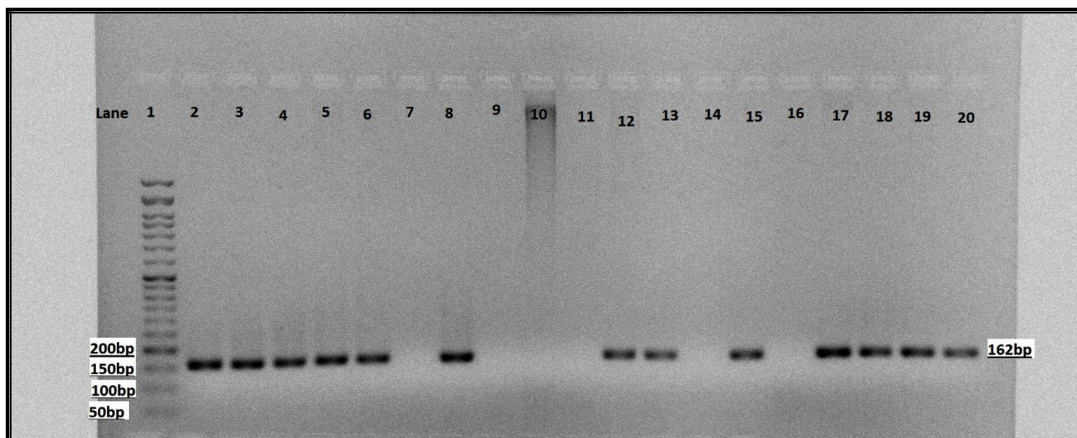


Figure 2. Showing PCR Amplification of *mecA* gene (162bp) for MRSA
 Lane 1: 50 bp ladder
 Lane 2 & 7: Positive Control & Negative Control for *mecA* gene of MRSA isolates.
 Lane 9-11,14,16: Negative for *mecA* gene of MRSA isolates.
 Lane 3-6,8,12,13,15,17-20: MRSA isolates Positive for *mecA* gene respectively

DISCUSSION

Staphylococcus aureus with the *mecA* gene or a minimum inhibitory concentration (MIC) of oxacillin $\geq 4\mu\text{g/ml}$ has been associated with MRSA. However, certain clinical isolates are oxacillin susceptible and *mecA*-positive. Therefore, we have investigated and found a total of 10 isolates with oxacillin MIC $\leq 2\mu\text{g/ml}$ but cefoxitin resistant. A similar result was found in the recent study of Loren Pardo et al., where 27 *mecA* positive OS-MRSA were reported.⁵ In another study by Roushan Liu et al., here a total of fourteen OS-MRSA isolates were reported out of 1200 *S. aureus* by VITEK2 system, oxacillin broth micro-dilution methods, BD Phonenix-100, and all the isolates were verified to be positive with *mecA* gene. Only 3 isolates out of 14 OS-MRSA isolates were cefoxitin resistant.⁶

In two different studies from China, reliably higher OS-MRSA isolates were detected in the year 2021. The first study was by Mingbiao Ma et al., in China where oxacillin susceptibility was detected by Vitek 2 automated method and E-test method. A total of 45 OS-MRSA isolates were detected out of a total of 499 *S. aureus* isolates. The second study was by Liu J-L et al., in China where they identified a total of 17 OS-MRSA out of 956 *S. aureus* isolates. In both studies, OS-MRSA isolates were positive for the *mecA*-gene by PCR method.^{7,8}

The PCR technique confirmed that the *mecA*-gene was present in a total of 160 isolates in the present study, of which 22 (13.75%) isolates were oxacillin sensitive which is comparable to the study of Tanit Boonsiri et al., reported a total of 43 *mecA* positive OS-MRSA.⁹ Similar result found in two different studies in 2019 by Teresa Conceic et al. where 17.7% (n=29/164) were *mecA* positive OS-MRSA and Sahar Zeinalpour Ahrabi et al., where OS-MRSA found in 6.25% of the students, and all together 54.54% (n=36/60) of the *S. aureus* isolates were *mecA* positive and 11.67% of the *S. aureus* isolates were resistant to oxacillin.^{10,11}

In the study of K. Saeed et al., oxacillin MIC with $\leq 0.25\mu\text{g/ml}$ was found in 63% of the clinical isolates, while 32.5 % of isolates had oxacillin MIC values between $\leq 0.25\mu\text{g/ml}$ and $> 0.5\mu\text{g/ml}$ for the remaining of isolates, 4.5 %

had the MIC ranged between ≤ 0.5 to $\leq 1.5\mu\text{g/ml}$. Despite overt cefoxitin and oxacillin sensitivities (MIC of $\leq 0.25\mu\text{g/ml}$), six of the isolates (1.2%), while having overt cefoxitin and oxacillin sensitivity (MIC of $\leq 0.25\mu\text{g/ml}$), confirmed positive for the *mecA*-gene, approving OS-MRSA.¹² This result also substantiates the present study, where one isolate was detected with oxacillin MIC $\leq 0.25\mu\text{g/ml}$ and another one with $0.5\mu\text{g/ml}$. Both the isolates were sensitive to the oxacillin disk diffusion method with the zone of inhibition 18mm and 11mm. This result can also be compared with the study of Marilyn Chung et al., where MRSA strains exhibited low oxacillin MICs $\leq 0.75\mu\text{g/ml}$.¹³ Another study by V. Anil Kumar et al., also reported 2 MRSA isolates out of 30 *S. aureus*, which was oxacillin sensitive by Vitek 2 system.¹⁴

In our investigation, one OS-MRSA isolate exhibited oxacillin MIC $2\mu\text{g/ml}$ while also being sensitive to the oxacillin disc diffusion method, which is comparable to the findings of Alexandros Ikonomidis et al., who found that the oxacillin MIC for two isolates with *mecA* positive was less than $2\mu\text{g/ml}$.¹⁵ Similar findings were made by Y. Hososaka et al., who reported 57 isolates had an oxacillin MIC of less than $2\mu\text{g/ml}$, out of 437 MRSA isolates and presence of *mecA*-gene was confirmed in six strains by pulse field gel electrophoresis assays.¹⁶

CONCLUSION

Since oxacillin susceptible-MRSA is a variation of MRSA, traditional phenotypic approaches based on susceptibility of oxacillin can potentially misidentify OS-MRSA for MSSA. Since OS-MRSA might be present in the collection of *S. aureus* isolates, we must examine the effectiveness of distinctive detection methods for *S. aureus* isolates that contain OSMRSA. Although, the extensively used Vitek2 system's capacity to generate findings faster than other methods, it has been observed a contradiction in distinguishing some of the OS-MRSA in the present analysis. Based on our sensitivity and specificity results, we suggest that even OSMRSA results can be interpreted by Vitek2 as MSSA, hence extra vigilance and confirmatory testing for oxacillin susceptible isolates should be employed

in clinical practice due to the incident of OS-MRSA in the oxacillin susceptible population. Cefoxitin has been reflected a more accurate interpreter of the existence of *mecA* than oxacillin since cefoxitin disc diffusion exhibited notable sensitivity for the identification of *Staphylococcus aureus* exhibit oxacillin susceptible isolates.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All data sets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, JSS Medical College, JSSAHER, Mysore, Karnataka, India, with reference number JSSMC/IEC/260822/38NCT/2022-23 dated 01-09-2022.

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