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Evaluation of methods for detection of β-lactamase production in MSSA

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Abstract

Objectives: Correct determination of penicillin susceptibility is pivotal for using penicillin in the treatment of *Staphylococcus aureus* infections. This study examines the performance of MIC determination, disc diffusion and a range of confirmatory tests for detection of penicillin susceptibility in *S. aureus*.

Methods: A total of 286 consecutive penicillin-susceptible *S. aureus* blood culture isolates as well as a challenge set of 62 MSSA isolates were investigated for the presence of the *blaZ* gene by PCR and subjected to penicillin-susceptibility testing using broth microdilution MIC determination, disc diffusion including reading of the zone edge, two nitrocefin tests and the cloverleaf test.

Results: Using PCR-based detection of *blaZ* as the gold standard, both broth microdilution MIC testing and disc diffusion testing resulted in a relatively low accuracy (82%–93%) with a sensitivity ranging from 49%–93%. Among the confirmatory tests, the cloverleaf test performed with 100% accuracy, while zone edge interpretation and nitrocefin-based tests increased the sensitivity of β-lactamase detection to 96%–98% and 82%–96% when using MIC determination or disc diffusion as primary test, respectively.

Conclusions: This investigation showed that reliable and accurate detection of β-lactamase production in *S. aureus* can be obtained by MIC determination or penicillin disc diffusion followed by interpretation of the zone edge as a confirmatory test for apparently penicillin-susceptible

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Transparency declarations

None to declare.

Disclaimer

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Supplementary data

Supplementary Materials and methods, Supplementary Results and Table S1 are available as Supplementary data at *JAC* Online.

isolates. The more cumbersome cloverleaf test can also be used. Nitrocefin-based tests should not be used as the only test for confirmation of a presumptive β-lactamase-negative isolate.

Introduction

Staphylococcus aureus is one of the most frequent and important causes of human infections. Penicillin was from the beginning the drug of choice against *S. aureus* infections and even though most *S. aureus* isolates are resistant to penicillin today, it is still a preferred antibiotic for penicillin-susceptible *S. aureus* (PSSA) isolates due to its high bactericidal activity and low toxicity, especially for severe infections requiring long treatment.[1]

In *S. aureus*, the two major resistance mechanisms to penicillin are: (i) *blaZ*-mediated production of β-lactamase (also called penicillinase); and (ii) resistance encoded by the *mec* genes (in particular *mecA*, but also *mecB* and *mecC*) leading to production of an additional altered penicillin-binding protein, PBP2a, that compared with native PBP2 has low affinity for not only penicillins, but also the majority of β-lactam antibiotics, including methicillin [hence the name methicillin-resistant *S. aureus* (MRSA)].[2] In addition, overproduction of the native PBPs may lead to variable degrees of resistance to β-lactam antibiotics,[3] but this mechanism does not constitute a significant clinical problem because it is relatively rare. This paper deals specifically with detection of penicillin resistance due to β-lactamase production. Resistance due to PBP2a or overproduction of native PBPs will not be discussed further here.

β-Lactamase-producing *S. aureus* was first described in 1944 by Kirby,[4] which was followed by a rapid increase in the prevalence of penicillin-resistant *S. aureus*, reaching almost 60% in some hospitals in the late 1940s.[5] During the following decades, the prevalence of penicillin resistance increased to 85%–90% among *S. aureus* isolates causing both community and hospital-acquired infections.[6] However, the trend seems to have reversed in recent years. Since 2015 almost 30% of *S. aureus* isolates from Danish patients with bacteraemia have been found to be penicillin susceptible.[7] Similar data were recently published in other European countries, Canada and the USA.[8–10] For example in the USA, a large multicentre survey revealed that 26% of the MSSA isolates were also susceptible to penicillin[10] In Sweden, an even higher frequency of PSSA (57%) of prospectively collected *S. aureus* isolates from bloodstream infections has recently been published.[11]

S. aureus isolates are defined as penicillin susceptible if the penicillin MIC is ≤0.125 mg/L and penicillin resistant if the penicillin MIC is >0.125 mg/L, according to the guidelines from both CLSI[12] and EUCAST.[13] However, some *S. aureus* isolates produce β-lactamase despite penicillin MICs of 0.125 or 0.064 mg/L.[10,14,15] This has led to mistrust of penicillin-susceptibility testing results in *S. aureus*. Thus, CLSI guidelines recommend testing for β-lactamase production and/or to confirm the absence of *blaZ* by PCR testing in all apparently penicillin-susceptible isolates.[12]

In the attempt to solve the problem of misclassifying β-lactamase-producing *S. aureus* isolates as penicillin susceptible, several phenotypic assays have been developed over the years, including both quantitative measurements of β-lactamase production (i.e. iodometric,

acidometric quantification), qualitative tests (i.e. hydrolysis of nitrocefin by cefinase, cloverleaf testing, or reading of the penicillin inhibition zone edge) and use of surrogate testing (i.e. against mecillinam).[16–20] The quantitative tests are cumbersome and have never found their way into routine laboratories, whereas the nitrocefin test has been used by many laboratories due to its ease, although the test has been reported to have a low sensitivity.[9,14,21,22] The cloverleaf test and the zone edge test both require that testing personnel are trained well in performing the tests, which may be the reason for the reported discrepancies in test performance results between different institutions.[9,14,21–24] At Statens Serum Institut in Denmark, both the cloverleaf test and interpretation of the zone edge have been used routinely for many years with high inter-test concordance (R. Skov and A. Rhod Larsen, unpublished data).

PCR-based detection of the *blaZ* gene is an alternative approach for detection of β-lactamase production in *S. aureus*. However, four different *S. aureus* β-lactamase isoenzymes (A–D) have been described, and published primers have shown variable sensitivities and specificities in detecting the corresponding genes.[25] In 2008, Kaase *et al.*[14] published PCR primers that were designed to cover all 279 *blaZ* sequences available in GenBank at that time[23] and Pereira *et al.*[26] subsequently published a real-time PCR method with similar performance as the method described by Kaase *et al.*[14]

Today, most clinical laboratories test *S. aureus* for susceptibility towards penicillin using MIC determination or disc diffusion as the primary method with or without using a second confirmatory method. In this study, we investigated the reliability of different phenotypic methods, including the cloverleaf and the zone edge test, for evaluating β-lactamase production in *S. aureus* using PCR-based detection of the *blaZ* gene as the gold standard.

Materials and methods

Strains

A total of 286 consecutive phenotypically PSSA blood culture isolates from the Massachusetts General Hospital (MGH), Boston, MA, USA (N = 199) and Aalborg University Hospital (AUH) in Denmark (N = 87) were investigated. The 199 isolates from MGH were collected in 2010 and had penicillin MICs of ≤0.125 mg/L using the Vitek 2 system (bioMerieux, Durham, NC, USA). The 87 isolates from AUH were collected between 2008 and 2010 and were found to be susceptible to penicillin based on disc diffusion and the cloverleaf test. In addition, a challenge set of 62 MSSA isolates from CDC, Atlanta, GA, USA, was also tested. This set included isolates that had previously given inconclusive or conflicting results by different phenotypic and genotypic tests. Two isolates from the AUH collection and one isolate from the CDC challenge collection were *blaZ* positive by PCR, but penicillin susceptible in all phenotypic tests. These isolates were investigated by WGS, which showed that all three isolates carried a non-functional *blaZ* gene [Supplementary Materials and methods, Supplementary Results and Table S1 (Supplementary Materials and methods, Supplementary Results and Table S1 are available as Supplementary data at *JAC* Online)]. These three isolates were excluded from the analysis, resulting in a final dataset of 345 isolates (284 consecutive PSSA blood culture

isolates, including 199 from MGH and 85 from AUH, and 61 challenge MSSA isolates from CDC).

All tests were initially performed at Statens Serum Institut. A subset of isolates ($N = 15$) with divergent phenotypic and/or difficult-to-interpret results were sent to CDC and MGH, where all tests were repeated. *S. aureus* ATCC 25923 (penicillin susceptible) and ATCC 29213 (penicillin resistant) were included for quality control assessment in all experiments.

PCR-based detection of the blaZ gene

All isolates were investigated for the presence of the *blaZ* gene by PCR, as described by Kaase *et al.*[14] using the following primers: stau-blaZ-fwd (5′-CAAAGATGATATAGTTGCTTATTCTCC) and stau-blaZ-rev (5′-TGCTTGACCACTTTTATCAGC).

Phenotypic assays

Penicillin MICs were determined by broth microdilution (BMD) using 2-fold dilutions of penicillin (0.004 to 8 mg/L), as described in the CLSI and EUCAST guidelines.[27,28] Disc diffusion tests with 1 U (EUCAST) and 10 U (CLSI) benzylpenicillin discs (Oxoid, Basingstoke, UK) were performed on Mueller–Hinton agar (MHA) plates (Becton Dickinson, Heidelberg, Germany) using an inoculum adjusted to a 0.5 McFarland turbidity standard. After incubation at 35°C for 18 ± 2 h, inhibition zone diameters were measured using calipers.[27,28]

Detection of β-lactamase production was further evaluated using three qualitative assays: (i) the nitrocefin test; (ii) the cloverleaf test; and (iii) interpretation of the zone edge. Two commercial versions of the nitrocefin test, BBL Cefinase™ (Becton Dickinson, Franklin Lakes, NJ, USA) and DrySlide™ Nitrocefin (Becton Dickinson), were included. The nitrocefin tests were performed using several colonies taken from the zone edge of a 1 U benzylpenicillin disc on an MHA plate incubated overnight. Test results were read every 5 min for up to 60 min. All isolates were tested using the same batch of Cefinase™ and DrySlide™ Nitrocefin. Isolates giving discrepant results were retested using colonies from the zone edge of both a cefoxitin 30 μg and an oxacillin 1 μg disc.

The cloverleaf test was performed on 5% sheep blood agar plates using a 1 U benzylpenicillin disc. The pre-seeded lawn of *S. aureus* ATCC 25923 (β-lactamase negative) was prepared the day before use. The lawn was made by flooding the plates with 2 mL of a 1:10 dilution of *S. aureus* ATCC 25923 in Mueller–Hinton broth that was originally matched to a 0.5 McFarland turbidity standard. The flooding was followed by immediate suction of excess fluid, drying and followed by storage at 4°C overnight, resulting in an even confluent lawn when used in the assay.[20] The test strains were streaked as a cross on the pre-seeded plate and the benzylpenicillin disc was placed at the centre of the cross. Indentation of growth where the test strain crossed the inhibition zone edge was interpreted as β-lactamase production. Photographs of the cloverleaf test for *S. aureus* AAH 7867 (β-lactamase negative) and *S. aureus* ATCC 29213 (β-lactamase positive) are shown in Figure 1. Fifty of the isolates were also tested on MHA plates without blood supplementation using both a 1 U and a 10 U benzylpenicillin disc.

For the zone edge test, isolates were tested on MHA plates using both the 1 U and 10 U benzylpenicillin discs.[27,28] The edge of the inhibition zone was interpreted for both discs as described by Gill *et al.*[18] Isolates that produce β-lactamase make a sharp demarcation zone with full-sized colonies right at the edge (i.e. a 'cliff' pattern), whereas isolates that do not produce β-lactamase exhibit a fuzzy demarcation of the zone edge due to growth of minute colonies, which become smaller and smaller towards the centre (i.e. a 'beach' or 'fuzzy' pattern).[18] The patterns for *S. aureus* AAH 7867 (β-lactamase negative) and *S. aureus* ATCC 29213 (β-lactamase positive) are shown in Figure 2.

Results

By PCR, 45 isolates were *blaZ* positive and 300 isolates were *blaZ* negative. The results of the different phenotypic tests for the consecutive, challenge and total sets of isolates versus their *blaZ* status are shown in Table 1. Of note, 19 of the 45 *blaZ*-positive isolates were from the consecutive set, which comprises isolates that were previously found to be penicillin susceptible by MIC determination at MGH (18 isolates), or by disc diffusion and the cloverleaf test (1 isolate) at AUH.

Detailed data for MIC determination and disc diffusion, according to CLSI and EUCAST, are shown in Tables 2–4, while Table 5 shows the obtained sensitivity of the various test combinations. Neither MIC determination nor disc diffusion using a 10 U or 1 U benzylpenicillin disc performed with high accuracy, with the number of correctly classified isolates being 321 (93%), 322 (93%) and 282 (82%), respectively. Both MIC determination and disc diffusion using a 10 U benzylpenicillin disc had a low level of detection of β-lactamase production (49% and 51%, respectively, with a specificity of 99.7% for both assays), whereas disc diffusion using a 1 U benzylpenicillin disc had a relatively high sensitivity of 93%, but a low specificity of 80%. Interestingly, two β-lactamase-producing isolates had an MIC of 0.064 mg/L, and an additional β-lactamase-positive isolate displayed an MIC of 0.032 mg/L. These results emphasize that both MIC determination and disc diffusion require the use of a second confirmatory test for correct detection of β-lactamase production in *S. aureus*. This conclusion is further supported by our finding that MIC determination at MGH misclassified 18 *blaZ*-positive isolates as penicillin susceptible.

Among the confirmatory tests, the cloverleaf assay performed best, demonstrating 100% sensitivity and specificity when using a 1 U benzylpenicillin disc. As mentioned above, one of the *blaZ*-positive isolates had initially been found to be penicillin susceptible by disc diffusion and the cloverleaf test at AUH. In the present investigation, the isolate only had a very subtle flattening of the zone, which was only visible because the test strain was streaked in two directions as a cross.

Repeated testing of 50 isolates on MHA plates using 1 U and 10 U benzylpenicillin disc gave the same interpretation, although reading the tests was easier for the combination of 5% sheep blood agar plate and 1 U benzylpenicillin disc (data not shown). The zone edge test showed 100% specificity, but missed two of the *blaZ*-positive isolates (having a 'beach' zone edge) from the consecutive set of isolates, resulting in a sensitivity of 89% and 96% for the consecutive and total sets of isolates, respectively. Using the zone edge test in combination

with either MIC determination or disc diffusion resulted in detection of 43–44 of the 45 *blaZ*-positive isolates (Table 5).

The results further showed that both the Cefinase™ (Becton Dickinson) and the DrySlide Nitrocefin™ (Becton Dickinson) tests had relatively low sensitivities when used alone (78% and 87%, respectively, on the total set of isolates). The sensitivities increased considerably when used in combination with MIC determination, or with disc diffusion using a 10 U benzylpenicillin disc, but not with disc diffusion using a 1 U benzylpenicillin disc (Table 5). In all cases, DrySlide™ (Becton Dickinson) and Cefinase™ (Becton Dickinson) did not perform as well as the zone edge test (Table 5).

There was a high sensitivity of 98% for detection of β-lactamase-producing isolates when both the zone edge test and one of the nitrocefin tests were used as confirmatory tests following initial MIC determination or disc diffusion testing (Table 5). The results of the initial tests at Statens Serum Institut and repeated tests at CDC and MGH were in accordance (data not shown).

Discussion

Penicillin is an attractive treatment option for infections caused by PSSA both from an efficiency as well as from an adverse effect and safety point of view, especially for cases requiring prolonged treatment. However, correct determination of susceptibility to penicillin and trust in the overall interpretation of all test results are pivotal for the clinical decision of whether to use penicillin for treating *S. aureus* infections. As several alternative antibiotics are available for treatment, it is especially important not to misclassify isolates with β-lactamase production as susceptible (i.e. sensitivity is the most important parameter). Development of algorithms for accurate determination of whether an isolate is penicillin susceptible or not is thus of great clinical importance.

In this investigation, we used both consecutively collected clinical PSSA isolates and challenge isolates to investigate the ability of different phenotypic methods to detect β-lactamase production, both alone as well as in combination, using PCR-based detection of the *blaZ* gene as the gold standard.

Clinical laboratories most frequently use either MIC determination or disc diffusion as the primary susceptibility testing method. In this investigation, we used BMD for penicillin MIC determination, which is generally considered the gold standard for susceptibility testing. We found that 63% of the *blaZ*-positive isolates from the consecutive set of isolates had an MIC of ≤0.125 mg/L (i.e. false susceptible) with one isolate showing an MIC as low as 0.032 mg/L. *blaZ*-positive isolates with penicillin MICs in the susceptible range have also been found in other studies.[10,14,21,23] Richter *et al.*[10] found that 32% of isolates having an MIC of 0.125 mg/L and 3.8% of isolates with an MIC of 0.06 mg/L were *blaZ* positive, whereas none of the *blaZ*-positive isolates had MICs as low as 0.03 mg/L. Our investigation also confirmed previous findings that disc diffusion using the size of the inhibition zones cannot reliably separate *blaZ*-negative from *blaZ*-positive isolates.

From this and other investigations, it is clear that regardless of whether MIC determination or disc diffusion is used as an initial test, there is a need for additional confirmatory tests. In our study, the cloverleaf test demonstrated both a high sensitivity (100%) and specificity (100%) for detection of β-lactamase production. Other studies of the cloverleaf test have shown a mixed range of sensitivities, from 68% to 100%.[11,14,16,22] It should be noted that the cloverleaf test on 5% sheep blood agar with a 1 U benzylpenicillin disc and inoculation by flooding of the plate made the interpretation much easier than when it was performed on MHA plates as originally suggested by Ørstavik and Ødegaard.[20] This may explain the better results found in our investigation. However, since the cloverleaf test is relatively cumbersome, the other alternatives may be preferred.

Interpretation of the shape (sharp or fuzzy) of the zone edge had a high correlation with the *blaZ* status with a sensitivity of 96% and a specificity of 100% (total set of isolates), both when using a 1 U and a 10 U benzylpenicillin disc. This correlation is considerably higher than those found by other recent investigations, which all used the 10 U benzylpenicillin disc.[10,14,21,23,26] Interestingly, Papanicolas *et al.*[21] found 100% sensitivity when using a 1 U benzylpenicillin disc compared with 89% when using a 10 U benzylpenicillin disc.

Increased sensitivity for both MIC determination and disc diffusion using a 10 U benzylpenicillin disc could be obtained by nitrocefin tests, but not as much as the zone edge test. However, given the importance of detecting β-lactamase-positive *S. aureus*, these nitrocefin-based tests should not be used alone as a definitive confirmatory test for exclusion of β-lactamase production. Several other investigators have also reported unacceptably low sensitivities ranging from approximately 30% to 85%.[14,21,22] The present CLSI guidelines recommend confirming penicillin susceptibility in *S. aureus* with a β-lactamase test, but also to consider testing the isolate for the *blaZ* gene by PCR for serious infections if the β-lactamase test is negative.[12]

S. aureus isolates exhibiting borderline resistance to oxacillin (BORSA) were not included in this study. The BORSA phenotype can be caused by at least two different mechanisms: (i) hyperproduction of β-lactamase; and (ii) alterations in/or hyperproduction of the native PBPs.[29] Most β-lactamase hyperproducers do not produce an inhibition zone, and the zone edge can therefore not be evaluated. They are known to exhibit marked indents in the cloverleaf test and are strongly positive in the different cefinase tests. For BORSA isolates with alterations in/or hyperproduction of native PBPs, the result of the tests depends on whether the isolate is also producing β-lactamase. β-Lactamase producers will be positive in the cloverleaf and cefinase tests and produce a ‘cliff’ pattern in the zone edge test, whereas β-lactamase-negative isolates do not exhibit indents in the cloverleaf test, will be negative in the different cefinase tests and will often produce a variable but reduced zone diameter without a ‘cliff’ pattern.

Based on the data presented in this study, we suggest that routine laboratories can use one of the following algorithms for phenotypic testing. (A) Use disc diffusion as an initial test and report isolates with an inhibition zone diameter of ≤28 mm (10 U benzylpenicillin disc) or ≤25 mm (1 U benzylpenicillin disc) as penicillin resistant. For isolates with an inhibition zone diameter of ≥29 mm (10 U benzylpenicillin disc) or ≥26 mm (1 U benzylpenicillin

disc), the edge of the inhibition zone needs to be clarified as either fuzzy or sharp by the reader. If the edge is sharp, the isolate is reported as resistant, regardless of the zone size. If the zone edge is fuzzy, then the zone diameter cut-off values can be trusted. (B) Use MIC determination as an initial test and report isolates with an MIC of ≥0.25 mg/L as penicillin resistant. For isolates with an MIC ≤0.125 mg/L, perform a nitrocefin test and report them as penicillin resistant if positive. For nitrocefin-negative isolates, disc diffusion should be performed and interpreted as described in algorithm A. Using either of these algorithms, a sensitivity of 98% can be reached by using MIC determination or disc diffusion as an initial test. Even though the cloverleaf test performed with 100% accuracy in this investigation, we do not recommend the usage in routine laboratories as it is too cumbersome. PCR-based detection of *blaZ*, as described by Kaase *et al.*,[14] can also be used as a confirmatory test.

On a final note, this study has shown that isolates with a non-functional *blaZ* gene do occur. These isolates were removed from the analysis as the purpose of this paper was to evaluate phenotypic methods. Misclassification of such isolates as β-lactamase producers has limited consequences in clinical practice, as such isolates are presumed to be quite rare and there are several other treatment options available.

In conclusion, this investigation found that phenotypic methods can be used to accurately test *S. aureus* isolates for penicillin susceptibility when interpretation of the zone edge of the penicillin inhibition zone is used as a confirmatory test. The nitrocefin-based tests can be used as part of the susceptibility testing algorithm, especially when used in combination with MIC determination. However, due to the low sensitivity of nitrocefin-based tests, they cannot be used alone and nitrocefin-negative isolates should always be further examined. The cloverleaf test can be used as a confirmatory test, but it is cumbersome. If the cloverleaf test is used, we recommend that it is performed on a 5% sheep blood agar plate using a 1 U benzylpenicillin disc, with the test strain applied in both directions making a full cross. Finally, PCR-based detection of the *blaZ* gene can be used to confirm the PSSA phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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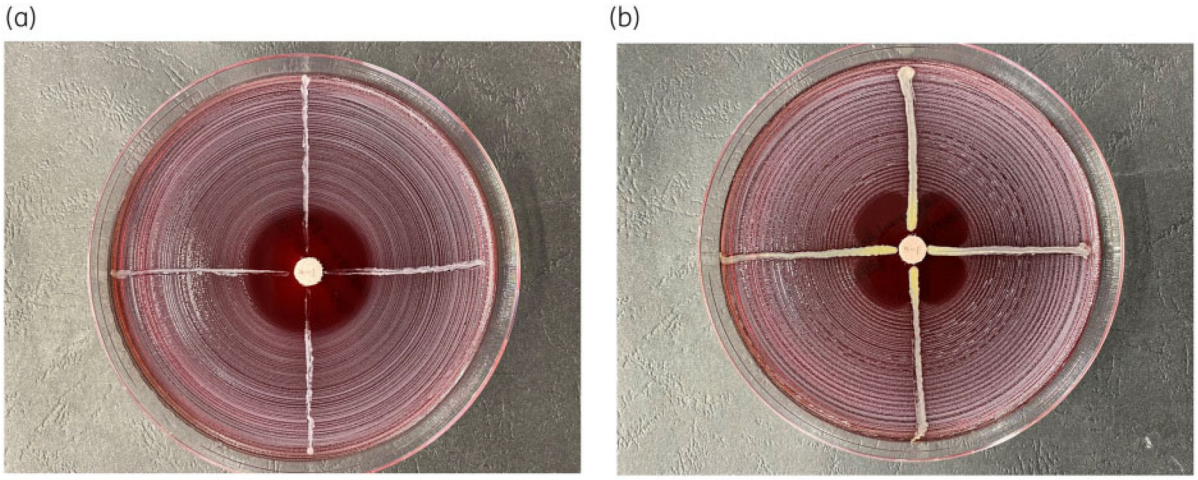

Figure 1.
Cloverleaf patterns for a β-lactamase-negative isolate and a β-lactamase-positive isolate. (a) β-Lactamase-negative isolate. (b) β-Lactamase-positive isolate. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

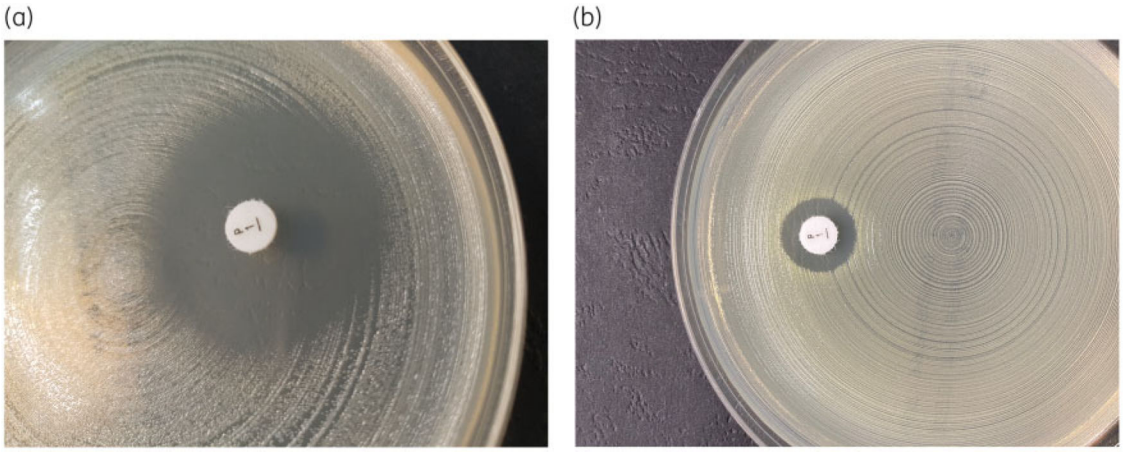

Figure 2.
Zone edge patterns for a β-lactamase-negative isolate and a β-lactamase-positive isolate. (a) β-Lactamase-negative isolate. (b) β-Lactamase-positive isolate. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 1.

Results of the different phenotypic tests in relation to *blaZ* gene status for the consecutive, challenge and total sets of MSSA isolates (*N* = 345)

		Consecutive set (*N*=284)		Challenge set (*N*=61)		Total strain set (*N*=345)	
Test	**Interpretation**	***blaZ* neg (*N* =265) *n* (%)**	***blaZ* pos (*N*=19) *n* (%)**	***blaZ* neg (*N* = 35) *n* (%)**	***blaZ* pos (*N* = 26) *n* (%)**	***blaZ* neg (*N* = 300) *n* (%)**	***blaZ* pos (*N* = 45) *n* (%)**
MIC	S ≤0.125 mg/L	264(99.6)	12(63)	35 (100)	11 (42)	299 (99)	23(51)
	R >0.125 mg/L	1 (0.4)	7(37)	0(–)	15 (58)	1 (0.3)	22 (49)
DD (10 U disc)[a]	S ≥29 mm	264(99.6)	9 (47)	35 (100)	13 (50)	299 (99)	22 (49)
	R ≤28 mm	1 (0.4)	10(53)	0(–)	13 (50)	1 (0.3)	23(51)
DD (1 U disc)[b]	S ≥26 mm	208(78)	3(16)	32 (91)	0(–)	240 (80)	3(7)
	R ≤25 mm	57 (22)	16 (84)	3 (9)	26 (100)	60(20)	42 (93)
Cefinase™	negative	265 (100)	4(21)	35 (100)	6(23)	300(100)	10(22)
	positive	0(–)	15 (79)	0(–)	20(77)	0(–)	35 (78)
DrySlide™	negative	265 (100)	4(21)	35 (100)	2 (8)	300(100)	6(13)
	positive	0(–)	15 (79)	0(–)	24 (92)	0(–)	39 (87)
Zone edge[c]	fuzzy	265 (100)	2 (11)	35 (100)	0(–)	300(100)	2 (4)
	sharp	0(–)	17 (89)	0(–)	26 (100)	0(–)	43 (96)
Cloverleaf	no	265 (100)	0(–)	35 (100)	0(–)	300(100)	0(–)
	yes	0(–)	19 (100)	0(–)	26 (100)	0(–)	45(100)

MIC, minimum inhibitory concentration as determined by broth microdilution; DD, disc diffusion; neg, negative; pos, positive; S, susceptible; R, resistant.

[a]Penicillin disc content recommended by CLSI.[12]

[b]Penicillin disc content recommended by EUCAST.[13]

[c]Same results were obtained whether using the 1 U or the 10 U benzylpenicillin disc.

Table 2.

Penicillin MIC distribution for the total set of MSSA isolates (N = 345) in relation to their *blaZ* status

	MIC (mg/L)										
	0.008	**0.016**	**0.032**	**0.064**	**0.125 j**	**0.25**	**0.5**	**1**	**2**	**4**	**Total**
blaZ gene negative	2	15	178	89	15	1	0	0	0	0	300
blaZ gene positive	0	0	1	5	17	15	4	0	**2**	1	45

The penicillin clinical breakpoint according to both CLSI[12] and EUCAST[13] is indicated by a broken line.

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Table 3.

Zone diameter distribution for the total set of MSSA isolates ($N = 345$) using CLSI methodology (10 U benzylpenicillin disc) in relation to their *blaZ* status

	Zone diameter (mm)																			Total
	≤21	22	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	>39	
blaZ gene negative							1		4	4	13	14	33	35	44	47	30	34	41	300
blaZ gene positive	6	4	2	2	4	3	2	3	4	5	6	1		1		2				45

The breakpoint according to CLSI[12] is indicated by a broken line.

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Table 4.

Zone diameter distribution for the total set of MSSA isolates (N = 345) using EUCAST methodology (1 U benzylpenicillin disc) in relation to their *blaZ* status

	Zone diameter (mm)																	
	≤16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**>31**	**Total**
blaZ gene negative						1	8	4	12	35	57	59	48	31	26	13	6	300
blaZ gene positive	8	1	4	8	4	3	1	3	9	1	2	1						45

The breakpoint according to EUCAST[13] is indicated by a broken line.

Table 5.

Sensitivity of different phenotypic test combinations to determine penicillin resistance in *blaZ*-positive *S. aureus* isolates ($N = 45$) using penicillin MIC or disc diffusion as an initial test

Test combination	No. of isolates classified as penicillin resistant	Sensitivity (%)
MIC	22	49
MIC + Cefinase™	37	82
MIC + DrySlide™	40	89
MIC + zone edge (10 U disc)	43	96
MIC + zone edge (1 U disc)	44	98
MIC + zone edge[a] + Cefinase™	44	98
MIC + zone edge[a] + DrySlide Nitrocefin™	44	98
DD (10 U disc)	23	51
DD (10 U disc) + Cefinase™	38	84
DD (10U disc) + DrySlide Nitrocefin™	40	89
DD (10 U disc) + zone edge	43	96
DD (10 U disc) + zone edge + nitrocefin[b]	44	98
DD (1 U disc)	42	93
DD (1 U disc) + Cefinase™	43	96
DD (1 U disc) + DrySlide Nitrocefin™	42	93
DD (1 U disc) + zone edge	44	98
DD (1 U disc) + zone edge + nitrocefin[b]	44	98

DD, disc diffusion.

[a]Same results were obtained whether using the 1 U or the 10 U benzylpenicillin disc.

[b]Same results were obtained whether using BBL Cefinase™ or DrySlide™.