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COMPARISON OF CEFOXITIN DISC DIFFUSION TEST, OXACILLIN DISC DIFFUSION TEST, OXACILLIN SCREEN AGAR AND PCR FOR *MEC*A GENE FOR DETECTION OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA)

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ABSTRACT

Cefoxitin is an efficient inducer of *mec*A-mediated methicillin resistance (MR) in methicillin-resistant *S. aureus* (MRSA). It is recommended by Clinical and Laboratory Standards Institute (CLSI) guideline (2010)/ different studies to use cefoxitin in disc diffusion method for the detection of MRSA. Therefore, the aim of this study to evaluate the efficiency of cefoxitin disc diffusion in comparison to oxacillin disc diffusion and oxacillin screening agarin the detection of mecA-mediated MR in *S. aureus* where PCR for *mec*A gene detection was 'gold standard'. This cross-sectional study was carried out in the Department of Microbiology and Immunology at Bangabandhu Sheikh Mujib Medical University, Dhaka from January, 2010 to December, 2010 for a period of one (01) year. *S. aureus* isolates were collected from different clinical samples including wound swab, pus, blood, urine, tracheal aspirate, throat swab, aural swab etc. *Staphylococcus aureus (S.aureus)* were isolated and confirmed by staing, biochemical tests. Routine antimicrobial susceptibility testing was performed cefoxitin discs diffusion test. PCR was performed for detection of the *mecA* gene for MRSA. In this study 120 isolated strains of *S. aureus* was included from different clinical specimens and antimicrobial susceptibility was performed according to CLSI guidelines. The sensitivity and specificity of oxacillin disc diffusion was 84.2% & 66.2%, oxacillin screen agar was 100.0% & 33.3% respectively where in cefoxitin disc diffusion both was 100.0% and matches with the result of PCR. So, the cefoxitin disc diffusion can be the alternative to technically demanding PCR in the detection of MRSA.

KEYWORDS: methicillin-resistant *S. aureu; mec*A gene; cefoxitin disc diffusion test; oxacillin disc diffusion test; oxacillin screen agar; PCR.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) constitute a major health care problem with a strong potential for dissemination and high rate of mortality and morbidity. Methicillin resistant *S. aureus* (MRSA) strains emerged soon after the introduction of methicillin into clinical practice.[1] Quick and reliable detection methods are required to obtain information in relation to MRSA isolates and to allow faster implementation of appropriate control measures. Therefore, the availability of sensitive and specific methods for the accurate detection of antibiotic resistance in these bacteria has become an important tool in clinical diagnosis.

Methicillin/oxacillin-resistant *Staphylococci* are heterogeneous in their expression of resistance to β-lactam agents and the test conditions have a major effect on the expression and therefore the detection of resistance. Conflicting recommendations regarding the most reliable method for routine use are partly related to differences between strains and there may be a variable interaction between the factors affecting the expression of resistance, including the agent tested, the medium, the NaCl concentration, the inoculum, temperature and period of incubation and the reading of endpoints. 'Borderline' resistant strains may have altered PBPs or be penicillinase hyperproducers, and these can be difficult to distinguish from resistant strains that carry

the *mec*A gene. Recommended methods for MIC and disc diffusion testing are described, although it is unlikely that any single method will detect all resistant strains. Some rapid or automated methods are also available, including latex agglutination techniques for the detection of PBP2a. The gold standard method for the detection of resistance mediated by *mec*A is PCR, which is most commonly used as a reference method at present.

Therefore, the aim of this study to evaluate the efficiency of cefoxitin disc diffusion in comparison to oxacillin disc diffusion and oxacillin screening agar in the detection of *mec*A-mediated MR in *S. aureus* where PCR for *mec*A gene detection was 'gold standard'.

MATERIALS AND METHODS

This cross-sectional study was carried out in the Department of Microbiology and Immunology at Bangabandhu Sheikh Mujib Medical University, Dhaka from January, 2010 to December, 2010 for a period of one (01) year. *S. aureus* isolates were collected from different clinical samples including wound swab, pus, blood, urine, tracheal aspirate, throat swab, aural swab etc. *Staphylococcus aureus (S.aureus)* were isolated and confirmed by staining, biochemical tests. Routine antimicrobial susceptibility testing was performed cefoxitin discs diffusion test. PCR was performed for detection of the *mecA* gene for MRSA. A total of 120 *Staphylococcus aureus (S.aureus)* were isolated and confirmed from three hospitals, namely Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka Medical College Hospital (DMCH) 10 and rest 30 collected from Popular Diagnostic Center (PDC), Dhaka. The *S. aureus* isolates were collected from different clinical samples including wound swab, pus, blood, urine, tracheal aspirate, throat swab, aural swab etc. Routine antimicrobial susceptibility testing was performed including oxacillin and cefoxitin discs, oxacillin screen agar plates. PCR was performed for detection of the *mecA* gene for MRSA. All specimens were collected aseptically and inoculated into appropriate media, incubated aerobically at 37^0C for 24 hours and colonies identified for Staphylococcus. Suspected colonies of *S. aureus* species were confirmed by wet film preparation, Gram staining, colony morphology, haemolytic status, pigment production, mannitol fermentation test, motility test and other relevant biochemical tests, catalase test, coagulase test (both slide and tube test) as per standard methods[2-4]. Routine antimicrobial susceptibility testing was performed bu Kirby-Bauer disc diffusion method. The antibiotic sensitivity discs from MAST, OXOID and HIMEDIA were used for sensitivity testing with corresponding strength. Oxacillin (1 µg), Cefoxitin (30 µg), Vancomycin (30 µg), Linezolid (30 µg), Penicillin-G(10µg), Ampicillin (10µg), Amoxicillin/Clavulanic acid (20/10 µg), Cloxacillin (1 µg), Cotrimoxazole (1.25/23.75 µg), Gentamycin (10 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), Erythromycin (15 µg), and Clindamycin (2µg). Representative of each cartridge of antimicrobial disc were tested for its potency using methicillin resistant *S. aureus* (MRSA) ATCC 43300 strain and methicillin sensitive *S. aureus*(MSSA) strain collected from BSMMU Laboratory used as negative control.

Oxacillin disc diffusion test: After adjusting to 0.5 McFarland standards the colony suspension was lawn cultured on Mueller-Hinton agar plate. A 1-µg oxacillin disc was placed on it and the plate was incubated at 37°C and result recorded after overnight incubation (16-18 h). Isolates showed the zone diameter measured ≤10 mm reported as oxacillin resistant and ≥13 mm as oxacillin sensitive according to Clinical and Laboratory Standards Institute (CLSI; previously National Committee for Clinical Laboratory Standards) guideline.[2,5]

Cefoxitin disc diffusion test: All the isolates were subjected to cefoxitin disc diffusion test using a 30 µg disc. A 0.5 McFarland standard suspension of the isolates were made and lawn culture on Mueller-Hinton agar plate. Plates were incubated at 37°C and zone diameters were measured after overnight incubation (16-18 h). A inhibition zone diameter measured of ≤19 mm was reported as cefoxitin resistant and ≥20 mm was as cefoxitin sensitive.[5]

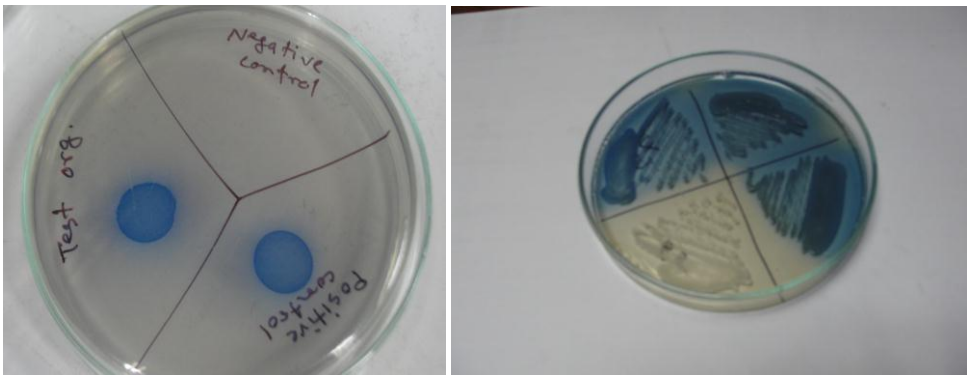
Oxacillin screen agar: Mueller-Hinton agar (MHA) plates supplemented with 4%(w/v) NaCl was prepared. Oxacillin at a concentration of 6µg/ml was added following CLSI guideline (2004). *S.aureus* suspension (10µL) matching No. 0.5 McFarland tube was inoculated on to each quadrant of plate and incubated at 35°C for 24 hours. Plates were observed carefully in transmitted light after 24 hours of incubation and result noted as sensitive or resistant.[5]

Detection of *mecA* gene by Polymerase Chain Reaction (PCR): DNA extraction was done by heat block. The mecA gene was amplified using primer *mec*A1-F - 5′ TGGCTATCGTGTCACAATCG 3′ (positions 885 to 905) and *mec*A2-R - 5′ CTGGAACTTGTTGAGCAGAG 3′ (positions 1174 to 1194) producing a 309-bp amplicon. The 25µl reaction mixture consisted of Master mix 10µl, Forward primer (F) 1.5µl, Reverse primer (R) 1.5 µl, Distilled water 10 µl, DNA 2 µl. PCR reactions were performed in a Thermocycler and the cycling conditions were initial denaturation for 10 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 extension was for 7 minutes at 72°C. PCR products were visualized on 2% agarose gel staining with ethidium bromide dye under UV transilluminator. Amplicons of 309 bp were consistent with mecA gene amplification.[6]

RESULTS

Of total 22 suspected isolates 19 were *mec*A positive by PCR which is 'gold standard'. Out of these 19, 16(84.2%) were resistant by oxacillin disc diffusion and

3(15.8%) were sensitive, on the cefoxitin disc diffusion all of them were resistant 19(100.0%) and they were also resistant on oxacillin screen agar.



A **B**

Figure 1: Growth (Blue colored) on Oxacillin screen agar. (A) by dropping method, (B) by streaking method.

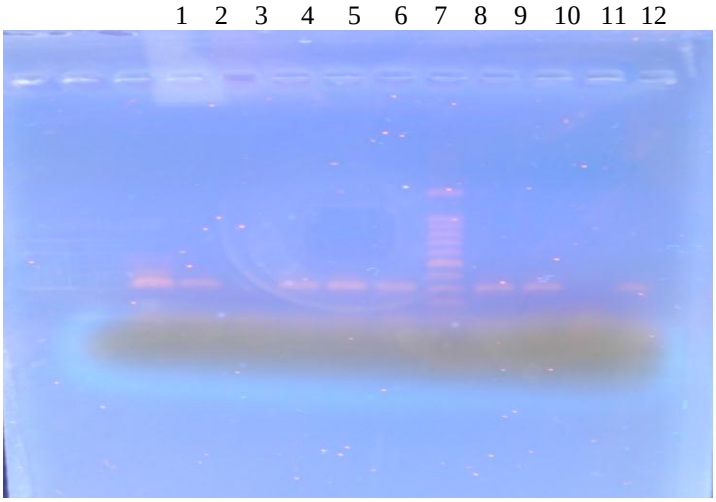


Figure 10: PCR amplification of *mecA* gene demonstrating the expected 309 bp products for some tested samples.

Lane 1: PCR negative control (sterile water).
Lane 2: Positive control (ATCC)
Lane 3: Clinical isolate of Staphylococcus aureus (Positive)
Lane 4: Clinical isolate of Staphylococcus aureus (Negative)
Lane 5: Clinical isolate of Staphylococcus aureus (Positive)
Lane 6: Clinical isolate of Staphylococcus aureus (Positive)
Lane 7: Clinical isolate of Staphylococcus aureus (Positive)
Lane 8: Molecular weight standard (DNA marker/Ladder)
Lane 9: Clinical isolate of Staphylococcus aureus (Positive)
Lane 10: Clinical isolate of Staphylococcus aureus (Positive)
Lane 11: Clinical isolate of Staphylococcus aureus (Negative)
Lane 12: Clinical isolate of Staphylococcus aureus (Positive)

Table 1: Sensitivity, specificity, accuracy, positive and negative predictive values of the Oxacillin disc diffusion, Oxacillin agar screen and Cefoxitin disc diffusion in diagnosis of MRSA (n=22).

Methods	Sensitivity	Specificity	Accuracy	PPV	NPV
Oxacillin disc diffusion	84.2	66.2	81.8	94.1	40.0
Oxacillin Screen agar	100.0	33.3	90.9	90.5	100.0
Cefoxitin disc diffusion	100.0	100.0	100.0	100.0	100.0
PCR	100.0	100.0	100.0	100.0	100.0

PPV- positive predictive values; NPV- negative predictive values

The 3 strains of *S. aureus* negative by PCR for *mec*A gene, of which 2(66.7%) were resistant on oxacillin screen agar and 1(33.3%) was resistant on oxacillin disc diffusion method while all 3 were sensitive by cefoxitin

disc diffusion. In the present study the sensitivity and specificity of oxacillin disc diffusion is 84.2% and 66.2%, cefoxitin disc diffusion both is 100% and oxacillin screen agar 100.0% and 33.3% respectively (Table 1).

DISCUSSION

Detection of mecA gene for its products, penicillin binding protein (PBP2a), is considered the gold standard for MRSA confirmation.[7] But PCR is time consuming and expensive method[8], it is not available in most of the routine laboratories; besides, its application, maintenance and reproducibility seems to be difficult because of its complicated procedure and skill needed. A currently available phenotypic method for the detection of methicillin resistance in *S. aureus* is problematic because of the heterogeneous resistance displayed by many clinical isolates.[4,8] To overcome this problem many phenotypic & genotypic method have been used. Therefore, quick and reliable identification procedures are required to obtain information on the MRSA isolates and allow faster implementation of appropriate control measures.

Current studies indicate that disc diffusion testing using cefoxitin disc is far superior to most of the currently recommended phenotypic methods like oxacillin disc diffusion and oxacillin screen agar testing and is now an accepted method for the detection of MRSA by many referencing groups including CLSI.[9] So, the aim of this study is to evaluate the cefoxitin disc diffusion test as a rapid, reliable, sensitive and more specific than oxacillin disc diffusion and oxacillin screen agar for the detection of methicillin resistance compare to PCR *mecA* gene detection which is considered as 'gold standard'.

Implementing the susceptibility testing by oxacillin screen agar and disc diffusion technic according to CLSI guidelines in this study, the observations of cefoxitin disc diffusion result was highly sensitive. The sensitivity and specificity of it was 100% and coincides with PCR. As compared to high specificity of cefoxitin disc diffusion method oxacillin disc diffusion was only 66.2% specific. Similar results were quoted by Mathew et al.[10] The false positivity of the oxacillin disc diffusion method in this study may be due to hyper production of beta-lactamase which may lead to phenotypic expression of resistance but sensitive to cefoxitin and negative for mecA gene. These isolates were reported as methicillin sensitive based on cefoxitin sensitivity and all the patients infected with these isolates responded well to cephalosporin and cloxacillin.[10] On the contrary, *S. aureus* strains naturally are either heterogenous and homogenous in their expression of resistance.[11] Cells expressing hetero-resistance grow more slowly than the oxacillin-susceptible population and may be missed at temperature above 35^0C. So, the CLSI recommendation is incubating isolates being tested against oxacillin, methicillin or nafcillin at 33-35^0C (maximum 35^0C) for a full 24 hours before reading.[12]

Among the methods tested the sensitivity of oxacillin screen agar test for detection of MRSA was excellent. Conversely, the specificity among susceptible strains tested were good unless strains with borderline MICs were included.[5,6]

CONCLUSION

Antimicrobial resistance results in increased morbidity, mortality and costs of health care. Prevention of emergence of alarmingly high incidence of MRSA will reduce these adverse effects and their attendant costs. Cefoxitin is a surrogate marker for detection of *mec*A-gene-mediated methicillin resistance. In the present study the result of cefoxitin disc diffusion have shown 100% sensitivity and specificity in comparison to *mec*A gene detection by PCR. Hence, it can be used as an alternative to technically demanding PCR.

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