

Molecular Typing of *Enterococcus faecalis* from Persistent Endodontic Infections

Tipificación Molecular de *Enterococcus faecalis* Provenientes de Infecciones Endodónticas Persistentes

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ABSTRACT: Molecular techniques that provide valuable information about the epidemiology of oral strains. The purpose of this study was to determine the genetic relatedness of 83 *Enterococcus faecalis* strains isolated from treated root canals. These strains were obtained from patients who were treated for persistent endodontic infections. *E. faecalis* isolates were molecularly typed by Pulsed Field Gel Electrophoresis using *Sma*I. Ten clonal groups and 13 pulse types with 38.7 % similarity for the less related strains were identified. Genetic heterogeneity among strains from different patients and a high level of genetic homogeneity among inpatient strains were observed. Therefore, restriction endonuclease fingerprinting of genomic DNA from *E. faecalis* strains confirmed the polyclonality of the isolates obtained from the root canals of patients diagnosed with persistent endodontic infections, compared with other reports. These results provide additional data for a better understanding of the epidemiological aspects of root canal infections by *E. faecalis*.

KEY WORDS: *Enterococcus faecalis*, pulsed field gel electrophoresis, apical periodontitis nonsuppurative.

INTRODUCTION

A primary goal of endodontic treatment is to eliminate microorganisms that colonize the dental pulp causing their necrosis (Atila-Pektas *et al.*, 2013) with appropriate instrumentation, irrigation and medication (Baca *et al.*, 2011; Hohscheidt *et al.*, 2013). Nevertheless, despite various therapeutic alternatives, the complete eradication of *E. faecalis* has not been possible (Rôças *et al.*, 2004; Kumar, 2013; Javidi *et al.*, 2014). This aerobic facultative Gram-positive bacterium is regarded as the main cause of persistent endodontic infection after root canal treatment, being the gold standard bacteria in endodontic microbiology studies. The microbiota that are associated with root canal treatment failure with periapical chronic periodontitis are different from those

in necrotic canals, and it is necessary to characterize their features (Vengerfeldt *et al.*, 2014). Resistance of *E. faecalis* strains has been well documented and has persisted after irrigation with disinfectants, such as sodium hypochlorite (Pladisai *et al.*, 2016). Molecular techniques that provide valuable information about the epidemiology of resistant bacterial strains, such as macrorestriction of genomic DNA and subsequent Pulsed Field Gel Electrophoresis (PFGE), are highly precise and reproducible typing methods for determining genetic relatedness among *E. faecalis* isolates (Castillo-Rojas *et al.*, 2013). That method has been used to characterize isolates obtained from clinical samples (Al-Ahmad *et al.*, 2010; Zhu *et al.*, 2010; Vidana *et al.*, 2011) and to

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type isolates obtained from root canals, providing differences in the epidemiological behavior of local strains. But there are few and very poorly detailed studies to reproduce the technique. Recently a study was published about diversity and similarity of *Enterococcus faecalis* genotype isolates from multiple oral sites using techniques, like repetitive sequence-based polymerase chain reaction and arbitrarily primed polymerase chain reaction (AP-PCR), which emphasizes the importance of typing (Delboni *et al.*, 2017). The aim of this study was to determine the genetic relatedness between the *E. faecalis* isolates from the root canals of patients who were undergoing treatment for persistent endodontic infections using a good description of PFGE, another technique to have information on this important topic.

MATERIAL AND METHOD

Bacterial Strains. The study was performed in compliance with the protocol approved by the Faculty Ethical Committee (C.I.Y.B number 08/14). Eighty-three *E. faecalis* strains isolated from root canals retreated for a diagnosis of Chronic Nonsuppurative Periapical Periodontitis were included. These strains were isolated from fourteen patients and a random non-correlative number was assigned to each patient. Clinical characteristics, strains isolation methodology and identification, at the species level by molecular methodologies, were previously published (Sánchez-Sanhueza *et al.*, 2015). Strains were stored until to molecular typing at -80 °C in a mixture of Trypticase soy broth (Oxoid Ltd., Basingstoke, Hampshire, England) and glycerol (50 % v/v) in a 2:1 ratio.

Molecular Typing. The genetic relatedness of the *E. faecalis* strains was assessed using PFGE in a CHEF-DR II apparatus (Bio-Rad, La Jolla, CA). *Salmonella enterica* subsp. *enterica* serotype Braenderup H9812 strain was used as a DNA molecular size control (kindly provided by Instituto de Salud Pública, Chile) and digested with the *Xba*I (5000 U) restriction enzyme (Roche Molecular Diagnostics, Pleasanton, CA) (Kainer *et al.*, 2007). The *Sma*I (1000 U) restriction enzyme (Roche Molecular Diagnostics, Pleasanton, CA) was used per the Unified Pulsed-Field Gel Electrophoresis (PFGE) Protocol for Gram-Positive Bacteria as described by the Centers for Disease Control and Prevention (2012). *E. faecalis* strains were grown in Columbia agar plates supplemented with 5 % bovine blood (BD, Heidelberg, Germany) and

incubated at 37 °C for 24 h under aerobic conditions. Colonies were inoculated in 3 mL of the suspension buffer (10 mM Tris; 0.1 mM EDTA; pH 8.0 for *E. faecalis* and 100 mM Tris; 100 mM EDTA; pH 8.0 for *S. Braenderup*), and the optical density at 660 nm was adjusted to 0.9-1.1 for *S. Braenderup* and to 0.6 for *E. faecalis* using an EPOCH spectrophotometer (BioTek®, USA). Subsequently, 400 µL of *E. faecalis* suspensions was transferred to a tube containing 20 µL of lysozyme (20 mg/mL) (Sigma Aldrich Chemical Co, MO, USA) and incubated for 30-45 min at 55 °C. Suspensions of *S. Braenderup* were treated with proteinase K (20 mg/mL) (Thermo Scientific, Massachusetts, USA) and incubated at 55 °C for 10 min. Then, 400 µL of 1 % Sea Kem Gold agarose (Lonza, Basilea, Suiza) in buffer TE (10 mM Tris EDTA, pH 8.0) was added to each bacterial suspension, and the suspensions were gently mixed. From each mixture, 300 µL were pipetted into plug molds. Agarose plugs were allowed to solidify at room temperature.

For cell lysis, plugs were transferred to Falcon tubes (Kima, Italy) containing 5 mL of the cell lysis buffer (50 mM Tris; 50 mM EDTA; pH 8.0; 1 % sarcosyl) and 25 µL of proteinase K (20 mg/mL) and were incubated for 2.5 h in a 54 °C shaking water bath, model 1083 (Thermolab®, Burgwedel, Germany) under continuous agitation at 150-175 rpm. Afterward, plugs were washed 3 times with 5 mL of MilliQ water at 54 °C for 10-15 min with agitation at 150-175 rpm. Three additional washes with 5 mL of TE buffer were performed, and the plugs were stored in 8 mL of TE buffer at 4 °C.

One-third of each plug was incubated with 100 µL of Tango buffer (Thermo Scientific, Massachusetts, USA) for 15 min at 25 °C for *E. faecalis* and 15 min at 37 °C for *S. Braenderup*. The predigestion solution was replaced with 100 µL of the digestion solution consisting of 88.5 µL of sterile distilled water, 10 µL of buffer Tango and 1.5 µL of *Sma*I (10 units/µL) for *E. faecalis* and 1.5 µL of *Xba*I (10 units/µL) for *S. Braenderup*. After a 4 h incubation at the optimum temperature for each enzyme, the digestion solution was removed, and plugs were loaded onto 1 % SeaKem® Gold agarose gels. The remaining plugs were stored up to 7 days in 200 µL of 0.5X Tris-borate-EDTA buffer (TBE, Thermo Scientific, Massachusetts, USA) at 4 °C.

PFGE was performed using 0.5X TBE buffer with 760 µL of the thiourea stock solution (10 mg/mL) under the following conditions: 14 °C, 6 V/cm, 19 h run-time, 3.5 to 23.5 s switch times, and 1 L/min flow rate (Ribeiro *et al.*, 2009). The gel was stained with ethidium bromide

(0.5 µg/mL) for 25 min, washed under agitation with MilliQ water for 20 min and visualized using a UV transilluminator (UVIttec system, Cambridge, UK).

Gel Analyses. An initial visual analysis of the gels (visible to the naked eye) for the 83 strains was carried out per the criteria proposed by Tenover *et al.* (1995), and a selected group of these strains were used for further analysis using the BioNumerics v.6.611 software (AppliedMaths, Inc., Austin, TX, USA) based on 2 or 3 strains for each distinct pattern. The band positions were normalized using the *S. Braenderup* strain as the standard marker in each gel. The PFGE pattern homology dendrogram was built using the Dice coefficient with a tolerance level of 1.5-2 % and with the UPGMA method, and 85 % similarity was established as the measure for classifying a group as closely related (Werner *et al.*, 2013).

RESULTS

In 6 patients (42.8 %), *E. faecalis* strains with at least two different fingerprints per the Tenover criteria, (Tenover *et al.*) were detected, including a non-genetically related strain. In 35.7 % of patients, the 6 strains were catalogued as indistinguishable, i.e., clones, and 21.4 % of the patients presented 5 clonal strains and one closely related strain (Fig. 1).

The *Sma*I digestions and PFGE of the DNA from the *E. faecalis* strains yielded 13 different fingerprints that included 14 to 16 bands, with sizes ranging from approximately 15 to 1200 kb (Fig. 2).

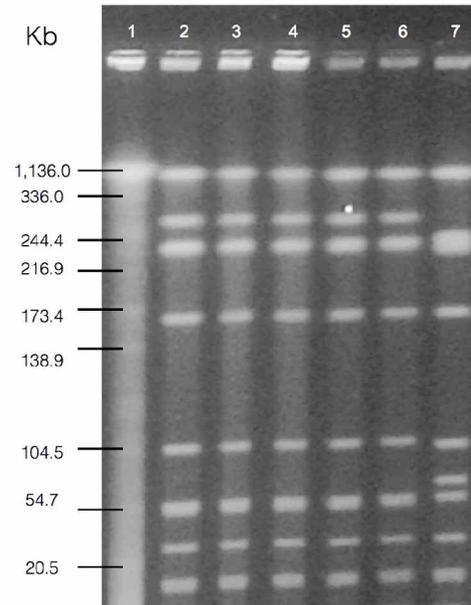


Fig. 2. Representative PFGE fingerprinting of *E. faecalis* strains isolates from a root canal in patient n° 8 diagnosed with a persistent endodontic infection. Lane 1: Gel size standard strain *Salmonella Braenderup*. Lanes 2 to 7: *E. faecalis* strains.

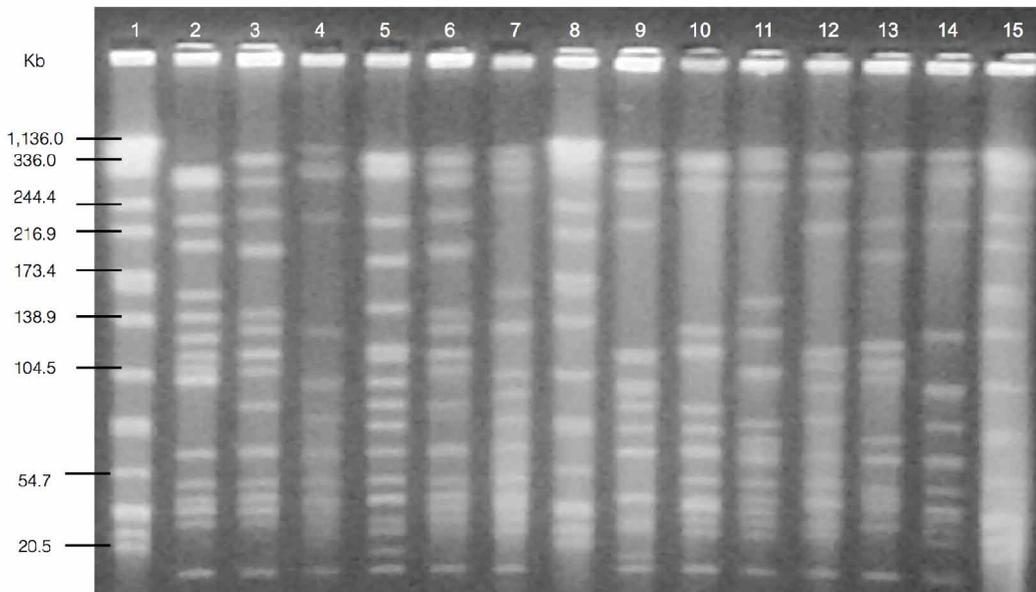


Fig. 1. Representative PFGE fingerprinting of *E. faecalis* strains isolated from root canals diagnosed with persistent endodontic infections. Lanes 1-8-15: Gel size standard strain *Salmonella Braenderup*. Lanes 2 to 7 and 9 to 14: *E. faecalis* strains.

The PFGE analysis by BioNumerics revealed 13 *Sma*I restriction patterns (referred to as pulse types) (A-M). The dendrogram (Fig. 3) was constructed with 35 *E. faecalis* strains that were selected, based on at least one strain per patient and the strains with more than four different bands, per the Tenover criteria.

Table I shows the distribution of the strains and patients for each PFGE fingerprint. Five pulse types (38.4 %) were present in 2 patients. Therefore, strains from different patients showed a high similarity (>85 %). Six pulse types (46.1 %) presented two closely related subtypes (A, I, J, K, L, M), and one of them (pattern M), with 38.7 % of similarity, separated the strains from patient 8 from the rest.

Table I. Distribution of strains and patients for each PFGE pattern.

PFGE pattern	n° of strains	n° of clonal strains	Patient
A	10	5(A1) 5(A2)	9 and 10
B	1	1(B1)	10
C	1	1(C1)	9
D	5	5(D1)	16
E	12	12(E1)	5 and 18
F	6	6(F1)	15
G	5	7(G1)	7
H	6	6(H1)	12
I	7	6(I1) 1(I2)	4 and 7
J	6	5(J1) 1(J2)	10
K	12	5(K1) 7(K2)	2 and 3
L	6	5(L1) 1(L2)	6 and 16
M	6	5(M1) 1(M2)	8

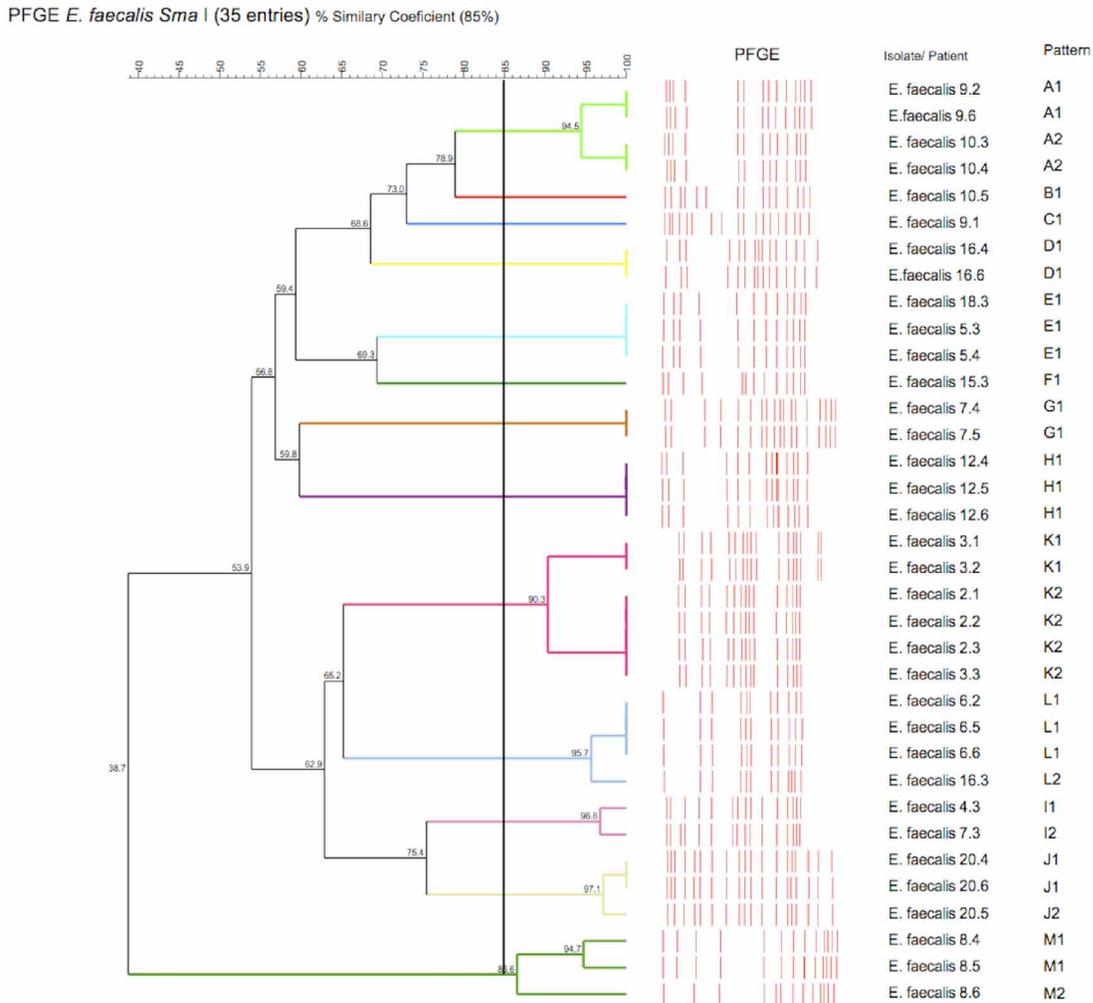


Fig. 3. Dendrogram of 35 *E. faecalis* strains isolated from the root canals of patients diagnosed with persistent endodontic infections. In the column for the isolate/patient, the first number refers to the patient, and the second refers to the strain.

DISCUSSION

Molecular typing is an important and useful tool for analyzing the epidemiology of microorganisms, and contributes to monitoring infections caused by resistant pathogens from various sources, including the oral cavity (Panesso *et al.*, 2010). Previous studies have evaluated the genetic relatedness of the *E. faecalis* strains from the oral cavity, food and other body places (Anderson *et al.*, 2016). A high genetic heterogeneity was revealed, which was a frequent finding in previous studies comparing strains from different times and places (Sedgley *et al.*, 2004; Vidana *et al.*; Castillo-Rojas *et al.*); these findings are consistent with our results. These studies support the hypothesis that molecular methods, such as PFGE, are highly valid and useful techniques for determining strain clonality. A supragingival isolate from one patient and a nosocomial infection isolate from another patient, obtained on different dates but in the same clinic, were closely related (Anderson *et al.*). This suggests that there is a possible connection in the circulation of strains in the same clinical environment. Our report describes findings of non-genetically related isolates in a single patient and indistinguishable isolates obtained from different patients on different dates. Moreover, findings of close relatedness and identical virulence genes have been reported for an endodontic strain taken from a German patient, and an isolate coming from a milk plant obtained on different dates (Anderson *et al.*). Together, these observations corroborate PFGE as a useful tool for complementing data from susceptibility assays and resistance profiles and confirm the importance of typing strains, of understanding the way they circulate in our population, and of their relationship with the presence of resistance determinants.

A high prevalence of *E. faecalis* in the root canals of teeth that are treated for persistent apical periodontitis has been shown by traditional culture and molecular methods and suggests that this species could be key to endodontic treatment failure (Zoletti *et al.*, 2011). Thus, it is important to perform microbial examinations of patients with this pathology to characterize the presence of this bacterial species. Moreover, previous studies have classified diverse isolates by PFGE and characterized virulence factors by REP-PCR to determine interindividual genetic diversity among the isolates obtained from endodontic infections, as observed in our study (Zoletti *et al.*). From 20 endodontic isolates of *E. faecalis*, 18 restriction profiles grouped in 14 different genotypes were

reported, with 50 % of isolates included in 5 genotypes (Zoletti *et al.*). In present study, which included 83 strains, revealed 19 profiles grouped in 13 different genotypes. Nevertheless, the previous analysis only used the Tenover criteria; 21.5 % of the patients presented strains with at least two different PFGE patterns (Zoletti *et al.*). A study by Zhu *et al.* showed that 62 % of the strains from different patients were closely related, and the isolates from the saliva and root canals were different in only one out of the 32 patients studied.

A study by Vidana *et al.* showed that inpatient strains from the root canals of Swedish patients were mostly identical or closely related. However, the inter patient strains were not related, differing from the present study's observation of genetic relatedness among strains from different patients. One probable cause of this difference may be that the previous study considered only a visual analysis, using the Tenover criteria, which emphasizes the importance of using Software as Bionumerics, since it offers readings more sensitive than just the use of the visual criterion. The BioNumerics software is a highly sensitive tool that is used and accepted worldwide for epidemiological studies of bacterial strain distributions that are associated with infections. It is important to note that in this study, the patients were a homogenous population, had no parental or social relationship, and only had the geographical location in which they lived in common (surface 37.068,7 km²); the only apparent differences involved the quality of the root canal filling (Sánchez-Sanhueza *et al.*). Nevertheless, it must be emphasized that to complement the information regarding the epidemiological aspects of *E. faecalis* infection in the root canal, it is necessary to isolate *E. faecalis* strains from the oral microbiota of healthy patients to establish whether the strains are genetically related to those of the root canal infection or to transient strains in the oral cavity that can colonize the root canal (Al-Ahmad *et al.*; Zhu *et al.*).

Enterococcus faecalis genotype isolates from multiple oral sites using other techniques like REP-PCR and AP-PCR, but with a similar software analysis, using the Dice coefficient, show genetic heterogeneity when compared among different subjects, corroborating the results of present study. REP-PCR shows very similar relatedness with 32.8 % similarity for the less related strains (Delboni *et al.*).

The limitations of the present findings are the lack of standardization of typing oral strains, to compare with other reports. Multiple online tools are currently available. For example, those offered by CDC PulseNet work by comparing the results of different studies and by obtaining information of the epidemiological behavior of *E. faecalis* infection worldwide, similarly to the tools used in the medical field for monitoring diverse bacteriological species. Therefore, it would be helpful to standardize the protocols for characterizing *E. faecalis* isolates of oral origins regarding the use of the size marker (lambda DNA ladder (Sedgley *et al.*), *Salmonella* Braenderup (Kainer *et al.*), *Staphylococcus aureus* (Anderson *et al.*) and software-based analysis.

Finally, *E. faecalis* American Type Culture Collection (ATCC) strains are a gold standard in endodontic research, for example to test new antimicrobials (Saatchi *et al.*, 2014; Silva *et al.*, 2014). But ATCC strains are highly susceptible, which may blind the results. Use of clinical *E. faecalis* strains and PFGE typing in microbiological research may improve knowledge in the field of endodontics.

CONCLUSION

E. faecalis strains with a diverse genetic similarity are circulating among patients treated for Persistent Endodontic Infections, including genetically related *E. faecalis* strains with >95 % similarity in different patients. This highlights the need for and importance of investigations that focus on the origins of root canal contamination with this bacterial species. PFGE is a recommended tool for typing strains from persistent intra-root canal infections, and further research is necessary to characterize the presence of resistance determinants that circulate in our population, in the different clonal types.

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RESUMEN: Las técnicas moleculares proporcionan información valiosa sobre la epidemiología de aislados orales. El propósito de este estudio fue determinar la relación genética de 83 cepas de *Enterococcus faecalis* aisladas de conductos radiculares tratados. Estas cepas se obtuvieron de pacientes que fueron tratados por infecciones endodónticas persistentes. Los aislados de *E. faecalis* se tipificaron molecularmente por electroforesis en gel de campo pulsado usando *Sma*I. Se identificaron diez grupos clonales y 13 pulsotipos con un 38,7 % de similitud para las cepas menos relacionadas. Se observó heterogeneidad genética entre las cepas de diferentes pacientes y un alto nivel de homogeneidad genética entre las cepas intrapacientes. Por lo tanto, la toma de huellas dactilares a través de restricción de ADN genómico de cepas de *E. faecalis* confirmó la policlonalidad de los aislados obtenidos de los conductos radiculares de pacientes diagnosticados con infecciones endodónticas persistentes, en comparación con otros informes. Estos resultados proporcionan datos adicionales para una mejor comprensión de los aspectos epidemiológicos de las infecciones del conducto radicular por *E. faecalis*.

PALABRAS CLAVE: *Enterococcus faecalis*, electroforesis de campo pulsado, periodontitis apical no supurativa.

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