

Original Article

Comparison of Susceptibility Testing Methods for the Detection of Methicillin/Oxacillin Resistance in *Staphylococcus Aureus*

Hanan Ahmed Habib Babay

Department of Pathology/Microbiology, King Khalid University Hospital, College of Medicine, Riyadh, Saudi Arabia

Kuwait Medical Journal 2006, 38 (3): 198-202

ABSTRACT

Objective: To compare the accuracy of disk diffusion method and E-test for the detection of methicillin resistance and low-level methicillin-resistance in *Staphylococcus aureus* (*S. aureus*) and the PBP2a latex agglutination test for confirmation.

Materials and Methods: A total of 76 methicillin resistant *S. aureus* (MRSA) isolates from different clinical specimens were tested by disk diffusion method. Disk diffusion method was performed using methicillin (MET) 5µg disk, oxacillin (OX) 1 µg disk, moxalactam (MOX), and cefoxitin (FOX) 30 µg each on Mueller Hinton agar (MHA) plates supplemented with 2% NaCl and incubated at 35 °C for 24 hours. Minimum inhibitory concentration (MIC) was performed by E-test for MET and OX on MHA plates containing 2% NaCl. Results for all tests were read according to NCCLS recommendations for zone of inhibition and break points. Low-level MRSA strains were confirmed by PBP2a latex agglutination test. All strains were tested for β-lactamase production.

Results: All MRSA strains were detected by disk diffusion methods using MET, OX and FOX (100%). Four (5.2%) strains were low-level MRSA by MOX disk. E-test detected 72 (94.7%) using MET and 74 (97.3%) MRSA strains using OX. No heterogeneous growth within the zones of inhibitions was noticed. One MRSA was misclassified as methicillin sensitive by MET E-test (MIC 6 µg /ml), but was 32 µg/ml by OX E-test. Two strains were low-level MRSA by E-tests but showed resistance by MET, OX and FOX disk diffusion method. One strain had MIC of 12 µg/ml both by OX and MET E-tests. All four strains showed low-level resistance by MOX disk and were positive for PBP2a latex agglutination test. All the strains produced β-lactamase.

Conclusion: Disk diffusion method using MET, OX, and FOX can reliably be used to detect methicillin resistance in *S. aureus*. MOX and E-test can be used to detect low-level methicillin resistance and these can further be confirmed by PBP2a latex agglutination test in diagnostic laboratories.

KEYWORDS: cefoxitin, E-test, latex agglutination test, low-level MRSA, moxalactam, oxacillin, PBP2a

INTRODUCTION

Methicillin /oxacillin resistant *S. aureus* is considered a major nosocomial and community acquired pathogen throughout the world^[1-3]. It is implicated in serious clinical conditions such as bacteremia, pneumonia, intra-abdominal infection and others^[2]. Accurate detection and confirmation of MRSA is essential for the institution of antimicrobial therapy and implementation of infection control measures. However, low-level (borderline) MRSA is often misdiagnosed as methicillin sensitive *S. aureus* (MSSA)^[4]. These strains are characterized by an OX MIC at or just above the susceptibility break point (4-8 µg/ml) and called borderline oxacillin resistant *S. aureus* (BORSA)^[5-7]. These strains may carry *mecA* and are extremely heterogeneous or produce PBP2a or be penicillinase hyperproducers^[5-7].

Bacterial populations that express the resistant phenotype may be heterogeneous and resistance expression may vary according to culture conditions^[5]. Several studies have focused on failure of conventional methods to identify low-level MRSA strains^[8-10]. This has led to the modification of laboratory protocols such as increased salt concentration in culture media, decreased temperature of incubation (30-35 °C) and increased incubation time (24 hrs) to enhance expression of resistance. These are presented by several tests such as OX agar screening test, OX disk diffusion, broth microdilution and rapid tests such as latex agglutination MRSA screen test, rapid ATB Staph and automated Vitek system^[9,11-13]. These differ in sensitivity and specificity. The molecular method, PCR detects *mecA* gene (the structural gene for penicillin binding protein 2a (PBP2a) which

Address correspondence to:

Dr. Hanan A.H. Babay, MD.KSFel Path (Mic), King Khalid University Hospital, Department of Pathology/Microbiology (32), P.O. Box 2925, Riyadh 11461, Saudi Arabia. Tel: 01-4672457, Fax: 01-4672462, E-mail: hahabib@ksu.edu.sa

is found in methicillin resistant *Staphylococcus* strains) and is considered the 'gold standard' method^[11,14]. However, it is not available in all clinical laboratories. Recent reports from Japan on the use of cephamycin, cefoxitin (FOX) and moxalactam (MOX) for routine detection of all classes of MRSA have proved to be good for the detection of low-level methicillin resistance in *S. aureus*.

The purpose of this study was to compare the accuracy of different methods for the detection of methicillin resistance and low-level methicillin resistance in *S. aureus* i.e., the METOX, FOX, and MOX disk diffusion method, E-test and confirmation by PBP2a latex agglutination test .

MATERIALS AND METHODS

Bacterial strains: This prospective study took place at King Khalid University Hospital, Riyadh, Saudi Arabia between 28/1/2003 and 31/8/2003 on 76 unselected clinical isolates of MRSA. The strains were recovered from different cultures of specimens (wound, skin, sputum, tracheal aspirates, nose swabs, blood, eyes, central lines and urine) of patients admitted to the hospital. Only one isolate was considered from each patient. Control used was a susceptible *S. aureus* ATCC 25923 strain (Remel, USA).

Susceptibility testing methods: Inocula: the inocula for susceptibility testing were made using suspensions from overnight cultures of MRSA on Mueller Hinton broth (Mueller Hinton, Becton Dickinson, USA). An inoculum equivalent to 0.5 McFarland (10^8 CFU/ml) turbidity standard was used for each test.

Disk diffusion method for MET, OX, FOX, and MOX: this was performed on MHA plates supplemented with 2% NaCl (Mueller Hinton, Becton Dickinson, USA). After inoculation with the MRSA strains, 5 µg MET, 1 µg OX, 30 µg each of FOX, and MOX disks (Oxoid Basingstoke, Hampshire, England) were applied on the surface of the plates and then incubated at 35 °C for 24 hours. Resistance was determined according to National Committee for Clinical Laboratory standards (NCCLS) where a zone diameter of < 9 mm was considered resistant for MET and < 10 mm for OX^[15,16]. For FOX and MOX a zone < 14 mm and < 19mm were respectively considered resistant^[16]. For all, no zone was also considered homogeneous resistance. Heterogeneous resistance was defined as the presence of small colonies in the circular growth inhibition area^[4].

E-test: E-test (AB Biodisk, Solna, Sweden) done to determine MICs for OX and MET were

Table 1: Percentages of MRSA and low-level MRSA strains obtained by different tests

Test	MRSA n (%)	Low level MRSA n (%)*
Disk Diffusion		
MET 5 µg	76 (100)	-
OX 1 µg	76 (100)	-
FOX 30 µg	76 (100)	-
MOX 30 µg	72 (94.7)	4 (5.2)
E-test		
MET	72 (94.7)	4 (5.2)
OX	74 (97.3)	2 (2.6)

*All low-level MRSA strains were positive for PBP2a latex agglutination test.

performed on MHA containing 2% NaCl (Mueller Hinton II, Becton Dickinson, USA) according to manufacturer's instructions. MIC for OX and MET susceptible strains were < 2 µg/ml and < 8 µg/ml respectively, according to NCCLS break points.

PBP2a latex agglutination test (Oxoid, Basingstoke, UK): This was performed for confirmation on isolates with low-level methicillin resistance .

β-lactamase test: All strains were tested for β-lactamase production by streaking colonies from the edge of *S. aureus* onto a nitrocefin disk (Cefinase, Becton Dickinson, USA). β-lactamase production was noticed by the appearance of pink color within a minute and no change in color indicated negative test.

RESULTS

MRSA strains in this study were isolated from wound and skin swabs 30 (39.4%), sputum / tracheal aspirate 20 (26.3%), nose swabs 14 (18.4%), blood and central lines four each (5.2%), eye swabs three (3.9%), and one urine specimen (1.3%). All MRSA isolates were detected by disk diffusion using MET, OX, and FOX disks but MOX detected 72 MRSA (94.7%) and four (5.2%) low-level MRSA isolates. Similarly, E-test detected 72 MET and 74 OX MRSA strains (sensitivity 94.7% and 97.3 % respectively, Table 1). Table 2 shows the results of susceptibility testing of 76 MRSA strains as determined by different methods. The range of inhibition zone diameters for FOX were between 5-18 mm for 11 (14.4%) of the strains and for MOX between 7-20 mm for 12 (15.7%) of the strains. All MRSA strains showed no zone with MET disk diffusion test (100%) and only two MRSA strains showed 9 and 10 mm zones with OX disk respectively (2.6%). No heterogeneous growth was observed within the inhibition zones. In the shadowed column, one MRSA (1.3%) strain was misclassified as MSSA by MET E-test, with an MIC of 6 µg/ml whilst the OX MIC was 32 µg/ml. Two strains were low-level MRSA by both MET and OX E-test (2.6%) and one strain had an MIC of 12

Table 2: Results of susceptibility testing of 76 MRSA isolates

No. of isolates	Disk diffusion (zone diameter)				E-test ($\mu\text{g/ml}$)	
	MET ($<9\text{mm}$)	OX ($<10\text{mm}$)	FOX ($<19\text{mm}$)	MOX ($<14\text{mm}$)	MET	OX
63	0*	0	0	0	>256	96->256
1	0	0	7	7	>256	>256
1	0	10	18	19	12	12
1	0	9	17	19	8	4
1	0	0	16	17	12	4
1	0	0	18	20	6	32
1	0	0	8	8	>256	32
1	0	0	6	8	>256	>256
1	0	0	14	10	>256	>256
1	0	0	0	9	>256	>256
1	0	0	0	10	>256	>256
1	0	0	14	14	>256	>256
1	0	0	5	0	>256	>256
1	0	0	13	14	128	>256
Control	Zone range (18-27mm)	Zone range (19-28mm)	Zone range (24-36mm)	Zone range (25-33mm)	MIC range (0.25-4 $\mu\text{g/ml}$) for control	0.25-2.5 $\mu\text{g/ml}$ for control

0* (no zone) homogeneous resistance; 2: low-level resistant MRSA, 1: MSSA and 1: intermediate resistant, all 4 were positive for PBP2a latex agglutination test

$\mu\text{g/ml}$ to both OX and MET (Table 1). PBP2a latex agglutination test was positive for these four low-level MRSA strains. In addition, these were isolated from wound and skin swabs. Seventy one (93%) of the strains had MET MIC > 256 $\mu\text{g/ml}$ and 70 (92.1%) had OX MIC > 256 $\mu\text{g/ml}$ which belonged to one or the other of the minor classes of Tomasz *et al* classification of MRSA, where classes one to three were heterogeneous, and class four was homogeneous; the methicillin MIC for class four was > 800 mg/l. For the major populations of class one to three isolates, methicillin MICs were 1.5 to 100 mg/l, respectively, and for the minor populations, 10^{-8} to 10^{-2} , respectively methicillin MICs were 100 mg/l^[17]. All strains produced β -lactamase.

DISCUSSION

Many laboratories have problems with MRSA and/or low-level MRSA detection, even for those that do use more than one method for detection or screening. However, most diagnostic laboratories used disk diffusion method for the detection of MRSA^[1,4,7,8,18]. In one study involving 40 laboratories, the sensitivity of detection of MRSA by disk diffusion using 1 μg OX disk was 100% and 97.2% with 5 μg MET disk whilst a sensitivity of 99% was reported for both MET and OX agar screening methods^[19]. However, other laboratories have reported a failure rate of 64% in detecting MRSA using 1 μg OX disk^[10]. Most laboratories used OX rather than MET in USA where OX has replaced MET because of its instability^[6]. However, one

should be mindful of the study of Van Griethuysen *et al*, which showed the sensitivity of OX screen agar to be only 93.6% and lower than the sensitivity of MRSA screen test and concluded that the risk of misclassification of MRSA as MSSA was 4.3 times higher by OX agar screen test^[13]. Although most of the references in this paper used OX 6 $\mu\text{g/ml}$ as against OX 1 $\mu\text{g/ml}$ in our work, our results are quite similar to the referenced ones^[4,12,13,20]. Mackenzie *et al*, suggest that differences in media of different manufacturers are important for disk diffusion test in which there is no supplemental salt^[18]. Bowers *et al* used mannitol salt agar, Baird-Parker agar with ciprofloxacin and bromocresol purple for the isolation of *S.aureus* as a preliminary step to testing for MRSA and found that all selective media performed equally well with 80% MRSA isolation rate^[21]. Atoum *et al*, compared the disk diffusion with PCR and microdilution methods and reported least sensitivity with disk diffusion method^[14]. Similarly, Chambers in 1997 stated that disk diffusion suffers from low specificity averaging 80% relative to other methods^[5]. Although the NCCLS recommend the use of OX disk method on a swab inoculate using MHA plate supplemented with 2% NaCl at 35 °C and OX agar screen test using MHA with 4% NaCl, most laboratories do not comply with these recommendations^[4]. Mackenzie *et al*, in two studies, reported that there is no correlation between the accuracy of the results and compliance with NCCLS recommendations and he recommended the use of low-expression class MRSA strain as a control for the NCCLS disk test^[8,18].

Cephamecins were extensively used in early 1980s in Japan and resulted in some MSSA and MRSA became resistant to FOX^[4]. Moriyasu *et al* (1994) and Okonogi *et al* (1989) reported that FOX induced production of PBP2a *in vitro* in MSSA for which FOX MIC were high and proved that disk diffusion with cephamycins is a good assay for detection of low-level MRSA in Japan^[22,23]. In addition, cephamycins have good affinity for *S. aureus* PBP4 which is involved in cell wall cross linkage^[24]. Although our sample was small, the results with the use of FOX and MOX were comparable to OX and MET disk diffusion methods and to the results of Felten *et al*, although MOX detected low-level MRSA compared to FOX in our study^[4]. Felten *et al*, found that FOX and MOX disk diffusion methods are suitable for detection of MRSA of all classes, have 100 % specificity and can be useful alternatives to OX^[4]. Skov *et al* used FOX 5 and 10 μg discs and all *Staphylococcus* isolates were tested on Iso-sensitest agar and MHA plates and the results were superior to OX with > 99% sensitivity and specificity for both discs^[25]. In a

study comparable to our study, Velasco *et al*, tested 51 MRSA isolates using OX and FOX in addition to cefazolin, cefotaxime and imipenem discs. The results showed 100% sensitivity for FOX disc and reported to be the best predictor of methicillin resistance in *S.aureus* whilst other discs showed 100% specificity^[26]. However, Chambers reported that the use of β - lactam antibiotics other than MET or OX especially, cephamycin is not recommended since it further reduces the accuracy of the test^[5]. Frebourg *et al* reported that OX E-test is reliable alternative to conventional agar or broth dilution methods^[9]. It showed 98.4% agreement with OX agar screen plate test in their study and is considered a very reliable method by the NCCLS although it has a maximum sensitivity of 95.9% according to Van Griethuysen *et al*^[13]. E-test with MET was less sensitive than OX E-test for the detection of low-level MRSA in our study.

All our isolates produced β - lactamase. The role of β - lactamase is unclear, however, it has been reported that even in low-level resistance, β - lactamase stable antibiotics could be hydrolysed by *Staphylococcus* β - lactamase, and over- production of β - lactamase could result in borderline MIC^[5]. It has been observed that culture conditions used to enhance MRSA also favor production of β - lactamase^[5]. Chambers reported that borderline strains that hyperproduce β - lactamase are mecA negative, show high levels of β - lactamase activity , and lowered the MIC into susceptible range upon addition of β - lactamase inhibitors^[5].

Most of our MRSA and low-level MRSA strains came from wound and skin swabs. This is similar to the study of Felten *et al*, in which it was reported that MSSA isolates from skin lesions probably acquired the mecA gene by horizontal transfer from other skin *Staphylococcus* species^[4,27]. The clinical implication of low-level MRSA is the possibility of fatal community acquired invasive sepsis as reported by the Centers for Disease Control and Prevention^[28]. Fortunately, our isolates did not result in serious infections or death.

PBP2a latex agglutination test is latex particles sensitized with a monoclonal antibody against PBP2a and react specifically with MRSA to cause agglutination in three minutes. It is reported to have a 97.6% sensitivity and it distinguishes between very low-level MRSA from MSSA^[4,17]. Bowers *et al*, reported MRSA-latex agglutination test as a reliable and rapid detection test from both pure culture and selective media as well as being a reliable alternative to mec A PCR for the definitive diagnosis of MRSA^[21]. A problem was raised by Atoum *et al* when they reported strains of negative mecA being MET resistant and positive mecA being MET sensitive. These observations are explained as

being due to non-functional mecA gene or non-active PBP2a protein. Consequently, they recommended the use of a combination of both molecular and microbiological methods for detection of MRSA^[4]. However, PBP2a latex agglutination method has demonstrated 100% agreement for both mecA-positive and negative strains^[6]. Furthermore, most diagnostic laboratories do not have efficient resources to provide molecular techniques on routine basis.

In conclusion, disk diffusion method using MET, OX, and FOX disks is reliable for detection of MRSA. For low-level MRSA, MOX disk diffusion and E-test OX and MET are reliable and for confirmation PBP2a latex agglutination test can be used. Laboratories should be aware of the shortcomings of tests available to detect MRSA and low-level MRSA. The best approach would be to have several methods available and use an alternative test when resistance is suspected but not detected by routine methods.

ACKNOWLEDGMENT

We would like to thank Dr Kingsley Twum-Danso for revising the manuscript and Mr. Kutubu Manneh for technical assistance.

REFERENCES

1. Seal JB, Moreira B, Bethel CD, Daum RS. Antimicrobial resistance in *Staphylococcus aureus* at the University of Chicago Hospitals: a 15-years longitudinal assessment in a large university hospital. *Infect Control Hosp Epidemiol* 2003; 24:403-408.
2. Lowy FD. *Staphylococcus aureus* infections. *New Eng J Med* 1998; 339:520-532.
3. Said-Salim B, Mathema B, Kreiswirth BN. Community - acquired methicillin - resistant *Staphylococcus aureus* : an emerging pathogen. *Infect Control Hosp Epidemiol* 2003; 24:451-455.
4. Felten A, Grandry B, Lagrange PH, Casin I. Evaluation of three techniques for detection of low-level methicillin-resistant *Staphylococcus aureus* (MRSA) : a disk diffusion method with cefoxitin and moxalactam, the Vitek 2 system, and the MRSA - screen latex agglutination test. *J Clin Microbiol* 2002; 40:2766-2771.
5. Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin Microbiol Rev* 1997; 10:781-791.
6. Hall GS. MRSA: Lab detection, epidemiology, and infection control. *Microbiology Frontline* 2003; 3:1-6.
7. Brown DFJ. Detection of methicillin / oxacillin resistance in staphylococci. *J Antimicrob Chemother* 2001; 48S1:65-70.
8. Mackenzie AMR, Richardson H, Missett P, Wood DE, and Groves DJ. Accuracy of reporting of methicillin-resistant *Staphylococcus aureus* in a provincial quality control program: A 9-year study. *Clin Microbiol* 1993; 31:1275-1279.
9. Frebourg NB, Nouet D, Lemee L, Martin E, and Lemeland JF. Comparison of ATB Staph, Rapid ATB Staph, Vitek, and E-test methods for detection of oxacillin heteroresistance in staphylococci possessing mecA. *J Clin Microbiol* 1998; 36:52-57.

10. Aldridge KE, Janney A, Sanders CV, and Marier RL. Interlaboratory variation of antibiograms of methicillin-resistant and methicillin - susceptible *Staphylococcus aureus* strains with conventional and commercial testing systems. *J Clin Microbiol* 1983; 18:1226-1236.
11. Fang H, and Hedin G. Rapid screening and identification of methicillin-resistant *Staphylococcus aureus* from clinical samples by selective - broth and Real-Time PCR Assay. *J Clin Microbiol* 2003; 41:2894-2899.
12. Sakoulas G, Gold H, Venkatarman L, DeGirolami PC, Eliopoulos GM, Qian Q. Methicillin-resistant *Staphylococcus aureus*: comparison of susceptibility testing methods and analysis of mecA-positive susceptible strains. *J Clin Microbiol* 2001; 39:3946-3951.
13. Van Griethuysen A, Pouw M, VanLeeuwen N, Heck M, Willemse P, Buiting A, and Kluytmans J. Rapid slide latex agglutination test for detection of methicillin resistance in *Staphylococcus aureus*. *J Clin Microbiol* 1999; 37:2789-2792.
14. Atoum MF, Akel H, Battikhi MN. Comparison of PCR and disc diffusion methods in detecting methicillin resistance among *Staphylococcus species* from nosocomial infections. *Saudi Med J* 2003; 24:1410-1412.
15. Performance standards for antimicrobial susceptibility testing. NCCLS document 1999. Ninth informational supplement, M100-S9, Wayne, Pa.
16. Clinical Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing 2005. CLSI approved standard M100-S15. Clinical and Laboratory Standards Institute, Wayne, PA.
17. Yamazumi T, Marshall SA, Wilke WW, Diekema DJ, Pfaller MA, and Jones RN. Comparison of Vitek gram positive susceptibility 106 card and the MRSA-screen latex agglutination test for detecting oxacillin resistance in clinical bloodstream isolates of *Staphylococcus aureus*. *J Clin Microbiol* 2001; 39:53-56.
18. Mackenzie AMR, Richardson H, Lannigan R, and Wood D. Evidence that the National Committee for Clinical Laboratory standards disk test is less sensitive than the screen plate for detection of low-expression - class methicillin - resistant *Staphylococcus aureus*. *J Clin Microbiol* 1995; 33:1909-1911.
19. Jones RN, Barry AL, Gardiner RV, Packer RR. The prevalence of Staphylococcal resistance to penicillinase - resistant penicillins. A retrospective and prospective national surveillance trial of isolates from 40 medical centers. *Diagn Microbiol Infect Dis* 1989; 12:385-394.
20. Louie L, Matsumura SO, Choi E, Louie M, Simor AE. Evaluation of three rapid methods for detection of methicillin-resistance in *Staphylococcus aureus*. *J Clin Microbiol* 2000; 38:2170-2173.
21. Bowers KM, Wren MWD, Shetty NP. Screening for methicillin resistance in *Staphylococcus aureus* and coagulase- negative staphylococci : an evaluation of three selective media and Mastalex-MRSA latex agglutination. *British J Biomed Science* 2003; 60:71-74.
22. Moriyasu I, Igari J, Yamane N, Oguri T, Takahashi A, Tosaka M, Takemori K, Toyoshima S, and Minamide W. Multicenter evaluation of Showa cefizoxime susceptibility test to discriminate between the strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and those susceptible (MSSA). *Rinsho Byori* 1994; 42:271-277.
23. Okonogi K, Nogi Y, Kondo M, Imada M, and Yokota T. Emergence of methicillin - resistant clones from cephamycin-resistant *S. aureus*. *J antimicrob Chemother* 1989; 24:637-645.
24. Murakami K, Nomura K, Doi M, and Yoshida T. Increased susceptibility to cephamycin-type antibiotics of methicillin - resistant *Staphylococcus aureus* defective in penicillin binding protein 2. *Antimicrob Agents Chemother* 1987; 31:1423-1425.
25. Skov R, Smyth R, Larsen AR, Frimodt-Moller N, Kahlmeter G. Evaluation of ceftiofex 5 and 10 {micro} g discs for the detection of methicillin resistance in staphylococci. *J Antimicrob Chemother* 2005; 55:157-161.
26. Velasco D, Del Mar Tomas M, Cartelle M, Beceiro A, Perez A, Molina F, Moure R, Villanueva R, Bou G. Evaluation of different methods for detecting methicillin (oxacillin) resistance in *Staphylococcus aureus*. *J Antimicrob Chemother* 2005; 55:379-382.
27. Hiramatsu K. Molecular evolution of MRSA. *Microbiol Immunol* 1995; 39:531-543.
28. Centers for Disease Control and Prevention: Four pediatric deaths from community - acquired methicillin - resistant *Staphylococcus aureus* - Minnesota and North Dakota, 1997 - 1999. *JAMA*; 282:1123-1125.