Performance of Phenotypic Tests to detect Penicillinase in Australian Staphylococcus aureus Isolates

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Running Title: Penicillinase detection in S. aureus

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ABSTRACT
Recent studies have shown that chromogenic cephalosporin tests are inferior to disc zone edge tests in detecting penicillinase in *Staphylococcus aureus* isolates resulting in a change to CLSI and EUCAST guidelines in 2012. We sought to confirm these findings using Australian isolates and compare the performance of the CLSI and EUCAST methods which use different disc strengths, penicillin 10 unit (P10) and 1 unit (P1) respectively. Using *blaZ* PCR as the reference standard the sensitivities of the tests were as follows: Cefinase Disc test 24/38 (63%); P10 disc zone edge test: 34/38 (89%); P10 disc diameter: 25/38 (66%); P1 disc zone edge: 38/38 (100%); P1 disc diameter: 38/38 (100%). We also found that reading of the P10 disc zone edge test was interpreted differently by the clinical laboratory and the study investigators in 11% of instances. Our findings support previous studies showing chromogenic cephalosporin based β-lactamase tests are inferior to disc methods in detecting *S. aureus* penicillinase. We also conclude that the EUCAST method using the P1 disc has the best performance, particularly because reading of the P1 disc zone diameter closely correlated with penicillinase production and is a less subjective test than zone edge reading.
INTRODUCTION

A proportion of *Staphylococcus aureus* isolates remain susceptible to penicillin, despite the rapid emergence of resistance following the introduction of penicillin in the 1940s (1). In Australia 14% of both community-based and hospital-acquired *S. aureus* isolates are considered penicillin susceptible (2,3). Penicillin remains the antimicrobial of choice for patients infected with susceptible isolates due to its narrow spectrum and low MIC (1). However, detection and reporting of penicillin resistance in the microbiology laboratory has not been straightforward.

The penicillin disc zone edge, which appears “sharp” or “heaped”, has been known to correlate with production of penicillinase for many years (4). Recent studies (5,6) demonstrated that chromogenic cephalosporin based $\beta$-lactamase tests are less sensitive than disc zone edge interpretation when compared with detection of *blaZ* by PCR. This resulted in a change to international guidelines in 2012 to recommend that disc zone edge reading – using the penicillin 10 unit (P10) disc in the CLSI guidelines and the penicillin 1 unit (P1) disc in the EUCAST guidelines - be used as the primary method for detecting $\beta$-lactamase production (7,8,9).

The aims of this study are threefold. Firstly, to confirm the findings that the appearance of the penicillin disc zone edge is more sensitive than chromogenic cephalosporin based $\beta$-lactamase testing in Australian strains. Secondly, to determine which disc strength (P10 recommended by CLSI or P1 recommended by EUCAST) was superior, including evaluation of zone diameter reading versus interpreting the zone edge appearance. Finally, we aimed to validate that the disc zone edge remains superior to chromogenic cephalosporin $\beta$-lactamase testing in the “real world” setting of a clinical diagnostic laboratory.

MATERIALS AND METHODS
Retrospectively collected *S. aureus* isolates. A database containing records of stored *S. aureus*
blood culture and sterile fluid isolates from March 2008 to March 2012 was interrogated to find isolates
that had been reported as penicillin susceptible. At the time of reporting, the penicillin susceptibility
was determined using an agar dilution breakpoint method (breakpoint of $\leq 0.12 \mu g/mL$) as per CLSI
guidelines. This was followed by a nitrocefin based β-lactamase test on isolates with penicillin MIC $\leq$
0.12 μg/ml (7). Neither examination of the penicillin zone edge nor *blaZ* molecular testing was done at
that time.

Prospectively collected *S. aureus* isolates. From April to July 2012, we collected *S. aureus*
isolates from the clinical laboratory that had been found to be penicillin susceptible (MIC $\leq 0.12$
μg/mL) by agar dilution and β-lactamase negative by a nitrocefin based test. These isolates also
underwent P10 disc testing according to the 2012 CLSI guidelines (8) in the clinical laboratory.
Both the retrospectively and prospectively collected isolates underwent further testing detailed
below:

**Identification confirmation.** All isolates were tested for DNase activity (DNase Test Agar
(OXOID, ThermoFisher Scientific) and had a rapid latex agglutination test (Staphaurex Plus* Remel,
ThermoFisher Scientific) performed to confirm identification.

**Penicillin susceptibility testing.** Penicillin MICs (0.002 to 1 μg/mL) were determined using
CLSI broth microdilution (BMD) reference method (10). Disc diffusion tests were performed on
Mueller-Hinton Agar (MHA) (OXOID) using both P1 and P10 (Oxoid, ThermoFisher Scientific) discs.
Zone diameters were interpreted according to EUCAST (P1 $< 26$ mm, resistant) and CLSI (P10 $\leq 29$
mm, resistant) criteria. Both zone diameter and the appearance of zone edge were recorded
independently by two investigators.

**Beta-lactamase Detection.** Phenotypic β-lactamase activity was determined using two different
chromogenic cephalosporin (nitrocefin) based methods: BBL™ Cefinase™ Discs (Becton Dickinson,
Sparks MD, USA) and Nitrocefin (OXOID SR112C, Basingstoke, UK). The Cefinase Disc test was
performed by taking the inoculum directly from the zone edge of a cefoxitin disc (30 µg) placed on the MHA. The Nitrocefin test was performed using the broth method technique as indicated by the manufacturer.

**Molecular confirmation.** PCR amplification of the _blaZ_ gene was performed using primers (5′-3′) _blaZ-F_, TTCAACACCTGCTGCTTTCG and _blaZ-R_, CCTTCATTACACTCTTGCCGGTTTC. PCR products (326 bp) were separated by electrophoresis in a 2% agarose gel, stained with Gel Red™ Nucleic Acid Gel Stain (Biotium, Hayward, CA) and the image visualised using a UV transilluminator (Gel-Doc™ 2000, Bio-Rad Laboratories Pty Ltd)

**Quality Control.** _S. aureus_ ATCC 29213 (β-lactamase positive) _S. aureus_ ATCC 25923 (β-lactamase negative) were used as quality control strains for broth microdilution and disc diffusion respectively; and for both the phenotypic and genotypic detection of β-lactamase.

**RESULTS**

**Retrospective study.** In total 100 of 1022 (9.8%) stored _S. aureus_ blood culture or sterile fluid isolates were reported as penicillin susceptible based on original agar dilution breakpoint and β-lactamase testing. Two isolates were not found in the frozen stock leaving 98 non-duplicate isolates that were retrieved. One isolate had a penicillin MIC >1 µg/mL on repeat broth microdilution testing and was positive for _blaZ_ and every other test for β-lactamase. Of the remaining 97 isolates which tested susceptible to penicillin by broth microdilution only four (4%) were _blaZ_ positive by PCR. All tests for these four isolates gave identical results on repeat testing. Table 1 shows the results of the phenotypic tests for these isolates. Of the _blaZ_ negative isolates, one (1%) isolate was called resistant using the P1 disc (25 mm). There were no false positive zone edge interpretations using the P10 disc.

**Prospective study.** Sixty three isolates for which the initial clinical laboratory findings showed a penicillin MIC ≤0.12 µg/mL by agar dilution were prospectively collected. Four isolates were
excluded from the final analysis as the organism identification could not be confirmed as *S. aureus*, leaving 59 isolates. In total 33 prospective isolates were *blaZ* positive by PCR. Using the P1 disc all isolates tested produced a sharp zone edge and all were resistant (≤26 mm) according to EUCAST interpretative guidelines. The P10 disc zone edge appearance detected 29 (88%) of the *blaZ* positive isolates and the CLSI zone diameter interpretation categorized 23 (70%) as resistant. All isolates that were negative for *blaZ* by PCR were also penicillinase negative by all phenotypic β-lactamase tests.

Of the two chromogenic cephalosporin based methods used, the Cefinase Disc detected 21 (64%) of β-lactamase producers confirmed by *blaZ* PCR, whilst the Nitrocefin broth only detected 15 (45%). All isolates that were β-lactamase positive by the Nitrocefin broth method were also positive with the Cefinase disc.

**Comparison of clinical diagnostic laboratory and investigator results.** Of the 59 prospectively collected isolates, 36 were reported as penicillin resistant by the clinical laboratory based on either a positive chromogenic cephalosporin test (n=11) or a sharp P10 zone edge (n= 25). The remaining 23 isolates were reported penicillin susceptible. One isolate, reported as resistant due to a positive Nitrocefin test, was negative by all β-lactamase tests performed by the investigators, including *blaZ* PCR. One isolate, reported as penicillin susceptible, was positive for *blaZ* with a resistant and sharp P1 zone but was susceptible with a fuzzy zone edge using the P10 disc.

There were 6 (11%) instances where the P10 disc zone edge test was interpreted differently by the investigators than by the clinical laboratory. In all cases the zone edge was called sharp by the clinical laboratory and fuzzy by the investigators. Three of these isolates tested positive by *blaZ* PCR and resistant by P1 disc edge and diameter; while the other three isolates were negative for all tests including *blaZ* PCR.
The combined results of the prospective and retrospective studies were used to calculate sensitivity, specificity, positive and negative predictive values of the phenotypic β-lactamase test methods compared to blaZ PCR as the reference standard. These results are summarised in Table 2.

**DISCUSSION**

The P1 zone diameter interpretation or P1 zone edge appearance, as outlined in the EUCAST guidelines, did not miss any β-lactamase producing S. aureus isolates in this study. The P10 zone edge, recommended by CLSI, performed well with a sensitivity of 89% but was inferior to the P1 zone edge. The P10 zone diameter was a poor predictor of β-lactamase production with a sensitivity of only 66% which was similar to the performance of the Cefinase disc test with a sensitivity of 63%. The Cefinase disc test was clearly superior to the Nitrocefin broth method (45% sensitivity) in detecting S. aureus β-lactamase. Differences in the concentration of nitrocefin (500 µg/ml in the Oxoid broth method but undefined in the Cefinase test) may account for the differences in test results. These results support previous findings of poor sensitivity of chromogenic cephalosporin tests in detecting S. aureus penicillinase (5,6).

Comparison of the clinical diagnostic laboratory and investigator’s results revealed one blaZ positive isolate that was reported as penicillin susceptible. This isolate had a resistant and sharp P1 zone but was susceptible with a fuzzy zone edge using the P10 disc. Therefore the clinical diagnostic laboratory would have correctly identified one additional isolate as penicillin resistant if the P1 disc had been utilized instead of the P10 disc.

The reading of the disc zone edge test was a new procedure in the laboratory at the time resulting in three isolates incorrectly identified as having sharp disc zone edges on the P10 disc. However, another three isolates were interpreted by the investigators as having fuzzy zone edges where the clinical laboratory interpretation of a sharp zone edge was in fact correct. In total there were six (11%) discrepant zone edge interpretations between the investigators and the clinical laboratory. As the
zone edges were read at the recommended time (16 to 18 h) in both laboratories, this suggests that even experienced staff have difficulty in interpreting zone edges due to the inherently subjective nature of the test.

One weakness of the study is that only one set of PCR primers was used and that no sequencing of the *bla*Z gene was performed to ensure that no mutant *bla*Z genes that were non-functional but still PCR positive were inadvertently counted as penicillinase producers. In addition it is possible that functional mutant *bla*Z genes were missed producing false negative *bla*Z PCR results (11).

In conclusion, the results of this study support previous data showing that chromogenic cephalosporin based β-lactamase tests have poor sensitivity in detecting *S. aureus* penicillinase producers. We found that the P1 zone edge appearance and zone diameter had the best correlation with *bla*Z detection. The P1 zone diameter interpretation performs just as well as the P1 zone edge in detecting penicillinase producers, although it does have slightly reduced specificity (99%) compared to other methods of β-lactamase detection. The zone diameter has the advantage of being a less subjective test with which laboratory staff are already familiar. We have also shown that reading zone edge appearance, at least in the early stage of implementation, can cause confusion and result in misinterpretation by laboratory staff. Therefore, using the P1 disc diameter interpretation in combination with the P1 disc edge test as recommended in the EUCAST guidelines is unlikely to miss penicillinase producers. As a result our clinical laboratory has now switched to using the P1 disc in accordance with the EUCAST guidelines to determine penicillinase production.

ACKNOWLEDGEMENTS

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REFERENCES


TABLE 1: Results from phenotypic tests from four retrospective isolates that were blaZ carriers but were susceptible to penicillin by broth microdilution

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>MIC (µg/mL)</th>
<th>Zone diameter, mm (interpretation)</th>
<th>β-lactamase test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 U</td>
<td>1 U</td>
</tr>
<tr>
<td>632</td>
<td>Blood</td>
<td>0.12</td>
<td></td>
<td>28 (R)</td>
</tr>
<tr>
<td>048</td>
<td>Blood</td>
<td>0.06</td>
<td></td>
<td>35 (S)</td>
</tr>
<tr>
<td>370</td>
<td>Blood</td>
<td>0.06</td>
<td></td>
<td>30 (S)</td>
</tr>
<tr>
<td>320</td>
<td>Joint fluid</td>
<td>0.06</td>
<td></td>
<td>30 (S)</td>
</tr>
</tbody>
</table>

R = resistant; S = susceptible; + = positive; - = negative
TABLE 2: Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of phenotypic β-lactamase tests using blaZ PCR as the reference standard for 157 Staphylococcus aureus isolates.

<table>
<thead>
<tr>
<th>β-lactamase Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefinase disc</td>
<td>63%</td>
<td>100%</td>
<td>100%</td>
<td>89%</td>
</tr>
<tr>
<td></td>
<td>(24/38)</td>
<td>(119/119)</td>
<td>(23/23)</td>
<td>(119/133)</td>
</tr>
<tr>
<td>P10 zone diameter interpretation</td>
<td>66%</td>
<td>100%</td>
<td>100%</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>(25/38)</td>
<td>(119/119)</td>
<td>(25/25)</td>
<td>(119/132)</td>
</tr>
<tr>
<td>P10 zone edge</td>
<td>89%</td>
<td>100%</td>
<td>100%</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>(34/38)</td>
<td>(119/119)</td>
<td>(34/34)</td>
<td>(119/123)</td>
</tr>
<tr>
<td>P1 zone diameter interpretation</td>
<td>100%</td>
<td>99%</td>
<td>97%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>(38/38)</td>
<td>(118/119)</td>
<td>(38/39)</td>
<td>(118/118)</td>
</tr>
<tr>
<td>P1 zone edge</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>(38/38)</td>
<td>(119/119)</td>
<td>(38/38)</td>
<td>(119/119)</td>
</tr>
</tbody>
</table>