Original Article

Microbiology

Comparison of Various Phenotypic Methods and *mecA* Based PCR for the Detection of MRSA

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ABSTRACT

Background: Methicillin resistant *Staphylococcus aureus* (MRSA) is the most commonly emerging pathogen in community and hospital acquired infections. Hence, an accurate detection is not only important for the control of the infection, but also to control the endemicity of MRSA.

Aim: To evaluate the efficacy of various phenotypic methods with *mecA* based PCR to detect MRSA. We also studied the resistance pattern of the MRSA isolates.

Settings and Design: This was a prospective study which was conducted at a tertiary care hospital

Materials and Methods: A total of 55 *S. aureus* strains which were isolated from patients with superficial and deep abscesses were included in this study. Methicillin resistance which was determined by oxacillin disc diffusion, cefoxitin disc diffusion and the oxacillin screen agar test was compared with *mecA* based PCR.

Results: Among the 55 *S.aureus* isolates, 20 (36.4%) isolates were positive for the *mec*A gene by PCR. Both the cefoxitin disc diffusion and the oxacillin screen agar test showed 100% sensitivity and 100% specificity, while oxacillin disc diffusion showed 90% sensitivity and 100% specificity. The resistance percentage of the MRSA isolates to erythromycin, ciprofloxacin and amikacin were 80%, 30% and 25%, respectively.

Conclusion: Conventional MRSA detection assays like the cefoxitin disc diffusion test and the oxacillin screen agar test are simple and relatively cheap and can be used as alternatives to PCR for the detection of MRSA in resource constraint settings. Also, most of the MRSA strains in this study showed co-resistance to many classes of antibiotics and thus they qualified as multi-drug resistant *S. aureus*.

Key Words: MRSA, cefoxitin disc diffusion, oxacillin screen agar, *mec*A gene

KEY MESSAGE

- 'MRSA' is a term which is used to describe the *Staphylococcus aureus* isolates that are resistant to all the available β-lactam antibiotics, including the penicillins and the cephalosporins.
- Methicillin resistance in *S. aureus* is primarily mediated by the *mec*A gene, which codes for the modified penicillin-binding protein 2a (PBP 2a or PBP 2')
- Strains that possess the mecA gene are either heterogeneous or homogeneous in their expression of resistance.
- The phenotypic expression of the resistance can vary, depending on the growth conditions (e.g. temperature, osmolarity and culture medium supplements such as NaCl or sucrose)
- The detection of the *mec*A gene by PCR is considered as the gold standard.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were first identified in 1961, immediately after the introduction of methicillin in the clinical settings. Subsequently, an increase in the resistance to methicillin among the *S. aureus* isolates has been observed globally [1]. MRSA is one of the major pathogens which are associated with serious nosocomial infections, because these strains generally show multiple drug resistance which limits the treatment possibilities. MRSA has become established outside the hospital environment and it is now appearing in community populations without any identifiable risk factors [2].

The methicillin resistance in Staphylococci is due to the acquisition of the *mec*A gene, which encodes the low-affinity penicillin-binding protein 2a [3]. The presence of the *mec*A gene in *S. aureus* defines methicillin resistance, while the absence of the gene indicates methicillin susceptibility [4]. Methicillin resistance can be difficult to detect, because the *mec*A-positive strains can differ in their level of expression of resistance. The resistance is typically heterogeneous, with only a few cells (one in 10^4 or 10^6) expressing the phenotype.

The *mec*A gene is highly conserved among the Staphylococcal species and therefore, presently, the detection of this gene by the polymerase chain reaction (PCR) is considered as the "gold

standard" for the detection of methicillin resistance in Staphylococci [5-7]. While PCR is considered as the gold standard assay for the detection of methicillin resistance, it still remains a time-consuming and expensive method; besides, it is not available in most of the routine laboratories.

The objective of the present study was to determine the methicillin resistance in *S. aureus* by *mec*A based PCR and to evaluate the usefulness of various phenotypic methods for the detection of MRSA in comparison to *mec*A based PCR. We also aimed to study the resistance pattern of the MRSA isolates.

MATERIALS AND METHODS

Clinical Isolates

A prospective study was conducted over a period of one year from May 2008 to June 2009 at a tertiary care hospital in south India. A total of 55 *S. aureus* strains which were isolated from patients with superficial and deep abscesses were included in this study. All the isolates were identified as *S. aureus* by their colony morphology, gram staining and their catalase and coagulase tests (both tube and slide tests). These 55 clinical isolates were tested for methicillin resistance by oxacillin disk diffusion, cefoxitin disc diffusion and the oxacillin screen agar test. From this collection, all the isolates were evaluated further by using *mec*A based PCR.

All the Staphylococcal isolates were tested for susceptibility to a predetermined battery of antibiotics by the Kirby-Bauer disc diffusion method. The antibiotics which were tested included Penicillin (10u), Ampicillin (10µg), Cefotaxime (30µg), Amikacin (30 µg), Erythromycin (15 µg), Ciprofloxacin (5µg) Oxacillin (1µg) and Vancomycin (30 µg). The zones of inhibition were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines [8].

Phenotypic Detection of MRSA

The Oxacillin Disk Diffusion Method: The Oxacillin disk (1 µg) diffusion method was carried out on Mueller-Hinton agar which was supplemented with 2% NaCl to detect MRSA according to the CLSI guidelines [8]. The plates were incubated at 35°C and the results were recorded after 24 hrs of incubation. The isolates were considered as resistant when the diameter of inhibition was ≤10 mm, as intermediately resistant when the diameter was 11-12 mm and as sensitive when the diameter was ≥13 mm [8].

The Cefoxitin Disc Diffusion Test: The Cefoxitin disc diffusion method was carried out on Mueller-Hinton agar by using a 30 µg cefoxitin disc. An inhibition zone diameter of ≤ 21 mm was reported as methicillin resistant and a diameter of ≥ 22 mm was considered as methicillin sensitive [8].

The Oxacillin Screen Agar Test: Muller-Hinton agar plates containing 4% NaCl and 6 µg/ml of oxacillin were prepared. To perform the oxacillin screen test, a swab which was dipped in 0.5 Mc Farland's suspension of the isolate was deposited as a spot on the agar surface and it was incubated at 35°C for 24 h. The plates were observed carefully in transmitted light for any growth. Any growth after 24 hr was interpreted as oxacillin resistance [9].

The Genotypic Detection of MRSA

Detection of the mecA Gene by Polymerase Chain Reaction: Bacterial DNA was obtained by the rapid cell lysis method as described by Unal *et al* [10]. For the DNA extraction, 0.1 mL of an overnight culture of bacteria in Mueller-Hinton broth was harvested by centrifuging the broth in a microcentrifuge tube at 16,000×g for 30 seconds. The precipitates were resuspended in 50 µL lysostaphin (100 µg/mL; Sigma) and they were incubated at 37°C for 10 minutes. Following the addition of 50 µL proteinase K (100 µg/mL; Sigma Aldrich) and 150 µL 100 mm Tris (pH 7.5), the suspension was incubated for 10 minutes at 37°C and then boiled for 5 minutes. After centrifugation at 13,000×g for 2 minutes, the supernatant which contained the extracted bacterial DNA was used in the PCR assay.

From this suspension, a 5µL volume was directly used as the template for the PCR amplification of the *mec* A gene fragments. The *mec* A1 (5′ – GTA GAA ATG ACT GAA CGT CCG ATA A – 3′) and the *mec* A2 (5′ – CCA ATT CCA CAT TGT TTC GGT CTA A – 3′) primers were used for the amplification of the 310 bp fragment of the methicillin-resistant gene (*mec* A) [11]. A 50 µl PCR reaction consisted of plus 45 µl of the master mix which contained the PCR buffer (1×), dNTP mix (0.2 mM of each), the primer (0.5 µM), Taq DNA polymerase (0.25 U), and $MgCl_2$ (1.5 mM) with 5 µL of the template DNA. The cycling conditions were as follows: 4 minutes at 94°C, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, extension at 72°C for 1 minute and the final extension step at 72°C for 3 minutes. The PCR products were visualized on an 0.8% agarose gel with ethidium bromide dye under a UV transilluminator. Amplicons of 310 bp were consistent with the *mecA* gene amplification.

Quality control

The quality control strains – methicillin sensitive *S. aureus* (MSSA) ATCC 25923 and methicillin resistant *S. aureus* (MRSA) ATCC 43300 – were used as the negative and positive controls, respectively.

Statistical Analysis

The percentages were calculated for the categorical variables. The sensitivity, specificity and the positive and negative predictive values were calculated by using the GraphPad InStat version 3.00 for Windows 95 and the GraphPad Software (San Diego, CA, USA) for determining the diagnostic value of the various phenotypic methods for detecting methicillin resistance.

RESULTS

Among the 55 *S . aureus* isolates, 20 (36.4%) were positive for the *mec*A gene by PCR. Methicillin resistance was detected by oxacillin disc diffusion, cefoxitin disc diffusion and the oxacillin screen agar test in 18, 20 and 20 isolates, respectively. The sensitivity, specificity and the positive and negative predictive values of the various phenotypic methods in comparison to PCR, for the detection of MRSA, are summarized in [Table/Fig-1].

In our study, the resistance percentage of the MRSA isolates were as follows: penicillin- 100%, ampicillin- 85%, cephotaxime and erythromycin- 80%, ciprofloxacin- 30%, amikacin- 25% and all the strains were 100% sensitive to vancomycin.

DISCUSSION

S. aureus is one of the most common causes of nosocomial as well as community-acquired infections. Methicillin resistance (as a result of the *mecA* gene which encodes the additional penicillin binding protein, PBP2a) renders *S. aureus* resistant to all the β-lactam antibiotics which is the most important group of antibiotics in the treatment of Staphylococcal infections.

Methods	No. of MRSA detected	Sensitivity (%)	Specificity (%)	Positive predictive value	Negative predictive value
Oxacillin Disc Diffusion	18	90	100	100	94.6
Cefoxitin Disc Diffusion	20	100	100	100	100
Oxacillin Screen Agar	20	100	100	100	100

[Table/Fig-1]: Comparison of results of various phenotypic methods for detection of MRSA (n=55)

The recent increase in the methicillin-resistant and multiple-resistant strains at large hospitals have started to pose a great difficulty in selecting anti-microbial agents for the management of the infections that they cause. Hence, an accurate and rapid detection of methicillin resistance in Staphylococci is therefore important, not only for choosing the appropriate antibiotic therapy for the individual patient, but also for the control of the endemicity of the MRSA.

Our study revealed that, overall rate of methicillin-resistance with *S. aureus* was nearly 36.4%. Similar rates which were reported in other studies from tertiary care centers all over the world [12,13] supported this high incidence which was found in our study.

In our study, the oxacillin disc diffusion method detected 18 of the 20 cases of MRSA with a sensitivity of 90% and a specificity of 100%, which was in concordance to the results of the previous study [14], which showed 87.5% sensitivity and 100% specificity by the oxacillin disc diffusion method. Although the oxacillin disc diffusion test has high sensitivity and specificity for detecting methicillin resistance, some difficulties have been encountered with it in detecting the hetero-resistant isolates of *S. aureus*. The accurate determination of methicillin resistance in Staphylococci by the oxacillin disc diffusion method may be affected by various components of medium, temperature, and the duration of incubation [15]. Hence, other phenotypic methods like the agar screen method and the cefoxitin disc diffusion method have been evaluated.

MRSA detection by the oxacillin screen agar method identified all the 20 MRSA with 100% sensitivity and 100% specificity. A similar study reported that the sensitivity of this method approached 100% for the detection of MRSA and 95% for the coagulase-negative *S. aureus* [16].

In this study, the cefoxitin disc diffusion method detected 20 (36.4%) out of the 55 isolates as MRSA, which accounted for 100% sensitivity and specificity as compared to the *mec*A-based PCR. Cefoxitin, a cephamycin, is a more potent inducer of the *mec*A regulatory system and an accurate surrogate marker for the detection of MRSA in the routine susceptibility testing. It has been suggested that no special medium or incubation temperature is required for cefoxitin as is required for oxacillin [17]. Recent studies have indicated that disc diffusion testing by using the cefoxitin disc is far superior to most of the currently recommended phenotypic methods like oxacillin disc diffusion and oxacillin screen agar testing and that it is now an accepted method for the detection of MRSA by many reference groups including CLSI [18].

In our study which was conducted to determine the resistance pattern of the MRSA isolates, the resistance to erythromycin, amikacin and ciprofloxacin were 85%, 40% and 30% respectively and no strains were found to be resistant to vancomycin, which was similar to the findings of several other studies [19,20]. The MRSA showed a high level of resistance to most of the antibiotics in comparison to the methicillin sensitive *S. aureus*. Also, most of the MRSA in this study showed co-resistance to many classes of antibiotics at the same time and thus they qualified as multi-drug resistant *S. aureus* (MDR-MRSA).

In conclusion, as has been shown in this as well as other studies, the oxacillin disk diffusion method was found to be less sensitive for the detection of MRSA. The results of the cefoxitin disc diffusion test and the oxacillin screen agar test were in concordance with the results of PCR for the *mec*A gene. Moreover, these conventional MRSA detection assays like the cefoxitin disc diffusion test and the oxacillin screen agar test are simple and relatively cheap and they can be used as an alternative to PCR for the detection of MRSA in resource constraint settings.

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