

Evaluation of penicillin G susceptibility testing methods for *Staphylococcus lugdunensis*

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Received 7 October 2019; returned 29 November 2019; revised 20 December 2019; accepted 30 December 2019

Background: *Staphylococcus lugdunensis* belongs to the CoNS group, but is regarded to be more virulent than most other CoNS. It is also remarkably susceptible to antibiotics, including penicillin G.

Objectives: To evaluate different methods for penicillin susceptibility testing, to assess penicillin susceptibility rates among *S. lugdunensis* and to describe the clinical presentation including antibiotic treatment.

Methods: Clinical isolates of *S. lugdunensis* were tested for penicillin susceptibility using disc diffusion according to CLSI (10 U disc) and EUCAST (1 U disc), assessment of zone-edge appearance, nitrocefin test and Etest for MIC determination. PCR of the *blaZ* gene was used as a reference method.

Results: Of the 112 isolates included in the study, 67% were susceptible to penicillin G according to *blaZ* PCR. The EUCAST disc diffusion test had 100% sensitivity, whereas the CLSI method had one very major error with a false-susceptible isolate. When zone-edge appearance was included in the assessment, the false-susceptible isolate was correctly classified as resistant. Foreign-body infection was the most common focus of infection, affecting 49% of the participants. Only 4% of the patients were treated with penicillin G.

Conclusions: Penicillin susceptibility is common in *S. lugdunensis* and the disc diffusion method according to EUCAST had a higher sensitivity than that of CLSI. Assessment of zone-edge appearance could increase the sensitivity of the disc diffusion test. Penicillin susceptibility testing and treatment should be considered in *S. lugdunensis* infections.

Introduction

Our normal skin inhabitants, the CoNS, are usually considered to be relatively harmless, but can cause opportunistic infections related to foreign-body materials. One of the exceptions in the CoNS group is *Staphylococcus lugdunensis*, which appears to be more virulent. The bacterium harbours several virulence factors similar to those in *Staphylococcus aureus*^{1,2} and it causes serious infections such as endocarditis, wound infections and prosthetic joint infections.^{3–5}

Unlike other CoNS, a large proportion of *S. lugdunensis* isolates are still susceptible to several narrow-spectrum antibiotics including isoxazolyl penicillins (e.g. oxacillin) and penicillin G.² Resistance to penicillin G is most commonly caused by the expression of a penicillinase encoded by the *blaZ* gene. Alternatively, the bacterium can modify a PBP and thereby become resistant to almost all β -lactam antibiotics (methicillin-resistant staphylococci).

According to the Sanford guidelines 2014, penicillin G is the drug of choice for the treatment of *S. lugdunensis*, when susceptible by testing.⁶ Recent research has addressed the need to use penicillin G instead of traditional staphylococcal antibiotics to reduce the risk of relapses and treatment failure for *S. lugdunensis* infections.⁷ However, in order to use a narrow-spectrum antibiotic, we must have reliable methods for susceptibility testing. The recommendations for penicillin susceptibility testing for *S. lugdunensis* differ in penicillin G disc content and zone diameter interpretations between the EUCAST and CLSI guidelines.^{8–10} Moreover, assessment of zone-edge appearance is recommended in the penicillin G susceptibility test for *S. aureus*,^{9,10} but not for CoNS.¹¹

The aim of this study was to retrospectively assess penicillin G susceptibility rates among *S. lugdunensis* isolates and to evaluate different methods for penicillin G susceptibility testing: disc diffusion according to CLSI and EUCAST guidelines, the nitrocefin test, MIC determination with Etest and assessment of zone-edge

appearance. A secondary aim was to describe the clinical presentation of *S. lugdunensis* isolates included in the study and to review the antibiotic treatment.

Materials and methods

Bacterial isolates

We retrospectively collected *S. lugdunensis* isolates from stored stocks at the Department of Medical Microbiology at Skåne University Hospital, Sweden. This laboratory serves nine hospitals in a region with approximately 1.3 million inhabitants. All stored isolates cultured from sterile locations between 2015 and 2017 were included. Duplicate isolates from the same patient were excluded. The identification of *S. lugdunensis* was performed at the Department of Medical Microbiology using MALDI-TOF MS.¹² Methicillin resistance was determined in the clinical routine process with disc diffusion using a ceftioxin disc.

PCR and sequencing

For each strain, 10–20 colonies grown on Mueller–Hinton agar plates were resuspended in 100 µL of water and incubated at 99°C for 5 min. The samples were then pelleted by centrifugation at 15 000 rpm for 5 min and the supernatant containing the DNA template was collected.

Amplification of the *blaZ* and *16S rDNA* genes was performed using primers described previously.^{13–15} The *16S rDNA* gene was amplified as an internal control to confirm that a PCR product could be obtained in *blaZ*-negative strains. DNA template (1 µL) was added to 24 µL of master mix containing 12.5 µL of KAPA2G Fast HotStart ReadyMix (Sigma, St Louis, MO, USA), 9 µL of sterile water and 2.5 µL of 10 µM forward and reverse primers. PCR products were separated on a 1% agarose gel (Sigma) with SYBR Safe DNA stain (Invitrogen, Waltham, MA, USA) and visualized in a Gel Doc EZ Imager (Bio-Rad, Hercules, CA, USA).

Sequencing of the *blaZ* gene was performed using primers located in the flanking regions of the gene: forward primer 5'-ACT TAG CCA TTT CAA CAC CTT C-3' and reverse primer 5'-TCA AAA TTA TAC ATG TCA ACG A-3'. The PCR product was sent for sequencing and compared with published *blaZ* sequences from *S. lugdunensis*.

Penicillin G susceptibility testing

Disc diffusion tests were performed on Mueller–Hinton agar plates using 1 and 10 U penicillin G discs (both Oxoid, Hampshire, UK) according to EUCAST

and CLSI guidelines.^{9,10} Bacterial cultures were suspended in sterile saline to a turbidity equivalent to that of a 0.5 McFarland standard and inoculated on Mueller–Hinton agar. Plates were incubated at 35°C in ambient air for 16–18 h (10 U) or 18±2 h (1 U). Zone diameters were then measured and all zone edges were assessed for fuzzy or sharp appearance (Figure 1). Interpretation of susceptibility was based on EUCAST (zone diameter ≥26 mm using 1 U penicillin G discs) and CLSI (zone diameter ≥29 mm using 10 U penicillin G discs) recommendations.^{8–10} Assessment of zone-edge appearance was made independently by two researchers, blinded to the *blaZ* PCR results.

The MIC of penicillin G was determined on Mueller–Hinton agar by Etest (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions.

The nitrocefin test was performed with a penicillin G-induced inoculum. Bacteria were applied to a nitrocefin disc (Oxoid) according to the manufacturer's recommendations and incubated for 1 h at room temperature. No colour change was considered negative for β-lactamase production and a conversion to red colour at the site of inoculation as positive for β-lactamase production.

Patient characteristics

Patient data were extracted retrospectively from medical records. The type of infection caused by *S. lugdunensis* was classified based on final diagnosis, comorbidity was assessed according to the Charlson comorbidity index¹⁶ and mortality was defined as mortality within 30 days after admission to hospital.

Ethics

The study was approved by the Medical Ethics Committee (Institutional Review Board) of Lund University (reference number 2017/237) and patient consent was not required.

Statistics

Sensitivity, specificity and predictive values were calculated from cross-tabulation tests. Comparisons between groups were made with the Mann–Whitney *U*-test. Two-tailed *P* < 0.05 was regarded as statistically significant. Analyses were performed using the SPSS software, version 25 (SPSS, Armonk, NY, USA).

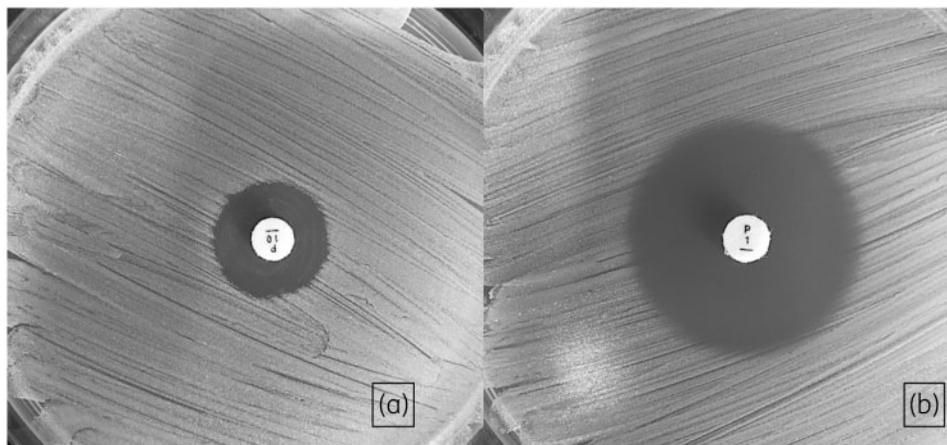


Figure 1. Sharp (a) and fuzzy (b) zone-edge appearance in *S. lugdunensis*. A sharp zone edge correlates with the presence of β-lactamase, whereas a fuzzy zone edge corresponds to the absence of β-lactamase.

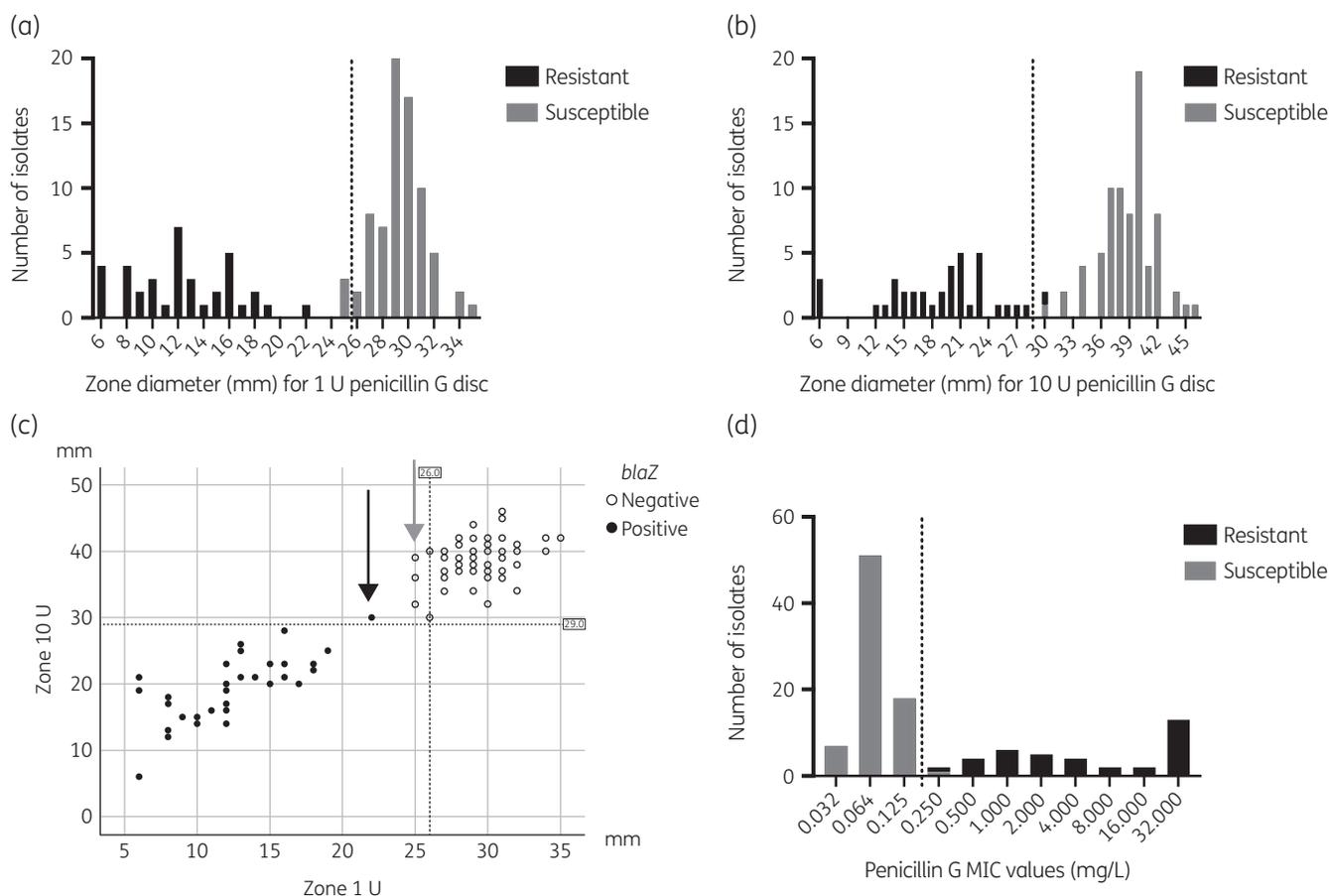


Figure 2. Histograms of zone diameters for *S. lugdunensis* isolates for the disc diffusion test using 1 U (a) and 10 U (b) penicillin G discs. Isolates are classified as resistant or susceptible based on the presence or absence of the *blaZ* gene. Six millimetres corresponds to the diameter of the disc and represents complete inhibition. Panel (c) shows how zone diameters for the 1 U penicillin G disc (x-axis) and the 10 U penicillin G disc (y-axis) are distributed among *blaZ*-positive and *blaZ*-negative isolates. The black arrow indicates a *blaZ*-positive isolate with a zone diameter ≥ 29 mm for the 10 U penicillin disc and the grey arrow indicates *blaZ*-negative strains with zone diameters < 26 mm for the 1 U penicillin disc. Panel (d) shows the MIC distribution using Etests. The dotted lines in panels (a) to (d) indicate breakpoints for resistant isolates based on zone diameters (≥ 26 mm for the 1 U disc and ≥ 29 mm for the 10 U disc) or MIC value.

Results

Collection of *S. lugdunensis* isolates

A total of 136 *S. lugdunensis* isolates were identified retrospectively from 2015 to 2017. Twenty-four isolates were excluded from analyses: 19 isolates were duplicates from the same patients and 5 isolates were not viable. Of the remaining 112 isolates included in the study, 1 isolate was resistant to methicillin, whereas all others were methicillin susceptible.

Performance of penicillin G susceptibility tests

Analysis of the *blaZ* gene, encoding the β -lactamase that degrades penicillin G, was used as a reference method for determining penicillin G resistance. Of all 112 unique isolates, 37 harboured the *blaZ* gene and were classified as resistant to penicillin G. One of these isolates was also resistant to methicillin. Consequently, 75 isolates (67%) were *blaZ* negative and considered to be penicillin G susceptible. None of the *blaZ*-negative isolates was resistant to methicillin.

Isolates were evaluated for penicillin G resistance with disc diffusion using a 1 U disc, according to EUCAST criteria, and a 10 U disc, according to CLSI criteria. When using the 1 U disc, *blaZ*-negative strains had a median zone diameter of 29 mm (range 25–35 mm) and *blaZ*-positive strains had a median zone diameter of 12 mm (range 6–22 mm) (Figure 2a), where 6 mm corresponded to complete inhibition. Three *blaZ*-negative isolates had a zone diameter of 25 mm and would be interpreted as resistant according to EUCAST. This corresponded to a sensitivity of 100% and specificity of 96% (Table 1). In addition, two *blaZ*-negative isolates had an inhibition zone of 26 mm, which is the lower limit for susceptible strains (Figure 2a). All three *blaZ*-negative isolates with a zone diameter below the cut-off for penicillin G resistance were susceptible to penicillin according to the other phenotypic tests. When tested with the 10 U disc, they were all well above the breakpoint, with inhibition zones of 32, 36 and 39 mm (indicated with a grey arrow in Figure 2c). Their MIC values were 0.125 and 0.064 mg/L and all three isolates had a fuzzy zone edge.

Table 1. Sensitivity, specificity and predictive values of phenotypic tests for the detection of penicillin resistance in *S. lugdunensis*

Penicillin G susceptibility test	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
1 U disc	100	96	100	92.5
10 U disc	97.2	100	98.7	100
10 U disc + zone edge	100	100	100	100
Nitrocefin	92.1	98.7	97.2	96.2
Etest	100	98.7	97.4	100

PCR of the *blaZ* gene was used as a reference method.

When using the 10U penicillin disc, the median zone diameter was 39 mm (range 30–46 mm) for *blaZ*-negative isolates and 20 mm (6–30 mm) for *blaZ*-positive isolates (Figure 2b). One *blaZ*-positive isolate had a diameter ≥ 29 mm when tested with the 10U penicillin disc, yielding a sensitivity of 97.2% and specificity of 100% (Table 1). This isolate was tested three times and the diameter was repeatedly over 29 mm and would therefore be falsely interpreted as susceptible. This isolate was classified as resistant by the other phenotypic tests. The zone diameter of this isolate with the 1 U disc was 22 mm (indicated with a black arrow in Figure 2c), the MIC value was 4 mg/L and the nitrocefin test was positive. The zone-edge appearance was sharp for both the 1 and 10U discs. In addition, the *blaZ* gene was sequenced and showed 100% identity to previously published *blaZ* sequences in *S. lugdunensis* (accession numbers CP020735.1, CP020769.1 and CP041722.1). Taken together, the results indicate that the isolate was able to produce a functional penicillinase. Figure 2(c) shows how *blaZ* results and zone diameters for the 1 and 10U discs correlate for individual isolates.

All *blaZ*-negative isolates had a fuzzy zone-edge appearance and all *blaZ*-positive isolates had a sharp zone edge, regardless of whether they were tested with the 1 or 10U penicillin G discs. When combining the 10U disc diffusion test with zone-edge appearance, all *blaZ*-positive isolates were correctly identified as resistant to penicillin G, thereby increasing the sensitivity to 100% (Table 1).

Three *blaZ*-positive isolates were negative in the nitrocefin test and would be falsely interpreted as susceptible, whereas one *blaZ*-negative isolate was nitrocefin positive. Thus, the nitrocefin test had three very major errors and was inferior to the disc diffusion tests (Table 1).

MIC distribution

The median MIC value for *blaZ*-positive strains was 4 mg/L (range 0.25–32 mg/L) and none was below 0.25 mg/L, which is the clinical breakpoint for penicillin G resistance in staphylococci according to both EUCAST and CLSI.^{8,9} The median MIC value for *blaZ*-negative isolates was 0.064 mg/L (range 0.032–0.25 mg/L). In the *blaZ*-negative population, one isolate had an MIC value of 0.25 mg/L and would be considered resistant in routine testing according to MIC interpretation.¹⁰ Furthermore, 17 isolates had an MIC value of 0.125 mg/L, just below the clinical breakpoint (Figure 2d).

Patient characteristics and antibiotic treatment

Next, medical records were reviewed for the 112 patients from whom *S. lugdunensis* was isolated. The median age was 72 years

Table 2. Patient characteristics and treatment; N = 112

Age (years), median (range)	72 (0–99)
Male gender, % (n)	69 (77)
30 day mortality, % (n)	11 (12)
Site of infection, % (n)	
foreign-body infection	49 (55)
osteitis	19 (21)
endocarditis	9 (10)
soft-tissue infection	7 (8)
other ^a	13 (14)
no infection	4 (4)
Treatment, % (n)	
cefotaxime	30 (34)
cloxacillin	16 (18)
vancomycin	11 (12)
penicillin G	4 (5)
combination or other	35 (39)
no treatment	4 (4)

^aSinus infection, urinary tract infection and infection of unknown origin.

(range 0–99 years) and 77 (69%) of the patients were male (Table 2). The most common focus of infection, affecting 55 (49%) of the patients, was infected foreign-body materials. Within this group, 31 patients (28%) suffered from prosthetic joint infections and 5 patients had infections in central venous access devices. Endocarditis was diagnosed in 9% of the study participants and skin and soft-tissue infections in 7% of the cases. In four patients, *S. lugdunensis* was regarded as a contaminant and left untreated. None of these patients had a relapse during the following 6 months. The 30 day all-cause mortality rate was 11% ($n=12$) and 58% ($n=7$) of these patients died during the first 7 days of hospitalization. The patients that died were significantly older (median 85 years) than those who survived (median 71 years) ($P=0.000$) and they had more comorbidities, assessed by the Charlson score index (median Charlson score 3 versus 1) ($P=0.002$).

A high proportion of the isolates in the study (111 of 112 isolates) were susceptible to isoxazolyl penicillins. Only 12 (11%) of the isolates were tested for penicillin G susceptibility in the clinical routine process and 8 of these were susceptible. Despite the high proportion of susceptibility to penicillins, cefotaxime was the most common single treatment, followed by cloxacillin and vancomycin

(Table 2). Only five (4%) patients received penicillin G as treatment. The only isolate resistant to isoxazolyl penicillins was treated with trimethoprim/sulfamethoxazole.

Discussion

In this retrospective study of *S. lugdunensis* infections, 67% of the isolates were susceptible to penicillin G according to the reference method *blaZ* PCR. Disc diffusion according to EUCAST and Etest had the highest sensitivity of the evaluated phenotypic tests for penicillin G susceptibility, whereas the nitrocefin test had the lowest sensitivity, with three very major errors.

The ideal test for antibiotic susceptibility should have 100% specificity and 100% sensitivity to ensure optimal treatment of the patient. Although a test with low specificity could limit the treatment options if an isolate is misclassified as resistant, a test with low sensitivity could lead to devastating consequences if an isolate is falsely interpreted as susceptible and the patient receives inadequate treatment. While the 1 U disc, according to EUCAST, had 100% sensitivity in our study, the 10 U disc, according to CLSI, had one very major error with an isolate that would be interpreted as susceptible according to zone diameter, although it harboured the *blaZ* gene and the ability to produce β -lactamase. In our hands, additional assessment of zone-edge appearance increased the sensitivity of the 10 U disc diffusion test to 100%. Both *blaZ* PCR and Etest could be used in times of uncertainty, but are more expensive and time-consuming.

Zone-edge appearance is included in the assessment of penicillin G susceptibility of *S. aureus*,¹⁴ but the test has previously been rejected for CoNS, when applied to *Staphylococcus epidermidis*¹¹ and in a recent study on *S. lugdunensis*.¹⁷ In our study, zone-edge assessment was performed by two independent researchers, blinded to the *blaZ* results, and the interpretation of zone-edge appearance was concordant in all isolates. However, this assessment needs a bit of training, which may in part explain the discrepancies between our results and the previous study by McHardy *et al.*¹⁷ Notably, *S. lugdunensis* is not always treated the same as the rest of the CoNS group in other susceptibility tests. For example, oxacillin susceptibility is interpreted in the same way for *S. lugdunensis* and *S. aureus*, but differently than for other CoNS.^{8–10} However, more studies are needed to verify the reliability of the zone-edge assessment.

Unlike other CoNS, findings of *S. lugdunensis* in blood cultures are often clinically relevant.¹⁸ In the present study, only four isolates were regarded as contaminants, whereas all others represented clinically important infections. In this respect, *S. lugdunensis* is more similar to *S. aureus* than to other CoNS. Moreover, there was a dominance of men in our study population, similar to invasive infections with *S. aureus*.¹⁴ In line with other reports, bone and prosthetic joint infections were the most common sources of infection.⁵ Endocarditis was only diagnosed in 9% of the patients. In comparison, other studies have reported an endocarditis frequency of 13.9% among patients with *S. lugdunensis* bacteraemia,¹⁹ which is comparable to the proportion of endocarditis in *S. aureus* bacteraemia.^{14,20} The different results may be explained by the small sample sizes in the studies and it could also be speculated that patients with *S. lugdunensis* bacteraemia undergo echocardiography less often than patients with *S. aureus*. Taken together, these data underline the clinical

relevance of *S. lugdunensis* and confirm that the clinical presentation of *S. lugdunensis* infections is more similar to that of *S. aureus* infections than of other CoNS infections.

S. lugdunensis has a remarkably conserved susceptibility to antibiotics compared with other CoNS. Similar to our data, studies from other geographical regions report relatively high levels of penicillin G susceptibility, ranging from 52% up to 85%.^{7,17,21,22} Penicillin G has many advantages compared with isoxazolyl penicillins, which are most commonly used for staphylococcal infections in Sweden today. Penicillin G has lower MIC values^{7,23} and a lower degree of protein binding compared with isoxazolyl penicillins such as cloxacillin (65% versus 94%). This results in a longer free time above the MIC and therefore a treatment advantage for penicillin G over isoxazolyl penicillins. Moreover, penicillin G has a narrow antimicrobial spectrum and is less associated with *Clostridioides difficile* infections compared with other broad-spectrum antibiotics.²⁴ Despite the high number of penicillin G-susceptible *S. lugdunensis* isolates in this study, a minority of isolates were tested for penicillin G in the clinical routine process and only 4% of the patients received treatment with penicillin G. As many as 30% of the patients were treated with cefotaxime, which has an unnecessarily broad spectrum of activity for this infection, and 10% of the patients were treated with vancomycin despite the fact that only 1 out of 112 isolates was methicillin resistant. These results show that we may improve the treatment of *S. lugdunensis* infections by routine penicillin G susceptibility testing in the laboratory and an increased awareness among clinicians of isoxazolyl penicillin and penicillin G utilization in *S. lugdunensis* infections.

The most important limitation of this study is the small sample size of only 112 isolates and patients. Another difficulty is the assessment of zone-edge appearance, where our results differ markedly from other studies. The method is subjective and user dependent and it is therefore difficult to evaluate how our assessment differs from others. However, more work is needed to verify the reliability of the test.

In conclusion, this study shows that penicillin G susceptibility among *S. lugdunensis* isolates is high. Penicillin G susceptibility testing with the 1 U disc diffusion test according to EUCAST has the highest accuracy and concomitant assessment of zone-edge appearance can increase the sensitivity of the disc diffusion test, according to CLSI, with a 10 U penicillin G disc. *S. lugdunensis* can cause severe infections, for which penicillin G testing and treatment should be considered as routine practice.

Acknowledgements

Parts of the results were presented at the Annual Swedish Meeting for Infection and Microbiology, Jönköping, Sweden, 2019 (Poster INF-P018).

We wish to thank Gisela Hovold for technical assistance, Lena Hyllebusk for database support and Linnea Polland for valuable input.

Funding

This work was funded by Swedish Government Funds for Clinical Research (ALF), Grant Number: 40231; Regional Research Funds, Grant Number: 40254; Skåne University Hospital Foundations, Grant Number: 91202; the Alfred Österlund Foundation, Grant Number: F2018/2008; and the Swedish Heart and Lung Foundation, Grant Number: F2016/783.

Transparency declarations

None to declare.

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