Comparison of Two Different Sensitivity Testing Agars for Detecting Methicillin Resistance in Staphylococcus Aureus

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ABSTRACT

Objective: To compare the accuracy of Mueller-Hinton agar and Isosensitest agar using cefoxitin disc for detecting methicillin resistant Staphylococcus aureus using mecA gene PCR assay as gold standard.

Study Design: Comparative study.

Place and Duration of Study: Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi, from May 2006 to January 2007.

Patients and Methods: One hundred clinical isolates of Staphylococcus aureus were evaluated; 64 MRSA (methicillin resistant Staphylococcus aureus) and 36 MSSA (methicillin sensitive Staphylococcus aureus) by mecA PCR assay. All the isolates were tested with cefoxitin 30 µg disc using semi-confluent growth on Mueller-Hinton agar as well as on Iso-sensitest agar in ambient air at 35-37°C after an overnight incubation as per recommendations of Clinical and Laboratory Standard Institute.

Results: Following diameters provided the best sensitivity and specificity without substantial overlapping between the zones of resistant and sensitive isolates; Mueller-Hinton agar: R ≤ 20 mm (sensitivity 100% and specificity 100%), S ≥ 22 mm (sensitivity 97.2% and specificity 100%), and Iso-sensitest agar: R ≤ 26 mm (sensitivity 100% and specificity 100%), S ≥ 26 mm (sensitivity 100% and specificity 100%). High accuracy was obtained with cefoxitin disc on both media.

Conclusion: Performance of both media was equally convincing for reliable prediction of methicillin resistance in Staphylococcus aureus by placing cefoxitin 30 µg disc on either of these in routine susceptibility testing.

Key words: Iso-sensitest agar. Mueller-Hinton agar. MRSA. mecA gene. Cefoxitin.
using cefoxitin 30 µg disc and semi-confluent growth for the detection of MRSA in terms of sensitivity and specificity associated with proposed zone sizes for reliable and accurate detection of MRSA.

PATIENTS AND METHODS

The study was conducted at the Microbiology department, Armed Forces Institute of Pathology, Rawalpindi, from May 2006 to January 2007. One-hundred clinical isolates of *Staphylococcus aureus* were collected from patients irrespective of age and gender by non-probability convenience sampling. Exclusion criteria incorporated were MRSA isolates from outbreaks and repeat samples from the same patients. All strains were identified as *Staphylococcus aureus* by biochemical procedures (Gram's staining, Catalase, Coagulase and DNase test). Sixty-four strains harbouring the *mecA* gene and 36 lacking this gene were detected by PCR.7 The methicillin susceptible *S. aureus* strain ATCC 29213 (Oxacillin MIC ≤ 0.5 µg/ml), ATCC 25923 and MRSA ATCC 51153 were used as controls. After identification, the isolates were sub-cultured on slopes of Mueller-Hinton agar (Oxoid, Basingstoke, UK) and stored at -70°C. Prior to further testing, they were sub-cultured on sheep blood agar plates and incubated at 35°C for 24 hours.

The break point MICs were performed according to CLSI (formerly NCCLS) recommended standards. Spot inoculation of colony suspension (10⁶ cfu/spot) was done on M-H plates (Oxoid, Basingstoke, UK) using Denley's spot inoculator. The agar was supplemented with sodium chloride 2% (NaCl) and Oxacillin (Oxoid, Basingstoke, UK) concentrations of 2 µg/ml and 4 µg/ml. The plates were incubated at 33°C for 24 hours and then examined for any colonies or a film of growth indicating oxacillin resistance. Controls were included with each batch of MIC determination.8

Cefoxitin disc 30 µg (Oxoid, Basingstoke, UK) was placed on M-H and ISA plates (Oxoid, Basingstoke, UK) overlaid with a standard inoculum (10⁶ cfu/ml) yielding semi-confluent growth of *Staphylococcus aureus* and incubated at 35-36°C in ambient air. Zone diameters were measured between 18-20 hours, using reflected light. For cefoxitin on M-H agar, CLSI recommendations were followed. For ISA cefoxitin sensitivity of 100% was the criterion for determining the interpretative zone diameter of resistance. Using calipers, inhibition zone diameters were measured to the nearest millimeter at the inner zone edge.6

The *mecA* gene PCR was performed in accordance with the procedure described by Sakoulas *et al.*9 Bacterial DNA was extracted from overnight growth of *Staphylococcus aureus* in BHI using DNA extraction kit (Symbiosis Asti, Italy). The chromosomal DNA was used as template in the PCR.

The primer pair used was (5'-CTCAGGTACTGCTATCCACC-3') and (5'-CACCTGGTATATCTTCACC-3') (Gene link, Hawthorne, NY). Amplification of reaction mixture was carried out in thermal cycler (Master cycler, Eppendorf, Hamburg, Germany). The 50 µl PCR mixture consisted of 5 µl of the extracted bacterial DNA, each nucleotide (MBI, Fermantas, USA) at a concentration of 0.2 mM, 2.5 mM MgCl₂, 0.25 µM of each primer and 0.25 U of Taq polymerase (MBI, Fermantas, USA). PCR buffer and 2.5 mM MgCl₂ were supplied by the manufacturer. The PCR program consisted of DNA denaturation of 5 minutes at 95°C; 30 cycles with a 30 seconds denaturation step at 94°C; 30 seconds annealing step at 42°C; 30 seconds extension at 72°C and a final 10 minutes extension step at 72°C. The amplified product of *mecA* gene (448-bp DNA fragment) was detected by 1% agarose gel electrophoresis with ethidium bromide staining and observing under UV light.

Data was compiled and analysed by using SPSS for window release 14.0 (SPSS Inc; Chicago, IL, USA) based on the *mecA* gene analysis, *S. aureus* isolates were classified as resistant or sensitive. The means of zone sizes (mm) around 30 µg cefoxitin disc of the isolates were compared by applying independent t-test. The sample means, standard deviations and the ranges of zone sizes (mm) of both the resistant and sensitive isolates were calculated. The data was quantitative and normally distributed. P-values of < 0.05 were interpreted as significant. Sensitivity, specificity, positive predictive values and negative predictive values for zone sizes on both the media were also calculated using *mecA* gene PCR assay as gold standard.

RESULTS

PCR detected *mecA* gene amplified product of 448-bp DNA (Figure 1) in 64 of the hundred *S. aureus* isolates thus confirmed as MRSA and 36 isolates without this gene as MSSA. All the isolates had their breakpoint MICs within the CLSI defined range i.e. R ≥ 4 µg/ml and S ≤ 2 µg/ml (Figure 1).

Cefoxitin disc on ISA had an overall sensitivity and specificity of 100%, the Positive Predictive Value (PPV) and the Negative Predictive Value (NPV) were again 100% with highest accuracy, considering the proposed zone diameter breakpoints of R < 26 mm and S > 26 mm. By the proposed zone diameters breakpoint of R < 20 mm and S > 22 mm for cefoxitin disc on M-H agar yielded 100% sensitivity and specificity, identifying all the MRSA isolates accurately. The overall accuracy of the cefoxitin disc was calculated to be 100% for MRSA isolates keeping PCR as gold standard.
The mean, SD, ranges of the zone sizes and confidence intervals of all the sensitive and resistant *Staphylococcus aureus* isolates are presented in Table I.

No significant difference was detected between the zones of resistant isolates on M-H agar and ISA (p=0.158) while a significant difference was noted between the zone sizes of sensitive isolates (p=0.0001) on the two media (Table I).

**Table I**: Zones of inhibition around cefoxitin disc 30 µg against *mecA* positive and *mecA* negative *Staphylococcus aureus* isolates (n=100) on Mueller-Hinton agar and Iso-sensitest agar.

<table>
<thead>
<tr>
<th>meca</th>
<th>Positive MRSA (n=64)</th>
<th>meca</th>
<th>Negative MSSA (n=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-H</td>
<td>Range (CI)</td>
<td>Mean ± SD</td>
<td>P-value</td>
</tr>
<tr>
<td></td>
<td>0-18 (1.69-4.38)</td>
<td>3.03 ± 5.38</td>
<td>0.158</td>
</tr>
<tr>
<td></td>
<td>ISA</td>
<td>0-26 (2.73-6.77)</td>
<td>4.75 ± 8.07</td>
</tr>
</tbody>
</table>


For cefoxitin disc on M-H agar, the ranges of the inhibition zones around the antibiotic disc for the MRSA isolates were measured as 6 mm (no zone), while 17 isolates had their zones between 10-18 mm. Among the MSSA isolates, 25 isolates yielded the zones higher than 30 mm whereas 10 isolates yielded between 22-29 mm (Figure 2). One isolate yielded a zone of 15 mm. Moreover, no considerable zone variation was seen between zone sizes taken at 24 hours and 48 hours.

In case of ISA, zones of inhibition for MRSA isolates were between 0-26 mm, with 45 isolates showing no zones at all, rest of the isolates had zone diameters between 8-26 mm. Among MSSA isolates, the zone diameters were between 30-38 mm with fair margin for the proposed zone diameters breakpoints (Figure 3).

**DISCUSSION**

Accurate and prompt identification of MRSA is the need of the time. With a rapid increase in number of MRSA infections as well as increase in their heterogeneity, many microbiology laboratories in Pakistan now require reliable routine phenotypic methods to be introduced in the absence of genotypic facilities. The mainstay is simplicity and cost effectiveness.

PCR for *mecA* gene is considered to be the gold standard for detecting MRSA. Countries overwhelmed by MRSA infection, including Pakistan, require a co-ordinated effort to bring MRSA under control. The enormous investment in PCR facilities suggest that for all practical purposes the phenotypic methods should be moulded for maximum benefit.

Most of the European as well as American antimicrobial susceptibility testing societies have recommended semi-confluent growth for routine antibiotic susceptibility testing agars for methicillin resistance in *Staphylococcus aureus*.
testing. For the identification of MRSA, confluent growth is recommended requiring separate medium, separate plate, and different temperature, up to 35°C. To overcome this extra effort, an inoculum-yielding semi-confluent growth for cefoxitin, on routine susceptibility testing, 9 cm agar plate was used thereby decreasing the workload and reducing the cost.\textsuperscript{2,5}

The study suggested that semi-confluent growth and standard susceptibility testing conditions, as for other antibiotics, worked equally well for cefoxitin,\textsuperscript{3,11} as it detected MRSA with reliability on both media. Moreover, results of cefoxitin on ISA were rather more convincing as it predicted MRSA and MSSA with extreme accuracy. These results were rather convincing for using ISA for routine laboratory susceptibility testing and correlated very well with the results of Felton et al. on M-H agar. The zone size difference noted on ISA could be attributed to the difference in the nature of the media.\textsuperscript{2}

The only disadvantage seen with ISA was the zones of inhibition, which were large enough\textsuperscript{3} to restrict its utility with other drugs for routine susceptibility testing on a 9 cm plate. This could be overcome by putting lesser number of discs on the plate.

Considering the sensitivity and specificity, following zone diameter breakpoints are suggested for cefoxitin (30 µg) disc on both the media with semi-confluent growth and 18-20 hours incubation in ambient air: M-H, R\textsuperscript{<20} mm and S \textsuperscript{>22} mm\textsuperscript{12} and ISA, R\textsuperscript{<26} mm and S \textsuperscript{>26} mm. Although our isolates had zones of inhibition well within the CLSI defined range, this results proposal seems rather more acceptable as supported by the findings of previous investigators (Table II) in which all the mec\textsubscript{A} positive isolates had their zones \textsuperscript{\leq19} mm and mec\textsubscript{A} negative with zone diameters \textsuperscript{\geq22} mm thus accommodating the outliers.\textsuperscript{4,13-15}

\textbf{Table II: CLSI published zone diameter ranges for mec\textsubscript{A} positive and mec\textsubscript{A} negative S. aureus isolates.}

<table>
<thead>
<tr>
<th>Reference</th>
<th>Total isolates</th>
<th>mec\textsubscript{A} positive</th>
<th>mec\textsubscript{A} positive outlier</th>
<th>mec\textsubscript{A} negative</th>
<th>mec\textsubscript{A} negative outlier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cauweiler et al.</td>
<td>73/82</td>
<td>6-19</td>
<td>23</td>
<td>24-34</td>
<td>-</td>
</tr>
<tr>
<td>Swenson and Tenover</td>
<td>202/309</td>
<td>6-19</td>
<td>21,28</td>
<td>22- &gt; 28</td>
<td>-</td>
</tr>
<tr>
<td>Velasco et al.</td>
<td>51/51</td>
<td>6-14</td>
<td>-</td>
<td>\geq 25</td>
<td>17</td>
</tr>
<tr>
<td>Pottumarthy et al.</td>
<td>103/100</td>
<td>6-17</td>
<td>-</td>
<td>23- &gt; 35</td>
<td>21</td>
</tr>
</tbody>
</table>

P: mec\textsubscript{A} Positive; N: mec\textsubscript{A} Negative.

Hence, the use of cefoxitin disc on M-H agar as well as on ISA proved equally beneficial and proved a robust addition in the arsenal of tests for MRSA detection. As the results were obtained with the inoculum recommended for routine susceptibility testing of antibiotics for \textit{S. aureus}, this method would adjust well in the laboratory routine and could be used for the prompt and accurate identification of MRSA from routine specimens as well as screening swabs from high dependency units.

\section*{CONCLUSION}

The study proves cefoxitin as a surrogate marker for the detection of MRSA on M-H as well as ISA, and suggests that zone diameters breakpoint for cefoxitin (30 µg) disc as defined by the CLSI should be adjusted for resistant isolates by one mm increase on M-H agar and 3 mm decrease on ISA.

\section*{REFERENCES}


4. Swenson JM, Tenover FC. Results of disk diffusion testing with cefoxitin correlate with presence of mec\textsubscript{A} in \textit{Staphylococcus} spp. \textit{J Clin Microbiol} 2005; \textbf{43}: 3818-23.


