Antimicrobial resistance among faecal enterococci from healthy individuals in Portugal
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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–dalofopristin. The vanA (two Tn1546 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

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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 Tn1546 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

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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 vanCl+ 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 Tn1546 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(3′)-Ia (n = 5) or both aac(6′)-aph(3′)-Ia and aph(3′)-Ila (n = 8). Isolates showing only high-level kanamycin resistance (40 samples, 44 isolates) contained mostly aph(3′)-IIda (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.
Streptomycin located in gene clusters associated with specific antibiotics. Vancomycin-resistant \( E. \) \( fæcium \) 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 \( E. \) \( fæcium \) 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 a connection between these two sources.

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

<table>
<thead>
<tr>
<th>Basis of isolate selection</th>
<th>Samples resistant to the antibiotics used in selection</th>
<th>No. of resistant isolates of each species selected in the different plates</th>
<th>Non-susceptibility* to</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antibiotic</td>
<td>H 82; E 67; GM 3; STR 30; KAN 20; NIT 41; Q/D 91</td>
<td>E. ( fæcium ) (n = 52)</td>
<td>VA MIC TE 21 TEC 20 TEC 21 AMP MIC 25 AMP MIC 25 AMIC 21 AMIC 21 CIP MIC 25 CIP MIC 25 CIP MIC 25 GM MIC 25 STR MIC 50 KAN MIC 25 KAN MIC 25 NIT MIC 25 Q/D MIC 25</td>
</tr>
<tr>
<td>Vancomycin (6 mg/L)</td>
<td>TEC 67; AMP 33; TE 67; E 83; CIP 67; C 33; GM 33; STR 33; KAN 33; Q/D 90</td>
<td>E. ( fæcium ) (n = 4)</td>
<td>0/52 0/53 0/53 0/53 14/14 1/4 1/4 14/14 4/14 14/14 1/4 1/4</td>
</tr>
<tr>
<td>Gentamicin (125 mg/L)</td>
<td>VA 18; TEC 9; AMP 18; TE 100; E 91; CIP 64; C 64; STR 73; KAN 100; Q/D 100</td>
<td>E. ( fæcium ) (n = 6)</td>
<td>0/119 0/119 0/119 54/119 48/119 18/119 2/119 25/119 14/119 8/119 55/119</td>
</tr>
<tr>
<td>Streptomycin (500 mg/L)</td>
<td>TE 89; E 89; CIP 29; C 48; KAN 99; Q/D 95; NIT 5</td>
<td>E. ( fæcium ) (n = 41)</td>
<td>0/2/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2 0/2</td>
</tr>
<tr>
<td>Kanamycin (500 mg/L)</td>
<td>TE 85; E 77; CIP 56; C 33; STR 72; Q/D 100</td>
<td>E. ( fæcium ) (n = 25)</td>
<td>0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1</td>
</tr>
<tr>
<td>Total</td>
<td>n = 247</td>
<td></td>
<td>0/44 0/44 0/44 0/44 34/44 34/44 34/44 34/44 16/44 0/44 30/44 44/44 11/42 19/19</td>
</tr>
</tbody>
</table>

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.

*Resistant and intermediately-resistant enterococci were considered together; \( E. \) \( fæcals \) is inherently resistant to Q/D, all vancomycin-resistant \( E. \) \( fæcium \) isolates contained vanA, \( ^{\text{a}} \) all vancomycin-resistant \( E. \) \( gallinarum \) isolates contained \( \text{erm} \)C; \( ^{\text{b}} \) technical issues impaired the study of nitrofurantoin and quinupristin–dalfopristin susceptibility for all isolates.

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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 Tn1546 type D, associated with enterococci of animal origin, further supports this hypothesis [5,15]. Notably, Tn1546 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.

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