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Molecular Characterization of Glycopeptide-Resistant *Enterococcus faecium* Isolates from Portuguese Hospitals

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Fifty-one pulsed-field gel electrophoresis types and 17 Tn*1546* variants were identified among 101 *Enterococcus faecium* isolates recovered in three distant Portuguese hospitals. Intra- and interhospital dissemination of specific strains and Tn*1546* types was detected, which might largely contribute to the endemicity of vancomycin-resistant *E. faecium* in Portuguese hospitals, as happened previously in other geographical locations.

Vancomycin-resistant enterococci (VRE) have been increasingly reported worldwide since first described in 1987, although the epidemiology of these microorganisms varies widely in different geographical areas (1, 18, 23). In the United States, VRE have become established nosocomial pathogens in intensive care units and increasingly in many hospital wards (1, 5, 15, 23, 27). In Europe, they have been mainly recovered from the community setting (1, 32), with sporadic cases of nosocomial outbreaks involving different epidemiological situations (1, 13, 14). Recent studies showing rates of VRE above 10% in six countries (Annual Report of the European Antibiotic Resistance Surveillance System, 2002 [http//:www.earss.rivm.nl]) and intrahospital dissemination of particular strains in some institutions suggest a change in the epidemiology of VRE in Europe (8). The objective of the study was to collect updated data on the clonality, antibiotic resistance genotype, and diversity of Tn*1546* of vancomycin-resistant *Enterococcus faecium* (VREF) from Portuguese hospitals.

One hundred one VREF clinical isolates were studied among those isolated during 1996 to 2003 in three Portuguese hospitals (University Hospital in Coimbra [HUC], 54 isolates; Santo António Hospital [HSA] in Porto, 36 isolates; and São Teotónio Hospital in Viseu [HST], 11 isolates). The sample included all VRE detected in HUC, HSA, and HST isolated from January 2001 to April 2003 and a few VRE saved by microbiology laboratories in HUC from 1996 to 2000. Transfer of patients from HST to HUC occurs when specialized treatment is required. Sites of isolation of the 101 clinical isolates were urine (36%), blood (16%), wound (12%), abdominal (9%), catheter (8%), and respiratory tract (3%), and 15% were from unknown sources. Distribution of the *E. faecium* clinical isolates in hospital units is shown in Table 1. Of the 101 patients, 64% were in medical wards, 14% in surgical wards, and 16% in intensive care facilities, and 6% could not be classified.

Susceptibility testing was performed according to NCCLS guidelines, using the recommended breakpoints to define resistance (19). A multiplex PCR assay was used for species identification and vancomycin resistance gene detection (6). Genes coding for resistance to aminoglycosides and macrolides, the backbone structure of Tn*1546* harbored by VREF, and virulence factors (cytolysin [Cyl], gelatinase [Gel], aggregation substance [Agg], hyaluronidase [Hyl], and enterococcal surface protein [Esp]) were investigated by PCR as described previously (7, 17, 24, 29, 30, 32). Conjugation experiments were performed using *E. faecium* GE1 as the recipient with isolates representing each pulsed-field gel electrophoresis (PFGE) type and subtype (10). Strains were typed by PFGE using SmaI and I-CeuI as restriction enzymes (3, 12). The location of *vanA* was determined by hybridization of I-CeuI-digested genomic DNA with probes labeled with the ECL kit (Amersham Life Sciences, Uppsala, Sweden) for *vanA* and 23S rRNA genes as described previously (3). Clonal relationships were established according to standard criteria (26).

Fifty-one PFGE types were identified among the 101 VREF isolates studied, with types 70 (14/101; 14%) and 78 (17/101; 17%) being the more commonly found. Interhospital dissemination of strain 70, 76, 78, 88, or 100 (found in two hospitals) or strain 71 (detected in three hospitals) was observed. Most VREF isolates studied were also resistant to teicoplanin (96/101; 95%), ampicillin (100/101; 99%), erythromycin (99/101; 98%), and ciprofloxacin (96/101; 95%). A high level of resistance to kanamycin, gentamicin, or streptomycin was detected in 67% (68/101), 34% (34/101), and 28% (28/101) of isolates, respectively. Rates of resistance to tetracycline, nitrofurantoin, and chloramphenicol were 34%, 7%, and 8%, respectively. All isolates were susceptible to linezolid and daptomycin.

The *vanA* gene was identified in all except one (*vanB2*) VREF isolate. A high level of resistance to gentamicin was due to *aac*(6′)-*aph*(2″), and resistance to erythromycin was associated with *erm*(B). The simultaneous presence of *aac*(6′)-*aph*(2″) and *aph*(3′)-IIIa was detected in 10 isolates. *vanC1*,

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TABLE 1. Features of VRE *Enterococcus faecium* clones isolated in hospitals located in three different Portuguese areas[a]

Hospital	PFGE type[b]	PFGE subtype	Date of isolation[c]	No. of isolates	Unit[d] (n^e)	Source (n^e)	Antibiotic resistance profile[f,g]	Transfer frequency[h]	Virulence gene(s)[g]
HSA	**78**	**4**	?/01–09/03	9	NEFR(2), SURG (3), UROL (1), IMED(3)	Urine (5), wound (2), blood (1), abdominal (1)	VC, TC, AP, EM, CP, (KM)	10^{-2}–10^{-7}/–	
	85		?/01	1	IMED	Urine	VC, AP, EM, CP, SM, KM	–	
	96		?/01	1	IMED	Urine	VC, TC, AP, EM, KM	10^{-5}	*esp*
	101		?/01	1	IMED	Urine	VC, TC, AP, EM, CP, CL, GM, SM, KM	–	*esp*
	110		?/01	1	SURG	Urine	VC, TC, AP, EM, CL, SM, KM	10^{-5}	*esp*
	111		?/01	2	IMED, OBSER	Blood	VC, AP, EM, CP, KM	–/ND	*hyl*, *esp*
	114		?/01	1	NEF	Urine	VC, TC, AP, EM, CP, SM, KM	–	*esp*
	102		03/01	1	ICU	Wound	VC, TC, AP, EM, CP, GM, SM, KM	–	
	77		06/01	1	SURG	Urine	VC, TC, AP, TE, CP, GM, KM	–	*asa1*, *gel*, *esp*
	91		?–07/01	2	NEFR, OBSER	Urine, wound	VC, TC, AP, EM, CP, (SM), KM	10^{-5}–10^{-7}	*esp*
	86		07/01	1	SURG	Urine	VC, TC, AP, EM, CP, GM, KM	10^{-8}	
	88	1	07/01	2	NEFR, GASTR	Urine, abdominal	VC, TC, AP, EM, CP, GM, KM	–	*esp*
	109		07/01	1	NEF	Urine	VC, TC, AP, TE, EM, CP, SM, KM	10^{-4}	*esp*
	118		07/01	1	IMED	Urine	VC, TC, AP, EM, CP, SM, KM	–	*esp*
	120		01/02	1	ICU	Urine	VC, TC, AP, CP, GM, KM	–	*esp*
	115		02/02	1	IMED	Urine	VC, TC, AP, EM, CP, SM, KM	–	
	116		05/02	1	NEF	Urine	VC, TC, AP, EM, CP, SM, KM	–	
	76		06/02	1	SURG	Wound	VC, TC, EM	10^{-3}	
	73		08/02	1	NEF	Abdominal	VC, TC, AP, TE, EM, CP	–	
	119	2	09–12/02	2	IMED, PED	Urine	VC, (TC), AP, EM, CP, GM, KM	10^{-7}/–	*esp*
	100	**2**	09–12/02	3	IMED(2), UROL (1)	Urine	VC, TC, AP, EM, CP, GM, KM	10^{-3}/ND	(*esp*)
	71		12/02	1	IMED	Blood	VC, TC, AP, EM, CP, GM, KM	–	*hyl*
HST	121		09/01	1	ICU	Unknown	VC, AP, EM, CP, SM, KM	–	
	70	**4**	03–10/02	6	IMED(5), UKNOWN (1)	Unknown	VC, TC, AP, (TE), EM, CP	10^{-3}–10^{-8}/–	
	100		02/03	1	ICU	Urine	VC, TC, AP, EM, CP, GM, KM	–	*asa1*, *hyl*

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	71		03/03	1	ICU		VC, TC, AP, EM, CP, GM, KM	10^{-1}	
	104		03/03	1	NSURG	LCR	VC, TC, AP, EM, CP, GM, KM	10^{-8}	*esp*
	105		03/03	1	ICU	Unknown	VC, TC, AP, EM, CP, GM, KM	10^{-7}	*esp*
HUC	**70**	**6**	10/01–01/03	8	HEMAT (2), GASTR (2), IMED(2), Unknown (2)	Blood (3), abdominal (2), urine (1), unknown (2)	VC, TC, AP, TE, EM, CP, (CL), (GM), (KM)	10^{-3}/–	(*asa1*), (*gel*), (*esp*)
	75		Unknown	1	Unknown	Unknown	VC, TC, AP, TE, EM, CP, SM	–	
	95		Unknown	1	Unknown	Unknown	VC, TC, AP, EM, CP, KM	10^{-6}	
	97		Unknown	1	Unknown	Unknown	VC, TC, AP, EM, CP	–	
	83		?/96	1	SURG	Catheter	VC, TC, AP, TE, EM, R, GM, SM, KM	10^{-4}	
	90		?/97	1	NEF	Unknown	VC, TC, AP, TE, EM, SM, KM	10^{-8}	
	80		?/97	1	IMED	Urine	VC, TC, AP, EM, CP	–	*esp*
	112		01/98	1	NEF	Catheter	VC, TC, AP, EM, CP, KM	–	
	98		04/98	1	IMED	Urine	VC, TC, AP, TE, EM, CP, SM, KM	10^{-5}	
	88		04/99	1	LTU	Blood	VC, TC, AP, TE, EM, CP, GM, SM, KM	10^{-6}	
	99		08/99–11/00	6	LTU (5), SURG	Abdominal (3), respiratory (2), urine (1), catheter (1)	VC, TC, AP, EM, CP, (CL), GM, (SM), KM	10^{-7}/–	(*esp*)
	74		01/00	1	IMED	Catheter	VC, TC, AP, TE, EM, SM	10^{-4}	*esp*
	106		01/00	1	LTU	Abdominal	VC, TC, AP, TE, EM, CP, GM, KM	10^{-4}	
	84		03/00	1	IMED	Catheter	VC, TC, AP, EM, CP, GM, SM, KM	–	*esp*
	117		03/00	1	NEF	Respiratory	VC, TC, AP, TE, EM, CP, SM, KM	10^{-6}	*esp*
	92	2	10/00–03/02	2	SURG, PNEUM	Catheter, urine	VC, TC, AP, TE, EM, CP, KM	10^{-8}/–	
	108		11/00	1	ICU	Blood	VC, TC, AP, TE, EM, CP, SM, KM	–	*esp*
	82		04/01	1	HEMAT	Urine	VC, TC, AP, TE, EM, CP, GM, SM, KM	–	*esp*
	76		06/01–08/01	2	HEMAT	Urine, wound	VC, TC, AP, TE, EM, CP, (KM)	–	*esp*
	122		07/01	1	NEF	Urine	VC, TC, AP, TE, EM, GM, KM	–	*esp*

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TABLE 1—*Continued*

Hospital	PFGE type[b]	PFGE subtype	Date of isolation[c]	No. of isolates	Unit[d] (n^e)	Source (n^e)	Antibiotic resistance profile[f,g]	Transfer frequency[h]	Virulence gene(s)[g]
	107		08/01	1	HEMAT	Blood	VC, TC, AP, TE, EM, CP, CL, SM, KM	−	*esp*
	78		09/01–01/03	8	GASTR (2), IMED (2), SURG (2), LTU (1), NEF (1)	Catheter (2), blood (2), wound (2), abdominal	VC, TC, AP, EM, CP, (CL), (KM), (NT)	10^{-3}/– 10^{-5}/−	
	81		10/01	1	NEF	Wound	VC, TC, AP, EM, CP, CL	10^{-5}	
	113		10/01	1	IMED	Wound	VC, TC, AP, TE, EM, CP, CL, KM	−	*asa1*, *gel*
	87		03/02	1	LTU	Urine	VC, TC, AP, EM, CP, GM, KM	−	
	93		03/02	1	IDIS	Urine	VC, TC, AP, TE, EM, CP, KM	−	*esp*
	94		03/02	1	NEF	Blood	VC, TC, AP, TE, EM, CP, KM	10^{-5}	
	ND		04/02	1	GASTR	Unknown	VC, TC, AP, EM CP, KM, NT	10^{-6}	
	103	2	12/02–02/03	2	GASTR, SURG	Wound (2)	VC, TC, AP, EM, CP, GM, KM	$-/10^{-7}$	
	71	**1**	01/03	1	GASTR	Blood	VC, TC, AP, EM, CP, GM, SM, KM	−	*hyl*
	ND		03/03	1	IMED	Blood	VC, TC, AP, EM CP, KM	10^{-2}	
	72		04/03	1	IMED	Blood	VC, TC, AP, EM, CP, SM	10^{-3}	

[a] Clinical data, PFGE, antibiotic resistance and virulence profiles, Tn*1546* types, and frequency of transfer of studied traits are given.
[b] PFGE types identified in more than one hospital are in bold.
[c] Month/year–month/year or month–month/year.
[d] IMED, internal medicine; SURG, surgery; NSURG, neurosurgery; HAEM, hematology; NEFR, nephrology; UROL, urology; OBSER, observation; PED, pediatrics; LTU, liver transplant unit; IDIS, infectious diseases; GASTR, gastroenterology; PNEUM, pneumology; ICU, intensive care unit.
[e] *n*, no. of isolates.
[f] VC, vancomycin; TC, teicoplanin; AP, ampicillin; TE, tetracycline; EM, erythromycin; CP, ciprofloxacin; CL, chloramphenicol; GM, high level of resistance to gentamicin; SM, high level of resistance to streptomycin; KM, high level of resistance to kanamycin; ND, not done.
[g] Variable presence of a given virulence trait and resistance gene among isolates belonging to the same PFGE appear in parenthesis; underlining indicates antibiotic resistance transferred by conjugation.
[h] Value or range; − indicates unsuccessful conjugation.

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TABLE 2. Tn*1546* types found among *E. faecium* clinical isolates recovered at three different Portuguese hospitals (1996 to 2003)[a]

Type	PCR product for specific primer pair (annealing sites)										No. of isolates	Date[b]	City(ies)	PFGE type(s)[c]
	p1p2 (22–1330)	p3p4 (1222–2353)	p5p6 (2227–3525)	p7p8 (2769–4042)	p9p10 (3569–4793)	p11p12 (4675–6353)	p13p14 (6229–8021)	p15p16 (6979–8920)	p17p18 (8889–10473)	p19p1 (10403–10830)				
A	+	+	+	+	+	+	+	+	+	+	2	04/98–10/00	Coimbra	92, 98
D	−	+	+	+	+	+	+	+	+	+	1	06/02	Porto	76
X	−	−	−	−	−	+	+	−	−	+	1	03/00	Coimbra	84
PP-2	+	+	+	+	+	+	+	+	−	+	7	10/01–10/02	Coimbra, Viseu	**(70),** 92, 93
PP-3	+	+	+	+	+	+	+	+	−	−	1	03/02	Coimbra	94
PP-4	+	+	+	+	+	+	+	+	++[c]	+	11	?/97–04/02	Coimbra	**76, 78,** 80, 82, 88, 90, 95, 97, 108, 112, 122
PP-5	+	+	+	+	+	+	+	+	++[c]	−	15	09/02–03/03	Coimbra, Viseu, Porto	**70, 71, 78,** 99, **100,** 72, 103, 104
PP-9	+	−	−	+	+	+	+	+	++	+	2	01/00	Coimbra	74, 106
PP-10	−	+	+	+	+	+	+	−	+	+	1	?/96	Coimbra	83
PP-13	−	−	−	−	+	+	+	−	−	+	6	01–12–2002	Porto	**(78),** 86, 88, 91, 119
PP-15	−	−	−	−	+	+	+	+	+	+	1	?/01	Porto	96
PP-16	−	−	−	−	−	+	+	+	+	+	1	06/01	Porto	77
PP-20	−	−	−	−	−	−	+	−	−	+	1	?/01	Porto	85
PP-23	−	−	+	−	−	+	+	−	−	+	1	09/02	Porto	119
PP-24	−	+	+	+	+	+	+	−	++[c]	−	1	03/03	Coimbra	ND
PP-25	−	−	−	−	+	+	+	−	+	+	1	07/01	Porto	88
PP-27	+	+	+	+	+	+	−	−	+	−	1	03/02	Coimbra	87

[a] Tn*1546* types were designed according to the Woodford scheme (32). For those that did not have a specific previously described type, we used our own designation (PP from Portugal-Oporto followed by a number randomly chosen). +, amplification; −, no amplification; ++, amplification of sequences larger than those of the expected size.

[b] See footnote *b* of Table 1.

[c] Positive amplification using IS*Ef1*-F (5′-GGT GTT ACG ATG TCT GAA ATT GC-3′) and p18. The clones in which vancomycin resistance was transferred by conjugation appear underlined. Variable Tn*1546* transference to receptor strains among isolates belonging to the same PFGE appear in parenthesis. PFGE types identified in more than one hospital appear in bold.

vanC2, *aph*(*2″*)I-Ib, *aph*(*2″*)-*Ic*, *aph*(*2″*)-*Id*, *erm*(A), *erm*(C) or *mef*(A) was not found in any case. *vanA* transference to the recipient *E. faecium* strain GE1 by filter mating was achieved for 36 of the 82 selected isolates (44%). Conjugation frequency ranged from 10^{-1} to 10^{-8}. *erm*(B) was cotransferred in all except one erythromycin-resistant isolates. A high level of resistance to kanamycin encoded by *aph*(*3′*)-*IIIa* was cotransferred only in six isolates.

A subset of 54 *E. faecium* isolates was selected for Tn*1546* typing and included isolates within a particular PFGE cluster recovered from different hospitals or in the same hospital over time and also isolates representing unique PFGE types showing different virulence and/or antibiotic susceptibility profiles. Seventeen different variants of Tn*1546* were found among 54 VREF isolates screened (Table 2). Some Tn*1546* types were detected in different hospitals (PP-2 and PP-5), among isolates corresponding to different PFGE types (A, PP-2, PP-4, PP-5, PP-9, and PP-13), and during long time periods (PP-4 and PP-5). Interestingly, most Tn*1546* variants (13/17) showed alterations downstream of *vanA* (X, PP-2, PP-3, PP-4, PP-5; PP-9, PP-10, PP-13, PP-20, PP-23, PP-24, PP-25, and PP-27). The more prevalent, disseminated, and long-term-persistent Tn*1546*-variants, PP-4 and PP-5, contained an IS*Ef1* insertion located in the *vanX-vanY* region. They were frequently transferred by conjugation to different *E. faecium* PFGE types (Table 2), and they also have been found among *Enterococcus faecalis* clinical isolates from the same institutions (20). Hybridization of I-CeuI-digested genomic DNA from representative isolates containing PP-4 or PP-5 corresponding to distinct PFGE types with the *vanA* probe was mainly associated with a band around 97 kb, suggesting the presence of a common plasmid. For representative isolates harboring other Tn*1546* variants, hybridization of I-CeuI-digested genomic DNA with the *vanA* probe was associated with bands of high and/or low molecular weight of different lengths, suggesting the location of the *vanA* gene in different plasmids and/or chromosomal sites.

Gene coding for Esp, Gel, and Hyl was detected in 33%, 7%, and 4% of the isolates, respectively. These isolates belonged to several PFGE types and were collected in HUC and HSA between 1997 and 2003. Different combinations of putative virulence factors were detected: *asa1* positive, *gel* positive (n = 2), *asa1* positive, *hyl* positive (n = 1), *esp* positive, *hyl* positive (n = 2), *asa1* positive, *gel* positive, *esp* positive (n = 1), *hyl* positive (n = 4), and *esp* positive (n = 30). Isolates corresponding to the same clonal type (clones 70, 71, 76, 88, 99, and 100) contained variable virulence patterns as previously described (2). None of these putative virulence trait genes was cotransferred with genetic determinants coding for glycopeptide resistance.

Our study shows a genetically diverse VREF population from Portuguese hospitals with the occurrence of intra- and interhospital dissemination of specific VREF strains and Tn*1546* types/plasmids. These results suggest a wide dissemination of VREF colonizing humans but also a successful spread of particular strains which might largely contribute to the endemicity of VREF in Portuguese hospitals (15, 27). Acquisition of Tn*1546* by a few widely disseminated ampicillin-resistant *E. faecium* clones and further spread of specific vancomycin resistance genetic elements to multiple strains led to the increase of VRE in the United States in hospitals during the last two decades (5, 9, 15, 16, 28, 31). Nosocomial VRE outbreaks in European countries have been associated with very diverse epidemiological situations involving spread of specific strains, transposons, and/or plasmids (1, 8, 13, 14, 16, 25, 33). Intrahospital dissemination of particular strains generally has been successfully controlled (1), and to our knowledge, interhospital dissemination of VRE strains has been described only rarely in Europe (4, 20, 33). However, transfer of mobile elements has caused larger outbreaks in different European countries (13, 14, 25). The recent description of specific genetic elements carrying glycopeptide resistance able to persist with or without apparent selection is of concern, since they may locally and in the long term maintain antibiotic resistance and they can be efficiently transferred to multiple genetic backgrounds (11, 28). The predominance of the Tn*1546* types PP-5 and PP-4 (48% of the Tn*1546* types studied) among epidemic and nonepidemic VREF clones and among *E. faecalis* strains from the same institutions (20) indicates that horizontal gene transfer also plays a relevant role in the dissemination of glycopeptide resistance in Portuguese hospitals (13, 14, 25). The evidence of a common plasmid band in several PFGE types isolates carrying PP-4 and PP-5 supports this hypothesis. Additionally, the presence of these Tn*1546* types on distinct plasmids and chromosomal locations of *E. faecalis* strains from the same hospitals (20) and also differences in the transferability of specific Tn*1546* among VREF isolates suggest a complex epidemiology involving different genetic elements and dissemination mechanisms. Wide dissemination of both particular clones, plasmid and/or Tn*1546*, might amplify the spread of VRE, as previously shown in American and certain European institutions (13, 14, 15, 25, 28).

Geographical variations in the occurrence of putative virulence traits, such as those encoded by *esp* or *hyl*, have been reported and associated with the epidemicity of VREF (22). Our data show a lower prevalence of *esp*-positive isolates than of VREF strains from the United States or the United Kingdom (33% versus 65% and 61%, respectively) (22, 34). However, the absence of *esp* among most of the epidemic VREF isolates indicates that other factors are important in the dissemination of antibiotic-resistant *E. faecium*, as previously suggested (2, 31). Acquisition of *esp* among isolates of *E. faecium* in the nosocomial setting (2, 21) might increase the fitness of already-widespread *E. faecium* clones.

In summary, our study documents a high level of genetic diversity of VREF populations from Portuguese hospitals with the presence of intra- and interhospital dissemination of specific strains and Tn*1546* types. These results suggest both a wide dissemination of human-colonizing VREF and a successful spread of particular strains and Tn*1546* types/plasmids which might largely contribute to the endemicity of VREF Portuguese hospitals, as previously has happened in another geographical locations.

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