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ORIGINAL ARTICLE

Comparison of PCR and phenotypic methods for the detection of methicillin resistant *Staphylococcus aureus*

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ABSTRACT

Background and Objectives: Resistance to methicillin in methicillin resistant strains of *Staphylococcus aureus* (MRSA) is due to the presence of *mec-A* gene ,which encodes a low affinity penicillin binding protein (PBP)-2a or PBP2. Accurate and rapid identification of MRSA in clinical specimens is essential for timely decision on effective treatment. The aim of the study was to compare three different methods for detection of MRSA namely cefoxitin disc diffusion, CHROM agar MRSA and VITEK-2 susceptibility with PCR which is the gold standard reference method and to find the antibiotic susceptibility pattern of these isolates by VITEK-2.

Materials and Methods: A Total of 100 non-duplicate *S. aureus* isolates were collected from different clinical samples among both outpatient and inpatients. Detection of MRSA among these isolates was done by cefoxitin disc diffusion, VITEK-2, CHROM agar MRSA and PCR.

Results: The sensitivity and specificity of cefoxitin disc diffusion and Vitek was found to be 97.2% and 100%, while that of CHROM agar was found to be 100% and 78.6%. The overall prevalence of MRSA in our study by PCR was 72%.

Conclusion: Based on the findings in our study, isolates which show cefoxitin zone diameter < 22 mm can be reported as MRSA. However, those isolates which have a zone diameter between 22-24 mm, should ideally be confirmed by PCR.

Keywords: Methicillin resistant *Staphylococcus aureus*; Cefoxitin; Polymerase chain reaction; Chrom agar; Vitek

INTRODUCTION

Staphylococcus aureus is one of the most common bacterial pathogens causing a wide variety of clinical manifestations. Initially infections were being managed with penicillin but gradually the bacteria developed resistance to penicillin (1). Methicillin, a semisynthetic beta-lactam drug was introduced in the UK in 1959 to treat patients infected with pencillin resistant staphylococci (2). The first case of methicillin resistant *Staphylococcus aureus* (MRSA) was described in 1961 by Jevons et al. (3). Till the mid 1990s, MRSA infections were limited to the hospitals. However within the last 20 years, MRSA were reported in the healthy individuals without association to health care insitutions. These were due to new strains of MRSA known as CA-MRSA (community

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acquired-methicillin resistant *S. aureus*) (4). Rate of infections caused by MRSA has been on a rapid rise and the prevalence in India varies from 25% in western India to 50% in South India (5, 6).

Methicillin resistance in MRSA is due to the presence of *mec-A* gene, which encodes a low affinity penicillin binding protein (PBP)-2a or PBP2' (7). Other genes—such as *femA, femB* can also contribute to methicillin resistance in MRSA (8). Detection of MRSA has become extremely complicated due to many reasons. Methicillin resistance in *S. aureus* is heterogenous in majority of the isolates and these strains appear phenotypically sensitive to methicillin (9). Addition of sodium chloride or sucrose to culture medium, incubation at 30°C or passage in the presence of beta-lactam antibiotics enhances the expression of resistance (10). Accurate and rapid detection of MRSA results in effective antimicrobial therapy, immediate patient isolation and appropriate disinfection measures (11, 12). Methicillin resistance implies that the organism is resistant to pencillins, 1st-4th generation cephalosporins and carbapenems. Vancomycin has become the drug of choice for treatment of MRSA. False positive results of MRSA has lead to widespread use of vancomycin which inturn resulted in the emergence of VISA (vancomycin intermediate *S. aureus*) and VRSA (vancomycin resistant *S. aureus*) (13).

Conventional MRSA detection methods included oxacillin disc diffusion, oxacillin MIC (minimum inhibitory concentration) and oxaciilin screen agar methods. Oxacillin disc diffusion method is no longer used. CLSI (Clinical and Laboratory Standards Institute) recommends the use of the cefoxitin for MRSA detection as it is a better inducer of PBP-2a encoding *mec-A* gene (14). Other methods in the detection of MRSA include latex agglutination assay, CHROM agar , susceptibility testing by VITEK (15,-17). Detection of *mec-A* gene by PCR has become the gold standard method in the detection of MRSA. However this method is expensive, time consuming and will not detect novel resistance mechanisms such as *mecC* (18).

The objectives of this study were: i) to compare three different methods namely, cefoxitin disc diffusion, CHROM agar MRSA, VITEK-2 susceptibility with PCR which is the gold standard reference method and ii) to find the antibiotic susceptibility pattern of these isolates by VITEK-2.

MATERIALS AND METHODS

Study design and bacterial isolates. This was a prospective cross-sectional study conducted in the Department of Microbiology, Government TD Medical College, Alleppey from September 2018 to February 2019. A Total of 100 non-duplicate *S. aureus* isolates were collected from different clinical samples among both outpatient and inpatient like blood, urine, tracheal aspirate, sputum, wound swab, pleural fluid, peritoneal fluid. *S. aureus* was identified by characteristic haemolytic colonies on blood agar, Gram stain showing Gram-positive cocci in clusters and positive by catalase, slide and tube coagulase methods (19).

Ethical approval. The study was approved by the Institutional Ethical committee of Government Medical College, Alleppey.

Cefoxitin disc diffusion method. All 100 isolates of *S. aureus* were subjected to testing with cefoxitin (30 µg) discs (BD BBL Sensi-Disc, Becton, Dickinson and Company, U.S.A.). A 0.5 McFarland standard suspension of the isolate was made and lawn cultures were made on Muller Hinton agar (MHA) plates (Himedia, New Delhi, India). The zone of inhibition was measured after incubation at 35°C for 16-18 hrs. Zone size was interpreted according to CLSI 2019 criteria. Isolates which showed an inhibition zone ≤ 21 were considered to be MRSA, whereas isolates which showed an inhibition zone ≥ 22 were considered to be MSSA (methicillin sensitive *S. aureus*) (20). A standard strain of MRSA (ATCC 43300) and MSSA (ATCC 29213) were used as positive and negative controls respectively.

CHROM agar. CHROMagar (Hicrome™ Rapid MRSA Agar Plate- MP1974, Himedia, New Delhi, India) is a new chromogenic medium for the identification of MRSA. It is a ready made media which contains chromogenic mix, MRSA selective supplement and cefoxitin. The chromogenic mixture incorporated in the medium is specifically cleaved by MRSA to give green coloured colonies. For each isolate, a bacterial suspension adjusted to 0.5 McFarland was made and a swab was dipped into the suspension and streaked onto a CHROMagar plate. The growth of any green colony after incubation for 48 hrs was considered to be positive, indicating MRSA. A standard

strain of MRSA (ATCC 43300) and MSSA (ATCC 29213) were used as positive and negative controls respectively.

Vitek-2 susceptibilty system. All strains were subcultured on Blood agar before testing. A bacterial suspension equivalent to a 0.5 McFarland standard was prepared after 18 to 24 h of incubation on a blood agar plate. Vitek 2-AST-P628 cards (bioMe´rieux, Marcy l'Etoile, France) were inoculated according to the manufacturer's instructions. Isolates for which cefoxitin screen was positive and oxacillin MIC was ≥ 4 µg/ml were regarded as MRSA as per CLSI 2019 (20). Isolates for which cefoxitin screen was negative and oxacillin MIC was ≤ 2 µg/ml were regarded as MSSA as per CLSI 2019 (20). Antibiotic susceptibility pattern of the isolates was also interpreted from VITEK-2 system.

DNA extraction. Bacterial DNA was extracted from all the isolates grown on blood agar plate by using the Rapid lysis procedure with Lysostaphin (21, 22) .

PCR. PCR was performed to detect the presence of *mec-A* gene. The reaction mixture consisted of 5 µl of the 10× reaction buffer; 3 µl of 25 mM $MgCl_2$; 1 µl of 2.5 mMdNTPs (Promega); 1 µl *mecA1* primer 20 pmol/µl; 1 µl *mecA2* primer 20 pmol/µl; 0.2 µl *Taq* polymerase 5 U/µl (Promega); 10 µl DNA; and 28.8 µl H_2O. The reaction mixture and the primers used for detection of the *mecA* gene were F 5'TGGCTATCGTGTCACAATCG 3' (positions 885 to 905) and R 5' CTGGAACTTGTTGAGCAGAG 3' (positions 1174 to 1194) producing a 304-bp amplicon as described by Vannuffel et al. (23).

Amplification was performed as follows, initial denaturation for 5 minutes at 94°C followed by 30 cycles at 94°C for 1 minute, at 54°C for 1 minute, then at 72°C for 1 minute. Final annealing was set at 72°C for 7 minute (23). The PCR products (5 µl) were subjected to electrophoresis in agarose 3% and the band size was assessed by direct comparison with a 100-bp DNA marker (Takara). A standard strain of MRSA (ATCC 43300) and MSSA (ATCC 29213) were used as positive and negative controls respectively.

Statistical analysis. Statistical analysis was done using IBM SPSS 20. (SPSS Inc, Chicago, USA). Diagnostic measures such as sensitivity, specificity & positive and negative predictive values of each test and accuracy was computed. K2 test and kappa concordance measures were used for evaluating association & levels of concordance of the data respectively.

RESULTS

Among the 100 isolates of *S. aureus*, 72 were positive for *mec-A* gene by PCR. The overall prevalence of MRSA in our study was 72% by PCR, which is regarded as the gold standard method. Remaining 28 isolates were negative for *mec-A* gene and were regarded as MSSA. Prevalence of MRSA by cefoxitin disc diffusion, CHROM agar MRSA, Vitek-2 were 70%, 78%, 70% respectively. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the different methods is shown in Table 1. Antimicrobial susceptibility pattern of the *S. aureus* isolates (MRSA and MSSA) is shown in Table 2. PCR showing the presence of *mec-A* gene is shown in Fig. 1. Chrom agar showing green coloured colonies of MRSA are shown in Fig. 2.

DISCUSSION

Accurate and early detection of methicillin resistance is of immense importance in the prognosis of infections caused by *S. aureus.* Correct identification

Table 1. Comparison of Phenotypic methods for detection of MRSA

Method	No of False Positives	No of False Negatives	Sensitivity (%)	Specificity (%)	PPV	NPV	Concordance with PCR (%)
Cefoxitin disc diffusion	0	2	97.2	100	100	93.3	98
Vitek	0	2	97.2	100	100	93.3	95
Chrom Agar	6	0	100	78.6	92.3	100	94

PPV- Positive Predictive Value, NPV- Negative Predictive Value

Table 2. Antibiotic susceptibility pattern of *S. aureus* strains (n=100) by Vitek-2

Antibiotics tested	Susceptibility of MSSA (%) (n=28)	Susceptibility of MRSA (%) (n=72)
Ampicillin	21.4	NR
Cefazolin	100	NR
Cefotaxime	100	NR
Cefepime	100	NR
Gentamicin	100	78.6
Ciprofloxacin	28.6	38.6
Erythromycin	46.4	21.4
Clindamycin	85.7	40
Linezolid	100	100
Vancomycin	100	100
Daptomycin	100	100
Teicoplanin	100	100
Tigecycline	100	100
Rifampicin	100	100
Cotrimoxazole	89.3	65.7

NR-Not Required.

Fig. 1. Image of electrophoresis gel of *S. aureus*. Lane 1. Positive control strain ATCC 43300
Lane 2. PCR Ladder (100-1500 bp)
Lanes: 3, 4, 5 - Negative results
Lanes: 6-16 - Positive results (MRSA)

of MRSA by conventional methods is quite difficult, as isolates which appear sensitive to methicillin by one method may appear resistant by another method (24). Methicillin resistance is difficult to detect because *mec-A* positive strains differ in their level of expression of resistance. The resistance is usually heterogenous, with only a few cells (one in 10^4 or 10^6) expressing the phenotype. Accurate identification is needed not only for choosing the appropriate antibiotic but also to control the endemicity of MRSA (25). Eventhough detection of *mecA* gene by PCR is the gold standard method for identification of MRSA,

Fig. 2. CHROM agar showing green coloured colonies of MRSA

use of molecular methods for identification may not be feasible in a resource poor setting. Therefore it is necessary to implement an accurate, rapid and cost effective phenotypic method for detection of MRSA (26).

In our study among the 100 isolates, 72 were identified as MRSA by PCR. Disc diffusion by cefoxitin and oxacillin are the most commonly used phenotypic methods in laboratory for the detection of MRSA. Oxacillin disc diffusion is no longer recommended by CLSI for MRSA. Disc diffusion by cefoxitin was used in our study, which showed a sensitivity of 97.2% and specificity of 100%. High sensitivity of this method is attributed to the increased expression of *mecA* encoded protein PBP2a, cefoxitin being a potent inducer of *mecA* gene (27). Our finding is concordant with other studies around the world, which have also reported that disc diffusion by cefoxitin has high sensitivity and specificity (28-30). In our study prevalence of MRSA by cefoxitin disc diffusion was 70% and there were 2 isolates which showed false negative results. The two isolates which showed false negative results had a zone diameter of 23 and 24 mm respectively which is just above the cut off zone. However, both the isolates which gave false negative results by cefoxitin disc diffusion where found to be MRSA by Vitek and CHROM agar. CHROM agar was used in our study for the identification of MRSA and the sensitivity, specificity was found to be 100% and 78.6% respectively. Eventhough this method was highly sensitive, it gave six false posi-

tive results, resulting in a low specificity. These six isolates were false positive after 24 hrs of incubation and the sensitivity, specificity did not increase after 48 hrs of incubation. High sensitivity of this medium has also been reported by Diederen et al. and Datta et al. (16, 31). False positive results while using chromogenic medium for the detection of MRSA was also described by Stoakes et al. who reported three false positive results (32). The six false positive isolates by CHROM agar were found to be MSSA by Vitek and cefoxitin disc diffusion. Prevalence of MRSA by CHROM agar was 78%.

In our study Vitek-2 was used to detect methicillin resistance. All the isolates which were correctly identified as MRSA had oxacillin MIC ≥ 4 µg/ml, whereas isolates which were correctly identified as MSSA had oxacillin MIC ranging from 0.25-1 µg/ml. This method had a sensitivity of 97.2% and a specificity of 100%. By this method two isolates were falsely identified as MSSA and these isolates had oxacillin MIC of 0.5 µg/ml. Both isolates had zone of inhibition of 20 mm by cefoxitin disc diffusion and produced green colonies on CHROM agar. Overall prevalence of MRSA by Vitek-2 was 70%. Roisin et al. in her study also reported high sensitivity and specificity of 97.5% and 100% respectively for Vitek-2 in the detection of MRSA (33). One advantage of Vitek over cefoxitin disc diffusion is that while disc diffusion requires 16-18 hrs incubation, Vitek can classify the isolate as MRSA or MSSA within 8 hrs of growth in culture. Antimicrobial susceptibility pattern of the isolates by Vitek-2 showed that all the isolates were sensitive to vancomycin, teicoplanin, linezolid and rifampicin. Inducible clindamycin resistance was seen in 57.1% of MRSA isolates and in 10.7% of MSSA isolates.

CONCLUSION

This study showed an overall MRSA prevalence of 70% by PCR. Although cefoxitin disc diffusion and Vitek-2 are excellent methods to detect methicillin resistance in *S. aureus*, it still produced two false negative results. Our study showed that, while CHROM agar had a high sensitivity for MRSA detection, it showed poor specificity. Based on the findings in our study, isolates which show cefoxitin zone diameter < 22 mm can be reported as MRSA. However, those isolates which have a zone diameter between 22-24 mm, should ideally be confirmed by PCR.

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