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# Penicillin-resistant, ampicillin-susceptible *Enterococcus faecalis* of hospital origin: *pbp4* gene polymorphism and genetic diversity

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ABSTRACT

Despite the spread of penicillin-resistant, ampicillin-susceptible *Enterococcus faecalis* (PRASEF) isolates in diverse countries, the mechanisms leading to this unusual resistance phenotype have not yet been investigated. The aim of this study was to evaluate whether polymorphism in the *pbp4* gene is associated with penicillin resistance in PRASEF isolates and to determine their genetic diversity. *E. faecalis* isolates were recovered from different clinical specimens of hospitalized patients from February 2006 to June 2010. The β-lactam minimal inhibitory concentrations (MICs) were determined by E-test®. The PCR-amplified *pbp4* gene was sequenced with an automated sequencer. The genetic diversities of the isolates were established by PFGE (pulsed-field gel electrophoresis) and MLST (multilocus sequencing typing). Seventeen non-producing β-lactamase PRASEF and 10 penicillin-susceptible, ampicillin-susceptible *E. faecalis* (PSASEF) strains were analyzed. A single-amino-acid substitution (Asp-573 → Glu) in the penicillin-binding domain was significantly found in all PRASEF isolates by sequencing of the *pbp4* gene but not in the penicillin-susceptible isolates. In contrast to the PSASEF isolates, a majority of the PRASEFs had similar PFGE profiles. Six representative PRASEF isolates were resolved by MLST into ST9 and ST524 and belong to the globally dispersed clonal complex 9 (CC9). In conclusion, it appears quite likely that the amino acid alteration (Asp-573 → Glu) found in the PBP4 of the Brazilian PRASEF isolates may account for their reduced susceptibility to penicillin, although other resistance mechanisms remain to be investigated.



## 1. Introduction

Enterococci are effective opportunistic pathogens involved in hospital-acquired infections mainly due to their intrinsic resistance to several classes of antimicrobials, including aminoglycosides (low-level resistance) and β-lactams (cephalosporins and β-lactamase-resistant penicillins), and their remarkable ability to acquire new mechanisms of resistance (Teixeira et al., 2011; Hollenbeck and Rice, 2012). Resistance to penicillins and other β-lactam antibiotics is of concern because these compounds are clinically useful drugs that are commonly used for the treatment of enterococcal infections, either alone or in association with aminoglycosides because this synergistic combination results in the killing of the enterococci (Arias et al., 2010).

β-Lactam antibiotics inhibit the biosynthesis of the bacterial cell wall by covalently binding to penicillin-binding proteins (PBPs) with transpeptidase and carboxypeptidase activity involved in the final stages of peptidoglycan synthesis (Eliopoulos, 2008; Zapun et al., 2008). Resistance to β-lactams in enterococci is mediated either by the production of β-lactamases that hydrolyze and inactivate the drug or by alterations of PBPs, which are the drug targets (Hollenbeck and Rice, 2012). Six different PBPs are currently described in enterococci (Fontana et al., 1992). PBP4 and PBP5 have low affinity for β-lactam antibiotics and have been associated with resistance to these drugs due to their ability to replace the normal functions of other PBPs, such as PBPs 1, 2 and 3 (Fontana et al., 1992). Overproduction of the endogenous low-affinity PBPs and point mutations in the genes encoding these PBPs, which lead to a lower affinity, are the most common mechanisms of resistance to β-lactams among enterococci (Hollenbeck and Rice, 2012; Ono et al., 2005; Hiraga et al., 2008).

Enterococci usually show cross-susceptibility to β-lactamase-susceptible penicillins and imipenem (Eliopoulos, 2008; Weinstein

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et al., 2004). However, some studies have reported the emergence of ampicillin-susceptible and imipenem-resistant *Enterococcus faecium* associated with the overproduction of PBP5, which has a selective decreased affinity for imipenem (Amin et al., 2001). In 2005, penicillin-resistant, ampicillin-susceptible *Enterococcus faecalis* (PRASEF) isolates were reported in Greece (Metzidie et al., 2005), and this unusual penicillin-resistance phenotype was thereafter also found in hospitals of Denmark (Guardabassi et al., 2010) and Brazil (Conceição et al., 2012). Interestingly, the majority of PRASEF isolates have also shown resistance to imipenem and to gentamicin.

*E. faecalis* is the most prevalent enterococcal species, accounting for 80–90% of the clinical enterococcal isolates identified in hospital and community human infections worldwide (Teixeira et al., 2011). Recent molecular epidemiological studies using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) based on the sequencing of seven housekeeping genes have revealed the existence of specific lineages in hospital environments that carry multiple virulence and antibiotic resistance traits. Up to September January 2014, 526 sequence types (ST) grouped into diverse clonal complexes (CC) were identified by MLST analysis of 1201 *E. faecalis* isolates recovered from different sources and geographic locations (http://efaecalis.mlst.net/).

CC6 and CC9 are currently the major complexes comprising hospital-adapted lineages that are globally dispersed (Ruiz-Garbajosa et al., 2006; Kuch et al., 2012). In Brazil, the occurrence of CC6 among *E. faecalis* isolates recovered from hospitalized patients has also been reported (Penas et al., 2013). The CC6 that mostly comprises ST6 and ST2 was designated CC2 in previous studies; however, eBURST grouping showed that ST6 became increasingly prevalent and could be considered the initiatory ST in this clonal complex. Therefore, in this study, CC2 is formally designated CC6.

Although the CC of the PRASEF isolates from Greece (Metzidie et al., 2005) and Brazil (Conceição et al., 2012) are unknown, Guardabassi et al. (2010) have demonstrated that most PRASEF isolates from Denmark belong to ST6 (CC6) and show indistinguishable or closely related PFGE patterns.

Despite the spread of PRASEF isolates in diverse countries, the mechanisms leading to this unusual phenotype of penicillin resistance are completely unknown and have not yet been investigated. Therefore, the aim of the present study was to investigate whether polymorphism in the *pbp4* gene could be associated with penicillin resistance in PRASEF isolates. In addition, the genetic diversity of these isolates was assessed by PFGE and MLST.

## 2. Materials and methods

### 2.1. Bacterial isolates and species identification

Of a collection of 317 non-repetitive *E. faecalis* isolates recovered from hospitalized patients at a Brazilian tertiary hospital in the period of February 2006 to June 2010, 34 (10.7%) were penicillin resistant and ampicillin susceptible in the three susceptibility tests performed (Etest, broth dilution, and disk diffusion) (Conceição et al., 2011, 2012). Among the 34 PRASEF isolates, 17 were randomly selected to perform the present study. Penicillin-susceptible, ampicillin-susceptible *E. faecalis* (PSASEF) isolates ($n = 10$) were also evaluated for comparative purposes. The isolates were recovered from different clinical specimens, such as secretions (abdominal, pleural and ocular), urine, wounds and blood. All PRASEF and PSASEF isolates were susceptible to vancomycin, and 30% of the PSASEF isolates and most (82.4%) of the PRASEF ones were resistant to high levels of gentamicin by disk diffusion test. The species identification of isolates was based on phenotypic tests (Teixeira et al., 2011) and confirmed by PCR using specific primers as described elsewhere (Dutka-Malen et al., 1995).

This study was approved by the Research Ethics Committee of the Universidade Federal do Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil (document number 1365).

### 2.2. Antimicrobial susceptibility testing

The antimicrobial minimal inhibitory concentrations (MICs) for the β-lactams penicillin, ampicillin, amoxicillin, imipenem and piperacillin were determined by E-test® (AB bioMérieux, Solna, Sweden) and by the broth dilution method using Mueller–Hinton broth (Difco, Becton, Dickinson and Company Sparks, France) and solutions of antimicrobials prepared from powders of known potencies (Sigma–Aldrich Denmark A/S, Copenhagen, Denmark). Susceptibility tests were performed and interpreted according to CLSI guidelines (CLSI, 2012; CLSI, 2013). For all of the β-lactams tested, MICs $\geqslant$ 16 μg/mL indicated resistance. Quality-control testing was performed using *E. faecalis* ATCC 29212.

### 2.3. β-Lactamase production

β-Lactamase production was tested with the chromogenic nitrocefin disk (Becton, Dickinson and Company, Cefinase™, USA). *Staphylococcus aureus* ATCC 25923 (positive control) was used in the quality-control testing.

### 2.4. Amplification and sequencing of the pbp4 gene

Bacterial DNA from *E. faecalis* isolates and the reference strain (*E. faecalis* ATCC 29212) was extracted using the QIAamp® DNA Mini kit (Qiagen, Hilden, Germany). The complete *pbp4* gene encoding the *E. faecalis* PBP4 protein, which consists of 680 amino acids (corresponding to 2040 base pairs), was amplified by PCR using specific primers (Table 1). The PCR products were purified using a Wizard® PCR Preps DNA Purification System (Promega, Madison, WI, USA) and quantified by comparison with the Low Mass DNA Ladder (Invitrogen Carlsbad, CA, USA). The purified amplicons were directly sequenced in both strands by an automated sequencer (ABI PRISM 3130XL, Applied Biosystems, Foster City, CA, USA) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit. The sequencing primers were the same as those used in the PCR reactions.

The sequences were aligned using the Geneious version 5.6.4 software (Biomatters, Auckland, New Zealand), compared with the reference sequences and translated into amino acid sequences.

Pearson's chi-square test or Fisher's exact test was used to compare the polymorphism frequency between the PRASEF and PSASEF isolates. Differences were considered significant at $P \leqslant 0.05$.

### 2.5. Pulsed-field gel electrophoresis (PFGE) typing

The genomic DNA obtained after bacterial lysis was digested with *SmaI* as described previously and subjected to PFGE (21). Briefly, digest electrophoresis was performed in a CHEF-DR III apparatus (Bio-Rad Laboratories, Richmond, CA, USA) adjusted with pulses of 5 s to 30 s at 6 V/cm$^2$ for 20 h Campanile et al. (2003). The processed gels were stained with ethidium bromide, visualized with a UV transilluminator and photographed for the analysis of banding patterns. The isolate clonalities were analyzed using the BioNumerics software version 5.01 (Applied Maths, Sint-Martens-Latem, Belgium). The genomic similarity was determined using a dendrogram constructed by the unweighted pair group method with the arithmetic mean (UPGMA) algorithm, and the band similarity was compared by the Dice coefficient and a tolerance of 0.3%.

**Table 1**
Primers used in the amplification and sequencing of the *pbp4* gene.

| Primers | Sequences 5′–3′ | Position in *pbp4* gene* (pb) | PCR product size (pb) | Reference |
|---|---|---|---|---|
| EF PBP4-1F | CAACGAAAGCCTGATGAAATGG | 164 | 1.272 pb | Ono et al. (2005) |
| EF PBP4-1R | AATCGCCTTTTTGAGGATCGG | 1435 | | |
| EF PBP4-2F | CGATTGACAGTGTACAACAACAAGC | 1346 | 1.088 pb | Ono et al. (2005) |
| EF PBP4-2R | CGCTTCATTGTAGCACACTTTCCTTTTTC | 2433 | | |
| EF PBP4-3F | TTTGTACCAATCACAGTTG | 1021 | 769 pb | This study |
| EF PBP4-3R | CCCCCATCCGTAATGTTTG | 1789 | | |

* GenBank accession: AJ290435.1.

### 2.6. Multilocus sequence typing (MLST)

*E. faecalis* MLST was performed using seven conserved housekeeping genes (*gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL*) (Ruiz-Garbajosa et al., 2006). A detailed protocol of the MLST procedure, including the allelic type and sequence type (ST) assignment methods, is available in the online *E. faecalis* MLST database (http://efaecalis.mlst.net/). New alleles and STs submitted to the MLST website were approved.

The phylogenetic relationships among closely related STs were determined using the eBURST V3 algorithm (http://eburst.mlst.net/; 05 September 2013, date last accessed) following the criteria described by Pitondo-Silva et al. (2009).

### 2.7. Nucleotide sequence accession numbers

The *pbp4* gene sequences of three (20-06, 154-07, and 212-08) representative PRASEF isolates and the reference strain (*E. faecalis* ATCC 29213) evaluated in this study were deposited in the GenBank database (www.ncbi.nlm.nih.gov/Genbank) with accession numbers KF006328, KF026744, KF026745, and KF192636, respectively.

## 3. Results

### 3.1. Susceptibility to β-lactams and β-lactamase production

The MICs of the β-lactams obtained by E-test® against all of the PRASEF and PSASEF isolates tested in the present study are summarized in Table 2. The MIC values obtained by the E-test® and the broth dilution method (data not shown) were not significantly different. The PSASEF isolates were susceptible to all of the β-lactams tested. The penicillin MICs for the PRASEFs ranged from 16 to 64 μg/mL, and all of the isolates showed ampicillin and amoxicillin susceptibility. However, it should be noted that the MIC values of both β-lactams for the PRASEF isolates tended to be higher (2 to 8 μg/mL and 0.38 to 8 μg/mL, respectively) than those (0.25 to 1 μg/mL, for both) for the PSASEF isolates. All of the PRASEFs showed resistance to piperacillin, and 14 (82.3%) of them exhibited resistance to imipenem. None of the *E. faecalis* isolates included in this study produced β-lactamase.

### 3.2. Sequencing of the pbp4 gene

The PBP4 amino acid substitutions detected in the PRASEF and PSASEF isolates are described in Table 2.

In comparison to the reference strain (*E. faecalis* ATCC 29212), all PRASEF isolates in the study had the same amino acid substitution (Asp-573 → Glu). The 573 position is inside the penicillin-binding domain (PBD) of PBP4. In addition, three PRASEF isolates had other alterations. In two (212-08 and 291-09), the alterations were located outside the PBD (Lys-218 → Asn and Lys-218 → Tyr, respectively), whereas in the third one (154-07), the change found was in the PBD (Asp-632 → Glu). In contrast to the Asp-573 → Glu substitution, other amino acid alterations found in this study had no effect on the MICs of β-lactams against PRASEF isolates.

Different polymorphisms were observed in the *pbp4* gene of four PSASEF isolates, three of which showed amino acid alterations at PBD positions (Val-582 → Ile in two isolates and Ala-623 → Pro in the other one). Again, these amino acid substitutions located outside of the PBD (Ile-582 → Phe) had no effect on the MICs of β-lactams for these isolates.

Notably, only the Asp-573 → Glu substitution was significantly ($P < 0.001$) more frequent in penicillin-resistant compared to susceptible isolates.

### 3.3. PFGE and MLST

According to the PFGE dendrogram, the majority (16 out of 17; 94.1%) of PRASEF isolates showed similar PFGE profiles, which were considered to be of the same clonal type by a criterion of greater than 90% genomic similarity (Fig. 1). Eleven (64.7%) PRASEF isolates belonging to pulsotype A showed indistinguishable PFGE patterns, and five (29.4%) isolates with pulsotypes related to A were classified as A1 (3 isolates), A2 (isolate 154-07) and A3 (isolate 272-09). Only one PRASEF (80-06) was classified as pulsotype B.

Compared with the PRASEF ones, the PSASEF isolates showed different PFGE profiles and were considered unrelated between themselves.

Six representative PRASEF isolates were selected for MLST analysis; five were of different pulsotypes, and the choice of the sixth was based on the sequencing results of the *pbp*4 gene and the penicillin MIC. The six isolates were resolved by MLST into two STs (Fig. 2), four of which belong to ST9 (Table 2). The other two, as demonstrated for the first time in this study, were characterized as ST524. Both ST9 and ST524 belong to CC9 characterized as a multi-resistant high-risk enterococcal clonal complex. The PRASEF isolate 80-06 (pulsotype B) showing a PFGE profile unrelated to the others in the group was also grouped as CC9 because it belongs to ST9.

## 4. Discussion

To date, few published studies have reported the emergence of PRASEF isolates (Conceição et al., 2012; Guardabassi et al., 2010; Metzidie et al., 2005). However, resistance surveillance studies based on antimicrobial activity data that were conducted worldwide indicate an increased number of *E. faecalis* isolates with this atypical penicillin-resistance phenotype. For instance, in a study conducted in Poland (Kawalec et al., 2007), the percentage of penicillin-resistant *E. faecalis* isolates was much higher than the percentage of those resistant to ampicillin (8.0% *versus* 0.69%), as also reported for isolates collected in several countries of Latin America (38.5% *versus* 3.8%) (Sader et al., 2004). Additionally, a recent review noted discordant percentages of resistance to penicillin and ampicillin among clinical *E. faecalis* isolates from various other countries (Gawryszewska et al., 2012).

**Table 2**
Clinical characteristics, *pbp4* gene polymorphism, PFGE, MLST and MICs by E-test® of penicillin-resistant, ampicillin-susceptible *E. faecalis* ($n = 17$) and penicillin- and ampicillin-susceptible *E. faecalis* ($n = 10$) isolates.

| Resistance phenotype[a] | Isolate number[b] | Isolation date (DD/MM/YY) | Clinical specimen[c] | Ward[d] | PBP4 amino acid change at position[e] | | | PFGE | MLST[f] | | MIC[g] Etest® (μg/mL) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Non-PBD | PBD | | | CC | ST | PEN | AMP | AMX | IPM | PIP |
| | | | | | 218 | 573 | 632 | | | | | | | | |
| | ATCC | | | | Lys | Asp | Asp | | | | | | | | |
| PRASEF | 20-06 | 20/03/06 | Urine | HIU | | Glu | | A | 9 | 9 | 64 | 8 | 8 | 16 | >256 |
| | 38-06 | 18/05/06 | Wound | Surgical | | Glu | | A1 | 9 | 9 | 32 | 4 | 8 | >32 | >256 |
| | 80-06 | 01/11/06 | Wound | HIU | | Glu | | B | 9 | 9 | 16 | 2 | 1.5 | 8 | 96 |
| | 154-07 | 29/09/07 | Urine | C-ICU | | Glu | Glu | A2 | 9 | 9 | 24 | 4 | 1.5 | >32 | >256 |
| | 157-07 | 10/10/07 | Wound | Medical | | Glu | | A1 | NE | NE | 16 | 3 | 2 | >32 | >256 |
| | 212-08 | 17/06/08 | Urine | Medical | Asn | Glu | | A | 9 | 524* | 24 | 6 | 1 | 32 | >256 |
| | 234-08 | 03/09/08 | Wound | Surgical | | Glu | | A | NE | NE | 16 | 4 | 1.5 | 32 | >256 |
| | 236-08 | 05/09/08 | Wound | Surgical | | Glu | | A | NE | NE | 16 | 4 | 1.5 | 16 | >256 |
| | 240-08 | 22/10/08 | Blood | Surgical | | Glu | | A | NE | NE | 16 | 6 | 1 | 8 | >256 |
| | 245-08 | 25/09/08 | Secretion | Surgical | | Glu | | A | NE | NE | 16 | 4 | 1.5 | 16 | 64 |
| | 250-08 | 13/10/08 | Wound | Surgical | | Glu | | A | NE | NE | 32 | 6 | 1.5 | >32 | >256 |
| | 253-08 | 15/10/08 | Blood | Medical | | Glu | | A1 | NE | NE | 16 | 4 | 1.5 | 8 | >256 |
| | 269-08 | 29/12/08 | Wound | Surgical | | Glu | | A | NE | NE | 16 | 6 | 1.5 | 16 | >256 |
| | 272-09 | 08/01/09 | Wound | Surgical | | Glu | | A3 | 9 | 524* | 24 | 8 | 2 | >32 | >256 |
| | 291-09 | 06/03/09 | Secretion | A-ICU | Tyr | Glu | | A | NE | NE | 16 | 6 | 0.38 | >32 | >256 |
| | 313-09 | 08/04/09 | Wound | Surgical | | Glu | | A | NE | NE | 24 | 6 | 1 | >32 | >256 |
| | 427-10 | 10/03/10 | Urine | Surgical | | Glu | | A | NE | NE | 16 | 4 | 0.5 | >32 | >256 |
| | | | | | PBP4 amino acid change at position[e] | | | | | | | | | | |
| | | | | | Non-PBD | PBD | | | | | | | | | |
| | | | | | 243 | 582 | 623 | | | | | | | | |
| | ATCC | 06/03/06 | Wound | Surgical | Ile | Val | Ala | | | | | | | | |
| PSASEF | 07-06 | 10/05/06 | Urine | Surgical | | | | E | NE | NE | 0.5 | 0.25 | 0.38 | 0.38 | 1 |
| | 35-06 | 11/07/06 | Wound | Surgical | | | | F | NE | NE | 1 | 0.38 | 0.38 | 0.75 | 1.5 |
| | 58-06 | 18/10/06 | Secretion | Surgical | | | | G | NE | NE | 1.5 | 0.38 | 0.5 | 0.75 | 1 |
| | 74-06 | 06/01/07 | Secretion | P-ICU | Phe | | | I | NE | NE | 1 | 0.5 | 0.38 | 0.5 | 1 |
| | 88-07 | 04/10/07 | Wound | Medical | | Ile | | H | NE | NE | 1 | 0.5 | 1 | 0.38 | 1.5 |
| | 155-07 | 03/02/08 | Blood | P-ICU | | | | J | NE | NE | 1 | 0.25 | 0.25 | 0.38 | 1.5 |
| | 175-08 | 07/07/08 | Wound | Medical | | Ile | | L | NE | NE | 4 | 1 | 0.5 | 1 | 4 |
| | 221-08 | 17/08/08 | Urine | Medical | | | | C | NE | NE | 1 | 0.75 | 0.19 | 0.38 | 1 |
| | 228-08 | 19/01/09 | Secretion | Surgical | | | Pro | K | NE | NE | 1 | 0.38 | 0.5 | 0.5 | 1 |
| | 277-09 | 06/03/06 | Wound | Surgical | | | | D | NE | NE | 1.5 | 0.75 | 0.38 | 0.5 | 1.5 |

[a] PRASEF, penicillin-resistant, ampicillin-susceptible *E. faecalis*; PSASEF, penicillin, ampicillin-susceptible *E. faecalis*.
[b] ATCC (American Type Culture Collection) used as reference strain (*E. faecalis* ATCC 29212).
[c] Secretions were abdominal, pleural and ocular.
[d] A-ICU, adult intensive care unit; C-ICU, coronary intensive care unit; P-ICU, pediatric intensive care unit; HIU, hospital infection unit.
[e] PBD, penicillin-binding domain; Non-PBD, non-penicillin-binding-domain; Ala, alanine; Asp, aspartic acid; Phe, phenylalanine; Glu, glutamic acid; Ile, isoleucine; Lys, lysine; Pro, proline; Tyr, tyrosine; Val, valine.
[f] CC, clonal complex; ST, sequence type (*New ST); NE, not evaluated.
[g] AMP, ampicillin; PEN, penicillin; AMX, amoxicillin; IPM, imipenem; PIP, piperacillin.

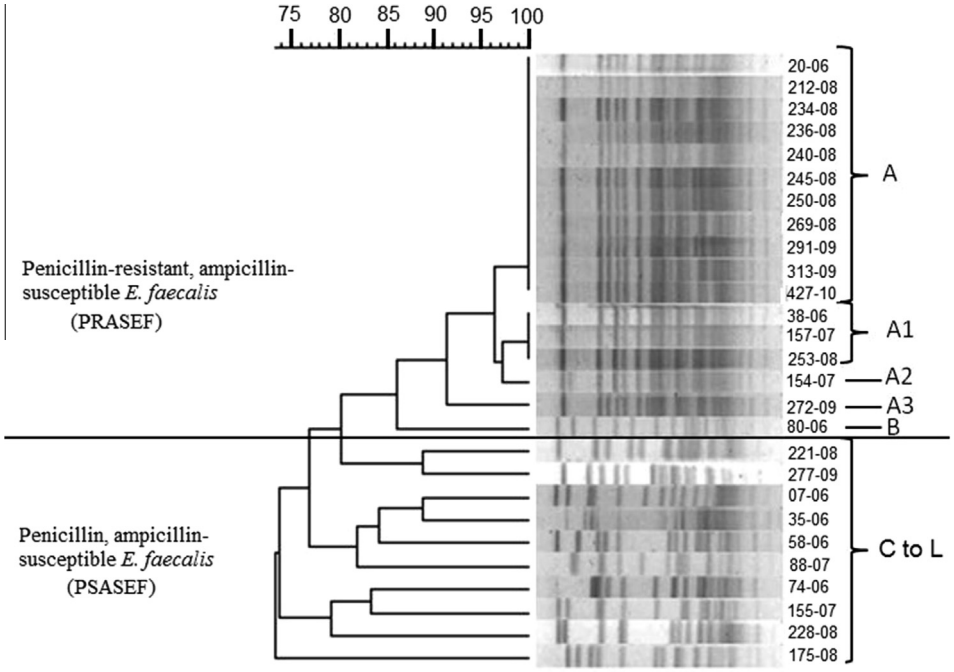


**Fig. 1.** Pulsed-field gel electrophoresis (PFGE) dendrogram of SmaI-digested DNA of penicillin-resistant, ampicillin-susceptible *E. faecalis* (PRASEF) and penicillin, ampicillin-susceptible *E. faecalis* (PSASEF). The PFGE profiles are represented by uppercase letters.

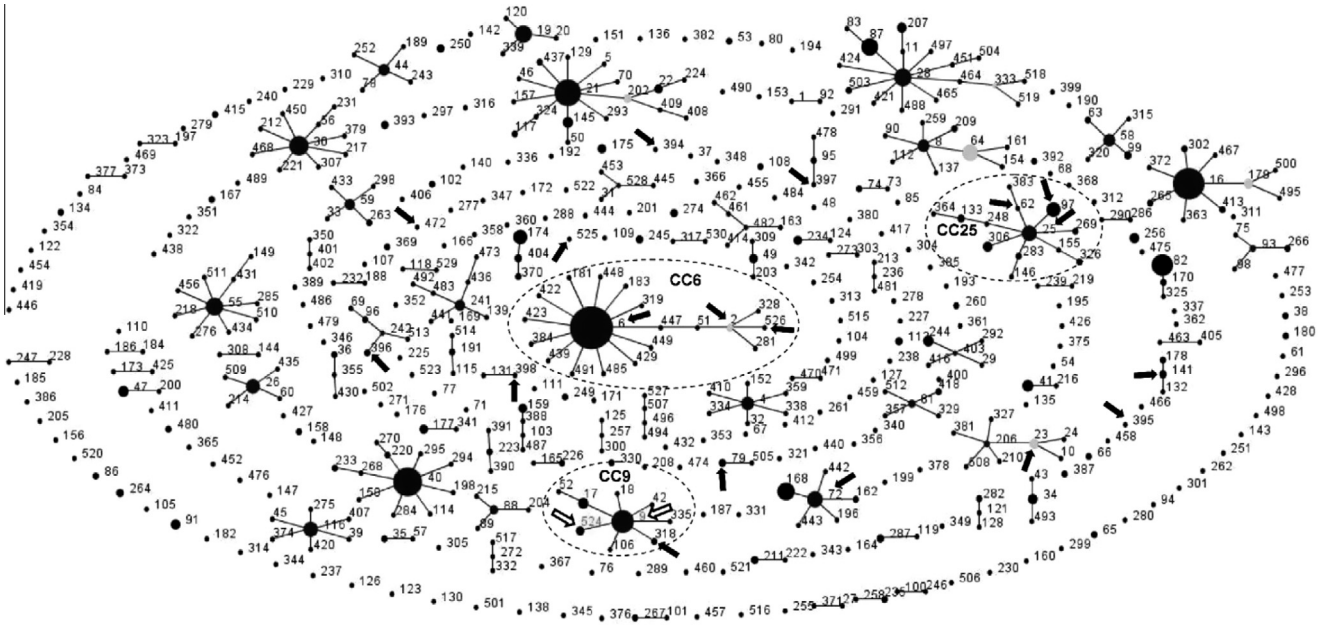


**Fig. 2.** eBURST diagram showing the analyzed sequence types (STs) of the Brazilian strains and all STs assigned to the entire public *E. faecalis* multilocus sequence type database (http://efaecalis.mlst.net/). Each ST is represented by dot sizes proportional to the number of *E. faecalis* involved considering all of the strains in the database. STs related to *E. faecalis* strains from Brazil are indicated by arrows, and white arrows indicate STs related to the strains tested in this study. The three main clonal complexes containing STs of Brazilian-related *E. faecalis* strains are indicated by broken circles.

The emergence of PRASEF isolates constitutes a challenge to current treatments of *E. faecalis* infections because the reduced susceptibility to penicillin of a majority of isolates is accompanied by resistance to other clinically useful antibiotics, such as imipenem, piperacillin and gentamicin (high-level), as demonstrated in this and previous studies (Conceição et al., 2012; Guardabassi et al., 2010; Metzidie et al., 2005). Furthermore, it is noteworthy that, although the ampicillin and amoxicillin MICs against PRASEF isolates are below the CLSI susceptible breakpoints, they are higher than the ones for PSASEF isolates. In general, the majority of penicillin-susceptible *E. faecalis* have penicillin MICs ranging from 2 to 8 μg/mL, whereas the MICs for ampicillin are one to twofold lower (Murray, 1990).

Similar to that reported in previous studies (Kuch et al., 2012; Ruiz-Garbajosa et al., 2006), the reduced susceptibility to penicillin of the PRASEF isolates from Brazil is not due to the production of β-lactamases. Indeed, the production of β-lactamase, which was first reported in 1983 in *E. faecalis* with acquired resistance to

penicillins, appears to be rare among enterococci and has been described predominantly in *E. faecalis* species (Murray, 1990).

Another resistance mechanism to β-lactam antibiotics found in enterococci, which was also assessed in this study, is the decreased affinity to the drugs due to point mutations in the gene encoding low-affinity PBPs. *E. faecalis* produces five PBPs, four high-molecular-mass and one low-molecular-mass PBPs (Duez et al., 2001). Among the high-molecular-mass PBPs, PBP4 is a low-affinity PPB and is sometimes called PBP5 (Duez et al., 2001; Zapun et al., 2008). PBP4 is closely related to other enterococcal PBPs described previously in *Enterococcus hirae* and *E. faecium* and is also related to staphylococcal PBP2 (Zapun et al., 2008). All of these proteins belong to the B class of multimodular PBPs, whose members have low affinity for β-lactams and are involved in resistance to these antibiotics (Sauvage et al., 2008). *E. faecalis* PBP4 has 680 amino acids distributed in three domains; the first comprises amino acids 1 to 39, which is the penicillin anchoring region in the cell wall; the second domain contains amino acids 40-349, and the third one, which is the penicillin-binding domain located at the C-terminal portion of the protein, involve amino acids 350 to 680 (Signoretto and Canepari, 2000). The latter domain harbors three specific catalytic motifs ($_{424}$SXXK$_{427}$, $_{482}$SDN$_{484}$ and $_{619}$KTG$_{621}$) that have been identified as the main targets of β-lactams.

The sequencing of the *pbp4* gene in the present study revealed that a single amino acid substitution (Asp-573 → Glu) located around two active-site-defining motifs (SDN and KTG) was significantly common in all PRASEF isolates but not in penicillin-susceptible clinical isolates. This amino acid substitution has never been described in *E. faecalis*, although a variety of other point mutations in low-affinity PBPs, also between motifs SDN and KTG in the penicillin-binding domain, have been associated with resistance to ampicillin and penicillin in *E. faecalis* (Ono et al., 2005) and *E. faecium* (Hsieh et al., 2006). In fact, the importance of alterations in low-affinity PBPs due to point mutations when expressing resistance to β-lactams is well illustrated in the latter species as resistance to penicillin and ampicillin but remains relatively rare in *E. faecalis.* Although little is known about the detailed functions of PBPs in *E. faecalis*, the role of PBP4 in the resistance to β-lactams is further supported by the fact that inactivation of the gene encoding this protein resulted in a significant decrease in the penicillin MIC value in the PBP4-deficient strain (Signoretto and Canepari, 2000).

Interestingly, a similar amino acid substitution (Asp → Glu) at position 520, as well as two other mutations, has been found in the PBP5 of imipenem-resistant, ampicillin-susceptible *E. faecium* isolates (Amin et al., 2001). In such isolates, the imipenem resistance was associated with the overproduction of PBP5, which exhibited a selectively decreased affinity for imipenem but unaltered affinity for ampicillin. It should be noted that PRASEFs and these *E. faecium* isolates show a similar dissociated resistance to β-lactams. This finding suggests that the amino acid alteration (Asp-573 → Glu) found in the PBP4 of PRASEF isolates may account for the decreased affinity for penicillin, imipenem and piperacillin and the unaltered affinity to ampicillin and amoxicillin. The amino acid substitution may selectively influence the binding of β-lactam antibiotics to one or both active sites of PBP4, which are close to the site of mutation. In fact, despite various kinetic studies and descriptions of several crystalline structures of PBPs in bacteria, the detailed mechanism of the interaction with β-lactam antibiotics at the molecular level remains subject to discussion. It appears quite probable that the mechanism differs between diverse PBPs and even for a single PBP and different β-lactams (Zapun et al., 2008).

One PRASEF isolate showed Asp → Glu substitutions at positions 573 and 632, but in contrast to other studies that suggest that two or more alterations within the penicillin-binding domain may result in a higher resistance to β-lactams, this isolate showed MIC values similar to those found for other PRASEFs with only one substitution (Ono et al., 2005). Two PRASEF isolates showed mutations at position 218 located outside the penicillin-binding domain of PBP, but the substitution was not associated with resistance to β-lactams in this and other studies (Ono et al., 2005; Hiraga et al., 2008).

PFGE analysis demonstrated that all but one of the PRASEF isolates that were recovered in a Brazilian hospital are closely related, which is different from the results found for the penicillin-susceptible isolates from the same hospital. Notably, eleven PRASEF isolates showing an indistinguishable PFGE profile (pulsotype A) were found throughout the four-year period of the study (2006 to 2010), characterizing an ability to adapt and persist in the hospital environment. The other five isolates, which showed closely related pulsotypes (A1, A2 and A3), were also recovered in different years. Indeed, previous studies have also reported the existence of a predominant clone among the 90 and 20 PRASEF isolates recovered from hospitals in Greece and Denmark, respectively, although a variety of other minor clones have also been found in the former hospital (Guardabassi et al., 2010; Metzidie et al., 2005).

Corroborating the PFGE data, the MLST analysis of representative PRASEF isolates from Brazil showed that they belong to a single clonal group, the CC9, comprising sequence types ST9 and the new ST524. The single PRASEF isolate with a different PFGE pattern (pulsotype B) was also shown by MLST to belong to CC9 and ST9, suggesting that the Brazilian PRASEF isolates share a common evolutionary history. A perfect correlation between PFGE and MLST is not always observed for *E. faecalis* and other bacterial species, although the ability of both techniques to discriminate clones is quite similar, as demonstrated in the present study (Ruiz-Garbajosa et al., 2006).

However, the current *E. faecalis* MLST scheme is highly reproducible and allows the study of long-term epidemiology and global comparisons of easily storable and exchangeable data (Ruiz-Garbajosa et al., 2006). In contrast to PRASEF isolates from Brazil that belong to CC9, most PRASEF isolates (17 out of 20; 85.0%) from Denmark have ST6 grouped into CC6 (formerly CC2, here reported as CC6 for the first time due to ST6, the initiatory member of the clonal complex) (Guardabassi et al., 2010). However, it is noteworthy that both CC9 and CC6 represent globally dispersed hospital-related lineages found in several countries and are characterized as multi-resistant high-risk enterococcal clonal complexes similar to well-known CC17 of *E. faecium* (Ruiz-Garbajosa et al., 2006; Kuch et al., 2012; Kawalec et al., 2007). This is the first report of Brazilian *E. faecalis* belonging to CC9, whereas other clinical isolates recovered from hospitalized patients have been shown to belong to CC6 (Penas et al., 2013).

In conclusion, it appears quite likely that the amino acid alteration (Asp-573 → Glu) located between the active-site-defining motifs SDN and KTG in the PBP4 of Brazilian PRASEF isolates may account for the reduced susceptibility to penicillin, although other resistance mechanisms remain to be investigated. Moreover, further studies are warranted to confirm whether this point mutation is also found in PRASEF isolates from other geographically located sites. In addition, it has been demonstrated that Brazilian PRASEF isolates are genetically related and belong to a single globally dispersed clonal complex, CC9, which characteristically comprises *E. faecalis* hospital-derived strains with a higher potential for accumulating antibiotic resistance and virulence traits. Therefore, it is necessary to monitor and prevent the further spread of penicillin-resistant *E. faecalis* in hospitals in Brazil and other countries.

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