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ARTIGO ORIGINAL

Características da constipação funcional em crianças de zero a doze anos atendidas em um ambulatório de gastroenterologia pediátrica

Characteristics of functional constipation in children from zero to twelve years old attended in a pediatric gastroenterology outpatient clinic

Características del estreñimiento funcional en niños de cero a doce años atendidos en ambulatorio de gastroenterología pediátrica

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RESUMO

Justificativa e Objetivos: *Staphylococcus aureus* resistente à meticilina (MRSA) é uma das causas mais frequentes de infecções relacionadas à assistência à saúde e comunitárias, e com seu avanço, a vancomicina tornou-se a principal opção terapêutica. Entretanto, o seu uso indiscriminado favoreceu o surgimento de MRSA com reduzida suscetibilidade à vancomicina, comumente associados com falhas no tratamento, bacteremia persistente, hospitalização prolongada e desfechos clínicos adversos. Este estudo avaliou a ocorrência de MRSA com reduzida suscetibilidade à vancomicina e determinou algumas características moleculares em comparação com MRSA suscetível à vancomicina (VS-MRSA). **Métodos:** Determinação do perfil de suscetibilidade aos antimicrobianos, a concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM) para vancomicina, tolerância à vancomicina, tipagem do SCC*mec* e *agr* foram realizadas em um total de 177 MRSA. Posteriormente, foram triados para hVISA por BHIA-3V e BHIA-6V e confirmados com a Análise do Perfil Populacional - Área Abaixo da Curva (PAP-AUC). **Resultados:** Os fenótipos VT-MRSA e hVISA foram encontrados em 13,6% e 5,1% dos isolados clínicos de MRSA, respectivamente, e a presença de hVISA foi estatisticamente significativa entre os isolados de VT-MRSA (p<0,05). Em VT-MRSA, SCCmec tipo II foi significativamente mais frequente do que em não-VT-MRSA, assim como a presença do agr grupo II. **Conclusão:** Características moleculares encontradas em MRSA são importantes para a epidemiologia, bem como para demonstrar um padrão em isolados com reduzida suscetibilidade à vancomicina. Testes não-convencionais para detecção destas características podem ser realizados para evitar a identificação errada de VS-MRSA que, consequentemente, resulta em falhas no tratamento com vancomicina.
Descritores: *agr. hVISA. SCCmec. VISA. VT-MRSA.*

ABSTRACT

Background and Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most frequent causes of healthcare-associated and community-acquired infections and with its advancement, vancomycin became the main therapeutic option. However, its indiscriminate use favored the emergence of MRSA with reduced susceptibility to vancomycin, commonly associated with vancomycin treatment failure, persistent bacteremia, prolonged hospitalization and adverse clinical outcome. This study evaluated the occurrence of MRSA with

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reduced vancomycin susceptibility and determined some molecular characteristics in comparison with vancomycin-susceptible MRSA (VS-MRSA). **Methods:** Determination of antimicrobial susceptibility profile, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for vancomycin, vancomycin-tolerance, SCC*mec* and *agr* typing were performed in a total of 177 MRSA. Thereafter, they were screened for hVISA by BHIA-3V and BHIA-6V and confirmed with population analysis profile - area under the curve method (PAP-AUC). **Results:** VT-MRSA and hVISA phenotypes were found in 13.6% and 5.1% of clinical isolates of MRSA, respectively, and the presence of hVISA was statistically significant among VT-MRSA isolates ($p<0.05$). In VT-MRSA, SCCmec type II was significantly more frequent than in non-VT-MRSA, as well as the presence of *agr* group II. **Conclusion:** Molecular characteristics found in MRSA are important for epidemiology, as well as demonstrate a pattern in reduced vancomycin susceptibility isolates. Non-conventional tests for detection of these characteristics might be performed to prevent misidentification of VS-MRSA that, consequently, results in vancomycin treatment failures.
Keywords: *agr. hVISA. SCCmec. VISA. VT-MRSA.*

RESUMEN

Justificación y objetivos: *Staphylococcus aureus* resistente a la meticilina (MRSA) es una de las causas más frecuentes de infecciones relacionadas con la asistencia sanitaria y comunitarias, y con su avance, a la vancomicina se ha convertido en la principal opción terapéutica. Sin embargo, su uso indiscriminado favoreció el surgimiento de MRSA con reducida susceptibilidad a la vancomicina, comúnmente asociados con fallas en el tratamiento, bacteriemia persistente, hospitalización prolongada y resultados clínicos adversos. Este estudio evaluó la ocurrencia de MRSA con reducida susceptibilidad a la vancomicina y determinó algunas características moleculares en comparación con MRSA susceptible a la vancomicina (VS-MRSA). **Métodos:** Determinación del perfil de susceptibilidad a los antimicrobianos, la concentración inhibitoria mínima (CIM) y la concentración bactericida mínima (CBM) para vancomicina, tolerancia a la vancomicina, tipificación del SCC*mec* y *agr* se realizaron en un total de 177 MRSA. **Resultados:** Los fenotipos VT-MRSA y hVISA se encontraron en el 13,6% y el 5,1% de los aislados clínicos de MRSA, respectivamente, y la presencia de hVISA fue estadísticamente significativa entre los aislados de VT-MRSA ($p<0.05$). En VT-MRSA, SCCmec tipo II fue significativamente más frecuente que en no-VT-MRSA, así como la presencia del agr grupo II. **Conclusión:** Características moleculares encontradas en MRSA son importantes para la epidemiología, así como para demostrar un patrón en aislados con reducida susceptibilidad a la vancomicina. Pruebas no convencionales para la detección de estas características pueden realizarse para evitar la identificación errónea de VS-MRSA que, consecuentemente, resulta en fallas en el tratamiento con vancomicina.
Palabras Clave: *agr. hVISA. SCCmec. VISA. VT-MRSA*

INTRODUCTION

The pathogenesis of *Staphylococcus aureus* infections is complex and depends on the host characteristics, expression of virulence factors and ability to develop resistance to antimicrobials.[1,2] Methicillin-resistant *S. aureus* (MRSA) is related to an advancement of healthcare- and community-acquired infections, being vancomycin the primary therapeutic option for the last fifty years, even with the availability of antimicrobials as linezolid, tigecycline, and daptomycin. Infections caused by vancomycin-susceptible MRSA (VS-MRSA) have been associated with vancomycin treatment failures and, also, an increase in mortality.[3,4] Hence, its excessive and inappropriate use has led to the emergence of *S. aureus* with reduced vancomycin susceptibility, as vancomycin-intermediate *S. aureus* (VISA), heterogeneous VISA (hVISA) and afterwards, vancomycin-tolerant MRSA (VT-MRSA), last two not yet detected routinely.[5-8]

Infections caused by hVISA and VISA are commonly associated with prolonged hospitalization, persistent bacteremia, and adverse clinical outcome. Although the relevance of hVISA persists unclear, studies speculate that these isolates could be a precursor of VISA phenotype.[9-11] MRSA strains also develop vancomycin tolerance, whose infections are even more difficult to treat, especially when they are invasive and present in immunocompromised patients.[7] Additionally, this reduced vancomycin susceptibility is associated with dysfunction in the accessory gene regulator (*agr*) locus, a quorum sensing system that controls the expression of genes encoding virulence factors.[12,13]

In this context, to understand the epidemiology and some molecular characteristics of MRSA with reduced vancomycin susceptibility is required to assist in the antimicrobial therapy and control the spread of these multiresistant microorganism. This study was designed to determine the prevalence of SCC*mec* and *agr* types among MRSA isolates, and to evaluate the association of these molecular characteristics with reduced susceptibility to vancomycin.

METHODS

Ethics

The cross-sectional observational study was approved by the institutional Ethics Committee, with permit number 1.212.043. The Central Laboratories from Hospital Mãe de Deus (HMD) and Hospital Nossa Senhora da Conceição (HNSC) provided de MRSA isolates for this study, stored at -20°C in our sample collection. According to the institutional ethical and type of investigation assessment, informed consent was not required.

Clinical isolates of MRSA

A collection of 177 MRSA clinical isolates from

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distinct hospitalized patients were analyzed. They were isolated from respiratory tract 72 (40.7%), blood 40 (22.6%), skin and soft tissue 39 (22.0%), bone and connective tissue 10 (5.6%), medical devices 9 (5.1%), urine 5 (2.8%), and others sites 2 (1.1%). *S. aureus* identification was confirmed in the laboratory using conventional methods, as Gram staining, catalase activity, plasma coagulase production and growth on brain heart infusion agar (BHIA; Oxoid, Basingstoke, England) supplemented with 15% NaCl. Also, methicillin resistance was confirmed by conventional PCR for *mecA* gene and by the traditional Kirby-Bauer method using cefoxitin (DME, São Paulo, Brazil), according to Clinical and Laboratory Standard Institute (CLSI) guidelines.[14,15]

Antimicrobial susceptibility

Antimicrobial susceptibility tests were performed by disk diffusion method for clindamycin (2 µg), erythromycin (15 µg), levofloxacin (5 µg), rifampicin (5 µg), teicoplanin (30 µg), tetracycline (30 µg), and trimethoprim/sulfamethoxazole (1.25 µg/ 23.75 µg) (DME, São Paulo, Brazil), and vancomycin susceptibility was determined by microdilution method in Mueller-Hinton broth (MHB; Oxoid, Basingstoke, England), according to the CLSI guidelines.[15] *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 strain was used as a control, respectively.

Vancomycin tolerance was determined by the minimum bactericidal concentration (MBC)/minimum inhibitory concentration (MIC) ratio of ≥ 32 µg/mL, as described by Cázares-Domínguez et al.[7] *S. aureus* ATCC 29213 was used as a control.

Screening for hVISA

All MRSA were submitted to a screening test for hVISA in brain heart infusion agar (BHIA; Oxoid, Basingstoke, England) containing 3 µg/mL vancomycin (BHIA-3V)[16] and 6 µg/mL vancomycin (BHIA-6V).[15] Briefly, 10 µL of a bacterial suspension (1 x 10^8 CFU/mL) was spread onto BHIA-3V and incubated for 24h at 37°C. Cultures that showed growth after 24h w confirmed by Population Analysis Profile - Area Under the Curve (PAP-AUC). hVISA Mu3 (ATCC 700698) was used as a control.

Population Analysis Profile – Area Under the Curve method

PAP/AUC was performed as previously described by Huang et al.[11] Briefly, a 0.5 McFarland standard suspension from an overnight culture in trypticase soy agar (TSA; Oxoid, Basingstoke, England) was prepared. Serial dilutions (10^{-1} a 10^{-5}) were prepared in sterile saline, 10 µL from each dilution was spread onto BHI agar plates containing increasing concentrations of vancomycin (0 µg/mL, 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL, 6 µg/mL and 8 µg/mL). The BHI agar plates were incubated at 37 °C for 48 h and after that period, the number of Colony Forming Units (CFU) were counted. The value of the dilutions was corrected and the result was graphed as $\log_{10}$ CFU/ml versus the vancomycin concentration using software Excel® (Microsoft, Windows 7, Redmond, WA, EUA). The following strains VSSA (ATCC 29213), hVISA Mu3 (ATCC 700698) and VISA Mu50 (ATCC 700699) were included as controls. Finally, AUC of the tested isolate/AUC of Mu3 ratio was calculated and interpreted as follows: for VSSA, less than 0.9; for hVISA, from 0.9 to 1.3; and for VISA, greater than 1.3.

DNA

Extraction and molecular typing

All MRSA were cultured on blood agar and incubated overnight at 37°C. DNA was extracted using Chelex®100 (Bio-Rad, Richmond, CA) and Proteinase K (Sigma-Aldrich, Poole, UK). DNA samples were stored at -20°C until use. Multiplex PCR was used to determine the SCC*mec* types I-X (including subtypes IVa, IVb, IVc and IVd) of all MRSA, as previously described.[14, 17] NCTC 10442 (SCC*mec* I), N315 (SCC*mec* II), 85/2082 (SCC*mec* III), JCSC 4474 (SCC*mec* IVa), JCSC 2172 (SCC*mec* IVb), JCSC 4488 (SCC*mec* IVc), JCSC 4469 (SCC*mec* IVd), WIS (SCC*mec* V), HDE 288 (SCC*mec* VI), JCSC 6082 (SCC*mec* VII), JCSC 6943 (SCC*mec* IX) and JCSC 6945 (SCC*mec* X) were included as controls. The *agr* typing was performed by multiplex PCR according to the method published by Wu et al.[18] *S. aureus* COL (*agr* I), *S. aureus* N315 (*agr* II), *S. aureus* ATCC 25923 (*agr* III) and *S. aureus* A920210 (*agr* IV) were included as controls.

Statistical analysis

Statistical analyzes were determined using SPSS version 23.0 software (SPSS, Chicago, IL, USA). Chi-square test or Fisher's exact test for categorical variables were used to calculate *p* values. A *p* value of < 0.05 was considered as statistically significant.

RESULTS

Antimicrobial susceptibility

Antimicrobial resistance profile showed lower levels of tetracycline resistance (22.0%), followed by trimethoprim/sulfamethoxazole (27.1%) and rifampicin (31.1%), among all 177 MRSA. On the other hand, higher resistance levels were observed for levofloxacin (49.7%), clindamycin (64.4%) and erythromycin (77.4%). Eighty-eight (49.7%) MRSA presented multidrug resistance (MDR, resistant to three or more antimicrobial classes). Teicoplanin resistance was not found among MRSA isolates.

All isolates were susceptible to vancomycin, with MIC values of 1 µg/mL (n=142; 80.2%) and 2 µg/mL (n=35; 19.8%). Screening for hVISA reveals that 67 (37.8%) isolates showed growth in BHIA-3V and, of these, only four were capable of growing in BHIA-6V. Population analysis profile confirmed that 9 (5.1%) were hVISA, and VISA phenotype was not detected by PAP-AUC (Figure 1). The phenotype VT-MRSA was found in 24 (13.6%) of MRSA isolates and all hVISA isolates were also classified as VT-MRSA (p<0.001) (Table 1).

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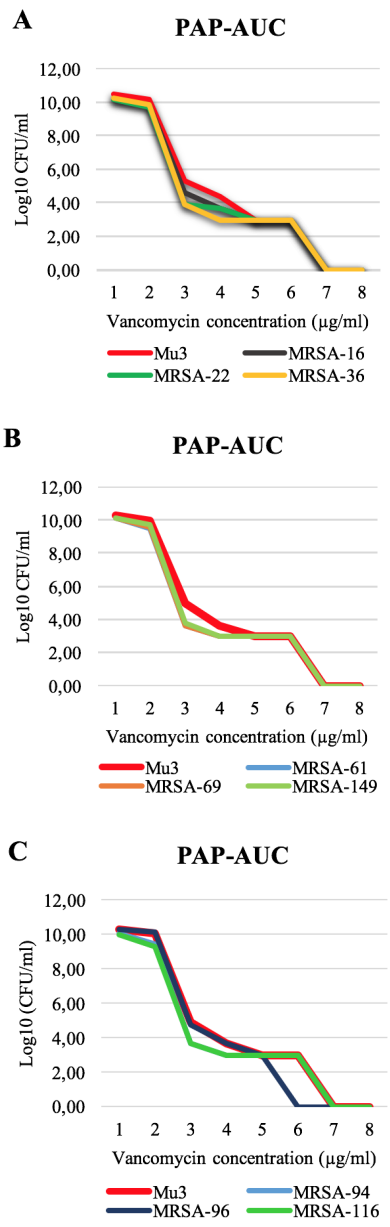

Figure 1. Growth curves of the nine isolates characterized as hVISA compared to the standard strain Mu3, by PAP-AUC method. All isolates demonstrated a ratio greater than 0.9. Graphs A, B, and C illustrate all hVISA isolates; the reference strain Mu3 is shown in all graphs.

Table 1. Characteristics of VT-MRSA and PAP-AUC confirmation.

Isolate	MIC	MBC	VT-MRSA	BHIA-3V/6V	PAP-AUC
MRSA-05	1	32	+	+	
MRSA-16	2	64	+	+/+	+
MRSA-22	2	64	+	+	+
MRSA-25	2	64	+	+	
MRSA-30	1	32	+	+	
MRSA-36	1	32	+	+/+	+
MRSA-46	1	32	+	+	
MRSA-61	1	64	+	+/+	+
MRSA-65	1	32	+	+	
MRSA-69	1	32	+	+	+
MRSA-73	2	64	+	+	
MRSA-91	1	32	+	+	
MRSA-94	1	32	+	+	+
MRSA-96	1	32	+	+	+
MRSA-106	1	32	+	+	
MRSA-116	2	64	+	+/+	+
MRSA-117	1	32	+	+	
MRSA-122	1	32	+	+	
MRSA-127	1	32	+	+	
MRSA-133	1	32	+	+	
MRSA-140	1	32	+	+	
MRSA-149	1	32	+	+	+
MRSA-163	1	32	+	+	
MRSA-166	2	64	+	+	

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; VT-MRSA = vancomycin tolerant methicillin-resistant Staphylococcus aureus; BHIA-3V/6V = BHIA with 3 or 6 µg/mL vancomycin; PAP-AUC = population analysis profile – area under the curve.

Significant differences in antimicrobial susceptibility pattern were observed between MRSA and VT-MRSA: all antimicrobial tested, except teicoplanin, demonstrated higher resistance rates in VT-MRSA when compared to MRSA. Also, hVISA demonstrated significantly higher resistance levels to rifampicin, trimethoprim/sulfamethoxazole and tetracycline than non-hVISA (Table 2). One of the possible reasons we could not find a significant difference in hVISA resistance levels for some antimicrobials is the low prevalence of these isolates in the study.

Table 2. Antimicrobial resistance rates of VT-MRSA and hVISA among MRSA isolates.

Antimicrobial agents	MRSA (n=153)	VT-MRSA (n=24)	*p**	Non-hVISA (n=168)	hVISA (n=9)	*p**
Clindamycin	91 (59.5%)	23 (95.8%)	0.002	106 (63.1%)	8 (88.9%)	0.133
Erythromycin	113 (73.9%)	24 (100.0%)	0.003	128 (76.2%)	9 (100.0%)	0.096
Levofloxacin	68 (44.4%)	20 (83.3%)	0.003	80 (47.6%)	8 (88.9%)	0.053
Rifampicin	37 (24.2%)	18 (75.0%)	<0.001	47 (28.0%)	8 (88.9%)	<0.001
Trimethoprim/sulfamethoxazole	30 (19.6%)	18 (75.0%)	<0.001	40 (23.8%)	8 (88.9%)	<0.001
Tetracycline	22 (14.4%)	17 (70.8%)	<0.001	31 (18.5%)	7 (77.8%)	<0.001

MRSA = methicillin-resistant Staphylococcus aureus; VT-MRSA = vancomycin tolerant methicillin-resistant Staphylococcus aureus; hVISA = heterogeneous vancomycin-intermediate Staphylococcus aureus. Number of isolates (percentage).
**Chi-square test or Fisher's exact test.*

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Molecular characteristics

SCC*mec* and *agr* typing are presented in Table 3. None of the isolates showed SCC*mec* types VII and X, and the prevalence of SCC*mec* IV subtypes was: 21 IVa, 2 IVb, 28 IVc and 6 IVd. Among VT-MRSA, the SCC*mec* type II was the most frequent followed by SCC*mec* type III. The presence of SCC*mec* type II in VT-MRSA was significantly higher than in non-VT-MRSA (p=0.025). Among hVISA isolates, the SCC*mec* type III was the most frequent followed by SCC*mec* II. There was no significant difference in the presence of SCC*mec* between hVISA and non-hVISA.

The majority of MRSA belonged to *agr* group II, followed by *agr* group I and III. *agr* group IV was not detected. Twenty-one isolates were untypeable by our assay. The presence of *agr* group II was significant in VT-MRSA (p=0.032) and hVISA (p=0.046) (Table 3).

Table 3. Molecular typing at the MRSA, VT-MRSA, and hVISA isolates.

Molecular typing	MRSA (n=177)	VT-MRSA (n= 24)	hVISA (n=9)*
SCCmec type			
I	21 (11.9%)	3 (12.5%)	1 (11.1%)
II	36 (20.3%)	10 (41.7%)	2 (22.2%)
III	54 (30.5%)	5 (20.8%)	5 (55.6%)
IV	49 (27.7%)	4 (16.7%)	1 (11.1%)
V	6 (3.4%)	2 (8.3%)	-
VI	1 (0.6%)	-	-
IX	1 (0.6%)	-	-
2/ +	9 (5.1%)	-	-
agr group			
I	48 (27.1%)	7 (29.2%)	2 (22.2%)
II	62 (35.0%)	14 (58.3%)	6 (66.6%)
III	39 (22.0%)	3 (12.5%)	1 (11.1%)
2/+	7 (4.0%)	-	-
NT	21 (11.9%)	-	-

MRSA = methicillin-resistant Staphylococcus aureus; VT-MRSA = vancomycin tolerant methicillin-resistant Staphylococcus aureus; hVISA = heterogeneous vancomycin--intermediate Staphylococcus aureus; SCCmec = staphylococcal cassette chromosome mec; agr = accessory gene regulator. Number of isolates (percentage).
**All hVISA isolates were also classified as VT-MRSA.*

DISCUSSION

Although vancomycin remains the main therapeutic option for the treatment of MRSA infections, this therapeutic management might be reconsidered regarding epidemiological studies worldwide. In our results, almost 15% of the MRSA isolates presented tolerance to vancomycin, which is in accordance with findings from other studies.[7, 19] The selection of VT-MRSA strains occurs likely due to exposure to suboptimal concentrations of vancomycin, which explains the development of hVISA and VISA strains and the association between VT-MRSA and hVISA.[3,7,19]

In this study, all MRSA were susceptible to vancomycin, with a MIC range of 1-2 µg/mL. Other studies have reported that infections caused by VS-MRSA have been associated with the increase in vancomycin treatment failures and mortality, particularly those isolates with MICs of 1.5 or 2 µg/mL.[3,4] Around 5% of MRSA isolates presented the hVISA phenotype. In previous reports, the frequency of the hVISA has ranged from 0 to 22.1% of MRSA.[11,20,21]

Their prevalence among MRSA varies significantly with the geographic area, the source of clinical isolates, and detection methods.[11,21] SCC*mec* III was the most prevalent type among the MRSA strains, as observed in other studies, and types III and II were more prevalent among isolates with reduced susceptibility to vancomycin (hVISA and VT-MRSA), as also observed in previous studies.[7,11,20-22] Regarding *agr* typing, group II was more frequent among MRSA isolates, as already seen in other analysis.[8,13] Its was also high among VT-MRSA and hVISA, being associated with reduced vancomycin susceptibility.[12,23,24] Many VISA are highly enriched for the *agr* group II. In this study, VISA was not detected, but hVISA and VT-MRSA demonstrated the same molecular characteristic previously described for VISA.[7] The *agr* group II has been strongly associated with vancomycin treatment failures in MRSA bacteremia, as well as the thickened cell wall in VT-MRSA that harbors SCC*mec* type II and *agr* group II.[7,25] It suggests that the vancomycin resistance is adaptative and could be inducible by molecular characteristics.

In summary, our study detected the prevalence of SCC*mec* types II and III and *agr* group II in MRSA with reduced susceptibility to vancomycin and demonstrated that in fact, these isolates showed higher resistance levels to antimicrobials than non-VT-MRSA and non-hVISA isolates. Conventional tests to detect vancomycin susceptibility do not comprehend the PAP-AUC method, a time-consuming and labor-intensive technique, and this is the scenario that treatment failures by vancomycin may occur since isolates are underestimated due to routinely characterization as susceptible to vancomycin.

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