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Identification of Enterococcus faecalis by PCR in saliva of pediatric patients

Identificação do Enterococcus faecalis por PCR em saliva de pacientes pediátricos

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Abstract

Objective: This study aimed to identify *E. faecalis* in saliva of patients that were divided into two groups: 10 patients with caries lesions and 10 caries-free patients. **Material and methods**: Saliva samples were collected with a sterile swab and inoculated in enterococcosel media for 48 hours. The positive samples were subcultured in broth agar - blood medium for storage and subsequent PCR analysis. The data were analyzed using the SPSS ($\chi 2$). **Results**: From the 20 cases included in the study, 3 were positive in the Enterococcosel medium, and both tests (culture and PCR) used confirmed that three of them belonged to the species *E. faecalis*. No samples were positive in Enterococcosel broth in the group of caries-free patients. From the three samples that were identified as Enterococcus in broth (positive bile esculin test), an amplified for *E. faecalis* PCR analysis ($p > 0.005$). **Conclusion**: Through the use of PCR, it was possible to identify the genus *Enterococcus* and the species *E. faecalis* in saliva of patients with carious lesions – the pathogen that may influence the prognosis of diseases of the oral cavity.

Keywords: *Enterococcus faecalis*, saliva, child, culture, PCR.

Resumo

***Objetivo**: Este estudo objetivou identificar E. faecalis em saliva de pacientes que foram divididos em dois grupos: 10 pacientes com lesões de cárie e 10 livres de cárie. **Material e métodos**: Amostras de saliva foram coletadas com um swab estéril e inoculadas em meios Enterococcosel por 48 horas. As amostras positivas foram repicadas em meio de caldo de agar - sangue para armazenamento e análise de PCR subsequente. Os dados foram analisados utilizando o SPSS. **Resultados**: Dos 20 casos incluídos no estudo, três foram positivos no médio Enterococcosel e ambos os testes (cultura e PCR) utilizados confirmaram que três deles pertenciam à espécie E. faecalis. Nenhuma amostra foi positiva em caldo Enterococcosel no grupo de pacientes livres de cáries. A partir das três amostras que foram identificadas como Enterococcus em caldo (teste positivo esculina biliar), foram amplificados por análise de PCR para o E. faecalis (p>0.005). **Conclusão**: Através da utilização de PCR, foi possível identificar gênero Enterococcus e a espécie de E. faecalis em saliva de pacientes com lesões de cárie – o agente patogênico que pode influenciar o prognóstico de doenças da cavidade oral.*

***Palavras-chave**: Enterococcus faecalis, saliva, criança, cultura, PCR.*

Introduction

Saliva is the fluid that moistens the mouth, being secreted by the salivary glands. It acts as protection of oral mucosa and teeth, helping the formation of food bolus in the beginning of digestion and regulating the pH of the oral environment (1). The composition of oral microflora is influenced by factors such as diet, host defense, antibiotic therapy, and other endogenous and exogenous factors (1, 2). Due to a dynamic balance resulting from the interaction between microorganisms and their host, the oral microflora diversity of the oral microflora can participate in the pathogenesis of several oral diseases (1). The dental plaque and gingival sulcus contain a flora that is unlike that from the saliva. Despite the fact that many variables and consequently many possibilities of error exist in the study of salivary microorganisms, there are nevertheless certain broad areas in which facts may be obtained and generalizations made. There is a great need for quantification of salivary organisms in order to gain precise information about their relative proportions (1). Moreover, more than 700 bacterial species have been detected in the oral cavity (2).

The most common pathogen in permanent teeth associated with failed root canal treatment is Enterococcus faecalis. E. faecalis is a facultative anaerobe, catalase-positive, gram-positive cocci that occurs in pairs or as a short chain. It is the most common bacteria isolated from the gastrointestinal tract of humans. They are also able to colonize the oral cavity (3). These microorganisms have been associated with oral mucosa lesions in immunocompromised patients (4), periodontitis (5), and root canal infections (6-9).

Analysis of the endodontic microbiota is still focused on the detection and identification of bacteria using different methods (culture, DNA-DNA hybridization, polymerase chain reaction-PCR, denaturing gradient gel electrophoresis fingerprint techniques-DGGE). Culture methods have provided a great contribution and have still much to offer in the elucidation of endodontic diseases. However, molecular approaches to detect and identify microbial species have several advantages when compared with culture (3). Molecular methods – particularly PCR – are more specific, accurate, sensitive, and rapid than culture, and can detect uncultivable and fastidious microorganisms (3, 10). PCR is a technique which uses a DNA polymerase enzyme to make a huge number of copies of virtually any given piece of DNA or gene. This technique can readily identify slow-growing as well as uncultivable bacteria (11). The aim of the present study was to investigate the presence of E. faecalis in saliva from children who were attendant in Pediatric Dentistry Clinic by culture and polymerase chain reaction methods.

Material and Methods

Case Description

This study was approved by the Research Ethics Committee of the Institute of Public Health (IESC)

UFRJ (No. 46/2010). The study was conducted in pediatric patients from the Pediatric Dentistry Department of the Federal University of Rio de Janeiro (UFRJ). The guardians of the patients signed an informed consent form to participate in the study.

During this study, 20 patients of both sexes were examined. All samples were collected by one of the calibrated authors. The patients were divided in two groups: 10 patients had some complaint concerning caries (G1) and 10 were caries-free patients (G2). All teeth that had no clinical evidence of necrosis and/or radiographically visible radiolucent areas in the region of the bone furcation and/or the periapical region. Saliva samples were collected from 20 children (aged from 1 to 8 years) who had been referred to the Pediatric Dentistry Clinic of a Public University for treatment in 2010. Patients who had received antibiotic therapy in the last 3 months or had a systemic condition were not included. The sequence used can be observed in Figure 1.

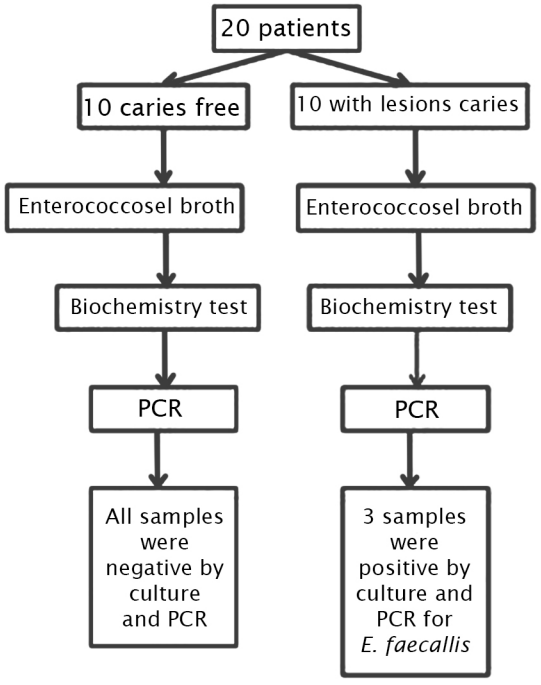

Figura 1 - Flowchart of the steps performed in this study

Sampling Procedures

All samples were collected by one of the authors. Sterile swabs were placed in the oral cavity, on the sites of mucosa (cheek and tongue) used to collect the material, and transferred to a tube containing Enterococcosel broth (Becton Dickinson Microbiology Systems, Cockeysville, MD), a selective medium with bile-esculin and sodium azide, and were incubated for 72 hours at 35 °C.

Culture Procedures

After incubation for 72 hours at 35 °C, samples that presented growth in Enterococcosel broth were plated onto trypticase soy agar plate containing 5% sheep blood, and incubated at 37 °C for 72 hours. Resulting pure colonies were submitted to conventional tests for identification of *Enterococcus* genus and *E. faecalis* species (18, 19). *E. faecalis* was identified by Gram staining, colony morphology, catalase test, growth in NaCl broth, hydrolysis of esculina in the presence of bile salts, hydrolysis of PCR, and LAP, hydrolysis of arginine, pyruvate utilization, motility, pigmentation production, and carbohydrate fermentation tests (arabinose, mannitol, metal-α-D-glucopyranoside, raffinose, sucrose, sorbitol, and sorbose). DNA was also extracted from the isolates and identification was confirmed by PCR using E. faecalis-specific primers.

PCR Procedures

Samples in TSB-DMSO were thawed to 37 °C for 10 minutes and vortexed for 30 seconds. Paper points were removed from the flasks and the microbial suspension was washed three times with 100 µL of ultrapure water by centrifugation for 2 minutes at 2,500 × g. Pellets were then resuspended in 100 µL of ultrapure water, boiled for 10 minutes, and cooled on ice. After centrifugation to remove cell debris for 10 seconds at 9,000 × g at 4 °C, the supernatant was collected and used as the template for PCR amplification. *E. faecalis* was identified using PCR amplification of signature sequences of the 16S rRNA gene (7). The oligonucleotide species--specific primers for E. faecalis were 5'-GTT TAT GCC GCA TGG CAT AAG AG-3' (forward primer, located at base position 165-187 of the *E.faecalis* 16S rDNA, GenBank accession No. Y18293) and 5'-CCG TCA GGG GAC GTT CAG-3' (reverse primer, located at base position 457-474 of the E. faecalis 16S rDNA,

GenBank accession No. Y18293), producing a PCR amplicon of 310 bp. A pair of universal primers (5'-GAT TAG ATA CCC TGG TAG TCC AC-3' and 5'-CCC GGG AAC GTA TTC ACC G-3') that match almost all bacterial 16S rRNA genes at the same position (base positions 786-1387, relative to Escherichia coli 16S rRNA gene sequence, accession No. J01695) were used as a positive control for the PCR reaction. It served to indicate the presence of bacteria in the clinical samples. The reported amplicon length was 602 bp (20). Aliquots of 5 µL of the DNA extracts were used as templates in a PCR reaction specific for detection of E. faecalis. PCR amplification was performed in the final volume of the 50 µL containing 1 µM of each species-specific primer, 5 µL of 10X PCR buffer, 2 mM of MgCl2, 1.25 U of Tth DNA polymerase (Biotools, Madrid, Spain), and 0.2 mM of each deoxynucleoside triphosphate (Biotools). Cycling parameters for PCR reactions using *E. faecalis-specific* primers or universal primers included an initial denaturation step at 95°C for 2 minutes, followed by 36 cycles of a denaturation step at 95 °C for 30 seconds, a primer annealing step at 60 °C for 1 minute, an extension step at 72 °C for 1 minute, and a final step at 72 °C for 2 minutes (19). PCR amplicons were separated by electrophoresis in a 2% agarose gel and visualized on an UV transilluminator.

Data Analysis

Prevalence of *E. faecalis* was recorded as the percentage of cases examined. All data were analyzed by using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Descriptive analysis was performed. Chi-square test was used to compare the data. The significance level was 5% ($p < 0.05$).

Results

The mean age of the patients involved in this study was 5.68 years (±2.26). From the 20 teeth included in the study, 10 cases had some complaint concerning caries (G1), and they revealed positive results in broth with the saliva samples from three patients (30%) in the group with caries lesions. All samples were negative in Enterococcosel broth in the group of caries-free patients. *E. faecalis* was detected by culture procedures and species-specific 16S rRNA gene-based PCR from samples and positive cultures (Enterococcosel broth) in 3 samples. Culture and PCR were as effective as each other in detecting this bacterial species.

Discussion

Enterococcus genus comprises species – including *E. faecalis* – that have participation in oral pathologies, such as caries, recurrent endodontic infections, and apical periodontites. Over 90% of human enterococcal infections are caused by *E. faecalis*, and the other, by *E. faecium* (21). Most studies of the *Enterococcus faecalis* emphasize the root canal contamination, and few authors investigate its presence in saliva and the possible damage. In fact, these bacteria can be part of the natural oral microflora. *Enterococcus* is a heterogeneous group of Gram-positive bacteria that are associated with endodontic infections in dentistry (21). No patient examined – even those with carious lesion – had any involvement in tooth pulp. In the present study, we found this pathogen in 30% of samples isolated from saliva in patients with caries lesions. Although these teeth presented primary infections without a retreatment evaluation, it is important to highlight that the presence of *E. faecalis* in deciduous teeth of children will add knowledge about the early presence of this pathogen in the oral cavity.

In this study, there was a positive correlation between the presence of Enterococcus in the presence of caries lesions, since three patients in the group with caries presented a positive test, and the bile esculin positive response was not observed in the bile esculin test of any patient of the caries-free group. This demonstrates that patients with caries lesions in the oral cavity appear to have increased susceptibility to *Enterococcus*, a pathogen which is not characteristic of the oral cavity. The only case that was positive in the PCR analysis, which has a small percentage of error, was in a patient of the group with caries.

Significant differences were found on the *E. faecalis* prevalence between saliva samples and root canal samples. The root canal system and the oral cavity are closely connected environments, especially in the presence of coronal leakage. Thus, it is feasible that *E. faecalis* species in the root canal originates from the oral cavity or that species in the

root canal spreads to the oral cavity. However, the exact origin of these *E. faecalis* organisms remains unknown (21). It is speculated then that the *E. faecalis* may be involved in diseases such as caries and as has been previously shown to affect diseases of the root canal. *E. faecalis* has the ability to penetrate into dentinal tubules, sometimes to a deep extent, which can enable this species to escape the effects of intracanal antimicrobial procedures (22). In addition, *E. faecalis* has been shown to be able to form biofilms in root canals, and this ability can be important for bacterial resistance to and persistence after endodontic procedures (23).

This is important for the treatment plan for the clinicians. If this pathogen is present in the oral cavity, the dentist has to anticipate the possible damage that the treatment he will propose will cause to the patient. The results of the present study confirmed that both culture and PCR methods were sensitive to detect *E. faecalis* in saliva. Our study showed that the *E. faecalis* is present in saliva, and this affirmation is based on PCR analysis allowing a molecular biology approach to be compared with a traditional culturing method is most reliable3.

Conclusion

Through the use of PCR, it was possible to identify the presence of the *Enterococcus* genus and the species *E. faecalis* in saliva of patients with caries lesions and absence in caries-free patients – the pathogen that may influence the prognosis of diseases of the oral cavity.

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