Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of Enterococcus faecalis from Portugal

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Objectives: The aim of this study was to assess the diversity of Enterococcus faecalis populations recovered in different regions of Portugal during the last decade (1996–2007) and to analyse their genetic elements associated with vancomycin resistance.

Methods: Forty E. faecalis isolates (22 vancomycin-susceptible and 18 vancomycin-resistant) representing disseminated and/or multiresistant strains from different sources (humans, animals and the environment) were characterized by PFGE and multilocus sequence typing. Genes encoding putative virulence markers and the backbone of Tn1546 were investigated by PCR. Plasmid analysis included determination of size, content (S1 hybridization) and comparison of restriction fragment length polymorphism patterns.

Results: The 40 E. faecalis isolates (22 PFGE types) mostly clustered within the worldwide-spread clonal complexes (CCs) CC2 (13 ST6 mostly corresponding to an epidemic strain, where ST stands for sequence type), CC21 (3 ST21, 1 ST22 and 1 ST224) and ST16 (n = 7), but also comprised ST159, ST35, ST19, ST26, ST30, ST41, ST55, ST59, ST117, ST160 and ST200. CC2 and CC21 were isolated from both hospital and community settings. Similar Tn1546-like elements encoding VanA were found on related plasmids within strains belonging to different clonal lineages and recovered in distinct hospitals over several years.

Conclusions: The predominance of E. faecalis CC2 is mainly due to the dissemination of a particular clone persistently recovered for 11 years. The presence in the community of specific strains belonging to major clonal lineages highlights the role of community-associated hosts as possible reservoirs of putative human pathogenic enterococci. Both clonal expansion and dissemination of epidemic conjugative VanA plasmids seem to join forces in the establishment of pathogenic E. faecalis strains.

Keywords: enterococci, MLST, CC2, CC21, ST16, VRE, mobile elements

Introduction

Enterococci are one of the most frequent causes of endocarditis, bacteraemia and urinary tract infections and have become one of the leading causes of nosocomial infections in recent years.1 The species most frequently identified in human infections is Enterococcus faecalis, followed by Enterococcus faecium; however, vancomycin resistance (VanA-Tn1546 and VanB-Tn1549) is more frequently associated with E. faecium than E. faecalis. Recent data show a progressive inversion of the ratio of E. faecalis/E. faecium causing infections, which seems to be associated with the increase in glycopeptide resistance of...
Population structure of *E. faecalis* from Portugal

*E. faecium.*

Despite vancomycin resistance genetic elements being more diffused among *E. faecium*, the successful transfer of Tn1546 from enterococci to more pathogenic bacteria such as *Staphylococcus aureus* has been associated with *E. faecalis.*

Molecular epidemiological methods have contributed to the gain in significant knowledge on the population structure of the enterococcal species most commonly recovered from humans. The contemporary *E. faecalis* population structure comprises a diversity of clones with overrepresentation of clonal complexes (CCs) CC2, CC9, CC10, CC21, CC40 and CC87 and the prominent singleton ST16 (where ST stands for sequence type). CC2, CC9 and CC87 are considered high-risk CCs, as they mostly include isolates causing infections in hospitalized patients. The available data indicate that *E. faecalis* has an epidemic population structure that apparently lacks relevant host specificity, and recombination seems to be the driving force in the major genetic diversity and evolution of this species.

The first nosocomial outbreak caused by vancomycin-resistant enterococci (VRE) detected in Portugal was associated with a single VanA *E. faecalis* clone that was widespread in different hospitals. Several VRE and vancomycin-susceptible enterococci (VSE) strains persistently recovered or widely disseminated in different ecological niches have been reported.

The main purposes of our study were to determine the genetic relationships among diverse and representative *E. faecalis* strains collected in Portugal during the last decade and to gain insights into antibiotic resistance, virulence traits and plasmid content among persistent and sporadic *E. faecalis* clones from Portugal.

### Material and methods

#### Bacterial strains

Fifty *E. faecalis* isolates (22 VSE and 18 VRE), representing disseminated, persistent and/or multiresistant strains recovered during 1996–2007 in different settings, were selected for multilocus sequence typing (MLST). They included: (i) 21 clinical VRE and VSE isolates from hospitalized patients in different cities (Oporto, Coimbra and Viseu) representative of all PFGE types detected to date in our collection, including the arbitrarily designated strains B and N disseminated in Portuguese hospitals and strains isolated from single patients; (ii) 4 isolates from swine excrement and 8 from environmental samples recently collected from 6 piggeries in rural areas of different regions of the country and selected as representative of multiresistant phenotypes, including 1 VRE and PFGE types disseminated in more than 1 piggery; (iii) 3 isolates from retail poultry of different commercial brands, including the only VRE detected among *E. faecalis* poultry isolates and 2 of the few vancomycin-susceptible *E. faecalis* recovered from this host; (iv) 3 faecal VSE isolates from healthy volunteers living in three different cities, comprising 1 strain recovered also from poultry (the same PFGE type) and 2 strains representative of clones expressing high-level resistance to gentamicin, as no vancomycin-resistant *E. faecalis* was detected in our collection of 124 *E. faecalis* faecal isolates from human volunteers during 1999–2001; and (v) 1 VRE isolate collected from hospital waste water corresponding to the hospital widespread strain B.

Species identification and vancomycin resistance gene detection were performed by a multiplex PCR assay. Antimicrobial susceptibility to 14 antibiotics (vancomycin, teicoplanin, erythromycin, tetracycline, ampicillin, ciprofloxacin, gentamicin, kanamycin, streptomycin, chloramphenicol, nitrofurantoin, daptomycin, linezolid and tigecycline) was determined by the standard agar dilution method. High-level resistance was determined for all aminoglycosides according to CLSI guidelines. A multidrug resistance phenotype was considered for isolates non-susceptible to three or more antimicrobials of different chemical groups.

#### Virulence traits

We searched for the presence of genes coding for the virulence markers enterococcal surface protein (*esp*), cytolysin/haemolysin (*cyl*), gelatinase (*gelE*), hyaluronidase (*hyl*) and aggregation substance (*agg*) by a multiplex PCR described previously.

#### Clonal relationship

The clonal relationship among isolates was established by PFGE and MLST as described previously. Clusters of related STs were grouped into CCs by using eBURST (http://www.mlst.net).

#### Genetic elements encoding vancomycin resistance

The backbone structure of Tn1546 was determined by the PCR overlapping assay described by Woodford et al. in all VRE isolates, some of which have been recently characterized. Plasmid size and content were determined in the clinical VRE isolates by the S1 nuclease method described by Barton et al. and the plasmid location of the *van* gene was confirmed by hybridization of S1-digested genomic DNA with an intragenic *vanA* probe. Plasmid extraction was performed by an alkaline lysis protocol, and the relationships among plasmids were established by comparisons of the profiles obtained after digestion with *EcoRI*, *ClaI* and *HindIII* by standard methods.

### Results

#### *E. faecalis* population structure

The 40 *E. faecalis* isolates studied were recovered from hospitalized patients (n = 21) and non-clinical sources (n = 19) that included 3 from healthy humans, 12 from swine excrements and piggery environments, 3 from poultry carcasses and 1 from hospital wastewater. All isolates were multidrug-resistant, with the exception of one isolate from poultry and one from swine. Vancomycin resistance was encoded by the *vanA* gene in 18 isolates (45%) and it was mainly observed among human clinical isolates (n = 15). They were divided into 22 PFGE types corresponding to 16 different STs including ST6 (n = 13), ST16 (n = 7), ST21 (n = 3), ST35 (n = 3), ST159 (n = 3) and ST19, ST22, ST26, ST30, ST41, ST55, ST59, ST117, ST160, ST200 and ST224 (one isolate each). The largest CCs found among *E. faecalis* isolates were CC2 (ST6) and CC21 (ST21, ST22 and ST224), previously identified as major *E. faecalis* CCs (Figure 1). This is the first description of the STs 159, 160, 200 and 224. Isolates belonging to a PFGE type were associated with a single ST, except for the PFGE type SN205 comprising isolates associated with unrelated genetic lineages (ST16 and ST224) by MLST. Detailed epidemiological data about these strains are provided in Table 1.

(i) CC2 (ST6). Thirteen isolates were classified into four PFGE types belonging to ST6 (11 VRE and 2 VSE) and were recovered...
The ST224 strain represents a widely disseminated strain and ST224 (swine; PFGE type SN205.4) clustering in CC21. ST21 (one from a hospitalized patient and two from a piggery; waste water (throughout Portugal in recent years8 and were also recovered rily designed clone B responsible for different hospital outbreaks from hospitalized patients (Figure 1. gelE virulence or streptomycin (46%). All ST6 isolates possessed the putative resistance to ciprofloxacin, gentamicin and kanamycin showed resistance to ciprofloxacin, gentamicin and kanamycin eventually associated with resistance to vancomycin and erythromycin (85% each), tetracycline and chloramphenicol (62% each) or streptomycin (46%). All ST6 isolates possessed the putative virulence gelE and agg genes, whereas cyl and esp were detected in 85% and 54% of isolates, respectively. More than half of the ST6 isolates were agg+/gelE+/cyl+/esp+ (54%), some were agg+/gelE+/cyl+ (31%) and only two isolates (15%) were agg+/gelE+. Different Tn1546 types were identified among ST6-vanA clinical isolates (PP-4, PP-5, PP-15 and PP-16) and Tn1546 type A was detected in the isolate recovered from swine.

(ii) CC21. Five isolates corresponding to three PFGE types recovered from hospital and swine samples were identified as ST21 (one from a hospitalized patient and two from a piggery; PFGE types ‘X’ and SN206), ST22 (hospital; PFGE type W) and ST224 (swine; PFGE type SN205.4) clustering in CC21. The ST224 strain represents a widely disseminated strain detected in five intensive piggeries. The three ST21 isolates recovered in a hospital and a piggery from 2000 to 2007 were resistant to erythromycin, exhibited variable resistance to glycopeptides and aminoglycosides, and showed highly related PFGE types arbitrarily designated as ‘X’. The remaining CC21 isolates were also resistant to erythromycin. All but one harboured gelE+ and some also contained agg (n=3) or esp (n=3) or cyl (n=1). The only VRE isolate within CC21 was a clinical strain containing the Tn1546 variant PP-4.

(iii) ST159. ST159 was assigned as a new ST and was identified in three clinical isolates corresponding to the PFGE type ‘N’ from hospitals in Coimbra (n=1 VRE in 1999) and Viseu (n=2, one VSE in 2001 and one VRE in 2002). These ST159 isolates were resistant to at least five antibiotics, and erythromycin and ciprofloxacin resistances were commonly detected. Their virulence profiles were agg+/gelE+/cyl+/esp+ (n=2) and agg+/gelE+ (n=1). Both vanA isolates harboured PP-4.

(iv) ST16. Three PFGE types comprising seven isolates from swine (n=5 VSE; one PFGE type), poultry (n=1 VRE) and healthy volunteer (n=1 VSE) samples collected during 2001–07 in different regions of Portugal were identified as ST16. Isolates corresponding to PFGE types SN205 and 22 were isolated from animals and recovered during 2 years from piggery samples of five scattered farms (2006–07) and poultry meat samples (1999–2001) of one commercial brand distributed at the national level, respectively. Resistances to tetracycline, erythromycin and
Table 1. Epidemiological features of *E. faecalis* isolates from human, swine, poultry and environmental samples in Portugal

<table>
<thead>
<tr>
<th>CC*</th>
<th>ST</th>
<th>PFGE type*b</th>
<th>Epidemiology/origin</th>
<th>Source (product)c</th>
<th>Region/year of isolation</th>
<th>Antibiotic resistance profiled,e</th>
<th>Virulence traitsf</th>
<th>Tn1546 typef</th>
<th>vanA plasmid(s)g</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>CC2</td>
<td>6</td>
<td>B-Bb ((n=8)</td>
<td>outbreak strain (n=37 isolates) widespread in six hospitals (1996–2008)</td>
<td>HPₐ (blood, n = 1; urine, n = 2), HP₈ (blood, n = 1), HP₅ (urine, n = 3; wound, n = 1)</td>
<td>Centre/1996–2001 North/2001–02</td>
<td>(VAN), (TEC), (TET), (ERY), CIP, GEN, KAN, (CHL)</td>
<td>agg, gel, (cyl), (esp)</td>
<td>PP-4 (n = 3), PP-5 (n = 1), PP-15 (n = 2), PP-16 (n = 1)</td>
<td>75–85 (n = 4), 100 (n = 3)</td>
<td>7; this study</td>
</tr>
<tr>
<td>CC2</td>
<td>6</td>
<td>B₂</td>
<td>from different sites of hospital sewage</td>
<td>HW₅C (waste water)</td>
<td>North/2002</td>
<td>VAN, TEC, ERY, CIP, GEN, STR, KAN, CHL</td>
<td>agg, gel, cyl</td>
<td>PP-16</td>
<td>100</td>
<td>8</td>
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<tr>
<td>CC2</td>
<td>6</td>
<td>B₈</td>
<td>from swine excrements of an intensive piggery</td>
<td>SWII (liquid manure)</td>
<td>South/2007</td>
<td>VAN, TEC, TET, ERY, CIP, GEN, KAN, CHL</td>
<td>agg</td>
<td>A</td>
<td>100</td>
<td>this study</td>
</tr>
<tr>
<td>CC2</td>
<td>6</td>
<td>C</td>
<td>clinical isolate</td>
<td>HP₅C (pus)</td>
<td>North/2001</td>
<td>VAN, TEC, ERY, CIP, GEN, STR, KAN, CHL</td>
<td>agg, gel, cyl</td>
<td>PP-15</td>
<td>40 + 100</td>
<td>this study</td>
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<tr>
<td>CC2</td>
<td>6</td>
<td>G</td>
<td>clinical isolate</td>
<td>HPₐ (blood)</td>
<td>Centre/2002</td>
<td>VAN, TEC, TET, ERY, CIP, GEN, KAN</td>
<td>agg, gel, cyl, esp</td>
<td>PP-4</td>
<td>75</td>
<td>7</td>
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<tr>
<td>CC2</td>
<td>6</td>
<td>R</td>
<td>clinical isolate</td>
<td>HP₈ (blood)</td>
<td>Centre/2002</td>
<td>ERY, CIP, GEN, STR, KAN</td>
<td>agg, gel, cyl, esp</td>
<td>—</td>
<td>—</td>
<td>this study</td>
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<tr>
<td>CC21</td>
<td>21</td>
<td>X</td>
<td>clinical isolate</td>
<td>HPₐ (unknown)</td>
<td>Centre/2000</td>
<td>VAN, TEC, ERY</td>
<td>agg, gel</td>
<td>PP-4</td>
<td>145</td>
<td>7</td>
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<tr>
<td>CC21</td>
<td>21</td>
<td>X₆</td>
<td>from one facility of an intensive piggery</td>
<td>PEIV (air)</td>
<td>North/2007</td>
<td>TET, ERY, GEN, STR, KAN</td>
<td>gel, esp</td>
<td>—</td>
<td>—</td>
<td>this study</td>
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<tr>
<td>CC21</td>
<td>21</td>
<td>SN206 (X₇)</td>
<td>from an intensive piggery; related to clone X (7 bands)</td>
<td>PEIV (septic tank)</td>
<td>North/2007</td>
<td>TET, ERY, GEN, STR, KAN</td>
<td>agg, gel, esp</td>
<td>—</td>
<td>—</td>
<td>this study</td>
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<tr>
<td>CC21</td>
<td>22</td>
<td>W</td>
<td>clinical isolate</td>
<td>HP₈ (unknown)</td>
<td>Centre/2002</td>
<td>ERY, CIP, STR</td>
<td>gel</td>
<td>—</td>
<td>—</td>
<td>this study</td>
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<tr>
<td>CC21</td>
<td>224</td>
<td>SN205.4</td>
<td>strain disseminated in five piggeries (2006–07)</td>
<td>SWII (solid manure)</td>
<td>South/2006</td>
<td>TET, ERY, GEN, STR, KAN, CHL</td>
<td>agg, cyl, esp</td>
<td>—</td>
<td>—</td>
<td>this study</td>
</tr>
<tr>
<td>CS</td>
<td>159</td>
<td>N-N₉ ((n=3)</td>
<td>outbreak strain disseminated in two hospitals (1999–2002)</td>
<td>HPₐ (blood, n = 1), HP₈ (urine, n = 2)</td>
<td>Centre/1999–2002</td>
<td>(VAN), (TEC), (TET), (ERY), CIP, (GEN), (KAN)</td>
<td>agg, gel, (cyl), (esp)</td>
<td>PP-4</td>
<td>85 (n = 2)</td>
<td>7; this study</td>
</tr>
<tr>
<td>ST</td>
<td>16</td>
<td>62</td>
<td>community surveillance</td>
<td>HV (faecal swab)</td>
<td>North/2001</td>
<td>TET, ERY, GEN, STR, KAN, CHL</td>
<td>agg, gel, cyl, esp</td>
<td>—</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>ST</td>
<td>16</td>
<td>22</td>
<td>from retail poultry samples commercialized at national level (1999–2001)</td>
<td>RP (carcass)</td>
<td>North/2001</td>
<td>VAN, TEC, TET, ERY, CIP, STR</td>
<td>agg, gel</td>
<td>A</td>
<td>ND</td>
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<th>CCa</th>
<th>ST</th>
<th>PFGE typeb</th>
<th>Epidemiology/origin</th>
<th>Region/year of isolation</th>
<th>Antibiotic resistance profilec,e</th>
<th>Virulence traitsf</th>
<th>Tn/546 typeg</th>
<th>vanA plasmid(s)h</th>
<th>Reference</th>
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<tr>
<td>ST 16</td>
<td>SN205-205.4</td>
<td>(n=5)</td>
<td>strain disseminated in five intensive piggeries (2006–07)</td>
<td>PEI (waste lagoon, n=1), SWIII (faeces, n=1), PEIV (food, n=1), PEV (manure, n=2)</td>
<td>South/2006, North/2007</td>
<td>TET, ERY, GEN, (STR), KAN, (CHL)</td>
<td>agg, (gel), cyl, (esp)</td>
<td>—</td>
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<tr>
<td>ST 35</td>
<td>51</td>
<td>from retail poultry samples</td>
<td>RP (carcass)</td>
<td>North/1999</td>
<td>TET, ERY, GEN, STR, KAN</td>
<td>agg, gel, cyl</td>
<td>—</td>
<td>—</td>
<td>9</td>
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<tr>
<td>ST 35</td>
<td>SN204 (n=2)</td>
<td>strain disseminated in two piggeries (2006–07)</td>
<td>SWIII (faeces, n=1), PEII (food, n=1)</td>
<td>South/2006–07</td>
<td>TET, ERY, CIP, GEN, STR, KAN, CHL</td>
<td>gel</td>
<td>—</td>
<td>—</td>
<td>this study</td>
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<tr>
<td>ST 19</td>
<td>I</td>
<td>clinical isolate</td>
<td>HPB (urine)</td>
<td>Centre/2001</td>
<td>TET, ERY, CIP, GEN, STR, KAN</td>
<td>agg, gel, esp</td>
<td>—</td>
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<td>this study</td>
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<tr>
<td>ST 26</td>
<td>61</td>
<td>community surveillance</td>
<td>HV (faecal swab)</td>
<td>North/2001</td>
<td>TET, ERY, GEN, STR, KAN, CHL</td>
<td>agg, gel, esp</td>
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<td>ST 30</td>
<td>K</td>
<td>clinical isolate</td>
<td>HPB (pus)</td>
<td>North/2001</td>
<td>VAN, TEC, TET, ERY</td>
<td>agg, gel, esp A</td>
<td>100</td>
<td>this study</td>
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<tr>
<td>ST 41</td>
<td>D</td>
<td>clinical isolate</td>
<td>HPB (urine)</td>
<td>Centre/2001</td>
<td>TET</td>
<td>agg, gel</td>
<td>—</td>
<td>—</td>
<td>this study</td>
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<tr>
<td>ST 55</td>
<td>Q</td>
<td>clinical isolate</td>
<td>HPB (blood)</td>
<td>Centre/2001</td>
<td>VAN, TEC, ERY, KAN</td>
<td>esp</td>
<td>PP-4 75</td>
<td>7</td>
<td></td>
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<tr>
<td>ST 59</td>
<td>45</td>
<td>from retail poultry samples</td>
<td>RP (carcass)</td>
<td>North/2001</td>
<td>TET, GEN, KAN</td>
<td>agg, gel</td>
<td>—</td>
<td>—</td>
<td>9</td>
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<tr>
<td>CS 117</td>
<td>F</td>
<td>clinical isolate</td>
<td>HPC (blood)</td>
<td>North/2002</td>
<td>VAN, TEC, ERY</td>
<td>agg, gel</td>
<td>PP-5 150 + 300</td>
<td>this study</td>
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<tr>
<td>CS 160</td>
<td>86</td>
<td>strain recovered from both HV and poultry during 2001</td>
<td>HV (faecal swab)</td>
<td>North/2001</td>
<td>TET, CIP, GEN, KAN</td>
<td>gel</td>
<td>—</td>
<td>—</td>
<td>10</td>
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<td>ST 200</td>
<td>SN201</td>
<td>from swine excrements of an intensive piggery</td>
<td>SWIII (solid manure)</td>
<td>South/2006</td>
<td>TET, ERY</td>
<td>agg, gel, esp</td>
<td>—</td>
<td>—</td>
<td>this study</td>
</tr>
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</table>

aCC, clonal complexes are shown according to eBURST clustering; CS, singletons; ST, sequence types not assigned to any particular CC.
bPersistent and/or disseminated PFGE types are shown in bold. Strains identified with different PFGE subtypes were included in some cases and their number is designated in parentheses.
cHP, hospitalized patients; HV, healthy volunteers; RP, retail poultry; SW, swine; PE, piggery environment; HW, hospital sewage. The different hospitals are designated by capital letters (A, Coimbra; B, Viseu; C, Porto) and the piggeries by Roman numerals (I–V).
dVAN, vancomycin; TEC, teicoplanin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; KAN, kanamycin; CHL, chloramphenicol.
eThe variable presence of a given antibiotic resistance gene or virulence trait among isolates belonging to the same PFGE type is indicated by parentheses.
ffTn1546 designation is based on the results obtained by a PCR overlapping assay described previously.17,18
gPlasmid size is shown in kilobases (kb); ND, not determined.
hPlasmid size is shown in kilobases (kb); ND, not determined.
aminoglycosides were expressed in all isolates, whereas those to vancomycin and ciprofloxacin were only detected in the poultry isolate. Different virulence profiles were observed: \textit{agg}/+\textit{cyt}+/\textit{esp}+ (four from piggeries), \textit{agg}/+\textit{gelE}/+\textit{cyt}+/\textit{esp}+ (one healthy volunteer), \textit{agg}/+\textit{gelE}/+\textit{cyt}+ (one swine) and \textit{agg}/+\textit{gelE}+ (one poultry). The \textit{vanA} transposon characterized in the poultry isolate corresponded to the variant A.

\textit{(v) ST35}. ST35 was identified in three VSE animal isolates from poultry, swine and a piggery (one each). The piggery isolate corresponded to a strain persisting in two different piggeries for 2 years. All were resistant to tetracycline, erythromycin, gentamicin, streptomycin and kanamycin, and the piggery isolate corresponded to the variant A.

\textit{(vi) Other \textit{E. faecalis} STs}. The STs ST19, ST26, ST30, ST41, ST55, ST59, ST117, ST160 and ST200 were represented by only one strain. They were recovered from hospitalized patients (ST19, ST30, ST41, ST55 and ST117), healthy humans (ST26 and ST160), poultry (ST59) and swine (ST200) from 2001 to 2006. Different antibiotic resistance and virulence marker profiles were observed within these STs (Table 1). Most STs contained \textit{agg} and \textit{gelE} with or without \textit{esp}. The \textit{Tn1546} types A, PP-4 and PP-5 were observed in ST30, ST55 and ST117 isolates, respectively.

\section*{Genetic elements encoding vancomycin resistance}

Five \textit{Tn1546} types, previously described by our group,\textsuperscript{18} were associated with the different \textit{Tn1546} types identified (Figure 2). Type I (75–85 kb), comprising four subtypes, was identified in four \textit{E. faecalis} clones clustering in CC2 (ST6; two PFGE types; \textit{n}=5), ST159 (\textit{n}=2) and ST55 (\textit{n}=1), and contained the most disseminated \textit{Tn1546} types (variants PP-4 and PP-5) identified in three hospitals during 1996–2002. Type II (100 kb), two subtypes, was identified in three ST6 isolates (PFGE type B) harbouring the related \textit{Tn1546} variants PP-15 and PP-16, which were recovered from the same hospital and respective hospital waste water in different years. Plasmid types III and IV (100 kb each) were recovered from two \textit{E. faecalis} strains of clinical and animal origins, both harbouring \textit{Tn1546} type A. Megaplasmids hybridizing with the \textit{vanA} gene were identified in clinical isolates of ST21 (145 kb) and ST117 (150 and 300 kb) (Table 1).

\section*{Discussion}

This study indicates the dominance in the Portuguese nosocomial setting of the major \textit{E. faecalis} clonal lineage CC2 that is globally associated with human infections, reveals the absence in Portugal of particular CCs (CC9, CC40 and CC87) that are widely spread in other EU countries\textsuperscript{5,7,22} and describes the role of horizontal transfer in the dissemination and persistence of VRE in Portuguese hospitals.

CC2 was identified among most clinical isolates studied, which mainly corresponded to a widely disseminated multidrug-resistant strain previously designated as ‘clone B’, recovered from Portuguese hospitals since 1996\textsuperscript{8} but also among \textit{vanA} isolates from swine manure and hospital sewage, indicating a community contamination by this \textit{E. faecalis} high-risk CC. Moreover, the diversity of the antibiogram and virulence gene profiles of CC2 isolates from extra-hospital settings further supports the suggestion that CC2 is particularly proficient at the DNA exchange highlighted by McBride et al.\textsuperscript{7} A recent study including clinical isolates from a hospital in Southern Portugal

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Plasmid type} & \textbf{Size (kb)} & \textbf{PFGE types (ST)} & \textbf{\textit{Tn1546} type} & \textbf{Origin (no. of isolates)} & \textbf{Date} \\
\hline
I & 85 & B (ST6); N (ST159) & PP-4; PP-5 & HP (\textit{n}=3) & 1999–2001 \\
Ia & 75 & B and G (ST6) & PP-4 & HP (\textit{n}=2) & 1996–2002 \\
Ib & 75 & B (ST6); Q (ST55) & PP-4 & HP (\textit{n}=2) & 1999–2001 \\
Ic & 75 & B (ST6) & PP-4 & HP (\textit{n}=1) & 2001 \\
II & 100 & B (ST6) & PP-15 & HP (\textit{n}=2) & 2001 \\
Iia & 100 & B (ST6) & PP-16 & HW (\textit{n}=1) & 2002 \\
Iib & 100 & B (ST6) & A & SW (\textit{n}=1) & 2007 \\
III & 100 & B (ST6) & K (ST30) & A & HP (\textit{n}=1) & 2001 \\
IV & 150 & K (ST30) & A & HP (\textit{n}=1) & 2001 \\
\hline
\end{tabular}
\caption{Gel electrophoresis (1\% agarose) of \textit{EcoRI}-digested \textit{vanA} plasmids isolated from representative VRE isolates. Lanes: lane 1, type I (HP; PFGE type B-ST6; PP-5); lane 2, type Ia (HP; PFGE type G-ST6; PP-4); lane 3, type Ib (HP; PFGE type Q-ST55; PP-4); lane 4, type Ic (HP; PFGE type B-ST6; PP-4); lane 5, type II (HP; PFGE type B-ST6; PP-15); lane 6, type Iia (HW; PFGE type B-ST6; PP-16); lane 7, type III (SW; PFGE type B-ST6; \textit{Tn1546} type A); lane 8, type IV (HP; PFGE type K-ST30; \textit{Tn1546} type A). HP, hospitalized patient; SW, swine; HW, hospital sewage.}
\end{table}
also described the predominance of \textit{E. faecalis} CC2 mainly associated with a dominant and epidemic clone,\textsuperscript{23} confirming the establishment of this clonal lineage in our country. Although we did not identify other high-risk CCs, the new singleton ST159 was persistently obtained from clinical isolates in different Portuguese cities during a 4 year period (1999–2002) and comprised both multiresistant VRE and VSE isolates mainly possessing \textit{exp} and \textit{cyl}. Interestingly, the double-locus variant of ST159 designated as ST103 (differing in two of the seven housekeeping genes) was also identified in clinical VRE isolates from the USA in 2002 (P. Ruiz-Garbajosa, A. R. Freitas, M. Zervos, S. Donabedian, R. Cantón, F. Baquero and T. M. Coque, unpublished results), which might reflect the possible emergence of a new epidemic ST in Portugal with a parallel emergence of related STs in other distant nations. Other STs identified from clinical isolates as ST19, ST30 and ST55 have also been associated with human strains from Spain, the Netherlands, the UK and the USA from the mid-1990s, but their relevance to the whole \textit{E. faecalis} population structure remains to be proved.\textsuperscript{4,7}

CC21 and ST16 isolates were mainly associated with widespread persistent clones from swine or poultry samples in Portugal. CC21 encompasses mostly animal and community surveillance isolates and only a few clinical strains.\textsuperscript{4} Moreover, an ST21 strain was recovered from human hospital and piggery samples, highlighting the dispersion of this CC in different ecological settings. Although previously characterized CC21 strains usually harboured less antibiotic resistance and fewer virulence traits than isolates of other CCs,\textsuperscript{5,7,22} the different genetic content exhibited by isolates of the Portuguese CC21 clones indicates successful acquisition of diverse genetic elements that might facilitate their persistence and spread in environments under selective antibiotic pressure. The presence of ST16 and ST160 strains in both poultry and faecal samples of healthy humans supports the spread of enterococcal strains between animals and humans via the food chain, which has also been observed in Denmark, New Zealand, Japan, Thailand and the USA, sometimes also associated with ST16.\textsuperscript{11,24–29} In addition, ST59 identified in one of the few Portuguese isolates from retail poultry included in this study was recently described in healthy animals from Spain,\textsuperscript{4} in chickens with arthritis and amyloid arthropathy from France\textsuperscript{30} and in human clinical samples from Poland.\textsuperscript{4} In our set of \textit{E. faecalis} isolates, \textit{cyl} was frequently associated with specific clonal lineages (CC2, ST159 and ST16), while \textit{esp} was widely disseminated among CC2, CC21 and most singletons, both in human and animal isolates. These observations support previous data from other authors in which the role of \textit{cyl} and \textit{esp} genes in the pathogenicity of enterococci is underscored.\textsuperscript{7,29}

The finding of similar \textit{vanA} plasmids among clonally related (ST6-CC2) or unrelated strains (ST6, ST55 and ST159) recovered from different hospitals over long periods of time highlights the role of horizontal gene transfer in the spread of vancomycin resistance among enterococci. The similarity of the restriction fragment length polymorphisms corresponding to plasmids containing similar Tn1546 variants suggests the evolution of specific plasmids by different genetic events.

In summary, our findings reflect an \textit{E. faecalis} population represented by CC2, CC21, ST159 and five unrelated STs among hospitalized humans, and CC21, ST16 and ST35 in the community setting in Portugal. The high prevalence of the worldwide-disseminated CC2 is associated with clonal expansion of a single strain in our area. In addition, plasmid dissemination seems to have greatly influenced the dissemination of vancomycin resistance among \textit{E. faecalis} in Portugal.

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\section*{Transparency declarations}

None to declare.

\section*{References}

Population structure of *E. faecalis* from Portugal


