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Detection of heterogeneous vancomycin intermediate resistance in MRSA isolates from Latin America

Betsy E. Castro[1], Maritza Berrio[2], Monica L. Vargas[1], Lina P. Carvajal[1], Lina V. Millan[1], Rafael Rios[1], Angie K. Hernandez[1], Sandra Rincon[1], Paola Cubides[1], Erika Forero[1], An Dinh[3], Carlos Seas[4], Jose M. Munita[3,5,6], Cesar A. Arias[1,3,7–9], Jinnethe Reyes[1,3] and Lorena Diaz[1,3,6]*

[1]Molecular Genetics and Antimicrobial Resistance Unit, International Center for Microbial Genomics, Universidad El Bosque, Bogotá, Colombia; [2]Instituto Nacional de Salud, Bogotá, Colombia; [3]Center for Antimicrobial Resistance and Microbial Genomics, McGovern Medical School, University of Texas Health Science Center, Houston, Texas, USA; [4]Universidad Peruana Cayetano Heredia, Lima, Peru; [5]Genomics and Resistant Microbes (GeRM) Group, Clínica Alemana de Santiago, Universidad del Desarrollo School of Medicine, Santiago, Chile; [6]Millennium Initiative for Collaborative Research On Bacterial Resistance (MICROB-R), Santiago, Chile; [7]Division of Infectious Diseases, Department of Internal Medicine, McGovern Medical School, University of Texas Health Science Center, Houston, Texas, USA; [8]Department of Microbiology and Molecular Genetics, McGovern Medical School, University of Texas Health Science Center, Houston, Texas, USA; [9]Center for Infectious Diseases, School of Public Health, University of Texas McGovern Medical School at Houston, Houston, Texas, USA

*Corresponding author. E-mail: diazsandra@unbosque.edu.co

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Background: Vancomycin is a common first-line option for MRSA infections. The heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) phenotype is associated with therapeutic failure. However, hVISA isolates are usually reported as vancomycin susceptible by routine susceptibility testing procedures.

Objectives: To detect and characterize the hVISA phenotype in MRSA isolates causing infections in nine Latin American countries.

Methods: We evaluated a total of 1189 vancomycin-susceptible MRSA isolates recovered during 2006–08 and 2011–14. After an initial screening of hVISA using glycopeptide-supplemented agar strategies, the detection of hVISA was performed by Etest (GRD) and Macro-method (MET). Isolates deemed to be hVISA were subjected to population analysis profile/AUC (PAP/AUC) and WGS for further characterization. Finally, we interrogated alterations in predicted proteins associated with the development of the VISA phenotype in both hVISA and vancomycin-susceptible *S. aureus* (VSSA) genomes.

Results: A total of 39 MRSA isolates (3.3%) were classified as hVISA (1.4% and 5.6% in MRSA recovered from 2006–08 and 2011–14, respectively). Most of the hVISA strains (95%) belonged to clonal complex (CC) 5. Only 6/39 hVISA isolates were categorized as hVISA by PAP/AUC, with 6 other isolates close (0.87–0.89) to the cut-off (0.9). The majority of the 39 hVISA isolates exhibited the Leu-14→Ile (90%) and VraT Glu-156→Gly (90%) amino acid substitutions in WalK. Additionally, we identified 10 substitutions present only in hVISA isolates, involving WalK, VraS, RpoB and RpoC proteins.

Conclusions: The hVISA phenotype exhibits low frequency in Latin America. Amino acid substitutions in proteins involved in cell envelope homeostasis and RNA synthesis were commonly identified. Our results suggest that Etest-based methods are an important alternative for the detection of hVISA clinical isolates.

Introduction

MRSA is a major human pathogen causing a broad spectrum of diseases ranging from mild skin infections to life-threatening bacteraemia.[1] The emergence and dissemination of MRSA are considered to be serious public health concerns. Recent data on the prevalence of *Staphylococcus aureus* bacteraemia in nine Latin American countries showed a highly heterogeneous prevalence of MRSA ranging from 22% to 62%.[2] Glycopeptides, particularly vancomycin, have been the treatment of choice for MRSA infections for decades. Unfortunately, the increasing rates of MRSA

infections and excessive use of vancomycin might have influenced the emergence of bacterial isolates with reduced susceptibility to this antibiotic.[3]

Decreased susceptibility to vancomycin was first identified in 1997, leading to therapeutic failure.[4,5] In 2002, the first *S. aureus* isolate with high-level resistance to vancomycin (VRSA) was reported in the USA and since then other cases have been described, mostly in the USA[6] and Brazil.[7,8] The VRSA phenotype is due to acquisition of the *vanA* cluster,[9,10] which is readily detected by conventional MIC methods.[11] In contrast, the mechanisms responsible for vancomycin-intermediate *S. aureus* (VISA) are complex and multifactorial, converging in a phenotype that is associated with thickening of the cell wall. Some of the most frequent genetic changes in VISA isolates are found in genes encoding cell envelope regulatory systems (*vraTSR, graSR* and *walKR*), membrane-associated (*tcaA*) and capsular polysaccharide (*capP*) proteins and those encoding the β subunit of the RNA polymerase (*rpoB* and *rpoC*).[12,13] Other phenotypic alterations observed in VISA strains include slow growth rate, high capsular expression, reduced autolysis and low peptidoglycan cross-linking, among others.[14] Of note, some of these phenotypic changes, particularly those associated with cell-wall thickening and changes in cell-wall electric charge,[15] may also affect other classes of antibiotics such as the lipopeptide daptomycin.[16]

Another distinct phenotype found in clinical isolates is the heterogeneous VISA (hVISA) phenotype. These isolates are classified as susceptible (vancomycin MICs $\leq$2 mg/L) through standard methodologies.[11] hVISA isolates contain cellular subpopulations (10^5 to 10^6 cfu/mL) with a vancomycin MIC within the intermediate range (4–8 mg/L) that are not detected by standard susceptibility methods such as automated methods, broth microdilution or Etest.[14] Due to the lack of detection of hVISA, its clinical impact remains poorly understood. However, studies have shown that infections caused by hVISA strains enhance the risk of therapeutic failure and carry higher hospital costs due to longer hospital stays compared with subjects infected with vancomycin-susceptible *S. aureus* strains (VSSA).[17–19]

Different methodologies have been proposed for detection of hVISA. Agar supplemented with glycopeptides (vancomycin or teicoplanin) may result in many false positives due to low specificity (53%–65%).[14] Additionally, Etest-based approaches, including the glycopeptide resistance detection (GRD) test and Etest macro method (MET) could facilitate detection of hVISA. However, these are not recommended by CLSI or EUCAST and are not usually implemented as routine techniques in clinical laboratories.[11,20,21] Currently, the most reliable method for hVISA detection is population analysis profile/AUC (PAP/AUC). This method detects and quantifies subpopulations able to grow at higher vancomycin concentrations when compared with the hVISA reference strain Mu3.[14,22] Unfortunately, PAP/AUC is cumbersome and expensive and it is not suitable to be implemented in a clinical laboratory as a routine diagnostic technique.

Previous reports suggest a general prevalence of hVISA from 0.2% up to 19.5%[23–25] in Latin America, although the actual prevalence of hVISA is unknown in most countries. During 2009–10, Argentina reported an hVISA prevalence of 3.3% in *S. aureus* causing bacteraemia in a teaching hospital.[26] Further, sporadic characterization of hVISA strains has been reported in isolates recovered from Chile, Venezuela and Brazil.[27–30] Here, using a large collection of bloodstream *S. aureus* isolates from patients in nine Latin American countries, we sought to identify the frequency of hVISA isolates. Furthermore, we developed a genomic framework to identify genetic features likely to be associated with the hVISA phenotype in these isolates.

Materials and methods

Bacterial isolates

From a total of 2755 *S. aureus* clinical isolates that were collected in two multicentre surveillance studies performed in Latin America (2006–08 and 2011–14), we included 1189 isolates confirmed as MRSA both phenotypically and molecularly. In the first study, 1570 *S. aureus* isolates causing infections in adult patients in 32 hospitals from Colombia, Ecuador, Peru and Venezuela were collected between 2006 and 2008, with 651 (41.5%) identified as MRSA.[31] In the second study, 1185 *S. aureus* isolates were recovered from patients with bacteraemia in 24 hospitals from Argentina, Brazil, Chile, Colombia, Ecuador, Guatemala, Mexico, Peru and Venezuela.[2] Of these, 538 (45%) were MRSA. Confirmation of *S. aureus* species and resistance to β-lactam antibiotics mediated by *mecA* was performed by multiplex PCR.[32] Antimicrobial susceptibility profiles were determined by the agar dilution method following CLSI recommendations.[11] The genetic relationships and MRSA lineages were determined by PFGE and MLST, as previously described.[33,34]

Screening for the VISA and hVISA phenotypes

We used brain heart infusion (BHI) agar supplemented with vancomycin 6 mg/L (BHI-V6) to screen for VRSA strains, as recommended by CLSI.[11] For VISA detection, we used previously described screening approaches.[14,35] Briefly, BHI agar with vancomycin 4 mg/L (BHI-V4) was inoculated with 10 μL of a 0.5 McFarland bacterial suspension. Simultaneously, Mueller–Hinton (MH) agar supplemented with teicoplanin 5 mg/L (MH-T5) was inoculated with 10 μL of bacterial suspension, using a 2.0 McFarland inoculum. Cultures were read at 48 h. A positive result was defined as the growth of $\geq$2 colonies on the agar.

GRD and MET methods

All isolates positive by at least one of the initial screening tests were further evaluated with the GRD strip. Positive isolates were then tested by MET. For GRD, an inoculum was prepared from an overnight culture in BHI broth. A bacterial suspension equivalent to a 0.5 McFarland standard was plated onto MH agar supplemented with 5% sheep blood and a double-ended Etest strip (bioMérieux® and Liofilchem®) with vancomycin and teicoplanin (0.5–32/0.5–32 mg/L) was applied. Plates were incubated at 37°C for 48 h.[36] For MET, 200 μL of a bacterial inoculum equivalent to 2.0 McFarland was plated onto BHI agar. Vancomycin and teicoplanin Etest strips (bioMérieux®) were applied and plates were incubated at 37°C for 48 h. A positive result was defined as the presence of any growth at concentrations of $\geq$8 mg/L for one or both antibiotics (vancomycin and teicoplanin).[36] *S. aureus* ATCC 700698 (Mu3; hVISA), ATCC 700699 (Mu50; VISA) and ATCC 29213 (VSSA) were used as control strains.

PAP/AUC

All GRD- and MET-positive isolates were subsequently tested by PAP/AUC using the microdilution method, as described previously.[22,37] In brief, a bacterial suspension equivalent to approximately 5.0×10^7 cfu/mL from an overnight culture was prepared. Serial dilutions (10^{-1} to 10^{-7}) were plated on BHI agar containing increasing concentrations of vancomycin (0 to 12 mg/L). To test the isolates from the second surveillance, the methodology was modified slightly by reducing the volume of the bacterial

suspension from 1 mL to 0.1 mL.[37] After 48 h of incubation at 37°C, cfu were manually counted and results were determined for each vancomycin concentration and expressed in $\log_{10}$ cfu/mL. This process was performed in triplicate for each strain. We also determined the AUC for each strain based on the average $\log_{10}$ cfu/mL at each concentration of vancomycin using the trapezoidal method. The PAP/AUC ratio was calculated as AUC_{strain}/AUC_{Mu3}. Ratios of 0.9 to 1.3 were considered as compatible with the hVISA phenotype and those >1.3 deemed compatible with a VISA strain.[22]

WGS, in silico typing and phylogenetics

A total of 39 MRSA isolates categorized as hVISA by GRD and MET ($n = 39$) and 305 vancomycin-susceptible MRSA were sequenced. Briefly, genomic DNA was extracted from overnight cultures using the DNeasy® Blood & Tissue kit (QIAGEN) after 30 min of lysostaphin treatment at 37°C. DNA libraries were prepared using Nextera XT DNA sample preparation (Illumina®, San Diego, CA, USA), according to the manufacturer recommendations. Raw reads were trimmed at their ends if their quality was below a Phred score of 30 using Trimmomatic[38] and *de novo* assembled using SPAdes v3.13.[39] SCC*mec* typing was performed *in silico* using reported primers.[40,41] The type of *agr* was determined using the reference sequences for *agr-I* (X52543.1), *agr-II* (AF001782.1), *agr-III* (AF001783.1) and *agr-IV* (AF288215.1). The resistome was determined using the ResFinder database[42] and mutations associated with resistance to fluoroquinolones (GyrA WP_000819088.1 and GrlA WP_000255586.1), rifampicin (RpoB CAG42275.1) and linezolid (G2576T in DNA coding the 23S rRNA and changes in RplC YP_000979/RplD YP_500978) were identified using the NCBI BLASTX tool.[43] Genome annotation was performed using the RAST server.[44] Sequencing data of the hVISA and VSSA isolates from this study are available in NCBI BioProjects: PRJNA595928, PRJNA291213, PRJNA393041 and PRJNA247399 (see Table S1, available as Supplementary data at *JAC* Online).

The genetic relationship of the hVISA isolates was determined by a phylogenetic tree based on the core genome from the sequenced genomes, as well as the genome sequences of *S. aureus* FPR3757, N315, Mu3, Mu50 and ATCC 29213 (NCBI GenBank accession numbers: NC_007793.1, NC_002745.2, NC_009782.1, NC_002758.2 and LHUS 02000001.1, respectively). Core genome was calculated with Roary.[45] The nucleotide sequences of each of the orthogroups defined in the core genome were aligned using MUSCLE[46] and these alignments were further concatenated to obtain a phylogenetic matrix. The matrix was used in RAxML[47] version 8.2.9 to reconstruct phylogeny of these strains, with a GAMMA model of rate heterogeneity selecting the best tree from 20 different runs and 1000 bootstrap resampling. The trees were edited using the interactive tree of life (iTol) tool (https://itol.embl.de/).[48]

Detection of changes potentially associated with VISA phenotype

Amino acid substitutions were identified in the sequences of WalKR (Q7A8E0.1 and Q7A4R9.1), VraTSR (OBY00462.1, Q99SZ7.1 and Q7A4R9.1), GraSR (Q7A6Z3.1 and Q99VW2.1), RpoB (BAB41731.1), RpoC (BAB41732.1), TcaA (BAB43448.1) and CapP (BAB41379.1) predicted proteins, which have been associated with the VISA phenotype. Amino acid sequences were compared through multiple sequence alignments using MUSCLE.[46] We included the genomes of VSSA strains ATCC 29213, N315 and FPR3757, as well as the hVISA and VISA prototypical isolates Mu3 and Mu50, respectively. An amino acid change was defined as the presence of a residue in a specific position that was different from those occurring in ATCC 29213, N315 and FPR3757. The chi-squared test was used to evaluate statistically significant differences between hVISA and VSSA isolates harbouring amino acid changes, using a significance level of 95% and the R programming language.[49]

Ethics

Ethics approval was obtained from the Ethics Review Committee, Universidad El Bosque (UEB 410-2016), Acta No. 012-2016.

Results

Low frequency of hVISA phenotype among clinical Latin American MRSA isolates

We defined hVISA as *S. aureus* isolates that were positive by both agar screening tests (BHI-V4 and/or MH-T5) and Etest-based methods (both GRD and MET). No VRSA were found in our collection using the BHI-V6 screening test. Additionally, we found that 43% of the isolates were positive using BHI-V4 and/or MH-T5. Table 1 shows the frequency of hVISA isolates in the two surveillance periods. In the initial surveillance period (2006–08), a total of 651 MRSA isolates were included and 27%, 26% and 8% were obtained from blood, surgical wounds and skin and soft tissue infections, respectively. Only 9 isolates (1.4%) were considered to be hVISA (by GRD and MET) (Table 1). In the second surveillance, which included bloodstream isolates only, (70% of which were collected from patients who received glycopeptide therapy), 30 (5.6%) MRSA were deemed to be hVISA (by GRD and MET). Interestingly, 25 of the 30 isolates (83%) were recovered from hospitals in Chile and Peru, which have a high prevalence of the clonal complex (CC)5 Chilean/Cordobes clone[2] (see below). Among the 39 hVISA MRSA, the majority (34; 87.2%) were obtained from blood, while 3 (7.6%) were recovered from bronchial aspirates, 1 (2.6%) from pleural fluid and 1 (2.6%) from a surgical wound.

The 39 hVISA isolates were subsequently examined by the PAP/AUC methodology.[22,37] Using this approach, only 6 (0.5%) isolates were confirmed as hVISA (PAP/AUC ratios between 1.03 and 1.19) (Figure 1). Of note, 6 (0.5%) other isolates exhibited a ratio between 0.87 and 0.89 when compared with Mu3, a value close to the lower limit of detection for hVISA (0.9) (Figure 2). These latter isolates exhibited high cfu counts ($>1\times10^6$ cfu/mL) at a vancomycin concentration of 1.5 mg/L (data not shown).

Table 1. Prevalence of hVISA phenotype (detected by GRD and MET) in Latin American MRSA isolates

	2006–08[a]		2011–14[a]	
Country	MRSA (*n*)	hVISA, *n* (%)	MRSA (*n*)	hVISA, *n* (%)
Brazil	—	—	126	1 (0.8)
Peru	178	6 (3.4)	84	16 (19.0)
Chile	—	—	74	9 (12.2)
Guatemala	—	—	74	ND
Argentina	—	—	60	3 (5.0)
Colombia	318	2 (0.6)	41	ND
Venezuela	69	—	33	ND
Ecuador	86	1 (1.2)	29	1 (3.4)
Mexico	—	—	17	ND
Total	651	9 (1.4)	538	30 (5.6)

ND, not determined.
[a]Period of time of multicentre study surveillance.

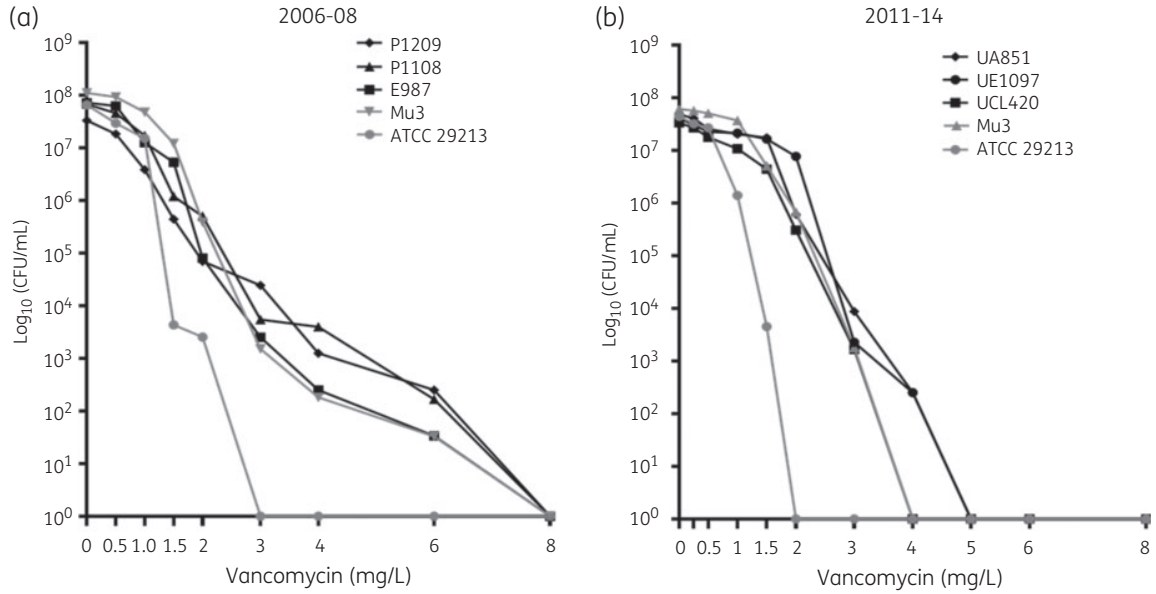


Figure 1. PAP of isolates confirmed as hVISA by PAP/AUC using vancomycin. cfu determinations at increasing vancomycin concentrations are shown. Results are representative of three independent experiments. Strains from the study with AUC ratio higher than 0.9 when compared with the AUC of *S. aureus* Mu3 are shown in black. Control strains of hVISA (ATCC 700698; Mu3) and VSSA (ATCC 29213) are represented by triangles and grey circles, respectively. Panels (a) and (b) include the results of strains collected during 2006–08 and 2011–14, respectively, which were performed by different methodologies.

MRSA isolates exhibiting the hVISA phenotype belong to CC5 lineage

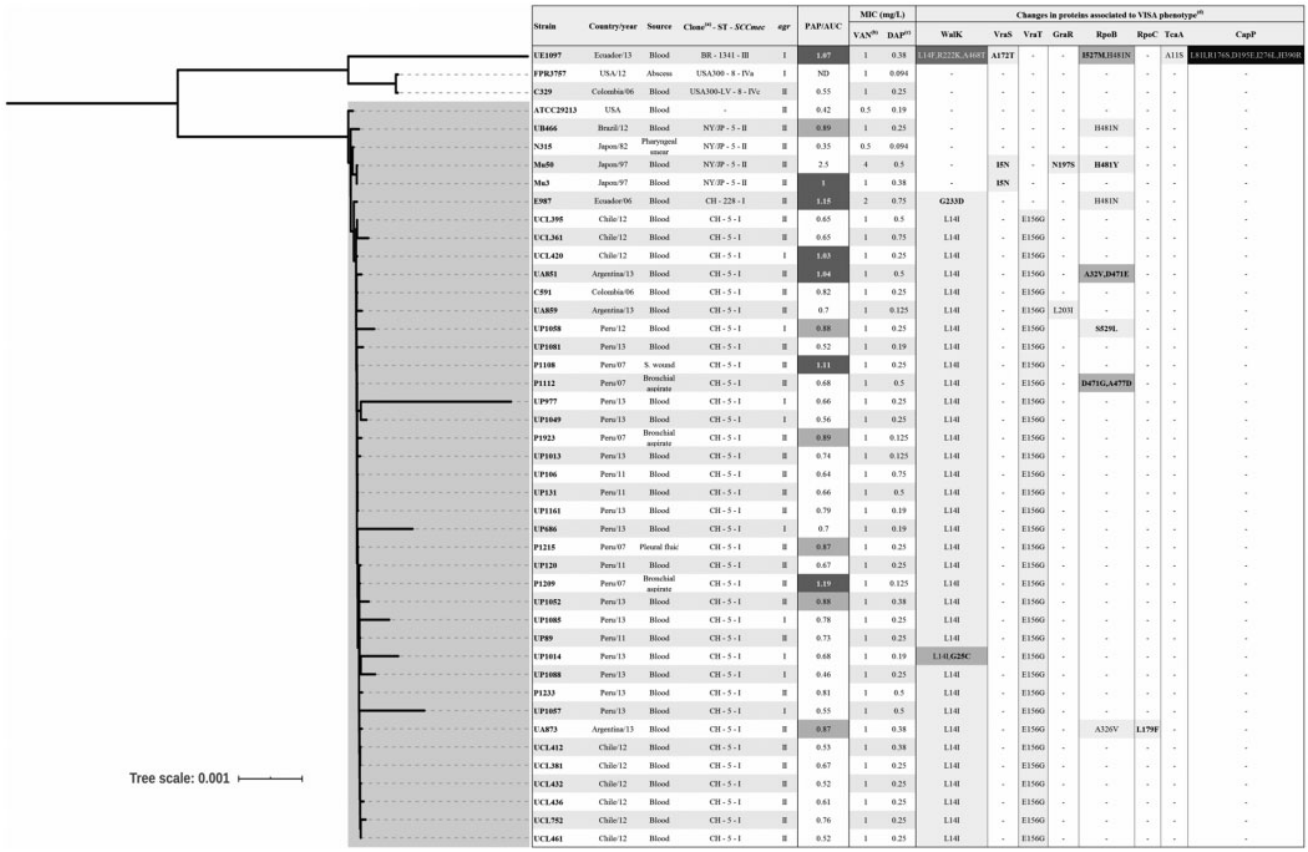
In Latin American hospitals, there is a wide diversity of MRSA genetic lineages circulating and clonal lineages CC5, CC8 and CC30 are the most prevalent.[2,3] Among the 39 hVISA, 95% belonged to CC5, showing PFGE profiles related to Chilean/Cordobes (SCC*mec*I, ST5/228) and New York/Japan clones (NY/JP-SCC*mec*II; ST5; 1 isolate). Further, among the hVISA isolates belonging to the CC5 Chilean/Cordobes clone, 35 isolates (97%) were ST5 and 1 isolate (3%) was ST228. Of note, only one hVISA isolate belonged to CC8, which was related to the USA300 Latin American variant (USA300-LV) (SCC*mec*-IVc, ST-8) harbouring *agr*-II. The other isolate belonged to CC239 (Brazilian clone; SCC*mec*-III, ST-1341, a single-locus variant of ST239), harbouring *agr*-I. The core genome-based phylogenetic reconstruction confirmed a genetic relationship among isolates belonging to CC5 (Figure 2). Of note, most of the hVISA isolates that had a PAP/AUC above the threshold (5/6) were not phylogenetically related although they belonged to CC5 (Figure 2).

Phenotypic resistance profiles indicated that the 39 hVISA isolates exhibited resistance to erythromycin, clindamycin, ciprofloxacin and gentamicin. Only 18% were resistant to chloramphenicol, 8% to tetracycline, 5% to rifampicin and 3% to trimethoprim/sulfamethoxazole (Table S2). None of the isolates were resistant to vancomycin, teicoplanin, linezolid, minocycline or daptomycin (daptomycin MIC_{90} of 0.5 mg/L). The resistome analysis is shown in Table S3. Most (82%) of the hVISA isolates carried *blaZ* and genes encoding aminoglycoside-modifying enzymes: *ant(9)-Ia* (97.4%); *ant(6)-Ia* (84.6%); and *aph(3′)-III* (84.6%). The *erm*(A) gene conferring macrolide, lincosamide and streptogramin B (MLS_B) resistance was present in 38 isolates (97.4%), while *erm*(C) was only identified in 2 isolates (5.1%). Changes associated with fluoroquinolone resistance in the putative GrlA and GyrA proteins were identified in the majority of the hVISA isolates (38/39; 97.4%). Of note, 16% of hVISA exhibited changes in RpoB associated with resistance to rifampicin. We also identified *tet*(M) and *fosD* in two isolates [one with *tet*(M) and the other with *fosD*].

Changes in components of WalKR and VraTRS systems potentially associated with hVISA phenotype in Latin American MRSA isolates

Next, we used the genomic information to interrogate changes in predicted proteins that have been previously postulated to contribute to the development of the VISA phenotype (including WalKR, VraTSR, GraSR, RpoB, RpoC, TcaA and CapP[12,13]) in the 39 hVISA isolate genomes (Figure 2). Overall, we did not observe a differential pattern of changes among the isolates confirmed by PAP/AUC. It was noticeable that the isolate UE1097 (PAP/AUC positive) exhibited the highest number of changes; however, this result could be influenced by the fact that the isolate belongs to a distant genetic lineage (non-CC5).

In order to identify the variations, the protein sequences from hVISA ($n = 39$) and VSSA ($n = 305$) isolates from our study were compared with those from VSSA reference genomes ATCC 29213, FPR3757 and N315. The majority of the hVISA isolates (95%; 37 out of 39) exhibited changes in WalK, with a Leu-14→Ile substitution present in 35 isolates. WalKR is a regulatory system involved in cell-wall homeostasis. Similarly, we identified a Glu-156→Gly substitution in VraT in 90% of the hVISA isolates. VraTSR is homologous to the LiaFSR systems in other Gram-positive organisms, functioning as a three-component regulatory system that orchestrates the cell envelope stress response.[50] Of note, an additional



Strain	Country/year	Source	Clone[a] - ST - *SCCmec*	*agr*	PAP/AUC	MIC (mg/L) VAN[c]	MIC (mg/L) DAP[d]	Changes in proteins associated to VISA phenotype[e] WalK	VraS	VraT	GraR	RpoB	RpoC	TcaA	CapP
UE1097	Ecuador/13	Blood	BR - 1341 - III	I	**1.07**	1	0.38	**L14F,R222K,A468T**	**A172T**	-	-	**I527M,H481N**	-	A11S	**L81L,R176S,D195E,I276L,H396R**
FPR3757	USA/12	Abscess	USA300 - 8 - IVa	I	ND	1	0.094	-	-	-	-	-	-	-	-
C329	Colombia/06	Blood	USA300-LV - 8 - IVc	II	0.55	1	0.25	-	-	-	-	-	-	-	-
ATCC29213	USA	Blood	-	II	0.42	0.5	0.19	-	-	-	-	-	-	-	-
UB466	Brazil/12	Blood	NY/JP - 5 - II	II	0.89	1	0.25	-	-	-	-	H481N	-	-	-
N315	Japon/82	Pharyngeal smear	NY/JP - 5 - II	II	0.35	0.5	0.094	-	-	-	-	-	-	-	-
Mu50	Japon/97	Blood	NY/JP - 5 - II	II	2.5	4	0.5	-	**I5N**	-	**N197S**	**H481Y**	-	-	-
Mu3	Japon/97	Blood	NY/JP - 5 - II	II	**1**	1	0.38	-	**I5N**	-	-	-	-	-	-
E987	Ecuador/06	Blood	CH - 228 - I	II	**1.15**	2	0.75	**G233D**	-	-	-	H481N	-	-	-
UCL395	Chile/12	Blood	CH - 5 - 1	II	0.65	1	0.5	L14I	-	E156G	-	-	-	-	-
UCL361	Chile/12	Blood	CH - 5 - 1	II	0.65	1	0.75	L14I	-	E156G	-	-	-	-	-
UCL420	Chile/12	Blood	CH - 5 - 1	I	**1.03**	1	0.25	L14I	-	E156G	-	-	-	-	-
UA851	Argentina/13	Blood	CH - 5 - 1	II	**1.04**	1	0.5	L14I	-	E156G	-	**A32V,D471E**	-	-	-
C591	Colombia/06	Blood	CH - 5 - 1	II	0.82	1	0.25	L14I	-	E156G	-	-	-	-	-
UA859	Argentina/13	Blood	CH - 5 - 1	II	0.7	1	0.125	L14I	-	E156G	L203I	-	-	-	-
UP1058	Peru/12	Blood	CH - 5 - 1	I	**0.88**	1	0.25	L14I	-	E156G	-	**S829L**	-	-	-
UP1081	Peru/13	Blood	CH - 5 - 1	II	0.52	1	0.19	L14I	-	E156G	-	-	-	-	-
P1108	Peru/07	S. wound	CH - 5 - 1	II	**1.11**	1	0.25	L14I	-	E156G	-	-	-	-	-
P1112	Peru/07	Bronchial aspirate	CH - 5 - 1	II	0.68	1	0.5	L14I	-	E156G	-	**D471G,A477D**	-	-	-
UP977	Peru/13	Blood	CH - 5 - 1	I	0.66	1	0.25	L14I	-	E156G	-	-	-	-	-
UP1049	Peru/13	Blood	CH - 5 - 1	I	0.56	1	0.25	L14I	-	E156G	-	-	-	-	-
P1923	Peru/07	Bronchial aspirate	CH - 5 - 1	II	**0.89**	1	0.125	L14I	-	E156G	-	-	-	-	-
UP1013	Peru/13	Blood	CH - 5 - 1	II	0.74	1	0.125	L14I	-	E156G	-	-	-	-	-
UP106	Peru/11	Blood	CH - 5 - 1	II	0.64	1	0.75	L14I	-	E156G	-	-	-	-	-
UP131	Peru/11	Blood	CH - 5 - 1	II	0.66	1	0.5	L14I	-	E156G	-	-	-	-	-
UP1161	Peru/13	Blood	CH - 5 - 1	II	0.79	1	0.19	L14I	-	E156G	-	-	-	-	-
UP686	Peru/13	Blood	CH - 5 - 1	I	0.7	1	0.19	L14I	-	E156G	-	-	-	-	-
P1215	Peru/07	Pleural fluid	CH - 5 - 1	II	**0.87**	1	0.25	L14I	-	E156G	-	-	-	-	-
UP120	Peru/11	Blood	CH - 5 - 1	II	0.67	1	0.25	L14I	-	E156G	-	-	-	-	-
P1209	Peru/07	Bronchial aspirate	CH - 5 - 1	II	**1.19**	1	0.125	L14I	-	E156G	-	-	-	-	-
UP1052	Peru/13	Blood	CH - 5 - 1	II	**0.88**	1	0.38	L14I	-	E156G	-	-	-	-	-
UP1085	Peru/13	Blood	CH - 5 - 1	I	0.78	1	0.25	L14I	-	E156G	-	-	-	-	-
UP89	Peru/11	Blood	CH - 5 - 1	II	0.73	1	0.25	L14I	-	E156G	-	-	-	-	-
UP1014	Peru/13	Blood	CH - 5 - 1	I	0.68	1	0.19	**L14I,G25C**	-	E156G	-	-	-	-	-
UP1088	Peru/13	Blood	CH - 5 - 1	I	0.46	1	0.25	L14I	-	E156G	-	-	-	-	-
P1233	Peru/13	Blood	CH - 5 - 1	II	0.81	1	0.5	L14I	-	E156G	-	-	-	-	-
UP1057	Peru/13	Blood	CH - 5 - 1	I	0.55	1	0.5	L14I	-	E156G	-	-	-	-	-
UA873	Argentina/13	Blood	CH - 5 - 1	II	**0.87**	1	0.38	L14I	-	E156G	-	A326V	**L179F**	-	-
UCL412	Chile/12	Blood	CH - 5 - 1	II	0.53	1	0.38	L14I	-	E156G	-	-	-	-	-
UCL381	Chile/12	Blood	CH - 5 - 1	II	0.67	1	0.25	L14I	-	E156G	-	-	-	-	-
UCL432	Chile/12	Blood	CH - 5 - 1	II	0.52	1	0.25	L14I	-	E156G	-	-	-	-	-
UCL436	Chile/12	Blood	CH - 5 - 1	II	0.61	1	0.25	L14I	-	E156G	-	-	-	-	-
UCL752	Chile/12	Blood	CH - 5 - 1	II	0.76	1	0.25	L14I	-	E156G	-	-	-	-	-
UCL461	Chile/12	Blood	CH - 5 - 1	II	0.52	1	0.25	L14I	-	E156G	-	-	-	-	-

Figure 2. Phylogenetic relationships and changes in proteins associated with the hVISA phenotype. The genetic relationship was determined by SNP-based phylogenetic tree using the core genome. The clade of isolates belonging to CC5 is highlighted in grey. [a]BR (Brazilian clone); CH (Chilean/Cordobes clone); NY/JP (New York/Japan clone). [b]PAP/AUC ratio values higher than 0.9 when compared with the Mu3 strain are highlighted in dark grey and isolates with ratio values between 0.87 and 0.89 in light grey. [c]Vancomycin MICs (mg/L) were determined by the agar dilution method. [d]Daptomycin MICs (mg/L) were determined by the Etest method. [e]Shade intensity is proportional to the accumulation of changes in the predicted protein. Changes in bold were not present in VSSA isolates.

isolate exhibited a change in VraS (Ala-172→Thr). Further, seven isolates exhibited changes in RpoB, including the His-481→Tyr substitution (observed in three isolates) that has been previously identified in Mu50.[51,52] Among the six isolates confirmed by PAP/AUC, all but one harboured the WalK change Leu-14→Ile/Phe. However, this unique isolate had another predicted substitution in WalK (Gly-233→Asp).

Finally, we sought to determine the frequency of the above amino acid changes in predicted proteins from 305 clinical isolates of MRSA (recovered from the multicentre study performed during 2011–14)[2] that were phenotypically non-hVISA by the two agar screening tests (BHI-V4 and MH-T5) (Table S3). Interestingly, both substitutions Leu-14→Ile and Glu-156→Gly in WalK and VraT, respectively, were found in 21% of the VSSA (63/305). In contrast, these changes were present in 90% (35/39) and 87% (34/39) of the hVISA isolates, respectively ($P < 0.01$). Additionally, other changes described above in WalK, VraS, RpoB and RpoC were exclusively present in the hVISA genomes (bold in Figure 2). Furthermore, we did not find significant differences in the presence of changes detected in the 6 hVISA PAP/AUC-positive (>0.9) isolates compared with those present in the 33 hVISA but PAP/AUC negative (<0.9) isolates (Table S4). When we compared the substitutions present in the PAP/AUC-positive isolates with those present in the 305 VSSA isolates (Table S5) plus the PAP/AUC-negative isolates (Table S6), the presence of changes in RpoB, VraS, Walk and VraT was still significantly higher in the PAP/AUC-positive isolates.

Discussion

To our knowledge, this is the first multicentre study attempting to evaluate the frequency of the hVISA phenotype in clinical isolates of MRSA from Latin American hospitals with temporal sampling (2006–08 and 2011–14). Our results show that the frequency of the hVISA phenotype (determined by GRD and MET) was low (3.3%). A previous meta-analysis by Zhang *et al.*[23] estimated a global hVISA prevalence of around 6%. Of note, hVISA prevalence ranged from 5.6% to 6.81% in different regions of the world.[23] In South America, previous reports suggested that the prevalence of hVISA in Brazil and Argentina approached 9.7%[53] and 3.3%,[26] respectively. Since there are no standardized laboratory tests for detecting this phenotype, these results should be interpreted with caution. Additionally, several factors seem to affect the hVISA

phenotype, including storage time and source of the isolates, among others.[23,24,54] In our study, we used a hierarchical approach to determine the phenotype and the results were supported by genomic information. Therefore, our findings suggest that the frequency of the hVISA phenotype among Latin American MRSA isolates seems to be low.

When analysing the genetic background of the hVISA isolates in our study, 95% belonged to CC5, (SCC*mec*I-ST5 and SCC*mec*II-ST5). Indeed, ST5 and ST239 are hospital-associated MRSA (HA-MRSA) lineages prevalent in Asia, South America and Eastern Europe and have been considered the most epidemic genotypes of hVISA/VISA strains.[23] Furthermore, the Chilean/Cordobes clone had a prevalence greater than 90% in countries such as Chile and Peru,[2,31] where a relatively high proportion of isolates exhibited the hVISA phenotype (12.2% and 19%, respectively). In contrast, only one hVISA isolate recovered in Colombia (2006–08) belonged to the USA300-LV clone, a unique genetic lineage circulating in that country.[2] Moreover, hVISA isolates in our study showed resistance to macrolides, lincosamides, fluoroquinolones and aminoglycosides (erythromycin, clindamycin, ciprofloxacin and gentamicin), a phenotype that is also associated with Latin American CC5 MRSA,[2] whereas tetracycline and trimethoprim/sulfamethoxazole resistance were 5.1% and 2.5%, respectively. Thus, our results support the notion that the genetic backgrounds of CC5 and ST239 may be prone to develop the hVISA phenotype, although it can occur in other MRSA lineages.

Interestingly, the majority of the hVISA isolates detected by Etest-based methods were negative by the PAP/AUC (only 6/39 were positive). Of note, an additional six isolates exhibited a PAP/AUC ratio very close to the hVISA definition. Moreover, we were able to detect alterations in proteins overrepresented in hVISA isolates (when compared with VSSA isolates), regardless of the PAP/AUC result. Since the PAP/AUC ratio is strictly based on the heterogeneous subpopulations of *S. aureus* Mu3, this technique might not detect some hVISA strains harbouring smaller subpopulations than those contained in the Mu3 strain. Considering that PAP is not an easy test to perform in clinical laboratories, our results suggest that Etest-based methods are an important alternative for the detection of hVISA clinical isolates in a faster and more reliable manner.

Our findings were supported by genomic analyses. Indeed, we found changes in amino acid sequences of predicted proteins previously associated with the VISA phenotype, such as changes in WalK (Arg-222→Lys), GraS (Leu-26→Phe, Ile-59→Leu and Thr-224→Ile) and RpoB (His-481→Asn).[14,15] However, these changes were not consistently found in all of our hVISA isolates, reflecting the complex and polygenic nature of this phenotype.[14,15] Of note, it has been shown that mutations in *walK* correlate with thickening of the cell wall, slow growth and decreased autolysis, which are phenotypic characteristics of hVISA isolates.[55] Indeed, the majority of hVISA isolates evaluated in this study exhibited WalK (Leu-14→Ile) and VraT (Glu-156→Gly) substitutions not previously reported. WalKR also controls autolytic activity, particularly of two *S. aureus* autolysins (Atl and LytM). Furthermore, WalKR also regulates the transcription of 13 genes involved in the metabolism and degradation of the cell wall.[56] Thus, our results are aligned with previous reports demonstrating heterogeneity of genetic patterns of hVISA isolates. Interestingly, a recent study by Liu *et al.*[57] evaluated mutations involved in the development of antibiotic tolerance and resistance and found that early alterations in RpoB and RpoC (associated with rifampicin resistance) played a potential role in the vancomycin/daptomycin-tolerant phenotype. Altogether, the data support the notion that RpoB and RpoC may be implicated in the adaptive cellular changes in *S. aureus* for the establishment of tolerance and heteroresistance.

In summary, our comprehensive study of MRSA clinical isolates in Latin America suggests that the overall prevalence of hVISA is low, but CC5 genetic lineages circulating in particular countries of the region may be predisposed to develop this phenotype. The difficulty in the epidemiological and clinical characterization of this phenotype makes it necessary to continue with prospective and molecular surveillance studies and detect the clinical impact in severe *S. aureus* infections.

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Supplementary data

Tables S1 to S6 are available as Supplementary data at *JAC* Online.

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