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ANALYSIS OF THE GENETIC DIVERSITY OF VANCOMYCIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MRCoNS) are among the most frequent causes of hospital infections worldwide, thus justifying the increasing use of vancomycin. In this study, we evaluated the presence of glycopeptide-resistant staphylococci, in 41 patients hospitalized in the Clinical Hospital of the Federal University of Uberlândia in Uberlândia, MG, who were being treated with vancomycin. All isolates were plated on Mueller-Hinton agar containing vancomycin. Vancomycin resistance was confirmed by surface growth after incubation for 24-48 h at 35ºC. Heteroresistance was evaluated by plating with a large inoculum (10^8 CFU/mL). One patient with nephritis who was on a hemodialysis program was diagnosed with the phenotype isolate of vancomycin-intermediate *Staphylococcus aureus* (VISA) (CIM = 8 μg/mL) and in eight patients, strains of heteroresistant *Staphylococcus* corresponding to the hVISA phenotype were isolated. In addition to the extended use of vancomycin, other risk factors associated with the presence of these microorganisms included the use of three or more antimicrobial agents, surgery, and three or more invasive procedures. Molecular analysis by randon amplified polymorphic DNA – polymerase chain reaction (RAPD-PCR) showed two clusters involving two samples each one of them, in surgical patients, with temporal and spatial relationship and isolates similarity concerning the susceptibility range to antimicrobial agents.

Key words: Hospital epidemiology, vancomycin-resistant *Staphylococcus*, RAPD-PCR

INTRODUCTION

Methicillin/oxacillin-resistant *Staphylococcus aureus* account for more than 50% of hospital staphylococci (3,18), and has led to an increase in the use of vancomycin (11,23). In Brazil, the situation is particularly serious because of the limited use of standard diagnostic microbiological procedures in hospitals (8) and also because of the empirical use of antimicrobial agents (5,14,17).

Staphylococcus aureus and mainly coagulase-negative staphylococci isolates resistant to methicillin/oxacillin and with a reduced susceptibility to glycopeptides were reported in Japan, United States, Europe and Asia at the end of the 1980s (13). The resistant phenotypes commonly found in most hospitals include VISA (vancomycin-intermediate *Staphylococcus aureus*) and VICoNS (vancomycin-intermediate coagulase negative *Staphylococcus*) which show intermediate resistance, VRSA (vancomycin-resistant *Staphylococcus aureus*) for which the minimum inhibitory concentration (MIC) of vancomycin is ≥ 32 μg/mL, and hVISA/hVICoNS a heteroresistant form with a MIC ≥ 8 μg/mL (13,15,23). Recently a VRSA isolate obtained from the catheter of a diabetic patient on hemodialysis showed clusters of *van*A resistance genes (4).

The first epidemiological report of these microorganisms in a Brazilian hospital was done in the city of São Paulo (6), followed by a report from Rio de Janeiro (16). An outbreak of VISA with four isolates from a burn unit in a hospital in São Paulo has also been described (17).

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The aim of this study was to assess the occurrence of glycopeptide-resistant strains of *Staphylococcus aureus* in hospitalized patients undergoing treatment with vancomycin.

MATERIALS AND METHODS

Hospital

The Clinical Hospital of the Federal University of Uberlândia (UFU) is a 450 bed hospital that also provides and holds tertiary services.

Study design

A prospective, longitudinal study of 41 patients, which included 20 children enrolled in the Pediatric Clinic and 21 adult surgical patients undergoing treatment with vancomycin, was done from December 2000 to March 2002. Bacterial colonization of the patient was monitored by microbiological screening throughout the study. All patients (or parents, in the case of children) provided verbal consent before being enrolled. A standard clinical form, which included demographic data, the clinical diagnosis, and intrinsic and extrinsic risk factors, was completed for each patient.

Microbiological techniques

Specimens were collected by swabing (Bionete, São Paulo, Brasil) oral and rectal cavities within the first 24 h of treatment and then at weekly intervals until discharge or death of the patient. The material was transported to the microbiology laboratory at UFU in test tubes containing brain heart infusion BHI agar (Difco, Sparks, USA). Primary cultures were done in mannitol salt agar (Difco, Sparks, USA) containing 4 µg of oxacillin/ mL (Sigma Chemical Co., St. Louis, USA) incubated for 48 h at 35ºC (11,12).

The isolates were identified to the level of genus/species using classic tests that included Gram staining, mannitol fermentation, and coagulase and catalase activities (16,17).

Screening for hetero-VRSA was performed by spreading 100 µL of the cell suspension adjusted to approximately 10^8 colony-forming units/mL, onto the agar plates containing vancomycin, followed by incubation for 48 h at 35ºC. Bacterial growth was inspected 24 and 48 hours after the beginnig of incubation (12, 17).

Molecular techniques

Genomic DNA was extracted according Santos *et al.* (21) and quantified at 260 nm in a Hitachi U-2000 spectrophotometer. The quality of the DNA was assessed in 1% agarose gel (20).

RAPD–PCR assays were done as described by Welsh and McClelland (24). Thirty-one primers were screened and seven informative primers were selected (OPT 6, 14, 114, 127, 167, 180 and 243). The reactions were amplified in MJ Research PTC–100 thermal cycler with two initial cycles of 94ºC for 1 min, 37ºC for 1 min and 72ºC for 2 min, followed by 33 cycles of 94ºC for 10 s, 40ºC for 20 s and 72ºC for 2 min and 4ºC for as long as necessary. The PCR products were separated in 1.5% agarose gels containing ethidium bromide (0.5 µg/mL). The bands were visualized and photographed in a VDS imaging system (Amersham Biosciences).

Statistical analysis

The electrophoretic profiles of the eight samples were compared with each other using a binary matrix based on the absence (0) and presence (1) of bands. The matrix generated by the Statistica 4.5[A] (1993) program was used to calculate of genetic distances and to run the cluster analysis. The genetic distances were calculated using the percentage disagreement method given by the formula N'_{AB}/N_T, where N'_{AB} is the number of polymorphic banding patterns and N_T is the total number of banding patterns (1). Cluster analysis was done with the unweighted pair-group method using arithmetic averages (UPGMA), in which samples are grouped based on their similarity. Epi Info (6.03) program were used for the epidemiological analysis (2).

RESULTS

Methicillin/oxacillin-resistant *Staphylococcus* were isolated from 32 (78.0%) out of 41 patients, including 21 (51.2%) and 18 (43.9%) individuals colonized by MRSA and MRCoNS, respectively. Eight patients (19.5%) had hVISA. One patient initially colonized with hVISA showed the phenotype VISA (MIC for vancomycin= 8 µg/mL) at a second sampling.

Six adults (average age of 43.5 years) and two children showed vancomycin-heteroresistant *Staphylococcus aureus*. The average duration of the treatment with vancomycin at the time of isolation was 15.3 days and the average time that these patients had been interned was 40.3 days.

Intrinsic rather than extrinsic factors such as three or more antimicrobial agents, surgery, and use of three or more invasive procedures, were related ($P<0.05$) with hVISA colonization (Table 1).

Table 2 shows the susceptibility of the MRSA (17) and MRCoNS (20) isolates to vancomycin based on the MIC and heteroresistance. One *Staphylococcus aureus* with MIC of 8 µg/mL behaved like VISA. The MIC_{90} for isolates of this microorganism was 4 µg/mL. Eight isolates showed heteroresistance and grew at a vancomycin concentration > 5 µg/mL.

Figs. 1 and 2 show the RAPD-PCR and the dendogram results for the eight hVISA isolates. RAPD-PCR identified two clusters with a dissimilarity of 63%. The first group included the genotypes A, B, D, E, G and D H, while the second group included the genotypes C and F, which had similar banding patterns (99.8% similarity). Both groups (D, H and C, F) showed

Table 1. Colonization by MRSA and MRCoNS heteroresistant to vancomycin in patients on vancomycin therapy at the Clinical Hospital, Uberlândia.

Variables	Colonized patients				
	Yes (N=32)			No (N=9)	*P*
	hVISA (8)	hVICoNS (4)	VSSA/VSCoNS (20)	(9)	
Adults	6	2	9	4	0.20
Children	2	2	11	5	0.20
Sex (M/F)	4/4	2/2	9/11	4/5	-
Age (variation)	33.4(2-79)	20.3(0.3-63)	25.6(0.5-79)	15.7(0.4-40)	-
Internment unit					
Pediatrics					
Onco-hematological	1	1	3	3	0.77
General	1	1	6	2	0.46
Surgical					
Neurological	2	1	5	2	0.95
Traumatological	3	1	3	2	0.26
Urological	1	0	3	0	0.84
Intrinsic risk factors					
Duration of internment	36.0(15-59)	40.7(18-60)	36.9(19-60)	23.5(11-38)	-
Re-internment	3	1	8	1	0.88
Immuno-compromising	4	1	4	2	0.17
Extrinsic risk factors					
≥ 3 antimicrobials	4	3	6	1	0.03
3rd generation cephalosporin	3	4	2	7	0.10
Surgery	7	3	10	4	0.04
Invasive procedures					
Central vascular catheter	4	1	7	4	0.82
Peripheral vascular catheter	8	4	19	5	0.12
Breathing	5	0	5	0	0.10
Drain	4	2	3	5	0.17
≥ 3 procedures	7	4	13	4	0.01

hVISA – heteroresistant vancomycin intermediate *Staphylococcus aureus*; hVICoNS – heteroresistant vancomycin intermediate coagulase-negative staphylococci; VSSA – vancomycin susceptible *Staphylococcus aureus*; VSCoNS – vancomycin susceptible coagulase-negative staphylococci.

differences according to classic epidemiology. This result was later confirmed by molecular study, suggesting horizontal transmission.

DISCUSSION

In this study, 19.5% of patients on prolonged treatment with vancomycin were colonized by *Staphylococcus aureus* resistant to this antimicrobial agent. These isolates belonged to the MRSA phenotype, as also reported in Japan (10,12), the United States (3,13), Europe (15) and Brazil (6,16). Risk factors such as the length of internment (34.3 days), surgery (58.5%), infection (100%), use of the wide-range cephalosporins (53.7%) and three or more invasive procedures (68.3%) were associated with colonization by vancomycin-resistant *Staphylococcus aureus*. The frequency of colonization by MRSA and/or MRCoNS (78.0%) reflected the seriousness of the situation and justifies more caution in hospitals. Infections by MRSA are endemic, accounting for almost half of infection by staphylococci in the Clinical Hospital at UFU (17). There observations along with the empirical use of antimicrobial agents in hospitals in developing countries (5,7,8,25), increase the risk of emergence of glycopeptide-resistant *Enterococcus* and *Staphylococcus*.

Table 2. MIC (=10^6 CFU/mL) and Heteroresistance (≥10^8 CFU/mL) of vancomycin in MRSA and MRCoNS samples isolated from patients in the Clinical Hospital, Uberlândia, from December 2000 to March 2002.

Vancomycin (μg/mL)	MRSA**		MRCoNS***	
MIC:	N	%	N	%
0.25	-	-	-	-
0.5	1	5.88	5	25
1	5	29.41	6	30
2	5	29.41	5	25
4	5	29.41	4	20
8	1	5.88	-	-
Heteroresistance*				
0.5	1	5.88	-	-
1	3	17.64	2	10
2	3	17.64	6	30
3	-	-	1	5
4	2	11.76	7	35
5	-	-	1	5
6	2	11.76	2	10
7	-	-	-	-
8	5	29.41	1	5
9	1	5.88	-	-
Total	17	100	20	100

* Inoculum ≥ 10^8 CFU/mL; ** MRSA – methicillin-resistant *Staphylococcus aureus*; *** MRCoNS – methicillin-resistant coagulase-negative staphylococci.

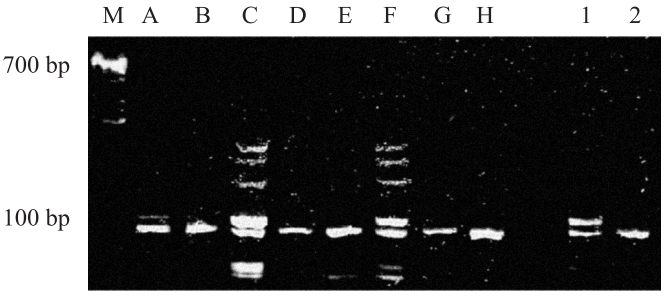

Figure 1. Electrophoresis (1.5% agarose gel) of products amplified with primer 180. M DNA molecular weight markers, A to H – samples of vancomycin-heteroresistant, 1 - *Staphylococcus aureus* ATCC 25923 and 2 - vancomycin resistant *Staphylococcus haemolyticus* 225r.

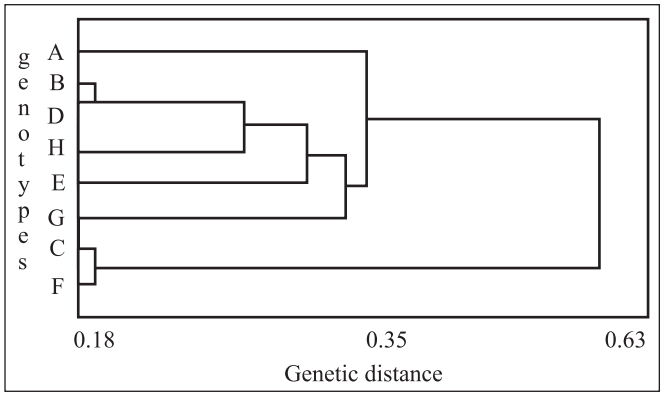

Figure 2. Dendogram featuring data of genetic distance by disagreement percentage and UPGMA method among the eight genotypes of *Staphylococcus aureus* based upon 58 RAPD markers obtained for short primers.

Exposure to vancomycin was the most evident risk factor in all patients from whom these microorganisms were isolated (13). The intrinsic risk factors include renal insufficiency and previous infection by MRSA (13,21). In our series, the only patient on hemodialysis had VISA and in the total eight patients (19.5%) were infected by hVISA; most patients (80%) were colonized by methicillin/oxacillin-resistant *Staphylococcus*.

Staphylococcus isolates with reduced susceptibility to vancomycin are not detected by the disk diffusion technique (3) and tests in BHI agar containing 4 or 6 μg of vancomycin use, determination of the MIC or E test are required (3,13,23). The E test was not used in the present investigation. The hVISA and hVICoNS isolates were detected using a larger inoculum; ≅10^8 UFC/mL in plates containing 1 μg - 10 μg of vancomycin/mL, according to Hiramatsu *et al.* (10).

The evidence that two clusters were associated with hVISA is a cause for concern mainly because of the precariousness of the measures for controlling infection and because of the indiscriminate use of antimicrobial agents in Brazilian hospitals (14). This situation raises the risk of the emergence of multi-resistance microorganisms emergency, including vancomycin-resistant *Staphylococcus* and *Enterococcus* (9).

In contrast to reports of microorganisms with outstanding epidemiological resistance, have been few reports of the risk factors associated with the appearance of *Staphylococcus aureus* that have reduced susceptibility to vancomycin, (10,22). Thus, the wise use of vancomycin and the adoption of adequate measures to control infection are necessary in order to avoid the emergence of glycopeptide-resistant microorganisms and their dissemination within hospitals.

The clinical cases of vancomycin-resistant *Staphylococcus aureus* (VRSA) reported here may be indicative of a higher and insidious prevalence of hVRSA and of hVRCoNS, in the Clinical Hospital at UFU. This relationship suggests transmission between adult patients interned in surgical units.

RESUMO

Análise da diversidade genética do *Staphylococcus aureus* resistente à vancomicina

Staphylococcus aureus resistente à meticilina (MRSA) e *Staphylococcus* coagulase negativo resistente à meticilina (MRCoNS) são os agentes mais freqüentes em infecções hospitalares mundialmente, justificando o incremento no uso de vancomicina. Neste estudo avaliamos a presença de *Staphylococcus* resistentes aos glicopeptideos em 41 pacientes, em uso de vancomicina, hospitalizados no Hospital de Clínicas da Universidade Federal de Uberlândia em Uberlândia-MG. Todos os isolados foram semeados em agar Mueller-Hinton acrescido do antimicrobiano. A resistência a vancomicina foi confirmada por crescimento após incubação por 24-48 horas a 35ºC. A heterorresistência foi avaliada por semeadura com inóculo mais denso (10^8 UFC/mL). Um paciente com nefrite, no programa de hemodiálise teve o fenótipo de *Staphylococcus aureus* com resistência intermediária à vancomicina (VISA) (CIM= 8 μg/mL) e em oito pacientes as amostras apresentaram heterorresistência (hVISA). Além do uso prévio de vancomicina outros fatores de risco incluindo três ou mais antimicrobianos, cirurgia e três ou mais procedimentos invasivos, foram observados. A análise molecular foi realizada por amplificação randômica de DNA polimórfico em reação em cadeia da polimerase (RAPD-PCR) mostrando dois *clusters* com duas amostras cada um, em pacientes cirúrgicos, com relação temporal espacial e com perfil de susceptibilidade semelhantes quando frente à vários outros antimicrobianos.

Palavras-chave: epidemiologia hospitalar, *Staphylococcus aureus* resistente à vancomicina, RAPD-PCR

REFERENCES

1. Cabral, T.A.T. Divergência genética entre genótipos de algodoeiro (*Gossypiu hirsutum*) estimada por análise de marcadores RAPD. Uberlândia, 1997, 54 p. (Master Thesis. Instituto de Ciências Biomédicas, UFU).
2. CENTER FOR DISEASES CONTROL AND PREVENTION (CDC) & World Health Organization (WHO). Epi info 6: a world processing, database and statistics program for public health. Vs 6.03. Atlanta and Geneve, Switzerland, 1996.
3. CENTER FOR DISEASES CONTROL AND PREVENTION. *Staphylococcus aureus* with reduced susceptibility to vancomycin. *MMWR Morb. Mortal. Wkly Rep.*, 46, 765-766, 1997.
4. CENTER FOR DISEASES CONTROL AND PREVENTION . *Staphylococccus aureus* resistant to vancomycin. *MMWR Morb. Mortal. Wkly Rep.*, 51, 565-567, 2002.
5. Couto, H.G.; Leles, C.C.V.; Lima, H.V.; Assunção, U.F.; Ribas; R.M.; Diogo Filho, A.; Gontijo Filho, P.P. Vancomycin use in a Brazilian university hospital. Comparison with hospital infection control practices advisory Committee guidelines. VI Congresso Brasileiro de Controle de Infecção e Epidemiologia Hospitalar, Rio de Janeiro, 1998. p. 188.
6. Del'Alamo, L.; Huband, M.D. Antimicrobial susceptibility of coagulase-negative staphylococci and characterization of isolates with reduced susceptibility to glycopeptides. *Diagn. Microbiol. Infect. Dis.*, 34, 185-191, 1999.
7. Goldmann, D.A.; Huskins, W.C. Control of nosocomial antimicrobial-resistant bacteria: a strategic priority for hospitals worldwide. *Clin. Infect. Dis.*, 24, 39-45, 1997.
8. Gontijo Filho, P.P. Definições de infecções hospitalares sem a utilização de critérios microbiológicos e sua consequência na vigilância epidemiológica no Brasil. *NewsLab*., 53, 120-124, 2002.
9. Guzmám-Blanco, M.; Casellas, J.M.; Sader, H.S. Bacterial resistance to antimicrobial agents in Latin America. *Infect. Dis. Clin. North Am.*, 14:67-81, 2000.
10. Hiramatsu, K.; Aritaka, N.; Hanaki, H.; Kawasaki, S.; Hosoda, Y.; Kobayashi, I. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet*., 350, 1670-1673, 1997.
11. Hiramatsu, K. Vancomycin resistance in staphylococci. *Drug Resist. Updat*., 1, 135-150, 1998.
12. Hiramatsu, K. Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. *Lancet Infect. Dis.*, 1, 147-155, 2001.
13. Laley, R.T.; Lanz, E.; Schrock, C.G.; Rapid control of an outbreak of *Staphylococcus aureus* on a neonatal intensive care department using standard infection control practices and nasal mupirocin, *Am. J. Infec. Control*., 32, 44-47, 2004.
14. Lutz, L.; Machado, A.; Kuplic, N.; Barth, A.L. Clinical failure of vancomicin treatment of *Staphylococcus aureus* infection in a tertiary care hospital in southern Brazil. *Braz. J. Infect. Dis.*, 7(3), 224-228, 2003.
15. Marchese, A.; Balistreri, G.; Torroli, E.; Schito, G.C. Heterogeneous vancomycin resistance in methicillin-resistant *Staphylococcus aureus* strains isolated in a large Italian hospital. *J. Clin. Microbiol.*, 38, 866-869, 2000.
16. Nunes, A.P.F. *Staphylococcus* coagulase-negativos: diferenciação em espécies, diversidade genômica de espécies resistentes à oxacilina e determinação da susceptibilidade à glicopeptídios. Rio de Janeiro, 2000, 97 p. (Master Thesis, Universidade Federal do Rio de Janeiro).
17. Oliveira, G.A.; Dell'Aquila, A.M., Masiero, R.L.; Levy, C.E.; Gomes, M.S.; Cui, L.; Hiramatsu, K.; Mamizuka, E.M. Isolation in Brazil of nosocomial *Staphylococcus aureus* with reduced susceptibility to vancomycin. *Infect. Control Hosp. Epidemiol.*, 22, 443-448, 2001.
18. Oteo, J.; Baquero, f.; Vindel, A.; Campos, J. Antibiotic rsistance in 3113 blood isolates of *Staphylococcus aureus* in 40 Spanish hospitals participating in the European Antimicrobial Resistance Surveillance System (2000-2002). *J. Antimicrob. Chemoth.*, 53, 1033-1038, 2004.
19. Sadoyama, G.; Gontijo Filho, P.P. Risk factors for methicillin resistant and sensitive *Staphylococcus aureus* infection in a Brazilian university hospital. *Braz. J. Infect. Dis.*, 4, 135-143, 2000.
20. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular cloning: a Laboratory Manual*. CSH Laboratory Press, 1989.
21. Santos, L.R.; Nascimento, V.P.; Oliveira, S.D.; Flores, M.L.; Pontes, A.P.; Salle, C.T.P.; Lopes, R.F.F. Polymerase chain reaction (PCR) for the detection of *Salmonella* in artificially inoculated chicken meat. *Rev. Inst. Med. Trop. Sao Paulo.*, 43, 247-250, 2001.
22. Srinivasan, A.; Dick, J.D.; Perl, T.M. Vancomycin resistance in staphylococci. *Clin. Microbiol. Rev.*, 15, 430-438, 2002.
23. Tenover, F.C. VRSA, VISA, and GISA: the dilemma behind the name game. *Clin. Microbiol. Newsl*., 22, 49-52, 2000.
24. Welsh, J.; McClelland, M. Fingerprint genomes using PCR with arbitrary primers. *Nucleic Acids Res.*, 18, 7213-7218, 1990.
25. Wey, S.B. Infection control in a country with annual inflation of 3,600%. *Infect. Control Hosp. Epidemiol.*, 16, 175-178, 1995.