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Phenotypic and genotypic identification of enterococci isolated from canals of root-filled teeth with periapical lesions

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The objectives of the present study were to identify enterococcal species isolated from the canals of root-filled teeth with periapical lesions using biochemical and molecular techniques, and to investigate the genetic diversity of the isolates. Twenty-two *Enterococcus* strains, isolated from the canals of root-filled teeth with persisting periapical lesions, were identified to species level using rapid ID 32 STREP galleries and partial 16S rDNA sequencing. To subtype the strains, genomic DNA from the isolates was analyzed by pulsed field gel electrophoresis (PFGE) after digestion with *Sma*I. Intragenic regions of two genes, *ace* and *salA*, were sequenced for further differentiation of the isolates. All strains were identified as *Enterococcus faecalis* by both commercial kit and partial 16S rDNA sequencing. PFGE with *Sma*I of 22 isolates demonstrated 18 macrorestriction profiles, whereas 13 distinct genotypes were identified after analysis of the *ace* and *salA* composite sequences. Most of the isolates from distinct patients had different PFGE profiles. Moreover, in two cases, different *E. faecalis* strains were found in different root-filled teeth from the same mouth. *E. faecalis* was the only enterococcal species isolated from the canals of root-filled teeth with periapical lesions. Genetic heterogeneity was observed among the *E. faecalis* isolates following PFGE and sequence-based typing method. Furthermore, the genetic diversity within root canal strains was similar to previous reports regarding *E. faecalis* isolates from different clinical and geographic origins.

Key words: bacterial typing; *Enterococcus faecalis*; genotype; root canal infections

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Enterococci are bile-tolerant, facultatively anaerobic, chaining gram-positive cocci that are common inhabitants of the human gastrointestinal and genitourinary tracts (19). Enterococci are also able to colonize a variety of other sites, including the oral cavity (36), where they have been associated with oral mucosal lesions in immunocompromised patients (43), periodontitis (28) and root canal infections (16, 25–27, 38). Of the enterococcal species associated with colonization and infection in humans, *Enterococcus faecalis* is the most common (18, 19).

Clinical studies have shown that *E. faecalis* is also the bacterial species most commonly isolated from the root canals of teeth with failed endodontic treatment. In such cases, their prevalence ranges from 23% to 77% of the infected root canals (3, 4, 11, 12, 16, 17, 25–27, 31, 34, 38). Virulence factors that may contribute to the high prevalence of *E. faecalis* in persistent endodontic infections have been studied. Several articles have described their resistance to intra-canal medication (5, 9, 35) and their capacity to invade dentinal tubules (9, 13). Recently, it has been demonstrated that *E. faecalis* has the capacity to endure prolonged nutrient limitation and to recover if serum is available (6). In addition, strain-specific variations of survival in nutrient-limited media were found (6). It is possible that other

virulence factors will also be strain-dependent. However, data regarding the nature of which *E. faecalis* strains are present in root canals with failed endodontic treatment are scarce.

Among the enterococcal molecular typing methods, pulsed field gel electrophoresis (PFGE) has been widely used in molecular epidemiological typing of enterococci from nosocomial infections (15, 23, 39). Moreover, it has been used as a reference method when different techniques are tested for typing enterococcal strains (15, 21, 39). PFGE allows the separation of large fragments (>50 kilobases) of a bacterial chromosome digested by rare-cutting (i.e. they cut in only a few places) restriction enzymes; such large fragments are impossible to separate by conventional gel electrophoresis (37). Nevertheless, the results obtained by PFGE are not readily transferable, making it difficult to compare results from different laboratories.

To overcome this problem, multi-locus sequence typing (MLST) has been proposed as a nucleotide sequence-based approach to the identification of pathogenic microorganisms (14). MLST characterizes isolates using intragenic sequences of multiple genes or loci that are between ~ 400 and 500 base pairs (bp) in length. The advantage of this technique is the unambiguity and portability of sequence data, which allow results from different laboratories to be compared using the internet (14, 42). Nallapareddy et al. (21) have suggested the use of MLST for differentiating isolates of *E. faecalis* based on the sequence of four genes: *ace*, *salA*, *efaA* and *pyrC*. They have demonstrated that this method is comparable to PFGE typing when differentiating outbreak isolates. Moreover, they have concluded that DNA sequencing of *ace* and *sal*A gene fragments is sufficient for distinguishing the isolates studied.

Therefore, the objectives of the present study were to identify *Enterococcus* species isolated from the canals of root-filled teeth with periapical lesions and to investigate the genetic diversity of the isolates by PFGE and by DNA sequencing of the *ace* and *sal*A genes.

Materials and methods

Clinical material

A total of 22 enterococcal strains were isolated from the canals of teeth with failed root canal treatment. The root canal samples were taken from 41 teeth in 35 patients who attended the Piracicaba Dental School, São Paulo, Brazil needing non-surgical endodontic re-treatment. Failure of root canal treatment was determined on the basis of clinical and radiographical examinations. All previously root-filled teeth showed radiographic evidence of apical periodontitis. Most of the teeth (92.7%) had been root-canal-treated more than 4 years previously; in three cases the teeth had been root-filled more than 2 years previously and the patients presented with persistent symptoms and/or discomfort to percussion.

Twenty-two teeth from 18 patients harbored enterococcal strains. Among the 18 subjects, 16 had only one tooth treated and each provided one isolate. The enterococcal strains were named by the letter E, followed by the patient number (1–18). Two patients had more than one tooth treated, providing four (E5.11, E5.12, E5.13 and E5.21) and two strains (E6.12 and E6.22) each. In the latter cases, strains were named by the patient number followed by the tooth number.

Isolation and phenotypic identification

The sampling procedure, microbial isolation and species determination were performed as previously described (7, 8, 26, 27). All coronal restorations, posts and carious defects were removed. After access cavity preparation, the teeth were individually isolated from the oral cavity with a rubber dam, and disinfection was carried out using firstly 30% hydrogen peroxide and then 2.5% sodium hypochlorite. The sterility of the operation field was checked after inactivation of the solution with 5% sodium thiosulfate. Aseptic techniques were used throughout endodontic therapy and sample acquisition. The root filling was removed using Gates Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland) and endodontic files without the use of chemical solvents. Irrigation with sterile saline solution was performed to remove any remaining materials and to moisten the canal before sample collection. For microbial sampling, a sterile paper point was introduced into the full length of the canal (as determined with a preoperative radiograph), and kept in place for 60 s. The paper point samples from the root canals were transferred to a VMGA III transport medium (Viability Medium Götenberg Agar) (2, 17).

The samples were inoculated onto non-selective blood agar plates and incubated in aerobic and anaerobic conditions. After incubation, each plate was examined and the different colony types were subcultured onto plates to obtain pure cultures. Enterococcal identification was performed using colonial morphology, oxygen tolerance, Gram-staining characteristics, and rapid ID 32 STREP (Bio Merieux, Marcy-l'Etoile, France).

Genotypic identification

Partial 16S rDNA gene sequencing

All enterococcal strains were subjected to partial 16S rDNA sequencing to confirm their identity as determined by rapid ID 32 STREP. For chromosomal DNA isolation, Wizard Genomic DNA solutions (Promega, Madison, WI, USA) were used with the modifications described by Ulrich & Hughes (41). Polymerase chain reactions (PCR) were performed in a total volume of 50 µl containing 5 U *Taq* DNA polymerase (Bioline, London, UK), 5 µl of 10 × PCR buffer plus 3 mM $MgCl_2$ (provided with *Taq* DNA polymerase), 10 µl Q-Solution (Qiagen, Crawley, UK), 0.2 mM concentrations of combined deoxynucleoside triphosphates (Bioline, London, UK), and 0.25 µM concentrations of each primer. PCR and sequence reactions were performed with the general bacterial primers RE-RTU3 and RE-TPU1 (Invitrogen, Paisley, UK) (30). The PCR conditions were as follow: 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 5 min. The reactions were performed on a Perkin Elmer GeneAmp PCR system 2400 thermal cycler (Perkin Elmer, Wellesley, MA, USA). Electrophoresis of PCR products confirmed the presence of a 500-bp fragment, which was cleaned using a QIAquick PCR Purification Kit (Qiagen). The amount of DNA was estimated visually by gel electrophoresis using a quantitative DNA Ladder (Hyperladder IV; Bioline). Sequence reactions were performed in a 20-µl volume with 3.2 pmol primer, 20 ng template and 4 µl BigDye Terminator Cycle Sequencing Ready Reaction mix (version 1.1; Applied Biosystems, Foster City, CA). The product was ethanol-precipitated, dried, and analyzed with ABI Prism 377 DNA sequencer.

PFGE

Chromosomal DNA was prepared in agarose blocks and was cleaved with *Sma*I by the method of Turabelidze et al. (40). Bacteria from overnight cultures were

harvested and washed twice with cell suspension buffer (100 mM Tris–HCl, pH 8.0, and 100 mM EDTA), and the suspensions were diluted to a final optical density at 610 nm of 3.7–4.0 (ca. 2.5×10^9 colony-forming units/ml). Aliquots (0.2 ml) of the suspensions were treated with lysis solution [50 mM Tris–HCl (pH 8.0), 50 mM EDTA, mutanolysin (1250 U/ml), lysozyme (2.5 mg/ml), and proteinase K (1.5 mg/ml)] for 10 min at 37°C. An equal volume of 1.2% molten Incert agarose (FMC BioProducts, Rockland, ME, USA) containing 1% sodium dodecyl sulfate was added to the suspension, and the mixtures were poured into a CHEF Disposable Plug Mold (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to solidify at 4°C for 10 min. The proteolysis was performed with 0.5 M EDTA, 1% sarcosyl and 400 μg proteinase K/ml; for 2 h at 55°C. The plugs were washed three times in H_2O, 50°C, for 10 min each; and three times in TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA), 50°C, for 10 min each. Digestion was performed with 30 U of *Sma*I (New England Biolabs, Hitchin, UK) for 2 h at 30°C, with a preincubation step of 10 min at 30°C.

After digestion, one-third of the agarose plug was loaded into the wells of 1.2% agarose gels (SeaKem Gold agarose; Cambrex Bio Science, Workingham, UK). The gels were processed by using the contour homogeneous electric fields device (CHEF-DR III) from Bio-Rad; the pulse time was increased from 5 to 35 s over 30 h at 6 V/cm (200 V), as suggested by Murray et al. (20). Concatenated bacteriophage λ DNA (New England Biolabs) was used to provide molecular size markers. Gels were then subjected to staining with ethidium bromide followed by destaining in distilled water and were then photographed under UV illumination.

DNA sequencing of ace and salA genes

To further differentiate *E. faecalis* isolates, intragenic regions of two antigen-encoding genes (*ace*, which encodes a collagen and laminin adhesin protein; and *salA*, which encodes a cell-wall associated antigen) were sequenced as described by Nallapareddy et al. (21). The PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 s, and a final extension of 72°C for 7 min. The PCR amplicons were purified and sequencing was performed using BigDye Terminator Cycle Sequencing Ready Reaction mix as described above.

Data analysis

Partial 16S rDNA gene sequencing

The sequence data were examined using the program Chromas version 1.45 (Griffith University, Qld, Australia), and then analyzed using the blast software of the National Center for Biotechnology Information for species determination.

PFGE

The macrorestriction fingerprints generated by PFGE were analyzed on Windows, version 5.0, Phoretix 1D Advanced software (Nonlinear Dynamics, Newcastle upon Tyne, UK). For the computer-assisted analysis, the gel photographs were scanned and saved as tiff files, which were imported into the software. An internal control strain was included in every gel in addition to the molecular size standards; this provided quality control and made the gel reproducible. A tolerance in the band position of 2% was applied during the comparison of PFGE patterns. The similarities of isolates were determined using the Dice coefficient from the PFGE binary matrix data, using SPSS for Windows, version 11.5. The Dice coefficient calculates the number of matching size fragments multiplied by two and divided by the total number of fragments (37). Matrices of similarity coefficients between all possible pairs of strains were obtained and clustered by the unweighted method with arithmetic averages (UPGMA). Dendrograms were constructed to reflect the similarities between strains in the matrix.

DNA sequencing of the ace and salA genes

The chromatograms of the *ace* and *salA* fragment sequences were exported into Gene Tool Lite software (Bio Tools Incorporated, Edmonton, Canada). The sequences of each gene were assembled, and the gene fragments *ace* (959 bp) and *salA* (919 or 922 bp because of a 3-bp in-frame deletion in some isolates) were spliced together to obtain a concatenated DNA sequence for each isolate (1881 or 1878 bp). When calculating the percentage of identity or divergence, in-frame insertions or deletions were not taken into account. The concatenated DNA sequences of the 22 isolates were aligned (pileup program), a distance matrix was calculated with no corrections (distances program), and the tree was generated by UPGMA (growtree program). All programs were computed using the Genetics Computer Group Wisconsin Package (Madison, WI, USA). Trees were then visualized using Treeview (24).

To correlate the sequence data of the 22 isolates studied with the data that were previously reported, the sequences of each gene fragment were initially compared to the corresponding sequences in *E. faecalis* strain OG1RF; and each gene sequence that differed by one or more nucleotides was considered to be a different allele (21). Nallapareddy et al. reported nine alleles each for genes *ace* and *salA*, which were represented by the letters A to I. In the present study, the alleles that were identical to those previously described were given the same letter; and the different alleles were given subsequent letters. Moreover, the concatenated DNA sequences of the isolates studied were compared directly with the concatenated DNA sequences (of the *ace* and *salA* fragments) of the isolates reported by Nallapareddy et al. (21). All sequences were aligned and a distance matrix was calculated as described above.

Results

Phenotypic identification

Microorganisms were recovered from 35 root canals examined after root filling removal; six root canals had no cultivable bacteria. Enterococcal strains were found in 22 (62.8%) of the 35 canals with positive culture. All enterococcal isolates were identified as *E. faecalis* by commercial kits, with high probabilities of correct identification (>90%). The biochemical patterns of the isolates were very similar. All strains showed positive results for the following tests: pyrrolidonyl-β-naphthylamide, esculine and arginine. All the isolates produced acid from sorbitol and mannose, but did not produce acid from L-arabinose and raffinose. The results were also negative for the enzymes: α-galactosidase, β-glucosidase and β-galactosidase.

In 15 samples, *E. faecalis* was present as the only isolate; in two samples, it was associated with one other bacterial species; and in five samples, *E. faecalis* was present in polymicrobial infections of three or more species per canal (Table 1).

Genotypic identification

Partial 16S rDNA gene sequencing

Molecular identification by 16S rDNA sequencing confirmed the phenotypic results. All strains were identified at the species level based on the *E. faecalis* V583 genome sequence (ref. NC 004668.1), showing 100% identity.

Table 1. Accompanying microflora isolated together with *Enterococcus faecalis* strains E1–E18

Strain	Accompanying microflora
E1	*Propionibacterium acnes*
E2	*Prevotella buccae*, *Peptostreptococcus micros*, *Lactobacillus acidophilus*
E3	*Prevotella intermedia*, *Prevotella melaninogenica*, *Prevotella corporis*, *Fusobacterium necrophorum*, *Peptostreptococcus prevotii*, *Peptostreptococcus magnus*, *Streptococcus constellatus*, *Staphylococcus lentus*
E4	Pure culture
E5.11	Pure culture
E5.12	*P. prevotii*, *Streptococcus sanguis*
E5.13	Pure culture
E5.21	Pure culture
E6.12	Pure culture
E6.22	Pure culture
E7	Pure culture
E8	Pure culture
E9	*P. buccae*, *P. micros*, *P. prevotii*, *Fusobacterium nucleatum*
E10	Pure culture
E11	*Propionibacterium propionicum*, *Veillonella* spp., *Actinomyces naeslundii*, *Candida* spp.
E12	Pure culture
E13	Pure culture
E14	Pure culture
E15	Pure culture
E16	Pure culture
E17	*P. intermedia*
E18	Pure culture

PFGE

Pulsed field gel electrophoresis with *Sma*I of 22 *E. faecalis* isolates demonstrated 18 macrorestriction profiles. The genetic relatedness of all isolates ranged from 20% to 100%. Most of the strains isolated from distinct patients had different PFGE profiles (Figs 1 and 2). However, strains E2, E3 and E5.13, isolated from different subjects, had identical restriction patterns. Likewise, strain E8, from one patient, showed a similar profile to strains E5.11 and E5.12, both isolated from another patient. On the other hand, even though E5.11 and E5.12 had identical patterns, isolates E5.13 and E5.21, from the same patient, had different profiles (Fig. 2). Similarly, another patient harbored different *E. faecalis* strains in different root-filled teeth (E6.12 and E6.22).

DNA sequencing of ace and salA genes

The composite sequence-based analysis of the 22 isolates identified 13 different genotypes. The values for % similarity for the 22 composite sequences were found to be between 99.3% and 100% (Fig. 1). The numbers of variable nucleotide sites were 34 and 24 in *ace* and *salA* gene fragments, respectively. Fourteen variable nucleotide sites were newly identified in this study (Fig. 3A); the others had been previously reported (21). Nine and eleven alleles were identified in *ace* and *salA*, respectively; the alleles that differed by a single nucleotide variation were nominated as SNV alleles (Table 2).

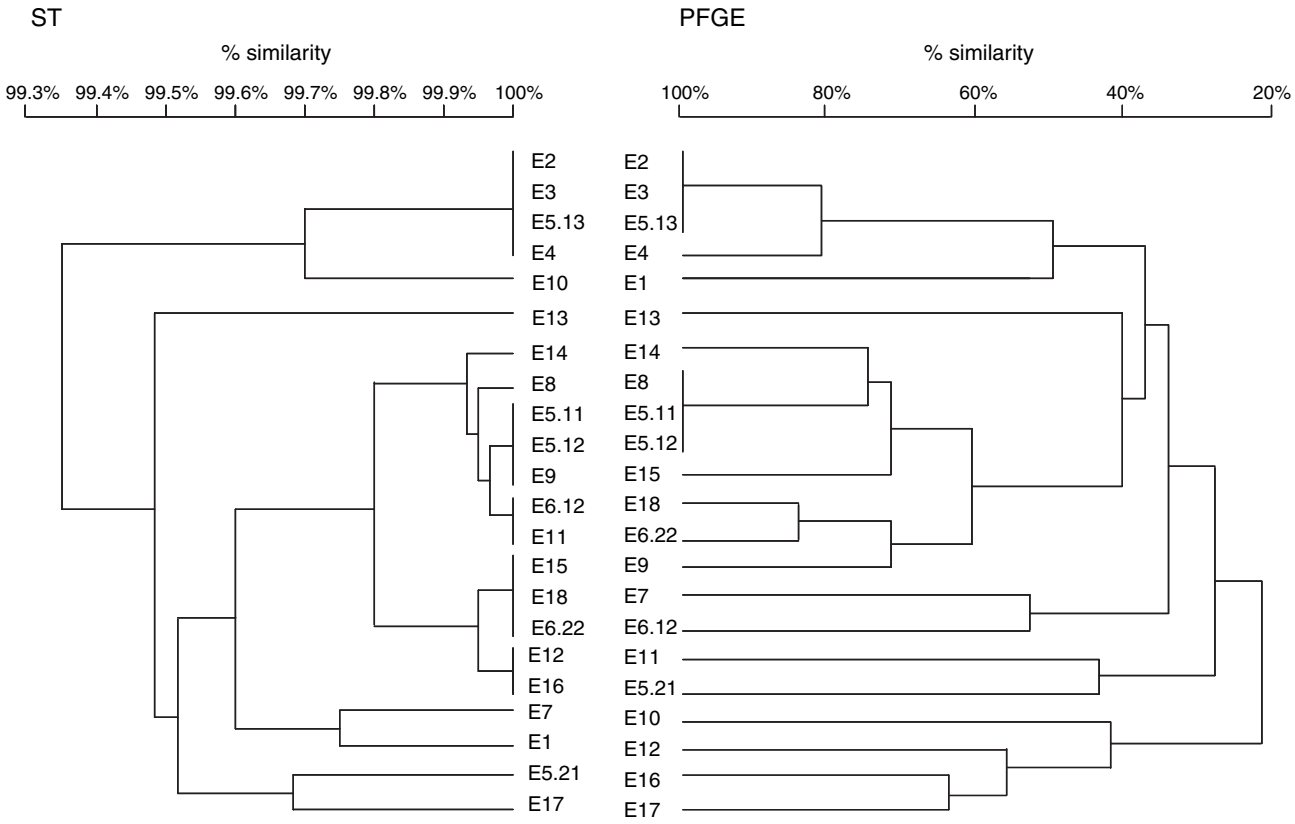

Fig. 1. Dendrograms showing similarity of 22 *Enterococcus faecalis* strains constructed with the UPGMA clustering method. Sequencing typing (ST) tree shows similarity of the concatenated DNA sequences of the *ace* and *salA* genes, whereas PFGE tree shows the *Sma*I pattern similarity of the strains.

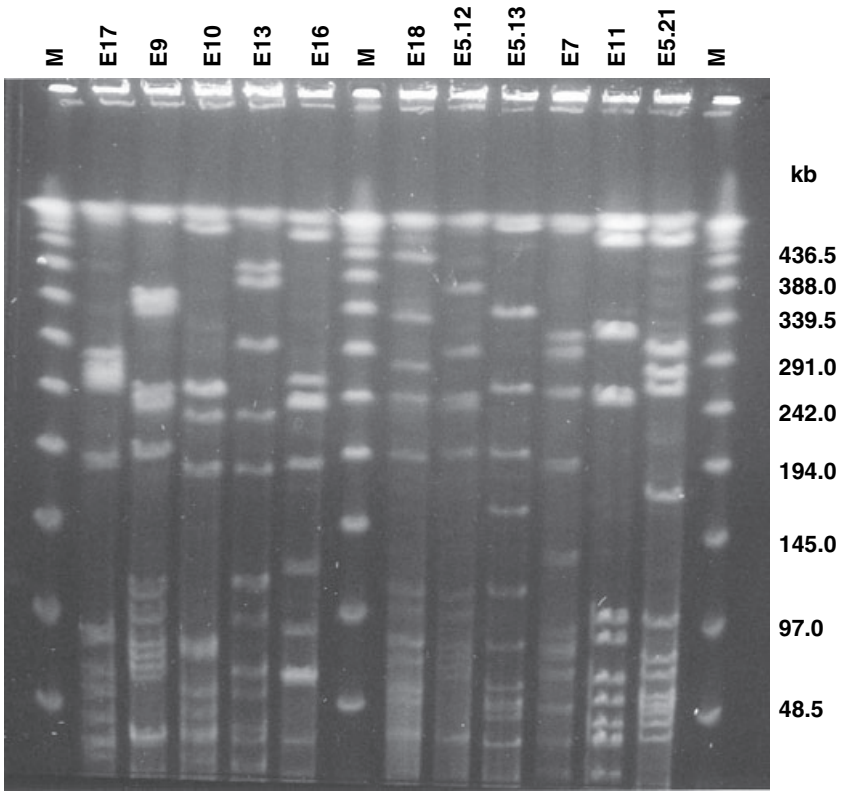

Fig. 2. Pulsed field gel electrophoresis of *Sma*I restriction fragments from *Enterococcus faecalis* strains isolated from infected root canals. M represents the molecular size marker (bacteriophage λ ladder); the sizes of which are shown in kilobases on the right. Strains E17, E9, E10, E13, E16, E18, E7 and E11, isolates from different patients; and strains E5.12, E5.13 and E5.2, isolates from the same patient, show different PFGE patterns.

The variations that did not change the amino acid sequence were indicated as synonymous base substitutions, and those that changed the amino acid sequence were indicated as non-synonymous base substitutions (Fig. 3; Table 2). To compare the isolates in this study with those reported by Nallapareddy et al. (21), a distance matrix of the composite sequences, including isolates from both studies, was created. The strains clustered together despite their clinical and geographical sources. Genetic clusters containing predominantly root canal isolates or extra-oral isolates were not identified by the dendrogram.

Discussion

Commercial identification kits are often used by clinical laboratories to identify enterococcal species from diverse sources of human infections, including the oral cavity (33, 36) and root canal infections (8, 26, 27). However, research has shown discordance between the results of the commercial kits and molecular identification methods, especially in cases of atypical enterococcal strains (1). Therefore, in the present study, the results of the biochemical identification performed with commercial kits were compared to the results of 16S rDNA sequencing. All enterococcal isolates were identified as *E. faecalis* by both commercial kits and 16S rDNA sequencing. This result further confirmed previous findings that had identified *E. faecalis* as the most common enterococcal species isolated from the canals of root-filled teeth with persisting periapical lesions; other enterococci are rarely found (3, 4, 11, 12, 16, 17, 25–27, 31, 34, 38).

The results of PFGE and sequenced-based typing methods demonstrated genetic diversity of the strains studied; no specific *E. faecalis* strains were involved in infections of root-filled teeth with periapical lesions. Similar findings have been reported for *Candida albicans* strains in root canals with persistent infections, showing that the root canal as an ecological environment, even after the use of antiseptic solutions and medicaments, may not have an impact on strain selection (44).

In the present study, genetic diversity was found among the strains despite the fact that they had been isolated from a single clinical source and a single geographic location. The genetic heterogeneity observed in the present study is in accordance with previous reports on root canal infections (33) and extra-oral *E. faecalis* isolates from different geographic locales (21).

Pulsed field gel electrophoresis has been considered to be an excellent method for typing *E. faecalis* strains, mainly because of its reproducibility and discriminatory power (15). This high level of discrimination was also observed in the present study, with 81.8% (18 out 22) of the strains yielding distinct PFGE patterns.

Most of the patients had only one tooth treated and provided one isolate each, with unique PFGE profiles in the majority of cases. However, different patients yielded strains with similar macrorestriction profile in some cases. This may have occurred as a result of a limitation of the PFGE technique, which only detects variations that take place in the cleavage sites of the restriction enzyme. In this study, we used the enzyme *Sma*I, as recommended by previous studies (20, 40). The use of an additional restriction enzyme with a different recognition sequence could have provided more information about the genome and may have improved the discriminatory power of the technique.

Two patients had different teeth needing root canal re-treatment: one patient yielded four root-filled teeth with periapical lesions and the other had two. Although similarity in the bacterial species isolated from root canals of different teeth in the same patient has already been demonstrated (7), diversity within the *E. faecalis* species was found in this study. Most *E. faecalis* isolates from different teeth in the same patient were differentiated by the molecular typing methods used. It has been considered that the oral cavity is the main source of microorganisms found in infected dental root canals. The presence of different strains of *E. faecalis* in different teeth in the same patient may suggest that a single individual can harbor different *E. faecalis* strains in different niches of the oral cavity. However, the number of patients with more than one root canal treatment in this study was very limited. Future studies, investigating multiple isolates from both oral cavity and root canals, are needed to establish whether or not a subject would yield different *E. faecalis* strains in such habitats.

A

ace

	1 1 1 1 1 2 2 **2** 3 3 **3** **4** 6 6 6 6 6 6 7 7 7 7 7 8 8 8 **8** 9 9
	3 4 7 9 2 2 2 5 5 2 5 **8** 0 1 **1** **9** 4 4 5 5 6 7 7 2 3 3 5 9 4 4 6 **9** 2 3
	6 8 5 6 1 3 7 1 3 8 8 **2** 3 2 **5** **8** 2 5 4 7 3 7 9 9 5 8 0 8 6 9 1 **0** 1 8
Allele A	A A A A G C C G T C T **A** G C **G** **A** C G T C T G A A A C A C A C C **A** T C
Allele C	. . . . . A . . . . T T . . . A . . C . G . . . .
Allele F	G C . . A T T . C T . . . . . T . C . . A G . . . . T . A .
Allele J	G C . . A T T . C T . **G** . . . . T . C . . A G . . . . T . A .
Allele K	. . . . A T T . C . C . . T . . . A . T . . G . . . . T . . .
Allele L	G C G C A T T . C T . . . T **A** **T** . . . . . G G G G G . . . . . .
Allele M	G . G . A T T . C . . . . T **A** . . A . . . . G . . . . . C T . **G** A .
Allele N	G . G . A T T . C . . . . T **A** . . A . . . . G . G T G A . . . . **.** A .
Allele O	. . . C A T T . C T . . . T . . . A . . . . G G . . . . **.** A T
	S S S N S S N S S S S S S S S S S S S N N S S S S N N S S S N N

salA

	3 3 **4** 4 4 **4** 4 **5** 5 **5** 6 **6** **7** 7 7 **7** 7 7 **7** **7** 8 8 8 8
	7 7 **0** 4 6 **8** 8 **1** 5 **7** 3 **5** **1** 2 4 **5** 5 6 **7** **8** 7 7 7 8
	5 8 **8** 1 8 **1** 9 **6** 2 **3** 9 **2** **2** 4 1 **1** 9 2 **9** **8** 7 8 9 0
Allele A	**C** C **C** T A **T** C **G** G **G** C **G** **G** C C **G** A C **C** **T** * * * G
Allele H	**.** T **.** . . **.** T **.** A **.** T **.** **.** A . **.** C . **.** **.** G T G .
Allele J	**.** . **.** . . **.** T **.** A **.** . **.** **.** . . **.** T T **.** **.** * * * .
Allele K	**.** . **.** . . **.** . **.** A **.** . **.** **.** . . **.** T T **.** **.** * * * .
Allele L	**.** . **.** . . **.** T **.** A **.** . **A** **.** . . **.** T T **.** **.** * * * .
Allele M	**.** . **.** . . **.** T **.** A **.** . **.** **.** . . **.** T T **G** **.** * * * .
Allele N	**.** T **T** . . **.** T **.** A **.** . **.** **.** A . **.** T T **.** **C** * * * .
Allele O	**.** . **.** A C **.** . **A** A **T** . . **A** A . **.** T . **.** **.** * * * .
Allele P	**.** . **.** . C **.** . **.** . **.** . **.** **.** A . **.** T . **.** **.** * * * .
Allele Q	**.** . **.** . . **C** . **.** . **.** . **.** **.** . . **.** . **.** **C** G T G .
Alllele R	**T** . **.** A C **.** . **.** A . **.** . **.** **.** . A **T** . **.** **.** G T G A
	S S S S S S S S S S S N N N S N S S N N * * * ***N***

B

Isolates	Allelic profile for *ace* and *salA*
E1	O, A
E2	C, R
E3	C, R
E4	C, R
E5.13	C, R
E5.11	F, M
E5.12	F, M
E5.21	M, P
E6.12	F, J
E6.22	F, A
E7	K, Q
E8	F, K
E9	F, M
E10	C, H
E11	F, J
E12	F, Q
E13	L, N
E14	J, L
E15	F, A
E16	F, Q
E17	N, O
E18	F, A

Fig. 3. (A) Variable nucleotide sites and alleles identified in *ace* and *salA* genes. The numbers in the vertical format represent the position of the variable nucleotides within the sequenced fragments. The numbers in bold represent the nucleotide variable sites newly identified in this study; the others were reported previously (18). The nucleotide sites that are identical in all the alleles are not shown. Allele A shows nucleotides of *Enterococcus faecalis* OG1RF (18). Alleles A, C and F (*ace*), and alleles A and H (*salA*), found in this study, were identical to those previously reported (18), whereas alleles J to O (*ace*) and J to R (*sal A*) were different. Whether the variations are synonymous (*S*) or non-synonymous (*N*) base substitutions is also shown. (B) The allelic profile for *ace* and *salA* genes of the isolates studied.

Table 2. Genetic variation in genes *ace* and *salA* of 22 *Enterococcus faecalis* isolates from root canals

Gene	Fragment size (bp)	No.of alleles	No. of variable sites	No. of synonymous base substitutions	SNV alleles
ace	959	9	34	26	F, J
salA	919, 922	11	22	14	J, K; J, L; J, M

Single nucleotide variation (SNV) are alleles that differ by a single nucleotide.

In this study, *E. faecalis* strains were also differentiated by the sequence typing of two genes, *ace* and *salA*. Thirteen different types were identified among the 22 isolates studied. In general, similarities in the patterns of *Sma*I fragments were observed among the isolates that were identical or appeared to be relatively closely related on the tree constructed from the sequence similarity data (namely, strains E2, E3, E5.13 and E4; E14, E8, E5.11 and E5.12; and E18 and E22) (Fig. 1). However, sequence-based typing and PFGE results were incongruent in two pairs of isolates (E6.12 and E11; E16 and E12). The latter were indistinguishable by the sequence of *ace* and *salA* genes, and were not closely related based on PFGE analysis. These differences may be the result of DNA rearrangements causing the PFGE patterns to change. It has been shown that the restriction fragment patterns observed in *E. faecalis* isolates are more frequently the result of DNA rearrangements than of point mutations (10). Furthermore, in the present study, whereas PFGE examined the entire genome, the sequence-based typing method only analyzed nucleotides within two genes, *ace* and *salA*. It is possible that the number of variable nucleotide sites within these genes was very limited to provide further differentiation of the strains studied. In addition, the nucleotide variations responsible for strain differentiation may be found outside the two gene fragments analyzed in this study. The fact that the results of different typing methods may not always be congruent has been previously reported in studies of different microorganisms (22, 29, 32).

Pulsed field gel electrophoresis has proved to have utility as a typing method for *E. faecalis*; however, it has some limitations (15). Although technical protocols have been used for optimization (40), the technique remains time-consuming. Moreover, without the aid of digital analysis, visual comparison of a great number of samples may be difficult (39). In addition, the results obtained by PFGE are not readily transportable and comparable among laboratories. On the other hand, such comparisons could be achieved by using sequence-based analysis because of the unambiguity and portability of the data (14, 42).

In this study, the results of DNA sequencing of *ace* and *salA* gene

fragments of 22 *E. faecalis* isolates were compared with those from Nallapareddy et al. (21), who also studied a total of 22 strains. The isolates studied were found to be less heterogeneous than in the previous report; the numbers of variable nucleotide sites in *ace* and *salA* fragments were 34 and 24, respectively, compared with 54 and 21 reported by Nallapareddy et al. (21). Note that the latter study examined isolates from different clinical sources (blood, urine, foot ulcer, sputum, gastric fluid, subdiaphragmatic abscess and catheter); and also different countries (USA, Thailand, Spain, Argentina and Lebanon). Therefore, more variation between the strains would be expected in that study than we found in the present one, where the isolates were all from dental root canals in a Brazilian population. When comparing the concatenated DNA sequence of the genes *ace* and *salA* of the strains analyzed in both studies, on the basis of clinical and geographical sources, no distinct clustering was observed. Strains from dental root canals and extra-oral sources of different countries clustered together.

In conclusion, all the enterococcal strains isolated from the canals of root-filled teeth with persisting periapical lesions were identified as *E. faecalis*. Genetic heterogeneity was observed among the *E. faecalis* isolates from dental root canals in a Brazilian population, which is in accordance with previous reports from oral and extra-oral isolates in different countries. PFGE and sequence-based typing methods proved to be useful for differentiation of *E. faecalis* strains isolated from root canal infections. PFGE had higher discriminatory power, whereas the sequence-based typing method permitted a study-to-study comparison of *E. faecalis* isolates from the root canals with isolates from various body sites. Future studies should investigate the presence and similarity of *E. faecalis* strains isolated from the oral cavity and root canal infections.

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