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Recovery of *Enterococcus faecalis* in root canal lumen of patients with primary and secondary endodontic lesions

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SUMMARY

The presence of *Enterococcus faecalis* in root canal teeth affected by primary and secondary periapical lesions was studied using polymerase chain reaction (PCR) assays. The association between presence of *E. faecalis* with clinical signs of apical lesions was assessed to evaluate a possible relationship between clinical findings.
Microbial samples were obtained from healthy patients affected by different periapical lesions, 79 teeth with primary periapical lesion and 23 with secondary periapical lesion. For each tooth, clinical symptoms and X-ray appearance were examined.
E. faecalis was detected in 6 of 79 samples with primary lesion (7.6%), and in 9 of 23 with secondary lesion (39.1%). Suggested association was found between *E. faecalis* and secondary apical lesions. As regard specific signs and symptoms *E. faecalis* was more associated with asymptomatic lesions (all $p<0.05$) than with symptomatic apical lesions.
The study confirms the high presence of *E. faecalis* in secondary apical lesions. However, its effective role in endodontic pathogenesis such as bone periapical lesions needs to be clarified.

KEY WORDS: Clinical signs, *Enterococcus faecalis*, PCR, Endodontic lesion

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INTRODUCTION

In dentistry, Enterococci have long been implicated in secondary or persistent root canal infection (Sedgley *et al.*, 2005). *Enterococcus faecalis* is a persistent micro-organism that is probably able to survive in the root canal as a single organism or as a major component of the flora (Evans *et al.*, 2002; Portenier *et al.*, 2003). It has been suggested that this species is involved in the pathogenesis of secondary endodontic apical lesions (Tronstad and Sunde, 2003). Nevertheless, there are some reports in the literature that have demonstrated that *Enterococci* can also be found in root-filled teeth with no apical (periapical) lesions (Zoletti *et al*., 2006) and also in primary endodontic lesions (Ferrari *et al.* 2005, Siqueira, 2002; Sakamoto *et al*., 2006). Finally, the association of *E. faecalis* with the specific signs and symptoms of periapical lesions is not well defined. The typical symptoms associated with apical lesions are pain to percussion, swelling and tenderness to percussion. Apical radiolucency detected by intra-oral Rx is more frequent in chronic apical lesion or in re-exacerbated apical lesions and are caused by a localized bone defect in the

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root apical region (Nair *et al*., 2005). Apical bone defects are more common in chronic lesions than in acute symptomatic lesions (Nair *et al*., 2005 and 2006).
Only few clinical studies have been performed in an Italian population detecting the presence of this pathogen in primary and in secondary endodontic lesions (D'Arcangelo *et al.*, 1999).
The aim of the present study was to use PCR techniques to investigate the correlation between *E faecalis*, identified within root canals in primary and secondary endodontic lesions, and the presence of signs and symptoms. The role of this micro-organism in patients with primary and secondary apical endodontic lesions with and without bone lesions has to be clarified.

MATERIAL AND METHODS

Patients

The study population consisted of 102 patients presenting at the Endodontic Clinical Section of the Department of Dental Science-University of Bologna, Italy for endodontic treatments. Medical histories revealed that all patients were in good general health and had no important systemic diseases such as diabetes. Patients that had received antibiotic therapy during the last two months before root canal therapy were excluded from the study. The patient ages ranged from 16 to 73 years, mean ± SD: 36.7 ± 15.6 years. During the first visit, written informed consent was obtained from each patient before inclusion in the study.
Only third molars were excluded from the study for anatomical reasons, but all the other types of teeth (i.e. molars, canines etc.) were included. Lesions with periodontal pocket probing greater than 4.0 mm were excluded due to possible endodontic-periodontal infection. Another exclusion criterion was teeth in which proper rubber dam isolation could not be achieved during the sampling procedures and followed endodontic retreatment. We collected 79 primary endodontic (peri)apical lesions and 23 secondary (peri)apical endodontic lesions.

Clinical signs and symptoms

Clinical features were recorded for each tooth. The following clinical data were collected: presence of previous root canal filling, pain, tenderness to percussion or palpation, swelling, and periapical radiolucency.
For all teeth the presence of a periapical radiolucency was assessed using the periapical index (PAI), determined with a paralleling X-ray technique, according to Orstavik *et al*. (1986). Teeth with a PAI score equal to or greater than '3' (signs of structural changes of bone periapical structure with mineral loss and anatomical lesion) were considered to be affected by (peri)apical bone lesions.

Specimen sampling

Endodontic samplings from teeth of different patients were obtained during the first visit for root canal therapy. After anaesthesia, a rubber dam was placed and surface disinfection of intact enamel was carried out using a small cotton pellet immersed in NaOCl 5.25% (Niclor 5, Ogna, Muggiò, Italy) as described by Ng *et al*. (2003). The antimicrobial solution was soaked up with a second dry sterile cotton pellet. No rubber dam leakage was observed during the access cavity procedure. Access cavity preparations were made using sterile burs with sterile water spray supplied by Logos Junior and Duo dental units (Castellini S.p.A., Castel Maggiore, Italy), equipped with an Autosteril system (Montebugnoli & Dolci, 2002). The patency of each canal was assessed by inserting a sterile #10 or 15 K-file (Dentsply-Maillefer, Ballaigues, CH) so that the tip was approximately 2-4 mm short from the apex, previously measured on the pre-operative radiograph. In cases of previously filled root canals (secondary apical lesions group), gutta-percha was preliminary removed without chemical solvents with the use of # 4, 3 and 2 Gates Glidden burs (Dentsply-Maillefer, Ballaigues, CH) and # 10-15 K-files. To obtain microbial samples, two or more paper points (ADA products-Mynol, Milwaukee, WI, USA) were placed into the root canal and retained inside for 40 seconds. The paper points were then immediately transferred to sterile 1.5 ml tubes (Eppendorf AG, Hamburg, Germany) containing 500 µl of sterile phosphate buffered saline (PBS) solution. Samples were frozen immediately at -20°C and stored up to one-two months until assayed by PCR.

PCR assays

DNA extraction of samples was performed using the QIAamp DNA Blood Mini Kit (Qiagen GmbH,

Hilden, Germany) according to the manufacturer's instructions. To control for the efficiency of DNA extraction and the absence of PCR inhibitors, a partial region of the human *Hfe* gene (390 bp) was amplified for each sample using a specific pair of primers (Hfe1 5'-TGGCAAGGGTAAACAAGATCC-3', Hfe2 5'-CTCAGGCACTCCTCTCAACC-3').
In addition, the presence of different *Enterococcus* species within the root canal samples was first investigated by amplifying the *Enterococcus* spp. *tuf* gene with genus-specific primers (Table 1). The samples yielding a positive result for the presence of *Enterococcus* spp. were further investigated for *E. faecalis* using specific primers targeting the *ddl* gene (Table 1). The DNA extracted from two clinical isolates of *E. faecium* and *E. faecalis* respectively was amplified as a positive control. The specificity of each primer-pair was confirmed using the BLAST software available on-line at http://www.ncbi.nlm.nih.gov/blast. Primers were custom synthesized by PRIMM (Milan, Italy). The amplifications were performed in 30 µl total final volume, containing 10 mM Tris-HCl, 50 mM KCl, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1.5 U Taq polymerase (Takara, Shiga, Japan) and a specific primer pair.
The concentration of primer was 0.4 µM for *Enterococcus* spp. and the human *Hfe* gene. For each sample 10 µl of extracted DNA was added to the reaction mixture, and PCRs were performed in a Mastercycler thermalcycler (Eppendorf, Hamburg, Germany) under optimized conditions as reported in Table 1. For detection of the *Hfe* gene, 35 amplification cycles were used (1 min at 95°C, 1 min at 61°C and 1 min at 72°C). An initial denaturation step of 3 min at 95°C preceded the amplification cycles, followed by a final extension step of 3 min at 72°C in each PCR reaction. The amplification products were analyzed by 2% agarose gel electrophoresis in TBE buffer (Tris-borate EDTA) at 100V for 2h. The gels were stained with ethidium bromide (0.5 µg/ml) and the PCR products were visualized under UV light with a TFX-20M Gibco BRL (Gaithersburg, MD, USA) UV Transilluminator. The identity of each band was inferred by comparison with a molecular weight ladder (DNA Marker IV, Roche, Penzberg, Germany) using the 1D image analysis software (Kodak Digital Science, Rochester, NY, USA).

Data analysis
Data collected for each sample were recorded on an electronic data spreadsheet and analyzed with SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistical analysis was performed using the Pearson Chi-square test or the one-sided Fisher's Exact test, as appropriate. The null hypothesis was that there was no correlation between different clinical signs of apical lesions and the detection of specific bacteria strains in sampled root canals.

RESULTS

Table 2 shows the incidence of cases in the study groups, according to their different clinical categories. Specifically, 79/102 teeth presented primary endodontic infection, while 23/102 pre-

TABLE 1 - *PCR primers, with expected amplicon size and thermocycling parameters, for endodontic pathogens investigated in the present study.*

Bacterial species	*Primers sequence*	*Amplicon size*	*Amplification*	*Reference*
	(from 5' to 3')	*(bp)*	*cycles*	
Enteroccoccus species	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112 bp	35 cycles 95°C 30 s 58°C 45 s 72°C 20 s	Ke et al. (1999)
E. faecalis	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941 bp	36 cycles: 95°C 30 s 47°C 45 s 72°C 40 s	Dutka-Malen et al. (1995)

TABLE 2 - *Distribution and percentage of e.faecalis in primary and secondary apical lesion groups according to different clinical signs and symptoms.*

Signs and symptoms	*Detected (yes/no)*	*Primary apical lesion (n=79)*		*Secondary apical lesion (n=23)*	
		E. faecalis positive (7.6%)	*E. faecalis negative (92.4%)*	*E. faecalis positive (39.1%)*	*E. faecalis negative (60.9%)*
Pain	Yes	0%	49.4%	8.7%	30.4%
	No	7.6%	43.0%	30.4%	30.5%
Periapical radiolucency	Yes	6.3%	49.4%	30.4%	47.8%
	No	1.3%	43.0%	8.7%	13.1%
Swelling	Yes	8.7%	24.1%	8.7%	8.7%
	No	5.1%	68.3%	30.4%	43.5%
Tenderness to percussion	Yes	2.5%	53.2%	8.7%	34.8%
	No	5.1%	39.2%	30.4%	26.1%

sented secondary endodontic lesions.
Suggested association were found between *E. faecalis* and secondary apical lesions ($p<0.05$). *E. faecalis* resulted associated with a large number of asymptomatic apical periodontitis ($p<0.05$) in primary apical lesions.

DISCUSSION

The purpose of this study was to evaluate the presence of *E faecalis* in the root canals of teeth with endodontic apical lesions and to associate its presence with clinical symptoms.
Bone defect is the principal condition determining a diagnosis of primary or secondary apical lesions (Nair 2006). In many cases the bone defect is radiographically detectable as an apical radiolucency. Bacteria, toxins, foreign bodies have been considered responsible for apical lesions such as apical granulomas and apical cysts (Nair 2006). The presence of radiographically detected apical bone resorption is indicative of a complex pathogenic mechanism which involves large numbers of bacteria at root apical level and in the proximity of root apical bone (Fabricious *et al.*, 1982) for a sufficient period of time to stimulate bone destruction and resorption and other complex immunological activities (Siqueira *et al.*, 2004; Siqueira & Rocas 2004; Nair *et al.*, 2005). In an innovative study, Sunde *et al.* (2003) revealed microorganisms directly in the apical region using fluorescent *in situ* hybridization techniques. These microorganisms were assumed to play a major role in the development of clinical symptoms (Jacinto *et al.*, 2003) and in tissue alterations and resorption (Siqueira *et al.*, 2004; Siqueira & Rocas 2004). Hancock *et al.* (2001) examined root filled teeth with persistent apical radiolucencies (considered secondary apical lesions) and found that as well as Enterococcus other genera, viz. Peptostreptococccus, Actinomyces and Streptococcus predominated.
Our results confirm that *E. faecalis* is associated with secondary apical lesions (i.e. previous treatment failures). No relationship was suggested with the symptoms studied both in primary and in secondary endodontic infections.
Using the DNA-DNA checkerboard technique, an 8.0% prevalence of *E. faecalis* was also reported in primary endodontic infections (Siqueira *et al.*, 2002), which agrees well with our study (7.6%). Another molecular-based study revealed the concurrent presence of *E. faecalis* and other bacteria (*Pseudoramibacter, Proprionibacterium, Dialister*, and *Filifactor*) (Siqueira *et al.*, 2004) in these types of lesions in asymptomatic patients.
It is not currently possible to consider *E. faecalis* responsible for the bone lesions, but it is evident that they may be only partially involved in the formation of the bone damage. It may be a sort of "in vivo index" and may be present in the api-

cal biofilm with other bacteria and play a critical support role (Johnson *et al*., 2006). It has been demonstrated that dentinal tubules may represent a long-term *nidus* for secondary subsequent root canal infection and subsequent apical bone infection. Hence, these bacteria *may* reside not only in the canal lumen but also may invade the dentinal tubules for more than 200 microns. Hence, these structures may act as a reservoir for future dental and systemic infections (Oguntebi 1994; Peters *et al*., 2001; Matsuo *et al*., 2003).

To explain the reason for a high percentage of positive samples only in secondary lesions, *E. faecalis* survival is favoured during therapy, and can also persist for a long time inside dentinal tubules before initiating secondary disease (Pinheiro *et al*., 2003). Adhesion to the dentin surface is an essential step determining the pathogenic potential of *E. faecalis* in the medicated root canal: serine protease and Ace aid *E. faecalis* binding to dentin (Hubble *et al.*, 2003). Therefore dentinal tubules may work as a great reservoir of bacteria completely outside immunological control.

Clearly, more effective clinical methodologies for disinfection of root canals must be established to eradicate this pathogen in the course of endodontic treatment. *E. faecalis*, may survive in the smear layer and in debris inside the root canal (and inside the lateral canals and dentinal tubules) and may be extremely difficult to remove by irrigation and instrumentation (Yang *et al*., 2006, Estrela *et al*., 2007). For these reasons, it is important to consider that when an *E faecalis* infection is suspected a different type of irrigation must be used in the root canal. Chlorexidine has a broad-spectrum antimicrobial effect and kills *E faecalis* in the dentinal tubules more effectively than other irrigations and disinfectants (Schafer and Bossman, 2005). Alternatively, ultrasound mechanical preparation and other sonic procedures must be used the remove and kill pathogen bacteria (Gulabivara *et al*., 2004).

Lastly, the presence of these pathogens inside the root canal may increase the risk for iatrogenic exacerbations (flare ups) when infected dentin debris is transported into the apical region (Siqueira, 2001).

Based on the ubiquitous occurrence of enterococci in many food products, such as cheeses and milk derivates, it can be speculated that niches such as root canal lumens and dentinal tubules may favour their survival and long-standing local infection (Razavi *et al.,* 2007). The bacteria inside the root canal could be the consequence of a coronal colonization after contaminated food ingestion.

In conclusion, the present study confirms that *E. faecalis* inside root canal may be detected in teeth with secondary apical lesions (treatment failures). Surprisingly, signs and symptoms are not correlated to bacteria presence. We could speculate that coaggregation interactions between this and other bacterial species could play a major role in endodontic infection.

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