ANALYSIS OF *ENTEROCOCCUS FAECALIS* IN SAMPLES FROM TURKISH PATIENTS WITH PRIMARY ENDODONTIC INFECTIONS AND FAILED ENDODONTIC TREATMENT BY REAL-TIME PCR SYBR GREEN METHOD

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ABSTRACT

*O*bjective: The aims of this study were to investigate the presence of *Enterococcus faecalis* in primary endodontic infections and failed endodontic treatments using real-time PCR and to determine the statistical importance of the presence of *E. faecalis* in a Turkish population with endodontic infections. Material and Methods: *E. faecalis* was investigated from 79 microbial samples collected from patients who were treated at the Endodontic Clinic of the Dental School of Atatürk University (Erzurum, Turkey). Microbial samples were taken from 43 patients (Group 1) with failed endodontic treatments and 36 patients (Group 2) with chronic apical periodontitis (primary endodontic infections). DNA was extracted from the samples by using a QIAamp® DNA mini-kit and analyzed with real-time PCR SYBR Green. Results: *E. faecalis* was detected in 41 out of 79 patients, suggesting that it exists in not less than 61% of all endodontic infections when the proportion test (z= -1.645, <x= 0.05) was applied. Real-time PCR SYBR Green allowed for the detection of *E. faecalis* in 32 out of 43 (74.4%) in Group 1, and in 9 out of 36 (25%) in Group 2. Conclusions: These results suggest that *E. faecalis* is a frequent isolate for endodontic infections in Turkish patients, and is more often associated with failed endodontic treatments than primary endodontic infections.

Key Words: Endodontic infection. *Enterococcus faecalis*. Failed endodontic treatment. Chronic apical periodontitis. Real-time PCR.

INTRODUCTION

Enterococci are facultative anaerobic, Gram-positive coccus, and part of the normal flora in the oral cavity and gastrointestinal tract. Enterococci possess a number of virulence factors such as aggregation substance, enterococcal surface proteins, gelatinase, extracellular superoxide production, capsular polysaccharides and antibiotic resistance determinant[14,20]. They are recognized as potential human pathogens causing 12% of nosocomial infections. Of the enterococcus species, *Enterococcus faecalis* is the most frequently isolated species from endodontic infections[14,20].

Three distinct clinical categories may be defined in periapical disease: acute apical periodontitis, chronic apical periodontitis, and exacerbated apical periodontitis[5,23]. Chronic apical periodontitis frequently develops and enlarges without any subjective signs and symptoms. This condition is usually associated with periradicular radiolucent changes. These changes range from thickening of the periodontal ligament and resorption of the lamina dura to destruction of apical bone resulting in a well demarcated radiolucency[28].

A strong predominance of strictly anaerobic bacteria is typical of primary endodontic infections (no previous endodontic treatment with necrotic pulp) together with some facultative anaerobes such as streptococci[13]. *E. faecalis* has been found occasionally in cases of primary endodontic infections[1,29]. In contrast, in cases of failed endodontic treatments, *E. faecalis* has been frequently isolated[9,13]. Traditionally, identification of enterococci in diverse sites has been performed by culture methods[11,27].

Cultivation and other traditional identification methods have been demonstrated to have several limitations with respect to microbiological diagnosis. Therefore, techniques that are more sensitive may be necessary to accurately characterize the microbial composition of root-filled teeth with periapical lesions[6]. Recently, molecular genetic

approaches have been used for the identification of enterococci in infections of endodontic origin. Among molecular techniques, the polymerase chain reaction (PCR) technique have been widely used to detect bacteria in primary endodontic infections[2], few studies exist in the literature using PCR to investigate the bacteria causing endodontic treatment failure in Turkish population. For this reason, the purpose of the present study was to investigate the presence of *E. faecalis* in both primary endodontic infections and failed endodontic treatments using a real time PCR with SYBR Green method in a Turkish population.

MATERIAL AND METHODS

Patients and Sampling

Seventy-nine patients (43 patients with failed endodontic treatments in Group 1 and 36 with primary endodontic infections in Group 2) who were referred for endodontic treatment to the Department of Endodontics of the Dental School of Atatürk University (Erzurum, Turkey) were enrolled in this study. A detailed medical and dental history was obtained from each patient. Patients who had received antibiotic treatment during the last 3 months or had a general disease were excluded from the study. Cases with a periodontal pocket probing depth greater than 4 mm and teeth in which proper rubber dam isolation could not be achieved were excluded from the study, as well.

Age, gender, tooth type, coronal restorations, if present; the presence of previous root canal filling, and the presence of periapical radiolucency were recorded for each patient. Clinical symptoms and signs included history of previous pain, tenderness to percussion, pain on palpation, mobility, presence of a sinus tract and its origin (endodontic or periodontal), probing depth of any periodontal pockets.

The diagnosis of chronic apical periodontitis was defined as the presence of periapical radiolucency, where no other clinical symptom had been present in the previous 3 months[5]. Failure of root canal treatment was determined on the basis of clinical and radiographic examinations. Coronal restorations were categorized as sound if they clinically and radiographically appeared intact; and as defective if there were open margins, fracture, or recurrent decay[8]. The teeth with failed endodontic treatments had been root canal treated more than 3 years previously. The previous root canal treatment of the teeth investigated in this study was carried out by unknown operators. Approval to undertake the study was given by the Research Ethics Committee of the Dental School of Atatürk University and informed consent was obtained from all patients.

Samples from cases of chronic apical periodontitis (Group 2) were obtained from the root canals as follows. Initially, each tooth was cleansed with pumice and isolated with a rubber dam. The tooth and the surrounding field were then cleansed with 3% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite solution. Complete access preparations were made using sterile burs, without water spray. The operative field, including the pulp chamber, was then swabbed with 2.5% sodium hypochlorite. NaOCl solution was then inactivated by sterile 5% sodium thiosulfate. Samples were initially collected with a size 15 K-type file (Maillefer, Ballaigues, Switzerland) with the handle cut off. The file was introduced to a level approximately 1 mm short of the tooth apex, based on diagnostic radiographs, with a discrete filing motion. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal. Afterward,s two sequential sterile paper points were placed to the same level and used to soak up the fluid in the canal. Each paper point was retained in position for 1 min. The cut file and the two paper points were then transferred with sterile forceps to cryotubes containing 1 ml of 5% dimethyl sulfoxide (DMSO) in trypticase soy broth (TSB)[17]. Samples were then immediately frozen at -20ºC until they were processed.

Cases with failed endodontic treatments (Group 1) were sampled as follows. After plaque removal, isolation, and disinfection of the operative field as described above, coronal restorations were removed. Endodontic access was performed using sterile burs, without water spray. After completion of endodontic access, the tooth, clamp, and adjacent rubber dam were disinfected again with 2.5% NaOCl. This solution was inactivated with sterile 5% sodium thiosulfate. Preexisting root canal fillings were removed using Gates Glidden drills (Maillefer) and the apical material was retrieved using K- type files without the use of chemical solvents. Whenever possible, the retrieved material was transferred to cryotubes containing 1 ml of 5% dimethyl sulfoxide (DMSO) in trypticase soy broth (TSB). Radiographs were taken to ensure that all filling material had been removed. A small amount of sterile saline solution was then introduced into the root canal by sterile syringe, and the canal walls were filed so that material could be obtained. Root canal contents were then absorbed into at least three paper points. These paper points were then transferred to cryotubes containing 1 ml of 5% dimethyl sulfoxide (DMSO) in trypticase soy broth (TSB)[17]. Samples were then immediately frozen at -20ºC until they were processed.

The criteria used to choose the canal to be microbiologically investigated in the multirooted teeth were the presence of exudation, or in its absence, the largest canal or the canal associated with periapical radiolucency. Before sampling the selected canal of the multirooted teeth, the entrance of the other canals was closed with sterile cotton pellets[8].

Obtaining and culturing of the bacterial strain for a positive control

As a positive control, an *E. faecalis* strain from the bacterial stocks of the authors' laboratory at the Department of Microbiology and Clinical Microbiology was used. The microorganim had been previously identified based on fatty acid profiles using the MIDI Sherlock® Microbial Identification System (MIS) (MIDI, Inc., Newark, DE, USA). The strain was grown aerobically in Todd Hewitt Broth (Difco®, Sparks, MD, USA) for 24 h at 37ºC. After

growth was achieved, the bacteria were collected in 2 mL tubes containing TSB-DMSO.

DNA extraction from the positive control and patient samples

Microbiological samples and positive control were thawed and vortexed vigorously, centrifuged at 8,000 x g for 5 min, then the supernatants were removed and the pellets were used for DNA extraction by using a QIAamp® DNA mini-kit (QIAGEN Inc., GmbH, Hilden, Germany). The protocol recommended by the kit manufacturer for DNA extraction from the tissue samples was followed precisely.

Ubiquitous- and species-specific primers

All primers were designed as previously described by Sedgley, et al.[22] (2005). 16S rRNA-directed species-specific primers were a forward 5'-3' CCGAGTGCTTGCACTCAATTGG and a reverse 5'-3' CTCTTATGCCATGCGGCATAAAC. Ubiquitous primers directed to 16S rRNA were a forward 5'-3' TTAAACTCAAAGGAATTGACGG and a reverse 5'-3' CTCACGACACGAGCTGACGAC.

PCR amplification protocol

The amplification and detection of DNA with species-specific and ubiquitous primers by RT- PCR were performed with the iCycler iQ Multicolor Real-time PCR Detection System (BIO-RAD® Laboratories, Inc., Hercules, CA, USA). For each RT-PCR, iQ™ SYBR® Green Supermix (BIO-RAD® Laboratories, Inc.) supplemented 4.5% DMSO was used. A 50 μl total PCR amplification volume for each reaction was placed in each well of a 96-well MicroAmp Optical Reaction Plate and covered with Optical-Quality Sealing Tape (BIO-RAD® Laboratories, Inc.). The DNA amplification conditions for both PCR with ubiquitous primers and species-specific primers were 14 min initial denaturation at 95°C, followed by 50 consecutive cycles at 95°C for 30 s, 68°C for 45 s, 72°C for 30 s, and 75°C for 30 s for data collection.

The DNA amplification conditions for PCR with species-specific primers were 14 min initial denaturation at 95°C, followed by 50 consecutive cycles at 94°C for 1 minute, 54°C for 45 s, 72°C for 45 s and data collection and real-time analyses enabled at 60°C for 1 minute, followed by 75 consecutive cycles at 60°C for 15 s, set point temperature was increased after cycles 2 by 0.5°C, and melt curve data collection and analyses enabled.

PCR amplifications with species-specific primers were repeated one more time to confirm the results obtained during the first study.

Statistical Analyses

The hypothesis test for one proportion[30] was used to estimate the highest possible rate of *E. faecalis* in the endodontic infections. Fisher's exact chi-square test was applied using Microsoft® SPSS (Statistical Package for Social Science for Windows, version 13.0; SPSS Inc., Chicago, IL USA) to analyze a statistically significant difference between the primary endodontic infections and failed endodontic treatments groups as to the number of *E. faecalis*-positive samples. The significance level was set at 0.05.

RESULTS

Some signs and symptoms were present in 43 canals with failed endodontic treatment, as follows: 8 cases with spontaneous pain; 30 with tenderness to percussion; 3 with pain to palpation; 1 with sinus tract; 2 with purulent exudates. Most teeth with necrotic pulps presented caries (21/36) and amalgam or composite coronal restorations (10/36). Teeth with previous root canal treatment had amalgam (20/43) or composite (23/43) coronal restorations.

All amplifications were obtained using ubiquitous primers, suggesting that there was no PCR inhibitor in the DNA samples used (data not shown).

PCR amplifications with species-specific primers gave the similar results when they were repeated. 68°C of melting temperature for the PCR product obtaining with species specific primers was used to establish positive results. Also, 68°C of melting temperature was proved by amplification of DNA from *E. faecalis* used as positive control DNA.

E. faecalis was detected in 43 out of 79 (51.9%) patients with endodontic infections. According to the left-tailed hypothesis test for one proportion[30], the presence of *E. faecalis* in the endodontic infections was not lower than 61% statistically (z= -1.645, α= 0.05). The real-time PCR method enabled the detection of *E. faecalis* in 32 out of 43 (74.4%) failed endodontic treatments. Additionally, *E. faecalis* was detected in 9 out of 36 (25%) of the root canals associated with chronic apical periodontitis (primary endodontic infections). When these results were taken into consideration, a statistically important difference was estimated between the primary endodontic infection and failed endodontic treatment groups as to the existence of *E. faecalis*, indicating that *E. faecalis* is significantly more often associated with failed endodontic treatments than primer endodontic infections (p<0.01; Fisher's exact chi-square test).

DISCUSSION

Traditionally, endodontic bacteria have been studied by culture techniques, which rely on the isolation, growth, and laboratory identification according to morphology and biochemical tests. However, cultivation and other traditional identification methods have been demonstrated to have several limitations regarding microbiological diagnoses. The past decade has brought many advances in microbial molecular diagnostics, the most prolific of which are DNA-DNA hybridization, PCR technology, and its derivative[25].

The PCR method is based on the *in vitro* replication of DNA through repetitive cycles of denaturation, primer annealing, and extension steps. PCR has unrivalled sensitivity, being at least 10-100 times more sensitive than

the other more sensitive identification methods[25], 2005). Conventional PCR assays are qualitative or can be adjusted to be semi-quantitative; one exception is real-time PCR. Real-time PCR methods are very sensitive to reagent variables. The advantages of real-time-PCR include the rapidity of the assay, the ability to quantify and identify PCR products directly without the use of agarose gels, and the fact that contamination of the nucleic acids is limited because of avoidance of post-amplification manipulation[15].

In the present study, *E.* faecalis was detected in 25% of the samples taken from primary endodontic infections. Siqueira, et al.[26] (2002) analyzed the prevalence of *Actinomyces* spp., streptococci, and *E. faecalis* in primary root canal infections, of which 26 had asymptomatic, by using molecular genetics methods. Those authors found *E. faecalis* in 11.5% in asymptomatic lesions. Fouad, et al.[7] (2005) detected *E. faecalis* in 8% of primary endodontic infections using PCR. Another recent study[19] identified this microorganism in 89.3% of cases with primary endodontic infections by using checkerboard DNA-DNA hybridization, which are different results from those of the present experiment. The discrepancies may be caused by different molecular techniques employed and could be attributed to both the sensitivity of the molecular method and the nature of the clinical material selected for the present study. Real-time PCR was used hereby without quantification of the positive DNA amplified. Real-time PCR methods are very sensitive to reagent variables[3]. The higher sensitivity of the real-time PCR method could be attributed to the fact that it potentially targets free-floating DNA and DNA from non-viable, cultivable viable cells, and viable, but non-cultivable cells[21]. On the other hand, the findings of the present study agree with those of Roças, et al.[16] (2004), who detected *E.* faecalis in 18% of primary endodontic infections using a PCR method.

In the present investigation, *E. faecalis* was detected in 74.4% of root-filled teeth using real-time PCR, which disagree with the findings or previous studies[7,18]. Fouad, et al.[7] (2005) found *E. faecalis* in 22% of unsuccessfully treated teeth using PCR and Rolph, et al.[18] (2001) have not found this bacterium in their study using culture and PCR. The differences between our results and those of the two studies mentioned above are probably due to geographic differences and may also be due to differences in molecular techniques employed. Some researchers[8,16,17,24] using molecular methods, found *E. faecalis* in 64, 77, 67 and 76% of failed endodontic treatments, respectively, supporting our outcomes.

The high rate detection of *E. faecalis* by real-time PCR in root-filled teeth, as observed in the present study, may have been associated with the ability of *E. faecalis* to invade dentinal tubules and to adhere to collagen and the resistance of *E. faecalis* to calcium hydroxide, which is commonly used during intracanal medication[4].

There is consensus that intraradicular infection is the essential cause of primary apical periodontitis and the major cause of secondary endodontic infections. Therefore the goal of endodontic treatment has been to eliminate infectious agents or to substantially reduce the microbial load from the root canal and to prevent reinfection by root filling[12].

According to the obtained results, *E. faecalis* was shown to be significantly more often associated with secondary endodontic infections than primer endodontic infections should lead to the development of more effective antimicrobial strategies during root canal treatment and retreatment. Because of the resistance of *E. faecalis* to calcium hydroxide[12], other medicaments have been proposed. Sodium hypochlorite has become the irrigant of choice worldwide, but chlorhexidine has also been suggested as an irrigant, a 2% chlorhexidine solution has been proven more effective against *E. faecalis* than sodium hypochlorite[10].

In conclusion, column extraction method was used to obtain the DNA from samples. This was a powerful method because all DNA samples were amplified with ubiquitous primers, which indicates that all DNA aliquots were almost pure. Otherwise it would not be possible to obtain amplification by ubiquitous primers. Melting analysis by real-time PCR SYBR Green method was a very convenient method. All results obtained at the end of the PCR reaction and melting analysis without additional gel electrophoresis to see the specific DNA band. The findings of the study indicated that *E. faecalis* is an important microbial agent for endodontic infections in Turkish patients, and is more often associated with failed endodontic treatments than primary endodontic infections.

ACKNOWLEDGEMENTS

REFERENCES

1- Baumgartner JC, Falkler WA Jr. Bacteria in the apical 5mm of infected root canals. J Endod. 1991;17:380-3.

2- Baumgartner JC, Siquera JF Jr, Xia T, Roças IN. Geographical differences in bacteria detected in endodontic infections using polymerase chain reaction. J Endod. 2004;30:141-4.

3- Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol. 2002;29:23-39.

4- Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J. 2002;35:221-8.

5- Foschi F, Cavrini F, Montebugnoli L, Stashenko P, Sambri V, Prati C. Detection of bacteria in endodontic samples by polymerase chain reaction assays and association with defined clinical signs in Italian patients. Oral Microbiol Immunol. 2005;20:289-95.

6- Fouad AF, Barry J, Caimano M, Clawson M, Zhu Q, Carver R, et al. PCR-based identification of bacteria associated with endodontic infections. J Clin Microbiol. 2002;40:3223-31.

7- Fouad AF, Zerella J, Barry J, Spangberg LS. Molecular detection of Enterococcus species in root canals of therapy-resistant endodontic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2005;99:112-8.

8- Gomes BPFA, Pinheiro ET, Sousa ELR, Jacinto RC, Zaia AA, Ferraz CCR, 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 Endod. 2006;102:247-53.

9- Hancock HH, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001;91:579-86.

10- Himel VT, McSpadden JT, Goodis HE. Instruments, materials, and devices. In: Cohen S, Hargreaves KM, editors. Pathways of the pulp, ninth edition. St. Louis: Mosby; 2006. p. 233-89.

11- Molander A, Reit C, Dahlén G, Kvist T. Microbiological status of root filled teeth with apical periodontitis. Int Endod J. 1998;31:1-7.

12- Nair PN, Henry S, Cano V, Vera J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after 'one-visit' endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2005;99:231-52.

13- Peciuline V, Reynauld AH, Balciuniene I, Haapasalo M. Isolation of yeasts and enteric bacteria in root filled teeth with chronic apical periodontitis. Int Endod J. 2001;34:429-34.

14- Portenier I, Waltimo TMT, Haapasalo M. *Enterococcus faecalis* the root canal survivor and 'star' in post treatment disease. Endod Topics. 2003;6:135-59.

15- Raoult D, Fournier PE, Drancourt M. What does the future hold for clinical microbiology? Nat Rev Microbiol. 2004;2:151-9.

16- Roças IN, Jung IY, Lee CY, Siqueira JF Jr. Polymerase chain reaction identification of microorganisms in previously root-filled teeth in a South Korean population. J Endod. 2004;30:504-8.

17- Roças IN, Siqueira JF Jr, Santos KR. Association of *Enterococcus faecalis* with different forms of periradicular diseases. J Endod. 2004;30:315-20.

18- Rolph HJ, Lennon A, Riggio MP, Saunders WP, MacKenzie D, Coldero L, et al. Molecular identification of microorganisms from endodontic infections. J Clin Microbiol. 2001;39:3282-9.

19- Sassone L, Fidel R, Figueiredo L, Fidel S, Faveri M, Feres M. Evaluation of the microbiota of primary endodontic infections using checkerboard DNA-DNA hybridization. Oral Microbiol Immunol. 2007;22:390-7.

20- Schleifer KH, Klipper-Balz R. Transfer of *Streptococcal faecalis* and *Sterptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int J Syst Bacteriol. 1984;34:31-4.

21- Sedgley C, Nagel A, Dahlén G, Reit C, Molander A. Real-time quantitative polymerase chain reaction and culture analyses of *Enterococcus faecalis* in root canals. J Endod. 2006;32:173-7.

22- Sedgley C, Nagel A, Shelburne CE, Clewell DB, Appelbe O, Molander A. Quantitative real-time PCR detection of oral *Enterococcus faecalis* in humans. Arch Oral Biol. 2005;50:575-83.

23- Sigurdsson A. Pulpal diagnosis. Endod Topics. 2003;5:12-25.

24- Siqueira JF Jr, Roças IN. Polymerase chain reaction based analysis of microorganisms associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;97:85-94.

25- Siqueira JF Jr, Roças IN. Exploiting molecular methods to explore endodontic infections: Part 1-Current molecular technologies for microbiological diagnosis. J Endod. 2005;31:411-23.

26- Siqueira JF Jr, Roças IN, Souto R, Uzeda M, Colombo AP. Actinomyces species, streptococci, and *Enterococcus faecalis* in primary root canal infections. J Endod. 2002;28:168-72.

27- Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiological analysis of teeth with failed endodontic treatment and the outcome of conservative retreatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1998;85:86-93.

28- Torabinejad M, Walton RE. Periradicular lesions. In: Ingle JI, Bakland LK, editors. Endodontics. 5th ed. London: BC Decker Inc; 2002. p. 175-201.

29- Weiger R, Manncke B, Werner H, Löst C. Mibrobial flora of sinus tracts and root canals of non vital teeth. Endod Dent Traumatol. 1995;11:15-9.

30- Weiss NA, Hassett MJ. Introductory statistics. New York: Addison-Wesley Publishing Company; 1993.