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Original Research Article

Evaluation of Phenotypic and Genotypic Test Methods to Detect Methicillin Resistant *Staphylococcus Aureus*

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a prevalent cause of nosocomial and community-acquired infections. Because of this, accurate MRSA detection is essential for both implementing control measures and lowering MRSA endemicity. The current study set out to evaluate the performance of various phenotypic methods with mecA-based PCR for MRSA detection.

Methods: This investigation includes a total of 2000 *S. aureus* isolates. Oxacillin disc diffusion, oxacillin MIC, cefoxitin disc diffusion, and the oxacillin screen agar test were used to assess methicillin resistance, and they were compared to mecA-based PCR.

Results: According to Cefoxitin and Oxacillin susceptibility tests, 530 (26.50%) and 302 (30.1%) of the 2000 isolates from our hospital were determined to be MRSA. The Cefoxitin disc diffusion test for MRSA identification has a better correlation with the gold standard PCR approach than any other phenotypic method.

Conclusion: Our study demonstrated that the cefoxitin disc diffusion method has a high sensitivity and specificity when compared to other phenotypic methodologies for MRSA detection. As an alternative to PCR for the detection of MRSA, cefoxitin disc diffusion test results for MRSA prevalence are compatible with PCR results for the mecA gene.

Keywords: MRSA, mecA, Cefoxitin, Oxacillin MIC, PCR.

Introduction

Staphylococcus aureus, a common human pathogen, can cause a variety of diseases, from minor skin infections to catastrophic ones including endocarditis, pneumonia, and sepsis. It is more affected by the development of antibiotic resistance, most notably methicillin-resistant *S. aureus.* (MRSA). Penicillin and cephalosporins are examples of the large class of drugs known as -lactams, which also contains the *S. aureus* strain known as MRSA.[1]

Meticillin resistance in *S. aureus* is associated with the production of the altered penicillin-binding protein PBP2a, a 78 kDa protein with a low affinity for β-lactam antibiotics. These strains are resistant to a

wide range of antibiotics, which limits the number of treatment options to only a handful, such teicoplanin and vancomycin. In order to ensure that infected patients receive the proper antibiotic treatment and that MRSA isolates are controlled in hospital settings to stop the spread of these pathogens, it is therefore clinically essential to ascertain whether *S. aureus* isolates are methicillin resistant or not as soon as possible.

The identification of the mecA gene or its product, PBP2a, using PCR is the most reliable and widely accepted method for detecting MRSA. But the cost of this test is high, and not all laboratories employ molecular biology methods in daily clinical work. (Especially in developing nations).[2]

Therefore, it is essential to assess the phenotypic techniques that can quickly and accurately detect MRSA isolates in order to ensure correct antibiotic administration and avoid the spread of MRSA isolates in the hospital environment. Many traditional phenotypic methods, including oxacillin disc diffusion, oxacillin MIC and screen agar, cefoxitin disc diffusion, and latex agglutination, have been established for the fast detection of methicillin-resistant staphylococci.

The most effective detection technique is still up for dispute. Many of the methods are unable to simultaneously identify methicillin resistance and species, and the majority need cultivating on solid media. MRSA is challenging to detect due to a variety of factors. Remembering how important correct detection is comes from the detrimental effects that disparities in detection have had on patient management. The Clinical and Laboratory Standards Institute recently recommended the cefoxitin disc diffusion method for MRSA identification. (CLSI). The most efficient inducer of the mecA gene that produces PBP2a is the antibiotic cefoxitin, which belongs to the cephamycin class. The goal of our study was to compare several phenotypic test methods to the Gold Standard test of molecular detection of the mec a gene by PCR in order to evaluate the efficacy of these methods as MRSA markers.

Material and Methods

2000 nonduplicate *S. aureus* isolates were used by the microbiology department at Darbhanga Medical College, Laheriasarai, Bihar, between November 2021 and October 2022. These clinical samples included blood, pus, surgical sites, wounds, fracture sites, sputum, tracheal aspirates, and urine. The isolates were identified using traditional methods such colony morphology, Gram staining, Catalase test, tube coagulate and slide coagulase test, mannitol fermentation, and DNase test. The CLSI or manufacturer's instructions were followed for all testing and data interpretation in the current investigation. Phenotypic methods for detecting MRSA.

All *S. aureus* isolates underwent a cefoxitin disc diffusion test utilizing a 30 μg disc of cefoxitin on a Muller Hinton agar plate. On the agar plates, lawn culture of the bacterial suspension calibrated to 0.5 Mc Farland standards was performed. Zone diameters were determined after the plates had been incubated at 37°C for 18 to 24 hours. Methicillin resistance was observed for zone diameters 19mm, and methicillin sensitivity was recorded for zone diameters ≥22mm. The zones of inhibition were retested on colonies that expanded within the zones.

On Mueller Hinton agar with 4% NaCl, a disk diffusion test using 1μg of oxacillin per disk was conducted on all isolates of *S. aureus*. cultivated at 35°C. According to the CLSI, the zone size was determined to be vulnerable at ≥13 mm and resistant at 10 mm.[3]

Oxacillin was added to Muller-Hinton agar plates containing 4% NaCl and 6 μg/ml. On the same isolates, the Oxacillin screen agar

(OSA) test was carried out in accordance with CLSI recommendations using direct colony suspension and calibrated to 0.5 McFarland turbidity standards. On the surface of the agar inoculated with Oxacillin screen agar, the suspension of the isolate was placed as a spot. (OSA). At 35°C, plates were incubated. In transmitted light, the plates were carefully examined for any signs of development. Any colony growth after 24 hours was taken to indicate oxacillin resistance. Homogenous MRSA was defined as an isolate with confluent visible growth on OSA, while heterogonous MRSA was defined as an isolate with little growth after 24 hours of incubation that had transformed into perfect visible growth.

We used the agar dilution method to determine the minimum inhibitory concentration for Oxacillin. By emulsifying parts of 4-5 distinct colonies into 4-5 ml of nutritional broth and adjusting the opacity to McFarland standard 0.5, the bacterial suspension was created. Oxacillin was diluted twice at concentrations ranging from 0.25 to 256 µg/ml to create Muller-Hinton agar (MHA) gradient plates with 4% NaCl. Using a sterile cotton swab stick, a spot of around 5-8 mm in diameter was infected on the plates, which were then incubated for 24 hours at 350C. Oxacillin's MIC of ≤2 µg/ml showed that the strain was susceptible, whereas methicillin's MIC of ≥4 µg/ml showed resistance. (NCCLS 2003).

The NCCLS has not recommended the use of Cefoxitin in agar dilution assays to identify methicillin resistance. As a consequence of several phenotypic approaches, MRSA isolates were subjected to standard protocols for the molecular detection of the mecA gene by PCR. MRSA genotyping techniques for detection. The I.C.M.R. Regional Center's molecular laboratory uses the following technique for PCR to detect the mecA gene. *S. aureus* overnight cultures were used to extract bacterial DNA using the CTAB-NaCl technique.[4]

The quantity and quality of the isolated DNA were evaluated using the Nanodrop 1000 spectrophotometer at 260/280 nm and visually by horizontal gel electrophoresis in 1% agarose. The Unal et al. technique was used in the PCR for the detection of mecA.[5] MecA (F): Primer sequences are used to detect mecA. 5'- GTA MecA R 5' CCA ATT CCA CAT TGT TTC GGT CTA A 3' and GAA ATG ACT GAA CGT CCG ATA A-3'

Briefly, 24 µl of PCR amplification mix, including 16 µl of double distilled autoclaved water, 2.5 µl of 10X Taq buffer, 1 µl of 2.5 mM dNTP mix (Merck, India), 0.5 l of 3U/l Taq polymerase (Merck, India), and 0.5 mM of each primer, was added to 1 µl of 60 ng of the extracted DNA.

The primers (Sigma, India) as described by Jonas et al., 1999 were used to amplify the mecA gene.[11] Amplifications were performed in a thermal cycler under settings that included denaturing for 30 cycles at 940C for 45s, annealing for 30 cycles at 500C for 45s, and extending for 1 minute and 2 minutes total.

Amplicons of 310 base pairs were compatible with amplification of the mecA gene. Gel red dye was used to electrophorese the PCR results on agarose gels, and the Alpha Imager gel documentation system was used to capture photos. For all phenotypic assays and genotypic tests, *S. aureus* ATCC 25923 (mecA negative) and ATCC 43300 (mecA positive) were used as controls. The findings of the PCR test were used as the gold standard test to calculate the sensitivity and specificity of each test.

To understand the overall efficacy of phenotypic techniques in the identification of MRSA isolates, sensitivity, specificity, positive and negative predictivity values

were established in accordance with the mecA gene positivity of MRSA strains.

Result

Based on the Cefoxitin disc diffusion method, 530 (26.50%) of the 2000 *S. aureus* strains recovered in our hospital were determined to be MRSA.

Oxacillin screen agar, oxacillin MIC technique, and 30.1%, 27.7%, and 27% of the strains, respectively, were identified as MRSA. The findings of the disc diffusion susceptibility tests for oxacillin and cefoxitin were inconsistent for 72 isolates (3.6%), as can be seen here. The same 72 isolates that were sensitive to cefoxitin were revealed by PCR to be mec A gene negative.

There were no isolates that were Oxacillin susceptible but Cefoxitin resistant. The outcomes of the phenotypic and PCR approaches for the mec A gene are displayed in Table 1 below.

Table 1: Results of phenotypic methods combined with genotypic techniques for MRSA detection

Phenotypic methods	MRSA	MSSA
Mec A positive	478	1522
Oxacillin disk diffusion test	562	1438
Oxacillin MIC	494	1506
Oxacillin Screen agar	482	1518
Cefoxitin disk diffusion test	478	1522
Total numbers of *S. aureus*: 2000		

638 of the 2000 *S. aureus* strains underwent PCR mecA detection. The Cefoxitin disc diffusion test for MRSA identification has a stronger correlation with the gold standard PCR method than any of the phenotypic methods mentioned above.

Table 2: Phenotypic and genotypic approaches' sensitivity and specificity for detecting MRSA

Methods	Sensitivity	Specificity	PPV	NPV
Oxacillin Disc Diffusion	100%	95.10%	88.03%	100%
Cefoxitin Disc Diffusion	100%	100%	100%	100%
Oxacillin MIC	100%	98.36%	95.66%	100%
Oxacillin Screen Agar	100%	99.31%	98.14%	100%
PCR for mecA	100%	100%	100%	100%

MIC-minimum inhibitory concentration, PPV-Positive predictive value, NPV- Negative predictive value

Discussion

As highly virulent and serious human diseases that significantly increase morbidity and death in hospitals and the general population, MRSA are becoming harder to eliminate due to their development of medication resistance. Clinical microbiology laboratories play a crucial role in the timely and accurate detection of MRSA to prevent treatment failure. Methicillin resistance can be homogenous or heterogeneous in *S. aureus* isolates containing the mecA gene. Heterogeneous strains are made up of two types of cell groups: cells that are moderately susceptible and cells that are very resistant. Methicillin sensitivity is phenotypic in these strains.[6] These isolates have methicillin MICs that are at or just below the

susceptibility breakpoint; for instance, oxacillin MICs vary from 4 to 8 μg/ml.

The strains are known as BORSA strains. Borderline resistance bacteria lack the methicillin resistance gene, and resistance is thought to result from alterations in the normal PBP genes, their overexpression, or excessive staphylococcal β-lactamase production rather than from the synthesis of the mecA or PBP2a genes. When exposed to β-lactums in an in-vivo context, oxacillin-susceptible cells that were oxacillin-sensitive in vitro may turn into oxacillin-resistant cells.

These mecA gene negative, non-PBP2a-producing BORSA infections respond very well to treatment with β–lactam antibiotics, according to in vitro susceptibility tests, experimental evidence from animal studies, and some clinical data. [7,8] As a result, detecting the presence of the mecA gene is crucial for correctly classifying the strains as MRSA, making PCR a standard procedure in the majority of clinical laboratories.

It can be challenging to appropriately categorise some MRSA strains using established methods since a strain may appear susceptible in one aspect but borderline or resistant in another. [9,10] These characteristics have sparked the creation of several genetic methods for locating the mecA gene in clinical MRSA isolates.[11,12] However, it is not possible for clinical laboratories to implement genotypic testing widely using the reference technique of mecA gene identification by PCR.

The PCR method offers a variety of extra advantages over conventional methods. MRSA diagnosis by conventional methods takes 48–72 hours, whereas PCR only requires 18–24 hours. PCR is more expensive than other phenotypic methods, nevertheless. A PCR-based test's success is influenced by a variety of critical variables, including price, dependability, speed, accuracy, and sensitivity. Although PCR equipment is expensive and not readily available in all laboratories, it is rapid and has a high level of sensitivity and specificity.

Although there are numerous phenotypic methods, it is still unclear which is the most effective for detection. In a number of recently published publications, cefoxitin has been recommended as a stand-in marker for the detection of mecA gene-mediated methicillin-resistant *Staphylococcus aureus*. The PBP2 test for the mecA gene or the protein encoded by the mecA gene is the most reliable test to detect MRSA, according to CLDSI. Isolates that extract any one of these should be reported as oxacillin resistant because oxacillin resistance is caused by a rather unusual mechanism other than MecA.[13] The CLSI recommendations at the same time recommended using cefoxitin to identify MRSA. According to CLSI standards, a zone that is less than 19 mm in diameter or equal is considered a resistant strain and should be treated with a 30 □g cefoxitin disc.[14]

Surveillance statistics for MRSA are difficult to understand because there is currently no accepted method for the detection of the bacteria, and laboratories apply different SOPs and interpret breakpoint results in different ways.[15] According to reports, the varied manifestation of resistance is the main reason why conventional assays for the detection of methicillin resistance produce inconsistent results.[16] The phenotypic expression of resistance is influenced by additional factors, such as the addition of sodium chloride or sucrose to the culture media, incubation at 300C, or passage while being treated with b-lactam antibiotics. (Hartman & Tomasz, 1986). These factors further highlight the requirement for a simple, rapid, precise, and sensitive method for MRSA identification in routine diagnostic laboratories.

The disc diffusion method is a straightforward process used in microbiology labs to test for MRSA. As indicated before, the oxacillin disc diffusion test was the approach that identified MRSA with the lowest degree of accuracy.[17] The oxacillin screen agar test for MRSA detection in our experiment exhibited 100% sensitivity and 99.31% specificity. Swenson et al. (2001) found that when strains with borderline MIC were explored, specificity decreased while sensitivity decreased when diverse resistant bacteria were evaluated.

Numerous studies, including this one, have shown that the results of the cefoxitin disc diffusion test correlate more favourably with the presence of mecA than the results of the oxacillin disc diffusion test.[18] Cefoxitin might be a better disc diffusion agent for identifying diverse MRSA populations that express mecA since oxacillin, a subpar inducer of PBP2a synthesis, ineffectively stimulates mecA expression. This is believed to be the main mechanism underlying cefoxitin's superior sensitivity to oxacillin. The 100% sensitivity and specificity of the cefoxitin disc technique reported by Anand et al. is in agreement with our results.[19]

In that investigation, it was also discovered that the oxacillin disc had 100% and 95.10%, respectively, sensitivity and specificity. The decreased specificity in the current investigation could be attributed to the manufacturer's disc differences. In Sakoulas et al.'s inquiry, the oxacillin MIC technique's sensitivity and specificity were 99 and 98.10%, respectively, and the specificity finding was compatible with the results of the current study, which found that it was 100 and 98.36%.[20] Comparing the MIC approach to PCR, Wallet et al. discovered that it was somewhat less sensitive (96% vs. 98.10%).[21] A total of 36 strains were susceptible to cefoxitin DD but resistant to oxacillin DD, with MIC values of less than 8 mcg/ml. These germs are probably BORSA (Borderline resistant strains), which hyper-produce beta lactamase and lack the conventional genetic components of such resistance, even if they appear to be oxacillin resistant. This was confirmed by the finding that all isolates that were susceptible to cefoxitin but resistant to oxacillin had mec A identified by PCR.

In comparison to disc diffusion tests using oxacillin, Anand et al. and a large number of other research on cefoxitin disc diffusion discovered that these results closely correlate with the presence of mecA.[22,23] The oxacillin disc diffusion approach was demonstrated to be less sensitive for the identification of MRSA.

Conclusion

Our study discovered that the cefoxitin disc diffusion method had a high sensitivity and specificity when compared to other frequently used methods for MRSA detection. Cefoxitin is a more potent inducer of the mecA regulatory system and an efficient substitute marker for the identification of MRSA in conventional susceptibility testing. It may be preferred in clinical microbiology laboratories since it is easy to use, requires no specialised knowledge, incubation temperature, or medium preparation, and is more cost-effective than alternative procedures.

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