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Performance of Phenotypic Tests for Detection of Penicillinase in *Staphylococcus aureus* Isolates from Australia

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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 at 10 units (P10) and 1 unit (P1), respectively. Using *blaZ* PCR as the reference standard, the sensitivities of the tests for detection of penicillinase production were as follows: Cefinase disc test, 24/38 isolates (63%); P10 disc zone edge test, 34/38 isolates (89%); P10 disc diameter test, 25/38 isolates (66%); P1 disc zone edge test, 38/38 isolates (100%); and P1 disc diameter test, 38/38 isolates (100%). We also found that the P10 disc zone edge test reading was interpreted differently by the clinical laboratory and the study investigators in 11% of instances. Our findings support those of previous studies showing that 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 the P1 disc zone diameter reading closely correlated with penicillinase production and reading of the disc zone diameter is less subjective than reading of the zone edge.

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 have not been straightforward.

The penicillin disc zone edge, which appears sharp or heaped, has been known to correlate with the production of penicillinase for many years (4). Recent studies (5, 6) demonstrated that chromogenic cephalosporin-based β-lactamase tests are less sensitive than disc zone edge interpretation when they are 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 β-lactamase production (7–9).

The aims of this study are 3-fold. The first aim was to confirm the findings that a test determining the appearance of the penicillin disc zone edge is more sensitive than chromogenic cephalosporin-based β-lactamase testing in Australian strains. The second aim was to determine which disc strength (P10 recommended by CLSI or P1 recommended by EUCAST) is superior, including evaluation of the zone diameter reading versus interpretation of the zone edge appearance. Finally, we aimed to validate that the disc zone edge test remains superior to chromogenic cephalosporin β-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 to be penicillin susceptible. At the time of reporting, the penicillin susceptibility was determined using an agar dilution breakpoint method (breakpoint, ≤0.12 μg/ml) per CLSI guidelines. This was followed by a nitrocefin-based β-lactamase test on isolates with penicillin MICs of ≤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 from the clinical laboratory *S. aureus* isolates that had been found to be penicillin susceptible (MICs ≤ 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, as detailed below.

Identification confirmation. All isolates were tested for DNase activity (DNase test agar; Oxoid, Thermo Fisher Scientific) and had a rapid latex agglutination test (Staphaurex Plus*; Remel, Thermo Fisher Scientific) performed to confirm the identification.

Penicillin susceptibility testing. Penicillin MICs (0.002 to 1 μg/ml) were determined using the CLSI broth microdilution (BMD) reference method (10). Disc diffusion tests were performed on Mueller-Hinton agar (MHA; Oxoid) using both P1 and P10 discs (Oxoid, Thermo Fisher Scientific). Zone diameters were interpreted according to EUCAST criteria (isolates with a P1 diameter of <26 mm were considered resistant) and CLSI criteria (isolates with a P10 diameter of ≤29 mm were considered

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TABLE 1 Results from phenotypic tests from four retrospective isolates that were *blaZ* carriers but were susceptible to penicillin by BMD

Isolate	Source	MIC (μg/ml)	Zone diam[a] (mm)		β-Lactamase test result[b]			
			P10	P1	Zone edge	Nitrocefin broth	Cefinase disc	*blaZ* PCR
632	Blood	0.12	28 (R)	22 (R)	Sharp	+	+	+
048	Blood	0.06	35 (S)	25 (R)	Sharp	−	−	+
370	Blood	0.06	30 (S)	22 (R)	Sharp	−	+	+
320	Joint fluid	0.06	30 (S)	22 (R)	Sharp	−	−	+

[a] Interpretations are given in parentheses. R, resistant; S, susceptible.
[b] +, positive; −, negative.

resistant). Both the zone diameter and the appearance of the zone edge were recorded independently by two investigators.

β-Lactamase detection. Phenotypic β-lactamase activity was determined using two different chromogenic cephalosporin (nitrocefin)-based methods: with BBL Cefinase discs (Becton, Dickinson, Sparks, MD) and nitrocefin (SR112C; Oxoid, Basingstoke, United Kingdom). The Cefinase disc test was performed by taking the inoculum directly from the zone edge of a cefoxitin disc (30 μg) placed on 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 (TTCAACACCTGCTGCTTTCGG) and blaZ-R (CCTTCATTACACTCTTGGCGGTTTC). PCR products (326 bp) were separated by electrophoresis in a 2% agarose gel and stained with Gel Red nucleic acid gel stain (Biotium, Hayward, CA), and the image was visualized using a UV transilluminator (Gel-Doc 2000; Bio-Rad Laboratories Pty. Ltd.).

Quality control. *S. aureus* ATCC 29213 (β-lactamase positive) and *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 1,022 (9.8%) stored *S. aureus* blood culture or sterile fluid isolates were reported to be penicillin susceptible on the basis of the original agar dilution breakpoint and β-lactamase testing. Two isolates were not found in the frozen stock, leaving 98 nonduplicate isolates that were retrieved. One isolate had a penicillin MIC of >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, 4 (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 of ≤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 to be *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 (diameter, <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 the β-lactamase producers confirmed by *blaZ* PCR, while the nitrocefin broth-based method detected only 15 (45%). All isolates that were β-lactamase positive by the nitrocefin broth-based 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 to be penicillin resistant by the clinical laboratory on the basis of either a positive chromogenic cephalosporin test ($n = 11$) or a sharp P10 zone edge ($n = 25$). The remaining 23 isolates were reported to be penicillin susceptible. One isolate reported to be resistant due to a positive nitrocefin test result was negative by all β-lactamase tests performed by the investigators, including *blaZ* PCR. One isolate reported to be penicillin susceptible was positive for *blaZ* and was resistant with a sharp zone edge using the P1 disc but was susceptible with a fuzzy zone edge using the P10 disc.

There were six (11%) instances where the P10 disc zone edge test interpretation by the investigators was different from that 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 appearance and diameter interpretation, while the other three isolates were negative by all tests, including *blaZ* PCR.

The combined results of the prospective and retrospective studies were used to calculate the sensitivity, specificity, and positive and negative predictive values of the phenotypic β-lactamase test methods when their results were compared to those of the *blaZ* PCR as the reference standard. These results are summarized 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 test, recommended by CLSI, performed well with a sensitivity of 89% but was inferior to the P1 zone edge test. 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 the poor sensitivity of chromogenic cephalosporin tests in detecting *S. aureus* penicillinase (5, 6).

Comparison of the clinical diagnostic laboratory and investi-

TABLE 2 Sensitivity, specificity, and positive and negative predictive values of phenotypic β-lactamase tests using *blaZ* PCR as the reference standard for 157 *Staphylococcus aureus* isolates[a]

β-Lactamase test	Sensitivity (%)[a]	Specificity (%)[b]	PPV (%)[c]	NPV (%)[d]
Cefinase disc	63 (24/38)	100 (119/119)	100 (24/24)	89 (119/133)
P10 zone diam interpretation	66 (25/38)	100 (119/119)	100 (25/25)	90 (119/132)
P10 zone edge	89 (34/38)	100 (119/119)	100 (34/34)	97 (119/123)
P1 zone diam interpretation	100 (38/38)	99 (118/119)	97 (38/39)	100 (118/118)
P1 zone edge	100 (38/38)	100 (119/119)	100 (38/38)	100 (119/119)

[a] Data in parentheses represent the number of isolates with true-positive results/number of isolates positive by *blaZ* PCR.
[b] Data in parentheses represent the number of isolates with true-negative results/number of isolates negative by *blaZ* PCR.
[c] PPV, positive predictive value. Data in parentheses represent the number of isolates with true-positive results/total number of isolates with positive results.
[d] NPV, negative predictive value. Data in parentheses represent the number of isolates with true-negative results/total number of isolates with negative results.

gator's results revealed one *blaZ*-positive isolate that was reported to be penicillin susceptible. This isolate was resistant with a sharp zone edge using the P1 disc 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 when 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 *blaZ* gene was performed to ensure that no isolates with mutant *blaZ* genes that were nonfunctional but still PCR positive were inadvertently counted as penicillinase producers. In addition, it is possible that functional mutant *blaZ* genes were missed, producing false-negative *blaZ* 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 *blaZ* detection. The P1 zone diameter interpretation performs just as well as the P1 zone edge appearance in detecting penicillinase producers, although it does have slightly reduced specificity (99%) compared to that of 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 the 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.

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