Heterogeneous Resistance to Vancomycin and Teicoplanin Among *Staphylococcus* spp. Isolated from Bacteremia

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This study evaluated the BHIA screening method with 4 or 6 μg/mL of vancomycin to detect glycopeptides heteroresistant staphylococci strains isolated from bacteremia. A total of 213 staphylococci strains were isolated from 106 patients between October/2001 and November/2002 in a tertiary hospital in Rio de Janeiro city. Fifty-seven (53.8%) patients presented *Staphylococcus aureus*, while coagulase-negative staphylococci (CNS) were isolated from 49 (46.2%). Resistance rates for oxacillin of 26.3% and 81.6% were found for the staphylococci isolates, respectively. Thirteen CNS isolated from nine (8.5%) patients grew on agar screening with 4 μg/mL of vancomycin and showed heterogeneous profiles of resistance for vancomycin and teicoplanin by the population analysis profile method. Only 30.8% of them grew at the concentration 6 μg/mL. Bacterial infection and use of antimicrobial therapy were common among these patients. Alert about the emergence of oxacillin-resistant staphylococci presenting heteroresistance to glycopeptides is important in order to achieve judicious use of antimicrobials. Vancomycin agar screening test could help to confirm the presence of these isolates in hospitals.

Key- Words: Staphylococci, vancomycin and teicoplanin heteroresistance, population analysis profile, vancomycin agar screen.

Antimicrobial therapy used in infections by multiresistant staphylococci has been limited since the resistance to oxacillin is associated with resistance to all β-lactams agents and, with frequency, to the other drugs. Thus, the vancomycin comes being widely used as last resource in the treatment of these infections [1].

Resistance to glycopeptides in coagulase-negative staphylococci (CNS) comes already being shown since the end of the years 80 [2,3]. However, the interest on the acquisition of resistance to glycopeptides increased only after the isolation of *Staphylococcus aureus* strains presenting intermediate resistance to vancomycin (VISA strains) worldwide [4,5]. Studies with VISA isolates from several countries indicated that these strains present resistance to low levels of vancomycin (minimal inhibitory concentration [MIC] of 8 μg/mL) that is not associated with the presence of *van* genes responsible for resistance to glycopeptides in enterococci [1,5,6].

Studies of population analysis profile (PAP) have shown phenotypes of heterogeneous susceptibility to glycopeptides in clinical strains of *S. aureus* [7] and CNS [8]. These strains present susceptibility to vancomycin (MIC ≤ 4 μg/mL) or teicoplanin (MIC ≤ 8 μg/mL) when tested by the conventional methods [9,10]. However, they possess subpopulations capable to grow in higher concentrations of vancomycin and/or teicoplanin [6,8,11,12]. The disk diffusion test is not able to detect these types of strains, while the methods based on the determination of MICs have shown good accuracy to detect VISA or CNS strains with intermediate resistance to vancomycin (MIC = 8 μg/mL) or resistance to teicoplanin (MIC ≥ 32 μg/mL). However, these methods fail in the detection of glycopeptides heteroresistant strains [13,14]. In this case PAP is considered a standard detection method [11]. However, its use in laboratorial routine is not advisable since it is a hard method. Thus, the BHIA (brain heart infusion agar) with 4 μg/mL [11] or 6 μg/mL [15] of vancomycin have been recommended to routines use. Nevertheless, studies that show the usefulness of the agar screening with vancomycin to detect heteroresistant staphylococci are scarce.

Staphylococci are considered important agents of hospital bacteremia [16] and glycopeptides have often been used in both rational and empirical therapy [1]. The clinical significance of heteroresistance to glycopeptides in *Staphylococcus* spp. is not completely established, but it has been considered that the presence of these strains could be associated with failure in the treatment [6,12,17,18]. In a previous study [19] we confirmed that the vancomycin heteroresistance in CNS is similar to the one described in *S. aureus* and is associated with an increase in cell wall thickness probably contributing to make difficult the therapy. Here, we evaluated the accuracy of the BHIA screening method with 4 or 6 μg/mL of vancomycin to detect staphylococci strains with heteroresistance to glycopeptides isolated from patients with bacteremia in a tertiary hospital in Rio de Janeiro, Brazil.

Materials and Methods

Bacterial Strains

Two hundred and thirteen staphylococci strains isolated from blood cultures of 106 patients between October/2001 and November/2002 at the Marcílio Dias Naval Hospital

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(MDNH), a tertiary hospital with 500 beds, were analyzed. The professional of the Commission of Hospital Infection Control as clinical considered all the bacteremias evaluated significant [20]. *Staphylococcus* identification and determination of the antimicrobial susceptibility profiles were performed by the Microscan WalkAway automated system (Dade Behring).

Oxacillin, Vancomycin and Teicoplanin Susceptibility Testing

Susceptibility to oxacillin, vancomycin and teicoplanin was initially evaluated by the Microscan WalAway system for 106 staphylococci strains (one isolate for each patient). Resistance to oxacillin (Sigma Chemical Company, St Louis, USA) was confirmed by the agar screening method containing 6 μg/mL of the drug for *S. aureus* [15] and 4 μg/mL for CNS isolates [21] and by the PCR method to detection of the *mecA* gene, according to Ferreira and coworkers [21]. Vancomycin (Sigma) and teicoplanin (provided by Marion Marrel Dow, Research Institute Cincinnati, Ohio, USA) MICs were obtained by the agar dilution method in Müeller-Hinton agar (MHA, Difco) in accordance with the CLSI [10]. Concentrations of 1, 2, 4, 6, 8, 10, 12, 14 and 16 μg/mL were used for vancomycin, while for teicoplanin the concentrations ranged from 1 to 256 μg/mL. Vancomycin and teicoplanin resistances, including intermediate resistance were defined as MIC ≥ 8 μg/mL and ≥ 16 μg/mL, respectively [15].

Vancomycin Agar Screen Method

All the 213 staphylococci strains isolated from blood cultures (1 to 3 strains for each patient) were cultured on BHIA screening plates with 4 μg/mL of vancomycin to detection of heteroresistant strains [11]. The isolates were inoculated with swab, using an inoculum from a bacterial suspension in saline that was adjusted at 0.5 McFarland turbidity (~10^8 CFU/mL). The plates were maintained at 35°C for 24 and 48 h. BHIA screening with 6 μg/mL of vancomycin recommended by the CLSI [10] was carried out in parallel for comparative analysis.

Population Analysis Profile (PAP) for Vancomycin and Teicoplanin

Bacterial subpopulations selected from the BHIA plates with 4 μg/mL of vancomycin were identified by classical tests to confirm the *Staphylococcus* species [22]. Heteroresistance phenotype to glycopeptides in subpopulations was confirmed by the PAP method for vancomycin and teicoplanin as recommended by Kim and coworkers [6], with modifications. Aliquot (100 μL) of a starting cell suspension at 0.5 McFarland turbidity and serial 10-fold dilutions were cultured on BHIA plates containing vancomycin at the concentrations 2, 4, 6, 8, 10, 12, 14 and 16 μg/mL or teicoplanin at the concentrations 3, 6, 12, 18, 20, 25, 50 and 100 μg/mL. After incubation at 35°C for 48 h, the number of colonies on plate in each concentration of drug was counted and plotted on a semi logarithmic scale. *S. aureus* 14a (without growth on BHI agar plates with vancomycin) and Mu50 (VISA) strains were used as controls [11]. The PAP experiment was performed at least twice for each strain to ensure reproducibility.

Results

In Table 1 are shown the 106 strains of *Staphylococcus*, distributed in species, isolated from bacteremias of 106 patients between October/2001 and November/2002 at the MDNH. Vancomycin and teicoplanin MICs were determined by the agar dilution method (CLSI, 2005), while the results found by the Microscan WalkAway automated method were only reported for vancomycin. In the period of the study, 57 (53.8%) patients presented *S. aureus* bacteremia, while CNS were isolated from 49 (46.2%) patients, including the species: *S. epidermidis* (21 strains), *S. haemolyticus* (14 strains), *S. hominis* (7 strains), *S. capitis* (3 strains) and *S. lugdunensis* (1 strain). Oxacillin resistance rates were 26.3% for *S. aureus* and 81.6% for CNS. All the isolates presented MICs for vancomycin <1 μg/mL (68 strains) or ranging from 1 to 2 μg/mL (38 strains) by the agar dilution method. Four *S. epidermidis*, three *S. haemolyticus* and two *S. hominis* isolates presented MICs of 8 μg/mL to teicoplanin. One *S. haemolyticus* isolate was resistant to teicoplanin (MIC of 32 μg/mL). By the automated system one isolate of each *S. aureus*, *S. epidermidis* and *S. haemolyticus* and, two isolates of *S. hominis* showed vancomycin MIC of 4 μg/mL.

From 213 isolates submitted to the vancomycin agar screening method, 13 (6.1%) CNS isolated from nine patients grew on the BHIA containing 4 μg/mL of vancomycin. Four of them also grew on the BHIA with 6 μg/mL of drug (Table 2). From five patients, the same CNS species were detected in two blood specimens. Isolates that grew on BHIA with 4 μg/mL of vancomycin were selected as subpopulations and were submitted to the tests to confirm the heteroresistance phenotype to glycopeptides. Isolates from the patient 115 presented MICs of 12 μg/mL for vancomycin, while the strains isolated from the patients 19, 31, 53 and 127 presented MICs of 16, 16, 32 and 32 μg/mL for teicoplanin, respectively. *Staphylococcus aureus* isolates were not detected by the vancomycin agar screening method. All oxacillin-resistant staphylococci isolates presented the *mecA* gene.

All the CNS isolates that grew on the vancomycin agar screening presented heterogeneous profile for vancomycin and/or teicoplanin by the PAP method (Figure 1). Isolates 4a and 4b presented subpopulations able to grow in 8 μg/mL of vancomycin, while the isolates of the patients 19, 53 and 120 presented subpopulations that grew in 6 μg/mL of the drug (Figure 1A). The remaining presented strains grew in 4 μg/mL of vancomycin. Concerning to the teicoplanin heteroresistance, some CNS species presented subpopulations able to grow in higher concentrations than 20 μg/mL of the drug (isolates of the patients 4, 19, 31, 53, 115 and 127) (Figure 1B). The clinical isolates of *S. aureus* (14a) that did not grow on the vancomycin agar screening and the Mu50 strain (VISA) were used as susceptible and resistant controls, respectively.

Table 1. Species distribution and, vancomycin and teicoplanin MICs obtained by agar dilution and automated methods in 106 strains of staphylococci isolated from bacteremia

Species (No. of strains)	Vancomycin MICs[a] range (µg/mL) Agar dilution test	Vancomycin MICs[a] range (µg/mL) Microscan WalkAway system[b]	Teicoplanin MICs range (µg/mL) Agar dilution test
Oxacillin R (n = 55)			
S. aureus (15)	< 1	≤ 2	1-2
S. epidermidis (18)	1-2	2-4	1-8
S. haemolyticus (11)	1-2	2-4	1-32
S. hominis (6)	1-2	2-4	1-8
S. capitis (2)	< 1	≤ 2	1-2
Staphylococcus spp. (3)	1-2	≤ 2	1-4
Oxacillin S (n = 51)			
S. aureus (42)	< 1	2-4	1-4
S. epidermidis (3)	< 1	≤ 2	1-4
S. haemolyticus (3)	< 1	≤ 2	1-2
S. hominis (1)	< 1	≤ 2	< 1
S. capitis (1)	< 1	≤ 2	< 1
S. lugdunensis (1)	< 1	≤ 2	< 1

[a] MIC=minimum inhibitory concentration breakpoints for susceptibility, intermediate resistance, and resistance, respectively: teicoplanin, 8, 16 and 32 µg/mL; vancomycin, 4, 8-16, and 32 µg/mL according to CLSI [9]. [b]Automated system for vancomycin, only. R=resistant; S=sensible.

Table 2. Characteristics of 13 clinical strains of CNS that grew on vancomycin agar screen plate

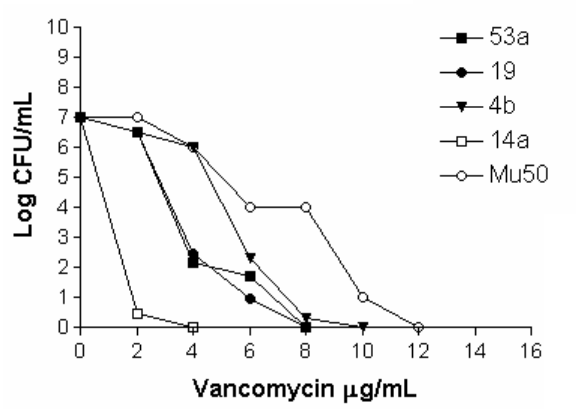
Patient Number	Strain Number	Staphylococcal species	Resistance to oxacillin (gene *mecA*)	Vancomycin MIC (automated system)	Vancomycin and teicoplanin MICs [a] (agar dilution method)		Growth on vancomycin agar with:		MIC from subpopulation gr on vancomycin	
					VC	TC	4 µg/mL	6 µg/mL	VC	TC
4	4a	S. hominis subsp novobiosepticus	+	4	2	4	+	-	2	4
	4b	*S. hominis subsp novobiosepticus*	+	4	2	4	+	-	2	4
19	19a	*S. haemolyticus*	+	≤ 2	2	32	+	-	2	16
31	31a	*S. haemolyticus*	+	≤ 2	< 1	8	+	-	2	16
53	53a	*S. haemolyticus*	+	≤ 2	< 1	4	+	-	2	32
	53b	*S. haemolyticus*	+	≤ 2	< 1	2	+	+	4	8
56	56a	*S. hominis subsp novobiosepticus*	+	≤ 2	2	4	+	-	2	8
115	115a	*S. hominis subsp hominis*	+	≤ 2	2	4	+	+	12	8
	115b	*S. hominis subsp hominis*	+	≤ 2	2	4	+	-	12	8
120	120a	*S. hominis subsp novobiosepticus*	+	≤ 2	< 1	< 1	+*	-	nd	nd
	120b	*S. hominis subsp novobios epticus*	+	≤ 2	2	8	+	+	4	4
127	127a	*S. haemolyticus*	+	4	2	8	+	-	2	32
168	168a	*S. epidermidis*	+	≤ 2	2	8	+	+	4	8

Table 3. Characteristics associated with nine patients presenting bacteremias by glycopeptides heteroresistant CNS

Patient number	Age (yr)	Sex	Ward	Underlying disease	Bacetremia source	Previous antimicrobial therapy	Outcome
4	84	M	Medical unit	Community pneumonia	Catheter related	Ceftriaxone	Discharged
19	65	M	Medical unit	Community pneumonia	Septicemia	Ciprofloxacin	Discharged
31	0.6	F	Pediatric	Cellulites of neck	Septicemia	Cephalothin, oxacillin	Discharged
53	78	M	ICU	Community pneumonia	Septicemia	Ceftazidime, amikacin	Died
56	79	F	ICU	Community pneumonia	Catheter related	Imipenem	Died
115	74	M	Medical unit	Community pneumonia	Septicemia	Vancomycin, meropenem	Discharged
120	83	F	ICU	Diverticulitis	Catheter related	Ciprofloxacin, metronidazole	Died
127	80	F	Medical unit	Rectovaginal fistula	Septicemia	Ciprofloxacin, gatifloxacin, trimethoprim/ sulfamethoxazole	Died
168	67	F	Medical unit	Brain tumour	Catheter related	Ciprofloxacin, amikacin	Died

F=female; M=male; ICU=intensive care unit.

Figure 1. Population analysis profiles (PAPs) of *Staphylococus haemolyticus* (53a and 19 strains) and *S. hominis* subs. *novobiosepticus* (4b strain) presenting reduced susceptibility to glycopeptides and of *Staphylococcus aureus* control strains (open symbols; 14a, susceptible control and Mu50, resistant control) with (A) vancomycin and (B) teicoplanin. The curves are representative of at least two experiments with each strain. CFU – colony-forming units.



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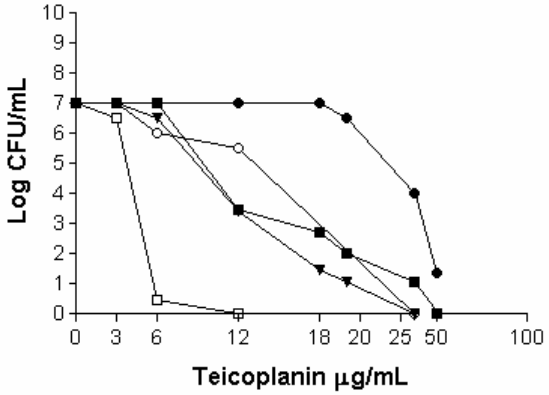

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Table 3 summarizes the characteristics associated with the nine patients presenting bacteremias by glycopeptides heteroresistant CNS. Infectious disease as underlying disease was common among these patients and all used previous antibiotics (five used β-lactams and four used quinolones) as therapy.

Discussion

The isolation of VISA strains has been rare in the last years and only 20 strains have been reported to date [4]. Susceptibility methods as microdilution in broth, agar screening with 6 µg/mL of vancomycin or E-test have shown accuracy in the detection of this type of strain, including *S. aureus* strains with full vancomycin resistance (MIC ≥ 32 µg/mL) [23,24]. However, isolates of *Staphylococcus* presenting heteroresistance to vancomycin have not been detected by these methods. Moreover, the best method to detection of vancomycin heteroresistance in *Staphylococcus* is not consensus [13].

The lack of a standardized method to detect heteroresistance to glycopeptides in staphylococci makes difficult the determination of the clinical significance of this resistance type in the course of the treatment of an infection with vancomycin, where the therapeutic failures associated with the underlying conditions of the patient is relatively common [25]. Studies on prevalence of vancomycin-heteroresistant *S. aureus* have shown variable rates, probably reflecting different criteria and/or methods used to detection of these strains [4].

Recently, our group evaluated the susceptibility to glycopeptides among 84 clinical strains of *Staphylococcus* by different methods [14]. A total of 20 (23.8%) strains of CNS grew on BHIA containing 4 and/or 6 µg/mL of vancomycin. Amongst these CNS, three isolates from the species *S. haemolyticus*, *S. epidermidis* and *S. warneri* were selected and analyzed by the PAP method, which confirmed its heteroresistance phenotypes. Respective subpopulations

selected in higher concentrations of vancomycin and teicoplanin presented cell wall thickness, main characteristic associated with resistance to glycopeptides [19].

In the present study, 213 staphylococci isolated from 106 patients presenting bacteremia were submitted to the agar screening with 4 and 6 μg/mL of vancomycin. Thirteen (6.1%) CNS strains from nine patients grew on the BHIA plate containing 4 μg/mL of vancomycin and four of them also grew on the BHIA with 6 μg/mL. Hiramatsu [11] suggests that a subpopulation growing on the BHIA plate with 4 μg/mL of vancomycin and presenting MIC ≥ 8 μg/mL for this drug might be considered as a vancomycin-heteroresistant strain. By this criterion, a patient (case 115) would have presented infection by vancomycin-heteroresistant staphylococci since the two strains (115a and 115b) isolated from him presented subpopulations with MICs of 12 μg/mL for vancomycin. Although the other isolates had presented vancomycin MICs < 8 μg/mL, these strains had subpopulations capable to grow in concentrations ranging from 4 to 8 μg/mL as showed by the PAP method in comparison to the control strains (14a and 150a) that presented subpopulations growing up with 2 μg/mL of vancomycin. Probably, the analysis of the bacterial subpopulations carried out after its storage in glycerol had a influence on the low MICs obtained, since the phenotype of resistance tends to revert in the absence of drug. Amongst 13 isolates grown on 4 μg/mL of vancomycin, only four (30.7%) grew on the BHIA with 6 μg/mL, as recommended by CLSI [10]. It is the concern, since the detection of these strains could have been compromised if these isolates were submitted only to selection in 6 μg/mL of vancomycin.

Teicoplanin is used as alternative therapy to vancomycin only in Europe [26,27]. However, some studies have shown an increase in the isolation of CNS with reduced susceptibility to teicoplanin worldwide. Schlegel and coworkers [28] isolated CNS strains presenting reduced susceptibility to teicoplanin from 6.3% of patients from an intensive care unit, while Lallemand and coworkers [29] found this type of strains in 31% of patients with bacteremias. The authors showed that the found rates were not associated with the increased use of teicoplanin in the hospitals analyzed. In addition, for some patients, the isolation of this type of strain was not related to the previous use of glycopeptides, indicating that CNS present large ability to develop glycopeptides resistance. Experiments *in vitro* about the resistance to glycopeptides in *Staphylococcus* have shown that the acquisition of teicoplanin resistance would be a previous stage for the development of vancomycin resistance [8,11]. Recently, Bertin and coworkers [30] showed that the incidence of CNS with decreased susceptibility to teicoplanin (32.1%) isolated at a French hospital was significantly associated with the use of vancomycin in medical units. To verify a possible correlation between heteroresistance to vancomycin and heteroresistance to teicoplanin, we determined the PAPs for teicoplanin of 12 isolates that showed heteroresistance to vancomycin (Table 2). All the isolates presented heterogeneous profiles for teicoplanin with subpopulations capable to grow in concentrations ranging from 6 μg/mL to 50 μg/mL. Moreover, the strain of the patient 53 presented full resistance to teicoplanin (MIC of 32 μg/mL) although the MIC 2 μg/mL for vancomycin, while four isolates showed intermediate resistance to teicoplanin. Thus, the determination of the teicoplanin MIC in routine laboratories could help to detect staphylococcal strains presenting heteroresistance to glycopeptides. The detection of this resistance type would be indicating that the strains possess vancomycin-resistant subpopulations, which could be selected during the treatment with this drug.

In this study we found a prevalence of 8.5 % (9/106 patients) for glycopeptides-heteroresistant staphylococci isolated from bacteremias. Wong and coworkers [31] also found a similar rate (7.4%) of CNS strains with reduced susceptibility to glycopeptides isolated from bloodstream among 203 patients analyzed. If we considered only bacteremias by CNS the prevalence found in our study would increase for 18.37% (9/49 patients). This result is worrisome because the CNS are the most frequent Gram-positive agents isolated from nosocomial bacteremias [18] and are also prevalent as endogenous microbiota in humans [22]. These facts in association with antibiotic pressure, especially by the use of vancomycin owing to the high prevalence of oxacillin resistance among these organisms, probably would be contributing to the establishment and maintenance of the glycopeptides resistance in hospitals.

Bacterial infection and use of antimicrobial therapy were common characteristics among the nine patients that presented glycopeptides heteroresistant CNS isolates. Marcilio Dias naval hospital is a tertiary hospital with an average of 13,000 admissions per year where patients with severe underlying disease are referred. Thus, our results may also reflect the severity of the patients admitted in this hospital. Wong and coworkers [31] in a case-control study showed that the admission to the intensive care unit, prior use of vancomycin and/or β-lactams and isolation of methicillin-resistant staphylococci were common characteristics among patients with bacteremia due to staphylococci with heteroresistance to vancomycin.

In conclusion, our results showed the emergence of glycopeptides heteroresistance in oxacillin-resistant CNS isolated from bacteremias. Alert about this emergence is important in order to achieve judicious use of antimicrobials. Moreover, the use of methods, as vancomycin agar screening in routine to detect these staphylococci would be crucial to confirm the presence of this pathogen in hospitals.

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