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Is prediffusion test an alternative to improve accuracy in screening hVISA strains and to detect susceptibility to glycopeptides/lipopeptides?

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ABSTRACT

The characterization of heteroresistant vancomycin-intermediate *Staphylococcus aureus* strains (hVISA) is even more challenging, as no routine standardized laboratory methods are available. A total of 124 *S. aureus* isolates recovered from inpatients attended in hospitals of Santa Catarina State, Southern Brazil, were evaluated. The MIC of vancomycin, teicoplanin, and daptomycin was determined by Etest and prediffusion tests using NeoSensitabs® tablets. All isolates were susceptible to vancomycin (MICs: 0.5–3 μg/mL) by Etest. However, according to prediffusion test, 17 isolates presented reduced susceptibility to vancomycin, and of these, 12 were confirmed as hVISA using populational analysis. Considering daptomycin, prediffusion results were in agreement with susceptibility data (MICs), as all isolates were susceptible. Considering that characterizing hVISA is challenging and that MIC determination is not adequate to characterize this phenotype, prediffusion test was a viable alternative to screening hVISA and reduced susceptibility to vancomycin. It was simple and low cost, with accuracy comparable to other well-established methods.

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1. Introduction

Vancomycin is a glycopeptide known since 1955, when it was placed at the disposal of medicine through the work of McCormick (McCormick et al., 1955). Initially, due to the success of methicillin, oxacillin, and other isoxazolepenicillins, it was not often used, though with the emergence of methicillin resistance in the 1960's, it came to be used quite often. In 1996, a strain of *Staphylococcus aureus* intermediate to vancomycin (vancomycin-resistant *S. aureus* [VISA]) was identified in Japan (Hiramatsu, 1997). This phenotype did not have a wide dissemination, and the drug most frequently used to treat infections caused by methicillin-resistant *S. aureus* (MRSA) is still vancomycin, as well as the daptomycin and linezolid (Jones, 2006; Van Hal and Fowler, 2013).

Detection of *in vitro* susceptibility of isolates to these drugs is challenging. Since 2009, the CLSI (2013) no longer recommends disk diffusion to determine vancomycin susceptibility due to its high molecular weight resulting in decreased diffusion in culture media (Heather et al., 2010). Based on current CLSI recommendations, susceptibility to linezolid and teicoplanin may be determined by disk diffusion method, while vancomycin and daptomycin susceptibilities should be assessed by dilution methods or some specific diffusion tests in agar. These tests are extremely laborious and expensive, making them difficult to implement, especially in developing countries.

Taking into account the seriousness of MRSA infections, early and aggressive antimicrobial therapy is an important part to reduce mortality. Treatment failures in infections caused by susceptible isolates are primarily due to heteroresistant vancomycin-intermediate *S. aureus* strains (hVISA), a subpopulation with reduced susceptibility to vancomycin. Thus, *in vitro* susceptibility may not be enough for the physician to achieve treatment success. The evaluation of tolerance to glycopeptide antibiotics is important for predicting treatment failure (Van Hal and Paterson, 2011) and can be considered the first step to preventing and controlling the emergence of vancomycin resistance in *S. aureus* (Howden et al., 2010). As the hVISA resistance phenotype manifests itself heterogeneously and is a minor component of the bacterial population (1 in 10^6 microorganisms), the methods commonly used in clinical microbiology laboratories (MIC determination by Etest or microdilution) fail to detect potential resistance, and vancomycin therapy may fail (Satola et al., 2011).

NeoSensitabs® (Rosco Diagnostica, Taastrup, Denmark) are tablets containing antimicrobial (9 mm diameter and 1.5 mm thick) stable at room temperature, which were developed to evaluate bacterial

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susceptibility to high-molecular-weight drugs such as vancomycin, teicoplanin, daptomycin, and colistin. The initial prediffusion allows a homogeneous diffusion of the antibiotic in the culture medium, making possible to discriminate strains with reduced susceptibility to these agents. The aim of this study was to evaluate the prediffusion methodology for the detection of decreased susceptibility to glycopeptides and daptomycin as well as its use in the detection of hVISA isolates.

2. Methods

2.1. Samples

Clinical isolates (n = 124) were collected (March 2009 to February 2013) from inpatients in three hospitals in Florianópolis (Hospital de Caridade, Hospital Governador Celso Ramos, and Cardio SOS) and a hospital in Blumenau (Hospital Santa Isabel), all located in Santa Catarina state, Southern Brazil. One isolate per patient was considered. All isolates were used, and there was no selection bias. Identification was done using the following testes: Gram staining, catalase production, mannitol fermentation, coagulase, and DNase production. Susceptibility to methicillin was determined by cefoxitin disk diffusion, according to the interpretive criteria of CLSI (M100-S23).

2.2. Phenotypic tests for screening and confirmation of hVISA

Three screening tests were used for the detection of hVISA strains. BHI agar plates containing 4 μg/mL of vancomycin and 16 g/L of pancreatic digest of casein were inoculated with a prepared 0.5 McFarland standard inoculum. After 24 h of incubation at 35–37 °C, the growth of more than 20 colonies was considered a positive test for hVISA (Satola et al., 2011). Using Etest® Glycopeptide Resistance Detection (GRD) (bioMerieux, Durham, NC, USA), the isolate was considered hVISA when the MIC for teicoplanin is 12 μg/mL or 8 μg/mL for teicoplanin and vancomycin after 48 h (Van Hal et al., 2011). The Etest® macromethod (bioMerieux) was performed using a 2.0 McFarland inoculum on BHI agar plates and readings taken at 24 and 48 h, where an MIC of 8 μg/mL to vancomycin identified hVISA isolates (Van Hal et al., 2011).

All isolates with at least 1 positive screening test were confirmed through population analysis profile/area under the curve (PAP-AUC). The area under the curve (AUC) was calculated using Mu3 (hVISA) as a control strain. For confirmation of an hVISA isolate, the ratio of AUC for the isolate divided by that of Mu3 should be greater than or equal to 0.9 and non-hVISA isolates had a PAP-AUC <0.9 (Wootton et al., 2001).

2.3. Minimum inhibitory concentration (MIC)

MIC values to vancomycin, teicoplanin, and daptomycin were obtained by the Etest® methodology (bioMerieux). Interpretation was performed, following CLSI (M100-S23) guideline.

2.4. Prediffusion tests

Neosensitabs® containing 30 μg vancomycin, 30 μg teicoplanin, or 30 μg daptomycin/100 μg calcium (Rosco Diagnostica) and prediffusion tests were performed following the manufacturer's guidelines (Supplement User's Guide, 2010). The tablets were placed on the surface of Mueller Hinton agar (bioMerieux). They were inverted and incubated for 2 h at room temperature. After this period, plates were incubated for further 18–22 h at room temperature to ensure the complete diffusion of antibiotics. Then, plates were inoculated with bacteria and incubated at 35 °C for 24 h and inhibition evaluated. Isolates with vancomycin ≤22 mm and/or teicoplanin <20 mm inhibition zones were considered VISA/hVISA. For daptomycin, isolates <2 mm were defined as resistant.

2.5. Quality control

To ensure the quality and accuracy of the test results, *S. aureus* strains ATCC 29213 (MSSA), ATCC 43300 (MRSA), ATCC 700698 (hVISA), and ATCC 700699 (VISA) were used.

3. Results

All isolates were susceptible to vancomycin, considering E-test results. MICs were 0.5 μg/mL (0.8%), 0.75 μg/mL (0.8%), 1.0 μg/mL (17.7%), 1.5 μg/mL (42.7%), 2.0 μg/mL (32.3%), and 3.0 μg/mL (5.6%) (Fig. 1). The isolates that had an MIC of 3 μg/mL were considered susceptible to vancomycin since all had MIC obtained by broth macrodilution values ≤2 μg/mL. Considering these data, all isolates would be characterized according to CLSI criteria as susceptible to vancomycin. Seventeen isolates had discrepant results for MIC and prediffusion: they were considered susceptible by the CLSI breakpoints. Among the 17 discrepant isolates, 2 had an MIC of 1.5 μg/mL, 10 presented MIC of 2.0 μg/mL, and 5 MIC of 3.0 μg/mL.

Teicoplanin data indicated MICs of 0.19 μg/mL (0.8%), 0.25 μg/mL (0.8%), 0.38 μg/mL (1.6%), 0.5 μg/mL (3.2), 0.75 μg/mL (1.6%), 1.0 μg/mL (4.8%), 1.5 μg/mL (17.7%), 2.0 μg/mL (16.9%), 3.0 μg/mL (33.9%), 4.0 μg/mL (13.7%), 6.0 μg/mL (1.6%), 8.0 μg/mL (2.4%), and 12.0 μg/mL (0.8%) (Fig. 2). Discrepancies were observed for 14 isolates, which were classified as intermediate according to prediffusion testing but considered susceptible according to CLSI criteria. Among them, 1 had an MIC of 1.5 μg/mL, 7 at 3.0 μg/mL, 4 at 4.0 μg/mL, and 2 at 8.0 μg/mL.

Vancomycin and teicoplanin susceptibility data demonstrated that 21 clinical isolates were intermediately susceptible to vancomycin and/or teicoplanin, which indicated these isolates were VISA using the prediffusion method.

All isolates were submitted to screening tests for the hVISA phenotype (GRD, Etest® macromethod, and agar screening with vancomycin), and of those that tested positive for any of the 3 screening tests, the phenotype was confirmed by PAP-AUC. Of the 124 isolates, 12 (9.7%) were characterized as hVISA (Table 1).

Based on the above data, 91.7% sensitivity, 83.1% specificity, a positive predictive value of 52.4%, a negative predictive value of 97.1%, and an accuracy of 89.5% were established for prediffusion test (Table 2).

Daptomycin data indicated MIC values of 0.125 μg/mL (3.2%), 0.19 μg/mL (5.6%), 0.25 μg/mL (8.9%), 0.38 μg/mL (23.4%), 0.5 μg/mL (33.9%), 0.75 μg/mL (21%), and 1.0 μg/mL (4%) (Fig. 3). All isolates presented, therefore, a susceptible phenotype.

Prediffusion results for ATCC700698 (hVISA) and ATCC 700699 (VISA) are demonstrated in Fig. 4, as well as the results of 2 clinical isolates (SI11 and L10).

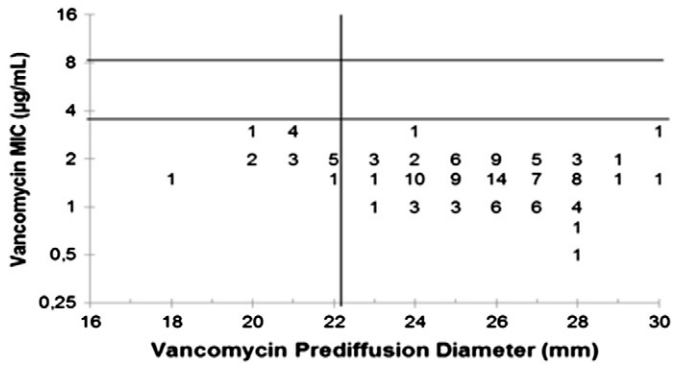

Fig. 1. Scattergram with distribution of vancomycin prediffusion results compared to MICs. Horizontal lines represent the CLSI breakpoints for susceptibility (<4 μg/mL) and resistance (>8 μg/mL); vertical line represents the breakpoint for a prediffusion-positive result (susceptible >22 mm).

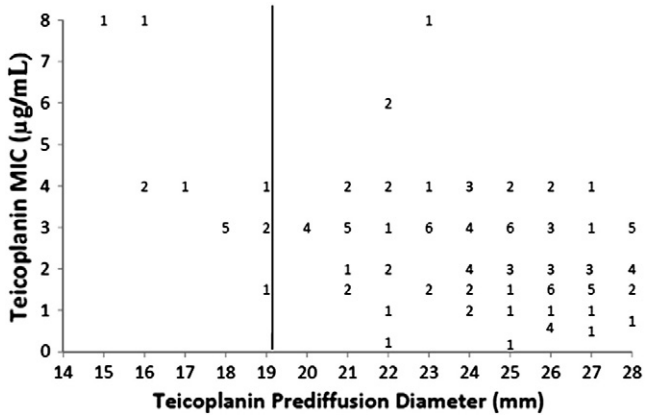

Fig. 2. Scattergram with the distribution of teicoplanin prediffusion results compared to MICs. Vertical line represents the breakpoint for a prediffusion-positive result (susceptible >19 mm).

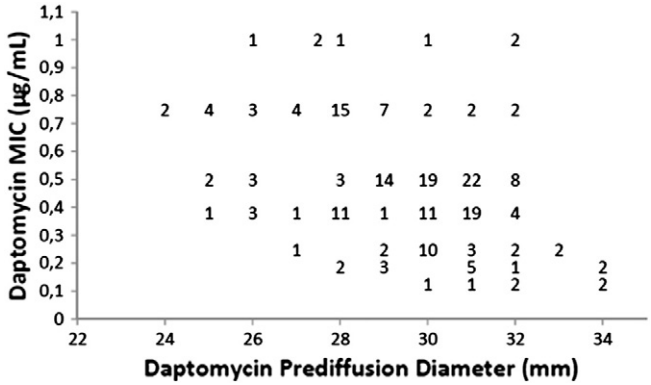

Fig. 3. Scattergram with the distribution of daptomycin prediffusion results compared to MICs. All isolates were susceptible.

Table 1
Comparison of PAP results (hVISA and non-hVISA) with prediffusion results.

	hVISA	Non-hVISA	Total
Positive prediffusion	11	10	21
Negative prediffusion	1	102	103
Total	12	112	124

4. Discussion

The prediffusion method is an alternative to conventional methods, allowing antimicrobials with high molecular weight to be evaluated by this alternative disk diffusion, since by conventional methodology, they do not have cutoffs in CLSI guidelines.

Surprisingly, few studies have been published using prediffusion for the assessment of *S. aureus* susceptibility to glycopeptides and daptomycin (Katz et al., 2008; Nielsen and Casals, 2005). The major disadvantage of prediffusion considering glycopeptide susceptibility is that the methodology is unable to differentiate between hVISA and VISA isolates. Besides, it is a qualitative methodology without the possibility to determine MICs. Its qualitative data have presented a good correlation with MIC results, suggesting this test may be used as an alternative test.

In this study, prediffusion data for daptomycin were also highly consistent with MIC results, which were also observed by other authors (Katz et al., 2008). Altogether, these data may support the clinical use of prediffusion test. However, more data must be generated to confirm this hypothesis.

Since 2009, CLSI no longer recommends disk diffusion methodology for vancomycin, requiring clinical laboratories to determine the MIC. Despite being a microdilution reference test, MIC testing is somehow laborious and requires validation. Therefore, several laboratories use the Etest® as an alternative methodology to determine MIC, which is more expensive and tends to overestimate the MIC (Van Hal et al., 2011). However, it is practical and shows a strong correlation with clinical results, justifying its wide usage.

A meta-analysis published in 2012 correlated vancomycin MIC values determined by Etest® with therapeutic failure. The study found a significant correlation between isolates having an MIC ≥ 1.5 µg/mL and therapeutic failure, with an odds ratio of 1.74 (95% confidence interval: 1.34–2.21; $P < 0.01$) (Van Hal et al., 2012). Thus, we used the MICs determined by Etest, in an attempt to find a correlation between the results of prediffusion and Etest.

Several phenotypic tests have been used to detect hVISA as the heteroresistant phenotype has no reliable molecular characterization marker(s) and required the use of phenotypic tests with varying sensitivities and specificities. Satola et al. (2011) reported that screening on BHI agar with 4 µg/mL of vancomycin presents 91% sensitivity and 94% specificity rates, while the Etest® macromethod shows a sensitivity of 57% and specificity of 96% and Etest ® GRD shows a sensitivity of 57% and specificity of 97%.

This study has some limitations. First, the methodology used to determine the MIC was the Etest®, even though the gold standard is the broth microdilution. Second, all isolates tested were susceptible to daptomycin, making it necessary to evaluate the performance of the prediffusion-resistant isolates to determine if the method may present good accuracy for this antimicrobial agent.

These data indicated that when comparing the prediffusion method used for detecting hVISA, the specificity (83.1%) and sensitivity (91.7%) rates from this study were similar to other widely used tests. The results demonstrated that, despite being infrequently used, this test could be viable and effective in screening clinical isolates for an hVISA phenotype. The high negative predictive value (97.1%) allows for testing negative, excluding the possibility of that

Table 2
Results showed that a disagreement between the prediffusion and screening tests for hVISA.

Isolate	Vancomycin (mm)	Teicoplanin (mm)	Etest GRD vancomycin/teicoplanin (µg/mL)	Agar screening	Etest macromethod (µg/mL)	PAP-AUC	Interpretation
SI1	21	21	1.5/4	No	8	0.8	False positive
SI5	22	18	2/6	No	8	0.77	False positive
SI9	22	19	1/1.5	No	6	0.72	False positive
SI10	21	17	1.5/6	No	8	0.86	False positive
SI12	21	16	2/8	Yes	12	0.89	False positive
SI26	22	18	1.5/8	Yes	6	0.78	False positive
SI29	21	19	3/8	Yes	8	0.84	False positive
L4	25	18	1.5/2	Yes	4	0.63	False positive
L10	23	20	2/8	Yes	4	0.92	False negative
L64	21	20	1.5/4	No	8	0.79	False positive
L83	24	19	1/4	No	3	0.71	False positive

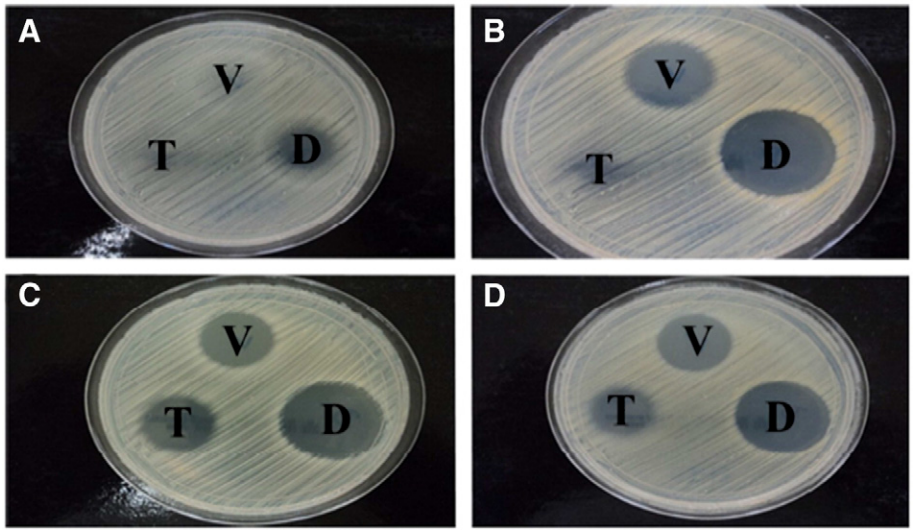

Fig. 4. The pictures above illustrate prediffusion, highlighting the control strains: (A) Mu50 ATCC 700699 (VISA), (B) Mu3 ATCC 700698 (hVISA), and 2 clinical isolates, (C) SI11 and (D) L10, both having the hVISA phenotype. V = vancomycin; T = teicoplanin; D = daptomycin.

the phenotype. Taking into account that all positive screening tests should be confirmed with PAP-AUC, the prediffusion method, as a simple and low-cost test, should be considered not only as a useful test to assess the susceptibility of *S. aureus* to glycopeptides and daptomycin, but also as a screening test for hVISA.

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