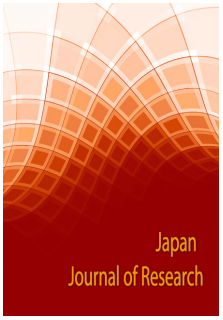
Japan Journal of Research

Original Article



Exploring Genetic Diversity in Enterococcus Faecalis Strains of Endodontic Origin: Pilot Study

Verónica D Hurtado-Narváez[2], Nidia E de León-Frías[1,3], Catalina Méndez-De la Espriella[2], Adriana Rodríguez-Ciodaro[2], Oscar E Gomez-Cárdenas[4], Hugo Díez-Ortega[1*]

[1]Grupo de Enfermedades Infecciosas, Departamento de Microbiología, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, Colombia

[2]Centro de investigaciones odontológicas, Facultad de Odontología, Pontificia Universidad Javeriana, Bogotá, Colombia.

[3]Faculty of Health Sciences, School of Dentistry, Universidad Nacional Pedro Henríquez Ureña, Distrito Nacional, República Dominicana.

[4]Departamento de Ciencias Fisiológicas, Facultad de Medicina, Universidad Javeriana, Facultad de Medicina Bogotá, D.C., Colombia.

Correspondence

Díez-Ortega, Hugo, Bact., M.Sc., Ph.D.
Professor, Departamento de Microbiología, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, D.C., Colombia

- Received Date: 23 June 2025
- Accepted Date: 01 July 2025
- Publication Date: 04 July 2025

Keywords

Enterococcus faecalis; biofilm; phylogeny; genetic clustering; virulence genes

Abstract

Objective: *The variability in reports associating Enterococcus faecalis with endodontic treatment failure is due to its remarkable genetic plasticity and clonal diversity facilitating the adaptation to adverse microenvironments.*

Materials and Methods: *Present study approximated the genetic diversity of Enterococcus faecalis isolates from oral cavity samples of endodontic origin; the detection involves genes Asa, Esp, EfaA, Ace, Asa373, GelE, and CylA, and mature biofilm formation.*

Results: *On the other side, the genetic clusters were analysed based on the sequence alignment of the amplified product of a 16S rDNA PCR. Results showed that the genetic clustering of Enterococcus faecalis isolates was distinct between patients but with a high degree of similarity between samples from the same individual.*

Conclusion: *The virulence genes presence and biofilm formation resulted independent of genetic clustering but related to sample origin.*

Introduction

The main factor associated with endodontic treatment failure is the persistence of microbial infection in the root canal system. The microorganisms involved may survive the biomechanical procedures performed during the execution of the treatment or may invade the canals as a result of leakage from the crown of the teeth [1-3]. Several studies have revealed that the microorganisms found in endodontic infection and teeth that fail endodontic treatment are predominantly facultative anaerobes and Gram-positive, with *Enterococcus faecalis* (*E. faecalis*) being the most frequently isolated species. Virulence factors related to the survival capacity of *E. faecalis* are collagen-binding protein (Ace), aggregation substance (Asa and its variant Asa373), haemolysin activator (CylA), endocarditis antigen (EfaA), surface protein (Esp), Gelatinase enzyme (GelE) and biofilm formation; however, some authors also associate it with antimicrobial resistance [4-6]. Once the microorganism reaches the root canal, differential expression of these genes occurs to adhere to the collagen of dentine, bone and other tissues, penetrate the dentinal tubules and resist drugs such as calcium hydroxide, survive irrigation with sodium hypochlorite, due to the formation of biofilms, both at the intra and extra-radicular levels, causing persistent periapical lesions [3,7]. The pathogenicity and ability to survive in adverse environments are associated with the genetic plasticity of the microorganism to modulate the expression or suppression of virulence genes when inhabiting different host biological niches. For this reason, studies of virulence genes are often heterogeneous and suggest that *E. faecalis* has a high genetic diversity. Understanding gene expression in endodontic infections is fundamental to explaining host-host interactions, understanding the association of pathogenic bacteria with disease and implementing more effective therapeutic measures to treat endodontic diseases [7-9]. Although genetic diversity can be analysed from phylogenetic trees resulting from methodologies such as Pulsed Field Gel Electrophoresis (PFGE), restriction enzyme analysis, Amplified Fragment Length Polymorphism (AFLP) and bioinformatic analysis of fragment or whole gene sequencing, among others [10], analysis of 16S rDNA combined with nucleic acid sequencing is an efficient tool for cluster analysis, gene

Citation: Hurtado-Narváez VD, de León-Frías NE, Espriella CMD, Rodríguez-Ciodaro A, Gomez-Cárdenas OE, Díez-Ortega H. Exploring Genetic Diversity in Enterococcus Faecalis Strains of Endodontic Origin: Pilot Study. Japan J Res. 2025;6(7):131.

clustering and clonality of microorganisms in the oral cavity [11].

The present work is a pilot study that evaluated whether a population of *E. faecalis* isolated from different oral samples taken from patients attending endodontic consultations, there is any genetic clustering and whether the diversity of this clustering is associated with the presence of virulence genes, biofilm formation or the type of sample from which they were isolated.

Materials and methods

Type of study, population and biological simples

Present was an observational and descriptive study with patients attending endodontic procedures, of legal age, with informed consent, who were not on antibiotic treatment in the last three months, and who did not have systemic or underlying pathologies. Only *E. faecalis*-positive samples entered the study. A total of 30 viable and pure isolates came from Ejectors (*n*=6), K-files (*n*=6), paper cones (*n*=6), gutta-percha filling material (*n*=6), and mucosal swabs (*n*=6). Sampling followed previously described protocols [12].

Isolation, microbiological identification

Genus and species identification was initially performed by traditional methodology, by primary isolation from Enterococcus Broth (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), culture on Chromocult® Enterococcus Agar (Merck, Darmstadt, Germany) and microbiological identification with the Dade/MicroScan Pos ID PC34 system (West Sacramento C.A., USA) [13]. Isolates confirmation was by MALDI-TOF-MS Biotyperidentification by Bruker, Daltonics, Germany and the standard Bruker Taxonomy Database Version 3.3.1. and following the protocol for Gram-positive bacteria [14], spectra analysis to identify genus and species was performed with BioExplorer 1.0 software following the previously proposed methodology [15]. Assays performed in duplicate involved *E. faecalis* ATCC 29212, *E. faecalis* ATCC 700802, and *E. faecalis* F ATCC 12030 strains, serving as positive and negative internal controls.

Identification of virulence genes

Bacteria were cultured in BHI Broth - Brain Heart Infusion (Sigma-Aldrich) at 37 °C until reached a concentration of $2x10^9$ Bact/mL (OD approx. 0.200). They were then centrifuged at 6000 *g* for 1 minute to obtain the bacterial pellet from which DNA extraction was with the commercial DNeasy Blood & Tissue 69504 kit (Qiagen, Santa Clarita, California) for Gram-positive bacteria. Virulence gene identification occurred through a PCR primer specific for *Asa*, *Asa*373, *Esp*, *EfaA*, *Ace*, *Gel*E and *Cyl*A genes (Table 1); amplification products visualisation was on 1 % (w/v) agarose gels in TBE-1X - Syber Green and analysed on a ChemiDocTM MP Imaging System BioRad Laboratories Inc. photo-documenter.

Mature biofilm formation

For the biofilm generation study, a microtiter plate following the methodology proposed by Al-Ahmad et al., (2014) with some modifications [19] served. For the technique, 20 µL of the inoculum plus 180 µL of BHI broth was seeded in a 96-well polystyrene plate (Nunc Maxisort, Life Technologies Ltd., CA, USA) and incubated at 37 °C for 48 hours. The liquid medium was aspirated to remove it, and the unattached cells were washed three times with 300 µL of 1X PBS (PBS, Sigma-Aldrich Chemie GmbH) removed. After the plate was dried at room temperature for 2 hours, were added 200 µL of 0.1 % (w/v) crystal violet (Median Diagnostics GmbH, Dunningen, Switzerland) for 10 minutes. The dye was decanted and washed three times with distilled water after drying the plates at 60 °C for 10 min, 50 µL ethanol (99.9 %, v/v), absolute for analysis; Merck, Darmstadt, Germany) was added to each well until the crystal violet was solubilised (approximately 30 min). The OD_{595nm} measurement was on a Labsystems Multiskan plate reader.

The assay was in quadruplicate and double-blinded, and the mean values were calculated for each sample. Negative controls without microorganisms, non-biofilm-forming negative control isolates, biofilm-forming positive control isolates (ATCC 29212), and positive internal controls from biofilm-positive clinical specimens (verified by other methodologies) serve as test blanks. Mature biofilm formation was by "*Ex Vivo*" modelling - Scanning Electron Microscopy confirmed in parallel work.

Table 1. PCR primers and amplification products that served to detect virulence genes in *E. faecalis isolates.*

Detected genes	Primers (5`-3`)	Amplicon size (bp)	References
Asa (aggregation)	F: GCA CGC TAT TAC GAA CTA TGA	375	[16]
	R: TAA GAA AGA ACA TCA CCA CGA		
Asa_{373} (mutant for aggregation)	F: GGA CGC ACG TAC ACA AAG CTAC	619	
	R: CTG GGT GTG ATT CCG CTG TTA		
Ace (collagen binding)	F: AAA GTA GAA TTA GAT CAC AC	320	
	R: TCT ATC ACA TTC GGT TGC G		
*Gel*E (gelatinase)	F: AGT TCA TGT CTA TTT TCT TCA C	402	
	R: CTT CAT TAT TTA CAC GTT TG		
Esp (surface protein)	F: TTG CTA ATG CTA GTC CAC GAC C	933	[17]
	R: GCG TCA ACA CTT GCA TTG CCG AA		
*Cyl*A	F: ACT CGG GGA TTG ATA GGC	688	[18]
	R: GCT GCT AAA GCT GCG CTT		
	R: GAC TGA CGT CCA AGT TTC CAA		
EfaA	F: CGT GAG AAA GAA ATG GAG GA	499	[16]
	R: CTA CTA ACA CGT CAC GAA TG		

Genetic diversity of the isolates

To determine the clustering types in the 30 *E. faecalis* isolates from the extracted DNA, a PCR for the 16S subunit of the bacterial ribosome (16S rDNA) was performed using primers 16S forward 5' GGA GGA GGA AGG TGG GGA TGA CG 3' and 16S reverse 5' ATG GTG TGA CGG GCG GTG GTG TG 3' [20]. A reaction mixture composed of 0.5 X Buffer, dNTPs 200 µM, 16S rDNA primers 0.5 µM, $MgCl_2$ 2 µM, Taq 0.45 U for a final volume of 25 µL allowed the amplification under the following conditions: 30 cycles at 95 °C; 30 s at 52 °C; 1 min at 72 °C, with a further extension of 10 min at 72 °C. The amplified DNA was observed by electrophoresis in 1 % (w/v) agarose with five aliquots of the PCR product using a negative control with unamplified DNA. The rDNA products were cut from the agarose by fine cutting with a sterile scalpel, and the DNA was purified with the QIAquic Gel Extraction Kits (Qiagen, USA) [21].

Relationship between virulence factors, mature biofilm and genetic diversity

PCR products of sequencing 16S rDNA were then compared with the Gen Bank database using BLASTn software. For the bioinformatics analysis, the MEGA V6 software [22] allowed performing the multiple alignments (Clustal- Multiple Sequence Alignments) for the construction of the tree from the evolutionary distances using the UPGMA methodology (Unweighted Pair Group Method with Arithmetic Mean) [23]. Exploration of possible relationships between these variables involved non-parametric tests, Fisher's exact test with an r-value > 0.05 as statistically significant and analyses performed through Stata 14. Data are shown descriptively and in percentages.

Results

Microbiological identification of *Efa*

All 30 isolates showed 100 % viability and purity, and 100 % of the *E. faecalis* were identified by MicroScan System and confirmed by MALDI TOF MS, compared against the spectral basis of 16S ribosomal proteins and wall glycoproteins of 5000 species referencing markers from 2000 to 20000 daltons and according to the established cut-off points to identify genus and species, all 30 isolates registered in the BrukerTaxonomy database program version 3. 3.1 had consistent and high probability scores for *E. faecalis* isolate identification [24].

Detection of virulence genes and mature biofilm formation

At least one gene amplification occurred in 100 % of the isolates. The most frequently occurring gene was *Ace* 100 % (30/30 isolates), followed by *EfaA* 96.7 % (29/30 isolates) and *Asa* 80 % (24/30 isolates). The gene found in the lowest isolates was *Asa*373 (3.3 %, 1/30 isolates).

When analysing gene frequency by sample type, several genes were in all samples with a similar number of cases in each sample. The samples with the highest frequency of detected genes were gutta-percha and mucosa, each with 28 detected genes; the sample with the lowest frequency of detected genes was K-files with 19 genes (Table 2).

An OD_{595nm} cut-off point of 0.040 obtained suggested that 63.3 % (19/30 isolates) stimulated biofilm formation, with the highest number of biofilm-forming isolates coming from mucosa and paper cone, each with 16.7 % (5/30 isolates); those with the lowest number were K-files 6.6 % (2/30 isolates)

Genetic diversity

In the 16S rDNA PCR, an amplicon of 960 bp resulted, and when it was sequenced and BLASTn analysed, more than 98.5 % was identical to more than 100 isolates of EF reported in Genbank. The chromatogram served for multiple alignments (Figure 1).

The tree showed that, in samples from different patients, there was genetic diversity in the isolates and grouped into five clusters, named A, B, C, D, and E. However, in different origins samples from the same patient, there was a high degree of homology in some of them, such as 4EF/30EF, 15EF/28EF, and 16EF/24EF (dotted lines), for others isolates partial homology detected, starting from the same ancestor, such as samples 7EF/29EF in Cluster D and 17EF/20EF/27EF in Cluster A.

Isolates analysed according to the sample origin showed heterogeneous behaviour. Only samples from the Ejector (1EF to 6EF) started from a common ancestor (Cluster A), while

Table 2. *Distribution frequency distribution of virulence genes and mature biofilm formation in the 5 samples used for the isolation of Enterococcus faecalis.*

Origen	Gene *Ace*		Gene *Esp*		Gene *Asa*		Gene Asa_{373}		Gene *Cyl*A		Gene *Gel*E		Gene *Efa*A		Mature biofilm	
	(+)	%	(+)	%	(+)	%	(+)	%	(+)	%	(+)		(+)	%	(+)	%
Ejector (n=6)	6	100	3	50.0	4	66.6	0	0,0	1	16.6	3	50.0	6	100.0	3	50.0
Lima K (n=6)	6	100	1	16.6	3	50.0	0	0,0	2	33.3	1	16.6	6	100.0	2	33.3
Paper cone (n=6)	6	100	4	66.6	6	100	0	0,0	2	33.3	3	50.0	6	100.0	5	83.3
Gutta-percha (n=6)	6	100	3	50.0	6	100	1	16,6	2	33.3	4	66.6	6	100.0	4	66.6
Mucosal (n=6)	6	100	3	50.0	5	83.3	0	0,0	4	66.6	5	83.3	5	83.3	5	83.3
Total (n=30)	30	100	14	46.7	24	80.0	1	3,3	11	36.7	16	53.3	29	96.7	19	63.3

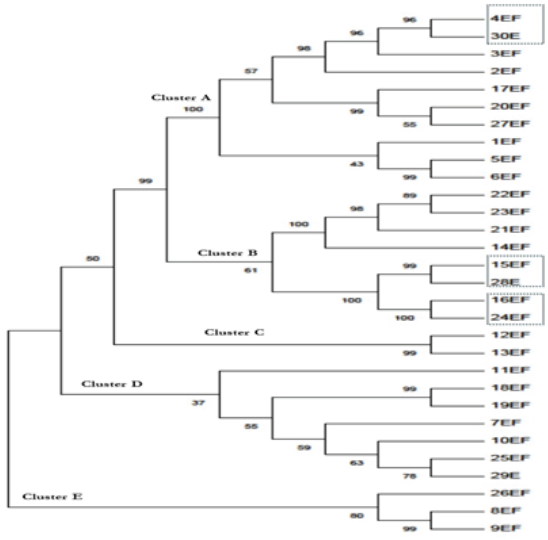


Figure 1. Genetic groups: phylogenetic tree of 16S rDNA PCR product sequences showing five main groupings among the study isolates of Eph isolates. Study groups: Ejectors 1EF to 6EF, K-files (7EF to 12EF), Paper cones (13EF to 18EF), Gutta-percha filling material (19EF to 24EF) and mucosal swab (25EF to 30EF).
**EF= E. faecalñis strains*

Table 3. Relationship of the oral and endodontic group to the presence of virulence genes and biofilm formation.

Detected genes	PCR	Endodontic origin samples (%)	Samples origin Oral (%)	p-value Fisher´s exact
Asa	Negative	5/24 (21)	1/6 (17)	1.0
	Positive	19/24 (79)	5/6 (83)	
Esp	Negative	13/24 (54)	3/6 (50)	1.0
	Positive	11/24 (46)	3/6 (50)	
*Efa*A	Negative	0/24 (0)	1/6 (17)	0.20
	Positive	24/24 (100)	5/6 (83)	
Ace	Negative	0/24 (0)	0/6 (0)	0.19
	Positive	24/24 (100)	6/6 (100)	
Asa_{373}	Negative	23/24 (96)	6/6 (100)	1.0
	Positive	1/24 (4)	0/6 (0)	
*Gel*E	Negative	13/24 (54)	1/6 (17)	0.18
	Positive	11/24 (46)	5/6 (83)	
*Cyl*A	Negative	17/24 (71)	2/6 (33)	0.16
	Positive	7/24 (29)	4/6 (67)	
Biofilm formation	Negative	10/24 (42)	1/6 (17)	0.37
	Positive	14/24 (58)	5/6 (83)	

Table 4. *Individual relationship between sample origin and virulence genes detected by PCR.*

Detected genes	Factor	Paper cone (%)	Ejector (%)	Gutta-percha (%)	Lima K (%)	Mucosal (%)	p-value Fisher´s exact
Asa	Negative	0/6 (0)	2/6 (33)	0/6 (0)	3/6 (50)	1/6 (17)	0.22
	Positive	6/6 (100)	4/6 (67)	6/6 (100)	3/6 (50)	5 (83)	
Esp	Negative	2/6 (33)	3/6 (50)	3/6 (50)	5/6 (87)	3 (50)	0.59
	Positive	4/6 (67)	3/6 (50)	3/6 (50)	1/6 (13)	3 (50)	
*Efa*A	Negative	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	1 (17)	1.0
	Positive	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	5 (83)	
Ace	Negative	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	0 (0)	1.0
	Positive	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6 (100)	
Asa_{373}	Negative	6/6 (100)	6/6 (100)	5/6 (87)	6/6 (100)	6 (100)	1.0
	Positive	0/6 (0)	0/6 (0)	1/6 (13)	0/6 (0)	0 (0)	
*Gel*E	Negative	3/6 (50)	3/6 (50)	2/6 (33)	5/6 (87)	1 (17)	0.26
	Positive	3/6 (50)	3/6 (50)	4/6 (67)	1/6 (13)	5 (83)	
*Cyl*A	Negative	4/6 (67)	5/6 (87)	4/6 (67)	4/6 (67)	2 (33)	0.61
	Positive	2/6 (33)	1/6 (13)	2/6 (33)	2/6 (33)	4 (67)	
Biofilm madure	Negative	1/6 (17)	3/6 (50)	2/6 (33)	4/6 (67)	1 (17)	
	Positive	5/6 (83)	3/6 (50)	4/6 (67)	2/6 (33)	5 (83)	

samples from other origins were placed in three Clusters as observed in K-file (7EF to 12EF), paper cones (13EF to 18EF), gutta-percha (19EF to 24EF) and mucosa (25EF to 30EF).

Relationship between genetic groups and study variables

The relationship between the samples, virulence factors and biofilm formation were analysed individually according to the origin of each sample and as a group; the samples were Clustered as Oral Group, those taken from the mucosa and as Endodontic Group all those that made contact with the root canal such as paper cones, gutta-percha, K-files and ejectors.

Relationship of oral and endodontic groups to the presence of virulence genes and biofilm formation

No statistically significant association occurred ($p < 0.05$) in any of the cases (Table 3). However, when analysing the behaviour of the groups, some trends were observed. About the presence of genes, *Ace*, *EfaA* and *Asa* were expressed in similar percentages in both groups, ranging from 79 to 100 %, and *Gel*E and *Cyl*A genes had higher expression at the level of the oral group with 83 and 67 %, respectively.

Concerning the mature biofilm formation, although there was no statistically significant difference ($p > 0.05$), it was clear that samples of endodontic origin had the highest number of biofilm-forming isolates of 14/30 (46.7 %).

Individual relationship of sample origin with virulence and biofilm formation genes

Once each sample was individually analysed, there was no statistically significant association ($p > 0.05$) for the proposed variables (Table 4). However, there was a clear trend in the behaviour of the data, where *Ace* and *EfaA* were involved in all sample types (83-100 %), *Asa* 373 was only present in gutta-percha, *Cyl*A with 67 % and *Gel*E 83 % predominated in mucosa and biofilm formation presented a heterogeneous behaviour, being slightly higher in gutta-percha (67 %) and paper/mucosa cones (83 %) (Table 4).

Relationship of variables with genetic Clustering

Relationship of the genetic Clusters with the sample origin (oral and endodontic) showed that Cluster A grouped the highest number of cases from both origins (endodontic and oral); the endodontic origin samples were not present in Cluster D, while oral samples were not into Cluster B (Table 5).

Analysing the relationship between the individual origin samples with the genetic groups resulted in a power significant association ($p = 0.001$) (Table 6) between the sampling site and the heterogeneity cluster, which suggested that the grouping for each sample depends on its genetic characteristics. Additionally,

Table 5. *Relationship of sample origin to genetic clustering.*

Gene clustering	Endodontic samples (%)	Oral samples (%)	p-value Fisher´s exact
Cluster A	9 (38)	3 (50)	0.22
Cluster B	6 (25)	0 (0)	
Cluster C	7 (29)	1 (17)	
Cluster D	0 (0)	1 (17)	
Cluster E	2 (8)	1 (17)	

100 % of K-files were in Cluster A, the highest number of Ejectors in Cluster B (67 %), and gutta-percha in Cluster C (67 %) (Table 6).

Discussion

E. faecalis is a ubiquitous microorganism isolated from different human biological samples, including samples from the oral cavity such as mucosa, tongue, saliva, teeth, gingival, periodontal, periapical and untreated necrotic pulps. Although the frequency of isolates varies widely [25-27], isolates reported in obturated root canals are of particular importance, which is one of the possible causes of endodontic treatment failure [28]. The diversity of data reported on ubiquity is due to the genetic variability of *E. faecalis*, which allows it to adapt to different ecological niches, while the wide ranges of isolates reported by researchers are due to the diversity of microbiological and molecular methods used to identify the microorganism. In this study, the use of high confidence, sensitivity and reproducibility techniques such as the MicroScan panel and MALDI-TOF-MS [29] allowed the identification of *E. faecalis* in the oral cavity and dental devices used for endodontic treatment such as K-files, gutta-percha filling material, paper cones and ejectors; results obtained for similar methodologies are congruent with other reports [30,31], and the microorganism's isolation in these devices is due to the ability to survive in adverse conditions. Kayaoglu et al., (2004) showed how this survival capacity has already been reported for more than three decades, demonstrating that *E. faecalis* survives high concentrations of chemical compounds such as sodium dodecyl sulphate, bile salts, ethanol, hydrogen peroxide, sodium hypochlorite, acids and basics environment and can grow in extreme physiological conditions such as hyperosmolarity, heat, and UV irradiation among others [28]. An important feature that explains the survival of *E. faecalis* in root canals and abiotic and inert materials used during endodontic treatment (as evidenced in the present study) is the ability of the bacterium to survive in nutrient-limited media and adverse environmental conditions by

Table 6. *Relationship of sample origin to genetic clustering.*

Gene clustering	Paper cone (%)	Ejector (%)	Gutta-percha (%)	Lima K (%)	Mucosal (%)	p-value Fisher´s exact
Cluster A	2 (33)	0 (0)	1 (17)	6 (100)	3 (50)	<0.001
Cluster B	1 (17)	4 (67)	1 (17)	0 (0)	0 (0)	
Cluster C	3 (50)	0 (0)	4 (67)	0 (0)	1 (17)	
Cluster D	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	
Cluster E	0 (0)	2 (33)	0 (0)	0 (0)	1 (17)	

a mechanism called viable but non-cultivable (VBNC), where *E. faecalis* can remain dormant under environment stress and regain growth when conditions become favourable [32].

Pathologies at the level of periapical tissues are associated with a triad that includes the immunological conditions of the host, the size of the inoculum, the presence of virulence factors and the formation of biofilm in the anatomical site where it resides. Several studies have identified the main virulence factors, the most studied being the extracellular surface protein (Esp), the collagen-binding adhesin (Ace), the aggregation substance (Asa) and its variants Asa 48 and Asa 373; Cytolysin (CylA), Gelatinase (GelE), endocarditis-specific antigen A (EfaA), hyaluronidase (Hy), immune evasion factors such as enterococcal polysaccharide capsule (Cps), superoxide ions (O_2^-) and complementary factors such as lipoproteins, proteolytic enzymes and wall carbohydrates among others [13,33,34]. The endodontic disease model proposes that the *E. faecalis*, through different phases: adherence, colonisation, tissue invasion, maturation and dispersal, potentiates virulence factors to cause infection, originate the biofilm and maintain the inflammatory state detrimental to the host [28,35]. Various proteins are involved in the adherence phase, with Esp and Ace being relevant. The study showed a frequency of 46 % (14/30 isolates) for *Esp* and 100 % (30/30 isolates) for *Ace*, distributed similarly in both the oral and endodontic groups, data that are in agreement with other reports [5]. The Esp protein is involved in adherence, subsequent colonisation and evasion; it is also a cofactor for biofilm formation. Ace protein is crucial for *E. faecalis* because it is an adhesin that mediates binding to type I and IV collagen, laminin and dentin [5,8], a reason it is expressed by most virulent *E. faecalis* strains. The colonisation phase involves aggregation-facilitating proteins where Asa and its variants are essential. The results showed 80 % (24/30 isolates) expression for *Asa* and 3.3 % (1/30 isolate) for Asa_{373}, similar data to that showed by Sedgley et al., (2006) and Zhu et al., (2010) [27,36]. *Asa* gene expression was high in both groups and for all samples with a high case number. These results are due to the *E. faecalis* need to bind to extracellular matrix proteins, including type I collagen, allowing bacterial aggregation and serving as protection against macrophage lysis, a mechanism serving it to reach the dentin duct. Additionally, Asa facilitates the antibiotic resistance plasmids exchange and invasion-type cytolysins plasmids, characteristics that increase the microorganism virulence degree and its expression in adverse environments. A variant of the *Asa* gene is Asa_{373}, a little studied and reported in lower frequency, whose expression product is a reinforcement molecule for adhesion in dentinal tubules [28]. In the phase of tissue invasion, inflammatory response and tissue damage associated with virulence genes, a large group of proteins are included, including *EfaA*, *Gel*E and *Cyl*A. In the study, *EfaA* was in 86.7 % (29/30 isolates), *Gel*E 53 % (16/30 isolates) and *Cyl*A 37 % (11/30 isolates) predominating in samples of oral-mucosal origin, data similar to those reported by other authors [27,36]. These proteins are expressed more on biotic surfaces such as mucosa, tongue, and root canal cellular detritus, where they find the substrates to act, originating diverse degradation products that serve the bacteria as nutrients.

GelE is a Zinc-dependent metalloprotease with proteolytic properties that hydrolyses endothelin-1, gelatin, collagen, fibrin, fibrinogen, haemoglobin, casein and complement factors C3, C3a, C5a, endothelin-1 and low molecular weight peptides. CylA is a bacteriocin of plasmid origin (pAD1 sex pheromone plasmid), consisting of two subunits associated with the Quorum-Sensing mechanism and synthesised by an 8-gene structural operon, capable of lysing phagocytes, red blood cells and Gran-negative bacteria; the latter, a mechanism that generates extracellular DNA, is essential for the synthesis of biofilms. EfaA at the dental level is crucial because it is the solute-binding protein receptor for a manganese transport system, a necessary ion for bacterial survival under adverse conditions [28,35].

Endodontic treatment failure is related to the ability of *E. faecalis* to form a calcified biofilm on root canal dentin, which contributes to its persistence after endodontic treatment, blocks the immune response and prevents antibiotic entry. In this study, 63.3 % (19/30 isolates) showed biofilm formation, and the results indicate no significant difference ($p > 0.05$) between the ability of *E. faecalis* to form biofilms and the source of isolation. This extensively studied characteristic by different authors who have demonstrated the various types of biofilms that form in endodontics at intracanal, extra-radicular, periapical, foreign body-centred biofilm and the impact of *E. faecalis* in treated and untreated root canals, where apical periodontitis can be considered a disease caused by biofilm formation [37-39].

The development of new therapeutic strategies against infections associated with biofilms caused by *E. faecalis* is only possible if the targets of new drugs are the genes and proteins responsible for biofilm formation, which is why there are reports of a large number of studies that demonstrate the individual function of the product of each gene and their interaction to generate the biofilm, exacerbate the virulence of the microorganism and even carry out horizontal gene transfer, mainly antibiotic resistance [35,37-40]. Concerning the *E. faecalis* pathogenic effect, the biofilm formation is associated with the interaction of virulence factors, and they can be by their functions classified into cell aggregation surface factors Asa, Esp, Ace, and secreted virulence factors CylA, GelE, HylA [41]. However, few studies discuss the impact of environment, host conditions and colonising microorganisms on biofilm formation [6].

In this context, the present study analysed a new variable, the relationship between the biological sample with virulence genes and mature biofilm formation. The results showed that gene expression and biofilm formation are independent of the type of sample (mucosa, K-files, ejectors, gutta-percha material and paper cones) where *E. faecalis* was isolated. However, it is necessary to note that at least one gene was present in all samples and that more than one isolate with biofilm-forming capacity was evident in all samples. These data confirm the pathogenic potential of the microorganism and its ability to form biofilms on both biotic and abiotic surfaces to survive in adverse environments or to remain in the VBNC state [28,32,40].

This ability to adapt to different environments, even adverse ones, and to differentially express virulence genes is related to the genetic plasticity of the micro-organism. However, research is needed to confirm this condition or to analyse whether the process is associated with various genetic clusters or circulating clones. As relevant data, the study showed a genetic diversity in the isolates, which grouped into five different clusters; very heterogeneous groupings when compared between different subjects, but with similarity between isolates from samples from the same individual. These results are congruent with those reported by Gómez et al., (2006) which show the applicability of the 16S rDNA technique to identify *E. faecalis*, and the

studies by Delboni et al., (2017), which show genetic diversity by establishing three different clusters at the root canal, the pulp chamber and saliva levels [42,43]. Although the present study is limited by the low number of isolates, by not including all known virulence genes, and by the fact that results did not show statistically significant associations between genetic clustering and virulence factors, the genetic diversity was evident. An observation was that a low number of individuals be colonised by the same type of microorganism, as was the case for samples 4EF/30EF, 15EF/28EF, and 16EF/24EF and that genetic Clusters can be associated with abiotic environmental niches as in the case of Ejectors where all samples belonged to a single Cluster. However, the main finding is the genetic diversity concerning the colonising strains between patients and about the strains from the different biological samples, indicating the circulation of more than one strain of *Enterococcus faecalis*; This suggests the existence of a mechanism by which microorganisms adapt to a specific environment, modulating the expression of genes and biofilm formation, and that when analysing the genetic clusters to the sample from which the *E. faecalis* isolate originates, statistically significant differences can be found (Table 7). However, to determine the specific genetic cluster or possible circulating clone and its impact on the sources of transmission and the development of endodontic infection or therapeutic failure in obturation, further research is needed, using more robust methodologies such as MLST (Multilocus Sequence Typing) that would allow evaluation of the population structure and the mechanisms involved in genetic diversity [44,45].

Conclusion

In conclusion, these first pilot data showed a genetic diversity in *E. faecalis* strains associated with endodontic treatment failure, where the genetic diversity observed among *E. faecalis* isolates from different patients and variability is probably influenced by the environment where the bacteria grow.

Declarations

Conflict of interest statement

The authors declare no potential conflicts of interest concerning the research, authorship or publication of this article..

Funding

This research was sponsored by the “Vicerrectoría de Investigación de la Pontificia Universidad Javeriana”, ID: 00007914 - SIAP 00007699. The study had the institutional endorsement of the Ethics Committee of the Faculty of Sciences and the Faculty of Dentistry. All included patients signed an informed consent.

Acknowledgements

The authors thank the “Centro de Investigaciones Odontológicas de la Pontificia Universidad Javeriana, Bogotá, D.C., Colombia”, for providing the samples for the study and Dr Raúl A. Poutou-Piñales, PhD, for English editing.

References

1. Alghamdi F, Shakir M. The influence of Enterococcus faecalis as a dental root canal pathogen on endodontic treatment: A systematic review. Cureus. 2020;12(3):e7257. doi:10.7759/cureus.7257
2. Nemer Molina NP, Centeno Dávila MC, Artieda Sáenz JG, Claure Venegas DC. Factores de resistencia microbiana de Enterococcus faecalis asociado a los fracasos endodónticos: Revisión de literatura. Canal Abierto. 2022;46:24-28.
3. Rodríguez-Niklitschek C, Oporto VGH. Implicancias clínicas de la contaminación microbiana por Enterococcus faecalis en canales radiculares de dientes desvitalizados: Revisión de la literatura. Rev Odontol Mex. 2015;19(3):181-186. doi:10.1016/j.rodmex.2015.04.002
4. Barbosa-Ribeiro M, Gomes BPFA, Arruda-Vasconcelos R, et al. Antibiotic resistance profile of clinical strains of Enterococci from secondary/persistent endodontic infections: What do we know? A systematic review of clinical studies. J Endod. 2024;50(3):299-309. doi:10.1016/j.joen.2023.12.007
5. Barbosa-Ribeiro M, De-Jesus-Soares A, Zaia AA, Ferraz CC, Almeida JF, Gomes BPFA. Antimicrobial susceptibility and characterization of virulence genes of Enterococcus faecalis isolates from teeth with failure of the endodontic treatment. J Endod. 2016;42(7):1022-1028. doi:10.1016/j.joen.2016.03.015
6. Ch'ng JH, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection by enterococci. Nat Rev Microbiol. 2019;17(2):82-94. doi:10.1038/s41579-018-0107-z
7. Prada I, Mico-Munoz P, Giner-Lluesma T, et al. Influence of microbiology on endodontic failure: Literature review. Med Oral Patol Oral Cir Bucal. 2019;24(3):e364-e372. doi:10.4317/medoral.22907
8. Fisher K, Phillips C. The ecology, epidemiology and virulence of Enterococcus. Microbiology. 2009;155(Pt 6):1749-1757. doi:10.1099/mic.0.026385-0
9. Siqueira JF Jr. Taxonomic changes of bacteria associated with endodontic infections. J Endod. 2003;29(10):619-623. doi:10.1097/00004770-200310000-00001
10. Cortés-López NG, Ordóñez-Baquera PL, Domínguez-Viveros J. Herramientas moleculares utilizadas para el análisis metagenómico: Revisión. Rev Mex Cienc Pecu. 2020;11(4):1150-1173. doi:10.22319/rmcp.v11i4.5202
11. Tannerm A, Maiden MFJ, Paster BJ, Dewhirst FE. The impact of 16s ribosomal RNA-based phylogeny on the taxonomy of oral bacteria. Periodontol 2000. 1994;5:26-51.
12. Rivas MA, Shadia Y, Díaz C, et al. Frecuencia de aislamiento y susceptibilidad de Enterococcus faecalis en pacientes endodónticos. Rev Odontol Andes. 2012;7(1):15-23.
13. Carrero C, González MC, Martínez MA, et al. Baja frecuencia de Enterococcus faecalis en mucosa oral de sujetos que acuden a consulta odontológica. Rev Fac Odontol Univ Antioquia. 2015;26(2):261-270.
14. Clark AE, Kaleta EJ, Arora A, Wolk DM. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev. 2013;26(3):547-603. doi:10.1128/CMR.00072-12
15. van Veen SQ, Claas EC, Kuijper EJ. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J Clin Microbiol. 2010;48(3):900-907. doi:10.1128/JCM.02071-09
16. Dupre I, Zanetti S, Schito AM, Fadda G, Sechi LA. Incidence of virulence determinants in clinical Enterococcus faecium and Enterococcus faecalis isolates collected in Sardinia (Italy). J Med Microbiol. 2003;52(Pt 6):491-498. doi:10.1099/jmm.0.05038-0
17. Leavis HL, Willems RJL, Top J, et al. Epidemic and nonepidemic multidrug-resistant Enterococcus faecium. Emerg Infect Dis. 2003;9(9):1108-1115.
18. Vankerckhoven V, Van Autgaerden T, Vael C, et al. Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. J Clin Microbiol. 2004;42(10):4473-4479. doi:10.1128/JCM.42.10.4473-4479.2004
19. Al-Ahmad A, Ameen H, Pelz K, et al. Antibiotic resistance and

capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth. J Endod. 2014;40(2):223-230. doi:10.1016/j.joen.2013.07.023

20. Kumar A, Kumar D. Characterization of Lactobacillus isolated from dairy samples for probiotic properties. Anaerobe. 2015;33:117-123. doi:10.1016/j.anaerobe.2015.03.004
21. Scheidegger EMD, Fracalanzza SAP, Teixeira LM, Cardarelli-Leite P. RFLP analysis of a PCR-amplified fragment of the 16S rRNA gene as a tool to identify Enterococcus strains. Mem Inst Oswaldo Cruz. 2009;104(7):1003-1008.
22. Tamura K, Stecher G, Kumar S. MEGA11: Molecular evolutionary genetics analysis version 11. Mol Biol Evol. 2021;38(7):3022-3027. doi:10.1093/molbev/msab120
23. Sneath PHA, Sokal RR. Numerical Taxonomy: The Principles and Practice of Numerical Classification. San Francisco, CA: W.H. Freeman; 1973.
24. García P, Allende F, Legarraga P, Huilcaman M, Solari S. Identificación bacteriana basada en el espectro de masas de proteínas: Una nueva mirada a la microbiología del siglo XXI. Rev Chilena Infectol. 2012;29(3):263-272.
25. Pinheiro ET, Gomes BPFA, Ferraz CCR, et al. Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility. Oral Microbiol Immunol. 2003;18:100-103.
26. Sánchez-Sanhueza G, Villegas C, Ulloa MI, et al. Molecular typing of Enterococcus faecalis from persistent endodontic infections. Int J Odontostomat. 2018;12(1).
27. Sedgley C, Nagel A, Dahlen G, Reit C, Molander A. Real-time quantitative polymerase chain reaction and culture analyses of Enterococcus faecalis in root canals. J Endod. 2006;32(3):173-177. doi:10.1016/j.joen.2005.10.037
28. Kayaoglu G, Orstavik D. Virulence factors of Enterococcus faecalis: Relationship to endodontic disease. Crit Rev Oral Biol Med. 2004;15(5):308-320. doi:10.1177/154411130401500506
29. Maldonado N, Robledo C, Roble J. La espectrometría de masas MALDI-TOF en el laboratorio de microbiología clínica. Infectio. 2018;22(1):35-45.
30. Saha S, Meenawat A, Sahu C, et al. Bacterial identification and monitoring around two-piece dental implants by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). J Lab Physicians. 2020;12(1):49-55. doi:10.1055/s-0040-1714934
31. Srivastava A, Saha S, Sahu C. Early and accurate detection of bacterial isolates from dental plaque in subjects with primary, mixed, and permanent dentition by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technique. J Indian Soc Periodontol. 2020;24(2):104-108. doi:10.4103/jisp.jisp_303_19
32. Lleò MM, Bonato B, Tai MC, et al. Resuscitation rate in different enterococcal species in the viable but non-culturable state. J Appl Microbiol. 2001;91:1095-1102.
33. Zoletti GO, Pereira EM, Schuenck RP, et al. Characterization of virulence factors and clonal diversity of Enterococcus faecalis isolates from treated dental root canals. Res Microbiol. 2011;162(2):151-158. doi:10.1016/j.resmic.2010.09.018
34. Saffari F, Sobhanipoor MH, Shahravan A, Ahmadrajabi R. Virulence genes, antibiotic resistance and capsule locus polymorphisms in Enterococcus faecalis isolated from canals of root-filled teeth with periapical lesions. Infect Chemother. 2018;50(4):340-345. doi:10.3947/ic.2018.50.4.340
35. Schiopu P, Toc DA, Colosi IA, et al. An overview of the factors involved in biofilm production by the Enterococcus genus. Int J Mol Sci. 2023;24(14):11577. doi:10.3390/ijms241411577
36. Zhu X, Wang Q, Zhang C, et al. Prevalence, phenotype, and genotype of Enterococcus faecalis isolated from saliva and root canals in patients with persistent apical periodontitis. J Endod. 2010;36(12):1950-1955. doi:10.1016/j.joen.2010.08.053
37. Duggan JM, Sedgley CM. Biofilm formation of oral and endodontic Enterococcus faecalis. J Endod. 2007;33(7):815-818. doi:10.1016/j.joen.2007.02.016
38. Al-Ahmad A, Muller N, Wiedmann-Al-Ahmad M, et al. Endodontic and salivary isolates of Enterococcus faecalis integrate into biofilm from human salivary bacteria cultivated in vitro. J Endod. 2009;35(7):986-991. doi:10.1016/j.joen.2009.04.013
39. Jhajharia K, Parolia A, Shetty KV, Mehta LK. Biofilm in endodontics: A review. J Int Soc Prev Community Dent. 2015;5(1):1-12.
40. Covo Morales E, Díaz Caballero A, Simancas Pallares M. Expresión del gen esp (enterococcus surface protein) de Enterococcus faecalis en un modelo in vitro de dientes extraídos. Av Odontoestomatol. 2016;32(4):195-204.
41. Too E, Masila E. The interconnection between virulence factors, biofilm formation, and horizontal gene transfer in Enterococcus: A review. In: Téllez-Isaías G, Graham D, El-Ashram S, eds. Infectious Diseases. London, UK: IntechOpen; 2024: In press. doi:10.5772/intechopen.115000 (placeholder DOI—replace with actual if known)
42. Delboni MG, Gomes BPFA, Francisco PA, Teixeira FB, Drake D. Diversity of Enterococcus faecalis genotypes from multiple oral sites associated with endodontic failure using repetitive sequence-based polymerase chain reaction and arbitrarily primed polymerase chain reaction. J Endod. 2017;43(3):377-382. doi:10.1016/j.joen.2016.10.042
43. Gomes BPFA, Pinheiro ET, Sousa EL, et al. Enterococcus faecalis in dental root canals detected by culture and by polymerase chain reaction analysis. Oral Surg Oral Med Oral Pathol Oral Radiol. 2006;102(2):247-253. doi:10.1016/j.tripleo.2005.11.031
44. Ruiz-Garbajosa P, Coque TM, Cantón R, Willems RJL, Baquero F, del Campo R. Los complejos clonales de alto riesgo CC2 y CC9 están ampliamente representados en cepas hospitalarias de Enterococcus faecalis aisladas en España. Enferm Infecc Microbiol Clin. 2007;25(8):513-518.
45. Monteiro Marques J, Coelho M, Santana AR, Pinto D, Semedo-Lemsaddek T. Dissemination of enterococcal genetic lineages: A One Health perspective. Antibiotics (Basel). 2023;12(7):1140. doi:10.3390/antibiotics12071140