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Research Article

Evaluation of the Accuracy of Phenotypic Methods for the Detection of Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus* (hVISA)

Silveira ACO[1,2]*, Da Cunha GR[1], Caierão J[1], De Cordova CMM[2], and d'Azevedo PA[1]

[1]*Department of Health Sciences, Federal University of Health Sciences of Porto Alegre, Brazil*

[2]*Department of Pharmaceutical Sciences, Regional University of Blumenau, Brazil*

***Corresponding author**

Alessandro Conrado de Oliveira Silveira, Department of Health Sciences, Federal University of Health Sciences of Porto Alegre, Department of Pharmaceutical Sciences, Regional University of Blumenau, Rua São Paulo, 2171 Blumenau SC 89030-001, Brazil, Tel: +55-47-3321-7318; Email: alessandrocosilveira@gmail.com

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Abstract

The detection of hVISA is challenging because these strains are susceptible to vancomycin *in vitro* (minimum inhibitory concentration (MIC) < 4 µg/mL) and are therefore categorized as susceptible by the usual laboratory methods. A total of 124 methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from hospitals in the state of Santa Catarina, southern Brazil, with vancomycin MIC between 0.5 and 2 µg/mL were evaluated. The Etest glycopeptide resistance detection (GRD) was done according to the manufacturer's instructions. Etest macro method was performed using 2.0 McFarland inoculum on BHI agar plates, using vancomycin Etest strips. Heteroresistant was defined as MICs for vancomycin of ≥ 8 µg/mL. Population analysis profile-area under the curve (PAP-AUC) was performed as described by Wootton et al., of the 124 MRSA tested, 21 (16.9%) had positive results for at least one hVISA detection tests. Twelve were confirmed as hVISA (prevalence of 9.7%). The Etest GRD had a sensitivity of 66.7% and specificity of 97.3%. For the Etest macro method sensitivity was 75% with a specificity of 94.6%. The screening with Brain Heart Infusion agar (BHI) showed a sensitivity of 90.9% and specificity of 93.8%. PAP-AUC confirmed 12 (8.8%) as hVISA, with ratios of 0.93 to 1.17. The methods used routinely to detect vancomycin resistance vary in sensitivity and specificity, and may fail to detect hVISA. The combination of the three methods may be the best alternative, since the ones with vancomycin MIC < 4 µg/mL, may have hetero-resistance. The correct characterization of hVISA may impact directly in the therapeutic success.

ABBREVIATIONS

VISA: Vancomycin-intermediate *Staphylococcus aureus;* hVISA: Heteroresistant Vancomycin-Intermediate *S. aureus;* MIC: Minimum Inhibitory Concentration; GRD: Etest Glycopeptide Resistance Detection; PAP-AUC: Population Analysis Profile-Area under the Curve; MRSA: Methicillin-Resistant *S. aureus;* CLSI: Clinical and Laboratory Standards Institute; ATCC: American Type Culture Collection

INTRODUCTION

Vancomycin-intermediate *Staphylococcus aureus* (VISA), a phenotype characterized by cell wall thickness, is becoming increasingly common during prolonged therapy with glycopeptides [1].

Colonies of heteroresistant vancomycin-intermediate *S. aureus* (hVISA) are heterogeneous in appearance and pigmentation, giving the impression of contamination and potentially confusing microbiologists [2]. The mechanism of hVISA resistance is associated with the activation of cell wall synthesis, which increases the production of waste mucopeptide and reduces the amount of antibiotic that reaches the site of action, resulting in cell wall thickening and subsequent sequestration of the drug [3]. It has been speculated that hVISA may be a precursor of VISA; after prolonged exposure to antimicrobials, the selection of a homogenous population of hVISA cells may occur, leading to the expression of a VISA phenotype [4].

The detection of hVISA is challenging because these strains are susceptible to vancomycin *in vitro* (minimum inhibitory concentration (MIC) ≤ 2 μg/mL) and are therefore categorized as susceptible by the usual laboratory methods. However, the presence of a subpopulation representing 1 in 10^6 bacterial cells that can grow in the presence of 4 μg/mL vancomycin may lead to treatment failure in patients treated with vancomycin [5,6].

A meta-analysis published in 2011 revealed that the rates of treatment failure (designated as persistent infection or bacteremia) related to hVISA were 2-fold greater than to infections caused by vancomycin-susceptible *S. aureus* (Odds-Ratio: 2.37, 95 % CI: 1.53-3.67) [6]. Therefore, the development of accurate and practical methods for the detection of hVISA is of increasing importance [7].

Reference methods used to evaluate susceptibility, such as broth microdilution, fail to detect hVISA, partly due to the small size of the inoculum, the relatively poor growth of hVISA on Mueller-Hinton agar, and the short incubation period of only 24 hours. The inoculum size is critical for the detection of subpopulations of resistant cells. In addition, hVISA strains are characteristically slow growing, with thicker cell walls and unique pleomorphic characteristics, and produce colonies of varying sizes and nutritional requirements [8].

Other screening methods, such as the macro Etest, Etest glycopeptides resistance detection (GRD) and agar screening, use enriched media, a denser bacterial inoculum (2 McFarland scale) and prolonged incubation (48 hours) but have variable sensitivity and specificity; thus, it is difficult to obtain an accurate diagnosis using a single test. Because hVISA is a heterogeneous, minority subpopulation, there are no reliable molecular markers to detect hetero resistance [9].

Population analysis profile-area under the curve (PAP-AUC) has been the most reliable and reproducible approach and is considered to be the gold standard for hVISA confirmation. PAP-AUC was specifically designed for discriminating hVISA and VISA. This method analyzes the presence of different subpopulations using serial concentrations of vancomycin to quantify the viable bacterial populations at each vancomycin concentration. PAP-AUC is a very laborious and expensive method and is inappropriate for routine use in clinical laboratories [10].

Knowledge of local epidemiological data and early detection of hVISA may assist in the selection of appropriate antimicrobial agent for treatment, decreasing the morbidity and mortality associated with infection caused by hVISA [11].

The objective of this study was to analyze the performance of the main phenotypic tests available to characterize hetero resistance to vancomycin.

MATERIAL AND METHODS

Bacterial samples

We used 124 clinical isolates of methicillin-resistant *S. aureus* (MRSA) obtained from various anatomical sites from patients in three hospitals in Florianópolis and a hospital in Blumenau, all located in the state of Santa Catarina in southern Brazil. Samples were collected from February 2009 to February 2013. We discarded isolates from the same patient. All isolates were used and there was no selection bias. The biochemical identification was confirmed by Gram, catalase, mannitol, coagulase and Dnase.

MIC determination

Vancomicin and teicoplanin MICs were determined by Etest® (BioMérieux, Marcy l'Etoile, France), following the manufacturer's instruction and Clinical and Laboratory Standards Institute (CLSI) interpretative criteria [12]. To ensure the quality and accuracy of the test results, *Staphylococcus aureus* strains ATCC 29213 (MSSA), ATCC 43300 (MRSA), ATCC 700698 (hVISA) and ATCC 700699 (VISA) were used.

Agar screening

To verify the ability of the isolates to grow in the presence of vancomycin, BHI agar plates containing 4 μg/mL vancomycin and 16 g/L pancreatic digest of casein were used. A 10-μL aliquot of a 2.0 McFarland was inoculated on the plates and incubated at 35°C for 48 hours. The growth of 2 or more colonies was considered to be a positive test for hVISA [13].

Etest GRD

The Etest GRD® method (BioMérieux, Marcy l'Etoile, France) utilizes different concentration gradients (0.5 to 32 μg/mL) of vancomycin and teicoplanin. The inoculum was adjusted to the 0.5 McFarland standard and inoculated on Mueller-Hinton agar containing 5% sheep blood, according to the manufacturer's recommendations. Readings were obtained at 24 and 48 hours and considered to be positive for hVISA isolates with a MIC of 8 μg/mL for teicoplanin and vancomycin [14].

Etest macro method

A high bacterial inoculum (2 McFarland scale) was used to inoculate nutritionally enriched medium (BHI), followed by prolonged incubation (48 hours). A 200-μL aliquot of the bacterial suspension was seeded onto a BHI agar plate. Etest®(BioMerieux, Marcy l'Etoile, France) vancomycin strips were added, and the plates were incubated at 35°C for 48 hours. Atest was considered to be positive for hVISA when the MIC for teicoplanin was 12 μg/mL or when an MIC of 8 μg/mL for teicoplanin and vancomycin was obtained [14].

PAP-AUC

After incubation on solid medium, the bacteria were diluted in sterile saline at dilutions ranging from 10^{-1} to $10^{-8}$and subsequently spotted as 10-μL spots on BHI agar plates containing 0, 0.5, 1, 2, 3, 4, 5, 6 and 8 μg/mL of vancomycin, respectively. The plates were incubated for 48 hours, and the colonies were counted to determine the $\log_{10}$CFU/mL; these data were then plotted on a graph as a function of the vancomycin concentration. The AUC was calculated using thestrain Mu3 (ATCC 700698) as a control. To confirm the designation as hVISA, the ratio of the AUC of the isolate to that of the Mu3 strain was required to be greater or equal to 0.9 and non-hVISA isolates had a PAP-AUC < 0.9 [10,13].

RESULTS AND DISCUSSION

For the antimicrobials vancomycin and teicoplanin, all 124

isolates were susceptible (Figure 1). All vancomycin MICs were equal to or greater than 0.5 µg/mL, reaching 3 µg/mL for seven isolates. To confirm these 7 isolates, the MICs were determined by the broth microdilution. Thus, three showed a MIC of 1 µg/mL and 4 with a MIC of 2 µg/mL. Therefore, all were considered to be susceptible to vancomycin (MIC ≤ 2 µg/mL).The MICs of teicoplanin were much higher than those of vancomycin, with MICs up to 12 µg/mL. The isolate with MIC 12 µg/mL for teicoplanin was regarded as sensitive as the MIC obtained by broth microdilution was 4 µg/mL (MIC ≤ 8 µg/mL).

Screening tests for the detection of hVISA isolates were performed. The results for the isolates that were confirmed as hVISA are listed in Table (1).

Based on the above results, the sensitivity, specificity, positive and negative predictive values, and accuracy of each test were calculated (Table 2). No test yielded optimum values for all variables; the agar screening had the best sensitivity (90.9%) and negative predictive value (99.1%), while the GRD had higher specificity (97.3%), positive predictive value (72.3 %) and accuracy (94.3%). Although the Etest macro method was not superior to the other tests, its accuracy was very similar (92.7%).

Because hetero resistance is usually associated with previous vancomycin use, *S. aureus* with elevated MICs (≥ 2 µg/mL) is among the main risk factors for the development of this phenotype. The selection of an appropriate treatment strategy depends on the methodology because the Etest has a tendency to overestimate MICs [15]. In this study, the hVISA isolates had an MIC ≥ 1.5 µg/mL(Etest). Some studies [16,17] have demonstrated a relationship between an MIC ≥ 1.5 µg/mL and the development of hVISA, using the Etest. Other studies using the same methodology [18,19] have presented data demonstrating an association ofan MIC < 1.0 µg/mL with hVISA. Thus, a higher MIC (< 4.0 µg/mL) is associated with an increased likelihood of the hVISA phenotype. Isolates with an MIC > 4.0 µg/mL are considered to be VISA.

Because the characteristics of hVISAare heterogeneous and constitute a minority of the bacterial population, there are no recommended methods for molecular detection. Phenotypic methods with large bacterial inoculums, enriched culture media and prolonged incubation times are needed [8]. These requirements can be fulfilled through the use of the three methods utilizedin this study in combination.

The Etest GRD uses rich medium (sheep blood) and a prolonged incubation time but a traditional inoculum (0.5 McFarland scale). Cost is a disadvantage of the Etest GRD, but its ease of standardization justifies its use. It has a sensitivity of 57-93% and a specificity of 82-97%, depending on the study [13,14,20]. We observed a sensitivity of 66.7% and a specificity of 97.3%, in agreement with international studies with larger samples.

The Etest macro method combines the three key features for the detection of hVISA: large inoculum, prolonged incubation time and nutrient medium (BHI). It is a fast and simple method that utilizes strips of vancomycin and/or teicoplanin to evaluate glycopeptides susceptibility. This method has a sensitivity of 57-89% and a specificity of 55-96%study [13,14,20, 21]. Our data indicated a sensitivity of 75% and a specificity of 94.6%.

Finally, agar screening in BHI with 4 µg/ml vancomycin and 16 g/L pancreatic digest of casein can be used with an inoculum of 0.5 or 2 McFarland standard and an incubation time of 48 hours in enriched medium. This test yields the best sensitivity and specificity, 91% and 94%, respectively [13], similar to the results obtained in our study (90.9% sensitivity and 93.8% specificity). These characteristics permit the use of this method for hVISA screening in association with (PAP-AUC) confirmatory testing. A disadvantage of this method is the need for standardization; these standards must be prepared in house, and thus the concentration of vancomycin can vary, thus negatively affecting test performance.

Because the performance of these screening tests is inconsistent, some authors [8,14] have recommended using these tests in combination to improve sensitivity—a strategy that may increase costs [14]. Alternatively, a test with high sensitivity could be combined with a test with high specificity.

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 a MIC ≥ 1.5 µg/mL and therapeutic failure, with an odds ratio of 1.74 (95% CI: 1.34 to 2.21; p < 0:01) [17].

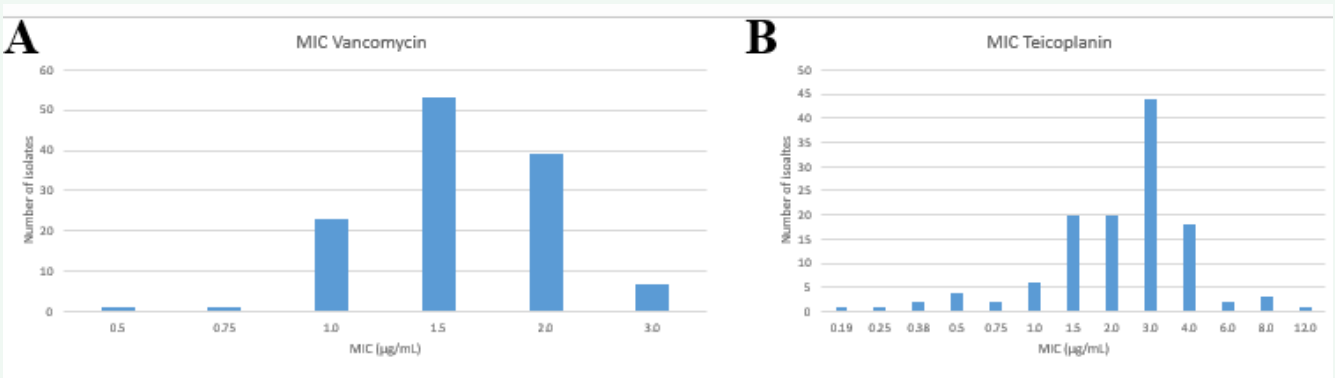

Figure 1 MIC to vancomycin and teicoplanin determined by Etest. In A, the results for vancomycin are shown. Seven isolates would be considered VISA by the Etest methodology, but their MICs determined by the reference methodology were ≤ 2 µg/mL. In B, susceptibility to teicoplanin is shown, with only one isolate presenting an MIC> 8 µg/mL, which was not confirmed by broth microdilution.

Table 1: Results of screening tests and PAP-AUC for the confirmation of hVISA (PAP-AUC values higher than 0.9 confirm this phenotype.

Isolate number	screening[a] / macromethod[b] / GRD[c]	AUC ratio[d]
SI4	negative/positive/negative	1.14
SI11	positive/positive/positive	0.99
SI13	positive/negative/positive	1.19
L10	positive/negative/positive	0.92
L36	positive/negative/negative	1.02
L43	positive/positive/negative	0.98
L54	positive/positive/positive	1.08
L69	positive/negative/positive	0.93
L74	positive/positive/positive	1.17
L80	negative/positive/positive	1.12
L84	positive/negative/positive	1.11
L92	negative/positive/postive	0.99

[a] – agar screening in brain-heart infusion (BHI) with 4 µg/mL vancomycin and 16 g/L pancreatic digest of casein
[b] – Etest macro method
[c] – Etest glycopeptides resistance detection®
[d] – ratio of isolate AUC/Mu3 AUC

Table 2: Parameters of the main screening tests for the detection of hVISA.

Methodology	Sensitivity	Specificity	PPV[a]	NPV[b]	Accuracy
Etest GRD[c]	66.7%	97.3%	72.3%	96.5%	94.3%
Etestmacromethod	75%	94.6%	60%	97.2%	92.7%
Agar screening[d]	90.9%	93.8%	58.8%	99.1%	93.5%

[a]– Positive predictive value
[b]– Negative predictive value
[c]– Etest glycopeptides resistance detection®
[d]– Agar screening in brain-heart infusion (BHI) with 4 µg/mL vancomycin and 16 g/L pancreatic digest of casein

Based on the results of this study and those of studies with larger population sizes, we suggest a flowchart for screening and confirming hVISA isolates (Figure 2). Due to its high sensitivity and high negative predictive value, the agar screening method is an excellent screening test because truly negative samples can be identified. The agar screening method is a simple, inexpensiveand easily employable method in routine diagnostic laboratories, including small laboratories. How the entire suspected hVISA should be confirmed by PAP-AUC, the screening agar can be an excellent alternative.

CONCLUSIONS

We conclude that the detection of hVISA, although challenging, is essential to the selection of the correct antibiotic therapy and the replacement of vancomycin with some other drug, such as linezolid or daptomycin. The methods used routinely to detect vancomycin resistance vary in sensitivity and specificity, and may fail to detect hVISA. The appropriate use of screening tests will depend on the prevalence rates of hVISA in each institution, and the use of a single screening test will yield poor results. A viable alternative would be to establish a flowchart for processing samples that includes a choice of tests that are suitable for routine epidemiology and are inexpensive. The combination of the three methods may be the best alternative because the ones with vancomycin MIC ≤ 2 µg/mL, may have hetero resistance and, in these cases, the correct characterization of hVISA may impact directly in the therapeutic success.

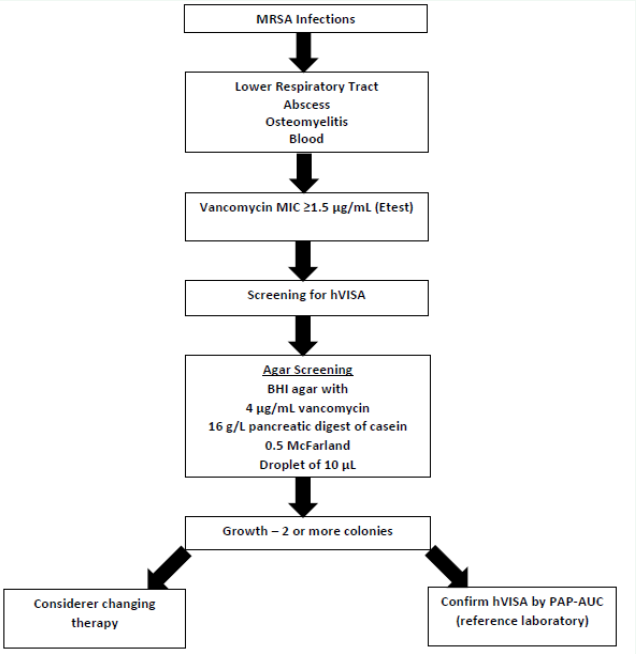

Figure 2 Flowchart for hVISA screening. Assuming that hVISA is more common in chronic infections and/or anatomical regions with high bacterial inoculum, it is likely that bone and lung infections are a major cause of hVISA infections. Because bloodstream infections are those with the highest mortality rates, the criteria of anatomical site and vancomycin MIC can be used to indicate the need for hVISA screening tests. Isolates with an MIC < 1.5 µg/mL can be excluded because they are associated with a lower prevalence of hVISA. The agar screening test should be used, and positive results should be reported to the doctor immediately to permit a change in therapy. Because performing PAP-AUC as a confirmatory test is impractical for routine laboratories, suspected hVISA isolates should be referred to a reference laboratory (adapted from [8,14,21]).

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