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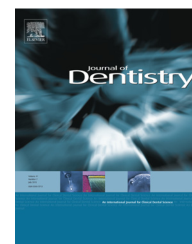

Antimicrobial resistance and virulence traits of *Enterococcus faecalis* from primary endodontic infections

Renata Ximenes Lins [a,*], *Aurimar de Oliveira Andrade* [a], *Raphael Hirata Junior* [b], *Melanie J. Wilson* [c], *Michael A.O. Lewis* [c], *David W. Williams* [c], *Rivail Antonio Sergio Fidel* [a]

[a] School of Dentistry, Rio de Janeiro State University, Rio de Janeiro, Brazil
[b] School of Medical Sciences, Rio de Janeiro State University, Rio de Janeiro, Brazil
[c] School of Dentistry, College of Biomedical and Life Sciences, Cardiff University, Cardiff, Wales, United Kingdom

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ABSTRACT

Objectives: To determine the phenotypic and molecular characteristics of *Enterococcus faecalis* recovered from primary endodontic infections in Brazilian patients.
Methods: Twenty isolates of *E. faecalis* recovered from 43 Brazilian patients with primary endodontic infections were identified by biochemical profiling (API20Strep) and 16S rDNA sequencing. Antimicrobial susceptibility was ascertained by agar dilution, using the recommended protocol of the Clinical and Laboratory Standards Institute (CLSI). PCR with validated primers was used to detect genes associated with antibiotic resistance and specific virulence factors.
Results: All isolates were deemed susceptible to penicillin G, erythromycin and vancomycin. However, nine isolates had a minimum inhibitory concentration of 4 μg/mL to vancomycin (the resistance breakpoint). Fourteen isolates (70% of isolates) were also resistant to tetracycline with MICs of >64 μg/mL. PCR products for tetracycline resistance genes were detected in test isolates, while erythromycin and vancomycin resistance genes were not evident. Gelatinase, aggregation substance and enteroccocal surface protein genes were detected in 20, 18 and 12 isolates, respectively.
Conclusions: Endodontic *E. faecalis* isolates exhibit high level of resistance to tetracycline, an antibiotic that has use in local treatment of dental infections. This opens up a much-needed debate on the role and efficacy of this antibiotic for oral infections. Furthermore, these isolates were shown to possess genes that could contribute to pathogenicity in the pulp cavity.

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1. Introduction

The microbiome of primary and post treatment endodontic infections has been extensively studied, although the main aetiological agents remain unclear. Ozok et al.[1] suggested that endodontic infections were more complex than previously reported with more than 600 bacterial taxa associated with infected root canals. Although a single aetiological agent is unlikely, there is evidence supporting the association of

* *Corresponding author at*: Av Afranio de Mello Franco, 141/207 Leblon, CEP 22430-060, Rio de Janeiro, RJ, Brazil. Tel.: +55 21 2294 8268.
E-mail addresses: ximenes@uerj.br, endodontia@renataximenes.com.br (R.X. Lins).
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enterococci, particularly *Enterococcus faecalis* with both primary and secondary endodontic infection.[2–8]

Enterococci are Gram-positive, coccus-shaped bacteria and frequent colonisers of the human gastrointestinal and genitourinary tracts. In the past, these bacteria were not considered particularly virulent. However, recent years have seen an increase in nosocomial infections caused by enterococcal species largely attributed to their antimicrobial resistance profiles.[9] Of the group, *E. faecalis* is considered the most significant species, both in terms of frequency of isolation from sites of infection and in the transfer of antibiotic resistance.[10]

Enterococci are regarded as antibiotic-resistant, opportunistic pathogens frequently recovered from patients who have received multiple courses of antibiotics and who have been hospitalized for prolonged periods. These bacteria exhibit intrinsic resistance to several antibiotics, as well as an ability to rapidly acquire antibiotic resistance. Enterococci are not generally regarded as normal inhabitants of the oral cavity, but may colonise transiently, particularly in debilitated individuals.[11] *E. faecalis* has, however, been isolated from a range of oral conditions including carious lesions, chronic periodontitis, and has been associated with persistent apical periodontitis.[12] *E. faecalis* has been detected primarily at sites of persistent endodontic infections,[9,13–15] and has also been found in high numbers in some primary endodontic infections.[7,8,16]

Neither the source, nor the role of enterococci in the pathogenesis of endodontic infections is understood.[15,17] Oral *E. faecalis* has been shown to possess a range of virulence factors including gelatinase activity, expression of haemolysin, response to pheromones, bacteriocin production, biofilm development, expression of adherence factors, aggregation substances and resistance to several antibiotics.[12,18–21] Importantly, enterococci have been shown to transfer certain virulence traits to related species in root canals *ex vivo*, highlighting the need to more fully understand the role of these pathogens in infection in general.[22]

Finally, it is not unrealistic to expect that enterococci resistant to multiple antimicrobials may be able to colonise the root canal environment. Although Vancomycin Resistant Enterococci (VRE) have not been isolated in root canal infections, Nandakumar et al.[23] identified proteins from the *tet* and *van* operons in all the patient cases where enterococci were detected by genomic analysis of endodontic infections. Additionally, in the patient sample from which the VanE protein was identified, *E. faecalis* was highly prevalent. This is important from a treatment perspective because antibiotics are known not to be very effective in treating chronic or localised acute endodontic infections or in preventing recurrent episodes of infection. Besides, it is an alert to the potential increase in antibiotic resistance amongst oral isolates.

All this information justifies the isolation, identification and characterisation of virulence factors and antimicrobials resistant determinants of endodontic enterococci. Genetic analysis can be used as an initial screening tool, and although detection of the target gene itself does not necessarily mean that the encoded protein will subsequently be expressed, it does indicate the potential for expression.

The aims of this study were therefore to analyse isolates of *E. faecalis* from patients with primary endodontic infections for the presence of defined virulence genes and susceptibility to antibiotics commonly used in dentistry and other areas of clinical importance. Through enhanced awareness of the characteristics of *E. faecalis* involved in endodontic infection, we can identify optimal treatment strategies to improve patients' health and potentially limit dissemination of antimicrobial resistance.

2. Materials and methods

2.1. Patient selection and sample collection

A total of 43 patients with primary endodontic infection were recruited with informed consent for participation in this study. Only necrotic pulps without oral exposition were included. All patients were otherwise healthy and had not been hospitalised or had received antibiotics for at least 6 months prior to the study. Pregnancy or active periodontal disease were additional exclusion criteria. The research was approved by the Research Ethics Committee of Rio de Janeiro State University (COEP051/2009).

After plaque removal and rubber dam isolation, the tooth and the operative field was cleansed with 3% (v/v) hydrogen peroxide and then disinfected with 2.5% NaOCl solution. Coronal access was made using sterile round burs without water spray. Samples were collected from the root canal using a #15 H-type file (Dentsply/Maillefer, Ballaigues, Switzerland) and two sterile paper points, as previously described.[24] Both file and paper points were then transferred to tubes containing Enterococcosel broth (Becton Dickinson Microbiology Systems, MD, USA) and incubated for 48 h at 37 °C to selectively recover enterococci. Isolated colonies were then subcultured on to blood agar plates (Becton Dickinson Microbiology Systems) and pure colonies subjected to identification tests. A sterile paper point without previous contact with the root canal was included in all sampling procedures and served as a negative control.

2.2. Culture and identification of E. faecalis

All isolates were presumptively identified as enterococci based on growth in the presence of bile and azide, and esculin hydrolysis. Additional tests to further characterise these isolates included Gram-staining, catalase activity, motility testing, pyruvate fermentation, and an ability to grow in Tryptic Soy Agar (Merk Darmstadt, DE) supplemented with 6.5% NaCl at 42 °C. A commercial biochemical profiling test (API 20 Strep identification kit; Analytical Profile Index; bioMérieux SA, Marcy-Etoile, France) was also used, and *E. faecalis* ATCC 29212 served as a positive control. The prevalence of *E. faecalis* was recorded as the percentage of cases examined.

2.3. Confirmation of E. faecalis identity by sequencing of 16S ribosomal DNA

Total DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Paisley, UK) and the manufacturer's

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