Comparison of BD Phoenix nitrocefin, a cefinase disk test, and Phoenix MIC for the detection of hyper β-lactamase in Staphylococcus saprophyticus

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ABSTRACT
Background: Staphylococcus saprophyticus is a common cause of urinary tract infection. Resistance to some of the agents commonly used to treat infections has been found, including resistance to trimethoprim, amoxicillin and meth/flucloraxillin. However, true resistance prevalence may be under estimated as susceptibility testing is not recommended by the Clinical and Laboratory Standards Institute. In addition, testing and determination of resistance to β-lactams has not been clearly defined and in-house testing has resulted in discrepancies between phenotypic methods for the detection of β-lactamase. The study objective was to compare Phoenix nitrocefin penicillinase detection, BBL chromogenic cefinase disk, Phoenix penicillin MIC, and ampicillin 2µg disk diffusion for the detection of β-lactam resistance in S. saprophyticus.

Methods: 111 consecutive S. saprophyticus isolates, collected between May to August 2013 at Canterbury Health Laboratories, New Zealand, were routinely analysed in BD Phoenix, using PMIC-ID-78 panels. Isolate identification was confirmed with MALDI-TOF and novobiocin disk diffusion. All study isolates were then repeated in Phoenix, using AST panel type PMIC-84. Chromogenic cefinase was performed using BBL discs, and ampicillin 2µg disk diffusion was performed according to EUCAST. On three isolates determined to be penicillinase hyper-producers, MICs to penicillin and ampicillin were performed by E-test, and an in-house PCR was used to determine blaZ and mecA status.

Results: 64/111 (57.7%) isolates were classified as β-lactamase positive by Phoenix nitrocefin, with only 28 of these isolates also positive by cefinase disk test. Three isolates were cefinase positive/Phoenix nitrocefin negative. 44/111 isolates were negative by both methods. Evaluation of Phoenix penicillin MIC values revealed three isolates with elevated MICs (≥2mg/L) and by E-test (≥1mg/L). Each of these 3 isolates had ampicillin disk diffusion zones of 14-15mm, which is below the EUCAST epidemiology cut-off limit of <18mm, and ampicillin MICs of 0.5mg/L. In addition, all three isolates were blaZ PCR positive but mecA negative. Resistance to other antimicrobial agents among all of the study isolates was found in trimethoprim (3.6%), trimethoprim/sulfamethoxazole (2.7%), tetracycline (12.6%) and erythromycin (17.1%). All isolates were susceptible to amoxicillin-clavulanate, ciprofloxacin, nitrofurantoin, and vancomycin.

Conclusions: Phoenix nitrocefin test classified 57.7% of S. saprophyticus isolates as β-lactamase positive, which was significantly more than the 27.9% positive by cefinase disk test. Whereas only three of these isolates showed penicillinase hyper-production, with penicillin MIC levels ≥2mg/L, ampicillin MICs of 0.5mg/L, ampicillin disk diffusion <18mm, and positive for the blaZ gene. This study has shown that penicillin MIC is a more reliable indicator of β-lactamase positive in S. saprophyticus than Phoenix nitrocefin or cefinase disk test, and that these isolates should be reported as resistant to penicillin and amoxicillin. Furthermore, resistance was found among other oral antimicrobials, indicating a need to routinely perform antimicrobial susceptibility testing on S. saprophyticus in order to detect emerging resistance and possible treatment failure.

Key words: Staphylococcus saprophyticus, urinary tract infection, β-lactamase, cefinase disk test, penicillinase, cefinase.


INTRODUCTION
In the clinical laboratory Staphylococcus saprophyticus is most frequently found as a uropathogen, being one of the leading causes of community acquired urinary tract infection (UTI) in young females (1,2). Most often the isolate will be susceptible to antibiotics commonly used to treat uncomplicated UTI, such as amoxicillin, trimethoprim, trimethoprim/sulfamethoxazole, nitrofurantoin, and meth/flucloraxillin (3). However, this does not tell the full story, in that serious infections such as bacteraemia have been reported to be caused by S. saprophyticus (1,4,5). In addition, isolates that are multi-drug resistant, including meth/flucloraxillin resistance due to mecA acquisition, have been reported in several studies (6-8).

Since 2001, the Clinical and Laboratory Standards Institute (CLSI) Guidelines has recommended that “Routine testing of urine isolates of S. saprophyticus is not advised, because infections respond to concentrations achieved in urine of antimicrobial agents commonly used to treat acute, uncomplicated urinary tract infections (eg. nitrofurantoin, trimethoprim ± sulfamethoxazole, or a fluoroquinolone)” (9). Hence many laboratories may not routinely perform susceptibility testing on S. saprophyticus, perhaps under-estimating the true resistance prevalence. The testing and determination of resistance to β-lactam agents such as penicillin, amoxicillin and amoxicillin-clavulanate has not been clearly defined and these antibiotics are not mentioned in the CLSI statement. Yet they can be a common first line empirical choice for UTI treatment, particularly in a community setting. Furthermore, studies using different detection methods have resulted in varying levels of β-lactam resistance in S. saprophyticus as well as recognising problems with method accuracy (6,10-12).
In contrast to the CLSI, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints v3.1, 2013, included ampicillin disk diffusion (DD) breakpoints for S. saprophyticus, but with a resistant breakpoint of <15mm, and noted that S. saprophyticus was the exception as the majority of other staphylococci are penicillinase producers (13). EUCAST also noted that this breakpoint could be used to screen for mecA, in that any ampicillin-susceptible isolates were mecA negative. However, the breakpoints were not correlated to β-lactamase production.

The presence of β-lactamase confers resistance to penicillin-labile penicillins (e.g. penicillin and amoxicillin); whereas the presence of mecA expands resistance to also include flucloxacillin, β-lactamase inhibitor combinations (e.g. amoxicillin-clavulanate), and most other β-lactam antibiotics. A recent study at our institution revealed discrepancies between Becton Dickinson (BD) Phoenix penicillinase detection and a chromogenic cefinase disk method for the determination of penicillin resistance (14). In order to resolve these discrepancies, the objective of this study was to compare detection of penicillinase in clinical isolates of S. saprophyticus by the Phoenix nitrocefin test and the BD-BBL chromogenic cefinase disk test methods, and to correlate those results with Phoenix penicillin MIC and ampicillin 2μg DD. Production of clinically significant levels of penicillinase was confirmed with blaz PCR. We aimed to provide a practical method for Phoenix users to enable the interpretation of β-lactam results and enhance the reporting of antimicrobial resistances that could guide treatment outcomes.

**METHODS AND MATERIALS**

**Bacterial isolates**

A total of 111 consecutive, non-duplicate S. saprophyticus isolates were collected between May to August 2013 at Canterbury Health Laboratories (Christchurch Hospital) and stored at -80°C in PROTECT cryopreservative fluid. Fifty of these isolates were referred from Christchurch Southern Community Laboratories (CSCL). All isolates originated from urine cultures. The majority of isolates were from females (104/111; 93.7%), with only seven (6.3%) from male patients.

**Susceptibility methods**

Study isolates were routinely analysed in BD Phoenix™ (Becton Dickinson Diagnostic Systems, Australia), using PMIC/ID-78 Combo panels which include a nitrocefin test to determine β-lactamase production. Results were stored on BD EpiCenter software database. Organism identification was confirmed with duplicate spots on a Bruker MALDI-TOF (Science Directions Limited, New Zealand) as well as novobiocin resistance by disk diffusion. All study isolates were retrospectively repeated in Phoenix, using AST panel type PMIC-84. Chromogenic cefinase was performed using BD-BBL discs (Fort Richard Laboratories Ltd, Auckland). Ampicillin 2μg DD was performed in duplicate, with interpretation according to EUCAST criteria, breakpoint table v3.1. Etest (bioMérieux) was also used to determine the minimum inhibitory concentration (MIC) of penicillin and ampicillin on three isolates classified as penicillinase hyper-producers. The PCR method of Kaase et al. was used to determine blaz status and an in house PCR was used to detect mecA (15). S. aureus ATCC 25923 (β-lactamase negative) and ATCC 29213 (weak β-lactamase positive) were used as controls.

**RESULTS**

Overall, 67 (60.4%) of the 111 S. saprophyticus isolates were classified as β-lactamase positive by either Phoenix nitrocefin or BBL cefinase tests. Sixty-four isolates were β-lactamase positive by nitrocefin test, with only 28 of these 64 also positive by cefinase disk test. A positive nitrocefin test means that the Phoenix BDXpert rules override any results of penicillinase-labile penicillins, hence potentially changing a susceptible MIC for penicillin to an interpretation category of resistant. Three isolates were cefinase positive/nitrocefin negative. Forty-four out of 111 isolates were negative by both methods. Results are summarised in Table 1.

**Table 1.** Comparison of Phoenix nitrocefin and BBL cefinase tests for β-lactamase production in 111 S. saprophyticus isolates.

<table>
<thead>
<tr>
<th></th>
<th>BBL cefinase positive</th>
<th>BBL cefinase negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phoenix nitrocefin positive</td>
<td>28</td>
<td>36</td>
<td>64 (57.7%)</td>
</tr>
<tr>
<td>Phoenix nitrocefin negative</td>
<td>3</td>
<td>44</td>
<td>47 (42.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>31 (27.9%)</td>
<td>80 (72.1%)</td>
<td></td>
</tr>
</tbody>
</table>

In comparison, evaluation of Phoenix penicillin MIC values showed that the majority of S. saprophyticus isolates had low penicillin MICs, with 84 (75.7%) having an MIC of 0.125mg/L and 24 (21.6%) having an MIC of 0.25mg/L. The remaining three isolates had elevated Phoenix penicillin MICs of 2, 4 and 8mg/L respectively, and were termed ‘hyper-penicillinase’ producers (Figure 1). Similarly, 108 (97.3%) isolates gave ampicillin MIC values of ≤0.25mg/L, with only the three hyper-penicillinase producers having a value of 0.5mg/L.

**Figure 1.** Frequency of penicillin MIC as determined by Phoenix.
For ampicillin DD, zones ranged between 14 and 31mm, with 85% of zones lying between 22 to 28mm. Three outlying strains, with zones of 14-15mm were the same three strains that gave elevated penicillin and ampicillin MICs. A graph of the zone size distribution is shown in Figure 2.

Figure 2. Ampicillin 2μg disk diffusion zone size distribution of 111 S. saprophyticus study isolates. The vertical straight line at <15mm represents the resistance breakpoint for EUCAST v3.1 2013. The dashed line at <18mm represents the current EUCAST v4.1 2014 table.

Etest evaluation of the three hyper-penicillinase strains produced penicillin MIC levels of 1mg/L and an ampicillin MIC of 1mg/L for two isolates and a value of 2mg/L for the remaining isolate. bla\text{Z} PCR was performed on these three isolates as well as a selection of nine study isolates that were positive by both nitrocefin and cefinase and had various resistances to other antibiotics. Only the three hyper-penicillinase producers were bla\text{Z} positive. mec\text{A} PCR was negative for all three strains. The data from this part of the study are shown in Table 2.

Table 2. Phenotypic and PCR results for three S. saprophyticus isolates determined to be hyper-penicillinase producers.

<table>
<thead>
<tr>
<th>Lab No.</th>
<th>Phoenix MIC (mg/L)</th>
<th>E-Test MIC (mg/L)</th>
<th>Disk diffusion (mm)</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PENI</td>
<td>AMPI</td>
<td>PENI</td>
<td>AMPI</td>
</tr>
<tr>
<td>SCL034</td>
<td>8.0</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>SCL041</td>
<td>2.0</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>SCL048</td>
<td>4.0</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The three bla\text{Z} positive isolates were also sent for analysis to the EUCAST Laboratory for Antimicrobial Susceptibility Testing, Sweden. The results from their investigations are detailed in Table 3.

Table 3. Phenotypic and PCR results as determined by the EUCAST laboratory for AST, Sweden.

<table>
<thead>
<tr>
<th>ID</th>
<th>MALDI-TOF ID</th>
<th>Score</th>
<th>FOX30</th>
<th>FOX MIC</th>
<th>AMP2</th>
<th>AMP MIC</th>
<th>mec\text{A}</th>
<th>PCG1</th>
<th>PCG MIC</th>
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<tbody>
<tr>
<td>SCL034</td>
<td>S. saprophyticus</td>
<td>2.190</td>
<td>28</td>
<td>4</td>
<td>16</td>
<td>1</td>
<td>Neg</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>SCL041</td>
<td>S. saprophyticus</td>
<td>2.095</td>
<td>28</td>
<td>4</td>
<td>16</td>
<td>1</td>
<td>Neg</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>SCL048</td>
<td>S. saprophyticus</td>
<td>2.103</td>
<td>28</td>
<td>4</td>
<td>16</td>
<td>1</td>
<td>Neg</td>
<td>12</td>
<td>1</td>
</tr>
</tbody>
</table>

FOX30 = cefoxitin 30μg; FOXMIC = cefoxitin Etest; AMP2 = ampicillin 2 μg; AMP MIC = ampicillin Etest; mec\text{A} = mec\text{A} status according to PCR; PCG1 = benzylpenicillin 1 unit; PCG MIC = benzylpenicillin Etest.

Resistance to other antimicrobial agents among all of the study isolates was also evaluated, revealing low level resistance to trimethoprim (3.6%) and trimethoprim/sulfamethoxazole (2.7%), but higher levels of resistance to tetracycline (12.6%) and erythromycin (17.1%). All study isolates were susceptible to amoxicillin-clavulanate, ciprofloxacin, nitrofurantoin, and vancomycin.
DISCUSSION

Many clinical laboratories use automated susceptibility testing analysers such as Phoenix, Vitek, and MicroScan for reliable, standardised and rapid throughput of common clinical pathogens. While there are many publications evaluating the performance of these analysers, limited information is available on how they perform with *S. saprophyticus*. A previous study at our institution found discrepancies between Phoenix nitrocefin and a chromogenic cephalosporin test in the determination of penicillinase activity (14), which led us to prospectively investigate these results and to compare results with Phoenix MIC values for penicillin and ampicillin and to compare results with ampicillin disk diffusion.

Our study of 111 *S. saprophyticus* clinical isolates suggests that the determination of penicillin resistance can be overestimated by the Phoenix nitrocefin test and the BBL cefinase test, with β-lactamase positive classifications of 57.7% and 27.9% respectively. However, only three of these isolates were positive by *bla*Z PCR for presence of the penicillinase enzyme, indicating that most of the nitrocefin and cefinase tests were false positive. Chromogenic cephalosporin tests can be difficult to read with the 1 hour reaction time required with staphylococci, and their use is not recommended by EUCAST. A similar finding with a chromogenic cephalosporin test was reported in the study by Ferreira et al., who found that 52/57 57 (91.2%) of *S. saprophyticus* isolates were classified as β-lactamase positive by the Vitek, compared to only 5.3% positive using a nitrocefin disk test (6). *bla*Z PCR was not performed to recently published a report of a newly discovered mecA variant problems with the Vitek 2 system erroneously reporting *S. saprophyticus* as methicillin resistant (16). They suggested not using Vitek 2 to perform susceptibility testing on *S. saprophyticus*, but instead to consider the detection of mecA by PCR. However, using this approach does not detect resistance to other antimicrobials.

Problems with the reliability of phenotypic and chromogenic cephalosporin detection methods for resistance to β-lactam agents in *S. saprophyticus* have also been recognised by other studies and is part of the reason why both CLSI and EUCAST have changed their guidelines over the years (9-11,13,17). *S. saprophyticus* may produce a low level penicillinase that is weak and difficult to reliably detect and is not picked up by the *bla*Z assay. Stratton et al. found the presence of penicillin binding protein (PBP 2a) in three *S. saprophyticus* strains that had high level methicillin resistance, but the authors demonstrated only negligible β-lactamase activity, despite using induction techniques (18). Alternatively, there may be other undefined mechanisms at play. For instance, Malyzko et al. recently published a report of a newly discovered mecC2, which conferred methicillin resistance to *S. saprophyticus* strains recovered from animals (19). It is possible that these strains could be passed onto humans through the food chain.

Our study also looked at penicillin and ampicillin MIC values on Phoenix and ampicillin DD in order to assess their ability to determine an interpretation for β-lactams. The use of ampicillin 2µg DD is recommended by EUCAST Clinical Breakpoint Table v3.1 as a way of screening for methicillin resistance in *S. saprophyticus*, with zones of <15mm indicating further investigation for the presence of mecA. EUCAST Note 1/A stated that “With the exception of Staphylococcus saprophyticus, most staphylococci are penicillinase producers”. This would imply that only mecA is responsible for ampicillin resistance in *S. saprophyticus*, rather than significant penicillinase production. At the beginning of 2014, EUCAST table v4.1 was updated to include a breakpoint interpretation for ampicillin of resistance at <18mm and the statement regarding *S. saprophyticus* not being a penicillinase producer was removed. Zones ≥18mm can be regarded as susceptible to both ampicillin and methicillin. No MIC breakpoint values were included.

The data from our study, shown in Figure 1, would indicate that a penicillin MIC value of ≥0.5mg/L could be used as a suitable screening method for the detection of hyper penicillinase, with values below these limits considered susceptible to penicillin, ampicillin, and methicillin. Raw MIC data can be obtained in Phoenix by temporarily changing the organism identification to “unidentified organism”. Once the MIC values for penicillin are obtained then the isolate identification can be corrected back to *S. saprophyticus*, with the penicillin and ampicillin category interpretation manually entered according to the penicillin MIC values obtained.

Our results from the ampicillin 2µg DD method would concur with the current EUCAST cut off and it is a useful method to confirm Phoenix results or as a standalone test for laboratories without automation. It is probably not necessary to confirm the ampicillin resistant isolates with *bla*Z, but it is necessary to check for mecA. Data (not shown) from our study agrees with previous investigations in that MIC values for cefoxitin and oxacillin are not specific for the detection of mecA with 3.6% of our isolates having a cefoxitin MIC of 4mg/L and 84.7% of isolates having an oxacillin MIC >0.25mg/L (considered positive for coagulase-negative Staphylococci). Yet all of these isolates, except for the three hyper-penicillinase producers, were susceptible to penicillin. The methicillin-resistant, mecA positive *S. saprophyticus* strains isolated so far (albeit infrequently) at our institution have all been highly resistant to all β-lactams as well as amoxicillin-clavulanate.

The clinical relevance of antibiotic resistance in *S. saprophyticus* has not been fully defined. It could be argued that antibiotics commonly used to treat UTI are concentrated in the urine at such high levels that most infections are treated despite _in-vitro_ resistance. In addition, many healthy adult females can sometimes clear a UTI in several days without antibiotic intervention. On the other hand, we have had anecdotal reports of treatment failure in patients with cultures growing *S. saprophyticus* that are resistant to the prescribed antibiotic, including amoxicillin and trimethoprim. In our opinion these hyper-penicillinase producing isolates should be reported as resistant to penicillin and amoxicillin as these first line antibiotics may be clinically ineffective. Furthermore, *S. saprophyticus* UTI can lead to serious complications such as septicaemia and this pathogen has been isolated from sites outside of the uro-genital tract. Surely it is the duty of a clinical laboratory to use the most reliable and accurate means in order to provide suitable treatment choices to clinicians and also to detect emerging resistance.

Limitations of this study include the inability to rule out duplicate isolates. Of the 50 isolates collected from CSCL, only gender and age were recorded, thus some of the strains could possibly be from the same patient who had a recurrent or relapsed infection.

In summary, the results of this study would suggest that the Phoenix nitrocefin and chromogenic cephalosporin tests should be discouraged for the determination of β-lactamase in *S. saprophyticus*. We have shown that penicillin MIC is a practical and more reliable indicator of penicillin hyper-production and that these isolates should be reported as resistant to penicillin and amoxicillin. Routine susceptibility testing of *S. saprophyticus* should be performed in order to enhance the reporting of antimicrobial susceptibilities that could guide successful treatment outcomes.

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