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Local Genetic Patterns within a Vancomycin-Resistant *Enterococcus faecalis* Clone Isolated in Three Hospitals in Portugal

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Eight pulsed-field gel electrophoresis subtypes and six Tn*1546* variants were identified among *Enterococcus faecalis* isolates of a single clone recovered in three geographically separate Portuguese hospitals. Some clonal subtypes were found in particular hospitals, and Tn*1546* variants were either widespread or confined to some of them. We also report on the first Tn*1546* transposon containing an IS*Ef1* insertion.

The epidemiology of enterococci is not fully understood since striking differences among resistant isolates of different species and resistant isolates from different geographic locations have been reported (1, 20). Besides clonal spread, a heterogeneous geographic distribution of different antibiotic resistance determinants, as different transposon types, is involved in resistance of vancomycin-resistant enterococci (VRE) (1, 4, 5, 8, 14, 27, 29). In Europe, a polyclonal enterococcal population structure with a large variety of Tn*1546* types was initially observed in the community (1, 27, 29). In the United States, the initial VRE population consisted of a few persisting clones harboring a few Tn*1546* variants (1, 5, 8, 12, 15, 16, 19). Recent studies have pointed out that the endemic susceptible clones could serve as substrates for the spread of VRE (11, 15, 19, 22; S. R. Nallapareddy, W. Huang, G. M. Weinstock, and B. E. Murray, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-2165, 2003). Also, there is an increasing trend toward the consideration of antibiotic resistance as a regional problem (21).

The dissemination of a single vancomycin-resistant *Enterococcus faecalis* clone among three geographically separate Portuguese hospitals and the characterization of antibiotic resistance genetic elements of isolates of this clone were studied in the work described here. Thirty-three VRE clinical isolates were detected from 1996 to 2002 in three Portuguese hospitals (University Hospital in Coimbra [HUC], 11 isolates; Santo António Hospital in Porto [HSA], 19 isolates; and São Teotónio Hospital in Viseu [HST], 3 isolates). The sample included all VRE detected during 2001 and 2002 in HUC, HSA, and HST and some VRE isolates saved by the microbiology laboratory in HUC from 1996 to 2000.

Susceptibility testing was performed according to NCCLS guidelines (17). A multiplex PCR assay was used for species identification and vancomycin resistance gene detection (6). Genes coding for resistance to aminoglycosides or macrolides were also investigated (13, 25). Conjugation experiments were performed with *E. faecalis* strain JH2-2 as the recipient (9). The backbone structure of the Tn*1546* transposon harbored by each VRE isolate was determined by the overlapping PCR assay described by Woodford et al. (29). Sequencing of specific fragments of Tn*1546* was performed in order to identify the insertions.

All PCR assays included suitable positive and negative controls, kindly provided by B. E. Murray, M. Zervos, and C. Torres. Strains and JH2-2 transconjugants were typed by pulsed-field gel electrophoresis (PFGE) with SmaI and I-CeuI as restriction enzymes (10). The location of *vanA* was determined by hybridization of I-CeuI-digested genomic DNA with probes labeled with an enhanced chemiluminescence kit (Amersham Life Sciences, Uppsala, Sweden) for *vanA* and 23S rRNA genes, as described previously (3). Clonal relationships were established by the criteria proposed by Tenover et al. (23). Clones were designated by capital letters. Subtypes were defined by a subindex that indicates the number of bands that differed from the number for the strain considered to be the initial PFGE type.

Nine PFGE types were identified among the 33 VRE isolates studied, with clone B being predominant (25 of 33 isolates [76%]). The first *vanA* isolate obtained in HUC (in 1996) was arbitrarily considered clone B (it was found in all three hospitals). Seven subtypes (which differed from each other by one to five bands) were detected in the subsequent years (Fig. 1) B2 (12 strains isolated from different patients in HSA); B5 (3 strains in HUC); B1 and B3 (2 strains each in HUC and HSA); and B1′, B3′, and B4 (1 strain each) (Table 1). Subtypes B2 and B5 persisted for 2 years in particular hospitals. Eight VRE PFGE types (types A, C, F, G, K, N, Q, and X) isolated from single patients were also detected.

Most of the VRE isolates were resistant to erythromycin (94%) and ciprofloxacin (88%) and had high-level resistance to gentamicin (HLRGm) (82%) or kanamycin (HLRKm) (82%) (Table 1). The *vanA* gene was detected in all VRE isolates. HLRGm and resistance to erythromycin were due to *aac*(6′)-*aph*(2′′) and *erm*(B), respectively. Both *aac*(6′)-aph(2′′) and *aph*(3′)-*IIIa* were detected in 13 isolates belong-

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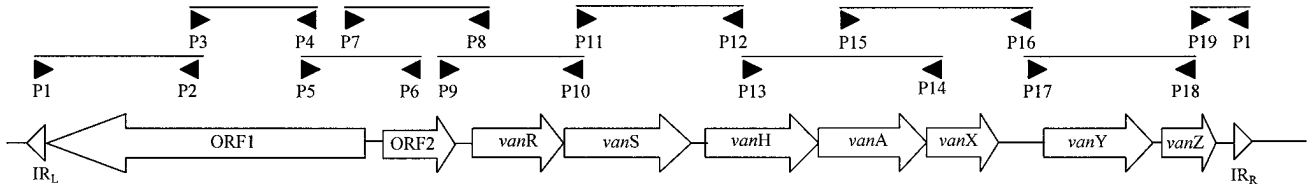
TABLE 1. Clinical data, PFGE types, antibiotic resistance and virulence profiles, Tn*1546* types, and frequency of transfer of studied traits for vancomycin-resistant *E. faecalis* clones isolated in hospitals located in three different geographical areas of Portugal[a]

Hospital and PFGE type	No. of isolates	Date of isolation (mo/yr)	Ward	Source[b]	Antibiotic resistance profile[c]									Transfer frequency[d]	Tn*1546* type
					VAN	TEC	ERY	CIP	GEN	KAN	STR	TET	CHL		
HSA															
B	1	6/01	ICU	Urine	R	R	R	R	R	R		R		10^{-5}	PP4
B2	9	1/01–10/02	Obstetrics, cardiac surgery, hematology, nephrology, general surgery, internal medicine	Urine (6), Pus (2), Blood (1)	R	R	R	R	R	R	R	R[f]	R[e]	10^{-7}–10^{-8}	PP15
B2	2	3/02–4/02	ICU, internal medicine B	Urine	R	R		R	R	R				10^{-6}–10^{-7}	PP16
B2	1	12/02	ICU	Blood	R	R	R	R	R	R	R			ND	PP5
B3	2	3/01–7/01	ICU, obstetrics	Pus, urine	R	R	R	R	R	R	R	R	R	10^{-7}–10^{-8}	PP15
B4	1	11/01	Urology	Urine	R	R	R	R	R	R	R		R	10^{-7}	PP15
K	1	9/01	Urology	Pus	R	R	R					R		10^{-4}	A
C	1	11/01	Urology	Pus	R	R	R	R	R	R	R		R	10^{-8}	PP15
F	1	11/02	Skull trauma	Blood	R	R	R							10^{-7}	PP5
HST															
B	1	8/01	Internal medicine 2A	Unknown			R	R	R	R		R	R	ND	
B	1	3/02	Internal medicine 1A	Unknown			R	R	R	R			R	ND	
B1′	1	11/01	Nephrology	Urine	R	R	R	R	R	R	R	R	R	10^{-4}	PP4
B3′′	1	3/02	Neurosurgery	Unknown			R	R				R	R	ND	
B3′	1	4/02	Internal medicine 2B	Urine	R	R	R	R				R	R	10^{-5}	PP4
A	1	1/02	Orthopedics	Urine	R	R	R	R				R		10^{-5}	PP4
HUC															
B	1	1996	Liver transplant	Unknown	R	R	R	R	R	R		R	R	10^{-8}	PP4
B	1	10/01	Hematology	Blood	R	R	R	R	R	R		R	R	10^{-6}	PP2
B1	1	7/01	Nephrology	Exudate	R	R	R	R	R	R		R	R	10^{-7}	PP4
B1	1	10/01	General surgery 3M	Urine	R	R	R	R	R	R		R	R	10^{-6}	PP5
B5	3	1/99–6/00	Nephrology	Unknown, blood (2)	R	R	R	R	R	R	R	R		10^{-8}	PP4
N	1	11/99	Nephrology	Blood	R	R	R	R	R	R		R		10^{-5}	PP4
X	1	2/00	Nephrology	Unknown	R	R	R							10^{-6}	PP4
Q	1	5/01	Nephrology	Blood	R	R	R							10^{-5}	PP4
G	1	4/02	Liver transplant	Blood	R	R	R	R	R	R		R		10^{-6}	PP4

[a] Abbreviations: R, resistant; ND, transfer experiments not done; ICU, intensive care unit; VAN, vancomycin; TEC, teicoplanin; ERY, erythromycin; CIP, ciprofloxacin; GEN, HLRGm; KAN, HLRKm; STR, high-level resistance to streptomycin; TET, tetracycline; CHL, chloramphenicol.
[b] The numbers of isolates are indicated in parentheses.
[c] Underscores indicate that antibiotic resistance was detected in transconjugants.
[d] Transfer frequency is expressed as the number of transconjugants per number of donors.
[e] All except two isolates were resistant.
[f] Only three isolates were resistant.

TABLE 2. Tn*1546* types found among *E. faecalis* clinical isolates recovered at three different Portuguese hospitals (1996 to 2002)



Type[a]	Amplification of PCR product with the following specific primer pair (annealing sites)[a]: p1-p2 (22–1330)	p3-p4 (1222–2353)	p5-p6 (2227–3525)	p7-p8 (2769–4042)	p9-p10 (3569–4793)	p11-p12 (4675–6353)	p13-p14 (6229–8021)	p15-p16 (6979–8920)	p17-p18 (8889–10473)	p19-p1 (10403–10830)	No. of isolates	Diversity of Tn*1546* among Portuguese isolates: Other source(s)[d]	Cities
A	+	+	+	+	+	+	+	+	+	+	1	HH, P (Efm, Efls)	Porto
PP2	+	+	+	+	+	+	+	+	−/++[b,c]	+	1	HH (Efm, Efls)	Coimbra, Viseu
PP4	+	+	+	+	+	+	+	+	++[c]	+	13	HH (Efm, Efls)	Coimbra, Viseu, Porto
PP5	+	+	+	+	+	+	+	+	++[c]	−	3	HH (Efm, Efls)	Coimbra, Porto
PP15	−	−	−	−	+	+	+	+	+	+	13	HH, P (Efm, Efls)	Porto
PP16	−	−	−	−	−	+	+	+	+	+	2	HH, SW (Efm, Efls)	Porto

[a] Tn*1546* types were designed according to the scheme of Woodford et al. (29). For those that did not have a specific previously described type, we used our own designation (PP [Portugal-Porto], followed by a randomly chosen number). +, amplification; −, no amplification; ++, amplification of sequences larger than those of the expected size.

[b] For PP2, amplification was negative with p17-p18 but positive with IS*Efl*-F (5′-GGT GTT ACG ATG TCT GAA ATT GC-3′) and p18.

[c] Sequencing of this fragment demonstrated the presence of IS*Efl* in the intergenic *vanX-vanY* region.

[d] Sources and species in which specific Tn*1546* types were found. HH, human isolates; P, poultry isolates; SW, isolates from sewage samples; Efm, *E. faecium*; Efls, *E. faecalis* (C. Novais, T. M. Coque, F. Baquero, and L. Peixe, Abstr. 14th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. 2430, 2004).

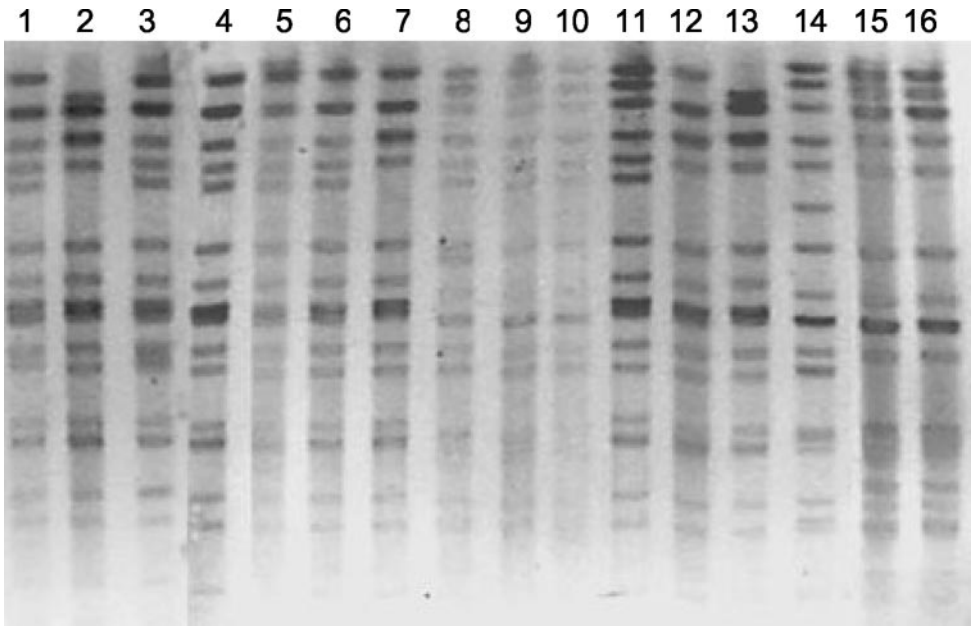


FIG. 1. SmaI-digested chromosomal DNA of vancomycin-susceptible and vancomycin-resistant *E. faecalis* isolates classified as clonal type B, as determined by PFGE. Lanes 1, 3, 4, 5, and 6, subtype B; lanes 7 and 12, subtype B1; lane 11, subtype B1′; lanes 9 and 10, subtype B2; lane 8, subtype B3; lane 13, subtype B3′; lane 14, subtype B4; lanes 15 and 16, subtype B5.

ing to four clone B subtypes and in one isolate classified as clone C.

Five different variants of Tn*1546* (variants PP2, PP4, PP5, PP15, and PP16) were found among clinical vancomycin-resistant *E. faecalis* isolates (Table 2). One of the more frequent variants, PP4, which contained an IS*Ef1* insertion sequence, was present among isolates ($n = 13$) of different PFGE types in all three hospitals: types B (subtypes B, B1, B1′, B3′, and B5), A, N, X, Q, and G. On the contrary, the PP15 variant, which was also present in 13 isolates of different PFGE types, types B (subtypes B2, B3, and B4) and C, was detected in only a single hospital (HSA). Some of these Tn*1546* types were also found in *E. faecalis* and *Enterococcus faecium* strains of different origins, indicating a wide distribution of specific transposon variants (Table 2) (C. Novais et al., unpublished data).

The transfer of *vanA* to *E. faecalis* strain JH2-2 was achieved with all VRE isolates. *erm*(B) was cotransferred with *vanA* in all cases. Transconjugants harboring *vanA*, *erm*(B), and *aph*(*3′*)-*IIIa* were detected for 7 of 12 VRE isolates with HLRKm at HSA (subtypes B2, B3, B4 and C) and 2 VRE isolates with HLRKm at HUC. They contained PP15 and PP4 variants of Tn*1546*, respectively (Table 1).

The patterns of I-CeuI-digested genomic DNA of strains and transconjugants harboring the more prevalent Tn*1546* variants, PP4 and PP15, mostly differed by a single band with a small molecular size that varied in length among the isolates. Hybridization of the *vanA* probe but not the 23S rDNA probe to these bands suggested that PP4 and PP15 are located on different plasmids.

This work focused on the dissemination and persistence of a particular *E. faecalis vanA* clone (clone B) isolated over 7 years in different Portuguese hospitals. Some clonal subtypes were recovered over prolonged periods of time in particular hospitals: as subtype B5 in Coimbra (HUC) or subtype B2 in Porto (HSA). The Tn*1546* types responsible for vancomycin resistance were also unevenly distributed: type PP4 was found in different clones detected in distinct hospitals, and type PP15 was found in different strains from a single hospital. The presence of specific Tn*1546* variants in selected PFGE types or subtypes with different geographic distributions may cast some light on the reason for differences in local genetic patterns of resistance. Vancomycin resistance determinants might be stably maintained only in selected clones, with some of them being widely disseminated in certain institutions. Locally prevalent clones of vancomycin-susceptible enterococci (VSE) have been suggested to be the leading force for the local spread of VRE (11, 22). Although VSE were not systematically studied, we were able to detect different VSE variants of the most prevalent VRE clone (clone B) during 2001 and 2002 in some of the hospitals studied, suggesting a possible longer persistence of this strain (Table 1). The intra- and interhospital transmissions of *E. faecalis* VSE clones have recently been reported in Spain, Sweden, The Netherlands, the United Kingdom, and Ireland (7, 26, 28; P. Ruíz-Garbajosa, R. Cantón, T. M. Coque, V. Pintado, F. Baquero, and R. del Campo, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. L-270, 2003), although the role of these widely disseminated VSE remains unknown. On the other hand, the acquisition of different Tn*1546* elements by a particular clone has scarcely been reported for either *E. faecium* or *E. faecalis* (24, 29).

The heterogeneity of Tn*1546* is mainly explained by the presence of different insertion sequences, which serve as hot spots for the rearrangement of genetic fragments (2, 9, 18). Some of these insertion sequences may be associated with certain geographic locations (4, 5, 14, 15, 27, 28). We report for the first time the presence of IS*Ef1* in Tn*1546* (variants PP2, PP4, and PP5), which was found to be widely distributed among Portuguese hospitals. Interestingly, this insertion se-

quence element is one of the most frequently detected insertion sequences in the chromosome of the sequenced genome of *E. faecalis* strain V583 (18), although its prevalence in the genomes of different *E. faecalis* strains remains unknown.

Our results indicate that the clonal and Tn*1546* patterns involved in vancomycin resistance among *E. faecalis* isolates from particular hospitals in different regions may differ and suggest that the analysis of local patterns might explain the differences in the occurrence and diversity of VRE in different institutions.

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