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


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 ºC 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 (BORA)[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[8]. 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 ºC) 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\textsuperscript{[11,14]. However, it is not available in all clinical laboratories. Recent reports from Japan on the use of cephaparin, 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 \textit{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 \textit{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 \textit{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\textsuperscript{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\textsuperscript{[15,16]}. For FOX and MOX a zone < 14 mm and < 19mm were respectively considered resistant\textsuperscript{[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\textsuperscript{[16]}.

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

\textit{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 \textit{MRSA} (94.7%) and four (5.2%) low-level MRSA isolates. Similarly, E-test detected 72 MET and 74 OX \textit{MRSA} strains (sensitivity 94.7% and 97.3 % respectively, Table 1). Table 2 shows the results of susceptibility testing of 76 \textit{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 \textit{MRSA} strains showed no zone with MET disk diffusion test (100%) and only two \textit{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 \textit{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 \textit{MRSA} by both MET and OX E-test (2.6%) and one strain had an MIC of 12

\begin{table}[h]
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\begin{tabular}{|l|c|}
\hline
\textbf{Test} & \textbf{MRSA n (%)} & \textbf{Low level MRSA n (%)}\tabularnewline \hline
\textbf{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) \\
\hline
\textbf{E-test} & & \\
MET & 72 (94.7) & 4 (5.2) \\
OX & 74 (97.3) & 2 (2.6) \\
\hline
\end{tabular}
\caption{Percentages of \textit{MRSA} and low-level \textit{MRSA} strains obtained by different tests}
\end{table}

\*All low-level MRSA strains were positive for PBP2a latex agglutination test.
Table 2: Results of susceptibility testing of 76 MRSA isolates

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Disk diffusion (zone diameter)</th>
<th>E-test (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>MET (≤9mm)</td>
<td>OX (≤10mm)</td>
</tr>
<tr>
<td>63</td>
<td>0*</td>
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<tr>
<td>1</td>
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<td>1</td>
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</table>

Control: Zone MIC range for control: 0.25–2.5 µg/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.

µg/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 µg/ml and 70 (92.1%) had OX MIC > 256 µg/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 µg/l. For the major populations of class one to three isolates, methicillin MICs were 1.5 to 100 µg/l, respectively, and for the minor populations, 10 to 100 µg/l, respectively methicillin MICs were 100 mg/l. 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[1,4,7,18,19]. 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[8]. However, other laboratories have reported a failure rate of 64% in detecting MRSA using 1 µg OX disk. Most laboratories used OX rather than MET in USA where OX has replaced MET because of its instability[8]. 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[8]. Although most of the references in this paper used OX 6 µg/ml as against OX 1 µg/ml in our work, our results are quite similar to the referenced ones[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[20]. 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[8]. Atoum et al, compared the disk diffusion with PCR and microdilution methods and reported least sensitivity with disk diffusion method[8]. Similarly, Chambers in 1997 stated that disk diffusion suffers from low specificity averaging 80% relative to other methods[8]. 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[8]. 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].

Cephamycins were extensively used in early 1980s in Japan and resulted in some MSSA and MRSA became resistant to FOX[8]. 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[8]. 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[8]. Skov et al used FOX 5 and 10 mug 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[20]. 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[20]. However, Chambers reported that the use of ß- lactam antibiotics other than MET or OX especially, cephapenem is not recommended since it further reduces the accuracy of the test[3]. Frebouerg et al reported that OX E-test is reliable alternative to conventional agar or broth dilution methods[6]. 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[10]. 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 ß- lactamase. The role of ß- lactamase is unclear, however, it has been reported that even in low-level resistance, ß-lactamase stable antibiotics could be hydrolysed by Staphylococcus ß- lactamase, and over-production of ß- lactamase could result in borderline MIC[5]. It has been observed that culture conditions used to enhance MRSA also favor production of ß- lactamase[5], Chambers reported that borderline strains that hyperproduce ß- lactamase are mecA negative, show high levels of ß- lactamase activity, and lowered the MIC into susceptible range upon addition of ß- 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[24][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 mecA 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[41]. However, PBP2a latex agglutination method has demonstrated 100% agreement for both mecA-positive and negative strains[49]. 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.

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REFERENCES


