The Brazilian Journal of
INFECTIOUS DISEASES

www.elsevier.com/locate/bjid

Original article

Molecular epidemiology of heteroresistant vancomycin-intermediate *Staphylococcus aureus* in Brazil

Alessandro Conrado de Oliveira Silveira[a,b,*], *Gabriela Rosa da Cunha*[a], *Juliana Caierão*[a], *Caio Mauricio Mendes de Cordova*[b], *Pedro Alves d'Azevedo*[a]

[a] *Laboratório de Cocos Gram Positivos, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil*
[b] *Departamento de Ciências Farmacêuticas, Fundação Universidade Regional de Blumenau, Blumenau, SC, Brazil*

ARTICLE INFO

Article history:
Received 23 April 2015
Accepted 12 June 2015
Available online 21 August 2015

Keywords:
MRSA
Small colony variants
Antimicrobial resistance

ABSTRACT

To determine the epidemiological and molecular characteristics of 12 *Staphylococcus aureus* isolates presenting heteroresistance to vancomycin in laboratories of two cities in Santa Catarina, southern Brazil. Epidemiological data, including the city of isolation, health institution, and date of isolation were considered, as well as the associated clinical specimen. For molecular characterization, we analyzed the staphylococcal cassette chromosome types, the *erm* gene presence, and the genomic diversity of isolates using pulsed-field gel electrophoresis. The 12 isolates of *S. aureus* were previously confirmed as heteroresistance to vancomycin using the population analysis profile–area under curve. Regarding genetic variability, two clones were detected: the main one (clone A) composed of four isolates and the clones B, with two isolates. For clone A, two isolates presented identical band patterns and were related to the same hospital, with an interval of 57 days between their isolation. The other isolates of this clone showed no epidemiological link between them because they were isolated in different hospitals and had no temporal relationship. The other clone showed no detectable epidemiological relationship. The heteroresistance to vancomycin recovered in Santa Catarina State from 2009 to 2012 had, in general, heterogeneous genomic patterns based on pulsed-field gel electrophoresis results, which is in accordance with the fact that these isolates had little or no epidemiological relationship among them. Due to the characteristic phenotypic instability and often prolonged vancomycin therapy for selection, clonal spread is not as common as for other resistance mechanisms disseminated through horizontal gene transfer.

* *Corresponding author.*
E-mail address: alessandrocosilveira@gmail.com (A.C.O. Silveira).
http://dx.doi.org/10.1016/j.bjid.2015.06.013

Introduction

Staphylococcus aureus can acquire, during prolonged therapy with glycopeptides, a very peculiar resistant phenotype. Through selective pressure and apparently without transfer of genetic material, it may undergo mutations in genes responsible for cell wall production, making it thicker and less susceptible to the action of antimicrobials.[1]

The heteroresistance of *S. aureus* to vancomycin (hVISA) causes changes in the macro-morphological features of the colonies, that present with heterogeneous appearance and pigmentation, giving the impression of contamination and may confuse the microbiologist.[2] Its mechanism of resistance is associated with activation of cell wall synthesis, which increases the production of waste mucopeptide and reduces the amount of antibiotic that reaches the site of action, thus causing cell wall thickening and subsequent drug imprisonment.[3] Speculation that hVISA could be regarded as precursors of VISA strains once, after prolonged exposure to antimicrobial could select a homogenous population of cells expressing the phenotype VISA. This phenotype is unstable.[4]

The detection of infections caused by hVISA represents a challenge for microbiologists, since these strains are considered susceptible to vancomycin *in vitro* (minimum inhibitory concentration (MIC) $\leq$2 μg/mL), and therefore categorized as susceptible by the usual laboratory methods.[1] However, as it contains subpopulations 1 in each 10^6 bacterial cells, that can grow in the presence of 4 μg/mL of vancomycin, it may lead to treatment failure[5,6] with vancomycin,.

Reference methods used to evaluate susceptibility as broth microdilution, Etest and automated methods fail to detect hVISA. Because the phenotype is a heterogeneous phenomenon, reliable molecular markers to confirm this phenotype have not yet been found. Partly due to the small inoculum, relatively poor growth on Mueller-Hinton agar, or incubation for only 24 h. The inoculum size is critical to the detection of subpopulation of resistant cells; furthermore hVISA strains are notoriously slow growing, with cell walls thicker and pleomorphic unique characteristics, with colonies of varying sizes and nutritionally exacting.[7]

The population analysis profile–area under the curve (PAP–AUC) has been the most reliable and reproducible approach, being considered the gold-standard test for hVISA.[8] It was specifically designed for discriminating hVISA and VISA. It is a method of analysis of modified sub-populations using serial concentrations of vancomycin, in order to quantify the viable bacterial populations in such concentrations. It is a very laborious, expensive, and inappropriate for routine use in clinical laboratories.[9]

In a meta-analysis published in 2011, the rates of treatment failure (designated as persistent infection or bacteremia) related to hVISA isolates were two times more common than in infections caused by *S. aureus* susceptible to vancomycin (OR: 2.37, 95% CI: 1.53–3.67).[6] Therefore, an accurate and practical laboratory method for the detection of hVISA isolated in clinical practice is of increasing importance.[10]

Despite the controversy between studies regarding the association of hVISA and mortality, knowledge of the epidemiological profile is very important in assisting the clinician when choosing the appropriate antibacterial therapy. The objective of this study was to evaluate the phenotypic and molecular epidemiology characteristics of hVISA isolates in the state of Santa Catarina, Brazil.

Methods

Bacterial samples

We used 12 clinical isolates of hVISA obtained from various anatomical sites of patients in hospitals in Florianópolis and hospital in Blumenau, all located in the state of Santa Catarina in southern Brazil. Samples were collected from November 2009 to October 2012.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method, according to the recommendations and interpretive criteria of the Clinical and Laboratory Standards Institute.[11] We also performed the D test for the detection of inducible resistance to clindamycin. Disks of erythromycin and clindamycin were placed 26 mm apart, and a flattening of the inhibition zone indicated a positive test, which was reported as clindamycin resistance. Vancomycin MICs were determined by macrodilution method.[11]

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. The plates were incubated for 48 h, 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 the strain 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 must be greater or equal to 0.9 and non-hVISA isolates had a PAP–AUC < 0.9.[7,8]

*Multiplex PCR for the detection of the staphylococcal cassette chromosome (SCC*mec*)*

The SCC*mec* type was determined using the multiplex PCR method according to the protocol developed by Zhang et al. The amplicons that were formed had the following sizes: I (613 bp), II (398 bp), III (280 bp), IVa (776 bp), IVb (493 bp), IVc (200 bp), IVd (881 bp), and V (325 bp).[12,13]

PCR for erm gene detection

For isolates with positive results in the phenotypic test for inducible resistance to clindamycin, *erm* gene PCR amplification was performed according to the multiplex PCR protocol developed by Khan et al.[14] The PCR products (610 bp for *erm*A and 520 bp for *erm*C) were analyzed by electrophoresis through a 1.5% agarose gel.[14,15]