

## Laboratory Evaluation of Phenotypic Detection Methods of Methicillin-Resistant *Staphylococcus Aureus*

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Although conventional antibiotic susceptibility tests are most commonly performed for methicillin-resistant *Staphylococcus aureus* (MRSA), the results of these phenotypic tests are dependent on the standardization of the culture conditions. The aim of the study was to evaluate the conventional phenotypic screening tests in comparison to the *mecA* gene polymerase chain reaction (PCR). One hundred and two clinical isolates of MRSA identified by the oxacillin disk diffusion were subjected to PCR for the *mecA* gene and by the cefoxitin disk diffusion test and culture on oxacillin screen agar, mannitol salt agar, and methicillin-resistant *Staphylococcus aureus* Agar (MeReSA) selective medium, for MRSA. Although all 102 isolates were resistant in oxacillin and cefoxitin disk diffusion, 92 (90.1%) isolates were positive for the *mecA* gene. The sensitivities of the mannitol salt agar, MeReSA agar, and oxacillin screen agar were 89.13, 97.82, and 98.91%, respectively. The oxacillin screen agar may be recommended for confirming methicillin resistance in the disk diffusion test in resource-poor settings, where molecular methods are not available.

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**Key words:** Cefoxitin disk diffusion, methicillin-resistant *Staphylococcus aureus*, *mecA* gene, oxacillin disk diffusion, oxacillin screen agar, polymerase chain reaction

Antibiotic therapy for patient care as well as hospital infection control measures essentially rely on the laboratory for accurate antimicrobial sensitivity results. Failure to report methicillin resistance in *Staphylococcus aureus* (*S. aureus*) may result in treatment failure, poor prognosis, increased cost of treatment, and dissemination of multi-drug resistant strains. Although molecular methods are considered to be the gold standard,<sup>[1]</sup> conventional antibiotic susceptibility tests are the mainstream of MRSA laboratory diagnosis in resource-limited settings. Phenotypic tests have their own disadvantages, as these are affected by culture conditions.<sup>[1-3]</sup> Various selective media containing methicillin, oxacillin, and cefoxitin are available commercially and commonly used for MRSA surveillance cultures. Currently, the Clinical and Laboratory Standards Institute (CLSI) recommends cefoxitin as the sole marker of *mecA* gene-mediated oxacillin resistance and use of oxacillin disk diffusion has been discontinued.<sup>[3]</sup> This study has been carried out to evaluate different conventional phenotypic screening tests for MRSA in comparison to PCR detection of the *mecA* gene.

### METHODS

This cross-sectional study was carried out in the Department of Microbiology, in a tertiary care hospital in South India after Institutional Ethical Committee clearance. All clinical samples were processed in the laboratory as per the standard guidelines. *S. aureus* isolates were identified by gram stain, colony morphology on blood agar, and MacConkey agar, catalase, and coagulase and deoxyribonuclease (DNase) test. The disk diffusion test (DDT) using a 1 µg oxacillin disk was employed as the initial screening test. Consecutive isolates from the same patient were excluded. Oxacillin-resistant *S. aureus* isolates were subjected to cefoxitin (30 µg) DDT, culture on oxacillin screen agar (containing 6 µg/ml oxacillin and 4% NaCl), mannitol salt agar with cefoxitin and MeReSA agar (HiMedia, Mumbai, India), and PCR for the *mecA* gene.

The phenol-chloroform extraction method was used for DNA extraction. Each PCR reaction mixture (25 µl) contained, 2 µl of 1 PCR buffer, 2.0 mM of each of the primers

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(1 µl), 15 mM of each deoxynucleotide triphosphate (1 µl), Taq DNA polymerase (1 µl), 14 µl of molecular grade water, and a DNA template (2 µl). The forward and reverse primers were 5'-AAAATCGATGGTAAAGGTTGGC-3' and 5'-AGTTCTGCAGTACCGGATTTGC-3', respectively. The amplification protocol included five minutes of initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 60 seconds, annealing at 54°C for 60 seconds, and extension for 60 seconds at 72°C, with a final extension at 72°C for 10 minutes. Following PCR, the reaction mixtures were analyzed for 533 base pair amplified products by electrophoresis on a 1% agarose gel containing ethidium bromide (0.2 mg/ml), in the presence of an appropriate DNA molecular weight marker.

*S. aureus* ATCC 25923 and ATCC 43300 were used as controls for the antibiotic susceptibility test and PCR. The viability of the test isolates was maintained by a periodic subculture in a semisolid nutrient agar. All data were entered in a Microsoft Excel 2007 spreadsheet and statistical analysis was done. For categorical variables, the proportions are represented as percentages. The sensitivity, specificity, and positive and negative predictive values were calculated. True positive (TP) is defined as positive results in the new test under evaluation, which are also positive by the gold standard test.<sup>[4]</sup> Similarly, true negative (TN) indicates negative results in the new test as well as by the gold standard test. False positive (FP) and false negative (FN) are positive and negative results, respectively, detected by the new test, which differ from the gold standard test results. Sensitivity is the ability of a test to correctly classify a test result as 'positive'. However, the ability of a test to correctly classify a test result as 'negative' is called specificity. A positive predictive value is the percentage of true positive results out of the total positives (both true and false positives) obtained by the test under evaluation. Likewise, a negative predictive value is the percentage of true negative results out of the total negatives (both true and false negatives) obtained by the new test. The following statistical formulas were used<sup>[4]</sup>

$$\text{Sensitivity} = \text{TP}/(\text{TP} + \text{FN})$$

$$\text{Specificity} = \text{TN}/(\text{FP} + \text{TN})$$

$$\text{Positive predictive value} = \text{TP}/(\text{TP} + \text{FP})$$

$$\text{Negative predictive value} = \text{TN}/(\text{TN} + \text{FN})$$

$$\text{Accuracy} = (\text{TP} + \text{TN})/(\text{TP} + \text{TN} + \text{FP} + \text{FN}).$$

## RESULTS

One hundred and two non-duplicate oxacillin-resistant *S. aureus* isolates from various clinical samples identified by oxacillin disk diffusion were included in this study. The comparison of various phenotypic methods with PCR for the *mecA* gene is represented in Table 1.

All 102 isolates were resistant in oxacillin and cefoxitin DDT. Among these isolates, 89 (87.2%), 97 (95.1%), and

92 (90.1%) isolates showed growth in the mannitol salt agar with cefoxitin, MeReSA agar, and OSA, respectively. The PCR assay for the *mecA* gene detected 92 (90.1%) *mecA* positive and 10 (9.8%) *mecA* negative isolates. Out of the 92 *mecA* PCR-positive isolates, 82, 90, and 91 strains were correctly detected as MRSA using the mannitol salt agar, MeReSA agar, and OSA, respectively. The sensitivity of MSA, MeReSA, and OSA selective media was 89.13, 97.82, and 98.91%, respectively. Among the 10 *mecA* negative isolates, seven were incorrectly identified as oxacillin-resistant by MSA and MeReSA. The low specificity of MSA and MeReSA may be attributed to these high false positive results. However, MeReSA was superior with lower false negative results ( $n = 2$ ) in comparison to MSA ( $n = 10$ ). OSA displayed only one false positive and one false negative result. Consequently, OSA had 98.91% sensitivity and 90% specificity. With respect to PCR as the gold standard test, the accuracy of MSA, MeReSA, and OSA was 83.33, 91.11, and 98.04%, respectively.

## DISCUSSION

Rapid and accurate detection of MRSA has a decisive role in hospital-infection control. MRSA can be detected either by demonstration of the *mecA* gene and its product PBP2a or by demonstration of phenotypic characters conferred by the *mecA* gene, namely, resistance to beta-lactam antibiotics in terms of increased minimum inhibitory concentration (MIC) or decreased zone of inhibition for oxacillin and cefoxitin.<sup>[1]</sup> Unlike the phenotypic tests, assays like PCR for the *mecA* gene, are rapid, accurate, commercially available, and thus, considered the gold standard.<sup>[1]</sup> However, cost constraints and greater technical and infrastructural requirements curtail their widespread use, especially in resource-poor settings. On the other hand, results of the phenotypic tests are dependent on the standardization of culture conditions like temperature, incubation period, salt concentration, inoculum size, and pH of the medium.<sup>[1-3]</sup> The antibiotic susceptibility testing for MRSA is further complicated by the presence of heteroresistance and induc-

**Table 1:** Comparison of mannitol salt agar, MeReSA agar, and oxacillin screen agar for MRSA

	MSA*	MeReSA†	OSA‡
True positive	82	90	91
False positive	7	7	1
True negative	3	3	9
False negative	10	2	1
Sensitivity (%)	89.13	97.82	98.91
Specificity (%)	30	30	90
Positive predictive value (%)	92.13	92.78	98.91
Negative predictive value (%)	23.07	60	90

Abbreviations: \*MSA: Mannitol salt agar with cefoxitin; †MeReSA: Commercial chromogenic selective media for MRSA; ‡OSA: Oxacillin screen agar

ible resistance seen among the different isolates.<sup>[1,5]</sup> These isolates are frequently misdiagnosed as methicillin-sensitive *S. aureus* (MSSA). Methicillin resistance is principally mediated by the *mecA* gene, which codes for an altered penicillin binding protein (PBP2a). However, isolates with alterations to the existing PBPs, known as moderately resistant *S. aureus* (MODSA) and penicillinase hyper-producer strains, referred to as 'borderline oxacillin-resistant *S. aureus*' (BORSA), were also described.<sup>[1,2]</sup> MRSA with the *mecC* gene, a novel homolog of *mecA*, has been reported in the human and bovine population in UK and Denmark.<sup>[6]</sup> These isolates are *mecA* negative in PCR, but resistant in oxacillin DDT.<sup>[7]</sup> Hence, there have been substantial changes in the CLSI disk diffusion interpretation criteria for MRSA in recent years. Previously cefoxitin DDT or MIC was recommended for isolates with oxacillin-intermediate zones.<sup>[3]</sup> However, the CLSI 2013 guideline no longer recommends the use of oxacillin DDT, as it may not effectively reflect the *mecA*-mediated resistance mechanism.<sup>[3]</sup>

In this present study, we compared three selective media for MRSA, namely, MSA, MeReSA, and OSA, with a genotypic method. PCR for *mecA* is the universally accepted gold standard detection method for MRSA. However, its sensitivity, specificity, and accuracy depend on the selection of primers, quality of reagents, and the amplification protocol used. In our study, we used a primer set that yielded 533 base pair amplified products, as described by Murakami *et al.*<sup>[8]</sup> Different primer sets and amplification protocols have been used in various studies.<sup>[2,9]</sup> We detected that 92 isolates were *mecA* positive and the remaining 10 were *mecA* negative. Compared to the previous studies, a higher proportion of oxacillin-resistant (oxacillin disk diffusion test) strains were found to be *mecA* PCR negative.<sup>[7,9]</sup> However, our results are in accordance with a similar study from South India where out of 75 oxacillin-resistant strains only 62 were *mecA* positive and 13 were *mecA* negative.<sup>[2]</sup> This inconsistency in results of various studies may be attributed to the difference in primers and amplification protocols as well as regional variation of the MRSA strains.

We found OSA to be the most suitable alternative for molecular methods, as it had 98.91% sensitivity. Among all three selective media used, mannitol salt agar showed the lowest sensitivity (89.13%). Several strains of MRSA might fail to grow on MSA due to its high salt concentration.<sup>[1]</sup> Out of 13 strains that failed to grow on MSA after 24 hours, 10 strains showed scanty growth after prolonged incubation for 48-72 hours. Other authors also reported similar findings.<sup>[2,10]</sup> Cefoxitin-supplemented MSA were reported to have better sensitivity and specificity than MSA and MSA with oxacillin.<sup>[10]</sup> Yamazumi *et al.*, reported 96.9, 98, 98, and 99% sensitivities and 100, 98, 100, and 99% specificities for MRSA-Screen agar, OSA, Vitek, and the microdilution test, respectively.<sup>[11]</sup> Cefoxitin has been reported to be superior to

oxacillin by authors on several occasions.<sup>[7,9,12,13]</sup> Unlike the oxacillin disk, the zones of inhibition of the cefoxitin disk are distinct, making it easier to interpret.<sup>[7]</sup> Broekema *et al.*, found that the sensitivity and specificity of cefoxitin DDT was 97.3 and 100% compared to the oxacillin disk.<sup>[7]</sup> In another study, cefoxitin DDT had 100% sensitivity in contrast to the 87.5 and 96.8% sensitivities of oxacillin DDT and OSA, respectively.<sup>[9]</sup> As per the current CLSI guideline cefoxitin DDT and agar dilution specifically identified *mecA*-mediated resistance. However, we found no difference in the results of cefoxitin and oxacillin DDT. All 102 isolates were resistant to both cefoxitin and oxacillin, and in both tests, 10 *mecA* negative isolates were falsely detected as resistant. These 10 false positive results could be attributed to the presence of alternate resistance mechanisms (MODSA and BORSA) in these isolates. However, there were no fundamental differences in the clinical course and treatment outcome of these cases. In this study, a substantial proportion of the isolates were *mecA* negative, which could express alternate resistance mechanisms. Although non-*mecA*-mediated methicillin resistance in *S. aureus* has been reported to be 1.3%,<sup>[7]</sup> there are infrequent reports from the Indian setup. However, further large-scale studies are essential to confirm it.

The limitation of the study was the small sample size. As we had selectively included MRSA strains by choosing only oxacillin-resistant isolates, the number of *mecA* negative isolates was very small. Hence, the specificity might not have an implication.

In conclusion, we found that a significant proportion of MRSA isolates detected by oxacillin and cefoxitin DDT was negative for the *mecA* gene. Although the clinical significance was unknown, these isolates might possess alternate resistance mechanisms like hyperproduction of penicillinase or alteration of the existing PBP.

We report the oxacillin screen agar as the best alternative for PCR and recommend using the oxacillin screen agar for confirming methicillin resistance in resource-limited settings.

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