Evaluation of various methods for the detection of methicillin-resistant *Staphylococcus aureus* strains and susceptibility patterns

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as one of the major pathogens in hospital as well as community settings. In India, the mean isolation rate of MRSA is 20–40 % and many studies have suggested an escalating rate of infections caused by this organism. Despite pharmaceutical and technological advancement, infections caused by MRSA still remain difficult to diagnose. The present study was undertaken to compare five phenotypic methods for the detection of MRSA. This involved examining 200 isolates of *S. aureus* by oxacillin disc diffusion, cefoxitin disc diffusion, oxacillin screen agar test, the latex agglutination test and growth on CHROMagar. PCR for *mecA* gene detection was taken as the gold standard. It was found that 35 % of all *S. aureus* infections were caused by MRSA. The cefoxitin disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute, was found to be a reliable method for MRSA detection but it should be supplemented with some other method like latex agglutination, CHROMagar or oxacillin screen agar testing so that no MRSA is missed. We recommend that along with cefoxitin disc diffusion, another method, preferably latex agglutination, should be routinely used in all hospitals to detect MRSA.

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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as one of the major pathogens in both hospital and community settings. The first case of MRSA was isolated way back in 1961 (Chambers, 1997). Since then, there has been an escalating rate of infections caused by MRSA worldwide resulting in increased mortality and morbidity statistics (Chambers, 1988).

In India, the prevalence of nosocomial infections caused by MRSA varies between 20 and 40 % (Mehta *et al.*, 1998; Verma *et al.*, 2000; Velasco *et al.*, 2005). Methicillin resistance in *S. aureus* is associated with production of an altered penicillin-binding protein, a 78 kDa protein termed PBP2a, which has a low affinity for β -lactam antibiotics. The production of PBP2a is mediated by the *mecA* gene present in MRSA (Chambers, 1988).

There are many factors which make the detection of MRSA complicated. Resistance to methicillin in *S. aureus* is heterogeneous in the majority of the isolates (Chambers, 1988). Thus, heterogeneous strains are composed of two populations of cells – relatively susceptible cells and highly resistant cells. These strains appear phenotypically sensitive

to methicillin (Chambers, 1988). Other factors also influence the phenotypic expression of resistance. Addition of sodium chloride or sucrose to culture medium, incubation at 30 °C or passage in the presence of β -lactam antibiotics enhances the expression of resistance (Hartman & Tomasz, 1986). These factors necessitate the requirement for a simple, rapid, accurate and sensitive method for the detection of MRSA in routine diagnostic laboratories.

Conventional methods for the detection of MRSA include oxacillin disc diffusion, oxacillin MIC and oxacillin screen agar methods. Recently, the Clinical and Laboratory Standards Institute (CLSI) recommended the use of the cefoxitin disc diffusion method for MRSA detection (CLSI, 2008). Cefoxitin is a cephamycin type antibiotic and has been described as an inducer of the PBP2a-encoding *mecA* gene (Velasco *et al.*, 2005). Various other methods for the detection of MRSA have been developed. The latex agglutination assay, developed by Denka Seiken Co., Japan, makes use of specific mAbs directed towards the PBP2a antigen (Hussain *et al.*, 2000). In addition, CHROMagar is another method, which utilizes a chromogenic medium for the identification of MRSA (Diederer *et al.*, 2005).

In recent years, PCR detection of the *mecA* gene has become the gold standard for MRSA detection. However, this is an expensive and time-consuming method, and use of this assay is restricted to reference centres and is not

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*.

routinely carried out in all laboratories (Velasco *et al.*, 2005).

The present study was undertaken to compare five phenotypic methods for the detection of MRSA, namely, the oxacillin disc diffusion method, the cefoxitin disc diffusion method, oxacillin screen agar, CHROMagar and the latex agglutination assay. PCR detection of the *mecA* gene was taken as the gold standard. The sensitivity and specificity of each test were determined in an attempt to find out the most suitable method, or combination thereof, for detection of MRSA in a routine diagnostic laboratory. Also, drug sensitivity patterns of MRSA and methicillin-sensitive *S. aureus* (MSSA) were evaluated to find the drug of choice for these pathogens.

METHODS

Strains. Between January 2008 and December 2008, a total of 200 strains of *S. aureus*, isolated consecutively from various clinical samples in the Department of Microbiology, Government Medical College Hospital, Chandigarh, India, was evaluated. The clinical samples from which the strains were isolated were wound swab, drain fluid, tracheal aspirate, peritoneal fluid, pleural fluid and high vaginal swab. *S. aureus* was identified by characteristic growth on blood agar, MacConkey agar, Gram staining and various biochemical tests, e.g. catalase test, free and bound coagulase test, and anaerobic mannitol fermentation (Baird, 2006).

PCR. All *S. aureus* isolates were subjected to DNA extraction followed by amplification of the *mecA* gene by PCR with primers *mec* 1 (5'-AAAATCGATGGTAAAGGTTGGC-3') and *mec* 2 (5'-AGTTCTGCAGTACCGCATTTGC-3') synthesized by GenxBio. The reaction mixture (25 µl) consisted of 100 pmol each primer, *Taq* polymerase (2.5 U), Mg²⁺ (2.5 mM), 2.5 µl PCR buffer and 3 µl template DNA. The PCR programme was as follows: 3 min at 94 °C; followed by 40 cycles of a 30 s denaturation step at 94 °C, a 30 s annealing step at 45 °C and a 30 s extension at 72 °C; and a final 10 min extension step at 72 °C. The amplified product was a 533 bp sequence, which was detected by 1% agarose gel electrophoresis with (0.5 µg ml⁻¹) ethidium bromide staining and observation under UV light. All strains positive for the *mecA* gene were designated MRSA (Skulnick *et al.*, 1992). For PCR, positive and negative control strains [ATCC 43300 (*mecA*-positive) and ATCC 29213 (*mecA*-negative), respectively] were used.

Phenotypic detection methods

All isolates of *S. aureus* were tested by oxacillin disc diffusion, cefoxitin disc diffusion, oxacillin screen agar and latex agglutination tests, and growth on CHROMagar. A standard strain of MSSA (ATCC 29213) and a PCR-positive control strain [ATCC 43300 (*mecA*-positive)] were used as controls for all methods.

Oxacillin disc diffusion method. All strains were tested with 1 µg oxacillin discs (Hi-Media) on Mueller–Hinton agar plates. For each strain, a bacterial suspension adjusted to 0.5 McFarland was used. The zone of inhibition was determined after 24 h incubation at 35 °C. Zone size was interpreted according to CLSI (2008) criteria: susceptible, ≥13 mm; intermediate, 11–12 mm; and resistant ≤10 mm.

Cefoxitin disc diffusion method. All strains were tested with 30 µg cefoxitin discs (Hi-Media) on Mueller–Hinton agar plates. For each strain, a bacterial suspension adjusted to 0.5 McFarland was used. The zone of inhibition was determined after 16–18 h incubation at

35 °C. Zone size was interpreted according to CLSI (2008) criteria: susceptible, ≥22 mm; resistant, ≤21 mm.

Oxacillin screen agar test. A bacterial inoculum of each strain was made and turbidity was adjusted to 0.5 McFarland. One drop of this suspension was inoculated on Mueller–Hinton agar containing 4% NaCl and 6 µg oxacillin ml⁻¹ (Hi-Media). Plates were incubated at 35 °C for 24 h. Any strains showing growth on the plate containing oxacillin were considered to be resistant to methicillin (Swenson *et al.*, 2001).

CHROMagar. CHROMagar (Hi-Media) is a new chromogenic medium for the identification of MRSA. For each strain, a bacterial suspension adjusted to 0.5 McFarland was used. Subsequently, a swab was dipped in the suspension and streaked onto a CHROMagar plate. The growth of any green colony was considered to be positive, indicating MRSA (Diederer *et al.*, 2005).

Latex agglutination test for detection of PBP2a. A latex agglutination MRSA screen test (Denka Seiken) was carried out for all strains according to the manufacturer's instructions (Hussain *et al.*, 2000; Van Leeuwen *et al.*, 1999).

Antimicrobial susceptibility testing. The Kirby Bauer disc diffusion method was used routinely to detect the sensitivity of all *S. aureus* isolates and interpretations were made according to CLSI (2008) guidelines. All discs were supplied by Hi-Media. For MRSA, cotrimoxazole (25 µg), erythromycin (15 µg), clindamycin (10 µg), ciprofloxacin (30 µg), netilmicin (30 µg), amikacin (10 µg), linezolid (30 µg), vancomycin (30 µg) and dalbapristin/quinapristin (15 µg) were tested. For MSSA, the same antibiotics as for MRSA were used, as well as ampicillin (10 µg), cephalixin (30 µg) and amoxicillin/clavulanic acid (30 µg).

RESULTS

A total of 200 clinical isolates of *S. aureus* was tested for the *mecA* gene by PCR. Out of the 200 strains, 70 were *mecA*-positive and were labelled as MRSA. Thus, the prevalence of MRSA in our institute was 35%. The remaining 130 strains were *mecA*-negative and were thus labelled as MSSA.

Generally, MRSA showed a high level of resistance to all antimicrobial agents compared to MSSA (Table 1). A comparative table showing sensitivities and specificities, along with negative and positive predictive values of all the methods is shown in Table 2.

DISCUSSION

In our hospital, 35% of all *S. aureus* infections are caused by MRSA. Susceptibility test profiles revealed a higher level of resistance to commonly prescribed antimicrobial agents among MRSA. All isolates were sensitive to vancomycin, linezolid and dalbapristin/quinapristin. These results were comparable to studies carried out by others (Anupurba *et al.*, 2003).

Accurate and early diagnosis of methicillin resistance is vital in the management of patients with infections caused by *S. aureus*. Although many phenotypic methods have been

Table 1. Antibiotic susceptibility patterns of *S. aureus* strains used in this study

Antibiotic	MSSA (%)	MRSA (%)
Ampicillin	98.7	—
Cephalexin	98.7	—
Amoxicillin/clavulanic acid	75	—
Cotrimoxazole	80.8	75.6
Erythromycin	82.9	55.4
Clindamycin	98.7	72.4
Ciprofloxacin	53.9	29.8
Netilmicin	98.7	93.7
Amikacin	98.7	63.8
Vancomycin	100	100
Linezolid	100	100
Dalfopristin/quinupristin	100	98

—, Antibiotic not tested.

developed to achieve this objective, the lacunae in the sensitivity of these tests in isolation may not ensure appropriate and timely treatment of all MRSA-infected patients. The current gold standard for MRSA detection is identification of the *mecA* gene. However, use of molecular methods for routine clinical practice may not be feasible in a budget-constrained setting. Therefore, it is desirable to identify an accurate, rapid and cost-effective phenotypic method for the detection of MRSA (Krishnan *et al.*, 2002).

In our study, the *mecA* gene PCR assay was used to classify 70 isolates as MRSA and the remaining 130 isolates as MSSA. Regarding the disc diffusion methods for detection of MRSA, the cefoxitin disc had a sensitivity of 98.5 %, whereas the oxacillin disc had a sensitivity of 91.4 %. Specificity for the cefoxitin disc was 100 %, whereas that for the oxacillin disc was 99.2 %. Various workers have shown that the cefoxitin disc method has better sensitivity than the oxacillin disc method for MRSA detection (Velasco *et al.*, 2005; Boutiba-Ben Boubaker *et al.*, 2004; Skov *et al.*, 2003). This higher sensitivity to cefoxitin can be explained by the increased expression of the *mecA*-encoded protein PBP2a, cefoxitin being an inducer of the *mecA* gene (Velasco *et al.*, 2005). Our study strengthens the point that cefoxitin is superior to oxacillin as an indicator of MRSA for the detection of methicillin resistance. The only

disadvantage with the cefoxitin disc is that the inhibition zones are very large and interfere with the zones of adjacent discs (Skov *et al.*, 2003).

The oxacillin screen agar test showed 97.1 % sensitivity and 100 % specificity for MRSA detection in our study. Swenson *et al.* (2001) noted that sensitivity decreased when heterogeneous resistant strains were tested and specificity decreased with strains having borderline MIC. Among the recently developed methods, CHROMagar showed 97.1 % sensitivity and 99.2 % specificity. This sensitivity could be increased to 100 % by increasing the incubation period of CHROMagar from 24 to 48 h (Diederer *et al.*, 2005). However, the delay in obtaining the information will reduce the efficacy of this method.

This study found that, for the detection of MRSA, the latex agglutination test had 100 % sensitivity and 99.2 % specificity. Many recent studies have reported the sensitivity of the latex agglutination test to be ≥ 97 % (Cavassini *et al.*, 1999; Louie *et al.*, 2000; Udo *et al.*, 2000). Latex agglutination has the advantages of being rapid, giving results on the same day, and easy to perform with very good sensitivity. This method could detect even low levels of PBP2a that are usually missed in routine disc diffusion methods. The only disadvantage is the cost factor. A study by Rohrer *et al.* (2001) proved that the sensitivity of the latex agglutination test can be improved (93.5 to 100 %) by induction with cefoxitin using growth from the edge of the inhibition zone of cefoxitin to perform the test.

One of the strains that was negative for *mecA* by PCR tested positive in the latex agglutination test. The result was confirmed by repeating both the tests. Sakoulas *et al.* (2001) faced the same discrepancy with one of their strains. They suggested the use of multiple colonies for PCR instead of a single colony as reported by most authors. In this study, multiple colonies were used for PCR, but this strain was still *mecA*-negative. Gene instability and primer design could be proposed as one of the reasons for the discrepancy. The key message is that such discrepancies should alert the microbiologist to this possibility as well.

Laboratory errors in the detection of MRSA cause grave clinical consequences. False positivity results in the needless use of reserve second-line drugs like vancomycin and linezolid. This leads to more drug resistance and increases

Table 2. Comparison of various methods for MRSA detection

The total number of MRSA isolates was 70. NPV, Negative predictive value; PPV, positive predictive value.

Method	No. of false negatives	No. of false positives	Sensitivity (%)	Specificity (%)	NPV	PPV
Oxacillin disc diffusion	6	1	91.4	99.2	95.5	98.4
Cefoxitin disc diffusion	1	0	98.5	100	99.2	100
Oxacillin screen agar	2	0	97.1	100	98.4	100
CHROMagar	2	1	97.1	99.2	98.4	98.5
Latex agglutination	0	1	100	99.2	100	98.5

in health-care costs. False-negative reports of MRSA lead to treatment failure, and increased nosocomial and community spread of this deadly microbe.

We found that the cefoxitin disc, as recommended by the CLSI, is a good method for MRSA detection but it should be supplemented with some other method so that no MRSA is missed. It is always advisable to combine two methods, one with high sensitivity and the other with high specificity. According to our results, the best combination is the cefoxitin disc diffusion method and the latex agglutination test. Since the latex agglutination test is expensive, it cannot be applied to all tests. Therefore, isolates that give a zone diameter of less than 20 mm can be easily reported as MRSA and only those with zone diameters of 20–22 mm need to be confirmed by latex agglutination. In conclusion, clinical microbiology laboratories associated with health-care facilities should combine screening with the cefoxitin disc along with another method that is feasible for that institute to reliably detect MRSA and control its spread.

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