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Major Article

Occurrence of the *vanA* gene in *Staphylococcus epidermidis* from nasopharyngeal secretion of Health-Care Workers, Recife, Brazil

***Armando Monteiro Bezerra Neto*[1], *Marcelle Aquino Rabelo*[1], *Jailton Lobo da Costa Lima*[1], *Stéfany Ojaimi Loibman*[2], *Nilma Cintra Leal*[3] *and Maria Amélia Vieira Maciel*[1],[4]**

[1]. Programa de Pós-Graduação em Medicina Tropical, Departamento de Medicina Tropical, Universidade Federal de Pernambuco, Recife, PE, Brasil.
[2]. Centro de Biociências, Universidade Federal de Pernambuco, Recife, PE, Brasil.
[3]. Departamento de Microbiologia, Centro de Pesquisa Aggeu Magalhães, Fundação Oswaldo Cruz-PE, Recife, PE, Brasil.
[4]. Departamento de Microbiologia, Centro de Ciências da Saúde, Universidade Federal de Pernambuco, Recife, PE, Brasil.

Abstract

Introduction: The increasing reports of vancomycin-resistant *Staphylococcus* strains (VRS) haves caused concern worldwide, from the laboratory detection to patient management. This study aimed to identify the occurrence of VRS strains among healthcare professionals from a university hospital. **Methods:** A total of 102 *Staphylococcus sp.* isolates from healthcare professionals, obtained in a previous study were evaluated according to standard techniques for VRS detection. **Results:** After screening inoculation of plates containing 6μg/ml of vancomycin, 19 resistant isolates were identified. The susceptibility profile to other antimicrobials revealed 18 multidrug resistant isolates. The minimum inhibitory concentration (MIC) was determined by E-test and broth microdilution. According to E-tests, of 19 isolates grown in BHI-V6, four isolates presented MIC ≥ 128 μg/ml, seven with MIC ranging from 4 to 8 μg/ml, and eight with MIC ≤ 2μg/ml. By broth microdilution, 14 isolates presented MIC ≤ 2 μg/ml and five with MIC ≥ 16μg/ml. The presence of the gene *vanA* was determined by PCR in the five resistant isolates, and this gene was detected in one of the strains. Furthermore, among the 19 strains, the gene *mecA* was found in 13 (39,4%) isolates, including the strain carrying the gene *vanA*. **Conclusions:** Based on these results, we highlight the presence of one strain carrying both *vanA* and the *mecA* genes, as well as multidrug-resistant strains colonizing healthcare professionals, and their importance as potential vectors to spread strains carrying resistance genes in the hospital environment.

Keywords: *Staphylococcus*. Healthcare professionals. Vancomycin. Multiresistance. Methicillin.

INTRODUCTION

Infections due to methicillin-resistant staphylococcal strains (MRS) present increased risk of treatment failure, and for some time glycopeptides, such as vancomycin and teicoplanin, have been the only therapeutic option, which justifies the crescent use of this class of drugs[1,2]. Some antimicrobials that exhibit *in vitro* activity against *Staphylococcus aureus* remain active against strains resistant to glycopeptides such as rifampicin and fusidic acid[3]. In recent years, a limited number of new antimicrobials have been developed. Among them, the 5th generation cephalosporins, ceftaroline and ceftobiprole have been shown to be effective against MRSA isolates[4]. Other antimicrobials not belonging to the group of beta-lactams and with activity against these micro-organisms, including linezolid, daptomycin and tigecycline, have been available since the beginning of the 21st century and are widely employed in clinical practice[5].

The indiscriminate use of vancomycin therapy to treat hospital infections has allowed for the emergence of *S. aureus* isolates and other staphylococcal species with reduced sensitivity to vancomycin and other glycopeptides[6], such as vancomycin/glycopeptide intermediate resistant *S. aureus* (VISA/GISA) and vancomycin/glycopeptide resistant *S. aureus* (VRSA/GRSA)[3]. Studies have reported an increased number of glycopeptide-resistant *Staphylococcus epidermidis* (GRSE) worldwide[7].

Isolates of *S. aureus*, especially coagulase-negative *Staphylococci* (CoNS) resistant to methicillin/oxacillin isolates, with reduced susceptibility to glycopeptides have been reported in Japan, the United States (US), Europe, and Asia since the end of the 80s[3]. In 1997, two major categories of vancomycin-resistant *S. aureus* had been defined: (1) vancomycin-resistant *S. aureus* (VRSA), carrying the *vanA* gene, mediating

Corresponding author: Msc. Armando Monteiro Bezerra Neto.
e-mail: monteiro.armando10@gmail.com
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high-level resistance (MIC ≥ 16µg/ml) and (2) vancomycin-intermediate *S. aureus* (VISA) isolates with low-level resistance (MIC ≥ 4 to < 16µg/ml) by cell wall thickening[8].

In Brazil, staphylococcal strains with reduced susceptibility to glycopeptides were reported in hospitals in São Paulo and Rio de Janeiro a decade ago[9], and recently *S. aureus* containing the *vanA* and *vanB* genes were described in Brazil[10,11].

Colonization of diverse body sites by CoNS, and transient colonization by *S. aureus*, can be a source of infection for immunocompromised patients[12], highlighting microbiota colonization of health professionals as a source of dissemination of health care-associated infections (HAIs)[13].

The transmission chain of antimicrobial resistant microorganisms in the hospital environment involves health professionals as potential source of transmission to patients, co-workers, family and community, emphasizing their importance in the context of the HAIs[13,14].

In Brazil, resistant phenotypic profiles of different *Staphylococcus* species, with reduced resistance to vancomycin, were detected in microbiota samples of asymptomatic carriers [12]. Considering that *Staphylococcus* species are important pathogens associated with HAIs and there are few studies regarding vancomycin resistance in the colonizing microbiota of health professionals, the aims of this research were to identify the microorganisms colonizing the nasopharynx of health professionals and analyze the vancomycin resistance profile of these isolates by phenotypic and genetic methods.

METHODS

Study design and bacterial samples

An experimental-based study was carried out in April to December 2014 with staphylococcal isolates obtained in a previous study[15], originated from nasopharyngeal secretions of healthcare professionals of three sectors of the University Hospital of Pernambuco, Brazil: Intensive Care Unit (ICU), Surgical Clinics, and Hemodialysis Service/Nephrology.

A total of 102 *Staphylococcus* strains–kept as frozen stock in Brain Heart-Infusion (BHI) broth supplemented with glycerol (20%) at -20°C and in nutrient agar slants at 4ºC–were placed in BHI broth, inoculated in 5% sheep blood agar, and incubated for 24-48h at 35ºC. Colonies with macroscopic characteristics of the genus *Staphylococcus* were Gram stained. Following confirmation by morphology and staining, colonies were submitted for identification using deoxyribonuclease (DNase), catalase and coagulase tests, and manitol fermentation. The identification of the staphylococcal species was made through the automated system VITEK 2. Based on these readings, profile identification was established and interpreted according to a specific algorithm. The result of the profile was compared with the database, generating the identification of the unknown organism.

Vancomycin susceptibility testing

The isolates were screened by vancomycin susceptibility test using BHI supplemented with 6µg/mL of vancomycin (BHI-V6). Inoculums were adjusted to 0.5 McFarland turbidity[3].

Two methods were used to determine the MIC to vancomycin: broth microdilution and E-test (BIOMÉRIEUX), according to Clinical and Laboratory Standards Institute (CLSI) guidelines[16]. One clinical isolate of *Enterococcus faecium* harboring the *vanA* gene was used as a positive control[17] and the *Enterococcus faecalis* (ATCC 29212) strain as the negative control. The E-test was performed using a suspension of 0.5 McFarland turbidity plated onto Müller-Hinton medium and incubated at 35ºC for 24h. The interpretation was performed according to manufacturer's specifications and compared to CLSI cut off values[3].

For determination of MIC by Broth Microdilution, it was determined manually in Mueller Hinton broth, according to the recommendations of the CLSI[16]. Assays for vancomycin were performed in medium supplemented with Ca2 + (50mg/L). Initial inoculums of bacteria (0.5×10^5 CFU/mL) were plated onto 96-well polypropylene plates, exposed to 8 dilutions (1µg/mL to 128µg/mL) of the tested compound, and incubated for 18h at 35°C. The minimum inhibitory concentration was taken as the lowest concentration of the compound in which no visible bacterial growth was observed. According to CLSI recommendations, the bacterial isolates were categorized as resistant or susceptible using interpretive criteria[16].

Agreement between E-test and microdilution was defined as minimum inhibitory concentrations (MICs) that differed by ± 1-log2 dilutions or less. Categorical agreement was defined as test results within the same susceptibility. Errors were ranked as follows: very major error, false-susceptible result by the E-test; major error, false-resistant result produced by the E-test; and minor error, intermediate result by E-test method and a resistant or susceptible category for the reference method (microdilution test), according to CLSI guidelines[19].

Antimicrobial susceptibility testing

The *Staphylococcus* isolates were tested by disc diffusion in Mueller-Hinton agar, according to the CLSI guidelines[18], using the following antibiotics: penicillin (10U), gentamicin (10µg), clindamycin (2µg), sulfazotrim (1.25/23.75µg), ciprofloxacin (5µg), chloramphenicol (30µg), cefoxitin (30µg), erythromycin (15µg), and linezolid (5µg). After incubation for 24 hours at 35°C, the inhibition zones were measured using a caliper.

Concordance scale analysis of phenotypic vancomycin susceptibility tests

The agreement between the phenotypic tests to assess the vancomycin resistance profile were verified by the k (kappa) index[20].

Molecular techniques

Deoxyribonucleic acid (DNA) was extracted as described by Oliveira[21] from vancomycin-resistant isolates detected by the different phenotypic techniques. Subsequently, it was used in polymerase chain reaction (PCR) for amplification of the genes *vanA* and *mecA*. PCR was performed using the primers and conditions as previously described (forward–5′-TGAATAACATCGGCATTAC-3′ and reverse–5′-TTATTTAACGGGGAAATC-3′)[22] and (P1 5′-GGTCCCATTAACTCTGAAG-3′ and P3 5′-AGTTCTGCAGTACCGGATTTGC-3′)[23].

Sequencing of vanA gene

A positive PCR product for the *vanA* gene was purified by the Wizard® SV Gel kit and PCR Clean-Up System (Promega) according to the manufacturer's protocol. Following, it was quantified by spectrophotometry using the software Chromas Lite 2.1.1, Basic Local Alignment Research Tool (BLAST), and Expert Protein Analysis System (ExPASy) algorithm. The analyzed sequences of *vanA* were deposited in GenBank with the following accession number: KT581638.

RESULTS

In this study, we analyzed 102 isolates collected from health professionals, 31.4% (32/102) *S. aureus* and 68.6% (70/102) CoNS isolates. Approximately 43.1% (44/102) of the isolates were from the Surgical/Infectious and Parasitic Diseases sector, of which 22.7% (10/44) were *S. aureus* and 77.3% (34/44) were CoNS. Isolates from the ICU represented 20.6% (21/102), of which 38% (8/21) were *S. aureus* and 62% (13/21) were CoNS.

Concerning the vancomycin susceptibility, seven out of 19 isolates were *S. aureus* and 12 CoNS, which grew at the vancomycin screening test at a concentration of 6μg/mL (BHI-V6). The susceptibility profile to other antimicrobials showed that 18 isolates presented resistance to more than three classes of antimicrobials and were considered multi-drug resistant (MDR) strains. It is worth noting that 16 were resistant to erythromycin. Of these, 11 were also resistant to clindamycin, indicating resistance to macrolides, lincosamide, and streptogramin-B.

All 19 resistant strains, previously detected by the screening test, were evaluated by E-test for quantitative determination of the MIC to vancomycin. The seven isolates of *S. aureus* presented the following MIC ranges: two isolates with MIC ≤ 2μg/mL (sensitive), three with MIC between 4 and 8μg/mL, and two with MIC > 256μg/mL. In addition, of the 12 CoNS, 10 isolates presented MIC ≤ 4μg/mL and two isolates MIC ≥ 128μg/mL.

According to the MIC values obtained by broth microdilution, of the seven *S. aureus* isolates, five were sensitive (MIC ≤ 2μg/mL) and two were resistant (MIC ≥ 16μg/mL). Regarding the 12 CoNS isolates, nine showed MIC ≤ 2μg/mL and three MIC ≥ 32μg/mL, as shown in **Table 1.**

The kappa index coefficient of 0.96 was obtained by comparing the E-test to Broth Microdilution, indicating very good agreement between these two methods.

TABLE 1: Susceptibility profile of the *Staphylococcus* isolates.

Bacterial species	Susceptibility profile	Cefoxitin disc-diffusion	BHI-V6	E-test (μg/mL)	Broth microdiluition (μg/mL)	Gene *vanA*	Gene *mecA*
S. aureus	PEN, CLI, CFO, ERY	R	+	4	2	-	-
S. aureus	PEN, CLI, GM, CFO, RIF, ERY, CIP	R	+	1	1	-	-
S. aureus	PEN, CHL, CFO, ERY	R	+	4	2	-	-
S. aureus	PEN, CLI, ERY, CIP	R	+	> 256	128	-	+
S. aureus	PEN, CLI, CFO, LZD, RIF, ERY, CIP	R	+	> 256	32	-	-
S. aureus	PEN, CIP	R	+	2	2	-	+
S. aureus	PEN, GM, CFO, ERY, CIP	R	+	4	1	-	-
CoNS	PEN, CLI, SUF, CHL, CFO, ERY	R	+	128	128	-	+
CoNS	SUF, CHL, CFO, CIP	R	+	2	2	-	+
CoNS	PEN, CLI, SUF, CFO, LZD, RIF, ERY, CIP	R	+	4	2	-	+
CoNS	CLI, CHL, GM, CFO, ERY, CIP	R	+	2	2	-	+
CoNS	CLI, GM, CFO, ERY, CIP	R	+	4	2	-	+
CoNS	PEN, CLI, CFO, ERY, CIP	R	+	4	2	-	+
CoNS	PEN, CLI, CFO, ERY, CIP	R	+	2	2	-	+
CoNS	PEN, SUF, CFO, ERY	R	+	> 256	32	+	+
CoNS	PEN, CLI, SUF, GM, CFO, RIF, ERY, CIP	R	+	2	2	-	+
CoNS	CFO, LZD, CIP	R	+	2	2	-	-
CoNS	PEN, CLI, CHL, CFO, ERY, CIP	R	+	2	2	-	+
CoNS	PEN, CLI, GM, CFO, ERY, CIP	R	+	4	2	-	+

BHIV6: brain heart infusion agar; ***vanA****: gene;* ***mecA****: gene;* ***S.****: Staphylococcus;* **CoNS***:* coagulase-negative *Staphylococci;* **PEN:** penicillin; **CLI**: clindamycin; **CFO**: cefoxitin; **ERY:** erythromycin; **GM**: gentamicina; **RIF:** rifampicin; **CIP: c**iprofloxacin; **CHL**: chloramphenico; **LZD**: linezolide; **SUF:** sulfazotrim; **R***:* resistant; **+**: growth; **-**: growth.

The agreement within 1 two-fold dilution between E-test and the broth microdilution reference method was 84%. Seven (36%) minor errors were found comparing E-test with microdilution in broth for vancomycin. There was no occurrence of major or very major errors. The categorical concordance was 93%.

Of the five isolates with MICs considered resistant, only one isolate (*S. epidermidis*) carried the *vanA* gene, which was confirmed by sequencing and showed 100% of similarity with ten sequences from *Enterococcus* deposited in GenBank (KT581638).

In parallel to obtaining vancomycin susceptibility, a cefoxitin susceptibility test was performed using the disc-diffusion technique, from which it was possible to observe that all the isolates were resistant to cefoxitin. After the disc-diffusion cefoxitin test, the presence of the *mecA* gene was investigated.

In order to genetically characterize these isolates, *mecA* gene detection was performed by PCR, and the gene was found in 13/19 (39.4%) isolates, including one isolate of *S. aureus* (MRSA) from the Nephrology/Hemodialysis Service sector. Of the 12 MRCoNS, five were obtained from the Nephrology/Hemodialysis Service sector, four from Surgical/Infectious and Parasitic Diseases sector, and three from ICU. The isolate that presented the gene *vanA* also exhibited the gene *mecA*. As for the remaining noncarrier isolates of the *vanA* gene, five contained the *mecA* gene (all MRCoNS) and 13 did not present this gene (6 MSCoNS and 7 MSSA).

DISCUSSION

In this study, we found a higher incidence of *mecA* gene in CoNS strains. Regarding the susceptibility profile analyses, the MRS strains were more resistant to multiple classes of antimicrobial agents than MRSA. Similar results were obtained by Costa[4]; however, Fadeyi[25] described MDR in MRSA isolates colonizing the nasopharynx of health professionals in Nigeria.

The studies regarding the analysis of susceptibility to vancomycin started approximately three decades ago, when environmental strains of *S. aureus* were detected with reduced susceptibility (intermediate) to this drug[26]. Additionally, the emergence of hetero-VRSA strains occurred in the 80s after the introduction of vancomycin use for treatment of staphylococcal infections in Japan[27]. Several research studies related to the molecular analysis of heteroresistance vancomycin-intermediate *S. aureus* worldwide have been performed, following associations of these strains with persistent infections and treatment failure[28].

Results of MIC to vancomycin evaluated by E-test and broth microdilution techniques demonstrated divergence. Three isolates of *S. aureus* showed MICs between 4 and 8µg/mL (VISA) with the microdilution method but no isolates presented similar MIC with E-test. In addition, this technique is a screening tool for heterogeneous VISA (hVISA) and VISA, but does not apply to vancomycin or teicoplanin, and the results obtained with this technique should not be reported as true MIC[29].

In the present study, four resistant vancomycin isolates (two VRSA and two VRS) were detected by both the E-test and broth microdilution. There was reasonable correlation between these two methods. Using comparisons between Broth Microdilution and E-test MICs results for vancomycin, it was possible to observe that essential and categorical agreements presented at 84% and 93%, respectively. Yet, a minor error of 36% was detected: however, major error and very major error were 0%.

Genetic characterization these vancomycin resistant isolates was performed by PCR for the *vanA* gene. During the period that the present study was conducted, vancomycin resistance was not described in other genes in Brazil[10]. Among the isolates only one harbored the *vanA* gene, which was a specific isolate *Staphylococcus epidermidis* from HCW microbiota.

The presence of the *vanA* gene was not found in the four resistant vancomycin strains (VRSA and VRS), as a result, the precise genetic mechanism for vancomycin resistance in these staphylococcal strains awaits elucidation. The cell wall thickening has been reported for glycopeptide-resistant VRS and VRSA[12,17,28].

The nasopharynx microbiota of health professionals harboring resistant strains to vancomycin have already been described in the literature[13,31]; however, the first Brazilian report of *S. epidermidis*, harboring the *vanA* gene, and colonizing a health professional from ICU occurred at an University Hospital in Recife, Brazil[12]. Breves[11] reported the occurrence of one *S. aureus* isolate obtained from the hands of a health professional, which harbored resistance genes to vancomycin (*vanB*) and methicillin (*mecA*). On the other hand, there are many studies reporting methicillin-resistant staphylococcal isolates from nasopharyngeal of health professionals and microbiota of patients[32-34].

Cases reports regarding microbiota colonization of vancomycin-resistant staphylococcal strains obtained from patients at clinics or hospitals are scarce. In India, two studies have reported patients harboring *S. aureus* with the *vanA* gene[35,36].

The occurrence of HAIs caused by *Staphylococcus* isolates carrying the *vanA* gene has been reported worldwide, associated with different patterns of infections, mainly in the US and in Brazil[11,22,37-41,24].

Some studies correlate methicillin resistance to vancomycin tolerance due to vancomycin treatment failures in cases of infections caused by methicillin-resistant microorganisms[7,42-44]. In 2006, three strains were reported, two *S. aureus* and one CoNS, with resistance to both antibiotics vancomycin and methicillin[45].

A potential emergence of vancomycin resistance may occur in hospitals in Brazil, as reported in recent studies[9,28]. This suggests the need for constant monitoring of susceptibility patterns to vancomycin, application of molecular methods and heteroresistance detection, as well as adoption of control measures to avoid the spread of these strains in the hospital environment.

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Conflict of interest

The authors declare that there is no conflict of interest.

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