

# ASSESSMENT OF DIFFERENT TESTS TO DETECT METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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**Abstract.** The heterogeneous expression of methicillin resistance in *Staphylococcus aureus* affects the efficiency of tests available to detect it. Not all laboratories have access to accurate molecular tests used for this purpose. This study compares the performances of four phenotypic tests used to detect methicillin resistant *S. aureus* (MRSA) with the *mecA* gene polymerase chain reaction. Two hundred thirty-seven *S. aureus* isolates were isolated from different patients visiting Sir Sundar Lal Hospital, Banaras Hindu University, Varanasi, India and subjected to cefoxitin and oxacillin disc diffusion tests, oxacillin minimum inhibitory concentration (MIC) test, and oxacillin screen agar test. The tests showed the following sensitivities and specificities, respectively: cefoxitin disc diffusion (98.5% and 100%), oxacillin disc diffusion (77.3% and 84.6%), oxacillin MIC (89.4% and 87.2%), and oxacillin screen agar (87.9% and 94.9%). The cefoxitin disc diffusion test can be the best method for routine detection of MRSA when molecular techniques are not available. We recommend the Clinical Laboratory Standards Institute (CLSI) cut-off point for determining cefoxitin resistance be reexamined to see if it should be revised from  $\leq 19$  mm to  $\leq 20$  mm.

## INTRODUCTION

The clinical importance of methicillin resistant *Staphylococcus aureus* (MRSA)– a public health threat– is immense. MRSA has resistance not only to methicillin but also other  $\beta$ -lactams and most other commonly used antibiotics (Brumfitt and Hamilton-Miller, 1989). It is a common cause of nosocomial infection (Maple *et al*, 1989) and a community pathogen (O'Brien *et al*, 1999; Said-Salim *et al*, 2003). Therefore, prompt and precise detection of MRSA is important

for the correct treatment of patients and the successful execution of infection control measures. A cost-effective test to detect MRSA is vital to curb the empiric use of vancomycin caused by the prevalence of MRSA and lack of routine testing for it in developing countries. Such a screening test is needed to generate the antibiogram for *S. aureus* strains prevalent in a health care institution and the community it serves, so that clinicians can choose correct antibiotics for surgical prophylaxis.

Several methods are available to detect methicillin resistance: *mecA* gene PCR, penicillin binding protein (PBP2a) detection, an MIC test (E test, agar or broth dilution), and screening in a medium containing oxacillin (Geha *et al*, 1994; Van Leeuwen *et al*, 1999;

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Louie *et al*, 2000, 2001; Swenson *et al*, 2001). However, factors such as incubation temperature and salt concentration, and genes other than *mecA* influence its expression, giving rise to heterogeneous resistance, in which a proportion of cells exhibit resistance while the others remain susceptible (De Lencastre and Tomasz, 1994; Chambers, 1997). The specificity of the tests may be inadequate due to the production of penicillin binding proteins other than PBP2a that have less affinity for methicillin, hyper-production of  $\beta$ -lactamase (enzymes that inactivate methicillin) or as yet unidentified means (Chambers, 1992; Geha *et al*, 1994; Kolbert *et al*, 1995). None of the methods used to detect methicillin resistance are able to detect it in all resistant strains of both *S. aureus* and coagulase negative staphylococci (Unal *et al*, 1995; York *et al*, 1996; Araj *et al*, 1999).

Detection of the *mecA* gene is the reference method to determine methicillin resistance (Chambers, 1997). Not all laboratories, especially those in developing countries, have funds or the trained staff needed to set up molecular techniques. This problem compelled us to evaluate various conventional tests available to select the best one to use in place of the detection of the *mecA* gene.

## MATERIALS AND METHODS

### Bacterial strains

Two hundred thirty-seven isolates of *S. aureus* were isolated from specimens of different patients visiting Sir Sundar Lal Hospital, Banaras Hindu University, Varanasi, India from August 2003 to December 2005. All specimens, except urine, for which cystine lactose electrolyte deficient agar was used, were cultured on blood agar and MacConkey agar (HiMedia, India). *S. aureus* was identified on the basis of colony morphology, Gram's staining, the catalase

test, tube coagulase test using rabbit plasma, slidex staph plus (Biomerurix India, India), thermonuclease and acetoin production, and mannitol fermentation. Following growth on blood agar, all the isolates were subjected to the studied tests.

### *mecA* gene PCR

Staphylococcal DNA was isolated using a chloroform, phenol extraction method (Sambrook *et al*, 1986). The primers used for detection of the *mecA* gene were *mecA1* (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and *mecA2* (5'-CCA ATT CCA CAT TGT TTC GGT CTA A), as determined by a previous study (Geha *et al*, 1994). A Biometra DNA thermocycler was programmed for initial denaturation at 94°C for 4 minutes; 30 cycles of amplification (denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 30 seconds); and a final extension at 72°C for 2 minutes. For visualization, 10  $\mu$ l of the PCR amplicon was loaded in 2% agarose gel in TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) containing 0.5  $\mu$ l/ml of ethidium bromide and visualized by using UV transillumination at 300 nm. DNA fragments of 310 bp corresponded to the *mecA* gene.

### Cefoxitin and oxacillin disc diffusion tests

Mueller-Hinton agar (MHA) plates were overlaid with a saline suspension with the isolate (turbidity matching 0.5 McFarland standard), and cefoxitin (30  $\mu$ g) and oxacillin (1  $\mu$ g) discs were placed after 10 minutes (HiMedia, India). After 24 and 48 hours of incubation at 35°C, the plates were read using the CLSI cut-off points as reference:  $\leq 19$  mm for cefoxitin and  $\leq 10$  mm for oxacillin (NCCLS, 2003b).

### Oxacillin MIC test

Gradient plates of MHA containing 2% NaCl were prepared with doubling dilutions (from 0.25 mg/l to 256 mg/l) of oxacillin. Inoculum was prepared by diluting a 0.5

Table 1  
Comparison of four tests used to detect MRSA with the *mecA* gene PCR.

Test	True positive	True negative	Sensitivity (%)	Specificity (%)
Cefoxitin disc diffusion	195	39	98.5	100
Oxacillin disc diffusion	153	33	77.3	84.6
Oxacillin agar screen	174	37	87.9	94.9
Oxacillin MIC	177	34	89.4	87.2

McFarland equivalent suspension of a strain with sterile normal saline to a concentration of  $10^4$  cfu/ml. The plates were spot-inoculated and incubated at 35°C for 24 hours. An oxacillin MIC of  $\leq 2$  mg/l indicated the strain was susceptible and  $>2$  mg/l resistant (NCCLS, 2003a).

#### Oxacillin screen agar test

A saline suspension of the isolate (turbidity identical to a 0.5 McFarland tube) was spotted on a MHA plate containing 6 µg/ml oxacillin and 4% NaCl. Any visible growth after 24 or 48 hours of incubation at 35°C was indicative of resistance (NCCLS, 2003b).

*S. aureus* ATCC 25923 (*mecA* negative) and ATCC 43300 (*mecA* positive) were used as controls for all the tests. The sensitivity and specificity of each test were calculated using the PCR results as a reference.

## RESULTS

Of the 237 *S. aureus* isolates, 198 were *mecA* positive and 39 *mecA* negative. Table 1 shows the results for each of the susceptibility tests in reference to the *mecA* gene PCR test results.

The cefoxitin disc diffusion test detected 195 of the 198 *mecA* positive strains and all 39 *mecA* negative strains, thus yielding a sensitivity and specificity of 98.5% and 100%, respectively. There were three false negative results: two strains with inhibition zones of 20 mm each and one with 23 mm.

The oxacillin disc diffusion, oxacillin agar screen, and oxacillin MIC tests detected 153, 174, and 177 of the 198 *mecA* positive strains, respectively, thus yielding sensitivities of 77.3, 87.9, and 89.4%, respectively. They identified 33, 37, and 34 of 39 *mecA* negative strains, respectively, yielding specificities of 84.6, 94.9, and 87.2%, respectively (Table 1).

## DISCUSSION

The cefoxitin disc diffusion method yielded the greatest efficiency, its results were easy to read in both transmitted and reflected lights. This test was least affected by the heterogeneous nature of methicillin resistance expression and testing conditions, as evidenced by the widespread preference for cefoxitin to detect MRSA (Felten *et al*, 2002; Skov *et al*, 2003; Boubaker *et al*, 2004; Cauwelier *et al*, 2004). A sensitivity and specificity of 100% was seen in one study (Velasco *et al*, 2005). In our study, had the CLSI cut-off point been  $\leq 20$  mm, two more strains would have been read as resistant, making the test 99.5% sensitive. While a new cut-off point of  $\leq 21$  mm has been proposed (Skov *et al*, 2006), we recommend a cut-off point of  $\leq 20$  mm. More studies are needed to evaluate the need to change the cut-off point and to determine the best cut-off point.

Our study repudiates the value of the oxacillin disc diffusion method as the best test to detect MRSA. To avoid error during

reading, we checked the plates carefully in transmitted light for any growth. Our results for the oxacillin disc diffusion test are comparable with the sensitivity of 61.3% and specificity of 96.7% reported by Cavassini *et al* (1999), but dissimilar to the sensitivity of 100% reported by Swenson *et al* (2001). The latter study had, however, a smaller number of heteroresistant isolates.

The poor sensitivity but relatively good specificity for the oxacillin screen agar test seen in our study is similar to the sensitivity of 82.5% and specificity of 98.3% reported by Cavassini *et al* (1999). A sensitivity of 100% with the agar screen has been reported (Thornberry and McDougal, 1983; Hindler *et al*, 1987). This difference may be due to the inclusion in our study of more isolates and the greater probably of heteroresistance. Testing greatly heteroresistant strains leads to decreased sensitivity (Resende and Figueiredo, 1997; Cavassini *et al*, 1999).

The agar dilution method detected 100% of MRSA isolates in two previous studies (Hindler and Inderlied, 1985; Unal *et al*, 1994). Our findings do not concur with their findings because the first study did not incorporate NaCl in the MHA and incubated the plates at 30°C for 48 hours, and the second study tested only 10 isolates. Another study obtained a sensitivity of 100% using Columbia medium but only 67% using Iso-sensitest medium (Weller *et al*, 1997). Therefore, the agar dilution test is influenced by conditions such as salt concentration, incubation period and temperature, number of strains tested, and the type of medium used.

In conclusion, with high efficiency, low price, and technical simplicity, the cefoxitin disc diffusion test was the best method to detect MRSA in our study, although it may be necessary to adjust the CLSI cut-off point for cefoxitin disc to  $\leq 20$  mm. The efficacy of the oxacillin disc diffusion method was lower than the cefoxitin disc diffusion test

in our study. Its use is common in laboratories in India and Nepal, but should be abandoned. The results of the oxacillin screen agar test are conflicting and the agar dilution method is affected by so various factors making these tests not as reliable for this purpose.

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