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Impact of mutations in hVISA isolates on decreased susceptibility to vancomycin, through population analyses profile – area under curve (PAP-AUC)

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ABSTRACT

We analyzed sequences of *gra*SR, *vra*SR, *wal*KR and *rpo*B genes in hVISA from Brazil. Five isolates showed mutations in at least one gene. *rpo*B H481N and *gra*S T224I were the most frequent mutations, followed by *gra*R D148Q and *wal*K A468T. Our study reinforces the heterogeneity of genetic patterns among hVISA.

The vancomycin Intermediate *S. aureus* (VISA) appears to result from an adaptive process accompanied by mutations in different genes (Alam et al. 2014; Saito et al. 2014) leading to a significant thickening of the cell wall (Cui et al., 2006a). However, as this adaptation involves a high biological cost, reduced susceptibility to vancomycin frequently occurs heterogeneously in a bacterial population – hVISA (Hiramatsu et al. 1997).

Although the exact mechanism and genetic events responsible for hVISA phenotype are not clearly understood, modifications in *gra*SR, *vra*SR, *wal*KR, which are regulatory genes of two-components systems, and in the gene encoding the RNA polymerase beta subunit (*rpo*B) are most frequently associated with this phenotype (Hafer et al. 2012;. Howden et al. 2014). Our study aimed to analyze mutations in these targets among *S. aureus* recovered from hospitals in Brazil.

Eight non-clonal hVISA isolates were obtained from clinical samples collected from hospitals of Santa Catarina State, Brazil (November 2009 to October 2012).

Sequencing was performed in MiSeq (Illumina, Inc.). Quality was evaluated by using the Phred/Phrap program. One error per 1000 bp was considered acceptable. Sequences were analyzed and compared using the *BioEdit Sequence Alignment Editor Program* version 7.2.5. The vancomycin susceptible *S. aureus* (VSSA) isolate and N315 strain (GenBank BA00018.3) were used as references. The sequences corresponding to each gene were also compared to other sequences in GenBank under the following accession numbers: AP002394.1 (Mu3), BA000017.4 (Mu50), CP009361.1 (ATCC 25293-VSSA) and CP002120 .1 (JKD6008-VISA).

To induce vancomycin resistance, isolates were inoculated in BHI broth for 330 minutes at 37 °C until the exponential growth phase. Then, 50 μL from each tube was transferred to BHI tubes containing 1 μg/mL of vancomycin and incubated for 24 h at 37 °C. Tubes that had an absorbance at 625 nm greater than 2 were considered to have reached the stationary growth phase. Tubes that did not reached this parameter were re-incubated at same conditions. Those presenting the required turbidity were added to BHI with 2 μg/mL vancomycin, and so on. Vancomycin Minimal Inhibitory Concentration (MIC) were checked to ensure resistant phenotypes.

The hVISA phenotype was confirmed by PAP-AUC (Silveira et al. 2014). We also determined the VAN PAP MIC, which is the concentration of vancomycin, in PAP analysis, that inhibits bacterial growth (populations $<10^2$ CFU/mL). A VAN PAP MIC ≥4 μg/mL is generally highly associated with therapeutic failure in bloodstream infections.

To determine the Minimum Bactericidal Concentration (MBC), 10 μL from the well corresponding to MIC were plated on blood agar to

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Table 1
Mutations found and phenotypic characteristics of hVISA isolates.

	Mutations					ST	MIC	Days	MVC	PAP-AUC		MBC	MBC/MIC
	graS	*graR*	*walk*	*vra*R	*rpo*B					before	after		
SI4	-	-	-	-	H481N	5	2	30	20	1,14	1,22	64	32*
SI11	-	-	-	-	-	5	2	33	16	0,99	1,05	128	64*
SI13	L26F T224I	-	A117V	-	-	6	2	35	16	1,19	1,37	64	64*
10	L26I T224I	V136I D148Q S207G	A468T	-	V731A Y737F	30	1	28	16	0,92	1,09	64	64*
36	-	-	-	-	-	105	2	30	16	1,02	1,1	64	32*
43	L26F I59L T224I	D148Q	R222K A468T	E59D	H481N	239	1	23	20	0,98	1,17	32	32*
69	-	-	-	-	H481N	5	2	28	20	0,93	0,99	64	32*
80	-	-	-	-	-	105	2	29	18	1,12	1,25	128	64*

ST = Sequence type; MIC = minimum inhibitory concentration (μg/mL); Days = time required to reach MVC; MVC (maximum vancomycin concentration) = highest vancomycin concentration tolerated (μg/mL); PAP-AUC = before and after exposure; MBC = minimum bactericidal concentration (μg/mL);* indicates isolates tolerant to vancomycin.
Neither isolates presented mutations in *vra*S or *wal*R.

observe bacterial growth. Isolates with MBC/MIC ratio ≥32 were considered tolerant to vancomycin (Cázares-Dominguez et al. 2015).

Also, isolates were inoculated in BHI with 4 μg/mL vancomycin and growth was evaluated at 24, 48, 72 and 96 hours, with macroscopic visualization of colonies and determination of MIC. Late-growth isolates in medium containing vancomycin, with MIC ≥8 μg/mL, were considered slow VISA – sVISA (Saito et al. 2014).

This study investigated some potential genetic targets that may be involved in hVISA phenotype (Table 1). Point mutations in genes *gra*SR, *vra*R, *wal*K and *rpoB* were found in five out of eight hVISA isolates. Neither *vra*S nor *wal*R were mutated in any of our isolates.

As expected, all hVISA were tolerant to vancomycin. Three isolates (SI11, 10 and 43) were characterized as sVISA (Table 2), demonstrating the presence of small or punctate colonies and a MIC ≥8 μg/mL after 96 hours of incubation.

We found substitutions in *gra*SR already described by other authors; some of which were also observed in VSSA isolates, suggesting they are not reliable markers of hVISA phenotype. Nevertheless, the *gra*R D148Q mutation has been described in previous studies as missense mutation characteristic of isolates with reduced susceptibility of vancomycin, especially in isolates with high MIC values (Doddangoudar et al. 2011). Our results reinforce those findings.

The VraSR is an important stimulator of the expression of enzymes involved in the biosynthesis of peptidoglycan (Mccallum et al. 2010). As Doddangoudar et al. (2011), we did not find any mutations in *vra*S. The E59D substitution in *vra*R, the most prevalent pattern of mutations in VISA isolates observed by Yoo et al. (2013), was seen in one of our hVISA.

The importance of WalKR as a regulator in bacterial cells is well established (Dubrac et al. 2008). Its role in the hVISA/VISA has been demonstrated by many authors around the world (Hafer et al. 2012; Howden et al. 2014; Mwangi et al., 2007, Shoji et al. 2011, Watanabe et al. 2011; Hiramatsu et al. 2014). We observed two hVISA with the *wal*K A468T and one with the *wal*K R222K substitution. No mutation was found in *wal*R gene, corroborating data obtained by Shoji et al. (2011), which may establish a secondary role of this gene in hVISA. To the best of our knowledge, the A117V substitution in *wal*K gene (isolate SI13) was not previously described and may not be specifically linked to hVISA phenotype.

Mutations in *rpoB* may change the cell transcriptional profile drastically (Saito et al. 2014). Hiramatsu et al. (2014) demonstrated that the most prevalent mutations among VISA occur in *rpo*B. Four hVISA in our study presented *rpo*B mutated. A study by Watanabe et al. (2011) showed that the *rpo*B H481N mutation was detected in 10 of 38 VISA isolates from various countries. Three isolates presented this mutation in our study; in two of them it was the only mutation found. The *rpo*B mutations V731A and Y737F (isolated 10), do not seem to have great influence on the heteroresistance to vancomycin as they have been described in VSSA and hVISA samples (Hiramatsu et al. 2014; Watanabe et al. 2011). Among isolates presenting the sVISA phenotype, two (10 and 43) had mutations in *rpo*B, corroborating with results described elsewhere (Katayama et al. 2017; Matuso et al., 2015; Saito et al. 2014).

The PAP ratio for our isolate ranged from 0.92 to 1.19 and showed no correlation with the number of mutations. Interestingly, the isolate with the highest number of mutations (#10) had the lowest PAP ratio.

The absence of mutations in some of our isolates is understandable and reinforces the participation of numerous regulatory genes in controlling cell wall synthesis. Our study reinforced the heterogeneity of genetic patterns among hVISA. Although studies indicate that regulatory genes *gra*SR, *vra*SR, *wal*KR and *rpo*B are more often involved in this phenotype, to establish a single molecular marker as a predictor of resistance remains a nightmare.

Table 2
Detection of the sVISA phenotype and CIMs according to the incubation time.

Isolate	sVISA	Colony	MIC			
			24 h	48 h	72 h	96 h
SI4	no	L	2	3	3	3
SI11	yes	P	2	4	8	12
SI13	no	L	2	4	4	4
10	yes	P	1	1	4	16
36	no	L	2	2	4	4
43	yes	S	1	2	4	8
69	no	L	2	4	4	4
80	no	L	2	3	3	4

sVISA = slow VISA; colony: macroscopic aspect of the colony after 48 hours of incubation; S = small; P = puntacte; L = large; MIC = minimum inhibitory concentration of vancomycin (microdilution).

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References

Alam T, Petit RA, Crispell RK, Thornton TA, Conneely KN, Jyang Y, et al. Dissecting vancomycin-intermediate resistance in *Staphylococcus aureus* using genome-wide association. Genome Biol Evol 2014;6(5):1174–85.

Cázares-Dominguez V, Cruz-Córdova A, Ochoa SA, Escalona G, Arellano-Galindo J, Rodríguez-Leviz A, et al. Vancomycin tolerant, methicillin-resistant *Staphylococcus aureus* reveals the effects of vancomycin on Cell Wall thickening. PLOS One 2015; 10(3), e0118791.

Doddangoudar VC, Boost MV, Tsang NSC, O'Dfbbonoghue MM. Tracking changes in the *vra*SR and *gra*SR two component regulatory systems during the development and loss of vancomycin non-susceptibility in a clinical isolate. Clin Microbiol Infect 2011;17(8):1268–72.

Dubrac S, Bisicchia P, Devine KM, Msadek T. A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. Mol Microbiol 2008;70:1307–22.

Hafer C, Lin Y, Kornblum J, Lowy FD, Uhlemann AC. Contribution of selected gene mutations to resistance in clinical isolates of vancomycin-intermediate *Staphylococcus aureus*. Antimicrob Agents Chemother 2012;56(11):5845–51.

Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother 1997;40:135–46.

Hiramatsu K, Kayayama Y, Matsuo M, Aiba Y, Saito M, Hishinuma T, et al. Vancomycin-intermediate resistance in *Staphylococcus aureus*. J Glob Antimicrob Resist 2014;2:213–24.

Howden BP, Peleg AY, Stinear TP. The evolution of vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogenous-VISA. Infect Genet Evol 2014;21:575–82.

Katayama Y, Azechi T, Miyazaki M, Takata T, Sekine M, Matsui H et al. Prevalence of slow-growth vancomycin nonsusceptibility in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2017;61(2)e00425–14.

Mccallum N, Berger-Bachi B, Senn MM. Regulation of antibiotic resistance in *Staphylococcus aureus*. Int J Med Microbiol 2010;300:118–29.

Saito M, et al. "Slow VISA", a novel phenotype of vancomycin resistance , found in vitro in heterogeneous vancomycin-intermediate *Staphylococcus aureus* strain Mu3. Antimicrob Agents Chemother 2014;58(9):5024–35.

Shoji M, Cui L, Iizuka R, Komoto A, Neoh HM, Watanabe Y, et al. walK and clpP mutations confer reduced vancomycin susceptibility in *Staphylococcus aureus*. Antimicrob Agents Chemother 2011;55(8):3870–81.

Silveira ACO, Sambrano GE, Paim TG, Caierão J, Cordova CM, d'Azevedo PA. Is prediffusion test an alternative to improve accuracy in screening hVISA strains and to detect susceptibility to glycopeptides/lipopeptides? Diagn Microbiol Infect Dis 2014;79:401–4.

Watanabe Y, Cui L, Katayama Y, Kozue K, Hiramatsu K. Impact of *rpo*B mutations on reduced vancomycin susceptibility in *Staphylococcus aureus*. J Clin Microbiol 2011;49(7):2680–4.

Yoo JI, Kim JW, Kang GS, Kim HS, Yoo JS, Lee YS. Prevalence of amino acid changes in the *yvq*F, *vra*SR, *gra*SR, and *tca*RAB genes from vancomycin intermediate resistant *Staphylococcus aureus*. J Microbiol 2013;51(2):160–5.