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

Antonella Santona [a *], Elisa Taviani [a], Hoa Minh Hoang [a, b], Maura Fiamma [a], Massimo Deligios [a], Tram Viet Q Ngo [b], An Van Le [b], Pietro Cappuccinelli [a, b], Salvatore Rubino [a], Bianca Paglietti [a]

[a] Department of Biomedical Sciences, University of Sassari, Viale San Pietro 43/b, 07100 Sassari, Italy

[b] Department of Microbiology & Carlo Urbani Center, Hue University of Medicine & Pharmacy, Hue, Viet Nam

*** Corresponding author**

Antonella Santona

Department of Biomedical Sciences,

University of Sassari,

Viale San Pietro 43/b,

07100 Sassari, Italy

Phone +39 079 228300 – Fax +39 079 212345

E-mail address: asantona@uniss.it

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

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 IS*16* [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 Tn*1546* 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 Tn*1549*/Tn*5382*-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* ($vanY_BW_BH_BBX_B$)
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 IS*16, 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 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 Tn*1546* and Tn*1549* 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 ≥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

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 linezolide 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 ≥256 mg/L) and teicoplanin (MICs of ≥256 mg/L and
187 ≥48mg/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 ≥256 mg/L) and teicoplanin (MIC ≥64 mg/L) (Table 1).
192 Moreover, four VREf isolates consisting of 3 *vanB*$_2$-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 IS*16*, 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*$_2$ 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 $vanB_2$ 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

239 reference strain. In all strains the Tn*1549*-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*$_2$-type strains (VH16 and
247 VH17), Tn*1546*-harbouring 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 Tn*1546* 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*$_{pRUM}$ replicon and an identical Tn*1546-*
265 *vanA* operon containing the IS*1251* 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*$_2$ isolates shared 6 replicons (*rep*2, *rep*11, *rep*US12, *rep*17, *rep*18
272 and *rep*US15) while 3 were found only in the VH16 isolate (*rep*14, *rep*22, and *rep*US7).
273 The *rep17*$_{pRUM}$ 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/vanB2* 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$_2$ 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 Tn*1549*-like
313 transposon, with a novel highly mutated $vanB_2$-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 $VanS_B$ 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*$_2$-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*$_B$, *vanY*$_B$, *vanW*$_B$ and *vanB*
323 genes might be involved in the high MICs values of vancomycin and teicoplanin displayed
324 by our *vanB*$_2$-type strains, and also in the vancomycin heteroresistance observed in three
325 isolates.
326
327 The acquisition of Tn*1549* 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$_2$-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*$_2$–type ST17 strains (VH16 and VH17) carried an additional Tn*1546-*
334 *vanA*-like transposon on pJEG40-like plasmids generating the unusual *vanA/vanB*$_2$
335 genotype with high level glycopeptides MICs (vancomycin ≥256 mg/L and teicoplanin
336 ≥256 mg/L and ≥48 mg/L). A recent study conducted in Australia, associated the same
337 Tn*1546-vanA like* transposon present in our *vanA/vanB*$_2$ 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*$_2$ operon rather than the acquisition of the *vanA* operon itself,
340 were responsible for the higher resistance phenotype observed in our *vanA/vanB*$_2$ 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*$_2$-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

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*$_2$-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*$_2$ 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*$_2$-Tn*1549*-like transposon able to confer resistance to teicoplanin, which is for this
387 reason strongly inadvisable.
388
389 The presence of the Tn*1546*-like-pJEG40-like plasmids in two high related *vanB*$_2$–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**
400 **Funding:** Supported by a grant from the Italian Cooperation for Development (Carlo
401 Urbani Project AID 009922).
402 **Competing Interests:** No conflicts
403 **Ethical Approval:** Not required
404

405 **References**

406 [1] Cheah A. L. Y. , SpelmanT., D. Liew, T. Peel, B. P. Howden, D. Spelman, M. L.
407 Grayson RLN and DCMK. Enterococcal bacteraemia: factors influencing mortality,
408 length of stay and costs of hospitalization. Clin Microbiol Infect 2013;19:384.
409 doi:10.1111/1469-0691.12204.

410 [2] Arias CA, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance.
411 Nat Rev Microbiol 2012;10:266–78. doi:10.1038/nrmicro2761.

412 [3] Orsi GB, Ciorba V. Vancomycin resistant enterococci healthcare associated
413 infections. Ann Ig 2013:485–92. doi:10.7416/ai.2013.1948.

414 [4] Jack N Pendleton SPG& BFG. Clinical relevance of the ESKAPE pathogens. Expert
415 Rev Anti Infect Ther 2013;11:297–308.

416 [5] Leavis HL, Bonten MJ, Willems RJ. Identification of high-risk enterococcal clonal
417 complexes: global dispersion and antibiotic resistance. Curr Opin Microbiol
418 2006;9:454–60. doi:10.1016/j.mib.2006.07.001.

419 [6] Werner G, Fleige C, Geringer U, van Schaik W, Klare I, Witte W. IS element IS16
420 as a molecular screening tool to identify hospital-associated strains of Enterococcus
421 faecium. BMC Infect Dis 2011;11:80. doi:10.1186/1471-2334-11-80.

422 [7] Leavis HL, Willems RJL, van Wamel WJB, Schuren FH, Caspers MPM, Bonten
423 MJM. Insertion sequence-driven diversification creates a globally dispersed
424 emerging multiresistant subspecies of E. faecium. PLoS Pathog 2007;3:e7.
425 doi:10.1371/journal.ppat.0030007.

426 [8] Cattoir V, Leclercq R. Twenty-five years of shared life with vancomycin-resistant
427 enterococci: Is it time to divorce. J Antimicrob Chemother 2013;68:731–42.
428 doi:10.1093/jac/dks469.

429 [9] Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P. Modes and

430 modulations of antibiotic resistance gene expression. Clin Microbiol Rev
431 2007;20:79–114. doi:10.1128/CMR.00015-06.
432 [10] Lebreton F, Schaik W Van, Manson A. Emergence of Epidemic Multidrug-Resistant
433 Enterococcus faecium. Am Soc Microbiol 2013;4:1–10. doi:10.1128/mBio.00534-
434 13.Editor.
435 [11] Freitas AR, Tedim AP, Francia M V., Jensen LB, Novais C, Peixe L, et al.
436 Multilevel population genetic analysis of *vanA* and *vanB Enterococcus faecium*
437 causing nosocomial outbreaks in 27 countries (1986–2012). J Antimicrob
438 Chemother 2016;71:3351–66. doi:10.1093/jac/dkw312.
439 [12] Sivertsen A, Billstr??m H, Melefors ??jar, Liljequist BO lsson, Wisell KT egmark,
440 Ullberg M, et al. A multicentre hospital outbreak in Sweden caused by introduction
441 of a vanB2 transposon into a stably maintained pRUM-plasmid in an Enterococcus
442 faecium ST192 clone. PLoS One 2014;9:e103274.
443 doi:10.1371/journal.pone.0103274.
444 [13] Courvalin P. Vancomycin resistance in gram-positive cocci. Clin Infect Dis 2006;42
445 Suppl 1:S25-34. doi:10.1086/491711.
446 [14] Heikens E, Bonten MJM, Willems RJL. Enterococcal surface protein esp is
447 important for biofilm formation of Enterococcus faecium E1162. J Bacteriol
448 2007;189:8233–40. doi:10.1128/JB.01205-07.
449 [15] Panesso D, Montealegre MC, Rincón S, Mojica MF, Rice LB, Singh K V, et al. The
450 hyl Efm gene in pHyl Efm of Enterococcus faecium is not required in pathogenesis
451 of murine peritonitis. BMC Microbiol 2011;11:20.
452 [16] Howden B, Holt K, Lam M, Seemann T. Genomic Insights Howden, B., Holt, K.,
453 Lam, M., & Seemann, T. (2013). Genomic Insights to Control the Emergence of
454 Vancomycin-Resistant Enterococci. mBio. doi:10.1128/mBio.00412-13.Editorto

455 Control the Emergence of Vancomycin-Resistant Enterococci. MBio 2013;4.
456 doi:10.1128/mBio.00412-13.Editor.
457 [17] De Been M, Pinholt M, Top J, Bletz S, Mellmann A, Van Schaik W, et al. Core
458 genome multilocus sequence typing scheme for high-resolution typing of
459 enterococcus faecium. J Clin Microbiol 2015;53:3788–97. doi:10.1128/JCM.01946-
460 15.
461 [18] Willems RJL, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, et al.
462 Global spread of vancomycin-resistant Enterococcus faecium from distinct
463 nosocomial genetic complex. Emerg Infect Dis 2005;11:821–8.
464 doi:10.3201/eid1106.041204.
465 [19] Brodrick HJ, Raven KE, Harrison EM, Blane B, Reuter S, Török ME, et al. Whole-
466 genome sequencing reveals transmission of vancomycin-resistant Enterococcus
467 faecium in a healthcare network. Genome Med 2016;8:4. doi:10.1186/s13073-015-
468 0259-7.
469 [20] Weng P, Ramli R, Mariana Nor S, Yoke-Kqueen C, Rukman Awang H. High
470 genetic diversity of Enterococcus faecium and Enterococcus faecalis clinical isolates
471 by pulsed-field gel electrophoresis and multilocus sequence typing from a hospital
472 in Malaysia. Biomed Res Int 2013;938937.
473 [21] Dang STT, Petersen A, Van Truong D, Chu HTT, Dalsgaard A. Impact of
474 medicated feed on the development of antimicrobial resistance in bacteria at
475 integrated pig-fish farms in Vietnam. Appl Environ Microbiol 2011;77:4494–8.
476 doi:10.1128/AEM.02975-10.
477 [22] Poulsen LL, Bisgaard M, Son NT, Trung N V., An HM, Dalsgaard A. Enterococcus
478 and Streptococcus spp. associated with chronic and self-medicated urinary tract
479 infections in Vietnam. BMC Infect Dis 2012;12:1. doi:10.1186/1471-2334-12-320.

480 [23] Daniel DS, Lee SM, Dykes GA, Rahman S. Public health risks of multiple-drug-
481 resistant Enterococcus spp. in Southeast Asia. Appl Environ Microbiol
482 2015;81:6090–7. doi:10.1128/AEM.01741-15.
483 [24] Kariyama R, Mitsuhata R, Chow JW, Clewell B, Kumon H. Simple and Reliable
484 Multiplex PCR Assay for Surveillance Isolates of Simple and Reliable Multiplex
485 PCR Assay for Surveillance Isolates of Vancomycin-Resistant Enterococci. J Clin
486 Microbiol 2000;38:3092–5.
487 [25] Elsayed S, Hamilton N, Boyd D, Enterococcus V. Improved Primer Design for
488 Multiplex PCR Analysis of Vancomycin-Resistant Improved Primer Design for
489 Multiplex PCR Analysis of. J Clin Microbiol 2001;39:2367–9.
490 doi:10.1128/JCM.39.6.2367.
491 [26] Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, et
492 al. Development of a multiplex PCR for the detection of asaI, gelE, cylA, esp, and
493 hyl genes in enterococci and survey for virulence determinants among european
494 hospital isolates of Enterococcus faecium. J Clin Microbiol 2004;42:4473–9.
495 doi:10.1128/JCM.42.10.4473-4479.2004.
496 [27] Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J.
497 ACT: The Artemis comparison tool. Bioinformatics 2005;21:3422–3.
498 doi:10.1093/bioinformatics/bti553.
499 [28] Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al.
500 Versatile and open software for comparing large genomes. Genome Biol
501 2004;5:R12. doi:10.1186/gb-2004-5-2-r12.
502 [29] Okonechnikov K, Golosova O, Fursov M, Varlamov A, Vaskin Y, Efremov I, et al.
503 Unipro UGENE: A unified bioinformatics toolkit. Bioinformatics 2012;28:1166–7.
504 doi:10.1093/bioinformatics/bts091.

505 [30] Alikhan N-F, Petty NK, Ben Zakour NL, Beatson SA. BLAST Ring Image
506 Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics
507 2011;12:402. doi:10.1186/1471-2164-12-402.
508 [31] Gardner SN, Slezak T, Hall BG. kSNP3.0: SNP detection and phylogenetic analysis
509 of genomes without genome alignment or reference genome. Bioinformatics
510 2015;31:2877–8. doi:10.1093/bioinformatics/btv271.
511 [32] Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics
512 Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 2016;33:1870–4.
513 doi:10.1093/molbev/msw054.
514 [33] Pinholt M, Gumpert H, Bayliss S, Nielsen JB, Vorobieva V, Pedersen M, et al.
515 Genomic analysis of 495 vancomycin-resistant Enterococcus faecium reveals broad
516 dissemination of a vanA plasmid in more than 19 clones from Copenhagen,
517 Denmark. J Antimicrob Chemother 2017;72:40–7. doi:10.1093/jac/dkw360.
518 [34] van Hal SJ, Espedido BA, Coombs GW, Howden BP, Korman TM, Nimmo GR, et
519 al. Polyclonal emergence of vanA vancomycin-resistant Enterococcus faecium in
520 Australia. J Antimicrob Chemother 2017;72:998–1001. doi:10.1093/jac/dkw539.
521 [35] Papagiannitsis CC, Malli E, Florou Z, Medvecky M, Sarrou S, Hrabak J, et al. First
522 description in Europe of the emergence of Enterococcus faecium ST117 carrying
523 both vanA and vanB genes, isolated in Greece. J Glob Antimicrob Resist
524 2017;11:68–70. doi:10.1016/j.jgar.2017.07.010.
525 [36] Guzman Prieto AM, van Schaik W, Rogers MRC, Coque TM, Baquero F, Corander
526 J, et al. Global emergence and dissemination of enterococci as nosocomial
527 pathogens: Attack of the clones? Front Microbiol 2016;7:1–15.
528 doi:10.3389/fmicb.2016.00788.
529 [37] Willems R, Top J, Schaik W Van, Leavis H. Restricted gene flow among hospital

530 subpopulations of Enterococcus faecium. MBio 2012;3. doi:10.1128/mBio.00151-
531 12.Editor.
532 [38] Hayden MK, Trenholme GM, Schultz JE, Sahm DF. In Vivo Development of
533 Teicoplanin Resistance in a VanB Enterococcus faecium Isolate 1993;1992:1224–7.
534 [39] Hammerum AM. Enterococci of animal origin and their significance for public
535 health. Clin Microbiol Infect 2012;18:619–25. doi:10.1111/j.1469-
536 0691.2012.03829.x.
537 [40] López M, Sáenz Y, Alvarez-Martínez MJ, Marco F, Robredo B, Rojo-Bezares B, et
538 al. Tn1546 structures and multilocus sequence typing of vanA-containing
539 enterococci of animal, human and food origin. J Antimicrob Chemother
540 2010;65:1570–5. doi:10.1093/jac/dkq192.
541 [41] Ballard SA, Pertile KK, Lim M, Johnson PDR, Grayson ML. Molecular
542 characterization of vanB elements in naturally occurring gut anaerobes. Antimicrob
543 Agents Chemother 2005;49:1688–94. doi:10.1128/AAC.49.5.1688-1694.2005.
544 [42] Domingo M-C, Huletsky A, Giroux R, Boissinot K, Picard FJ, Lebel P, et al. High
545 prevalence of glycopeptide resistance genes *vanB*, *vanD*, and *vanG* not associated
546 with *enterococci* in human fecal flora. Antimicrob Agents Chemother
547 2005;49:4784–6. doi:10.1128/AAC.49.11.4784-4786.2005.
548 [43] Knight DR, Androga GO, Ballard SA, Howden BP, Riley T V. A Phenotypically
549 Silent vanB2 Operon Carried on a Tn1549-Like Element in Clostridium difficile.
550 MSphere 2016;1:e00177-16. doi:10.1128/mSphere.00177-16.
551 [44] DiazGranados CA, Zimmer SM, Mitchel K, Jernigan JA. Comparison of Mortality
552 Associated with Vancomycin-Resistant and Vancomycin-Susceptible Enterococcal
553 Bloodstream Infections: A Meta-analysis. Clin Infect Dis 2005;41:327–33.
554 doi:10.1086/430909.

555 [45] Eshaghi A, Shahinas D, Li A, Kariyawasam R, Banh P, Desjardins M, et al.
556 Characterization of an Enterococcus gallinarum isolate carrying a dual vanA and
557 vanB cassette. J Clin Microbiol 2015;53:2225–9. doi:10.1128/JCM.03267-14.
558 [46] Pinholt M, Larner-Svensson H, Littauer P, Moser CE, Pedersen M, Lemming LE, et
559 al. Multiple hospital outbreaks of vanA Enterococcus faecium in Denmark, 2012-13,
560 investigated by WGS, MLST and PFGE. J Antimicrob Chemother 2015;70:2474–
561 82. doi:10.1093/jac/dkv142.
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).

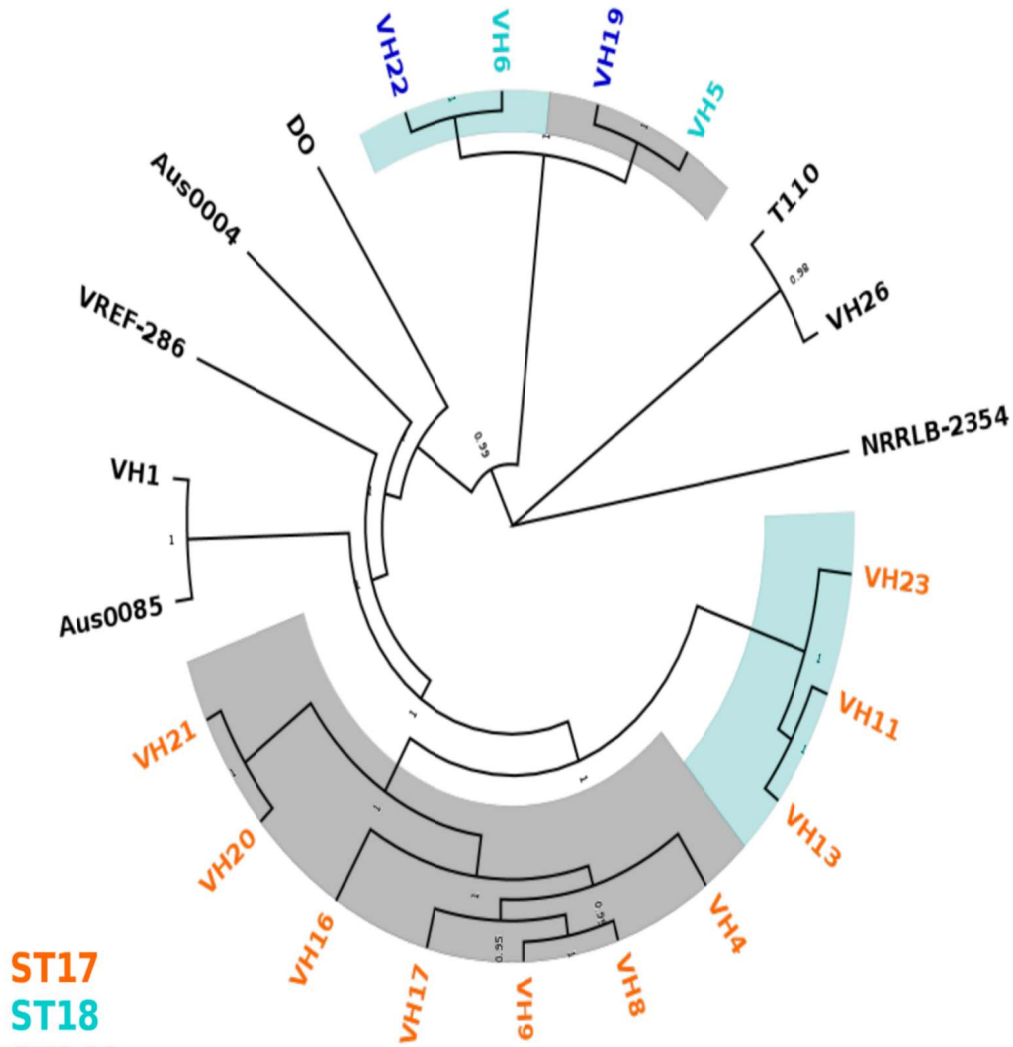
Table 1

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

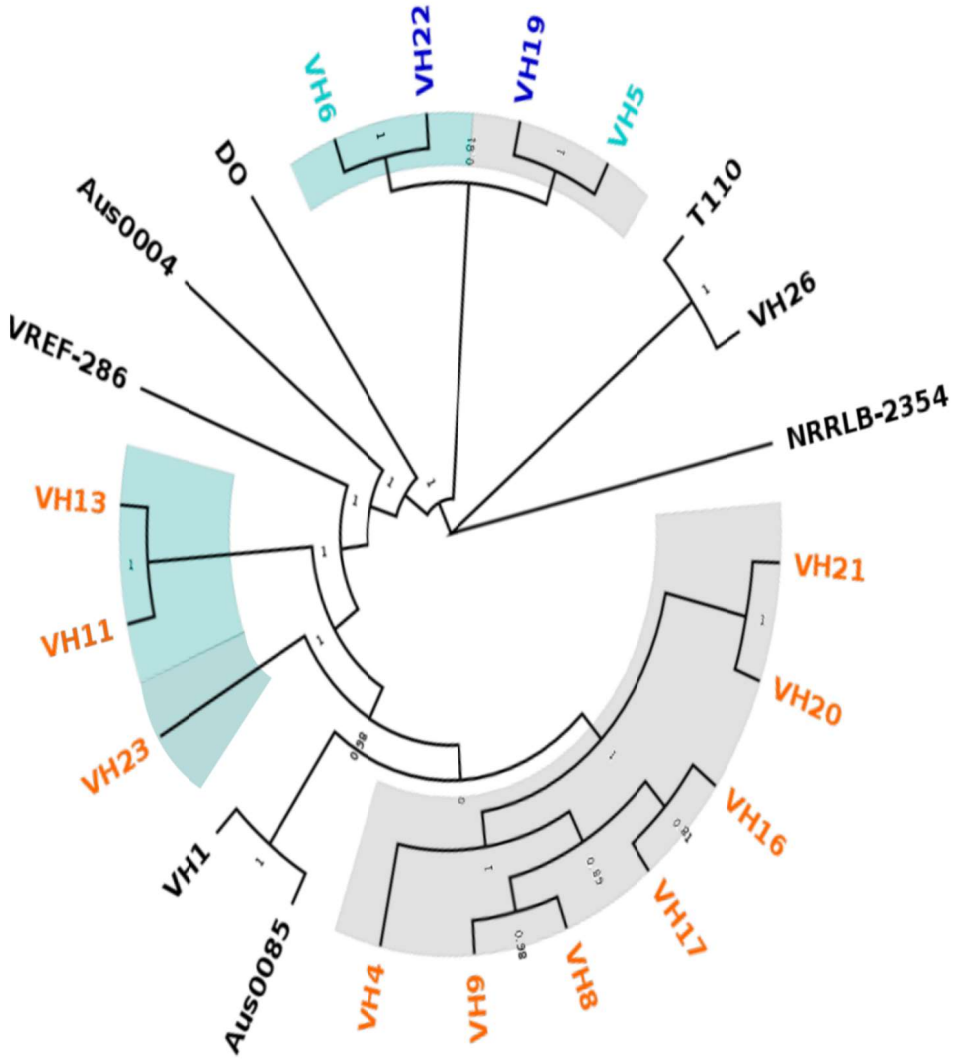
Strains	Date of isolation	Source	Wards	Floor_Building	First diagnosis	Amp	Van MIC	Tei MIC	Van Phenotype	van Genotype	ST	IS16	hyl	esp
VH18	10/01/2014	Blood	CH	B1	Acute leukemia	R >8	1.0	0.25	S	-	ST262	+	-	-
VH22	18/05/2014	Pus	CH	B1	CML	R>8	1.5	0.125	S	-	ST262	+	-	-
VH19	10/01/2014	Blood	CEI	Ground floor-B2	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	4th 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	6th floor - B2	Head trauma	R>8	0.50	0.25	S	-	ST78	+	-	+
VH9	26/08/2014	Pus	S-ICU	6th floor - B2	Postoperative ileus	R>8	≥256	0.125	VanB	$vanB_2$	ST17	+	+	+
VH10	31/08/2014	Pus	S-ICU	6th floor - B2	Pleuritis	R>8	0.50	0.25	S	-	ST17	+	-	+
VH28	19/06/2015	Pus	S-ICU	6th floor - B2	Abscess	S=1	1.0	0.25	S	-	ST1086	-	-	-
VH2	09/07/2014	Blood	C-ICU	6th floor - B2	Multiple injuries	R>8	1.0	0.25	S	-	ST78	+	-	+
VH3	11/07/2014	Blood	C-ICU	6th floor - B2	Head trauma	R>8	0.50	0.25	S	-	ST17	+	-	+
VH17	05/04/2015	Catheter	C-ICU	6th floor - B2	GBS	R>8	≥256	≥48	VanA	vanA/ $vanB_2$	ST17	+	+	+
VH20	28/03/2014	Blood	C-ICU	6th 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	4th floor - B3	PPL	R>8	1.0	0.25	S	-	ST18	+	-	-
VH12	09/09/2014	Urine	GS	4th floor - B3	Post Studer surgery	R>8	≥256	0.19	VanB	$vanB_2$	ST18	+	-	-
VH25	16/06/2015	Blood	GS	4th floor - B3	PFLCD	R>8	1.5	0.125	S	-	ST18	+	+	-
VH8*	24/08/2014	Urine	GS	4th floor - B3	Bladder tumor	R>8	≥256	0.25	VanB	$vanB_2$	ST17	+	+	+
VH11	08/09/2014	Pus	GS	4th floor - B3	Acute cholecystitis	R>8	0.50	0.25	S	-	ST17	+	-	+
VH15	05/12/2014	Blood	GS	4th floor - B3	Sepsis	R>8	≥256	0.50	VanB	$vanB_2$	ST17	+	-	+
VH21*	06/05/2014	Pus	OT	4th 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



A: wg SNPs



B: core SNPs

ST17
ST18
ST262
VSEf
VREf

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

Antonella Santona [a *], Elisa Taviani [a], Hoa Minh Hoang [a, b], Maura Fiamma [a], Massimo Deligios [a], Tram Viet Q Ngo [b], An Van Le [b], Pietro Cappuccinelli [a, b], Salvatore Rubino [a], Bianca Paglietti [a]

[a] Department of Biomedical Sciences, University of Sassari, Viale San Pietro 43/b, 07100 Sassari, Italy

[b] Department of Microbiology & Carlo Urbani Center, Hue University of Medicine & Pharmacy, Hue, Viet Nam

*** Corresponding author**

Antonella Santona

Department of Biomedical Sciences,

University of Sassari,

Viale San Pietro 43/b,

07100 Sassari, Italy

Phone +39 079 228300 – Fax +39 079 212345

E-mail address: asantona@uniss.it

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

Formatted: Highlight

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 IS*16* [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 Tn*1546* 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 Tn*1549*/Tn*5382*-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* ($vanY_BW_BH_BBX_B$)
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 IS*16, 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^{TM}-5-USA) and sequenced at the LMU Sequencing Service (Munich,
120 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

Formatted: Highlight

Formatted: Highlight

Field Code Changed

Formatted: English (United States)

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

Formatted: English (United States)

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 (38~~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,

Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Not Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight

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)~~.

Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight
Formatted: Highlight

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*.

Formatted: Highlight

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

239 reference strain. In all strains the Tn*1549*-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*$_2$-type strains (VH16 and
247 VH17), Tn*1546*-harbouring 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 Tn*1546* 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*$_{pRUM}$ replicon and an identical Tn*1546-*
265 *vanA* operon containing the IS*1251* 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*$_2$ isolates shared 6 replicons (*rep*2, *rep*11, *rep*US12, *rep*17, *rep*18
272 and *rep*US15) while 3 were found only in the VH16 isolate (*rep*14, *rep*22, and *rep*US7).
273 The *rep17*$_{pRUM}$ 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/vanB2* 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*$_2$ *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

Formatted: Subscript, Highlight

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 Tn*1549*-like
313 transposon, with a novel highly mutated $vanB_2$-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 $VanS_B$ 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*$_2$-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*$_B$, *vanY*$_B$, *vanW*$_B$ and *vanB*
323 genes might be involved in the high MICs values of vancomycin and teicoplanin displayed
324 by our *vanB*$_2$-type strains, and also in the vancomycin heteroresistance observed in three
325 isolates.
326
327 The acquisition of Tn*1549* 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$_2$-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*$_2$–type ST17 strains (VH16 and VH17) carried an additional Tn*1546*-
334 *vanA*-like transposon on pJEG40-like plasmids generating the unusual *vanA/vanB*$_2$
335 genotype with high level glycopeptides MICs (vancomycin ≥256 mg/L and teicoplanin
336 ≥256 mg/L and ≥48 mg/L). A recent study conducted in Australia, associated the same
337 Tn*1546-vanA like* transposon present in our *vanA/vanB*$_2$ 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*$_2$ operon rather than the acquisition of the *vanA* operon itself,
340 were responsible for the higher resistance phenotype observed in our *vanA/vanB*$_2$ 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*$_2$-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

Formatted: Highlight

Formatted: Highlight

Formatted: Subscript, Highlight

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*$_2$-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*$_2$ 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*$_2$-Tn*1549*-like transposon able to confer resistance to teicoplanin, which is for this
387 reason strongly inadvisable.
388
389 The presence of the Tn*1546*-like-pJEG40-like plasmids in two high related *vanB*$_2$–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

Formatted: Highlight

Formatted: English (United States)

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

Funding: Supported by a grant from the Italian Cooperation for Development (Carlo Urbani Project AID 009922).

Competing Interests: No conflicts

Ethical Approval: Not required

405 **References**

406 [1] Cheah A. L. Y. , SpelmanT., D. Liew, T. Peel, B. P. Howden, D. Spelman, M. L.
407 Grayson RLN and DCMK. Enterococcal bacteraemia: factors influencing mortality,
408 length of stay and costs of hospitalization. Clin Microbiol Infect 2013;19:384.
409 doi:10.1111/1469-0691.12204.

410 [2] Arias CA, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance.
411 Nat Rev Microbiol 2012;10:266–78. doi:10.1038/nrmicro2761.

412 [3] Orsi GB, Ciorba V. Vancomycin resistant enterococci healthcare associated
413 infections. Ann Ig 2013:485–92. doi:10.7416/ai.2013.1948.

414 [4] Jack N Pendleton SPG& BFG. Clinical relevance of the ESKAPE pathogens. Expert
415 Rev Anti Infect Ther 2013;11:297–308.

416 [5] Leavis HL, Bonten MJ, Willems RJ. Identification of high-risk enterococcal clonal
417 complexes: global dispersion and antibiotic resistance. Curr Opin Microbiol
418 2006;9:454–60. doi:10.1016/j.mib.2006.07.001.

419 [6] Werner G, Fleige C, Geringer U, van Schaik W, Klare I, Witte W. IS element IS16
420 as a molecular screening tool to identify hospital-associated strains of Enterococcus
421 faecium. BMC Infect Dis 2011;11:80. doi:10.1186/1471-2334-11-80.

422 [7] Leavis HL, Willems RJL, van Wamel WJB, Schuren FH, Caspers MPM, Bonten
423 MJM. Insertion sequence-driven diversification creates a globally dispersed
424 emerging multiresistant subspecies of E. faecium. PLoS Pathog 2007;3:e7.
425 doi:10.1371/journal.ppat.0030007.

426 [8] Cattoir V, Leclercq R. Twenty-five years of shared life with vancomycin-resistant
427 enterococci: Is it time to divorce. J Antimicrob Chemother 2013;68:731–42.
428 doi:10.1093/jac/dks469.

429 [9] Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P. Modes and

430 modulations of antibiotic resistance gene expression. Clin Microbiol Rev
431 2007;20:79–114. doi:10.1128/CMR.00015-06.
432 [10] Lebreton F, Schaik W Van, Manson A. Emergence of Epidemic Multidrug-Resistant
433 Enterococcus faecium. Am Soc Microbiol 2013;4:1–10. doi:10.1128/mBio.00534-
434 13.Editor.
435 [11] Freitas AR, Tedim AP, Francia M V., Jensen LB, Novais C, Peixe L, et al.
436 Multilevel population genetic analysis of *vanA* and *vanB Enterococcus faecium*
437 causing nosocomial outbreaks in 27 countries (1986–2012). J Antimicrob
438 Chemother 2016;71:3351–66. doi:10.1093/jac/dkw312.
439 [12] Sivertsen A, Billstr??m H, Melefors ??jar, Liljequist BO lsson, Wisell KT egmark,
440 Ullberg M, et al. A multicentre hospital outbreak in Sweden caused by introduction
441 of a vanB2 transposon into a stably maintained pRUM-plasmid in an Enterococcus
442 faecium ST192 clone. PLoS One 2014;9:e103274.
443 doi:10.1371/journal.pone.0103274.
444 [13] Courvalin P. Vancomycin resistance in gram-positive cocci. Clin Infect Dis 2006;42
445 Suppl 1:S25-34. doi:10.1086/491711.
446 [14] Heikens E, Bonten MJM, Willems RJL. Enterococcal surface protein esp is
447 important for biofilm formation of Enterococcus faecium E1162. J Bacteriol
448 2007;189:8233–40. doi:10.1128/JB.01205-07.
449 [15] Panesso D, Montealegre MC, Rincón S, Mojica MF, Rice LB, Singh K V, et al. The
450 hyl Efm gene in pHyl Efm of Enterococcus faecium is not required in pathogenesis
451 of murine peritonitis. BMC Microbiol 2011;11:20.
452 [16] Howden B, Holt K, Lam M, Seemann T. Genomic Insights Howden, B., Holt, K.,
453 Lam, M., & Seemann, T. (2013). Genomic Insights to Control the Emergence of
454 Vancomycin-Resistant Enterococci. mBio. doi:10.1128/mBio.00412-13.Editorto

455 Control the Emergence of Vancomycin-Resistant Enterococci. MBio 2013;4.
456 doi:10.1128/mBio.00412-13.Editor.
457 [17] De Been M, Pinholt M, Top J, Bletz S, Mellmann A, Van Schaik W, et al. Core
458 genome multilocus sequence typing scheme for high-resolution typing of
459 enterococcus faecium. J Clin Microbiol 2015;53:3788–97. doi:10.1128/JCM.01946-
460 15.
461 [18] Willems RJL, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, et al.
462 Global spread of vancomycin-resistant Enterococcus faecium from distinct
463 nosocomial genetic complex. Emerg Infect Dis 2005;11:821–8.
464 doi:10.3201/eid1106.041204.
465 [19] Brodrick HJ, Raven KE, Harrison EM, Blane B, Reuter S, Török ME, et al. Whole-
466 genome sequencing reveals transmission of vancomycin-resistant Enterococcus
467 faecium in a healthcare network. Genome Med 2016;8:4. doi:10.1186/s13073-015-
468 0259-7.
469 [20] Weng P, Ramli R, Mariana Nor S, Yoke-Kqueen C, Rukman Awang H. High
470 genetic diversity of Enterococcus faecium and Enterococcus faecalis clinical isolates
471 by pulsed-field gel electrophoresis and multilocus sequence typing from a hospital
472 in Malaysia. Biomed Res Int 2013;938937.
473 [21] Dang STT, Petersen A, Van Truong D, Chu HTT, Dalsgaard A. Impact of
474 medicated feed on the development of antimicrobial resistance in bacteria at
475 integrated pig-fish farms in Vietnam. Appl Environ Microbiol 2011;77:4494–8.
476 doi:10.1128/AEM.02975-10.
477 [22] Poulsen LL, Bisgaard M, Son NT, Trung N V., An HM, Dalsgaard A. Enterococcus
478 and Streptococcus spp. associated with chronic and self-medicated urinary tract
479 infections in Vietnam. BMC Infect Dis 2012;12:1. doi:10.1186/1471-2334-12-320.

480 [23] Daniel DS, Lee SM, Dykes GA, Rahman S. Public health risks of multiple-drug-
481 resistant Enterococcus spp. in Southeast Asia. Appl Environ Microbiol
482 2015;81:6090–7. doi:10.1128/AEM.01741-15.
483 [24] Kariyama R, Mitsuhata R, Chow JW, Clewell B, Kumon H. Simple and Reliable
484 Multiplex PCR Assay for Surveillance Isolates of Simple and Reliable Multiplex
485 PCR Assay for Surveillance Isolates of Vancomycin-Resistant Enterococci. J Clin
486 Microbiol 2000;38:3092–5.
487 [25] Elsayed S, Hamilton N, Boyd D, Enterococcus V. Improved Primer Design for
488 Multiplex PCR Analysis of Vancomycin-Resistant Improved Primer Design for
489 Multiplex PCR Analysis of. J Clin Microbiol 2001;39:2367–9.
490 doi:10.1128/JCM.39.6.2367.
491 [26] Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, et
492 al. Development of a multiplex PCR for the detection of asaI, gelE, cylA, esp, and
493 hyl genes in enterococci and survey for virulence determinants among european
494 hospital isolates of Enterococcus faecium. J Clin Microbiol 2004;42:4473–9.
495 doi:10.1128/JCM.42.10.4473-4479.2004.
496 [27] Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J.
497 ACT: The Artemis comparison tool. Bioinformatics 2005;21:3422–3.
498 doi:10.1093/bioinformatics/bti553.
499 [28] Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al.
500 Versatile and open software for comparing large genomes. Genome Biol
501 2004;5:R12. doi:10.1186/gb-2004-5-2-r12.
502 [29] Okonechnikov K, Golosova O, Fursov M, Varlamov A, Vaskin Y, Efremov I, et al.
503 Unipro UGENE: A unified bioinformatics toolkit. Bioinformatics 2012;28:1166–7.
504 doi:10.1093/bioinformatics/bts091.

505 [30] Alikhan N-F, Petty NK, Ben Zakour NL, Beatson SA. BLAST Ring Image
506 Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics
507 2011;12:402. doi:10.1186/1471-2164-12-402.
508 [31] Gardner SN, Slezak T, Hall BG. kSNP3.0: SNP detection and phylogenetic analysis
509 of genomes without genome alignment or reference genome. Bioinformatics
510 2015;31:2877–8. doi:10.1093/bioinformatics/btv271.
511 [32] Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics
512 Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 2016;33:1870–4.
513 doi:10.1093/molbev/msw054.
514 [33] Pinholt M, Gumpert H, Bayliss S, Nielsen JB, Vorobieva V, Pedersen M, et al.
515 Genomic analysis of 495 vancomycin-resistant Enterococcus faecium reveals broad
516 dissemination of a vanA plasmid in more than 19 clones from Copenhagen,
517 Denmark. J Antimicrob Chemother 2017;72:40–7. doi:10.1093/jac/dkw360.
518 [34] van Hal SJ, Espedido BA, Coombs GW, Howden BP, Korman TM, Nimmo GR, et
519 al. Polyclonal emergence of vanA vancomycin-resistant Enterococcus faecium in
520 Australia. J Antimicrob Chemother 2017;72:998–1001. doi:10.1093/jac/dkw539.
521 [35] Papagiannitsis CC, Malli E, Florou Z, Medvecky M, Sarrou S, Hrabak J, et al. First
522 description in Europe of the emergence of Enterococcus faecium ST117 carrying
523 both vanA and vanB genes, isolated in Greece. J Glob Antimicrob Resist
524 2017;11:68–70. doi:10.1016/j.jgar.2017.07.010.
525 [36] Guzman Prieto AM, van Schaik W, Rogers MRC, Coque TM, Baquero F, Corander
526 J, et al. Global emergence and dissemination of enterococci as nosocomial
527 pathogens: Attack of the clones? Front Microbiol 2016;7:1–15.
528 doi:10.3389/fmicb.2016.00788.
529 [37] Willems R, Top J, Schaik W Van, Leavis H. Restricted gene flow among hospital

530 subpopulations of Enterococcus faecium. MBio 2012;3. doi:10.1128/mBio.00151-
531 12.Editor.
532 [38] Hayden MK, Trenholme GM, Schultz JE, Sahm DF. In Vivo Development of
533 Teicoplanin Resistance in a VanB Enterococcus faecium Isolate 1993;1992:1224–7.
534 [39] Hammerum AM. Enterococci of animal origin and their significance for public
535 health. Clin Microbiol Infect 2012;18:619–25. doi:10.1111/j.1469-
536 0691.2012.03829.x.
537 [40] López M, Sáenz Y, Alvarez-Martínez MJ, Marco F, Robredo B, Rojo-Bezares B, et
538 al. Tn1546 structures and multilocus sequence typing of vanA-containing
539 enterococci of animal, human and food origin. J Antimicrob Chemother
540 2010;65:1570–5. doi:10.1093/jac/dkq192.
541 [41] Ballard SA, Pertile KK, Lim M, Johnson PDR, Grayson ML. Molecular
542 characterization of vanB elements in naturally occurring gut anaerobes. Antimicrob
543 Agents Chemother 2005;49:1688–94. doi:10.1128/AAC.49.5.1688-1694.2005.
544 [42] Domingo M-C, Huletsky A, Giroux R, Boissinot K, Picard FJ, Lebel P, et al. High
545 prevalence of glycopeptide resistance genes *vanB*, *vanD*, and *vanG* not associated
546 with *enterococci* in human fecal flora. Antimicrob Agents Chemother
547 2005;49:4784–6. doi:10.1128/AAC.49.11.4784-4786.2005.
548 [43] Knight DR, Androga GO, Ballard SA, Howden BP, Riley T V. A Phenotypically
549 Silent vanB2 Operon Carried on a Tn1549-Like Element in Clostridium difficile.
550 MSphere 2016;1:e00177-16. doi:10.1128/mSphere.00177-16.
551 [44] DiazGranados CA, Zimmer SM, Mitchel K, Jernigan JA. Comparison of Mortality
552 Associated with Vancomycin-Resistant and Vancomycin-Susceptible Enterococcal
553 Bloodstream Infections: A Meta-analysis. Clin Infect Dis 2005;41:327–33.
554 doi:10.1086/430909.

555 [45] Eshaghi A, Shahinas D, Li A, Kariyawasam R, Banh P, Desjardins M, et al.
556 Characterization of an Enterococcus gallinarum isolate carrying a dual vanA and
557 vanB cassette. J Clin Microbiol 2015;53:2225–9. doi:10.1128/JCM.03267-14.
558 [46] Pinholt M, Larner-Svensson H, Littauer P, Moser CE, Pedersen M, Lemming LE, et
559 al. Multiple hospital outbreaks of vanA Enterococcus faecium in Denmark, 2012-13,
560 investigated by WGS, MLST and PFGE. J Antimicrob Chemother 2015;70:2474–
561 82. doi:10.1093/jac/dkv142.
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).