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Correlation of phenotypic tests with the presence of the *blaZ* gene for detection of beta-lactamase

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ABSTRACT

Staphylococcus aureus and *Staphylococcus saprophyticus* are the most common and most important staphylococcal species associated with urinary tract infections. The objective of the present study was to compare and to evaluate the accuracy of four phenotypic methods for the detection of beta-lactamase production in *Staphylococcus* spp. Seventy-three strains produced a halo with a diameter ≤28 mm (penicillin resistant) and all of them were positive for the *blaZ* gene. Among the 28 susceptible strain (halo ≥29 mm), 23 carried the *blaZ* gene and five did not. The zone edge test was the most sensitive (90.3%), followed by MIC determination (85.5%), but the specificity of the former was low (40.0%). The nitrocefin test was the least sensitive (28.9%). However, the nitrocefin test together with the disk diffusion method showed the highest specificity (100%). The present results demonstrated that the zone edge test was the most sensitive phenotypic test for detection of beta-lactamase, although it is still not an ideal test to detect this type of resistance since its specificity was low. However, the inhibition halo diameter of the penicillin disk can be used together with the zone edge test since the same disk is employed in the two tests. Combined analysis of the two tests shows a sensitivity of 90.3% and specificity of 100%, proving better sensitivity, especially for *S. saprophyticus*. This is a low-cost test of easy application and interpretation that can be used in small and medium-sized laboratories where susceptibility testing is usually performed by the disk diffusion method.

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Introduction

The most common etiological agents involved in urinary tract infections (UTIs) include enterobacteria, non-fermenting Gram-negative bacilli, fungi, enterococci, and staphylococci. *Staphylococcus aureus* and *Staphylococcus saprophyticus* are the most common and most important staphylococcal species associated with UTIs; however, other coagulase-negative staphylococci have gained importance in recent years.

S. saprophyticus is the second most common causative agent of acute UTI in the community after *Escherichia coli* and is isolated mainly from urine of sexually active young women,[1,2] causing symptoms that are undistinguishable from those induced by *E. coli*. Cases of sepsis and pyelonephritis caused by this microorganism have also been reported.[3,4]

The occurrence of penicillin resistance in *S. aureus* isolates recovered from hospitalized patients has been reported shortly after the introduction of penicillin in 1941.[5,6] At the beginning of the 1970s, resistance to penicillin became common among nosocomial *S. aureus* isolates (85–90%) and shortly thereafter penicillinase-producing strains rapidly spread in the community.[7–9] Today, more than 90% of *S. aureus* strains isolated from humans are resistant to penicillin.[10–12]

Penicillin resistance in staphylococci is mediated by the production of a penicillinase encoded by the *blaZ* gene, which inactivates penicillin by hydrolysis of the beta-lactam ring.[13] Four types of penicillinases (A–D) have been described in *S. aureus*, all of them belonging to Ambler class A beta-lactamases.[14,15] Penicillinases type A, C and D are usually plasmid encoded, while type B is located on the chromosome.[16,17]

Penicillin-susceptible staphylococci are susceptible to all beta-lactam antibiotics. Penicillin-resistant, oxacillin-susceptible strains are resistant to penicillinase-labile penicillins, but are susceptible to other penicillinase-stable penicillins, beta-lactam/beta-lactamase inhibitor combinations, cephems, and carbapenems. Oxacillin-resistant staphylococci are resistant to all currently available beta-lactam antibiotics, except for new cephalosporins with anti-MRSA activity (ceftaroline and ceftobiprole).

At present, most staphylococci, either coagulase positive or coagulase negative, are resistant to penicillin G, as well as to penicillin V, ampicillin, amoxicillin, and carbenicillin.[18,19] Penicillin susceptibility testing of staphylococcal isolates is performed in most laboratories by the agar disk diffusion. The Clinical and Laboratory Standards Institute[20] defines penicillin susceptibility as a minimum inhibitory concentration (MIC) of penicillin $\leq$0.12 μg/mL or as the formation of an inhibition halo $\geq$29 mm by the disk diffusion method (Kirby-Bauer), in combination with a negative result in the chromogenic cephalosporin assay (nitrocefin disk) for detection of beta-lactamase production. Although the disk diffusion and nitrocefin tests are accepted as methods for determining penicillin susceptibility, studies suggest that these methods do not reliably detect beta-lactamase production.[21,22]

Other methods that have been proposed to improve the sensitivity of beta-lactamase detection in staphylococci include the clover-leaf test[23–26] and observation of the appearance of the inhibition zone edge around penicillin G disks (zone edge test). However, little information about the reliability of the latter phenomenon is available,[21,27] especially for coagulase-negative staphylococci.

In 2012, the CLSI[28] started to recommend the zone edge test. This test was found to be more sensitive than the nitrocefin method in detecting beta-lactamase production in *S. aureus* when only one test was performed. However, laboratories could choose to perform first the nitrocefin test and, if the test were positive, to report the isolate to be beta-lactamase positive or penicillin resistant. If the nitrocefin test were negative, the zone edge test should be performed before reporting an isolate to be penicillin susceptible.

In view of the high prevalence of penicillin resistance among staphylococci, penicillin is rarely used as antistaphylococcal treatment mainly UTIs, where there are several other antimicrobial agents that may be used. However, little information about these tests is available for coagulase-negative staphylococci and better assessment of the detection of beta-lactamase production in these species is required. The objective of the present study was to compare and to evaluate the accuracy of the following four phenotypic methods for the detection of beta-lactamase production in *Staphylococcus* spp.: appearance of the inhibition zone edge around the penicillin G disk, disk diffusion, MIC determination, and nitrocefin disk test. Detection of the *blaZ* gene by the polymerase chain reaction (PCR) was used as a reference.

Materials and methods

Strains

One hundred one *Staphylococcus* spp. strains isolated from urine samples of patients seen in wards, outpatient clinics, the emergency department and several health centers of Botucatu and region were used in this study. The isolates were sent to the Laboratory of Microbiology of the University Hospital (HC), Botucatu Medical School (FMB), between March 10 and November 14, 2008. The project was approved by the Ethics Committee of FMB, UNESP (Of.-416/08-CEP).

Inclusion and exclusion criteria

Patients of both genders and all ages, whose urine cultures were positive for *Staphylococcus* spp. and considered compatible with UTI, with a colony-forming unit count (CFU) $\geq 10^5$ per mL urine according to the criteria of Kass,[29] were included in the study.

Strains isolated from urine catheters, suprapubic punctures and positive urine cultures containing $<10^5$ CFU/mL were excluded.

Sample size calculation

The sample size was calculated using the formula of Fisher and Belle,[30] adopting a 95% confidence interval and precision of 5% for the expected prevalence of patients with UTI. The proportion of patients with UTI caused by *Staphylococcus* spp., which was 5% in a study conducted at the Laboratory of Microbiology of HC-FMB, was used as a basis.

Although the sample size calculation indicated 73 isolates as the minimum number, all *Staphylococcus* spp. strains isolated during the study period, which met the inclusion and exclusion criteria, were used. A total of 101 isolates were thus included.

Nucleic acid extraction

Total nucleic acid was extracted from *Staphylococcus* spp. strains cultured on blood agar, individually inoculated into BHI broth, and incubated at 37 °C for 24 h. The Illustra Kit (GE Healthcare, Chalfont, England) was used for extraction according to manufacturer recommendations and the extracted DNA was stored under refrigeration at −20 °C.

Genotypic identification of Staphylococcus spp.

The *Staphylococcus* spp. isolates were submitted to genotypic identification using primers targeting conserved sequences adjacent to the 16S and 23S genes. This method described by Barry et al.[31] and Couto et al.[32] is known as internal transcribed spacer-polymerase chain reaction (ITS-PCR). The technique was carried out as described by Couto et al.[32] using primers G1 (5′-GAA GTC GTA ACA AGG-3′) and L1 (5′-CAA GGC ATC CAC CGT-3′).

Antimicrobial susceptibility testing by the disk diffusion method

The susceptibility profile to penicillin G 10 UN (Oxoid, Basingstoke, England) was evaluated using the criteria recommended by the CLSI.[20]

For inoculum preparation, a direct suspension of colonies (pure 24-h culture) in 0.9% saline (Fresenius Kabi, Aquiraz, Brazil), corresponding to a 0.5 McFarland turbidity standard, was used. After homogenization of the suspension, a sterile swab was pressed against the inner wall of the tube for removal of excess inoculum and seeded in four different directions onto the surface of Mueller–Hinton agar plates (Oxoid, Basingstoke, England). The plates were left to stand for 5–15 min at room temperature to allow complete absorption of the inoculum by the agar before application of the disks. The plates were incubated for 24 h at 35 °C and the penicillin inhibition halos were measured after this period by illuminating the surface with reflected light.

Determination of the minimum inhibitory concentration of antimicrobials

The MIC against the *S. aureus* and coagulase-negative staphylococcal isolates was determined using E-test® strips (AB Biodisk, Solna, Sweden) for penicillin G at an MIC interval of 0.002–32.0 μg/mL. The procedures of inoculum preparation, plating and incubation were the same as those described for the disk diffusion method. The MIC was determined at the point of intersection between the strip's scale and the ellipse of the zone of bacterial growth inhibition. Values ≤0.12 μg/mL and ≥0.25 μg/mL were defined as susceptible and resistant, respectively.[20] Strains exhibiting MICs >0.12 μg/mL and <0.25 μg/mL were considered resistant.

Nitrocefin disk test for detection of beta-lactamase production

Production of beta-lactamase was detected using disks impregnated with nitrocefin (chromogenic cephalosporin) (Becton Dickinson, Sparks, USA). The test is based on the release of a chromogenic radical, which causes a color change when the beta-lactam ring is broken open by the action of beta-lactamase.

The disk was moistened with one or two drops of sterile distilled water and deposited near an oxacillin E-test® strip on a culture previously incubated for 24 h at 35 °C on a Mueller-Hinton agar plate. The disks were analyzed after 5 min. A positive reaction was defined by the development of a red color and a negative reaction by the lack of color change. In the case of beta-lactamase-negative strains, the reaction was reexamined after 1 h according to manufacturer recommendations. Positive (*S. aureus* ATCC 33591) and negative (*Staphylococcus xylosus* ATCC 29979) control strains were included.

Zone edge test for detection of beta-lactamase production

The production of beta-lactamase was evaluated based on the appearance of the inhibition zone edge around the penicillin G disk (Oxoid, Basingstoke, England) obtained by the disk diffusion method. The test was defined as negative when the appearance of the edge was fuzzy like a "beach" and as positive when the edge was sharp like a "cliff".[20]

Detection of the blaZ gene by PCR

The PCR mixtures contained, in a total volume of 50 μL, 200 μM of each dNTP, 1 μM of each primer (stau-blaZ-forward: 5′-CAAAGATGATATAGTTGCTTATTCTCC-3′ and stau-blaZ-reverse: 5′-TGCTTGACCACTTTTATCAGC-3′), 10 μL DNA, 1.25 U Taq polymerase (GE Healthcare, München, Germany), and reaction buffer provided by the manufacturer. Amplification was carried out in a PTC-100™ thermocycler (MJ Research, Watertown, USA) using the parameters described by Kaase et al.[15]: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min.

The reaction was positive when a 421-bp DNA fragment was observed. *blaZ*-positive (*S. aureus* ATCC 29213) and negative (*S. aureus* ATCC 25923) reference strains were included in all reactions.

Results

Among the 101 isolates analyzed by ITS-PCR, 57 were identified as *S. saprophyticus*, 17 as *S. aureus*, 16 as *Staphylococcus epidermidis*, eight as *Staphylococcus haemolyticus*, two as *Staphylococcus warneri*, and one as *Staphylococcus lugdunensis*.

Twenty-eight (27.7%) isolates were susceptible to penicillin by the disk diffusion method and 16 (15.8%) by the E-test®

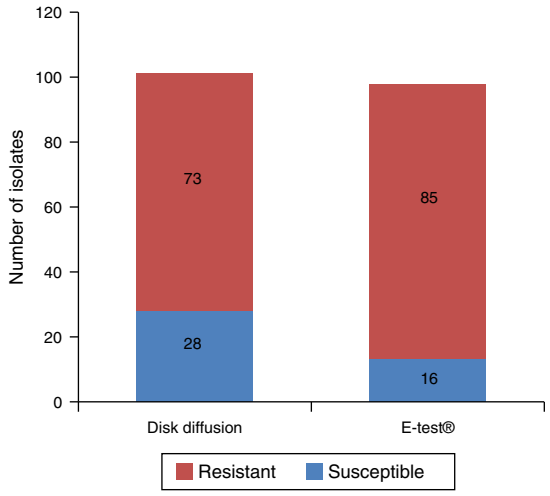

Fig. 1 – Resistance to penicillin by the disk diffusion method and E-test®.

(Fig. 1). The 73 remaining isolates (72.3%) were resistant to penicillin by the disk diffusion method, with an inhibition halo ≤28 mm and MIC ≥0.12 μg/mL, except for four isolates that exhibited MIC ≤0.12 μg/mL. The zone edge test identified three false-positive isolates and eight false-negative isolates among the 88 strains studied. Thirteen isolates could not be evaluated due to the lack of formation of an inhibition halo.

Among the 28 isolates susceptible to penicillin by the disk diffusion method, 22 (78.6%) were *S. saprophyticus*, three (10.7%) were *S. aureus*, two (7.1%)were *S. epidermidis*, and one (3.6%) was *S. lugdunensis*.

The nitrocefin method detected beta-lactamase production in only one (4.5%) *S. saprophyticus* isolate among the 28 isolates susceptible to penicillin by the disk diffusion method; however, the zone edge test was positive in 18 (81.8%) (Fig. 2). Fifteen (68.2%) *S. saprophyticus* isolates were resistant to penicillin by the E-test® and 19 carried the *blaZ* gene. Three isolates *S. aureus* and two *S. epidermidis* were susceptible to penicillin by the two methods (disk diffusion and E-test®) and tested negative in the nitrocefin and zone edge tests (Fig. 3). However, three isolates carried the *blaZ* gene (two *S. aureus* and one *S. epidermidis*). The *S. lugdunensis* was resistant to penicillin only by E-test® and *blaZ* gene positive although tested negative in the nitrocefin and zone edge tests (Table 1).

Seventy-three isolates exhibited a halo diameter ≤28 mm (resistant) for penicillin and all of them were positive for the *blaZ* gene (Fig. 4). Among the 28 susceptible isolates (halo ≥29 mm), 23 carried the *blaZ* gene and five did not (Fig. 5).

As can be seen in Table 2, the zone edge test was the most sensitive (90.3%), followed by MIC determination (85.5%). In contrast, the zone edge test showed low specificity (40%) and the nitrocefin test was the least sensitive (28.9%). The highest specificity (100%) was observed for the nitrocefin test in combination with the disk diffusion method.

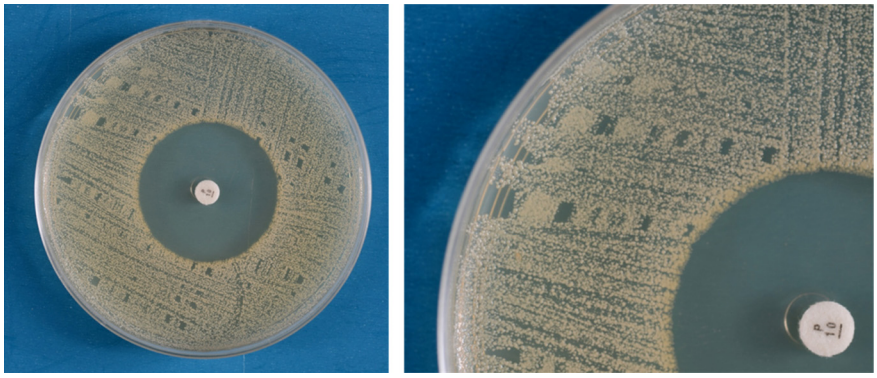

Fig. 2 – Positive zone edge test for the detection of beta-lactamase production (sharp edge).

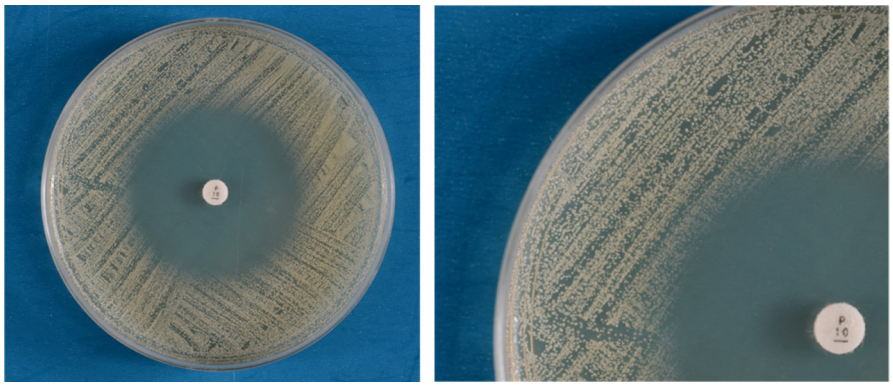

Fig. 3 – Negative zone edge test for the detection of beta-lactamase production (fuzzy edge).

Table 1 – Detection of beta-lactamase production by phenotypic and genotypic methods in isolates that were susceptible to penicillin by the disk diffusion method.

	Penicillin			Detection of beta-lactamase production		
	Disk (mm)	MIC (μg/mL)		Nitrocefin	Zone edge test	*blaZ*
Species	≥29[a]	≤0.12[b]	≥0.25[c]	Positive	Positive	Positive
S. saprophyticus	22 (78.6)	7 (31.8)	15 (68.2)	1 (4.5)	18 (81.8)	19 (86.4)
S. aureus	3 (10.7)	3 (100)	0 (0)	0 (0)	0 (0)	2 (66.7)
S. epidermidis	2 (7.1)	2 (100)	0 (0)	0 (0)	0 (0)	1 (50)
S. lugdunensis	1 (3.6)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)
Total	28 (100)	12 (42.9)	16 (57.1)	1 (3.6)	18 (64.3)	23 (82.1)

MIC, minimum inhibitory concentration.
[a] Halo diameter ≥29 mm = susceptible.
[b] MIC ≤0.12 μg/mL = susceptible.
[c] MIC ≥0.25 μg/mL = resistant.

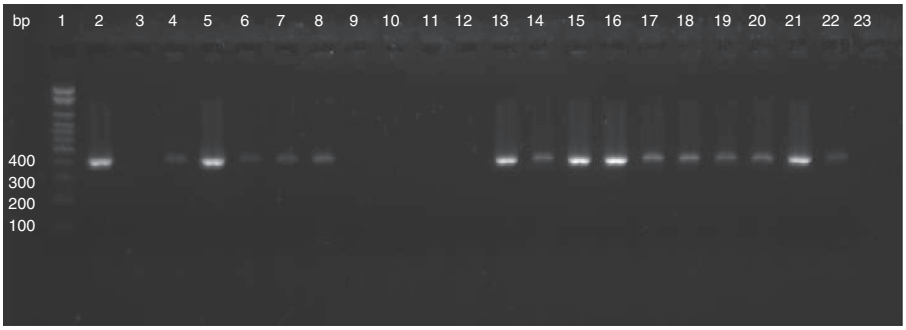

Fig. 4 – Agarose gel electrophoresis for detection of the *blaZ* gene (421 bp) by PCR. Lanes 4–8 and 13–22: positive isolates; line 9 is positive an 13 is negative: negative isolates; 3: negative control (*S. aureus* ATCC 25923); 2: positive control (*S. aureus* ATCC 29213); 23: water; 1: molecular weight marker (100 bp).

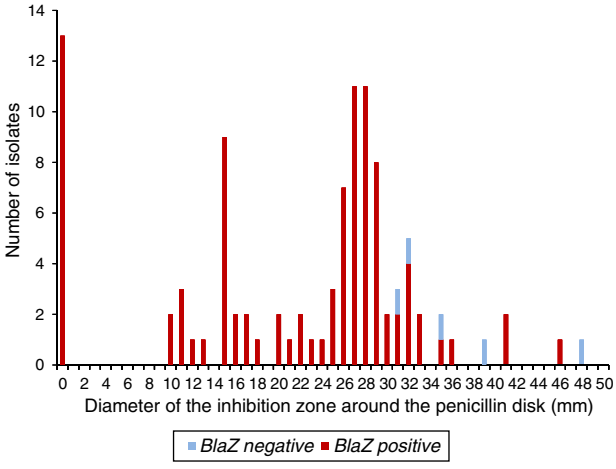

Fig. 5 – Correlation between halo size and presence of the *blaZ* gene.

Discussion

Although UTIs caused by *S. saprophyticus* have been documented, the antimicrobial resistance and dissemination of this species are not well studied. Today, most of the isolated staphylococcal species, either coagulase positive or coagulase negative, are resistant to penicillin. In view of the high prevalence of penicillin resistance in staphylococci, this antibiotic is rarely used as antistaphylococcal treatment. However, prolonged treatment with penicillin may be desirable in certain circumstances, as long as the susceptibility test result is reliable.

The best phenotypic method to detect penicillin resistance in *Staphylococcus* spp. continues to be a challenge. In this respect, the CLSI is evaluating recommendations for the detection of this type of resistance. Several methods have been proposed to improve the sensitivity of beta-lactamase detection in staphylococci, including the clover-leaf test, nitrocefin disk method, and zone edge test. However, little information

Table 2 – Sensitivity and specificity of the methods used for the detection of beta-lactamase in *Staphylococcus* spp.

Accuracy test	Disk diffusion	MIC	Nitrocefin	Zone edge test
Sensitivity (%)	72.2	85.5	28.9	90.3
Specificity (%)	100.0	80.0	100.0	40.0

MIC, minimum inhibitory concentration.

is available regarding the reliability of these tests, especially in coagulase-negative staphylococci.

In the present study, only 28 (27.7%) of the 101 isolates analyzed were susceptible to penicillin by the disk diffusion method and 12 (11.8%) by the E-test®. The remaining 73 (72.3%) penicillin-resistant isolates exhibited an inhibition halo ≤28 mm and MIC ≥0.12 μg/mL, demonstrating low susceptibility to this antimicrobial agent. This susceptibility is even lower when considering that 19 of the 28 susceptible isolates in the disk diffusion test carried the *bla*Z gene, a fact impairing any attempt of treatment of UTIs with this antimicrobial drug, especially empirical therapy.

Kaase et al.[15] studied 197 *S. aureus* isolates with MIC ≤0.12 μg/mL. Twenty-eight (14.2%) of these isolates were positive for the *bla*Z gene, including 20 (71.4%) detected by the zone edge test and 11 (39.3%) by the nitrocefin test. In the present study, 12 (11.8%) of the 28 isolates susceptible to penicillin by the disk diffusion method had MIC ≤0.12 μg/mL. However, 18 (64.3%) tested positive in the zone edge test and only one (3.6%) in the nitrocefin method. These values are similar to those reported by Kaase et al.[15] for the zone edge test, but are lower than those observed by these authors for the nitrocefin method.

The breakpoint recommended by the CLSI to classify penicillin-resistant isolates by the disk diffusion method showed a sensitivity of 72.2%, a value higher than the 57.1% reported by Kaase et al.[15] In this study, the sensitivity of the nitrocefin test (28.9%) was lower than that obtained by these authors (39.3%) and by others: 82.0%,[33] 86.2%,[34] 95.6%,[23] 70.8–97.9% depending on the induction method and agar used,[24] and 62.1–100% depending on the manufacturer.[26] The large difference in the sensitivity of the nitrocefin test between the present study and the studies cited may be related to the fact that we mainly evaluated *S. saprophyticus*.

McDougal and Thornsberry[35] evaluated the production of beta-lactamase in 66 *S. aureus* isolates using the nitrocefin disk method and all strains tested positive. However, the change in color was weak when the penicillin MIC was less than ≤16 μg/mL. In the present study, all *S. saprophyticus* isolates with a negative nitrocefin disk test exhibited penicillin MICs of 0.125–0.38 μg/mL, except for one isolate with an MIC of 0.94 μg/mL, while three of the two positive isolates had higher MICs (1.0 and 1.5 μg/mL) and one isolated had an MIC of 0.19 μg/mL.

More recently, Pitkälä et al.[26] used detection of the *bla*Z gene by PCR as a reference method to evaluate different commercial tests for detection of beta-lactamase production in *Staphylococcus* spp., including the nitrocefin disk method. The authors found 19 isolates with a very weak reaction to nitrocefin; of these, 15 were positive for the *bla*Z gene. In the present study, only one of the 22 *S. saprophyticus* isolates tested positive in the nitrocefin test, but 19 were positive for the *bla*Z gene. These results agree with the findings of Hovelius and Mardh[36] who showed that the chromogenic cephalosporin (nitrocefin) does not detect beta-lactamase production in *S. saprophyticus*, a fact explaining the low sensitivity of the nitrocefin disk in detecting beta-lactamase production observed here, especially in *S. saprophyticus*. In contrast, Haveri et al.[22] found high agreement between the nitrocefin test and the presence of the *bla*Z gene in *S. aureus*. Although the nitrocefin disk method is a commonly used test for beta-lactamase detection, the enzymatic reactions may be incomplete, leading to a large number of false-negative results. This fact may explain the large number of beta-lactamase-negative *S. saprophyticus* isolates.

Twenty-three (82.1%) of the 28 isolates that were susceptible to penicillin by the disk diffusion method were *bla*Z-positive. This rate is much higher than those reported by Kaase et al.[15] and by El Feghaly et al.[37] who evaluated 105 penicillin-susceptible *S. aureus* isolates. In those studies, 14.2% and 9.5% of the *S. aureus* isolates phenotypically susceptible to penicillin carried the *bla*Z gene, respectively. This marked difference is likely due to the large number of *S. saprophyticus* isolates tested in the present study.

All isolates with a halo diameter ≤28 mm carried the *bla*Z gene. However, a large inhibition halo did not rule out the presence of the *bla*Z gene, as also reported by Kaase et al.[15] and El Feghaly et al.[37] In the study of El Feghaly et al.,[37] *bla*Z-positive isolates by the disk diffusion method exhibited an average halo size of 34 mm, while the inhibition halo of *bla*Z-negative isolates was greater than 38 mm. However, we found three isolates with an inhibition halo >40 mm (one *S. aureus*, one *S. epidermidis*, and one *S. lugdunensis*), which were positive for the *bla*Z gene. These findings agree with Kaase et al.[15] and El Feghaly et al.[37] who suggested conventional (phenotypic) methods to be unable to adequately detect penicillin resistance in staphylococcal isolates.

El Feghaly et al.[37] also suggested that changing the breakpoint of the penicillin inhibition halo from 29 to 35 mm would improve sensitivity of the test. A similar observation was made by Latham et al.[38] for *S. saprophyticus*. These authors found that isolates with a diameter <31 mm were all beta-lactamase positive. The use of this new breakpoint would permit to detect penicillin resistance in all *S. saprophyticus* isolates of the present study, which exhibited inhibition halos ranging in size from 29 to 35 mm. However, three *bla*Z-negative isolates would be considered positive if this new criterion were used.

With respect to the MIC of penicillin, Kaase et al.[15] found that all isolates with an MIC of 0.03 μg/mL determined with the Vitek 2 system were *bla*Z-negative, while five (6.2%) of 81 isolates with an MIC of 0.06 μg/mL and 23 (23.2%) of 99 isolates with an MIC of 0.12 μg/mL were *bla*Z-positive. Among the *bla*Z-negative isolates, two *S. saprophyticus* strains exhibited an MIC of 0.125 μg/mL and one an MIC of 0.19 μg/mL, one *S. aureus* had an MIC of 0.032 μg/mL, and one *S. epidermidis* had an MIC of 0.012 μg/mL. The MIC of the other *bla*Z-positive isolates ranged from 0.012 to 0.94 μg/mL, demonstrating that determination of the MIC also does not reliably detect this type of resistance. This difficulty has also been reported by Haveri et al.[22] who found five *bla*Z-negative isolates with MIC ≥0.25 μg/mL and 26/211 (12.3%) susceptible isolates with MIC of 0.06–0.125 μg/mL that carried the *bla*Z gene.

In the present study, the zone edge test was the most sensitive phenotypic test (90.3%). A similar result has been reported by Kaase et al.[15] who evaluated the sensitivity of five phenotypic methods for detection of beta-lactamase and found a sensitivity of 71.4% for the zone edge test. In contrast, in the study of El Feghaly et al.,[37] the most sensitive phenotypic test was the clover-leaf test (60.0%). According to these authors, the zone edge test is highly subjective and its interpretation may vary depending on the examiner. However, the test was

analyzed by two examiners in the present study and there was no discrepancy between results.

The present results demonstrated that the zone edge test was the most sensitive phenotypic test for detection of beta-lactamase, although it is still not an ideal test to detect this type of resistance since its specificity was low. Determination of the MIC and the disk diffusion method provided better results than the nitrocefin test and the zone edge test. However, the inhibition halo diameter of the penicillin disk can be used together with the zone edge test since the same disk is employed in the two tests. Combined analysis of the two tests shows a sensitivity of 90.3% and specificity of 100%, proving better sensitivity, especially for *S. saprophyticus*. This is a low-cost test of easy application and interpretation that can be used in small and medium-sized laboratories where susceptibility testing is usually performed by the disk diffusion method.

Conflicts of interest

The authors declare no conflicts of interest.

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