Emergence of unusual vanA/vanB2 genotype in a highly mutated vanB2-vancomycin-resistant hospital-associated E. faecium background in Vietnam

Emergence of unusual *vanA/vanB*$_2$ genotype in a highly mutated *vanB*$_2$-Vancomycin Resistant Hospital Associated *E. faecium* background, in Vietnam

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Running title: Characterization of *vanB*$_2$- and *vanA/vanB*$_2$-types Hospital Associated Vancomycin Resistant *E. faecium*.

Abstract

Enterococcus faecium has become a globally disseminated nosocomial pathogen principally as the consequence of acquisition and diffusion of virulence factors and multidrug resistance determinants, including glycopeptides, one of the last resort antimicrobials used to treat more serious infections that usually occur in high-risk patients. In this study we investigated and molecular characterized Hospital-Associated (HA) *Enterococcus faecium* isolates collected at Hue Central Hospital, Vietnam.

Our results highlighted the spread among hospital wards of a surprisingly heterogeneous multi drugs resistant *E. faecium* population, composed by five different CC17-related STs, of which 46% VREf carrying the *vanB gene.* Whole genome Sequencing of selected *E. faecium* isolates showed that VREf from different STs carried the same chromosomal integrated Tn1549-like transposon, with a highly mutated vanB2-operon, showing an increased level of vancomycin resistance (VanB phenotype) and able, in one isolate, to confer resistance to teicoplanin (VanA incongruent phenotype).

Two unusual *vanA/vanB$_2$*-type strains were detected within the *vanB$_2$-type ST17* population, harbouring a Tn*1546*-vanA-like transposon in pJEG40-like plasmids. Wg-SNPs-based analysis evidenced the genetic relatedness of VSEf/VREf of same STs and suggested lateral exchange of the Tn*1549*-like element among isolates followed by clonal expansion. Particularly microevolution among ST17 isolates, including the vanA/vanB$_2$-type strains, and inter-wards VREf transmission were highlighted.

The use of teicoplanin is strongly discouraged in the study's hospital for the spreading of Tn*1549*–*vanB$_2$* associated to teicoplanin resistance. A rational use of glycopeptides and effective surveillance measures are always required to reduce nosocomial VSEF/VREf spread and to avoid the rise of unusual and misleading VREf genotypes.

Keywords: VSEf/VREF; WGS; Tn1547; Tn1546; vanA/vanB

1. Introduction

Due to the clinical significance [1] and the capability to spread by cross-contamination leading to local outbreaks [2,3]. Hospital Associated-Vancomycin Resistance *Enterococcus faecium* (HA-VREf) is currently cause of increasing concerns, and was recently included in the seven "ESKAPE" nosocomial bacteria under global surveillance [4]. The HA-VREf originates from a specific Vancomycin Susceptible *E. faecium* (VSEf) subpopulation, characterized by Ampicillin Resistance (HA-AREf) [2,5] and the carriage of IS*16* [6] insertion sequence which allows an enhanced genetic plasticity and an easier acquisition of mobile genetic elements (MGE), vehicles of antimicrobial resistance genes [7]. The potential acquisition of vancomycin resistance determinants leads to the rise of VREf, threatening our capability to treat more serious infections that can occur especially in high-risk patients [8]. In total, eight types of acquired vancomycin resistance genotypes are known in enterococci, with *vanA* and *vanB* genotypes being the most represented in clinical isolates [9–11]. The former confers high inducible resistance to both vancomycin (MIC >64 mg/L) and teicoplanin (MICs from 4 up to >64 mg/L) and is mainly associated with the Tn*1546* transposon, often found as a part of non-conjugative or conjugative plasmids, while the latter confers lower levels of inducible resistance only to vancomycin (MIC ranging from 4 to 32mg/L) and is predominantly found as integral part of the Tn*1549*/Tn*5382*-like conjugative transposons of chromosomal origin [12,13]. Resistance to glycopeptides results from the production of d-Ala-d-Lac instead of d-Ala-d-Ala in cell wall synthesis due to the presence of the *vanA* (*vanHAXYZ*) and *vanB* ($vanY_BW_BH_BBX_B$) operons regulated at the transcription level by two-component system genes (*vanR–vanS*) [9]. Hospital-associated *E. faecium* clones can also acquire several factors, increasing adaptation and out-competing *E. faecium* commensal clones, as the enterococcal surface

protein (*esp),* that enhance colonization ability promoting adhesion and biofilm formation [14], and the glycoside hydrolase (*hyl*) also involved in intestinal colonization [15]. Molecular epidemiology studies by Whole Genome Sequencing (WGS) have shown that HA-*E. faecium* clones are genetically grouped in a polyclonal cluster designed Clade A1 based on core genome single nucleotide polymorphisms (cgSNPs) [10,16] and cgMLST [17], which includes Sequence Types (STs) Clonal Complex 17 (CC17) related, based on MLST and EBurst analysis [18]. Phylogenetic analysis on cgSNPs and wgSNPs can deeply discriminate clones of the same STs detecting and tracking nosocomial outbreaks [19].

The importance to monitor the presence of high-risk HA-*E. faecium* clones, now endemic in many hospitals across the world, and to characterize their glycopeptides resistant determinants is mandatory for a fully understanding of dissemination and dynamics of evolving of this pathogen.

In this study, we aimed to assess the presence of HA-*E. faecium* at the Hue Central Hospital, Vietnam, and to molecular characterize isolates including WGS of representative isolates. To date, only few data on VREf are available from South-East Asian countries [20–23] and to our knowledge, this is the first study describing clinical VREf genotypes in Vietnam.

2. Material and Methods

2.1 Strains and antimicrobial susceptibility

All AREf strains isolated during January 2014 and June 2015 from patients admitted at the tertiary Hue Central Hospital Vietnam, and two *E. faecium* Ampicillin Susceptible strains (ASEf), were included in the study. Patient's location and source of isolation were collected from paper and electronic medical records. Test results of strain identification by API20 Strep and antimicrobial susceptibility by Kirby-Bauer performed at the Hue Central Hospital laboratory, were confirmed using MicroScan WalkAway plus System (Beckman Coulter, Inc.) following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The minimum inhibitory concentration (MIC) of vancomycin and teicoplanin were also determined by Etest (bioMerieux, Marcy l'Etoile, France).

2.2 DNA extraction and PCRs

Bacterial DNA was extracted with a DNeasy Blood & Tissue Kit (QIAGEN, Inc., Valencia, CA). A multiplex PCR was performed to detect the glycopeptide resistance genes *vanA*, *vanB*, *vanC1*, *vanC2/C3*, and the relevant *Enterococcus* species specific genes, by using the modified protocol of Kariyama et al. [24,25]. The presence of IS*16, esp* and *hyl* genes, was performed by PCR and multiplex-PCR respectively, as previously described [6,26].

2.3 MLST

MLST was carried out on isolates with primers included in the *E. faecium* MLST scheme (http://pubmlst.org/efaecium/). Specific amplicons were purified (DNA clean and concentrator^{TM}-5-USA) and sequenced at the LMU Sequencing Service (Munich, Germany). Sequences were analyzed using Geneious Pro 4.8.4 software

(http://www.geneious.com/). Allelic profiles and STs were assigned according to the *E. faecium* MLST database (http://pubmlst.org/efaecium/). CC17 related clones were identified by eBURST V3 analysis (http://eburst.mlst.net).

2.4 Whole Genome Sequencing (WGS) and analysis

Sixteen isolates (9 VREf and 7 VSEf), selected on the basis of their MLST STs were chosen to span the time period of the study and to include multiple wards, sources and genotype including the 2 *vanA/vanB* isolates, and then submitted to WGS using a HiScanSQ Illumina platform (Porto Conte Ricerche Srl, Tramariglio, Italy). Generated sequences were assembled *de novo* into contigs using Velvet 1.2.10 (http://www.ebi.ac.uk/~zerbino/velvet/). Contigs were reordered against the reference genome of *E. faecium* Aus0004 (Accession no. NC_017022) using Mauve. Genomic comparison with reference strains *E. faecium* Aus0004 and *E. faecium* Aus0085 (Accession no. CP006620) was performed using Artemis Comparative Tool (ACT) [27] and MUMmer [28]. Genomes were submitted to the RAST platform for annotation (http://rast.nmpdr.org). The NUCmer tool of the MUMmer software was used to search individual sequences within the genomes.

2.5 Transposons and plasmid assembly

The Tn*1546* and Tn*1549* harboring contigs identified using NUCmer were extracted from the de novo assemblies followed by a BLASTn search against publicly available plasmid sequences in GenBank. If a contig coexisted with other plasmid core elements and showed >99% identity to and >90% query coverage of a known plasmid, the contig was preliminarily classified as the reference-like plasmid (e.g. pJEG040 or pHvH-V24). The contigs were further aligned to putative references by NUCmer and visually inspected to

146 confirm the plasmid contents with Ugene version 1.27 [29]. Furthermore, BLASTn
147 comparisons of isolate's *de novo* assembly and the reference plasmid were conducted by
148 ACT and visualized by BRIG [30] and the presence of a reference-like plasmid was
149 defined as ≥98% sequence identity over ≥80% of the length of the reference. The presence
150 of plasmid replicons was performed *in silico* using available service (PlasmidFinder) from
151 Center of Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/).
152
153 *2.5 Phylogenetic analysis*
154 A core genome phylogenetic tree was inferred from SNPs identified by kSNP v 3.0 [31] by
155 using a k-mer length of 19 nucleotides. A total of 31255 core SNPs positions were
156 identified. Parsimony trees based on all SNPs and core SNPs were generated as consensus
157 trees of the equally most parsimonious trees from a sample of 100 trees based on an
158 Extended Majority Rule. SNPs-based analysis was further confirmed by a maximum-
159 likelihood tree build with MEGA 7 [32] with 100 bootstrap replicates. Furthermore, to
160 investigate cross-transmission of same clones among patients, SNPs calculation among
161 strains from same STs was performed by kSNP v 3.0.
162
163

3. Results

3.1 Epidemiological background

A total of 26 AREf isolates were identified from January 2014 to June 2015 from patients admitted at the Hue Central Hospital, Vietnam. Sources, wards origins and characteristics of isolates, are summarized in Table 1. The most common wards were Surgery wards (54%) and Intensive Care Units ICU (15%). Isolates were mainly from blood (31%) and pus (38%) and the rest from urine, catheter and ascitic fluid.

3.2 Antimicrobial susceptibility and PCRs

All AREf strains were also MDR showing resistance to at least three different classes of antibiotics in different combinations (data not shown). All were resistant to, high-level gentamicin, 81% were resistant to high-level streptomycin, 46% to vancomycin and 11% to teicoplanin. All isolates were susceptible to linezolide and quinupristin/dalfopristin. Susceptibility of isolates to ampicillin, vancomycin and teicoplanin are shown in Table 1.

Genotyping of the vancomycin resistance genes by multiplex PCR targeting *vanA,-B,-C* detected the *vanB* gene in all VREf strains and, unexpectedly, an additional *vanA* gene was found in two of them (Table 1). None of VREF isolates contained *vanC* genes.

The atypical *vanA/vanB* type VREf strains (VH16 and VH17) were isolated from two debilitated patients: strain VH16 was isolated from a cirrhotic patient admitted at the Internal Digest Department, while strain VH17 was isolated from a patient admitted at the ICU ward, suffering Guillain Barre Syndrome (Table 1). These isolates showed high-level resistance to vancomycin (MICs ≥256 mg/L) and teicoplanin (MICs of ≥256 mg/L and ≥48mg/L, respectively) by E-test (Table 1). The *vanB*-type isolates showed a VanB phenotype displaying variable levels of vancomycin MICs from 32 mg/L up to 264 mg/L,

and were all susceptible to teicoplanin with the exception of one isolate (VH20), which displayed a VanA incongruent phenotype, characterized by high level of resistance to vancomycin (MIC ≥256 mg/L) and teicoplanin (MIC ≥64 mg/L) (Table 1).

Moreover, four VREf isolates consisting of 3 *vanB*$_2$-type (VH8, VH21 and VH24) and 1 *vanA/vanB*-type (VH16), showed vancomycin heteroresistance, with a growth of sub colonies in the vancomycin inhibition zone of E-test strip corresponding to MICs of >256 mg/L, >96 mg/L, and >32 mg/L (Table 1).

All AREf isolates carried the HA marker IS*16*, which was absent in the two ASEf strains (VH27 and VH28) included in the study for comparison. The *esp* and the *hyl* genes were present in the 65% and 38% of isolates respectively (Table 1) and were not present in ASEf isolates.

3-3 MLST

To evaluate the relatedness of *E. faecium* under investigation, MLST and EBurst analysis were performed. Analysis results, included in Table 1, evidenced the nosocomial origin of the VSEf/VREf isolates that belonged to five different STs, all CC17 related, including ST17 (61;5%), ST18 (15%), ST262 (11,5%) a single locus variant (SLV) of ST18, ST78 (7,7%) and the new ST1085 (3,8%). On the contrary, the ASEf isolates from ST904 and the new ST1086, were not CC17 related. Both *vanA/vanB*$_2$ type isolates were of ST17. Nosocomial STs were isolated from numerous wards (Table 1), ST17 was mainly found in ICU and in other 9 wards, in the hospital building 2 and 3, ST18 in General Surgery and other 2 wards (Building 2 and 3), ST262 in Clinical Haematology and Cardiology Emergency wards (Building 1 and 2), while ST78 in Central-ICU and S-ICU (Building 2).

214 *3.4 WGS analysis of vancomycin resistant and susceptible E. faecium isolates*

215 Nine VREf (7 *vanB* positive and 2 isolates carrying both *vanA* and *vanB*), and 7 VSEf,
216 were selected on the basis of their STs and subjected to WGS. The STs were the following:
217 10 ST17 (6 VREf and 4 VSEf, including the 2 *vanA/vanB* types), 2 ST18 (1 VREf and 1
218 VSEf), 2 ST262 (1 VREf and 1 VSEf), 1 ST78 and the new ST1085 (Table 1). The WGS
219 of *E. faecium* strains were deposited on BioProject Accession *PRJNA419341.*

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221 *3.4.1 vanB operons and Tn1549 transposon*

222 Sequence analysis of *vanB* operons revealed in all VREf isolates a new highly mutated
223 $vanB_2$ operon in a chromosomal integrated Tn*1549*-like transposon, showing 99% of
224 identity with the Tn*1549*-$vanB_2$ transposon of reference strain AUS0004 (26612/26624 bp).
225 Comparative BLAST analysis of our $vanB_2$-operon sequence showed the highest base pair
226 coverage with sequences of *E. faecium* strains SAU16 (KF823968), followed by TSGH1
227 (AF310956), UW7606x64/3 TC1 (CP013009) and AUS0004 reference strain (CP003351).
228 It showed also 99% of identity with *Clostridium* spp (AY655720 and AH014495). The
229 vancomycin regulator and the sensor genes of the new $vanB_2$ operon showed mutations in
230 *vanR* (G27T) and *vanS* (G313A) genes, resulting in E9D and N105D amino acid changes
231 in the corresponding VanR and VanS proteins. The *vanY* gene showed a A65C mutation
232 (E66A) and a G insertion at position 123bp generating a stop codon and consequently a
233 truncated $VanY_B$ d,d-carboxypeptidase. The *vanW* gene had a T514C mutation translated
234 into a S172P. Moreover, the *vanB* gene showed a C953T substitution generating a P318L
235 amino acid change in the VanB resistance protein. Mutations of the $vanB_2$ operon were
236 further confirmed by conventional Sanger sequencing. Tn*1549* transposons showed 100%
237 of identity in all isolates, except for strain VH4 that showed a T356M mutation in the
238 hypothetical protein corresponding to the EFAU004_02789 gene of *E. faecium* AUS0004

reference strain. In all strains the Tn*1549*-like transposon had a common chromosomal insertion site, between genes EFAU004_00610 and EFAU004_00611 according to the reference genome *E. faecium* AUS0004 (Accession no. NC_017022). This hotspot is present in *E. faecium* VREN3305 strain isolated in United Kingdom (Accession no. FXHW01000001.1).

3.4.2. vanA operon, Tn1546 transposon and plasmids

To study the location of the *vanA* operon in the *vanA/vanB*$_2$-type strains (VH16 and VH17), Tn*1546*-harbouring contigs were mapped on the *E. faecium* plasmids pHvH-V24 (Accession no. KX574671) [33] and pJEG40 plasmid (Accession no. KX810025) [34]. The closure of *vanA* operon-harboring plasmid of strain *E. faecium* VH17 resulted in a sequence of 37459bp and showed 99% of sequence identity for 97% of the sequence to both the *E. faecium* pJEG40 and pHvH-V24 plasmids. Assembly of the *E. faecium* VH16 plasmid was not complete due to sequence breakage into many small contigs: a 32726bp sequence was assembled, which showed 99% sequence identity for 88% of the sequence to both the *E. faecium* pJEG40 and pHvH-V24 plasmids and 98% sequence identity for 88% of the sequence to the VH17 pJEG40-like plasmid. VH16 and VH17 pJEG40-like plasmids were also highly similar (98% sequence identity for 77% and 79% of the length, respectively) to the pA698 plasmid carrying the *vanA* resistance operon and described in a *vanA/vanB E. faecium* isolated in Greece [35].

Identity to the *E. faecium* p5357a (Accession no. GQ900435), previously associated as carrier of the same mutated Tn*1546* element, was 99% for only 32% and 36% of the sequence of VH17 and VH16 plasmids, respectively. For this reason, we refer to the newly assembled plasmids in strains VH16 and VH17 as pJEG40-like plasmids.

Both the pJEG40-like plasmids carried the *rep17*$_{pRUM}$ replicon and an identical Tn*1546*-*vanA* operon containing the IS*1251* between the *vanS-vanH* intergenic region, showing 100% of sequence identity with several isolates present in database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig 1b). In total, 87 plasmid replicons of 9 *rep*-families were detected by PlasmidFinder in the 16 *E. faecium* strains under study, with a median of 5 *rep* types per isolate (range, 2-9). Gradual increase of reps was found in VSEf/VREf isolates from same STs (Table S1). None of the *rep* types were VRE or VSE specific. The *vanA/vanB*$_2$ isolates shared 6 replicons (*rep*2, *rep*11, *rep*US12, *rep*17, *rep*18 and *rep*US15) while 3 were found only in the VH16 isolate (*rep*14, *rep*22, and *rep*US7). The *rep17*$_{pRUM}$ type replicon was also present in the VSEf VH1 isolate, with small pairing portions with pJEG40-like plasmids (less than 3000 bp).

3.5. WG-SNPs unrooted phylogenetic tree

The wg-SNPs unrooted phylogenetic tree, build with Vietnamese isolates and six reference strains evidenced distinct subclones within ST17, grouping in two genetically related ST17 lineages (Fig 1a), one lineage included VREf isolates (VH8, VH9, VH4, VH16, VH17 and VH20, VH21) and the other included the VSEf isolates (VH23 and VH11, VH13). Also, genetically distinct isolates of ST18 (VH5 and VH6) and ST262 (VH19 and VH22) clustered in 2 related VSEf (VH6 and VH22) and VREf (VH5 and VH19) lineages. The other VSEf isolates VH1 (ST78) and VH26 (ST1085) clustered with reference isolates AUS0085 (ST203) and T110 (ST810), respectively (Fig 1a). The wg-SNPs tree (Fig 1a) revealed that the *vanA/vanB2* type strains VH16 and VH17 were highly genetically related but did not cluster together (Fig.1a, Table S2), in contrast to how they did in the phylogenetic tree build solely on cg-SNPs (core genome) (Fig. 1b). Indeed when we compared the wgSNPs of strains within the ST17, 229 wgSNPs of difference were found

between the *vanA/vanB$_2$ strains,* while 29 wgSNPs between ST17 VSEf (VH11, VH13), and just 5 and 9 wgSNPs of difference in VREf isolates VH8-VH9 and VH20-VH21 (Table S2).

4. Discussion

During the last decades, VREf has emerged worldwide as a relevant nosocomial multidrug-resistant pathogen becoming a serious health concern [36]. This phenomenon is particularly worrisome in low-income countries as many South Asian countries, where the overuse of antibiotics, also in veterinary field and the hospital surveillance are not always adequate [23]. In this study we investigated ampicillin resistant *E faecium* strains isolated during 2014-15, finding a high proportion of multi-drug resistance including vancomycin (46%), with 35% of the isolates being from invasive infections (i.e. blood). This is worrisome and underlines that VREf is a pathogen causing severe hospital infections and compromising treatment options.

To our knowledge, this represents the first study in which Vietnamese nosocomial multi-resistant *E. faecium* isolates were genetically characterized. Our results highlighted the spread among hospital wards of a surprisingly heterogeneous population of HA-VSEf/VREf isolates, composed by five different MLST-CC17 related STs, including the ancestral ST17, ST18 and ST78, responsible of epidemics worldwide [37]. *E. faecium* isolates, particularly those resistant to vancomycin were mainly from ICU and Surgery wards. The study evidenced that the same chromosomal integrated Tn*1549*-like transposon, with a novel highly mutated *vanB*$_2$-operon, conferring high level of vancomycin resistance and the ability to resist to teicoplanin, has spread among *E. faecium* of different STs.

Previous studies showed that *vanB*-type strains resistant to teicoplanin can arise under glycopeptides antibiotic pressure, due to mutations in the N-terminal sensor portion of the $VanS_B$ kinase allowing the inducible expression of resistance by teicoplanin [9,38,39].

Here, we found a VanS mutation (N105D) in that the same sensor portion in all *vanB$_2$*-type isolates under study, suggesting its involvement in the onset of resistance to teicoplanin. Furthermore, the several other mutations detected in the *vanR$_B$, vanY$_B$, vanW$_B$* and *vanB* genes might be involved in the high MICs values of vancomycin and teicoplanin displayed by our *vanB$_2$*-type strains, and also in the vancomycin heteroresistance observed in three isolates.

The acquisition of Tn*1549* transposon may have different origins, acquired either from spreading HA-VREf or from community CA-VRE, but also from gut microbiota that can share also mutated vanB$_2$-like transposons (i. e. *Clostridium difficile*) [16,39–43]. Once acquired, VREf can spread easily increasing clinical treatment failures and mortality rate compared to VSEf, as previously described [44].

Moreover, two *vanB$_2$*–type ST17 strains (VH16 and VH17) carried an additional Tn*1546-vanA*-like transposon on pJEG40-like plasmids generating the unusual *vanA/vanB$_2$* genotype with high level glycopeptides MICs (vancomycin ≥256 mg/L and teicoplanin ≥256 mg/L and ≥48 mg/L). A recent study conducted in Australia, associated the same Tn*1546-vanA like* transposon present in our *vanA/vanB$_2$* isolates to a lower vancomycin and teicoplanin resistance (MICs 2 and 4 mg/L) [34]. These findings suggest that the mutations occurred in *vanB$_2$* operon rather than the acquisition of the *vanA* operon itself, were responsible for the higher resistance phenotype observed in our *vanA/vanB$_2$* isolates and as well in the VanA incongruent phenotype -*vanB*-type strain.

Finding both *vanA* and *vanB* determinants in clinical *E. faecium* isolates is unusual, and only few cases have been reported during the last 20 years [11]. Recently, *vanA/vanB$_2$*-type

345 isolates were described in two clinical isolates, an *E. gallinarum* strain isolated in Canada
346 [45] and an *E. faecium* strain detected in Greece [35], both showing high glycopeptides
347 resistance levels. These strains carried distinct mutated Tn*1549*-*vanB*$_2$ operon and the same
348 Tn*1546*-*vanA* operon present in our *vanA/vanB*$_2$ isolates, on plasmids pA6981 and
349 pA6981-like, which showed high similarity with the pJEG40-like plasmid here described,
350 suggesting the inclination of such plasmids to be acquired by Tn*1549*-*vanB*$_2$ isolates. The
351 comparative genetic analysis of the pJEG40-like plasmids combined with the phylogenetic
352 relationship of our *vanA/vanB*$_2$ isolates did not clarify if two different plasmid acquisition
353 events by two nosocomial related *vanB*$_2$–type ST17 clones, or a single plasmid acquisition,
354 followed by the evolution into a single ST17 *vanB*$_2$ clone, occurred. Additional studies are
355 necessary to clarify the origin of the Tn1546-*vanA* transposon and to better elucidate the
356 rise of the *vanA/vanB*$_2$ genotypes.
357
358 MLST typing and eBURST results of Vietnamese isolates were improved by deeper
359 genomic analysis carried out by wgSNPs and cgSNPs computations and cluster analysis of
360 isolates. The analysis revealed the genetic relatedness between VSEf and VREf isolates of
361 same ST, and evidenced the clonal expansion, particularly among ST17 population, with
362 subclones displaying different assortment of virulence genes and increased numbers of
363 plasmid replicons. In all VREf isolates the presence of the same Tn1549-like transposon
364 with common chromosomal insertion site suggested lateral exchange of Tn*1549*-like
365 element among isolates, with *de novo* generation of VREf, followed by clonal expansion.
366
367 Micro-evolution among ST17 isolates, including the vanA/vanB$_2$-type strains, was
368 highlighted by comparing wgSNPs between isolates, which also suggested, in accordance

with other reports [19,46], a VREf cross-transmission between patients from different wards, with isolates (VH8, VH9) showing just 5 wgSNPs of difference [46].

Notably, the presence of two highly related *vanB*$_2$-type ST17 isolates (wgSNPs=9) in two patients from different wards showing different glycopeptide resistant phenotypes (VH20/ICU/VanA, VH21/OT/VanB), suggested the involvement of the different antibiotic pressure in the two hospital wards in the regulation of the same mutated *vanB*$_2$ operon. Infection control strategy, such as improvement of hand hygiene and implementation of antibiotic stewardship (ABS) programs should be undertaken in this hospital to limit the VREf and other nosocomial MDR strains spread.

A limitation of this study resides in the selection of AREf *E. faecium* isolates, therefore other patients colonized with community-acquired *vanA*-VRE susceptible to ampicillin may have gone undetected. Further, future VRE surveillance programs should consider monitoring vancomycin resistant determinants on patients stool by PCR, including the screening of gut microbiota species, gaining the ability to detect possible vancomycin transposon donors.

In conclusion, this study described the spread among different HA-*E. faecium* STs, of a *vanB*$_2$-Tn*1549*-like transposon able to confer resistance to teicoplanin, which is for this reason strongly inadvisable.

The presence of the Tn*1546*-like-pJEG40-like plasmids in two high related *vanB*$_2$–type ST17 isolates contributed to the heterogeneity of VREf in this hospital. The de *novo* generation of VREf from VSEf strains suggests that, in addition to VREf, also VSEf (AREf) clones should be monitored in the patients and in the hospital environment.

Our findings provide insights useful for infection control of nosocomial *E. faecium* and more importantly for clinical practice. Rational use of glycopeptides and effective surveillance measures are highly required in this and all hospitals to reduce VSEF/VREf spread and to avoid the rise of unusual and misleading VREf genotypes.

Declarations

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Figure Legend

Figure. 1. Unrooted parsimony tree based on wgSNPs (A) and core SNPs (B). Trees are consensus trees of the equally most parsimonious trees from a sample of 100 trees based on an Extended Majority Rule. Internal node labels show the support for each node as calculated by FastTreeMP. Branch lengths are expressed in terms of changes per number of SNPs. Strains branching in the two major clades are color coded based on their ST. VREf and VSEf clades are highlighted in grey and cyan, respectively. Six reference strains were included in the comparison Aus0004 (Acc. no. CP00335), Aus0085 (Acc. no. CP006620), NRRL B-2354 (Acc. no. NCP004063), DO (Acc. no. CP003583), T110 (Acc. no. CP006030), VREF_286 (Acc. no. CP019992).

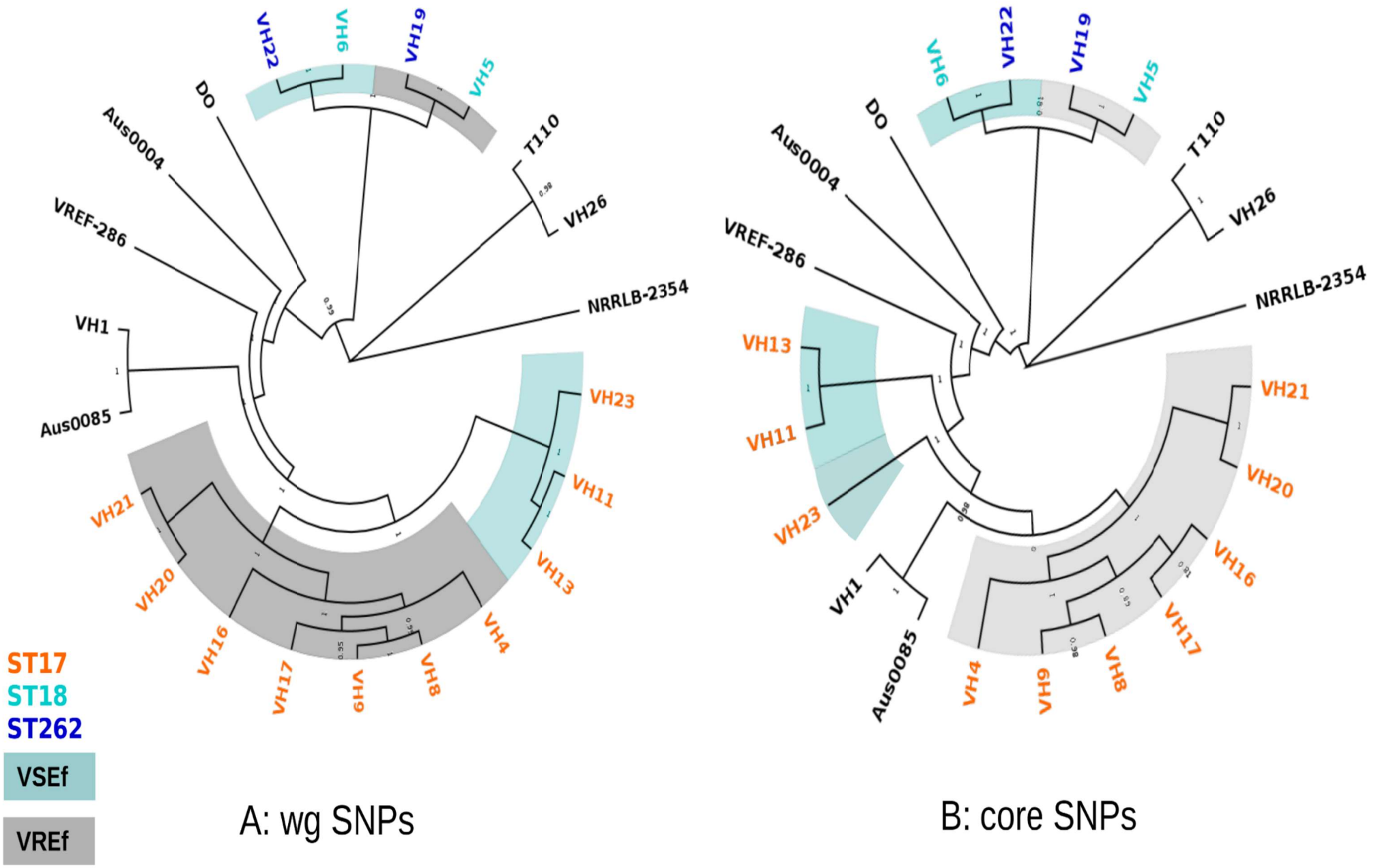
Table 1

Table 1. Origins and characteristics of Vietnamese *E. faecium* isolates.

Strains	Date of isolation	Source	Wards	Floor_Building	First diagnosis	Amp	Van MIC	Tei MIC	Van Phenotype	van Genotype	ST	IS16	hyl	esp
VH18	10/01/2014	Blood	CH	B1	Acute leukemia	R >8	1.0	0.25	S	-	ST262	+	-	-
VH22	18/05/2014	Pus	CH	B1	CML	R>8	1.5	0.125	S	-	ST262	+	-	-
VH19	10/01/2014	Blood	CEI	Ground floor-B2	FUO	R>8	≥256	0.25	VanB	$vanB_2$	ST262	+	-	-
VH14	20/10/2014	Pus	C	2th floor - B2	Stage3 heart failure	R>8	0.50	0.25	S	-	ST17	+	-	+
VH7	26/07/2014	Pus	Sb	4^{th} floor - B2	LTN	R>8	1.0	0.25	S	-	ST17	+	-	+
VH13	07/10/2014	Pus	Sa	5^{th} floor - B2	Ureteral perforation	R>8	1.5	0.125	S	-	ST17	+	-	+
VH1	23/06/2014	Pus	S-ICU	6^{th} floor - B2	Head trauma	R>8	0.50	0.25	S	-	ST78	+	-	+
VH9	26/08/2014	Pus	S-ICU	6^{th} floor - B2	Postoperative ileus	R>8	≥256	0.125	VanB	$vanB_2$	ST17	+	+	+
VH10	31/08/2014	Pus	S-ICU	6^{th} floor - B2	Pleuritis	R>8	0.50	0.25	S	-	ST17	+	-	+
VH28	19/06/2015	Pus	S-ICU	6^{th} floor - B2	Abscess	S=1	1.0	0.25	S	-	ST1086	-	-	-
VH2	09/07/2014	Blood	C-ICU	6^{th} floor - B2	Multiple injuries	R>8	1.0	0.25	S	-	ST78	+	-	+
VH3	11/07/2014	Blood	C-ICU	6^{th} floor - B2	Head trauma	R>8	0.50	0.25	S	-	ST17	+	-	+
VH17	05/04/2015	Catheter	C-ICU	6^{th} floor - B2	GBS	R>8	≥256	≥48	VanA	vanA/ $vanB_2$	ST17	+	+	+
VH20	28/03/2014	Blood	C-ICU	6^{th} floor - B2	Meningitis	R>8	≥256	R≥64	VanA	$vanB_2$	ST17	+	+	+
VH4	15/07/2014	Urine	UKS	2nd floor - B3	Bladder cancer	R>8	≥64	0.19	VanB	$vanB_2$	ST17	+	+	-
VH5	17/07/2014	Urine	UKS	2nd floor - B3	BPH	R>8	≥256	0.25	VanB	$vanB_2$	ST18	+	-	-
VH16*	25/12/2014	Ascites	ID	3th floor - B3	Cirrhosis	R>8	≥256	≥256	VanA	vanA / $vanB_2$	ST17	+	+	+
VH24*	23/05/2015	Urine	G	3th floor - B3	FUO	R>8	≥32	0.25	VanB	$vanB_2$	ST17	+	+	+
VH6	18/07/2014	Catheter	CS	4^{th} floor - B3	PPL	R>8	1.0	0.25	S	-	ST18	+	-	-
VH12	09/09/2014	Urine	GS	4^{th} floor - B3	Post Studer surgery	R>8	≥256	0.19	VanB	$vanB_2$	ST18	+	-	-
VH25	16/06/2015	Blood	GS	4^{th} floor - B3	PFLCD	R>8	1.5	0.125	S	-	ST18	+	+	-
VH8*	24/08/2014	Urine	GS	4^{th} floor - B3	Bladder tumor	R>8	≥256	0.25	VanB	$vanB_2$	ST17	+	+	+
VH11	08/09/2014	Pus	GS	4^{th} floor - B3	Acute cholecystitis	R>8	0.50	0.25	S	-	ST17	+	-	+
VH15	05/12/2014	Blood	GS	4^{th} floor - B3	Sepsis	R>8	≥256	0.50	VanB	$vanB_2$	ST17	+	-	+
VH21*	06/05/2014	Pus	OT	4^{th} floor - B3	Abscess	R>8	≥96	0.38	VanB	$vanB_2$	ST17	+	+	+
VH23	19/06/2014	Blood	NS	B3	Head trauma	R>8	1.0	0.25	S	-	ST17	+	-	+
VH26	16/06/2015	Pus	NPE	B4	Abscess	S=4	1.5	0.125	S	-	ST1085	+	+	-
VH27	19/06/2015	Blood	NPE	B4	Sepsis	R>8	0.50	0.25	S	-	ST944	-	-	-

CH = Clinical Hematology; CEI = Cardiology Emergency Intervention; C = Cardiology, Sb = Surgery b; Sa = Surgery a; S-ICU= Surgical Intensive Care Unit; C-ICU = Central Intensive Care Unit; UKS = Urological & Kidney surgery; ID = Internal Digest; G = Gastroenterology; CS = Cardiovascular Surgery; GS = General Surgery; OT = Orthopedics and traumatology; NS = Neurological Surgery; NPE = Neonatal Pediatric Emergency; CML = Chronic myelogenous leukemia; FUO = Fever of Unknown Origin; LTN = Left big toe necrosis; GBS = Guillain Barre Syndrome; BPH = Benign prostatic hyperplasia; PPL = Postoperative PCA ligation; PFLCD = Postoperative frontal lobe cyst drainage; Amp =Ampicillin susceptibility (by Microscan); Van MIC = Vancomycin Minimal Inhibitory Concentration obtained (by Etest); Tei MIC= Teicoplanin Minimal Inhibitory Concentration obtained (by Etest); ST= Sequence Type; *hyl* = glycoside hydrolase gene; *esp* = enterococcal surface protein gene; * = Vancomycin hetero-resistant strains by E-test; S= susceptible; in bold = fully sequenced *E. faecium* isolates.

Figure 1


VH22
VH6
VH19
VH5
DO
Aus0004
VREF-286
T110
VH26
NRRLB-2354
VH1
Aus0085
VH23
VH11
VH13
VH21
VH20
VH16
VH17
VH9
VH8
VH4
ST17
ST18
ST262
VSEf
VREf
A: wg SNPs
B: core SNPs

Supplementary data

Click here to download Supplementary data: Suppl Table S1 new.xlsx

Emergence of unusual *vanA/vanB*$_2$ genotype in a highly mutated *vanB*$_2$-Vancomycin Resistant Hospital Associated *E. faecium* background, in Vietnam

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Field Code Changed

Running title: Characterization of *vanB*$_2$- and *vanA/vanB*$_2$-types Hospital Associated Vancomycin Resistant *E. faecium*.

Abstract

Enterococcus faecium has become a globally disseminated nosocomial pathogen principally as the consequence of acquisition and diffusion of virulence factors and multidrug resistance determinants, including glycopeptides, one of the last resort antimicrobials used to treat more serious infections that usually occur in high-risk patients.

In this study we investigated and molecular characterized Hospital-Associated (HA) *Enterococcus faecium* isolates collected at Hue Central Hospital, Vietnam.

Our results highlighted the spread among hospital wards of a surprisingly heterogeneous multi drugs resistant *E. faecium* population, composed by five different CC17-related STs, of which 46% VREf carrying the *vanB gene.* Whole genome Sequencing of selected *E. faecium* isolates showed that VREf from different STs carried the same ~~cromosomal~~ chromosomal integrated Tn1549-like transposon, with a highly mutated vanB2-operon, showing an increased level of vancomycin resistance (VanB phenotype) and able, in one isolate, to confer resistance to teicoplanin (VanA incongruent phenotype).

Two unusual *vanA/vanB$_2$*-type strains were detected within the *vanB$_2$-type ST17* population, harbouring a Tn*1546*-vanA-like transposon in pJEG40-like plasmids. Wg-SNPs-based analysis evidenced the genetic relatedness of VSEf/VREf of same STs and suggested lateral exchange of the Tn*1549*-like element among isolates followed by clonal expansion. Particularly microevolution among ST17 isolates, including the vanA/vanB$_2$-type strains, and inter-wards VREf transmission were highlighted.

The use of teicoplanin is strongly discouraged in the study's hospital for the spreading of Tn*1549*–*vanB$_2$* associated to teicoplanin resistance. A rational use of glycopeptides and effective surveillance measures are always required to reduce nosocomial VSEF/VREf spread and to avoid the rise of unusual and misleading VREf genotypes.

Keywords: VSEf/VREF; WGS; Tn1547; Tn1546; vanA/vanB

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1. Introduction

Due to the clinical significance [1] and the capability to spread by cross-contamination leading to local outbreaks [2,3]. Hospital Associated-Vancomycin Resistance *Enterococcus faecium* (HA-VREf) is currently cause of increasing concerns, and was recently included in the seven "ESKAPE" nosocomial bacteria under global surveillance [4]. The HA-VREf originates from a specific Vancomycin Susceptible *E. faecium* (VSEf) subpopulation, characterized by Ampicillin Resistance (HA-AREf) [2,5] and the carriage of IS*16* [6] insertion sequence which allows an enhanced genetic plasticity and an easier acquisition of mobile genetic elements (MGE), vehicles of antimicrobial resistance genes [7]. The potential acquisition of vancomycin resistance determinants leads to the rise of VREf, threatening our capability to treat more serious infections that can occur especially in high-risk patients [8]. In total, eight types of acquired vancomycin resistance genotypes are known in enterococci, with *vanA* and *vanB* genotypes being the most represented in clinical isolates [9–11]. The former confers high inducible resistance to both vancomycin (MIC >64 mg/L) and teicoplanin (MICs from 4 up to >64 mg/L) and is mainly associated with the Tn*1546* transposon, often found as a part of non-conjugative or conjugative plasmids, while the latter confers lower levels of inducible resistance only to vancomycin (MIC ranging from 4 to 32mg/L) and is predominantly found as integral part of the Tn*1549*/Tn*5382*-like conjugative transposons of chromosomal origin [12,13]. Resistance to glycopeptides results from the production of d-Ala-d-Lac instead of d-Ala-d-Ala in cell wall synthesis due to the presence of the *vanA* (*vanHAXYZ*) and *vanB* ($vanY_BW_BH_BBX_B$) operons regulated at the transcription level by two-component system genes (*vanR–vanS*) [9]. Hospital-associated *E. faecium* clones can also acquire several factors, increasing adaptation and out-competing *E. faecium* commensal clones, as the enterococcal surface

protein (*esp),* that enhance colonization ability promoting adhesion and biofilm formation [14], and the glycoside hydrolase (*hyl*) also involved in intestinal colonization [15]. Molecular epidemiology studies by Whole Genome Sequencing (WGS) have shown that HA-*E. faecium* clones are genetically grouped in a polyclonal cluster designed Clade A1 based on core genome single nucleotide polymorphisms (cgSNPs) [10,16] and cgMLST [17], which includes Sequence Types (STs) Clonal Complex 17 (CC17) related, based on MLST and EBurst analysis [18]. Phylogenetic analysis on cgSNPs and wgSNPs can deeply discriminate clones of the same STs detecting and tracking nosocomial outbreaks [19].

The importance to monitor the presence of high-risk HA-*E. faecium* clones, now endemic in many hospitals across the world, and to characterize their glycopeptides resistant determinants is mandatory for a fully understanding of dissemination and dynamics of evolving of this pathogen.

In this study, we aimed to assess the presence of HA-*E. faecium* at the Hue Central Hospital, Vietnam, and to molecular characterize isolates including WGS of representative isolates. To date, only few data on VREf are available from South-East Asian countries [20–23] and to our knowledge, this is the first study describing clinical VREf genotypes in Vietnam.

2. Material and Methods

2.1 Strains and antimicrobial susceptibility

All AREf strains isolated during January 2014 and June 2015 from patients admitted at the tertiary Hue Central Hospital Vietnam, and two *E. faecium* Ampicillin Susceptible strains (ASEf), were included in the study. Patient's location and source of isolation were collected from paper and electronic medical records. Test results of strain identification by API20 Strep and antimicrobial susceptibility by Kirby-Bauer performed at the Hue Central Hospital laboratory, were confirmed using MicroScan WalkAway plus System (Beckman Coulter, Inc.) following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The minimum inhibitory concentration (MIC) of vancomycin and teicoplanin were also determined by Etest (bioMerieux, Marcy l'Etoile, France).

2.2 DNA extraction and PCRs

Bacterial DNA was extracted with a DNeasy Blood & Tissue Kit (QIAGEN, Inc., Valencia, CA). A multiplex PCR was performed to detect the glycopeptide resistance genes *vanA*, *vanB*, *vanC1*, *vanC2/C3*, and the relevant *Enterococcus* species specific genes, by using the modified protocol of Kariyama et al. [24,25]. The presence of IS*16, esp* and *hyl* genes, was performed by PCR and multiplex-PCR respectively, as previously described [6,26].

2.3 MLST

MLST was carried out on isolates with primers included in the *E. faecium* MLST scheme (http://pubmlst.org/efaecium/). Specific amplicons were purified (DNA clean and concentrator^{TM}-5-USA) and sequenced at the LMU Sequencing Service (Munich, Germany). Sequences were analyzed using Geneious Pro 4.8.4 software

(http://www.geneious.com/). Allelic profiles and STs were assigned according to the *E. faecium* MLST database (http://pubmlst.org/efaecium/). CC17 related clones were identified by ~~EBurst3v~~ eBURST V3 analysis (http://eburst.mlst.net).

2.4 Whole Genome Sequencing (WGS) and analysis

Sixteen isolates (9 VREf and 7 VSEf), selected on the basis of their MLST STs were chosen to span the time period of the study and to include multiple wards, sources and genotype including the 2 *vanA/vanB* isolates, and then submitted to WGS using a HiScanSQ Illumina platform (Porto Conte Ricerche Srl, Tramariglio, Italy). Generated sequences were assembled *de novo* into contigs using Velvet 1.2.10 (http://www.ebi.ac.uk/~zerbino/velvet/). Contigs were reordered against the reference genome of *E. faecium* Aus0004 (Accession no. NC_017022) using Mauve. Genomic comparison with reference strains *E. faecium* Aus0004 and *E. faecium* Aus0085 (Accession no. CP006620) was performed using Artemis Comparative Tool (ACT) [27] and MUMmer [28]. Genomes were submitted to the RAST platform for annotation (http://rast.nmpdr.org). The NUCmer tool of the MUMmer software was used to search individual sequences within the genomes.

2.5 Transposons and plasmid assembly

The Tn*1546* and Tn*1549* harboring contigs identified using NUCmer were extracted from the de novo assemblies followed by a BLASTn search against publicly available plasmid sequences in GenBank. If a contig coexisted with other plasmid core elements and showed >99% identity to and >90% query coverage of a known plasmid, the contig was preliminarily classified as the reference-like plasmid (e.g. pJEG040 or pHvH-V24). The contigs were further aligned to putative references by NUCmer and visually inspected to

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confirm the plasmid contents with Ugene version 1.27 [29]. Furthermore, BLASTn comparisons of isolate's *de novo* assembly and the reference plasmid were conducted by ACT and visualized by BRIG [30] and the presence of a reference-like plasmid was defined as ≥98% sequence identity over ≥80% of the length of the reference. The presence of -plasmid replicons was performed *in silico* using available service (PlasmidFinder) from Center of Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/).

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2.5 Phylogenetic analysis

A core genome phylogenetic tree was inferred from SNPs identified by kSNP v 3.0 [31] by using a k-mer length of 19 nucleotides. A total of 31255 core SNPs positions were identified. Parsimony trees based on all SNPs and core SNPs were generated as consensus trees of the equally most parsimonious trees from a sample of 100 trees based on an Extended Majority Rule. SNPs-based analysis was further confirmed by a maximum-likelihood tree build with MEGA 7 [32] with 100 bootstrap replicates. Furthermore, to investigate cross-transmission of same clones among patients, SNPs calculation among strains from same STs was performed by kSNP v 3.0.

3. Results

3.1 Epidemiological background

A total of 26 AREf isolates were identified from January 2014 to June 2015 from patients admitted at the Hue Central Hospital, Vietnam. Sources, wards origins and c~~C~~haracteristics of isolates, ~~and , susceptibility of isolates against the main antimicrobials and vancomycin phenotypes~~ are summarized in Table 1. The most common wards were Surgery wards (54%) and Intensive Care Units ICU (15%). Isolates were mainly from blood (31~~5~~%) and pus (3~~8~~5%) and the rest from urine, catheter and ascitic fluid.

3.2 Antimicrobial susceptibility and PCRs

All AREf strains were also MDR showing resistance to at least three different classes of antibiotics in different combinations ~~(Table 1~~ data not shown). All were resistant to ~~ciprofloxacin, levofloxacin~~, high-level gentamicin, ~~tetracycline and chloramphenicol~~, 81% were resistant to high-level streptomycin, 46% to vancomycin and 11% to teicoplanin. All isolates were susceptible to linezolide and quinupristin/dalfopristin. Susceptibility of isolates to ampicillin, vancomycin and teicoplanin are shown in Table 1.

Genotyping of the vancomycin resistance genes by multiplex PCR targeting *vanA,-B,-C* detected the *vanB* gene in all VREf strains and, unexpectedly, an additional *vanA* gene was found in two of them (Table ~~2~~1). None of VREF isolates contained *vanC* genes.

The atypical *vanA/vanB* type VREf strains (VH16 and VH17) were isolated from two debilitated patients: strain VH16 was isolated from a cirrhotic patient admitted at the Internal Digest Department, while strain VH17 was isolated from a patient admitted at the ICU ward, suffering Guillain Barre Syndrome (Table 1). These isolates showed high-level resistance to vancomycin (MICs ≥256 mg/L) and teicoplanin (MICs of ≥256 mg/L and ≥48mg/L, respectively) by E-test (Table ~~2~~1). The *vanB*-type isolates showed a VanB phenotype displaying variable levels of vancomycin MICs from 32 mg/L up to 264 mg/L,

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and were all susceptible to teicoplanin with the exception of one isolate (VH20), which displayed a VanA incongruent phenotype, characterized by high level of resistance to vancomycin (MIC ≥256 mg/L) and teicoplanin (MIC ≥64 mg/L) (Table ~~2~~1). Moreover, four VREf isolates consisting of 3 *vanB*$_2$-type (VH8, VH21 and VH24) and 1 *vanA/vanB*-type (VH16), showed vancomycin heteroresistance, with a growth of sub colonies in the vancomycin inhibition zone of E-test strip corresponding to MICs of >256 mg/L, >96 mg/L, and >32 mg/L (Table ~~2~~1).

All AREf isolates carried the HA marker IS*16*, which was absent in the two ASEf strains (VH27 and VH28) included in the study for comparison. The *esp* and the *hyl* genes were present in the 65% and 38% of isolates respectively (Table ~~2~~1) and were not present in ASEf isolates.

3-3 MLST

To evaluate the relatedness of *E. faecium* under investigation, MLST and EBurst analysis were performed. Analysis results, included in Table ~~2~~1, evidenced the nosocomial origin of the VSEf/VREf isolates that belonged to five different STs, all CC17 related, including ST17 (61,~~,~~5%), ST18 (15%), ST262 (11,5%) a single locus variant (SLV) of ST18, ST78 (7,7%) and the new ST1085 (3,8%). On the contrary, the ASEf isolates from ST904 and the new ST1086, were not CC17 related. Both *vanA/vanB*$_2$ type isolates were of ST17. Nosocomial STs were isolated from numerous wards (Table 1), ~~-~~ST17 was mainly found in ICU and in other 9 wards, in the hospital building 2 and 3, ST18 in General Surgery and other 2 wards (Building 2 and 3), ST262 in Clinical Haematology and Cardiology Emergency wards (Building 1 and 2), while ST78 in Central-ICU and S-ICU (Building 2) ~~(Table 2)~~.

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3.4 WGS analysis of vancomycin resistant and susceptible E. faecium isolates

Nine VREf (7 *vanB* positive and 2 isolates carrying both *vanA* and *vanB*), and 7 VSEf, were selected on the basis of their STs and subjected to WGS. The STs were the following: 10 ST17 (6 VREf and 4 VSEf, including the 2 *vanA/vanB* types), 2 ST18 (1 VREf and 1 VSEf), 2 ST262 (1 VREf and 1 VSEf), 1 ST78 and the new ST1085 (Table ~~2~~1). The WGS of *E. faecium* strains were deposited on BioProject Accession *PRJNA419341.*

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3.4.1 vanB operons and Tn1549 transposon

Sequence analysis of *vanB* operons revealed in all VREf isolates a new highly mutated *vanB*$_2$ operon in a chromosomal integrated Tn*1549*-like transposon, showing 99% of identity with the Tn*1549*-*vanB*$_2$ transposon of reference strain AUS0004 (26612/26624 bp). Comparative BLAST analysis of our *vanB*$_2$-operon sequence showed the highest base pair coverage with sequences of *E. faecium* strains SAU16 (KF823968), followed by TSGH1 (AF310956)~~-~~, UW7606x64/3 TC1 (CP013009) and AUS0004 reference strain (CP003351). It showed also 99% of identity with *Clostridium* spp (AY655720 and AH014495). The vancomycin regulator and the sensor genes of the new *vanB*$_2$ operon showed mutations in *vanR* (G27T) and *vanS* (G313A) genes, resulting in E9D and N105D amino acid changes in the corresponding VanR and VanS proteins. The *vanY* gene showed a A65C mutation (E66A) and a G insertion at position 123bp generating a stop codon and consequently a truncated VanY$_B$ d,d-carboxypeptidase. The *vanW* gene had a T514C mutation translated into a S172P. Moreover, the *vanB* gene showed a C953T substitution generating a P318L amino acid change in the VanB resistance protein. Mutations of the *vanB*$_2$ operon were further confirmed by conventional Sanger sequencing. Tn*1549* transposons showed 100% of identity in all isolates, except for strain VH4 that showed a T356M mutation in the hypothetical protein corresponding to the EFAU004_02789 gene of *E. faecium* AUS0004

reference strain. In all strains the Tn*1549*-like transposon had a common chromosomal insertion site, between genes EFAU004_00610 and EFAU004_00611 according to the reference genome *E. faecium* AUS0004 (Accession no. NC_017022). This hotspot is present in *E. faecium* VREN3305 strain isolated in United Kingdom (Accession no. FXHW01000001.1).

3.4.2. vanA operon, Tn1546 transposon and plasmids

To study the location of the *vanA* operon in the *vanA/vanB*$_2$-type strains (VH16 and VH17), Tn*1546*-harbouring contigs were mapped on the *E. faecium* plasmids pHvH-V24 (Accession no. KX574671) [33] and pJEG40 plasmid (Accession no. KX810025) [34]. The closure of *vanA* operon-harboring plasmid of strain *E. faecium* VH17 resulted in a sequence of 37459bp and showed 99% of sequence identity for 97% of the sequence to both the *E. faecium* pJEG40 and pHvH-V24 plasmids. Assembly of the *E. faecium* VH16 plasmid was not complete due to sequence breakage into many small contigs: a 32726bp sequence was assembled, which showed 99% sequence identity for 88% of the sequence to both the *E. faecium* pJEG40 and pHvH-V24 plasmids and 98% sequence identity for 88% of the sequence to the VH17 pJEG40-like plasmid. VH16 and VH17 pJEG40-like plasmids were also highly similar (98% sequence identity for 77% and 79% of the length, respectively) to the pA698 plasmid carrying the *vanA* resistance operon and described in a *vanA/vanB E. faecium* isolated in Greece [35].

Identity to the *E. faecium* p5357a (Accession no. GQ900435), previously associated as carrier of the same mutated Tn*1546* element, was 99% for only 32% and 36% of the sequence of VH17 and VH16 plasmids, respectively. For this reason, we refer to the newly assembled plasmids in strains VH16 and VH17 as pJEG40-like plasmids.

Both the pJEG40-like plasmids carried the *rep17*$_{pRUM}$ replicon and an identical Tn*1546*-*vanA* operon containing the IS*1251* between the *vanS*-*vanH* intergenic region, showing 100% of sequence identity with several isolates present in database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig 1b). In total, 87 plasmid replicons of 9 *rep*-families were detected by PlasmidFinder in the 16 *E. faecium* strains under study, with a median of 5 *rep* types per isolate (range, 2-9). Gradual increase of reps was found in VSEf/VREf isolates from same STs (Table S1). None of the *rep* types were VRE or VSE specific. The *vanA/vanB*$_2$ isolates shared 6 replicons (*rep*2, *rep*11, *rep*US12, *rep*17, *rep*18 and *rep*US15) while 3 were found only in the VH16 isolate (*rep*14, *rep*22, and *rep*US7). The *rep17*$_{pRUM}$ type replicon was also present in the VSEf VH1 isolate, with small pairing portions with pJEG40-like plasmids (less than 3000 bp).

3.5. WG-SNPs unrooted phylogenetic tree

The wg-SNPs unrooted phylogenetic tree, build with Vietnamese isolates and six reference strains evidenced distinct subclones within ST17, grouping in two genetically related ST17 lineages (Fig 1a), one lineage included VREf isolates (VH8, VH9, VH4, VH16, VH17 and VH20, VH21) and the other included the VSEf isolates (VH23 and VH11, VH13). Also, genetically distinct isolates of ST18 (VH5 and VH6) and ST262 (VH19 and VH22) clustered in 2 related VSEf (VH6 and VH22) and VREf (VH5 and VH19) lineages. The other VSEf isolates VH1 (ST78) and VH26 (ST1085) clustered with reference isolates AUS0085 (ST203) and T110 (ST810), respectively (Fig 1a). The wg-SNPs tree (Fig 1a) revealed that the *vanA/vanB2* type strains VH16 and VH17 were highly genetically related but did not cluster together (Fig.1a, Table S2), in contrast to how they did in the phylogenetic tree build solely on cg-SNPs (core genome) (Fig. 1b). Indeed when we compared the wgSNPs of strains within the ST17, 229 wgSNPs of difference were found

between the *vanA/vanB*$_2$ *strains,* while 29 wgSNPs between ST17 VSEf (VH11, VH13), and just 5 and 9 wgSNPs of difference in VREf isolates VH8-VH9 and VH20-VH21 (Table S2).

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4. Discussion

During the last decades, VREf has emerged worldwide as a relevant nosocomial multidrug-resistant pathogen becoming a serious health concern [36]. This phenomenon is particularly worrisome in low-income countries as many South Asian countries, where the overuse of antibiotics, also in veterinary field and the hospital surveillance are not always adequate [23]. In this study we investigated ampicillin resistant *E faecium* strains isolated during 2014-15, finding a high proportion of multi-drug resistance including vancomycin (46%), with 35% of the isolates being from invasive infections (i.e. blood). This is worrisome and underlines that VREf is a pathogen causing severe hospital infections and compromising treatment options.

To our knowledge, this represents the first study in which Vietnamese nosocomial multi-resistant *E. faecium* isolates were genetically characterized. Our results highlighted the spread among hospital wards of a surprisingly heterogeneous population of HA-VSEf/VREf isolates, composed by five different MLST-CC17 related STs, including the ancestral ST17, ST18 and ST78, responsible of epidemics worldwide [37]. *E. faecium* isolates, particularly those resistant to vancomycin were mainly from ICU and Surgery wards. The study evidenced that the same chromosomal integrated Tn*1549*-like transposon, with a novel highly mutated *vanB*$_2$-operon, conferring high level of vancomycin resistance and the ability to resist to teicoplanin, has spread among *E. faecium* of different STs.

Previous studies showed that *vanB*-type strains resistant to teicoplanin can arise under glycopeptides antibiotic pressure, due to mutations in the N-terminal sensor portion of the VanS$_B$ kinase allowing the inducible expression of resistance by teicoplanin [9,38,39].

Here, we found a VanS mutation (N105D) in that the same sensor portion in all *vanB*$_2$-type isolates under study, suggesting its involvement in the onset of resistance to teicoplanin. Furthermore, the several other mutations detected in the *vanR*$_B$, *vanY*$_B$, *vanW*$_B$ and *vanB* genes might be involved in the high MICs values of vancomycin and teicoplanin displayed by our *vanB*$_2$-type strains, and also in the vancomycin heteroresistance observed in three isolates.

The acquisition of Tn*1549* transposon may have different origins, acquired either from spreading HA-VREf or from community CA-VRE, but also from gut microbiota that can share also mutated vanB$_2$-like transposons (i. e. *Clostridium difficile*) [16,39–43]. Once acquired, VREf can spread easily increasing clinical treatment failures and mortality rate compared to VSEf, as previously described [44].

Moreover, two *vanB*$_2$–type ST17 strains (VH16 and VH17) carried an additional Tn*1546*-*vanA*-like transposon on pJEG40-like plasmids generating the unusual *vanA/vanB*$_2$ genotype with high level glycopeptides MICs (vancomycin ≥256 mg/L and teicoplanin ≥256 mg/L and ≥48 mg/L). A recent study conducted in Australia, associated the same Tn*1546-vanA like* transposon present in our *vanA/vanB*$_2$ isolates to a lower vancomycin and teicoplanin resistance (MICs 2 and 4 mg/L) [34]. These findings suggest that the mutations occurred in *vanB*$_2$ operon rather than the acquisition of the *vanA* operon itself, were responsible for the higher resistance phenotype observed in our *vanA/vanB*$_2$ isolates and as well in the VanA incongruent phenotype -*vanB*-type strain.

Finding both *vanA* and *vanB* determinants in clinical *E. faecium* isolates is unusual, and only few cases have been reported during the last 20 years [11]. Recently, *vanA/vanB*$_2$-type

isolates were described in two clinical isolates, an *E. gallinarum* strain isolated in Canada [45] and an *E. faecium* strain detected in Greece [35], both showing high glycopeptides resistance levels. These strains carried distinct mutated Tn*1549*-*vanB*$_2$ operon and the same Tn*1546*-*vanA* operon present in our *vanA/vanB*$_2$ isolates, on plasmids pA6981 and pA6981-like, which showed high similarity with the pJEG40-like plasmid here described, suggesting the inclination of such plasmids to be acquired by Tn*1549*-*vanB*$_2$ isolates. The comparative genetic analysis of the pJEG40-like plasmids combined with the phylogenetic relationship of our *vanA/vanB*$_2$ isolates did not clarify if two different plasmid acquisition events by two nosocomial related *vanB*$_2$–type ST17 clones, or a single plasmid acquisition, followed by the evolution into a single ST17 *vanB*$_2$ clone, occurred. Additional studies are necessary to clarify the origin of the Tn1546-*vanA* transposon and to better elucidate the rise of the *vanA/vanB*$_2$ genotypes.

MLST typing and eBURST ~~Eburst~~ results of Vietnamese isolates were improved by deeper genomic analysis carried out by wgSNPs and cgSNPs computations and cluster analysis of isolates~~, which~~ The analysis revealed the genetic relatedness between VSEf and VREf isolates of same ST, and evidenced the clonal expansion, particularly among ST17 population, with subclones displaying different assortment of virulence genes and increased numbers of plasmid replicons. In all VREf isolates the presence of the same Tn1549-like transposon with common chromosomal insertion site suggested lateral exchange of Tn*1549*-like element among isolates, with *de novo* generation of VREf, followed by clonal expansion.

Micro-evolution among ST17 isolates, including the vanA/vanB$_2$-type strains, was highlighted by comparing wgSNPs between isolates, which also suggested, in accordance

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with other reports [19,46], a VREf cross-transmission between patients from different wards, with isolates (VH8, VH9) showing just 5 wgSNPs of difference [46].

Notably, the presence of two highly related *vanB*$_2$-type ST17 isolates (wgSNPs=9) -in two patients from different wards showing different glycopeptide resistant phenotypes (VH20/ICU/VanA, VH21/OT/VanB), suggested the involvement of the different antibiotic pressure in the two hospital wards in the regulation of the same mutated *vanB*$_2$ operon.

Infection control strategy, such as improvement of hand hygiene and implementation of antibiotic stewardship (ABS) programs should be undertaken in this hospital to limit the VREf and other nosocomial MDR strains spread.

A limitation of this study resides in the selection of AREf *E. faecium* isolates, therefore other patients colonized with community-acquired *vanA*-VRE susceptible to ampicillin may have gone undetected. Further, future VRE surveillance programs should consider monitoring vancomycin resistant determinants on patients stool by PCR, including the screening of gut microbiota species, gaining the ability to detect possible vancomycin transposon donors.

In conclusion, this study described the spread among different HA-*E. faecium* STs, of a *vanB*$_2$-Tn*1549*-like transposon able to confer resistance to teicoplanin, which is for this reason strongly inadvisable.

The presence of the Tn*1546*-like-pJEG40-like plasmids in two high related *vanB*$_2$–type ST17 isolates contributed to the heterogeneity of VREf in this hospital. The de *novo* generation of VREf from VSEf strains suggests that, in addition to VREf, also VSEf (AREf) clones should be monitored in the patients and in the hospital environment.

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Our findings provide insights useful for infection control of nosocomial *E. faecium* and more importantly for clinical practice. Rational use of glycopeptides and effective surveillance measures are highly required in this and all hospitals to reduce VSEF/VREf spread and to avoid the rise of unusual and misleading VREf genotypes.

Declarations

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Competing Interests: No conflicts

Ethical Approval: Not required

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Figure Legend

Figure. 1. Unrooted parsimony tree based on wgSNPs (A) and core SNPs (B). Trees are consensus trees of the equally most parsimonious trees from a sample of 100 trees based on an Extended Majority Rule. Internal node labels show the support for each node as calculated by FastTreeMP. Branch lengths are expressed in terms of changes per number of SNPs. Strains branching in the two major clades are color coded based on their ST. VREf and VSEf clades are highlighted in grey and cyan, respectively. Six reference strains were included in the comparison Aus0004 (Acc. no. CP00335), Aus0085 (Acc. no. CP006620), NRRL B-2354 (Acc. no. NCP004063), DO (Acc. no. CP003583), T110 (Acc. no. CP006030), VREF_286 (Acc. no. CP019992).