

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

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1 **Emergence of unusual *vanA/vanB<sub>2</sub>* genotype in a highly mutated *vanB<sub>2</sub>*-**  
2 **Vancomycin Resistant Hospital Associated *E. faecium* background, in**  
3 **Vietnam**

4

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24 **Running title:** Characterization of *vanB<sub>2</sub>*- and *vanA/vanB<sub>2</sub>*-types Hospital Associated

25 Vancomycin Resistant *E. faecium*.

26 **Abstract**

27 *Enterococcus faecium* has become a globally disseminated nosocomial pathogen  
28 principally as the consequence of acquisition and diffusion of virulence factors and  
29 multidrug resistance determinants, including glycopeptides, one of the last resort  
30 antimicrobials used to treat more serious infections that usually occur in high-risk patients.  
31 In this study we investigated and molecular characterized Hospital-Associated (HA)  
32 *Enterococcus faecium* isolates collected at Hue Central Hospital, Vietnam.  
33 Our results highlighted the spread among hospital wards of a surprisingly heterogeneous  
34 multi drugs resistant *E. faecium* population, composed by five different CC17-related STs,  
35 of which 46% VREf carrying the *vanB* gene. Whole genome Sequencing of selected *E.*  
36 *faecium* isolates showed that VREf from different STs carried the same chromosomal  
37 integrated Tn1549-like transposon, with a highly mutated vanB2-operon, showing an  
38 increased level of vancomycin resistance (VanB phenotype) and able, in one isolate, to  
39 confer resistance to teicoplanin (VanA incongruent phenotype).  
40 Two unusual *vanA/vanB*<sub>2</sub>-type strains were detected within the *vanB*<sub>2</sub>-type ST17  
41 population, harbouring a Tn1546-*vanA*-like transposon in pJEG40-like plasmids. Wg-  
42 SNPs-based analysis evidenced the genetic relatedness of VSEf/VREf of same STs and  
43 suggested lateral exchange of the Tn1549-like element among isolates followed by clonal  
44 expansion. Particularly microevolution among ST17 isolates, including the *vanA/vanB*<sub>2</sub>-  
45 type strains, and inter-wards VREf transmission were highlighted.  
46 The use of teicoplanin is strongly discouraged in the study's hospital for the spreading of  
47 Tn1549-*vanB*<sub>2</sub> associated to teicoplanin resistance. A rational use of glycopeptides and  
48 effective surveillance measures are always required to reduce nosocomial VSEf/VREf  
49 spread and to avoid the rise of unusual and misleading VREf genotypes.

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

## 51 **1. Introduction**

52 Due to the clinical significance [1] and the capability to spread by cross-contamination  
53 leading to local outbreaks [2,3]. Hospital Associated-Vancomycin Resistance  
54 *Enterococcus faecium* (HA-VREf) is currently cause of increasing concerns, and was  
55 recently included in the seven “ESKAPE” nosocomial bacteria under global surveillance  
56 [4]. The HA-VREf originates from a specific Vancomycin Susceptible *E. faecium* (VSEf)  
57 subpopulation, characterized by Ampicillin Resistance (HA-AREf) [2,5] and the carriage  
58 of IS16 [6] insertion sequence which allows an enhanced genetic plasticity and an easier  
59 acquisition of mobile genetic elements (MGE), vehicles of antimicrobial resistance genes  
60 [7]. The potential acquisition of vancomycin resistance determinants leads to the rise of  
61 VREf, threatening our capability to treat more serious infections that can occur especially  
62 in high-risk patients [8]. In total, eight types of acquired vancomycin resistance genotypes  
63 are known in enterococci, with *vanA* and *vanB* genotypes being the most represented in  
64 clinical isolates [9–11]. The former confers high inducible resistance to both vancomycin  
65 (MIC >64 mg/L) and teicoplanin (MICs from 4 up to >64 mg/L) and is mainly associated  
66 with the Tn1546 transposon, often found as a part of non-conjugative or conjugative  
67 plasmids, while the latter confers lower levels of inducible resistance only to vancomycin  
68 (MIC ranging from 4 to 32mg/L) and is predominantly found as integral part of the  
69 Tn1549/Tn5382-like conjugative transposons of chromosomal origin [12,13]. Resistance to  
70 glycopeptides results from the production of d-Ala-d-Lac instead of d-Ala-d-Ala in cell  
71 wall synthesis due to the presence of the *vanA* (*vanHAXYZ*) and *vanB* (*vanY<sub>B</sub>W<sub>B</sub>H<sub>B</sub>X<sub>B</sub>*)  
72 operons regulated at the transcription level by two-component system genes (*vanR–vanS*)  
73 [9]. Hospital-associated *E. faecium* clones can also acquire several factors, increasing  
74 adaptation and out-competing *E. faecium* commensal clones, as the enterococcal surface

75 protein (*esp*), that enhance colonization ability promoting adhesion and biofilm formation  
76 [14], and the glycoside hydrolase (*hyl*) also involved in intestinal colonization [15].  
77 Molecular epidemiology studies by Whole Genome Sequencing (WGS) have shown that  
78 HA-*E. faecium* clones are genetically grouped in a polyclonal cluster designed Clade A1  
79 based on core genome single nucleotide polymorphisms (cgSNPs) [10,16] and cgMLST  
80 [17], which includes Sequence Types (STs) Clonal Complex 17 (CC17) related, based on  
81 MLST and EBurst analysis [18]. Phylogenetic analysis on cgSNPs and wgSNPs can deeply  
82 discriminate clones of the same STs detecting and tracking nosocomial outbreaks [19].

83

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

88

89 In this study, we aimed to assess the presence of HA-*E. faecium* at the Hue Central  
90 Hospital, Vietnam, and to molecularly characterize isolates including WGS of representative  
91 isolates. To date, only few data on VREf are available from South-East Asian countries  
92 [20–23] and to our knowledge, this is the first study describing clinical VREf genotypes in  
93 Vietnam.

94

95

96 **2. Material and Methods**

97 *2.1 Strains and antimicrobial susceptibility*

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

107

108 *2.2 DNA extraction and PCRs*

109 Bacterial DNA was extracted with a DNeasy Blood & Tissue Kit (QIAGEN, Inc.,  
110 Valencia, CA). A multiplex PCR was performed to detect the glycopeptide resistance  
111 genes *vanA*, *vanB*, *vanC1*, *vanC2/C3*, and the relevant *Enterococcus* species specific  
112 genes, by using the modified protocol of Kariyama et al. [24,25]. The presence of *IS16*, *esp*  
113 and *hyl* genes, was performed by PCR and multiplex-PCR respectively, as previously  
114 described [6,26].

115

116 *2.3 MLST*

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

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

124

#### 125 *2.4 Whole Genome Sequencing (WGS) and analysis*

126 Sixteen isolates (9 VREf and 7 VSEf), selected on the basis of their MLST STs were  
127 chosen to span the time period of the study and to include multiple wards, sources and  
128 genotype including the 2 *vanA/vanB* isolates, and then submitted to WGS using a  
129 HiScanSQ Illumina platform (Porto Conte Ricerche Srl, Tramariglio, Italy). Generated  
130 sequences were assembled *de novo* into contigs using Velvet 1.2.10  
131 (<http://www.ebi.ac.uk/~zerbino/velvet/>). Contigs were reordered against the reference  
132 genome of *E. faecium* Aus0004 (Accession no. NC\_017022) using Mauve. Genomic  
133 comparison with reference strains *E. faecium* Aus0004 and *E. faecium* Aus0085  
134 (Accession no. CP006620) was performed using Artemis Comparative Tool (ACT) [27]  
135 and MUMmer [28]. Genomes were submitted to the RAST platform for annotation  
136 (<http://rast.nmpdr.org>). The NUCmer tool of the MUMmer software was used to search  
137 individual sequences within the genomes.

138

#### 139 *2.5 Transposons and plasmid assembly*

140 The Tn1546 and Tn1549 harboring contigs identified using NUCmer were extracted from  
141 the *de novo* assemblies followed by a BLASTn search against publicly available plasmid  
142 sequences in GenBank. If a contig coexisted with other plasmid core elements and showed  
143 >99% identity to and >90% query coverage of a known plasmid, the contig was  
144 preliminarily classified as the reference-like plasmid (e.g. pJEG040 or pHvH-V24). The  
145 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  $\geq 98\%$  sequence identity over  $\geq 80\%$  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



164 **3. Results**

165 *3.1 Epidemiological background*

166 A total of 26 AREf isolates were identified from January 2014 to June 2015 from patients  
167 admitted at the Hue Central Hospital, Vietnam. Sources, wards origins and characteristics  
168 of isolates, are summarized in Table 1. The most common wards were Surgery wards  
169 (54%) and Intensive Care Units ICU (15%). Isolates were mainly from blood (31%) and  
170 pus (38%) and the rest from urine, catheter and ascitic fluid.

171

172 *3.2 Antimicrobial susceptibility and PCRs*

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

178

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

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

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

196

197 All AREf isolates carried the HA marker *IS16*, which was absent in the two ASEf strains  
198 (VH27 and VH28) included in the study for comparison. The *esp* and the *hyl* genes were  
199 present in the 65% and 38% of isolates respectively (Table 1) and were not present in  
200 ASEf isolates.

201

### 202 3-3 MLST

203 To evaluate the relatedness of *E. faecium* under investigation, MLST and EBurst analysis  
204 were performed. Analysis results, included in Table 1, evidenced the nosocomial origin of  
205 the VSEf/VREf isolates that belonged to five different STs, all CC17 related, including  
206 ST17 (61;5%), ST18 (15%), ST262 (11,5%) a single locus variant (SLV) of ST18, ST78  
207 (7,7%) and the new ST1085 (3,8%). On the contrary, the ASEf isolates from ST904 and  
208 the new ST1086, were not CC17 related. Both *vanA/vanB*<sub>2</sub> type isolates were of ST17.

209 Nosocomial STs were isolated from numerous wards (Table 1), ST17 was mainly found in  
210 ICU and in other 9 wards, in the hospital building 2 and 3, ST18 in General Surgery and  
211 other 2 wards (Building 2 and 3), ST262 in Clinical Haematology and Cardiology  
212 Emergency wards (Building 1 and 2), while ST78 in Central-ICU and S-ICU (Building 2).

213

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*.

220

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<sub>2</sub>* operon in a chromosomal integrated *Tn1549*-like transposon, showing 99% of  
224 identity with the *Tn1549-vanB<sub>2</sub>* transposon of reference strain AUS0004 (26612/26624 bp).  
225 Comparative BLAST analysis of our *vanB<sub>2</sub>* 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<sub>2</sub>* 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<sub>B</sub> 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<sub>2</sub>* operon were  
236 further confirmed by conventional Sanger sequencing. *Tn1549* 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

239 reference strain. In all strains the Tn1549-like transposon had a common chromosomal  
240 insertion site, between genes EFAU004\_00610 and EFAU004\_00611 according to the  
241 reference genome *E. faecium* AUS0004 (Accession no. NC\_017022). This hotspot is  
242 present in *E. faecium* VREN3305 strain isolated in United Kingdom (Accession no.  
243 FXHW01000001.1).

244

#### 245 3.4.2. *vanA* operon, Tn1546 transposon and plasmids

246 To study the location of the *vanA* operon in the *vanA/vanB*<sub>2</sub>-type strains (VH16 and  
247 VH17), Tn1546-harboured contigs were mapped on the *E. faecium* plasmids pHvH-V24  
248 (Accession no. KX574671) [33] and pJEG40 plasmid (Accession no. KX810025) [34].  
249 The closure of *vanA* operon-harboring plasmid of strain *E. faecium* VH17 resulted in a  
250 sequence of 37459bp and showed 99% of sequence identity for 97% of the sequence to  
251 both the *E. faecium* pJEG40 and pHvH-V24 plasmids. Assembly of the *E. faecium* VH16  
252 plasmid was not complete due to sequence breakage into many small contigs: a 32726bp  
253 sequence was assembled, which showed 99% sequence identity for 88% of the sequence to  
254 both the *E. faecium* pJEG40 and pHvH-V24 plasmids and 98% sequence identity for 88%  
255 of the sequence to the VH17 pJEG40-like plasmid. VH16 and VH17 pJEG40-like plasmids  
256 were also highly similar (98% sequence identity for 77% and 79% of the length,  
257 respectively) to the pA698 plasmid carrying the *vanA* resistance operon and described in a  
258 *vanA/vanB E. faecium* isolated in Greece [35].

259

260 Identity to the *E. faecium* p5357a (Accession no. GQ900435), previously associated as  
261 carrier of the same mutated Tn1546 element, was 99% for only 32% and 36% of the  
262 sequence of VH17 and VH16 plasmids, respectively. For this reason, we refer to the newly  
263 assembled plasmids in strains VH16 and VH17 as pJEG40-like plasmids.

264 Both the pJEG40-like plasmids carried the *repI7*<sub>PRUM</sub> replicon and an identical Tn1546-  
265 *vanA* operon containing the IS1251 between the *vanS-vanH* intergenic region, showing  
266 100% of sequence identity with several isolates present in database  
267 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Fig 1b). In total, 87 plasmid replicons of 9 *rep*-  
268 families were detected by PlasmidFinder in the 16 *E. faecium* strains under study, with a  
269 median of 5 *rep* types per isolate (range, 2-9). Gradual increase of reps was found in  
270 VSEf/VREf isolates from same STs (Table S1). None of the *rep* types were VRE or VSE  
271 specific. The *vanA/vanB*<sub>2</sub> isolates shared 6 replicons (*rep2*, *rep11*, *repUS12*, *rep17*, *rep18*  
272 and *repUS15*) while 3 were found only in the VH16 isolate (*rep14*, *rep22*, and *repUS7*).  
273 The *repI7*<sub>PRUM</sub> type replicon was also present in the VSEf VH1 isolate, with small pairing  
274 portions with pJEG40-like plasmids (less than 3000 bp).

275

### 276 3.5. WG-SNPs unrooted phylogenetic tree

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

289 between the *vanA/vanB<sub>2</sub>* strains, while 29 wgSNPs between ST17 VSEf (VH11, VH13),  
290 and just 5 and 9 wgSNPs of difference in VREf isolates VH8-VH9 and VH20-VH21  
291 (Table S2).

292

293

294

#### 295 4. Discussion

296 During the last decades, VREf has emerged worldwide as a relevant nosocomial  
297 multidrug-resistant pathogen becoming a serious health concern [36]. This phenomenon is  
298 particularly worrisome in low-income countries as many South Asian countries, where the  
299 overuse of antibiotics, also in veterinary field and the hospital surveillance are not always  
300 adequate [23]. In this study we investigated ampicillin resistant *E. faecium* strains isolated  
301 during 2014-15, finding a high proportion of multi-drug resistance including vancomycin  
302 (46%), with 35% of the isolates being from invasive infections (i.e. blood). This is  
303 worrisome and underlines that VREf is a pathogen causing severe hospital infections and  
304 compromising treatment options.

305

306 To our knowledge, this represents the first study in which Vietnamese nosocomial multi-  
307 resistant *E. faecium* isolates were genetically characterized. Our results highlighted the  
308 spread among hospital wards of a surprisingly heterogeneous population of HA-  
309 VSEf/VREf isolates, composed by five different MLST-CC17 related STs, including the  
310 ancestral ST17, ST18 and ST78, responsible of epidemics worldwide [37]. *E. faecium*  
311 isolates, particularly those resistant to vancomycin were mainly from ICU and Surgery  
312 wards. The study evidenced that the same chromosomal integrated Tn1549-like  
313 transposon, with a novel highly mutated *vanB*<sub>2</sub>-operon, conferring high level of  
314 vancomycin resistance and the ability to resist to teicoplanin, has spread among *E. faecium*  
315 of different STs.

316

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

320 Here, we found a VanS mutation (N105D) in that the same sensor portion in all *vanB*<sub>2</sub>-type  
321 isolates under study, suggesting its involvement in the onset of resistance to teicoplanin.  
322 Furthermore, the several other mutations detected in the *vanR*<sub>B</sub>, *vanY*<sub>B</sub>, *vanW*<sub>B</sub> and *vanB*  
323 genes might be involved in the high MICs values of vancomycin and teicoplanin displayed  
324 by our *vanB*<sub>2</sub>-type strains, and also in the vancomycin heteroresistance observed in three  
325 isolates.

326

327 The acquisition of Tn1549 transposon may have different origins, acquired either from  
328 spreading HA-VREf or from community CA-VRE, but also from gut microbiota that can  
329 share also mutated *vanB*<sub>2</sub>-like transposons (i. e. *Clostridium difficile*) [16,39–43]. Once  
330 acquired, VREf can spread easily increasing clinical treatment failures and mortality rate  
331 compared to VSEf, as previously described [44].

332

333 Moreover, two *vanB*<sub>2</sub>-type ST17 strains (VH16 and VH17) carried an additional Tn1546-  
334 *vanA*-like transposon on pJEG40-like plasmids generating the unusual *vanA/vanB*<sub>2</sub>  
335 genotype with high level glycopeptides MICs (vancomycin  $\geq 256$  mg/L and teicoplanin  
336  $\geq 256$  mg/L and  $\geq 48$  mg/L). A recent study conducted in Australia, associated the same  
337 Tn1546-*vanA* like transposon present in our *vanA/vanB*<sub>2</sub> isolates to a lower vancomycin  
338 and teicoplanin resistance (MICs 2 and 4 mg/L) [34]. These findings suggest that the  
339 mutations occurred in *vanB*<sub>2</sub> operon rather than the acquisition of the *vanA* operon itself,  
340 were responsible for the higher resistance phenotype observed in our *vanA/vanB*<sub>2</sub> isolates  
341 and as well in the VanA incongruent phenotype -*vanB*-type strain.

342

343 Finding both *vanA* and *vanB* determinants in clinical *E. faecium* isolates is unusual, and  
344 only few cases have been reported during the last 20 years [11]. Recently, *vanA/vanB*<sub>2</sub>-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 Tn1549-*vanB*<sub>2</sub> operon and the same  
348 Tn1546-*vanA* operon present in our *vanA/vanB*<sub>2</sub> 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 Tn1549-*vanB*<sub>2</sub> isolates. The  
351 comparative genetic analysis of the pJEG40-like plasmids combined with the phylogenetic  
352 relationship of our *vanA/vanB*<sub>2</sub> isolates did not clarify if two different plasmid acquisition  
353 events by two nosocomial related *vanB*<sub>2</sub>-type ST17 clones, or a single plasmid acquisition,  
354 followed by the evolution into a single ST17 *vanB*<sub>2</sub> 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*<sub>2</sub> 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 Tn1549-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*<sub>2</sub>-type strains, was  
368 highlighted by comparing wgSNPs between isolates, which also suggested, in accordance

369 with other reports [19,46], a VREf cross-transmission between patients from different  
370 wards, with isolates (VH8, VH9) showing just 5 wgSNPs of difference [46].  
371 Notably, the presence of two highly related *vanB*<sub>2</sub>-type ST17 isolates (wgSNPs=9) in two  
372 patients from different wards showing different glycopeptide resistant phenotypes  
373 (VH20/ICU/VanA, VH21/OT/VanB), suggested the involvement of the different antibiotic  
374 pressure in the two hospital wards in the regulation of the same mutated *vanB*<sub>2</sub> operon.  
375 Infection control strategy, such as improvement of hand hygiene and implementation of  
376 antibiotic stewardship (ABS) programs should be undertaken in this hospital to limit the  
377 VREf and other nosocomial MDR strains spread.

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

384

385 In conclusion, this study described the spread among different HA-*E. faecium* STs, of a  
386 *vanB*<sub>2</sub>-Tn1549-like transposon able to confer resistance to teicoplanin, which is for this  
387 reason strongly inadvisable.

388

389 The presence of the Tn1546-like-pJEG40-like plasmids in two high related *vanB*<sub>2</sub>-type  
390 ST17 isolates contributed to the heterogeneity of VREf in this hospital. The *de novo*  
391 generation of VREf from VSEf strains suggests that, in addition to VREf, also VSEf  
392 (AREf) clones should be monitored in the patients and in the hospital environment.

393

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

398

#### 399 **Declarations**

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402 **Competing Interests:** No conflicts

403 **Ethical Approval:** Not required

404

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- 562
- 563
- 564

565 **Figure Legend**

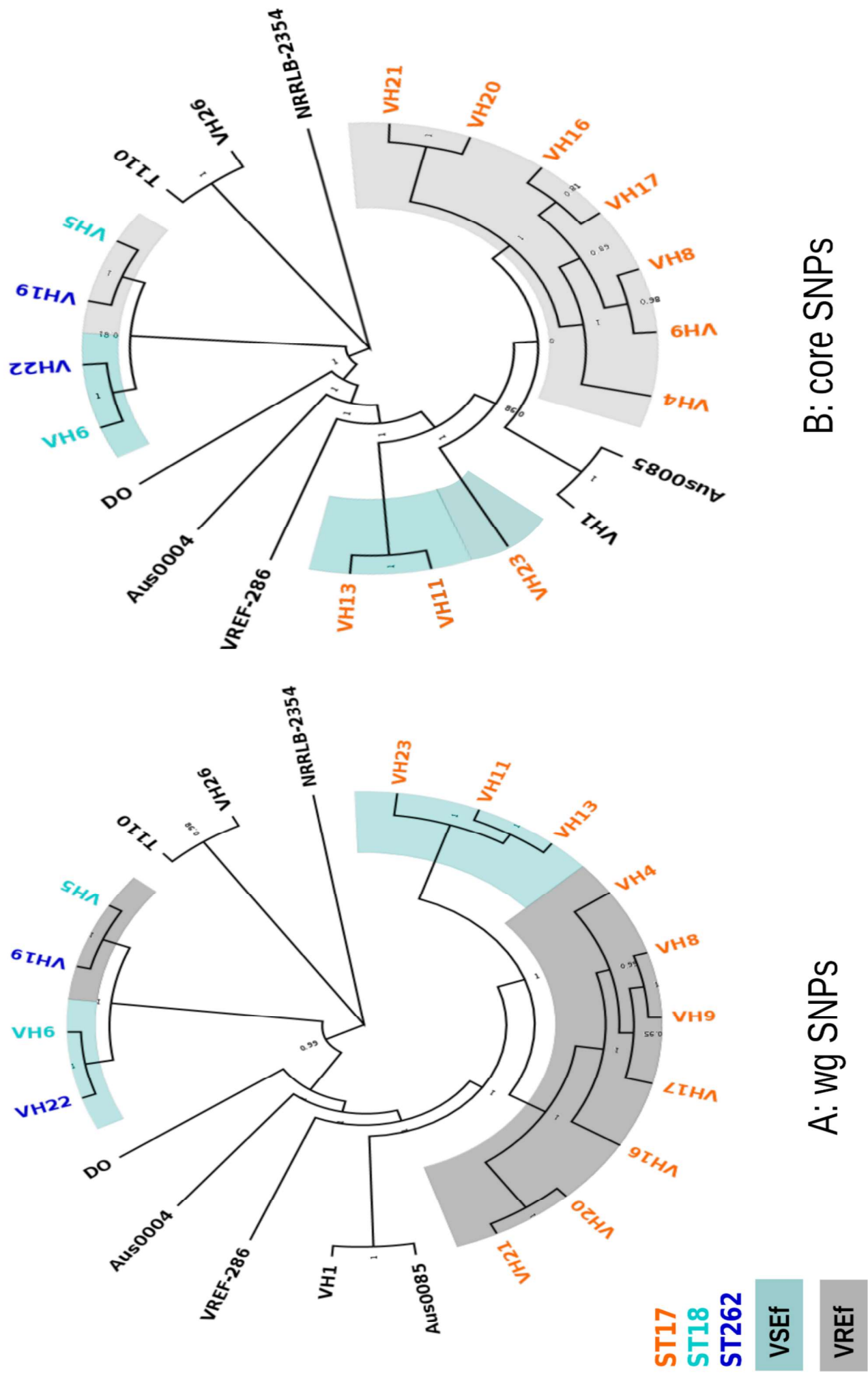
566 **Figure. 1.** Unrooted parsimony tree based on wgSNPs (A) and core SNPs (B). Trees are  
567 consensus trees of the equally most parsimonious trees from a sample of 100 trees based  
568 on an Extended Majority Rule. Internal node labels show the support for each node as  
569 calculated by FastTreeMP. Branch lengths are expressed in terms of changes per number  
570 of SNPs. Strains branching in the two major clades are color coded based on their ST.  
571 VREf and VSEf clades are highlighted in grey and cyan, respectively. Six reference  
572 strains were included in the comparison Aus0004 (Acc. no. CP00335), Aus0085 (Acc. no.  
573 CP006620), NRRL B-2354 (Acc. no. NCP004063), DO (Acc. no. CP003583), T110  
574 (Acc. no. CP006030), VREF\_286 (Acc. no. CP019992).

**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 <sub>2</sub>	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 <sup>th</sup> floor - B2	LTN	R >8	1.0	0.25	S	-	ST17	+	-	+
VH13	07/10/2014	Pus	Sa	5 <sup>th</sup> floor - B2	Ureteral perforation	R >8	1.5	0.125	S	-	ST17	+	-	+
VH1	23/06/2014	Pus	S-ICU	6 <sup>th</sup> floor - B2	Head trauma	R >8	0.50	0.25	S	-	ST78	+	-	+
VH9	26/08/2014	Pus	S-ICU	6 <sup>th</sup> floor - B2	Postoperative ileus	R >8	≥256	0.125	VanB	vanB <sub>2</sub>	ST17	+	+	+
VH10	31/08/2014	Pus	S-ICU	6 <sup>th</sup> floor - B2	Pleuritis	R >8	0.50	0.25	S	-	ST17	+	-	+
VH28	19/06/2015	Pus	S-ICU	6 <sup>th</sup> floor - B2	Abscess	S=1	1.0	0.25	S	-	ST1086	-	-	-
VH2	09/07/2014	Blood	C-ICU	6 <sup>th</sup> floor - B2	Multiple injuries	R >8	1.0	0.25	S	-	ST78	+	-	+
VH3	11/07/2014	Blood	C-ICU	6 <sup>th</sup> floor - B2	Head trauma	R >8	0.50	0.25	S	-	ST17	+	-	+
VH17	05/04/2015	Catheter	C-ICU	6 <sup>th</sup> floor - B2	GBS	R >8	≥256	≥48	VanA	vanA/ vanB <sub>2</sub>	ST17	+	+	+
VH20	28/03/2014	Blood	C-ICU	6 <sup>th</sup> floor - B2	Meningitis	R >8	≥256	R≥64	VanA	vanB <sub>2</sub>	ST17	+	+	+
VH4	15/07/2014	Urine	UKS	2nd floor - B3	Bladder cancer	R >8	≥64	0.19	VanB	vanB <sub>2</sub>	ST17	+	+	-
VH5	17/07/2014	Urine	UKS	2nd floor - B3	BPH	R >8	≥256	0.25	VanB	vanB <sub>2</sub>	ST18	+	-	-
VH16*	25/12/2014	Ascites	ID	3th floor - B3	Cirrhosis	R >8	≥256	≥256	VanA	vanA / vanB <sub>2</sub>	ST17	+	+	+
VH24*	23/05/2015	Urine	G	3th floor - B3	FUO	R >8	≥32	0.25	VanB	vanB <sub>2</sub>	ST17	+	+	+
VH6	18/07/2014	Catheter	CS	4 <sup>th</sup> floor - B3	PPL	R >8	1.0	0.25	S	-	ST18	+	-	-
VH12	09/09/2014	Urine	GS	4 <sup>th</sup> floor - B3	Post Studer surgery	R >8	≥256	0.19	VanB	vanB <sub>2</sub>	ST18	+	-	-
VH25	16/06/2015	Blood	GS	4 <sup>th</sup> floor - B3	PFLCD	R >8	1.5	0.125	S	-	ST18	+	+	-
VH8*	24/08/2014	Urine	GS	4 <sup>th</sup> floor - B3	Bladder tumor	R >8	≥256	0.25	VanB	vanB <sub>2</sub>	ST17	+	+	+
VH11	08/09/2014	Pus	GS	4 <sup>th</sup> floor - B3	Acute cholecystitis	R >8	0.50	0.25	S	-	ST17	+	-	+
VH15	05/12/2014	Blood	GS	4 <sup>th</sup> floor - B3	Sepsis	R >8	≥256	0.50	VanB	vanB <sub>2</sub>	ST17	+	-	+
VH21*	06/05/2014	Pus	OT	4 <sup>th</sup> floor - B3	Abscess	R >8	≥96	0.38	VanB	vanB <sub>2</sub>	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 = Neonatal Pediatric Emergency; NPE = Neonatal Pediatric Emergency; CML = Chronic myelogenous leukemia; FUIO = 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



**Supplementary data**

[Click here to download Supplementary data: Suppl Table S1 new.xlsx](#)

1 **Emergence of unusual *vanA/vanB*<sub>2</sub> genotype in a highly mutated *vanB*<sub>2</sub>-**  
2 **Vancomycin Resistant Hospital Associated *E. faecium* background, in**  
3 **Vietnam**

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24 **Running title:** Characterization of *vanB*<sub>2</sub>- and *vanA/vanB*<sub>2</sub>-types Hospital Associated  
25 Vancomycin Resistant *E. faecium*.

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26 **Abstract**

27 *Enterococcus faecium* has become a globally disseminated nosocomial pathogen  
28 principally as the consequence of acquisition and diffusion of virulence factors and  
29 multidrug resistance determinants, including glycopeptides, one of the last resort  
30 antimicrobials used to treat more serious infections that usually occur in high-risk patients.  
31 In this study we investigated and molecular characterized Hospital-Associated (HA)  
32 *Enterococcus faecium* isolates collected at Hue Central Hospital, Vietnam.  
33 Our results highlighted the spread among hospital wards of a surprisingly heterogeneous  
34 multi drugs resistant *E. faecium* population, composed by five different CC17-related STs,  
35 of which 46% VREf carrying the *vanB* gene. Whole genome Sequencing of selected *E.*  
36 *faecium* isolates showed that VREf from different STs carried the same **chromosomal**  
37 **chromosomal** integrated Tn1549-like transposon, with a highly mutated vanB2-operon,  
38 showing an increased level of vancomycin resistance (VanB phenotype) and able, in one  
39 isolate, to confer resistance to teicoplanin (VanA incongruent phenotype).  
40 Two unusual *vanA/vanB<sub>2</sub>*-type strains were detected within the *vanB<sub>2</sub>-type ST17*  
41 population, harbouring a Tn1546-*vanA*-like transposon in pJEG40-like plasmids. Wg-  
42 SNPs-based analysis evidenced the genetic relatedness of VSEf/VREf of same STs and  
43 suggested lateral exchange of the Tn1549-like element among isolates followed by clonal  
44 expansion. Particularly microevolution among ST17 isolates, including the *vanA/vanB<sub>2</sub>*-  
45 type strains, and inter-wards VREf transmission were highlighted.  
46 The use of teicoplanin is strongly discouraged in the study's hospital for the spreading of  
47 Tn1549-*vanB<sub>2</sub>* associated to teicoplanin resistance. A rational use of glycopeptides and  
48 effective surveillance measures are always required to reduce nosocomial VSEF/VREf  
49 spread and to avoid the rise of unusual and misleading VREf genotypes.

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

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51 **1. Introduction**

52 Due to the clinical significance [1] and the capability to spread by cross-contamination  
53 leading to local outbreaks [2,3]. Hospital Associated-Vancomycin Resistance  
54 *Enterococcus faecium* (HA-VREf) is currently cause of increasing concerns, and was  
55 recently included in the seven “ESKAPE” nosocomial bacteria under global surveillance  
56 [4]. The HA-VREf originates from a specific Vancomycin Susceptible *E. faecium* (VSEf)  
57 subpopulation, characterized by Ampicillin Resistance (HA-AREf) [2,5] and the carriage  
58 of IS16 [6] insertion sequence which allows an enhanced genetic plasticity and an easier  
59 acquisition of mobile genetic elements (MGE), vehicles of antimicrobial resistance genes  
60 [7]. The potential acquisition of vancomycin resistance determinants leads to the rise of  
61 VREf, threatening our capability to treat more serious infections that can occur especially  
62 in high-risk patients [8]. In total, eight types of acquired vancomycin resistance genotypes  
63 are known in enterococci, with *vanA* and *vanB* genotypes being the most represented in  
64 clinical isolates [9–11]. The former confers high inducible resistance to both vancomycin  
65 (MIC >64 mg/L) and teicoplanin (MICs from 4 up to >64 mg/L) and is mainly associated  
66 with the Tn1546 transposon, often found as a part of non-conjugative or conjugative  
67 plasmids, while the latter confers lower levels of inducible resistance only to vancomycin  
68 (MIC ranging from 4 to 32mg/L) and is predominantly found as integral part of the  
69 Tn1549/Tn5382-like conjugative transposons of chromosomal origin [12,13]. Resistance to  
70 glycopeptides results from the production of d-Ala-d-Lac instead of d-Ala-d-Ala in cell  
71 wall synthesis due to the presence of the *vanA* (*vanHAXYZ*) and *vanB* (*vanY<sub>B</sub>W<sub>B</sub>H<sub>B</sub>BX<sub>B</sub>*)  
72 operons regulated at the transcription level by two-component system genes (*vanR–vanS*)  
73 [9]. Hospital-associated *E. faecium* clones can also acquire several factors, increasing  
74 adaptation and out-competing *E. faecium* commensal clones, as the enterococcal surface

75 protein (*esp*), that enhance colonization ability promoting adhesion and biofilm formation  
76 [14], and the glycoside hydrolase (*hyl*) also involved in intestinal colonization [15].  
77 Molecular epidemiology studies by Whole Genome Sequencing (WGS) have shown that  
78 HA-*E. faecium* clones are genetically grouped in a polyclonal cluster designed Clade A1  
79 based on core genome single nucleotide polymorphisms (cgSNPs) [10,16] and cgMLST  
80 [17], which includes Sequence Types (STs) Clonal Complex 17 (CC17) related, based on  
81 MLST and EBurst analysis [18]. Phylogenetic analysis on cgSNPs and wgSNPs can deeply  
82 discriminate clones of the same STs detecting and tracking nosocomial outbreaks [19].

83

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

88

89 In this study, we aimed to assess the presence of HA-*E. faecium* at the Hue Central  
90 Hospital, Vietnam, and to molecular characterize isolates including WGS of representative  
91 isolates. To date, only few data on VREf are available from South-East Asian countries  
92 [20–23] and to our knowledge, this is the first study describing clinical VREf genotypes in  
93 Vietnam.

94

95

96 **2. Material and Methods**

97 *2.1 Strains and antimicrobial susceptibility*

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

107

108 *2.2 DNA extraction and PCRs*

109 Bacterial DNA was extracted with a DNeasy Blood & Tissue Kit (QIAGEN, Inc.,  
110 Valencia, CA). A multiplex PCR was performed to detect the glycopeptide resistance  
111 genes *vanA*, *vanB*, *vanC1*, *vanC2/C3*, and the relevant *Enterococcus* species specific  
112 genes, by using the modified protocol of Kariyama et al. [24,25]. The presence of *IS16*, *esp*  
113 and *hyl* genes, was performed by PCR and multiplex-PCR respectively, as previously  
114 described [6,26].

115

116 *2.3 MLST*

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

121 (<http://www.geneious.com/>). Allelic profiles and STs were assigned according to the *E.*  
122 *faecium* MLST database (<http://pubmlst.org/efaecium/>). CC17 related clones were  
123 identified by ~~EBurst3v~~ **EBURST\_V3** analysis (<http://eburst.mlst.net>).

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#### 125 2.4 Whole Genome Sequencing (WGS) and analysis


126 Sixteen isolates (9 VREf and 7 VSEf), selected on the basis of their MLST STs were  
127 chosen to span the time period of the study and to include multiple wards, sources and  
128 genotype including the 2 *vanA/vanB* isolates, and then submitted to WGS using a  
129 HiScanSQ Illumina platform (Porto Conte Ricerche Srl, Tramariglio, Italy). Generated  
130 sequences were assembled *de novo* into contigs using Velvet 1.2.10  
131 (<http://www.ebi.ac.uk/~zerbino/velvet/>). Contigs were reordered against the reference  
132 genome of *E. faecium* Aus0004 (Accession no. NC\_017022) using Mauve. Genomic  
133 comparison with reference strains *E. faecium* Aus0004 and *E. faecium* Aus0085  
134 (Accession no. CP006620) was performed using Artemis Comparative Tool (ACT) [27]  
135 and MUMmer [28]. Genomes were submitted to the RAST platform for annotation  
136 (<http://rast.nmpdr.org>). The NUCmer tool of the MUMmer software was used to search  
137 individual sequences within the genomes.

138

#### 139 2.5 Transposons and plasmid assembly

140 The Tn1546 and Tn1549 harboring contigs identified using NUCmer were extracted from  
141 the *de novo* assemblies followed by a BLASTn search against publicly available plasmid  
142 sequences in GenBank. If a contig coexisted with other plasmid core elements and showed  
143 >99% identity to and >90% query coverage of a known plasmid, the contig was  
144 preliminarily classified as the reference-like plasmid (e.g. pJEG040 or pHvH-V24). The  
145 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  $\geq 98\%$  sequence identity over  $\geq 80\%$  of the length of the reference. The presence  
150 of  $\phi$ -plasmid replicons was performed *in silico* using available service (PlasmidFinder) from  
151 Center of Genomic Epidemiology (CGE) (<http://www.genomicepidemiology.org/>).

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### 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

164 **3. Results**

165 *3.1 Epidemiological background*

166 A total of 26 AREf isolates were identified from January 2014 to June 2015 from patients

167 admitted at the Hue Central Hospital, Vietnam. **Sources, wards origins and c**Characteristics

168 of isolates, ~~and, susceptibility of isolates against the main antimicrobials and vancomycin~~

169 ~~phenotypes~~ are summarized in Table 1. The most common wards were Surgery wards

170 (54%) and Intensive Care Units ICU (15%). Isolates were mainly from blood (31.5%) and

171 pus (38.5%) and the rest from urine, catheter and ascitic fluid.

172 *3.2 Antimicrobial susceptibility and PCRs*

173 All AREf strains were also MDR showing resistance to at least three different classes of

174 antibiotics in different combinations-~~(Table 1, data not shown)~~. All were resistant to

175 **ciprofloxacin, levofloxacin**, high-level gentamicin, **tetracycline and chloramphenicol**, 81%

176 were resistant to high-level streptomycin, 46% to vancomycin and 11% to teicoplanin. All

177 isolates were susceptible to linezolid and quinupristin/dalfopristin. **Susceptibility of**

178 **isolates to ampicillin, vancomycin and teicoplanin are shown in Table 1.**

179 Genotyping of the vancomycin resistance genes by multiplex PCR targeting *vanA*,*-B*,*-C*

180 detected the *vanB* gene in all VREf strains and, unexpectedly, an additional *vanA* gene was

181 found in two of them (Table 2~~1~~). None of VREF isolates contained *vanC* genes.

182 The atypical *vanA/vanB* type VREf strains (VH16 and VH17) were isolated from two

183 debilitated patients: strain VH16 was isolated from a cirrhotic patient admitted at the

184 Internal Digest Department, while strain VH17 was isolated from a patient admitted at the

185 ICU ward, suffering Guillain Barre Syndrome (Table 1). These isolates showed high-level

186 resistance to vancomycin (MICs  $\geq 256$  mg/L) and teicoplanin (MICs of  $\geq 256$  mg/L and

187  $\geq 48$ mg/L, respectively) by E-test (Table 2~~1~~). The *vanB*-type isolates showed a VanB

188 phenotype displaying variable levels of vancomycin MICs from 32 mg/L up to 264 mg/L,

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189 and were all susceptible to teicoplanin with the exception of one isolate (VH20), which  
190 displayed a VanA incongruent phenotype, characterized by high level of resistance to  
191 vancomycin (MIC  $\geq$ 256 mg/L) and teicoplanin (MIC  $\geq$ 64 mg/L) (Table 21).

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192 Moreover, four VREf isolates consisting of 3 *vanB*<sub>2</sub>-type (VH8, VH21 and VH24) and 1  
193 *vanA/vanB*-type (VH16), showed vancomycin heteroresistance, with a growth of sub  
194 colonies in the vancomycin inhibition zone of E-test strip corresponding to MICs of >256  
195 mg/L, >96 mg/L, and >32 mg/L (Table 21).

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196  
197 All AREf isolates carried the HA marker IS16, which was absent in the two ASEf strains  
198 (VH27 and VH28) included in the study for comparison. The *esp* and the *hyl* genes were  
199 present in the 65% and 38% of isolates respectively (Table 21) and were not present in  
200 ASEf isolates.

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### 201 3-3 MLST

202  
203 To evaluate the relatedness of *E. faecium* under investigation, MLST and EBurst analysis  
204 were performed. Analysis results, included in Table 21, evidenced the nosocomial origin of  
205 the VSEf/VREf isolates that belonged to five different STs, all CC17 related, including  
206 ST17 (61,5%), ST18 (15%), ST262 (11,5%) a single locus variant (SLV) of ST18, ST78  
207 (7,7%) and the new ST1085 (3,8%). On the contrary, the ASEf isolates from ST904 and  
208 the new ST1086, were not CC17 related. Both *vanA/vanB*<sub>2</sub> type isolates were of ST17.

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209 Nosocomial STs were isolated from numerous wards (Table 1), ST17 was mainly found in  
210 ICU and in other 9 wards, in the hospital building 2 and 3, ST18 in General Surgery and  
211 other 2 wards (Building 2 and 3), ST262 in Clinical Haematology and Cardiology  
212 Emergency wards (Building 1 and 2), while ST78 in Central-ICU and S-ICU (Building 2)  
213 (Table 2).

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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 21). 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*<sub>2</sub> operon in a chromosomal integrated Tn1549-like transposon, showing 99% of  
224 identity with the Tn1549-*vanB*<sub>2</sub> transposon of reference strain AUS0004 (26612/26624 bp).  
225 Comparative BLAST analysis of our *vanB*<sub>2</sub> 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*<sub>2</sub> 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<sub>B,d,d</sub>-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*<sub>2</sub> operon were  
236 further confirmed by conventional Sanger sequencing. Tn1549 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

239 reference strain. In all strains the Tn1549-like transposon had a common chromosomal  
240 insertion site, between genes EFAU004\_00610 and EFAU004\_00611 according to the  
241 reference genome *E. faecium* AUS0004 (Accession no. NC\_017022). This hotspot is  
242 present in *E. faecium* VREN3305 strain isolated in United Kingdom (Accession no.  
243 FXHW01000001.1).

244

#### 245 3.4.2. *vanA* operon, Tn1546 transposon and plasmids

246 To study the location of the *vanA* operon in the *vanA/vanB*<sub>2</sub>-type strains (VH16 and  
247 VH17), Tn1546-harboring contigs were mapped on the *E. faecium* plasmids pHvH-V24  
248 (Accession no. KX574671) [33] and pJEG40 plasmid (Accession no. KX810025) [34].  
249 The closure of *vanA* operon-harboring plasmid of strain *E. faecium* VH17 resulted in a  
250 sequence of 37459bp and showed 99% of sequence identity for 97% of the sequence to  
251 both the *E. faecium* pJEG40 and pHvH-V24 plasmids. Assembly of the *E. faecium* VH16  
252 plasmid was not complete due to sequence breakage into many small contigs: a 32726bp  
253 sequence was assembled, which showed 99% sequence identity for 88% of the sequence to  
254 both the *E. faecium* pJEG40 and pHvH-V24 plasmids and 98% sequence identity for 88%  
255 of the sequence to the VH17 pJEG40-like plasmid. VH16 and VH17 pJEG40-like plasmids  
256 were also highly similar (98% sequence identity for 77% and 79% of the length,  
257 respectively) to the pA698 plasmid carrying the *vanA* resistance operon and described in a  
258 *vanA/vanB E. faecium* isolated in Greece [35].

259

260 Identity to the *E. faecium* p5357a (Accession no. GQ900435), previously associated as  
261 carrier of the same mutated Tn1546 element, was 99% for only 32% and 36% of the  
262 sequence of VH17 and VH16 plasmids, respectively. For this reason, we refer to the newly  
263 assembled plasmids in strains VH16 and VH17 as pJEG40-like plasmids.

264 Both the pJEG40-like plasmids carried the *rep17*<sub>pRUM</sub> replicon and an identical Tn1546-  
265 *vanA* operon containing the IS1251 between the *vanS-vanH* intergenic region, showing  
266 100% of sequence identity with several isolates present in database  
267 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Fig 1b). In total, 87 plasmid replicons of 9 *rep*-  
268 families were detected by PlasmidFinder in the 16 *E. faecium* strains under study, with a  
269 median of 5 *rep* types per isolate (range, 2-9). Gradual increase of reps was found in  
270 VSEf/VREf isolates from same STs (Table S1). None of the *rep* types were VRE or VSE  
271 specific. The *vanA/vanB*<sub>2</sub> isolates shared 6 replicons (*rep2*, *rep11*, *repUS12*, *rep17*, *rep18*  
272 and *repUS15*) while 3 were found only in the VH16 isolate (*rep14*, *rep22*, and *repUS7*).  
273 The *rep17*<sub>pRUM</sub> type replicon was also present in the VSEf VH1 isolate, with small pairing  
274 portions with pJEG40-like plasmids (less than 3000 bp).

275

### 276 3.5. WG-SNPs unrooted phylogenetic tree

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

289 | between the *vanA/vanB*<sub>2</sub> strains, while 29 wgSNPs between ST17 VSEf (VH11, VH13),  
290 | and just 5 and 9 wgSNPs of difference in VREf isolates VH8-VH9 and VH20-VH21  
291 | (Table S2).

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295 **4. Discussion**

296 During the last decades, VREf has emerged worldwide as a relevant nosocomial  
297 multidrug-resistant pathogen becoming a serious health concern [36]. This phenomenon is  
298 particularly worrisome in low-income countries as many South Asian countries, where the  
299 overuse of antibiotics, also in veterinary field and the hospital surveillance are not always  
300 adequate [23]. In this study we investigated ampicillin resistant *E. faecium* strains isolated  
301 during 2014-15, finding a high proportion of multi-drug resistance including vancomycin  
302 (46%), with 35% of the isolates being from invasive infections (i.e. blood). This is  
303 worrisome and underlines that VREf is a pathogen causing severe hospital infections and  
304 compromising treatment options.

305

306 To our knowledge, this represents the first study in which Vietnamese nosocomial multi-  
307 resistant *E. faecium* isolates were genetically characterized. Our results highlighted the  
308 spread among hospital wards of a surprisingly heterogeneous population of HA-  
309 VSEf/VREf isolates, composed by five different MLST-CC17 related STs, including the  
310 ancestral ST17, ST18 and ST78, responsible of epidemics worldwide [37]. *E. faecium*  
311 isolates, particularly those resistant to vancomycin were mainly from ICU and Surgery  
312 wards. The study evidenced that the same chromosomal integrated Tn1549-like  
313 transposon, with a novel highly mutated *vanB*<sub>2</sub>-operon, conferring high level of  
314 vancomycin resistance and the ability to resist to teicoplanin, has spread among *E. faecium*  
315 of different STs.

316

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

320 Here, we found a VanS mutation (N105D) in that the same sensor portion in all *vanB*<sub>2</sub>-type  
321 isolates under study, suggesting its involvement in the onset of resistance to teicoplanin.  
322 Furthermore, the several other mutations detected in the *vanR*<sub>B</sub>, *vanY*<sub>B</sub>, *vanW*<sub>B</sub> and *vanB*  
323 genes might be involved in the high MICs values of vancomycin and teicoplanin displayed  
324 by our *vanB*<sub>2</sub>-type strains, and also in the vancomycin heteroresistance observed in three  
325 isolates.

326

327 The acquisition of Tn1549 transposon may have different origins, acquired either from  
328 spreading HA-VREf or from community CA-VRE, but also from gut microbiota that can  
329 share also mutated *vanB*<sub>2</sub>-like transposons (i. e. *Clostridium difficile*) [16,39–43]. Once  
330 acquired, VREf can spread easily increasing clinical treatment failures and mortality rate  
331 compared to VSEf, as previously described [44].

332

333 Moreover, two *vanB*<sub>2</sub>-type ST17 strains (VH16 and VH17) carried an additional Tn1546-  
334 *vanA*-like transposon on pJEG40-like plasmids generating the unusual *vanA/vanB*<sub>2</sub>  
335 genotype with high level glycopeptides MICs (vancomycin  $\geq 256$  mg/L and teicoplanin  
336  $\geq 256$  mg/L and  $\geq 48$  mg/L). A recent study conducted in Australia, associated the same  
337 Tn1546-*vanA* like transposon present in our *vanA/vanB*<sub>2</sub> isolates to a lower vancomycin  
338 and teicoplanin resistance (MICs 2 and 4 mg/L) [34]. These findings suggest that the  
339 mutations occurred in *vanB*<sub>2</sub> operon rather than the acquisition of the *vanA* operon itself,  
340 were responsible for the higher resistance phenotype observed in our *vanA/vanB*<sub>2</sub> isolates  
341 and as well in the VanA incongruent phenotype -*vanB*-type strain.

342

343 Finding both *vanA* and *vanB* determinants in clinical *E. faecium* isolates is unusual, and  
344 only few cases have been reported during the last 20 years [11]. Recently, *vanA/vanB*<sub>2</sub>-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 Tn1549-*vanB*<sub>2</sub> operon and the same  
348 Tn1546-*vanA* operon present in our *vanA/vanB*<sub>2</sub> 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 Tn1549-*vanB*<sub>2</sub> isolates. The  
351 comparative genetic analysis of the pJEG40-like plasmids combined with the phylogenetic  
352 relationship of our *vanA/vanB*<sub>2</sub> isolates did not clarify if two different plasmid acquisition  
353 events by two nosocomial related *vanB*<sub>2</sub>-type ST17 clones, or a single plasmid acquisition,  
354 followed by the evolution into a single ST17 *vanB*<sub>2</sub> 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*<sub>2</sub> genotypes.

357

358 MLST typing and **eBURST** ~~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, ~~which~~ **The analysis** revealed the genetic relatedness between VSEf and VREf  
361 isolates of same ST, and evidenced the clonal expansion, particularly among ST17  
362 population, with subclones displaying different assortment of virulence genes and  
363 increased numbers of plasmid replicons. In all VREf isolates the presence of the same  
364 Tn1549-like transposon with common chromosomal insertion site suggested lateral  
365 exchange of Tn1549-like element among isolates, with *de novo* generation of VREf,  
366 followed by clonal expansion.

367 Micro-evolution among ST17 isolates, including the *vanA/vanB*<sub>2</sub>-type strains, was  
368 highlighted by comparing wgSNPs between isolates, which also suggested, in accordance

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369 with other reports [19,46], a VREf cross-transmission between patients from different  
370 wards, with isolates (VH8, VH9) showing just 5 wgSNPs of difference [46].  
371 Notably, the presence of two highly related *vanB*<sub>2</sub>-type ST17 isolates (wgSNPs=9) -in two  
372 patients from different wards showing different glycopeptide resistant phenotypes  
373 (VH20/ICU/VanA, VH21/OT/VanB), suggested the involvement of the different antibiotic  
374 pressure in the two hospital wards in the regulation of the same mutated *vanB*<sub>2</sub> operon.

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

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

384

385 In conclusion, this study described the spread among different HA-*E. faecium* STs, of a  
386 *vanB*<sub>2</sub>-Tn1549-like transposon able to confer resistance to teicoplanin, which is for this  
387 reason strongly inadvisable.

388

389 The presence of the Tn1546-like-pJEG40-like plasmids in two high related *vanB*<sub>2</sub>-type  
390 ST17 isolates contributed to the heterogeneity of VREf in this hospital. The *de novo*  
391 generation of VREf from VSEf strains suggests that, in addition to VREf, also VSEf  
392 (AREf) clones should be monitored in the patients and in the hospital environment.

393



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

398

399 **Declarations**

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402 **Competing Interests:** No conflicts

403 **Ethical Approval:** Not required

404

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565 **Figure Legend**

566 **Figure. 1.** Unrooted parsimony tree based on wgSNPs (A) and core SNPs (B). Trees are  
567 consensus trees of the equally most parsimonious trees from a sample of 100 trees based  
568 on an Extended Majority Rule. Internal node labels show the support for each node as  
569 calculated by FastTreeMP. Branch lengths are expressed in terms of changes per number  
570 of SNPs. Strains branching in the two major clades are color coded based on their ST.  
571 VREf and VSEf clades are highlighted in grey and cyan, respectively. Six reference  
572 strains were included in the comparison Aus0004 (Acc. no. CP00335), Aus0085 (Acc. no.  
573 CP006620), NRRL B-2354 (Acc. no. NCP004063), DO (Acc. no. CP003583), T110  
574 (Acc. no. CP006030), VREF\_286 (Acc. no. CP019992).