



ORIGINAL ARTICLE

Investigation of virulence factors of *Enterococcus faecalis* strains isolated in secondary/ persistent infections

Investigação de fatores de virulência de estirpes de *Enterococcus faecalis* isoladas em infecções secundárias persistentes

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ABSTRACT

Objective: More virulent strains may result from the acquisition of genes by genetic exchange, pathogenicity islands in several species encoding toxins, adhesion factors and other factors associated with virulence. The aim of this study was to investigate the prevalence of *E. faecalis* strains in secondary endodontic/ persistent using endodontic infection by culture and PCR techniques; and to investigate for the presence of virulence factor genes of gelatinase (gelE), cytolysin activator (Cyla), surface adhesin of *Enterococcus* (ESP) and collagen adhesin of *Enterococcus* (ACE). **Material and methods:** Microbial samples were obtained from 12 teeth with secondary/ persistent endodontic infection showing apical periodontitis. Culture techniques were used including serial dilution, plating, incubation, and biochemical identification. For PCR detection, samples were analyzed using a species-specific primer of the 16S rDNA and the downstream intergenic spacer region. **Results:** Culture and PCR detected the test species in 3/12 (25%) and 5/12 (41.6%) of teeth, respectively. A total of 38 *Enterococcus faecalis* strains were isolated and submitted to the virulence factor genes analysis. PCR products consistent with genes encoding surface adhesion (ESP), gelatinase (gelE) and collagen binding antigen (ACE) were found in 26/38 (68%), 31/38 (81%) and 38/38 (100%) of the isolates. The Cytolysin activator (Cyla) gene was not recovered from *E. faecalis* isolates. **Conclusions:** In conclusion, the present study revealed by culture and molecular methods revealed a high prevalence of *E. faecalis* in teeth with secondary/ persistent endodontic infection. Moreover, of a clinical relevance, we found different *E. faecalis* strains carrying different virulence determinants.

KEYWORDS

Bacteria; *E. faecalis*; Root canal, virulence.

RESUMO

Objetivos: O objetivo deste estudo foi investigar a prevalência de cepas de *E. faecalis* em canais com infecções endodônticas secundárias/persistentes por meio de cultura e PCR, além de analisar a presença de fatores de virulência genéticos como: gelatinase (gelE), ativador de citolisina (Cyla), adesina de superfície (ESP) e adesina de colágeno (ACE). **Material e métodos:** Foram coletadas amostras de 12 canais radiculares com infecção endodôntica secundária/persistente e presença de lesão periapical. Para a cultura microbiológica foi realizada diluição em série, incubação e identificação bioquímica dos microrganismos, enquanto que no PCR as amostras foram analisadas através de primers específicos 16S rDNA. Os casos com presença de *Enterococcus faecalis* foram selecionadas para realização de análise quanto aos fatores de virulência: gelE, Cyla, ESP e ACE. **Resultados:** *Enterococcus faecalis* foi detectado através de cultura e PCR em 3/12 (25%) e 5/12 (41,6%) dos casos, respectivamente. No total, foram isoladas 38 amostras com presença de *E. faecalis*. Os produtos de PCR consistentes com os genes ESP, gelE e ACE foram encontrados em 26 /38 (68%), 31 /38 (81%) e 38/38 (100%) dos isolados. Cyla não foi recuperado a partir de *E. faecalis* em nenhum dos isolados. **Conclusões:** O presente estudo revelou alta prevalência de *E. faecalis* em dentes com infecção endodôntica secundária/ persistente. Estes microrganismos apresentaram elevado índice de diferentes fatores de virulência.

PALAVRAS-CHAVE

Bactéria; Canais radiculares; *E. faecalis*; Fatores de virulência.

INTRODUCTION

The persistence of intracanal infections are one of the main causes of failure of endodontic treatment that is characterized by the persistence or emergence of apical periodontitis after root canal filling [1-3]. The microbiota of a tooth with apical periodontitis and endodontic failure proves to be different from that found in teeth with pulp necrosis predominating Gram-positive facultative anaerobic bacteria [3-7].

Enterococcus faecalis is a facultative anaerobic commensal species commonly detected in the root canal of teeth with secondary endodontic/persistent infection [1-4, 6-12], and can invade the root canal microleakage through the coronary or contamination during endodontic treatment prior [5].

Virulence factors of microorganism mean any component that is required to cause damage or to intensify the host immune response [13]. More virulent strains may result from the acquisition of genes by genetic exchange, pathogenicity islands in several species encoding toxins, adhesion factors and other factors associated with virulence [14]. Several are the characteristics that determine bacterial virulence, including adherence to host tissue invasion and abscess formation, modulation of the inflammatory response and secretion of toxic products [15]. Among the known virulence factors of enterococci, they are intrinsically resistant to commonly used antibiotics in the treatment of disease and other virulence factors can also be acquired by genetic exchange, such as: gelatinase (gelE), a cytolysins activator (Cyla) surface adhesins of *Enterococcus* (ESP) and the collagen adhesin *Enterococcus* (ACE) [11,14,16-21].

The aim of this study was to investigate the prevalence of *E. faecalis* strains in secondary endodontic/ persistent endodontic infection by culture and PCR techniques; and to investigate for the presence of virulence factor genes of gelatinase (gelE), cytolysin activator (Cyla),

surface adhesin of *Enterococcus* (ESP) and collagen adhesin of *Enterococcus* (ACE).

MATERIAL & METHODS

Patient Selection

A total of 12 patients who attended the Fluminense Federal University, Niteroi, Rio de Janeiro, Brazil, were included in the present study. They needed nonsurgical endodontic retreatment because of persistent or emergent apical periodontitis. A detailed dental history was obtained from each patient. Those who had received antibiotic treatment during the last 3 months or who had any general disease were excluded of the study.

All the selected teeth were single rooted, showing the presence of 1 root canal and the absence of periodontal pockets deeper than 4 mm. None of the patients reported spontaneous pain. Teeth that could not be isolated with a rubber dam were excluded. The failure of root canal treatment was determined on the basis of clinical and radiographic examinations. The reasons for retreatment were the presence of persistent apical radiolucent lesions; voids in or around the root canal filling; and persistent symptoms such as pain on palpation (POP), discomfort to percussion, and sinus tract presence. The quality of coronal restoration was evaluated in the present study.

The following clinical/radiographic features were collected for further analyses: tenderness to percussion (TTP), POP, exudation (EX), and the size of the radiolucent area > or #3 mm (SRA). Teeth with clinical symptoms were considered those with the presence of TTP and/or POP, whereas the asymptomatic ones were those showing no clinical symptomatology.

Sampling Procedures

All the materials used in this study were sterilized. The method used for the disinfection of the operative field has been previously described [7]. The sterility of the external surfaces of the crown was checked by taking a swab sample

from the crown surface and streaking it on blood agar plates, which were then incubated both aerobically and anaerobically.

A 2-stage access cavity preparation was made without the use of water spray but under manual irrigation with sterile/apyrogenic saline solution and by using a sterile/apyrogenic high-speed diamond bur. The first stage was performed to promote a major removal of contaminants, including carious lesions and restoration. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the protocol used previously [7]. The sterility of the internal surface of the access cavity was checked as previously described, and all procedures were performed aseptically.

After the sterility of the access cavity was checked, a new sterile bur was used with irrigation with sterile/endotoxin-free saline solution to access the canal. Root-filling materials were removed by rotary instrumentation (Gates-Glidden drills #5, 4, 3, and 2, Dentsply-Maillefer), a Hero-file #20.06 (MicroMega, Besanc on, France), and K-files in a crown-down technique without the use of a chemical solvent accompanied by irrigation with a sterile/ endotoxin-free saline solution. Next, endotoxin and microbial sampling procedures were performed as previously described [22].

***Enterococcus faecalis* isolation and Identification (Culture Analysis)**

In summary, after vortexing, 250 μ L were diluted in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) by using a 10 - fold serial dilution to 10⁻⁴. Fifty microliters of each dilution was spread onto 5% defibrinated sheep blood BHI agar plates (Oxoid). The plates were incubated aerobically at 37 °C. The same dilutions, also plated on 5% sheep blood BHI agar, were incubated at 37 °C in anaerobic atmosphere. All aerobic cultures were examined at 24 to 48 h, whereas anaerobic cultures were

kept for at least 2 weeks but examined for growth every 3 days.

From each bacterial plate, representative colonies of each morphologic type were subcultured. Pure cultures were initially characterized according to their Gram stain characteristic, ability to produce catalase, and gaseous requirements. Facultative gram-positive cocci, catalase negative, were then selected for further identification using the Rapid ID 32 Strep (Bio Merieux, Marcy-l'Etoile, France). Miniapi software (BioMérieux) was used to automatically read ID 32 tests. In order to confirm their identity, all enterococcal strains were subjected to partial 16S rDNA sequencing, and analyzed using the BLAST software of the National Center for Biotechnology Information (NCBI) for species determination. All strains were identified at the species level based on the *E. faecalis* V583 genome sequence (ref. NC 004668.1), showing 100% of identity.

***Enterococcus faecalis* Detection (PCR 16S rDNA)**

The bacterial DNA was extracted from endodontic samples as well as from ATCC bacteria and then purified with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration (absorbance at 260 nm) was determined by using a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE).

The PCR reaction was performed in a thermocycler (My- Cyclor; Bio-Rad, Hercules, CA) at a total volume of 25 mL containing 2.5 mL 10xTaq buffer (Invitrogen, Eugene, OR), 0.5 mL deoxyribonucleoside triphosphate mix (25 mmol/L each deoxyribonucleoside triphosphate [ie, dATP, dCTP, dGTP, and dTTP]; Invitrogen), 1.25 mL 25 mmol/L MgCl₂, 0.25 mL forward and reverse universal primers (0.2 mmol/L; Invitrogen), 1.5 mL sample DNA (1 mg/50 mL), 1.5 mL Taq DNA polymerase (1 U, Invitrogen), and 17.25 mL nuclease-free water. Primer

forward and reverse sequences as well as PCR cycling parameters used for the detection of *E. faecalis* species was: Primer sequence: Forward: CCG AGT GCT TGC ACT CAA TTG G; Reverse: CTC TTA TGC CAT GCG GCA TAA AC; Cycle Parameter: Initial denaturation 95° C for 2 min e 36 cycles: 95° C for 1 min, 57° C for 1min, 72° C for 1min and a final extension - 72° C for 7 min. [23]. Negative controls corresponded to the reaction mixture without DNA. Either the positive or negative detection of gram-negative target bacteria species was based on the presence of clear bands of expected molecular size. Negative controls corresponded to the reaction mixture without DNA. Either the positive or negative detection of gram-negative target bacteria species was based on the presence of clear bands of expected molecular size.

Detection of the virulence factor genes of ESP, Cyla, gelE and ACE (PCR analysis)

Primers targeting segments of the enterococcal virulence determinants, surface adhesion (ESP), cytolysin activator (Cyla), gelatinase (gelE) and collagen binding antigen (ACE) have been previously described [24,25] (Table I).

Table 1 - Oligonucleotides used in the present study.

Gene	Sequence	Product size (bp)
ACE	GGA ATG ACC GAGAAC GAT GGC	616
	GCT TGA TGT TGG CCT GCT TCC G	
Cyla	GAC TCG GGG ATT GAT AGG C	688
	GCT GCT AAA GCT GCG CTT AC	
ESP	TTG CTA ATG CTA GTC CAC GAC C	932
	GCG TCA ACA CTT GCA TTG CCG A	
gelE	ACC CCG TAT CAT TGG TTT	405
	ACG CAT TGC TTT TCC ATC	

PCR conditions were modified to accommodate a common annealing temperature profile of 58° C for all PCR reactions, based on initial temperature gradient PCR amplifications of each target. Briefly, 100–200 ng total DNA template was prepared for 30 µl PCR amplifications with the following: 6 pmol of each respective primer; 100 µm dNTPs (Invitrogen); 2 U HotStarTaq DNA polymerase (Qiagen); 3 µl 10X PCR buffer (Qiagen). The PCR conditions were as follows: 15 min initial enzyme activation/ DNA denaturation step at 95° C followed by 35 consecutive cycles at 94° C for 20 s; 58° C for 45 s; 72° C for 60 s.

Statistical analysis

The data collected for each case were typed onto a spreadsheet and statistically analyzed using SPSS for Windows (SPSS Inc., Chicago, Ill). The Pearson test or the 1-sided Fisher's exact test, as appropriate, was chosen to test the null hypothesis that there was no relationship between endodontic clinical symptoms and signs and the presence of *E. faecalis*.

RESULTS

Sterility samples taken from the external and internal surfaces of the crown and its surrounding structures, tested before and after entering the pulp chamber, showed no microbial growth. The following clinical/radiographic features were found in root canals with primary endodontic infections investigated: pain on palpation, 3 of 12, tenderness to percussion, 5 of 12, and size of radiolucent area > 3 mm, 7 of 12. Bacteria was recovered in 100% (12/12) of the root canal samples collected from teeth with secondary/ persistent endodontic infection showing apical periodontitis. Culture and molecular methods indicated the presence of *Enterococcus faecalis* in 3/12 (25%) and 5/12 (41.6%), respectively. A total of 38 *Enterococcus faecalis* strains were isolated and submitted to the virulence factor genes analysis. PCR products consistent with genes encoding collagen-binding

antigen (ACE) were found in all isolates 38/38 (100%) (Figure 1). An ESP gene was present in 26/38 isolates (68%). A *Cyla* gene was not recovered from the isolated; and the *gelE* was detected in 31/38 isolates (81%) (Figure 1).

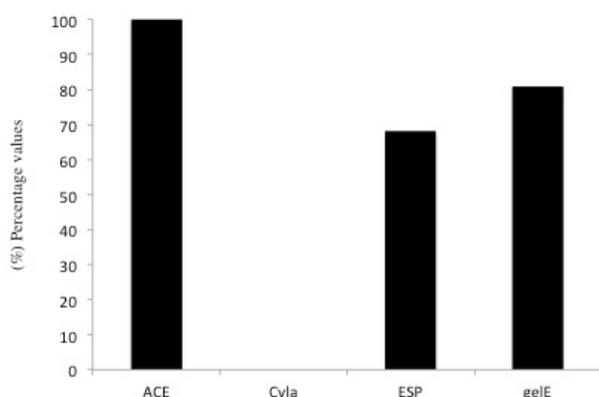


Figure 1 - Recovery of the virulence factor genes of gelatinase (*gelE*), cytolysin activator (*Cyla*), surface adhesin of *Enterococcus* (*ESP*) and collagen adhesin of *Enterococcus* (*ACE*) in 38 isolates strains from secondary/ persistente endodontic infection.

DISCUSSION

Data obtained in the present study revealed by culture and molecular methods revealed a high prevalence of *E. faecalis* in teeth with secondary/ persistent endodontic infection. Moreover, of a clinical relevance, we found different *E. faecalis* strains carrying different virulence determinants.

In the present study, using bacterial culture procedures, *E. faecalis* was recovered in 25% of the root canal samples collected from teeth with secondary/ persistent endodontic infection. This finding is in agreement with previous investigations [1,2,10,12,26,27] indicating its prevalence ranging from 24-70% by culture analysis.

Due to the higher sensitivity of the PCR technique over the bacterial culture, which can possibly underestimate the recovery of bacterial species from clinical samples, the present study considered the PCR analysis for the detection of

E. faecalis in secondary/ persistent endodontic infection. The hypothesis of culture analysis in understating the recovery of *E. faecalis* from secondary/ persistent endodontic infection was confirmed in our study. Whilst 25% of *E. faecalis* were indicated by the culture analysis, the PCR technique revealed a higher frequency of *E. faecalis* (in 41.6%). This difference relies on the detection limit of both techniques used. The sensitivity of culture is approximately 104 to 105 cells for target species using nonselective media, while for PCR varies from 10 to 102 cells depending on the technique used [28]. Moreover, PCR can detect nonviable or viable cells [29].

Currently, the PCR technique revealed the presence of *E. faecalis* in 41.6% of endodontic isolated. Our finding is in agreement with different authors [10, 12, 27, 30]. Authors [10,12] using molecular technique found *E. faecalis* in 77% and 67%, respectively. These findings disagree with those reported that found this microorganism in 22% of unsuccessfully endodontically treated teeth [27]. This variation in the recovery of *E. faecalis* from secondary/ persistent infection might be attributed to case selection (i.e. clinical features) as well as geographic location.

Of a clinical relevance, we found different *E. faecalis* strains carrying different combinations of virulence factors. The potential virulence factors investigated in the present study included the surface adhesion (*ESP*), cytolysin activator (*Cyla*), gelatinase (*gelE*) and collagen binding antigen (*ACE*).

A potential explanation for the persistence of *E. faecalis* in the root canal might be an association with expression of adherence factors. ACE (adhesin of collagen from enterococci) protein was present in all endodontic strains, similar to reports for 'medical' strains [15,24]. According to previously report, the ACE gene may aid binding of *E. faecalis* to dentin [11,31].

The *ESP* gene was found in 68% of endodontic isolates. Such finding is in agreement

with reported the ESP gene in 61% of endodontic isolates from secondary/ persistent endodontic infection [11]. The ESP protein is associated with colonization and persistence of *E. faecalis* in urinary tract infections in mice [20].

The gelE (gelatinases) was detected in 81% of endodontic isolated. The high detection of gelE in *E. faecalis* strains recovered from root canal infection was previously reported [11]. It is worth to point out that the Gelatinases are extracellular peptidases that hydrolyze gelatin, collagen and other proteins (peptides) [21,32]. They are produced by a large number of *E. faecalis* isolates from hospitalized patients or those with endocarditis. The test checks if gelatinase microorganisms in a culture medium are capable of displaying gelatinase activity in the presence of gelatin as substrate. The gel gene encodes an extracellular metalloendopeptidase, also known as gelatinase, which is an enzyme that hydrolyzes gelatin, collagen, casein, hemoglobin, and other bioactive compounds [33]. The gelatinase produced by *Enterococcus faecalis* subsp. *liquefaciens* had the sequence of its gene determined by Su et al [21]. Significant homology was found with this species of *Bacillus* proteinases and elastase of *Pseudomonas aeruginosa* [21].

Overall, the present study revealed by culture and molecular methods a high prevalence of *E. faecalis* in teeth with secondary/ persistent endodontic infection. Moreover, of a clinical relevance, we found different *E. faecalis* strains carrying different virulence determinants.

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