Antimicrobial resistance among faecal enterococci from healthy individuals in Portugal

C. Novais[1,2], T. M. Coque[3], J. C. Sousa[2] and L. V. Peixe[1]

[1]REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, [2]Laboratório de Microbiologia, Faculdade Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal and [3]Servicio de Microbiologia, Hospital Universitario Ramón y Cajal, Madrid, Spain

ABSTRACT

Analysis of 247 faecal enterococcal isolates from 99 healthy Portuguese individuals during 2001 revealed the presence of enterococci resistant to vancomycin (5%) and highly resistant to streptomycin (52%), kanamycin (40%) or gentamicin (11%). Most isolates were also resistant to tetracycline, erythromycin, ciprofloxacin and quinupristin–dalfopristin. The *vanA* (two Tn*1546* types), *vanC1*, *erm*(B), *aac*(*6′*)-*aph*(*2″*)-*Ia*, *aph*(*3′*)-*IIIa*, *vat*(E) and *vat*(D) genes were detected. *Enterococcus faecalis* and *Enterococcus faecium* isolates with high-level resistance to gentamicin were related to Portuguese poultry isolates described previously. *E. faecium* isolates that were highly resistant to vancomycin or gentamicin harboured different housekeeping *purK* alleles associated previously with different hosts.

Keywords Antimicrobial resistance, enterococci, faecal flora, Portugal, vancomycin resistance

Original Submission: 18 January 2006; **Revised Submission:** 24 April 2006; **Accepted:** 8 May 2006

Clin Microbiol Infect 2006; 12: 1131–1134
10.1111/j.1469-0691.2006.01542.x

Antibiotic-resistant enterococci (ABRE) have been reported previously in nosocomial and community settings following increased antibiotic usage in humans and animals [1]. Despite measures adopted in Europe, ABRE are still reported in healthy humans (HH) and animals from areas with no apparent selection pressure [2–4]. Recent studies have demonstrated host-specificity for particular enterococcal strains and genetic elements, which are sometimes able to persist for long periods in specific hosts [2,5,6]. However, similar strains and genes encoding resistance to antibiotics used in clinical practice have been observed in both humans and animals [2,7]. The present study analysed the presence of ABRE in faeces from HH, as well as their clonal relationship with strains from poultry in Portugal, a country with high rates of ABRE in hospitals and the community [8,9] (http://www.rivm.nl/earss).

Ninety-nine faecal swabs from HH (55 females, 44 males; age range 5–69 years, mean 30 years) were collected in northern and central Portugal (January–June 2001). Participants were questioned concerning their contact with antibiotics, hospitals, animal meat and live animals. Also included in the study were 149 Portuguese poultry isolates (103 vancomycin-resistant enterococci and 46 with high-level gentamicin resistance (HLGR) [8]). Swabs were enriched in brain–heart infusion broth, with and without vancomycin (6 mg/L), to improve the recovery of vancomycin-resistant enterococci. Enriched samples (100 μL) were plated on Slanetz–Bartley media, with and without vancomycin (6 mg/L), gentamicin (125 mg/L), streptomycin (1000 mg/L) or kanamycin (500 mg/L). Resistance to other antibiotics was considered to be associated with the agents used in selection plates in order to avoid over-estimation of results by repeatedly counting isolates on different selection media. For each sample, one colony of each morphological type and antibiotic susceptibility profile was studied. Susceptibility to 14 antibiotics was determined by the agar dilution method using *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 as control strains [10]. Species identification, detection of genes conferring resistance to glycopeptides, aminoglycosides, macrolides, streptogramins, and characterisation of Tn*1546* structures were performed by PCR with appropriate controls [11–15]. Conjugation experiments with vancomycin-resistant enterococcal isolates were performed by filter and/or broth mating methods [16]. *Enterococcus faecium* and *E. faecalis* isolates harbouring the *vanA* or *aac*(*6′*)-*aph*(*2″*) genes were tested for clonal relationship by

Corresponding author and reprint requests: L. Peixe, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 4050-030 Porto, Portugal
E-mail: lpeixe@ff.up.pt

pulsed-field gel electrophoresis [16]. Identification of *purK* alleles in vancomycin-resistant isolates and selected isolates with HLGR was performed by PCR amplification and sequencing [5].

Enterococci (124 *E. faecalis*, 96 *E. faecium*, three *Enterococcus gallinarum* and 24 unidentified *Enterococcus* spp.) were recovered from 93% (92/99) of HH samples. Most HH had not taken antibiotics (91/99; 92%) or had contact with hospitals (94/99; 95%) during the 3-month or 12-month periods, respectively, preceding sample collection. All except one individual had consumed meat, i.e., poultry (94/99; 95%), pork (90/99; 90%) or turkey (57/99; 57%), of more than one type in most cases (90/99; 90%). Regular meat consumption (more than five occasions per week) was recorded for 79% (78/99) of individuals. Contact with pets (mainly cats and dogs) or farm animals was recorded in 56% (55/99) and 17% (97/99) of HH, respectively.

A high percentage of samples contained enterococci with high-level streptomycin resistance (52%) or high-level kanamycin resistance (40%) and, to a lesser extent, HLGR (11%) or vancomycin resistance (5%). Resistance to daptomycin or to linezolid was not observed. Daptomycin breakpoints were those recommended by the manufacturers (Chiron, Uxbridge, UK) (i.e., MIC <8 mg/L indicates susceptibility). Linezolid breakpoints were those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.srga.org/eucastwt/MICTAB/MICoxazolidones.htm). Co-resistance to tetracycline, erythromycin, ciprofloxacin and quinupristin–dalfopristin was found in most enterococci grown on selective plates. Six vancomycin-resistant enterococcal isolates from five HH were recovered, i.e., four *vanA*$^+$ *E. faecium* and two *vanC1*$^+$ *E. gallinarum*. All HH *vanA* carriers had consumed more than one type of meat, but had not had contact with hospitals, antibiotics or farm animals. The Tn*1546* elements corresponded to the PP-5 and D types described previously, which have been associated with hospitals and animal isolates, respectively [15–17].

Isolates with HLGR from 11 HH harboured *aac*(6′)-*aph*(2″)-*Ia* (n = 5) or both *aac*(6′)-*aph*(2″)-*Ia* and *aph*(3′)-*IIIa* (n = 8). Isolates showing only high-level kanamycin resistance (40 samples, 44 isolates) contained mostly *aph*(3′)-*IIIa* (36 samples, 39 isolates). The *erm*(B) gene was detected in both erythromycin-resistant and erythromycin-non-resistant isolates (17/33 *E. faecium* from 22 samples). Interestingly, five erythromycin-resistant isolates (MICs 8 to >32 mg/L) did not harbour *erm*(B), and 18% of isolates containing *erm*(B) did not express it phenotypically (MICs 1–4 mg/L). The *vat*(E) and *vat*(D) genes were observed in one *E. faecium* isolate each. The *aph*(2″)-*Ib*, *aph*(2″)-*Ic*, *aph*(2″)-*Id*, *erm*(A), *erm*(C) and *mef*(A) genes were not detected. Transfer of *vanA* and co-transfer of *erm*(B) was achieved in all cases by filter mating (10^{-1}–10^{-8} transconjugants per donor or recipient).

Three pulsed-field gel electrophoresis types were observed among four *vanA*-carrying *E. faecium* isolates, and seven pulsed-field gel electrophoresis types among eight isolates with HLGR

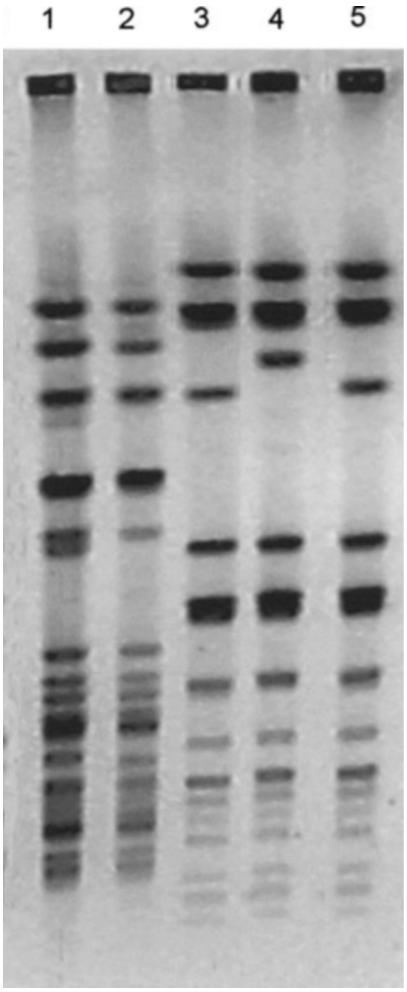

Fig. 1. Pulsed-field gel electrophoresis types of isolates with high-level gentamicin resistance from healthy humans (HH) and poultry (P). Lanes 1 (P) and 2 (HH): *Enterococcus faecium* clone 36. Lanes 3 (HH), 4 (P) and 5 (P): *Enterococcus faecalis* clone 56.

(two *E. faecium* and five *E. faecalis*). Two isolates with HLGR (one *E. faecalis* and one *E. faecium*) were related closely to poultry isolates described previously (clones 36 and 56) (Fig. 1) [8]. Vancomycin-resistant *E. faecium* harboured the *purK-1* (one isolate representative of one clone) or *purK-6* (two isolates representative of two clones) alleles, while isolates with HLGR recovered from both HH (one isolate) and poultry (one isolate) were related to clone 36 [8] containing the *purK-3* allele.

A high frequency of ABRE was detected among HHs with no hospital and/or antibiotic exposure, with the majority of isolates with vancomycin resistance or high-level aminoglycoside resistance also being resistant to erythromycin, tetracycline, ciprofloxacin and quinupristin–dalfopristin, as reported for enterococci of animal origin [8]. Most of these antibiotic resistance genes are frequently located in gene clusters associated with specific plasmids, transposons or other widely distributed transferable elements, which could explain the presence of different multiresistance patterns, even without selective enrichment [1,7]. The susceptibility results of this study are summarised in Table 1.

Resistance to several antibiotics has been reported in a previous Portuguese study including HHs [18]. The lower resistance rates observed for some agents in the previous study might be related to the absence of an enrichment method or antibiotic selection plates. This might also explain the low rate of ampicillin resistance revealed among the *E. faecium* population recovered in this study. Although Portugal uses large amounts of β-lactam agents [9], this observation is supported by other studies with HH and animals, suggesting that such strains are not disseminated widely in the Portuguese community [8,18]. The presence of strains with HLGR that are similar to those described previously in poultry [8] indicates

Table 1. Reduced susceptibility to 12 antimicrobial agents among 247 enterococcal isolates from 99 healthy humans in Portugal

				Non-susceptibility[a] to											
Basis of isolate selection	**Samples resistant to the antibiotics used in selection**	**% Co-resistance to other antibiotics[b,c,d]**	**No. of resistant isolates of each species selected in the different plates**	**VA MIC ≥8 mg/L**	**TEC MIC ≥8 mg/L**	**AMP MIC ≥16 mg/L**	**TE MIC ≥8 mg/L**	**E MIC ≥1 mg/L**	**CIP MIC ≥2 mg/L**	**C MIC ≥16 mg/L**	**GM MIC ≥500 mg/L**	**STR MIC ≥2000 mg/L**	**KAN MIC ≥2000 mg/L**	**NIT[e] MIC ≥64 mg/L**	**Q/D[b,e] MIC ≥2 mg/L**
No antibiotic		TE 55; E 67; CIP 53; C 22; GM 3; STR 30; KAN 20; NIT 41; Q/D 91	*E. faecalis* (n = 52)	0/52	0/52	0/52	25/52	16/52	17/52	11/52	1/52	9/52	5/52	0/52	27/27
			E. faecium (n = 53)	0/53	0/53	0/53	18/53	34/53	27/53	5/53	1/53	12/53	8/53	5/53	23/29
			Enterococcus spp. (n = 14)	0/14	0/14	0/14	3/14	4/14	4/14	2/14	0/14	4/14	1/14	3/14	5/5
				0/119	0/119	0/119	46/119	54/119	48/119	18/119	2/119	25/119	14/119	8/119	55/61
Vancomycin (6 mg/L)	5	TEC 67; AMP 33; TE 67; E 83; CIP 67; C 33; GM 33; STR 33; KAN 33; Q/D 50	*E. faecium* (n = 4)	4/4[b]	4/4	2/4	4/4	4/4	3/4	2/4	2/4	2/4	2/4	2/4	3/4
			E. gallinarum (n = 2)	2/2[c]	0/2	0/2	0/2	1/2	2/2	0/2	0/2	0/2	0/2	0/2	1/2
				6/6	4/6	2/6	4/6	3/6	5/6	2/6	2/6	2/6	2/6	2/6	4/6
Gentamicin (125 mg/L)	11	VA 18; TEC 9; AMP 18; TE 100; E 91; CIP 64; C 64; STR 73; KAN 100; Q/D 100	*E. faecalis* (n = 6)	0/6	0/6	0/6	6/6	4/6	3/6	3/6	6/6	5/6	6/6	0/6	4/4
			E. faecium (n = 3)	2/3	1/3	2/3	3/3	3/3	3/3	2/3	3/3	2/3	3/3	3/3	1/1
			E. gallinarum (n = 1)	1/1	0/1	0/1	1/1	1/1	1/1	1/1	1/1	0/1	1/1	1/1	ND
			Enterococcus spp. (n = 3)	0/3	0/3	0/3	2/3	3/3	1/3	1/3	3/3	2/3	3/3	0/3	1/1
				2/13	1/13	2/13	12/13	11/13	8/13	7/13	13/13	9/13	13/13	4/13	6/6
Streptomycin (1000 mg/L)	52	TE 89; E 89; CIP 29; C 48; KAN 59; Q/D 95; NIT 5	*E. faecalis* (n = 41)	0/41	0/41	0/41	38/41	36/41	20/41	22/41	0/41	41/41	26/41	2/41	18/18
			E. faecium (n = 18)	0/18	0/18	1/18	13/18	17/18	13/18	3/18	0/18	18/18	5/18	9/17	6/6
			Enterococcus spp. (n = 6)	0/6	0/6	0/6	3/6	4/6	3/6	2/6	0/6	6/6	2/6	0/6	ND
				0/65	0/65	1/65	54/65	57/65	36/65	27/65	0/65	65/65	33/65	11/64	24/24
Kanamycin (500 mg/L)	40	TE 85; E 77; CIP 56; C 33; STR 72; Q/D 100	*E. faecalis* (n = 25)	0/25	0/25	0/25	24/25	22/25	8/25	13/25	0/25	23/25	25/25	1/24	15/15
			E. faecium (n = 18)	0/18	0/18	0/18	11/18	12/18	16/18	3/18	0/18	6/18	18/18	10/17	4/4
			Enterococcus spp. (n = 1)	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	1/1	1/1	0/1	ND
				0/44	0/44	0/44	36/44	34/44	24/44	16/44	0/44	30/44	44/44	11/42	19/19
Total			n = 247												

VA, vancomycin; TEC, teicoplanin; AMP, ampicillin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; C, chloramphenicol; GM, high-level resistance to gentamicin; STR, high-level resistance to streptomycin; KAN, high level resistance to kanamycin; Q/D, quinupristin–dalfopristin; NIT, nitrofurantoin; ND, not determined.
[a]Resistant and intermediately-resistant enterococci were considered together; [b]*E. faecalis* is inherently resistant to Q/D; [c]all vancomycin-resistant *E. faecium* isolates contained *vanA*; [d]all vancomycin-resistant *E. gallinarum* isolates contained *vanC1*; [e]technical issues impaired the study of nitrofurantoin and quinupristin–dalfopristin susceptibility for all isolates.

clonal spread via the food chain, as has been suggested previously [4,19]. The presence in two HH vancomycin-resistant isolates of *purK-6* and Tn*1546* type D, associated with enterococci of animal origin, further supports this hypothesis [5,15]. Notably, Tn*1546* type PP-5 and *purK-1*, linked to strains from hospitalised patients [5,6,16,17], were found in an HH with no antibiotic or hospital exposure. The absence of common resistance genes in some isolates resistant to kanamycin, erythromycin or quinupristin–dalfopristin suggests that other unknown resistance mechanisms may exist in the community.

In summary, healthy Portuguese individuals are frequently colonised with ABRE. Selection of these strains by factors other than antibiotics, and/or possible strain/gene transmission from animal sources, might increase the human reservoir of clinically relevant ABRE outside of the hospital setting.

ACKNOWLEDGEMENTS

C. Novais was supported by a fellowship from the Fundação para a Ciência e Tecnologia (SFRH/BD/3372/2000). This work was supported, in part, by grants from the Fundação para a Ciência e Tecnologia (POCTI/SAU-ESP/61385/2004) and from the Ministerio of Educación y Ciencia of Spain, Programa Acciones Integradas Hispano-Portuguesas (H2004-0092). The authors would like to thank J. Oliveira for the development of the Excel macros used in data analysis.

REFERENCES

1. Bonten MJM, Willems RJ, Weinstein RA. Vancomycin resistant enterococci: why are they here, and where do they come from? *Lancet Infect Dis* 2001; **1**: 314–325.
2. Johnsen PJ, Osterhus JI, Sletvold H *et al*. Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated *vanA* element within a polyclonal *Enterococcus faecium* population. *Appl Environ Microbiol* 2005; **71**: 159–168.
3. Heuer OE, Pedersen K, Jensen LB, Madsen M, Olsen JE. Persistence of vancomycin-resistant enterococci (VRE) in broiler houses after the avoparcin ban. *Microb Drug Resist* 2002; **8**: 355–361.
4. Hammerum AM, Lester CH, Neimann J *et al*. A vancomycin-resistant *Enterococcus faecium* isolate from a Danish healthy volunteer, detected 7 years after the ban of avoparcin, is possibly related to pig isolates. *J Antimicrob Chemother* 2004; **53** (suppl): 547–549.
5. Homan WL, Tribe D, Poznanski S *et al*. Multilocus sequence typing scheme for *Enterococcus faecium*. *J Clin Microbiol* 2002; **40**: 1963–1971.
6. Willems RJ, Top J, van Santen M *et al*. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. *Emerg Infect Dis* 2005; **11**: 821–828.
7. Weaver KE, Rice LB, Churchward G. Plasmids and transposons. In: Gilmore MS, ed., *The enterococci: pathogenesis, molecular biology, and antibiotic resistance*, 1st edn. Washington, DC: American Society for Microbiology, 2002; 219–265.
8. Novais C, Coque TM, Costa MJ, Sousa JC, Baquero F, Peixe LV. High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal. *J Antimicrob Chemother* 2005; **56**: 1139–1143.
9. Goossens H, Ferech M, Vander Stichele R, Elseviers M, ESAC Project Group. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* 2005; **365** (suppl): 579–587.
10. National Committee for Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*. Approved standard M7-A5. Wayne, PA: NCCLS, 2000.
11. Dukta-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* 1995; **33**: 24–27.
12. Vakulenko SB, Donabedian SM, Voskresenskiy AM, Zervos MJ, Lerner SA, Chow JW. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrob Agents Chemother* 2003; **47**: 1423–1426.
13. Lim JA, Kwon AR, Kim SK, Chong Y, Lee K, Choi EC. Prevalence of resistance to macrolide, lincosamide and streptogramin antibiotics in Gram-positive cocci isolated in a Korean hospital. *J Antimicrob Chemother* 2002; **49**: 489–495.
14. Soltani M, Beighton D, Howard J, Woodford N. Mechanisms of resistance to quinupristin–dalfopristin among isolates of *Enterococcus faecium* from animals, raw meat, and hospital patients in Western Europe. *Antimicrob Agents Chemother* 2000; **44**: 433–436.
15. Woodford N, Adebiyi AMA, Palepou MFI, Cookson B. Diversity of VanA glycopeptide resistance elements in enterococci from humans and animals. *Antimicrob Agents Chemother* 1998; **42**: 502–508.
16. Novais C, Coque TM, Sousa JC, Baquero F, Peixe L. Local genetic patterns within a vancomycin-resistant *Enterococcus faecalis* clone isolated in three hospitals in Portugal. *Antimicrob Agents Chemother* 2004; **48**: 3613–3617.
17. Novais C, Coque TM, Sousa JC, Peixe L, Portuguese Resistance Study Group. Molecular characterization of glycopeptide-resistant *Enterococcus faecium* isolates from Portuguese hospitals. *Antimicrob Agents Chemother* 2005; **49**: 3073–3030.
18. Poeta P, Costa D, Rodrigues J, Torres C. Antimicrobial resistance and the mechanisms implicated in faecal enterococci from healthy humans, poultry and pets in Portugal. *Int J Antimicrob Agents* 2006; **27**: 131–137.
19. Donabedian SM, Thal LA, Hershberger E *et al*. Molecular characterization of gentamicin-resistant enterococci in the United States: evidence of spread from animals to humans through food. *J Clin Microbiol* 2003; **41**: 1109–1111.