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Original Research Article

Comparative evaluation of phenotypic methods to detect methicillin resistance with *mecA* gene detection by PCR in *Staphylococcus aureus*

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ABSTRACT

Background: It is crucial to employ precise, reliable, and rapid *methicillin resistant Staphylococcus aureus* detection methods to avoid the indiscriminate use of antimicrobial drugs and make informed decisions regarding appropriate treatment and the execution of efficient measures to prevent infections.
Materials and Methods: A group of 112 *Staphylococcus aureus* isolates from different clinical specimens, initially identified as *MRSA* using the cefoxitin disc diffusion test, underwent additional phenotypic tests such as the Oxacillin E strip and MRSA CHROM agar. These methods were then evaluated and compared with *mecA* gene detection via PCR, which is regarded as the gold standard.
Results: Of the 112 *MRSA* isolates identified by the cefoxitin disc diffusion test, 101 (90.2%) tested positive for the *mecA* gene. The Oxacillin E strip had a sensitivity of 98% and a specificity of 91%, while MRSA CHROM agar showed a sensitivity of 96.03% and a specificity of 82%. Among the 101 mec A-positive *MRSA* isolates, 44.5% met the CDC definition criteria for *HA MRSA*.
Conclusion: Our study concludes that phenotypic methods, including the cefoxitin disc diffusion test, are not completely reliable in detecting methicillin resistance in *S. aureus.* According to our results, combining the cefoxitin disc diffusion test with the oxacillin E strip is an effective approach for detecting *MRSA* in resource-constrained settings. Given the advantages of PCR, it is recommended to perform PCR for *mecA* gene detection on a regular basis to identify *MRSA* strains in important clinical specimens.

1. Introduction

Staphylococcus aureus is a frequently encountered bacterial agent in clinical settings, causing a variety of suppurative infections. It can thrive in diverse environments and, as a component of the normal human microbiota, establishes itself in regions such as the nostrils, perineum, underarms, and groin. With the potential to spread, particularly in hospitals, it has developed resistance to antimicrobials such as methicillin.[1]

Methicillin-resistant Staphylococcus aureus (MRSA) remains a substantial infection risk in both hospital and community settings. Over time, it has grown into a global problem, with prevalence ranging between 13% and 74%.[2] In India, the overall prevalence of MRSA stood at 37% with a combined prevalence of 49% in hospital environments and 27% in community settings from 2015 to 2019.[3] Infected patients in hospitals serve as conduits for MRSA strains to spread.

The main factor contributing to methicillin resistance is the presence of an altered penicillin-binding protein (PBP2a) that exhibits reduced affinity for most semi-synthetic penicillins Various phenotypic methods, such as the cefoxitin disc diffusion test, are used to detect MRSA in the laboratory. Cefoxitin is preferred over oxacillin as it is a potent activator of the *mecA* regulatory system. It

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is also highly effective in identifying *borderline oxacillin-resistant Staphylococcus aureus (BORSA)* strains and exhibits accuracy comparable to PCR.[4–6] This test, along with other methods such as oxacillin screen agar, oxacillin MIC, cefoxitin MIC, latex agglutination tests, CHROM agar, and automated systems like VITEK, aids in the accurate detection of *MRSA*.[7,8]

Although phenotypic methods are simpler, genotypic methods, such as the *mecA* polymerase chain reaction assay, are considered as the reference standard due to their 100% sensitivity. However, the genotypic method is costly and restricted to reference centres.[9] Errors in methicillin resistance detection can have serious clinical consequences, highlighting the importance of accurate and definitive methods.

The objective of this study was to evaluate and compare conventional methods for MRSA detection with the molecular approach of detecting *mecA* gene through PCR for accuracy, sensitivity and specificity. The main aim was to identify the best method or combination suitable for regular use in clinical laboratories. Additionally, the secondary objective was to determine the proportion of MRSA isolates originating from hospital and community sources.

2. Materials and Methods

A cross-sectional study was carried out in the Department of Microbiology at a tertiary care hospital in Thiruvananthapuram, spanning a duration of a year and a half, commencing in May 2021, after receiving ethics committee approval.

2.1. Inclusion criteria

MRSA isolates collected from diverse clinical specimens processed in the microbiology laboratory for routine culture and sensitivity throughout the study duration.

Sample size obtained using the equation:

$n = (z\alpha^2 \times p \times 1-p)/d^2$

zα= 1.96 for α at 0.05

P = sensitivity of cefoxitin disc diffusion method = 96.7%,

Based on the reference study[10]

d = absolute precision = 3.3%, Thus n = 112

Sample size is estimated to be 112 using nmaster sample size software developed by Christian Medical College, Vellore. All samples meeting the inclusion criteria were collected consecutively until the required sample size is obtained

2.2. Study procedure and data collection

Consecutive and non-duplicate *Staphylococcus aureus* isolates acquired from various clinical specimens underwent screening for *MRSA*. Organism identification involved the application of standard microbiological techniques like Gram staining, characteristic appearance on culture media, catalase test, coagulase tests and routine biochemical tests, following the established laboratory procedures.Antibiotic susceptibility testing was carried out on Mueller-Hinton agar through the Kirby Bauer standard disc-diffusion method, adhering to the current CLSI guidelines. The initial identification of methicillin resistance involved the cefoxitin disc diffusion test, followed by the subsequent application of other phenotypic methods and genotypic method.

2.3. Study variables

A semi-structured questionnaire was used to collect the patient's name, age, and gender, date of admission, specimen type, time and date of specimen collection, provisional diagnosis, significant medical and surgical history, duration of hospital stay, history of repeated hospitalisation and inter-hospital transfer, past medical history, history of any indwelling medical devices, and antibiotic use.

2.4. Phenotypic methods for MRSA detection

2.4.1. Cefoxitin disc diffusion test[11]

The cefoxitin disc diffusion test was performed on Muller-Hinton agar using a 30 μg cefoxitin disc. Each specimen was tested twice to confirm its susceptibility to cefoxitin. Following CLSI guidelines, a zone diameter of $\leq$ 21 mm was indicative of an *MRSA* isolate, whereas a diameter of $\geq$ 22 mm suggested methicillin sensitivity.

2.4.2. Oxacillin MIC by E test[11]

Oxacillin MIC testing was done with E-Strip (Hi-Media, Oxacillin EZY MIC strip 0.016-256mcg/ml) with a 0.5 McFarland inoculum, as per the directions provided by the manufacturer. Mueller-Hinton agar (MHA) plates with 2% NaCl were used. A lawn culture of a standardized bacterial suspension was applied to the MHA plate with a sterile cotton swab. The E-strip with preformed antibiotic gradient, was immediately placed on the agar surface. The plate was kept in incubation at 35°C for 24 hours.

The CLSI criteria categorise *Staphylococcus aureus* isolates as *methicillin-susceptible (MSSA)* if the oxacillin MIC is $\leq$ 2μg/ml and *methicillin-resistant (MRSA)* if the MIC is $\geq$4μg/ml.

2.4.3. MRSA CHROM agar[12]

CHROM agar (HiCrome Rapid MRSA agar plate, Hi - Media) is a chromogenic medium designed for the detection of *MRSA*. The chromogenic mixture incorporated in this medium is specifically cleaved by *MRSA* to give greenish yellow-coloured colonies.

2.5. MecA gene detection by polymerase chain reaction[6,10,13–15]

The PCR assay to detect the *mecA* gene was conducted at the Division of Pathogen Biology, Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala.

The positive control was *Staphylococcus aureus* ATCC 43300 *(MRSA*) while the negative control was ATCC 25923 *(MSSA).* The chromosomal DNA of the isolates was extracted by a simple lysis method. An isolated single colony was inoculated in 1 ml of Luria Bertani Broth (LB Broth Difco) in small Eppendorf tubes and incubated overnight at 37°C. The tubes with turbid broth are centrifuged at 6000 rpm for 5 minutes. The supernatant is discarded, and the cell pellet is obtained. The pellet is then resuspended in 400 μl of sterile nuclease-free water. This is heated at 85 for 15 minutes in a water bath and immediately transferred to -20, which causes cell lysis.

Master mix of a single reaction: A 20μL PCR reaction consisted of PCR buffer (2μL), $MgCl_2$ (2μL), dNTPs (1.5μL), *MecA* F primer (0.5μL), *MecA* R primer (0.5μL), Taq polymerase (0.3μL), Template DNA (1μL), and MilliQ H_2O (12.2μL).

The mecA F primer (5'-AAAATCGATGGTAAAGGTTGGC-3'), which corresponds to nucleotides 1282 to 1303, and the *mecA* R primer (5'-AGTTCTGCAGTACCGGATTTGC-3'), which is complementary to nucleotides 1581 to 1598 within the coding frames, were used for the amplification of the 533-base pair (bp) fragment of the *mecA* gene.(Figure 1) These primers were taken from a published sequence by Nam et al.[15] The PCR technique included a 5-minute denaturation stage at 95°C, followed by 30 amplification cycles. Each cycle consisted of a 60-second denaturation at 94°C, a 60-second annealing at 55°C, and a 90-second extension at 72 °C. The programme ended with a 10-minute extension at 72 degrees Celsius. The PCR products are observed on a 1% agarose gel containing ethidium bromide dye under a UV transilluminator (Gel Doc, Bio-Rad US). A 100-base-pair DNA ladder serves as the molecular-weight size marker.

3. Results

The study included 112 *MRSA* isolates detected by cefoxitin disc diffusion (CDD) in a variety of clinical specimens. These isolates were mostly from male patients (64.3%), with the majority falling into the 41–50 age range (25.9%). Of the total 112 isolates detected by CDD, 48.7% were obtained from the department of surgery, 23.9% from the orthopaedics department, and 10.6% from ENT. Thus, these three surgical specialties contributed to 83.1% of the total number of isolates included in the study. 61 (54.5%) isolates were from patients admitted to the hospital and 51 (45.5%) from patients consulted in the outpatient department. Notably, pus samples accounted for 79.49% of all *MRSA* isolates, followed by tissue samples (10.7%). (Table 1)

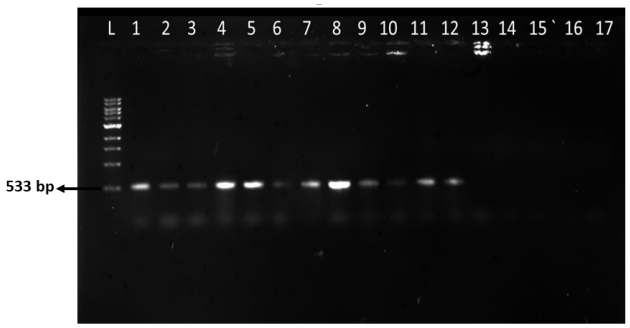

Figure 1: Result of PCR amplification of *mecA* gene
L – 100bp Ladder
Lane 1 – *S. aureus* ATCC 43300 (*mecA* positive)
Lane 2 to 12 – MRSA strains (533bp product)
Lane 13 to 16 – MSSA strains (*mecA* negative)
Lane 17 – *S. aureus* ATCC 25923 (*mecA* negative)

Out of 112 cefoxitin-resistant *S. aureus* isolates, 100 isolates had a MIC of ≥4 μg/ml with the Oxacillin E test, and 99 showed greenish-yellow colonies on MRSA CHROM agar. PCR assay for *mecA* gene identified 101 (90.2%) *mecA* positive and 11 (9.8%) *mecA* negative isolates. Out of 101 *mecA*-positive isolates, the Oxacillin E strip and MRSA CHROM agar accurately identified 99 and 97 isolates as *MRSA*, respectively. Each test had a sensitivity of 98% and 96.03%. The positive predictive value, negative predictive value and accuracy of Oxacillin E strip were found to be 99%,83.3% and 97.3% respectively and that of MRSA CHROM agar were found to be 98%, 69.23% and 95% respectively.(Tables 2 and 3)

Among 11 *mecA* negative isolates, one was incorrectly identified as *MRSA* by the Oxacillin E test and two by MRSA CHROM agar. Consequently, the specificity of each of the test were 91% and 82%.

Table 1: Sample wise distribution of isolates

Sample	Frequency	Percentage%
Pus	89	79.49
Tissue	12	10.7
Knee joint aspirate	3	2.69
Blood	2	1.78
Endotracheal aspirate	2	1.78
Pleural fluid	2	1.78
Pericardial fluid	1	0.89
Ascitic fluid	1	0.89

3.1. Proportion of HA MRSA and CA MRSA

Based on the length of hospital stay and various risk factors predisposing, the proportion of *HA MRSA* was analysed

Table 2: Comparison of oxacillin E strip with PCR

		mec A gene		
		Detected	**Not detected**	
Oxacillin E strip	Detected	99	1	100
	Not detected	2	10	12
	Total	101	11	

Table 3: Comparison of MRSA CHROM agar with PCR

		mec A gene		
		Detected	**Not detected**	
MRSA CHROM agar	Detected	97	2	99
	Not detected	4	9	13
		101	11	

from the *mecA* gene detected *MRSA* cases.

Out of the 101 *MRSA* isolates, 45 were obtained from patients who had been hospitalised for over 48 hours and had one or more risk factors associated with *healthcare-associated MRSA (HA MRSA),* aligning with the criteria defined by the Centre For Disease Control and Prevention (CDC) for *MRSA* acquired in a hospital setting.[16]

41 *HA MRSA strains (*91.1%) were isolated from patients who had surgical site infection and this was the most common single risk factor for *MRSA* infection.

3.2. Susceptibility to other class of antibiotics

Maximum resistance was seen to Erythromycin (83.9%), followed by Ciprofloxacin (72.3%). All 112 isolates (100%) demonstrated sensitivity to vancomycin and linezolid. Vancomycin sensitivity was demonstrated using vancomycin screen agar.[11] Additionally, 97.3% of isolates were sensitive to rifampicin, 95.5% to tetracycline, 93% to clindamycin, and 88.4% to cotrimoxazole.11.6% of isolates were D test positive (inducible MLS_B (iMLS_B) phenotype).

4. Discussion

Over the past three decades, *MRSA* has become a prevalent nosocomial pathogen and a significant contributor to infections in both healthcare facilities and the community. *MRSA* strains commonly exhibit resistance not only to beta-lactams and cephalosporins but also to a broad spectrum of antibiotics.

Inconsistencies in the identification of *MRSA* have a substantial influence on patient care, highlighting the importance of precision in detection. Therefore, techniques employed for detecting *MRSA* in clinical specimens should demonstrate elevated sensitivity and specificity while ensuring a quick turnaround of results. Presently, *mecA* amplification using PCR is accepted as the bench mark for detecting methicillin resistance in *S. aureus*.[9,17] Even

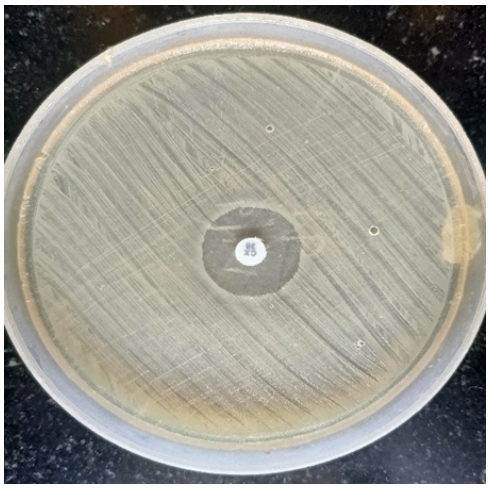

Figure 2: MRSA detection by Cefoxitin disc diffusion method

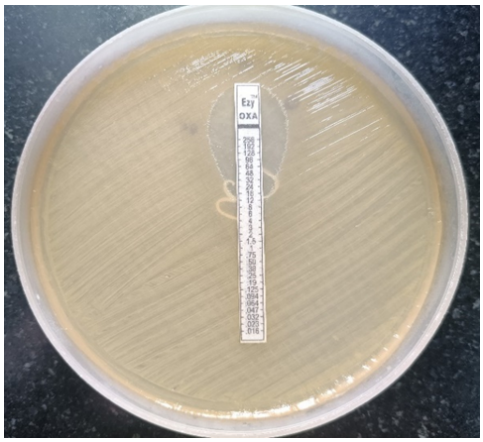

Figure 3: Oxacillin E strip: MIC 12 μg/ml

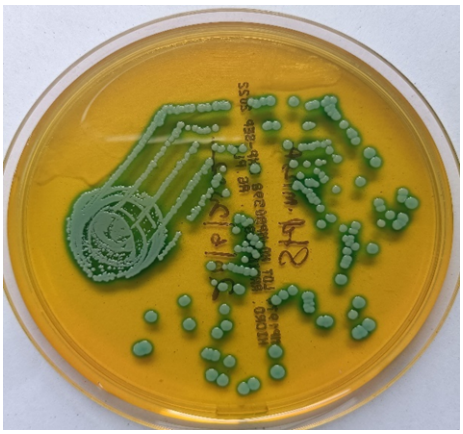

Figure 4: MRSA CHROM agar

Table 4: Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of phenotypic methods

Test	Sensitivity%	Specificity%	PPV%	NPV%	Accuracy%
Oxacillin E strip	98	91	99	83.3	97.3
MRSA CHROM agar	96.03	82	98	69.23	95

Table 5: Distribution of *HA- MRSA* cases based on predisposing factors

Predisposing factors	Frequency (Out of 45 HA MRSA isolates)	Percentage%
H/O surgery recently	41	91.1
Hospital admission within one year	15	33.3
Indwelling medical devices or catheters	26	57.7
Chronic renal/liver disease	3	6.6
Haemodialysis	1	2.2

though there is growing agreement in the literature about this method, not all clinical laboratories have access to it. For this reason, phenotypic approaches are still preferred in environments with limited resources. However, due to a variety of environmental factors and the presence of different strains within the *S. aureus* population, the performance of phenotypic methods in methicillin resistance detection is inconsistent, time-consuming, and faces challenges in identifying all resistant isolates.[18]

In this study, despite the resistance observed in all 112 isolates through the cefoxitin disc diffusion, only 101 isolates exhibited a positive result for *mecA*. Unlike several studies that reported 100% accuracy for cefoxitin disk diffusion,[4–6] our investigation found a significant number of false positives using this method, consistent with reports published by Bhutia et al. and Jain et al.[18,19]

Oxacillin E strip demonstrated a sensitivity of 98% and a specificity of 91% in our study. Among the 101 MRSA isolates positive for *mecA*, two exhibited inconsistent results for the oxacillin MIC and PCR, indicating *mecA* positivity but an oxacillin MIC of ≤ 2 μg/ml (1.5 μg/ml, 0.5 μg/ml). This disparity could potentially be clarified by the variation in *mecA* gene expression among different *Staphylococcus isolates*.[20]

Out of 11 *mecA*-negative isolates, 10 were found to be negative by the oxacillin E strip. One isolate showed false positivity, i.e., *mecA* negative and oxacillin MIC ≥4μg/ml. This inconsistency is attributed to the varied expression of methicillin resistance, such as modification of existing PBP (*MODSA*) or low-level resistance demonstrated by penicillinase hyperproducer isolates (*BORSA*) found in many strains.[7]

MRSA CHROM agar (Hi-Media), demonstrated a sensitivity of 96.03% and a specificity of 82% in this study. Some studies have assessed this medium, with a few reporting false-positive outcomes with the chromogenic medium. In our study, we had two isolates that produced greenish-yellow colonies on CHROM agar but were negative for the *mecA* gene, and four isolates were found to be falsely negative. A study by Datta et al.[3] found a sensitivity of 98.07% and a specificity of 99.2% for CHROM agar MRSA (Hi-Media), using *mecA* gene detection through PCR as the reference standard.

In this study, none of the tests, including cefoxitin, the oxacillin E test, or CHROM agar, achieved 100% accuracy when compared to PCR for MRSA detection. These results align with studies conducted by Bhutia et al.[18] As a result, a combination of tests is recommended, with the PCR method preferred for confirming resistance due to its ability to detect *mecA*-mediated resistance quickly and simultaneously.

Of the 45 HA MRSA isolates in our analysis, 33.3% were from patients who had been hospitalized at least once in the previous year. 91.1% of HA MRSA isolates in our study had a history of recent surgery, consistent with reports published by Chatterji et al. and Dhanalakshmi et al.[21,22] Studies indicate that there is variation in the HA-MRSA epidemiology across various regions of India. The biological features of the staphylococcal isolates, variations in patient populations, and infection control techniques can all be attributed to these disparate rates.[23]

MRSA, once confined to the hospitals, is now a rising community infection. Moreover, recent change in the epidemiological scenario, resulting in the presence of community-acquired MRSA within hospital environments. On occasions, it displaces the conventional MRSA strains isolated from hospitals. This transformation increases the risk of antibiotic resistance and complicates infection management.[24]

In the present study, 83.9% of all MRSA strains were resistant to erythromycin. This is comparable to the studies of Lohan et al.,[25] Mallick et al.,[26] and Joshi et al.,[27] which showed 76.5%, 74.5%, and 70.8% resistance to erythromycin, respectively. Additionally, higher resistance levels were observed for ciprofloxacin at 72.3% and doxycycline at 58%. Vancomycin and linezolid resistance were not detected.

5. Conclusion

Cefoxitin serves as a good surrogate marker for methicillin resistance. However, constant surveillance and breakpoint assessments are necessary. Our analysis shows that none of the phenotypic methods were as accurate as PCR for *MRSA* detection. Despite this, the oxacillin MIC by E test performed better and provided additional information for isolates with discrepant disk diffusion results. According to our results, Cefoxitin disk diffusion method and Oxacillin E strip are an effective combination for detecting *MRSA* in a resource constraint setting. Efforts should be made to implement regular PCR testing for the *mecA* gene, which detects *MRSA* strains from significant clinical specimens or special units like ICUs, given the benefits of timely detection and accuracy.

6. Ethics Approval

Approval was obtained from Human Ethics Committee, Medical college, Trivandrum, referenced as HEC.No.04/33/2021/MCT. dated 15^{th} march 2021.

7. Source of Funding

This study received no external funding.

8. Conflict of Interest

The authors declare that they have no competing interests.

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