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Comparison of different phenotypic methods of detection of methicillin resistance in staphylococcus aureus with the molecular detection of mec-a gene

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COMPARISON OF DIFFERENT PHENOTYPIC METHODS OF DETECTION OF METHICILLIN RESISTANCE IN *Staphylococcus aureus* WITH THE MOLECULAR DETECTION OF *mec-A* GENE

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ABSTRACT

Objective: To evaluate accuracy, cost-effectiveness and ease to perform different phenotypic methods i.e. Cefoxitin $30\,\mu g$ disc, Oxacillin $1\mu g$ disc and Oxacillin agar screening plate ($6\,\mu g$/ml) for early and accurate identification of MRSA by comparing with the detection of *mec-A* gene in our clinical isolates.

Design: A comparative study.

Place and Duration of Study: Clinical samples submitted in the Clinical Microbiology Laboratory at Aga Khan University Hospital, Karachi, from 1st August to 31st October 2006.

Material and Methods: Out of 200 clinical samples, conventional Polymerase Chain Reaction (PCR) was done on 62 pure biochemically identified *S. aureus* isolates for *mec-A* gene detection. Phenotypic methods for detecting methicillin sensitivity (Cefoxitin $30\,\mu g$ disc, Oxacillin $1\,\mu g$ disc and Oxacillin agar screening plate) were also used according to the recommended incubation time, duration and temperature on the same isolates.

Results: Out of 62 isolates of *S. aureus*, *mec-A* gene were detected (MRSA) in 32, whereas 30 were *mec-A* gene negative (MSSA). Cefoxitin disc and agar screening plate correctly identify all MRSA isolates with the sensitivity and specificity of 100%. Single isolate was false, positively detected as sensitive with Oxacillin $1\,\mu g$ disc, due to which, the sensitivity and negative predictive value of this method were reduced to 96.9% and 96.8% respectively, while positive predictive value and specificity remained 100%.

Conclusion: Comparing different phenotypic methods for MRSA screening in routine microbiology laboratory, Cefoxitin disc and Oxacillin agar screening has better sensitivity and specificity comparative to Oxacillin disc. However, Cefoxitin disc can be preferred especially for small laboratories because it is easy to perform, do not require special technique and media preparation is consequently more cost-effective.

KEY WORDS: Cefoxitin disc. Methicillin resistance. Oxacillin. Penicillin-binding protein-2a. Staphylococcus aureus.

INTRODUCTION

Methicillin Resistant *Staphylococcus aureus* (MRSA) is one of the most important etiologic agent of nosocomial infections. Increasing frequency of MRSA infections has been reported worldwide despite infection control measures.[1-2] Hospitals in Pakistan have also encountered worsening situation in the last ten years.[3,4] Moreover, recently MRSA is being increasingly recognized from infections in community settings.[5-6] Therefore, control and management of MRSA infection is a global challenge. In this regard, rapid and reliable detection of MRSA is crucial for both effective control and optimum therapeutic outcome.

Altered Penicillin Binding Protein PBP2a encoded by *mec-A* gene is mainly responsible for methicillin resistance in *Staphylococcus aureus*. Therefore, its detection by genotypic method Polymerase Chain Reaction (PCR) is considered as gold standard.[7-8] However, in developing countries like Pakistan, cost constraints and lack of basic laboratory facilities limits its use as routine diagnostic tool.

Phenotypic methods are used by most clinical laboratories for MRSA detection because it is easy to perform and interpret. Even with overall good sensitivity and specificity, the phenotypic methods have limitations in detecting Staphylococcal isolates with borderline resistance or heteroresistance, as they are affected by variables, such as inoculums size, incubation time, temperature, media, pH, salt concentration and interobserver variability.[9-11] Until 2006, Oxacillin disc and agar screening methods were used for detection of MRSA, however, in January 2006, Clinical Laboratory Standards Institute (CLSI) recommended use of Cefoxitin $30\,\mu g$ disc as standard marker for MRSA identification.[12] The shift towards use of Cefoxitin disc is emphasized because of its property to induce production of PBP2a *in-vitro*, thus it has better predictive value for detection

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of heteroresistance in MRSA isolates.[13] Moreover, test could be performed on Mueller-Hinton agar and incubate only for 24 hours.

Currently, the method employed for the detection of methicillin-resistance *Staphylococcus aureus* in Clinical Microbiology Laboratory of The Aga Khan University, is Oxacillin disc and oxacillin screening agar. Before implementing the new recommendation by CLSI in our laboratory, this study was conducted to compare the efficiency of the current and new phenotypic tests (Cefoxitin disc) for detection of MRSA in local strains isolated in our clinical samples.

The aim of this work was to evaluate sensitivity, specificity, PPV, NPV, cost-effectiveness and ease to perform different phenotypic methods i.e. Cefoxitin 30 μg disc, Oxacillin 1 μg disc and Oxacillin agar screening plate (6 μg/ml) for early and accurate identification of MRSA resistance by comparing with detection of *mec-A* gene in our clinical samples (local strains). Detection of *mec-A* gene through Polymerase Chain Reaction (PCR) was used as a gold standard.

We hypothesized that Cefoxitin disc is a better inducer of *mec-A* gene expression and, therefore, an efficient screening method for detecting heteroresistance in *Staphylococcus aureus* in clinical isolates as compared to other phenotypic methods.

MATERIAL AND METHODS

This comparative study was conducted at the Department of Microbiology and Jumma Research Laboratory at Aga Khan University, Karachi, Pakistan, from 1st August to 31st October 2006.

During the study period, only pure *Staphylococcus aureus* isolates from sheep blood agar plates were picked and identified using tube coagulase method, DNase (BD-Becton-Dickinson), Mannitol salt agar (Oxoid) and Phosphates agar (Phenolphthalein diphosphate-Sigma, Agar base -Oxoid). Out of 200 clinical specimens, including pus swabs, drain abscesses, urine, blood, ear swabs, antral washing, tracheal aspirates, umbilical swabs, synovial fluid, nasal swab, central line and eye swab, 62 isolated *S. aureus* colonies were used for further assessment. All were non-duplicate isolates from different patients. The methicillin susceptible and methicillin resistant *S. aureus* ATCC 29213 and ATCC 43300 respectively, were used as a control for all diagnostic procedure. All isolates were saved in glycerol phosphate buffer at -80°C.

PCR for *mec-A* gene detection was done in all the *Staphylococcus aureus* isolates, as described by Murakami *et al.*[14] with few modification.

Total DNA of the isolates were purified by using the Wizard genomic DNA purification kit (Promega-USA) as specified by the manufacturer with lysozyme at 10 mg/ml and lysostaphin at 2 mg/ml to achieve bacterial lysis. The purified DNA concentration was estimated with a spectrophotometer (Beckman, DU 650) and stock solution were diluted to a concentration of 30 ng/μl. A total of 30 ng of DNA were then used in each PCR.[14]

The oligonucleotides primers used in this study have been previously described and were obtained from a commercial source (Operon Biotechnologies). One of the primers (5' AAAATCGATGGTAAAGGTTGGC 3') corresponded to nucleotide 1282 to 1303, and the other (5' AGTTCTGC-AGTACCGGATTTGC 3') was complementary to nucleotides 1793 to 1814.[14]

With conventional PCR, amplifications were performed with PCR master mix (promega), in a 25 μl reaction mixture containing 1 μl of 30 ng of template DNA and 0.4 μM of each primer. Following Murakami *et al.* [14] with few modification, DNA amplification was carried out in a Perker-Elmer Gene Amp® 9700 Thermal Cycler for 30 cycles as follows: initial denaturation of 94°C for 3 min, denaturation for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. Ten microlitres of PCR products was analyzed by 2% agarose gel electrophoresis stained with 0.5 μg of ethidium bromide.

Disc diffusion test was performed on Mueller-Hinton Agar (BD-Becton-Dickinson) plates. For this MHA plates were overlaid with clinical strain of the *S. aureus* with an inoculum of 0.5 McFarland turbidity standard. Oxacillin 1μg disc and Cefoxitin 30 μg discs were used on same plate and incubated at 35°C for 24 hours.[12,18] Cut off zone diameters for Cefoxitin was according to CLSI recommendation i.e. ≤19 mm resistant and ≥20 mm sensitive, whereas for Oxacillin disc[12], diameter of ≥13 mm was considered as sensitive and ≤10 mm as resistant.[15] For quality control, ATCC controls strains for MRSA and MSSA were placed on the same plate.

Agar screening plates were prepared using 6 μg/ml Oxacillin powder with Mueller-Hinton Agar (Oxide). These plates were spot inoculated with 0.5 McFarland turbidity standard test isolates and with ATCC controls for MRSA and MSSA. Finally, these plates were incubated at 37°C for 24 hours.[16,18] Presence and absence of growth on plates was considered as resistant and sensitive respectively.

Receiver Operating Characteristic (ROC) curve technique was applied for the calculation of sample size. A sample size of 31 from positive group (MRSA) and 31 from negative group (MSSA) were required to detect minimum difference between the area under the null hypothesis of 0.8000 and an area under the alternative hypothesis of 0.9600 using two sided z-test at a significance level of 0.0500 with 80% power. The ratio of the standard deviation of the responses in the negative group to the standard deviation of the responses in the positive group was 1.00.

Recommended zone sizes were entered and susceptibility of each isolate was checked according to CLSI standards. To assess the significance of the new method (Cefoxitin disc method) McNemar's test were used. To observe the percentage agreement between the two methods beyond what was expected by chance alone, Kappa statistics were used. Results from all methods were analyzed using SPSS version 13.0.

RESULTS

A total of 62 clinical isolates of *S. aureus* were collected from different specimen types as given in Figure 1. All were evaluated for detecting methicillin resistance with the Cefoxitin disc, Oxacillin disc and agar screening for detection of MRSA.

Figure 1: Source of Staphylococcus aureus isolates from different anatomic sites

Based on PCR assay, 32 isolates of *S. aureus* were classified as *mec-A* was positive, while the remaining 30 were found to be *mec-A* gene negative. The overall comparison and statistical analysis of the results of MRSA detection through different phenotypic and genotypic methods is given in Table I.

Out of 32 *mec-A* gene positive, the zone size diameter of Oxacillin 1 μg disc for almost all MRSA was zero, except for the single isolate, which had zone size of 15 mm, while the average zone size diameter for MSSA was 20 mm. The single isolate that was misidentified as MSSA through Oxacillin 1 μg disc decreased the sensitivity of this assay to 96.9% and its negative predictive value to 96.8%. All isolates were correctly identified with Oxacillin agar screen method and, therefore, had 100% sensitivity, specificity, PPV and NPV (Table I).

Figure 3 shows the zone diameter of Cefoxitin 30 μg disc for MRSA and MSSA isolates. The average zone size diameter of Cefoxitin 30 μg disc for MSSA was 27 mm and for MRSA it was 4.5 mm. The results of Cefoxitin disc method were in complete conformity with the *mec-A* gene detection by PCR leading to 100% sensitivity, specificity, PPV and NPV for the identification of all S. aureus strains (Table I).

DISCUSSION

Early and accurate identification of MRSA is essential to restrict its spread. Oxacillin disc is being used in most of the laboratories in Pakistan. Cefoxitin has been suggested as new

marker for MRSA detection in comparison to Oxacillin[12] because of its ability to induce PBP2a. From practical point of view, this method is simple to read and have no special requirement for media and temperature.

This study was conducted as pilot project in the Department of Clinical Microbiology and Jumma Research Laboratory. We compared disc diffusion method (Oxacillin disc and Cefoxitin disc) and agar screening methods on each isolates for detection of MRSA. The identification through Cefoxitin 30 μg disc and Oxacillin agar screening plate (6 μg/ml) correctly correlate with the molecular method for the presence and absence of *mec-A* gene, so that the sensitivity and specificity of both these methods were 100% and with 100% negative and positive predictive value.

One of the important findings was the detection of a single isolate, which was *mec-A* gene positive, but Oxacillin 1 μg disc failed to correctly identify this isolate (zone diameter=15 mm) (Figure 2). Therefore, the sensitivity and negative predictive values of Oxacillin 1 μg disc decreased to 96.8% and 96.9% respectively.
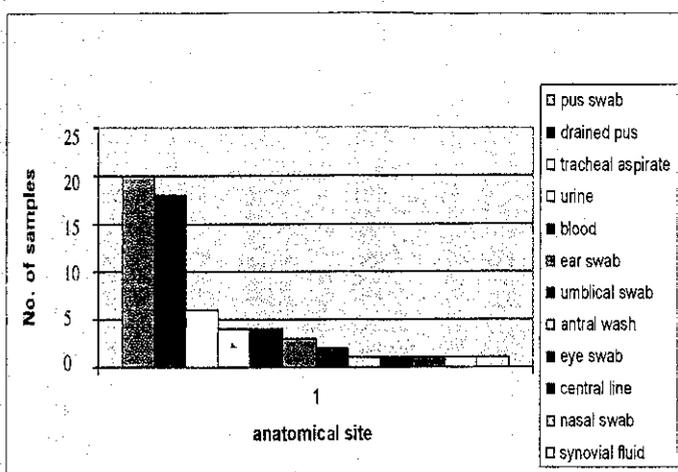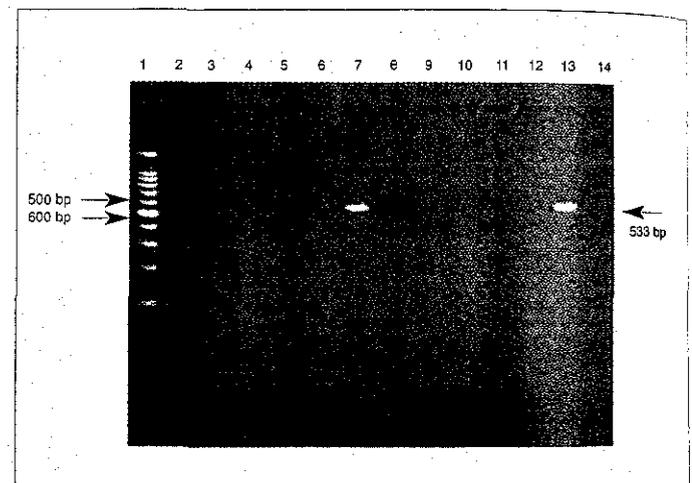

Figure 2: Agarose gel electrophoresis of amplified 533bp DNA fragment.
Lane 13: MRSA (phenotypically identified as MSSA with Oxacillin disc)
Lane 2-5 and 8-11: MSSA.
Lane 12: Blank.
Lane1: Molecular weight standard (100bp ladder).
Lane 6 &14: Negative control ATTC29213
Lane 7: Positive control ATTC 43300

The resistance in this isolate was perhaps due to the low level expression of *mec-A* gene, which according to Tomasz *et al.* is

Table I: Comparison between different phenotypic methods with Gold Standard for MRSA detection.

Phenotypic methods	(n=62)	mec A gene detected (MRSA)	mec A gene not detected (MSSA)	Sensitivity	Specificity	PPV	NPV
Cefoxitin 30 μg disc method	Susceptible	00	30	100 %	100 %	100 %	100 %
	Resistant	32	00				
Oxacillin 1 μg disc method	Susceptible	01	31	96.90 %	100 %	100 %	96.8 %
	Resistant	30	00				
Oxacillin agar screening method	Susceptible	00	30	100 %	100 %	100 %	100 %
	Resistant	32	00				

due to heteroresistance.[17] Since Oxacillin is relatively a weak inducer of *mec-A* gene expression as compared to Cefoxitin, therefore, it appeared sensitive on disc diffusion. Although the sample size in this study is not large enough to validate this hypothesis, this finding is important for laboratories that are now only using single phenotypic method i.e. Oxacillin disc for screening of MRSA. The low sensitivity of the Oxacillin disc diffusion method may also be due in part to the absence of salt supplements, which are currently not recommended by CLSI but which are known to promote staphylococcal growth incubation at +30°C and hypersalted agar medium could improve the MRSA recovery[18]; however, Mougeot *et al.* found out that despite the use of these methods, Cefoxitin disc still had good sensitivity and specificity relative to Oxacillin disc.[19] In the current study, we had not evaluated results of Oxacillin 1 μg disc by incubating at different temperature. The study conducted by Boubaker *et al.* also found that Cefoxitin disc method had better sensitivity and specificity relative to Oxacillin disc method if both disc plates were kept at same temperature (35°C) and for similar time duration (24 hours).[20] Velasco in their study also concluded that Cefoxitin disc method was a better predictor for MRSA relative to other discs method including Oxacillin disc.[21]

Regarding zone sizes of disc diffusion method in this study, the ranges of the inhibition zones for Cefoxitin disc against *mec-A* negative isolates were from 21 and 30 mm; whereas for *mec-A* positive isolates it ranged in between 0 and 15 mm (Figure 3). With the use of Cefoxitin 30 μg disc, wider and clear ranges in zone sizes of MRSA and MSSA was also observed by Velasco.[21] For *mec-A* gene negative isolates, it was more than 25 mm and for *mec-A* positive strain its range was in between 0 to 14 mm. Consequently, there was no discrepancy in labelling isolates as sensitive or resistant.
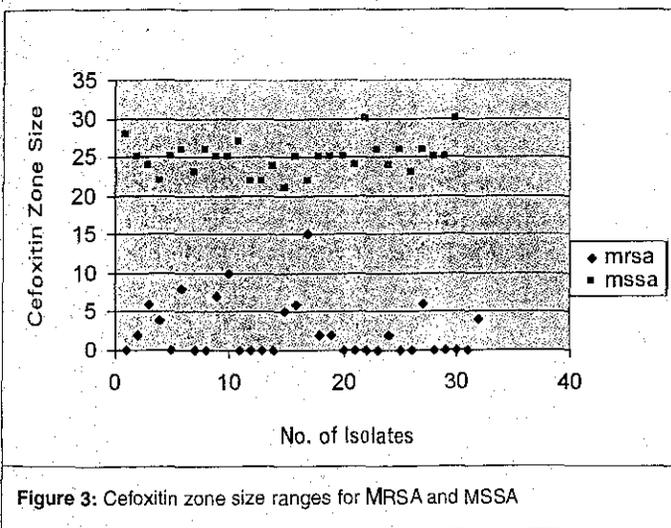

Figure 3: Cefoxitin zone size ranges for MRSA and MSSA

The method of Oxacillin agar screening plate (6 μg/ml) was also evaluated by Cauwelier and Velasco [21-23] in their studies, and they concluded that this method was less sensitive and 100% specific. This reduced sensitivity for the detection of heterogeneous MRSA populations, could attribute due to the high variability in PBP2a expression.[22] Only small subpopulations of bacterial cells that highly express PBP2a were able to grow on Oxacillin screen agar. Although this is an easy and inexpensive method, there is the probability of missing borderline Oxacillin resistant *S. aureus* limiting its use.

In the current study, the sensitivity and specificity of Oxacillin agar screening plate (6 μg/ml) was 100%. Although these results were good, it should be interpreted with caution due to small sample size.

The results of this study were in conformity with the CLSI recommendation. When tested on our local strains, it was found that Cefoxitin 30 μg disc a better alternate to Oxacillin 1 μg disc in predicting MRSA because it is not affected by salt, pH and temperature variability, which is very difficult to maintain, while working in resource limited laboratory. In addition, it is a strong inducer of the *mec-A* gene expression as compared to the Oxacillin disc method and is, therefore, better predictor of the heteroresistance. The results of Cefoxitin disc and agar screening plates were similar in this study, however, Cefoxitin disc could be preferable over agar screening plates specially in small and low budget laboratory because in agar screening plate preparation, it requires antibiotic in powder form, which should be reconstituted and diluted to the required concentration. The prepared stock solution should be stored at 4-8°C with the expiry duration of only 6 months. Hence, this is associated with high preparation cost, trained personnel and adequate storage facilities to maintain the quality of this test. This could be very difficult task, especially for laboratories with low budget and limited number of samples. Majority of laboratories in Pakistan are not using this method due to above practical problems and therefore, they prefer disc susceptibility method for MRSA detection. Cefoxitin disc could be easy, accurate and cost-effective alternative for these laboratories.

CONCLUSION

Comparing different phenotypic methods for MRSA screening in routine microbiology laboratory, Cefoxitin disc and Oxacillin agar screening has better sensitivity and specificity comparative to Oxacillin disc. However, Cefoxitin disc can be preferred especially for small laboratories because it is easy to perform, do not require special technique and media preparation is consequently more cost-effective.

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