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Detection of bacteria in endodontic samples by polymerase chain reaction assays and association with defined clinical signs in Italian patients

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Background/aims: The presence of selected bacteria (*Enterococcus faecalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis*, *Treponema denticola*) in infected root canals was studied using polymerase chain reaction (PCR) assays, and the association of bacteria with clinical signs of endodontic disease was assessed. The null hypothesis, that no difference could be observed between clinical signs of apical periodontitis and a specific bacterial strain, was tested.
Methods: Microbial samples were obtained from 62 teeth in 54 patients with endodontic disease. For each tooth, clinical data including patient symptoms were collected. Teeth were categorized by diagnosis as having acute apical periodontitis (AAP, teeth with clinical symptoms but no periapical radiolucency, $n = 22$), chronic apical periodontitis (CAP, teeth with radiolucency but no clinical symptoms, $n = 15$) or exacerbated apical periodontitis (EAP, teeth with symptoms and radiolucency, $n = 25$). Seventy-one percent of cases were primary endodontic infections, and 29% were recurrent ('secondary') endodontic infections (failing cases). PCR assays were used to detect the presence of the selected bacteria.
Results: *T. denticola* and *E. faecalis* were each detected in 15 of 62 samples (24%), *P. gingivalis* in 8 samples (13%), *P. intermedia* in 5 samples (8%), and *T. forsythensis* in 4 samples (7%). *T. denticola* was detected in 56% of teeth with EAP. *E. faecalis* was found in 60% of teeth with CAP and in 72% of teeth with secondary infection. Statistical analysis demonstrated an association of CAP and secondary endodontic infection with the presence of *E. faecalis*. ($P < 0.01$). EAP was associated with the presence of *T. denticola* ($P < 0.01$).
Conclusion: *T. denticola* was associated with symptomatic endodontic disease in the presence of apical bone resorption. *E. faecalis* was associated with treatment failures. We suggest that these species may play critical roles in endodontic pathology.

Key words: clinical signs; endodontic pathogens; *Enterococcus faecalis*; polymerase chain reaction; *Treponema denticola*

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Root canal infections are mixed and semispecific infections with a great predominance of obligate and facultative anaerobic bacteria (36, 47, 51). Bacterial infections and the immune responses they induce are the putative cause of periapical disease (54). Infections of the root canal space with gram-negative, facultative, and obligate anaerobic bacteria have been associated with different clinical signs and symptoms (12). Black-pigmented bacteria have been isolated from acute abscesses of odontogenic origin, suggesting an active role in the pathogenesis of acute symptoms (15, 45, 55). Another study demonstrated the simultaneous presence of black-pigmented bacteria and spirochetes in acute periradicular abscesses (53). Studies have also demonstrated that the ratio of obligate anaerobic microorganisms that colonize root canal increases when the infection persists for a sufficient period (6, 30, 51). These data suggest that the higher the number of bacteria inside the root canal, the greater the risk for the development of bone resorption in the periapical region (49). Bacteria may not only reside in the canal lumen but may also invade the dentinal tubules, and these structures may act as a reservoir for future dental and systemic infections (19, 25, 27). Sunde et al. revealed the presence of microorganisms directly in the periapical region using fluorescent *in situ* hybridization techniques (50). These microorganisms likely play a major role in the development of clinical symptoms (16) and in periapical bone resorption (49). It is therefore important to define the presence of specific bacteria, their role and their growth ability in the endodontic microenvironment.

Three distinct clinical categories may be defined in periapical disease: acute apical periodontitis (AAP), chronic apical periodontitis (CAP), and exacerbated apical periodontitis (EAP) (35). AAP presents with acute clinical symptoms but no periapical bone resorption. CAP is characterized by absent clinical symptoms with the presence of a periapical radiolucency. Teeth with EAP have both clinical symptoms and periapical resorption. These pathologic conditions are clearly interrelated since CAP and EAP represent an evolution from AAP (36). The occurrence of viruses (HCMV and EBV) has been recently suggested to contribute to the etiology of EAP (34).

Analysis of the endodontic microbiota is still focused on the detection and identification of bacteria using different methods, including cultural (17, 28, 55), DNA–DNA hybridization (47), polymerase chain reaction (PCR) (14, 44) or denaturing gradient gel electrophoresis fingerprint (46) techniques. PCR assays represent the newest and the most sensitive method applied to the study of endodontic bacteria (37, 40). This technique can readily identify slow growing as well as uncultivable bacteria, including heretofore undiscovered species (13, 43).

The aim of the present study was to investigate, using PCR techniques, the correlation between selected bacterial species, identified within root canals, and the presence of symptomatic or asymptomatic periradicular disease, in the presence or absence of periapical bone resorption, as well as the correlation between these bacteria and primary and secondary infections.

Material and methods

Patients

The study population consisted of 54 patients, presenting at the Endodontic unit of the Department of Dental Science of the University of Bologna, Italy, for endodontic treatment. 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 3 months before root canal therapy were excluded from the study. Patient ages ranged from 19 to 72 years (mean ± SD: 38.4 ± 15.6). During the first visit, written informed consent was obtained from each patient before enrolment in the study.

Clinical signs and symptoms

The following clinical features of each tooth were recorded: the type of restoration, if present; the presence of previous root canal filling; pain on occlusion; tenderness to percussion or palpation; swelling; and the presence of periapical radiolucency. Teeth in the study included molars, premolars, canines and incisors. Teeth presented with either primary or secondary endodontic infection. 'Secondary infection' described teeth that had failed endodontic treatment, which was diagnosed if the tooth remained symptomatic, became symptomatic or exhibited a nonhealing or expanding periapical bone lesion after the initial therapy. Teeth treated for primary endodontic infection presented with deep carious lesions or secondary caries associated with old/fractured composite or amalgam restorations, and complete pulpal necrosis. Cases with a periodontal pocket probing depth greater than 4 mm were excluded due to possible endodontic–periodontal infection. Another exclusion criterion was teeth in which proper rubber dam isolation could not be achieved.

The diagnosis of acute apical periodontitis was made whenever a patient suffered from acute clinical symptoms (pain on occlusion, tenderness to percussion or palpation, swelling), but lacked radiographic evidence of periapical radiolucency (35). Chronic apical periodontitis was defined as the presence of periapical radiolucency, where no other clinical symptom had been present in the previous 3 months (17). The concomitant presence of periapical radiolucency and one or more clinical symptoms was diagnosed as exacerbated apical periodontitis (35).

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. (26). Teeth with a PAI score equal to or greater than '3' (signs of structural changes of periapical structure with mineral loss) were considered to be affected by periapical bone resorption.

Specimen sampling

Sixty-two endodontic samplings, from different teeth, 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. (24). 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 cavities were prepared using sterile burs with sterile water spray supplied by a Logos Junior dental unit (Castellini S.p.A., Castel Maggiore, Italy), equipped with an Autosteril system (21). The patency of each canal was assessed by inserting a sterile #15 K-file (Dentsply Maillefer, Ballaigues, Switzerland) so that the tip was 2 mm short from the apex, previously measured on the preoperative radiograph. In cases of previously filled root canals with failing endodontic treatment, gutta-percha was removed without chemical solvents with Gates Glidden burs (Dentsply Maillefer) and K-files. In cases presenting with a dry root canal space (i.e. retreatment cases) a small quantity of sterile saline was introduced into the canal before sampling. To obtain microbial samples, two or more paper points (ADA

products-Mynol, Milwaukee, WI) were placed at the same level and used to soak up the fluid in the canal. Each paper point was retained in the canal for 40 s. The paper points were then 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 −80°C and stored up to 1 month 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′). To detect the presence of bacterial pathogens (*Prevotella intermedia*, *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythensis*), four species-specific pairs of primer pairs targeted to the 16S rRNA gene were used (Table 1) (5, 9, 18, 20, 44, 52). Each PCR was performed using as controls 1 μl of DNA extracted from the following bacterial strains: *T. denticola* (ATCC 35405), *P. intermedia* (ATCC 15032), *T. forsythensis* (ATCC 43037), and *P. gingivalis* (ATCC 33277). 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 that gave a positive result for the presence of Enterococcus spp. were further investigated for *Enterococcus faecalis* using specific primers targeting the *ddl* gene (Table 1). DNA extracted from two clinical isolates of *Enterococcus 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 each primer was 0.4 μM for *T. forsythensis*, *E. faecalis and T. denticola*, and 0.5 μM for *P. intermedia*, *P. gingivalis,* Enterococcus spp. and the human *Hfe* gene. For the detection of *T. denticola*, DNA was amplified in a reaction mixture containing 2 mM $MgCl_2$ and 0.6 U Taq polymerase. For each sample, 10 μl of extracted DNA was added to the reaction mixture, and PCRs were performed in a Mastercycler thermalcycler (Eppendorf) under optimized conditions (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 100 V for 2 h. The gels were stained with ethidium bromide (0.5 μg/ml) and the PCR products were visualized under UV light with a TFX-20 M Gibco BRL (Gaithersburg, MD) 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).

Data analysis

Data collected for each sample were recorded into an electronic data spreadsheet and analyzed with SPSS 12.0 (SPSS Inc., Chicago, IL). Descriptive statistical analysis was performed using the Pearson Chi-squared 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 periodontitis (AAP, CAP and EAP) and the detection of specific bacteria strains in sampled root canals.

Results

Table 2 shows the incidence and percentage of cases in the study group, according

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

Bacterial species	Primer sequences (from 5′ to 3′)	Amplicon size (bp)	Amplification cycles	Reference
T. denticola	TAATACCGAATGTGCTCATTTACAT TCAAAGAAGCATTCCCTCTTCTTCTTA	316 bp	36 cycles: 95°C 30 s 60°C 1 min 72°C 1 min	Siqueira et al. (44)
P. gingivalis	AGGCAGCTTGCCATACTGCG CTGTTAGCAACTACCGATGT	404 bp	35 cycles: 95°C 30 s 58°C 45 s 72°C 30 s	Mättö et al. (20)
P. intermedia	CGTGGACCAAAGATTCATCGGT CTTTACTCCCCAACAAAAGCA	256 bp	35 cycles: 95°C 30 s 58°C 45 s 72°C 20 s	Fouad et al. (9)
T. forsythensis	TACAGGGGAATAAAATGAGATACG ACGTCATCCCAACCTTCCTC	746 bp	35 cycles: 95°C 30 s 61°C 45 s 72°C 40 s	Tran & Rudney (52)
Enterococcus species	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112 bp	35 cycles: 95°C 30 s 58°C 45 s 72°C 20 s	Ke et al. (18)
E. faecalis	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941 bp	36 cycles: 95°C 30 s 47°C 45 s 72°C 40 s	Dutka-Malen et al. (5)

Table 2. Distribution of cases according to diagnosis, treatment and restorative status

	Tooth				Rx status		Restorative status		
Diagnosis	I	C	P	M	1°	2°	A	CR	D
AAP ($n = 22$)	3	2	9	8	22	0	1	8	13
CAP ($n = 15$)	1	3	5	6	4	11	6	7	2
EAP ($n = 25$)	3	2	4	16	18	7	13	6	6

AAP = acute apical periodontitis, CAP = chronic apical periodontitis, EAP = exacerbated apical periodontitis; I = incisor, C = canine; P = premolar; M = molar; 1° = primary infection, 2° = secondary infection (treatment failure); A = amalgam, CR = composite resin, D = decayed tooth, no restoration.

to their different clinical categories. Specifically, 44/62 teeth presented with primary endodontic infection and 18/62 presented with secondary endodontic infection, i.e. with failed treatment. Symptomatic cases (47/62, 76%) were categorized as AAP (clinical symptoms but no radiolucency) and EAP (symptoms with radiolucency), whereas asymptomatic teeth (15/62, 24%) were categorized as CAP (no symptoms with radiolucency). All teeth affected by AAP showed primary endodontic infection (22/22, 100%). In the AAP group, 13/22 teeth were affected by decay with no restoration present (59%). Among 15 teeth diagnosed with CAP, 11 presented with secondary endodontic infection (73%). In contrast, teeth with EAP showed mainly primary endodontic infection(18/25, 72%).

The incidence of detection of the selected bacterial species in the three clinical categories is summarized in Fig. 1. *T. denticola* was most frequently detected in cases of EAP (14/25), an association that was significant ($P < 0.01$), followed by *E. faecalis* (5/25). In contrast, *E. faecalis* was most frequently found in CAP (9/15) ($P < 0.05$). Overall, these two bacteria were the most frequently detected microorganisms regardless of clinical categorization (15/62). *P. intermedia* was found only in symptomatic cases; however, its incidence was low (5/47) (nonsignificant). No other strong associations were seen in this patient cohort. Table 3 shows the percentage and incidence of the five selected bacteria among symptomatic (AAP, EAP) and asymptomatic cases (CAP). As suggested above, *T. denticola* was significantly associated with clinical symptomatology ($P < 0.05$), whereas *E. faecalis* was associated with asymptomatic endodontic pathology ($P < 0.01$).

Figure 2 illustrates the incidence and distribution of the selected endodontic pathogens with respect to primary and secondary endodontic infection. Among failing cases with secondary infection, 72% were positive for *E. faecalis* ($P < 0.01$). *T. denticola* was strongly associated with primary endodontic infections ($P < 0.01$).

Fifty-six percent of teeth diagnosed with EAP were positive for *T. denticola* ($n = 14$). The presence of swelling originating from the sampled tooth was significantly related to the detection of this spirochete (12/16 cases; $P < 0.01$). Furthermore, a correlation was found between the identification of *T. denticola* and the presence of a periapical bone lesion (PAI > 2, 15/40; $P < 0.01$). Thus all samples positive for *T. denticola* presented with a periapical radiolucency.

No statistically significant association was found between the type of coronal restoration, if present (amalgam or composite), and the endodontic microbiota.

Discussion

The purpose of this study was to evaluate the presence of selected bacterial pathogens in the root canals of teeth affected by apical periodontitis, and to associate these species with clinical symptoms. The clinical symptoms evaluated included pain on

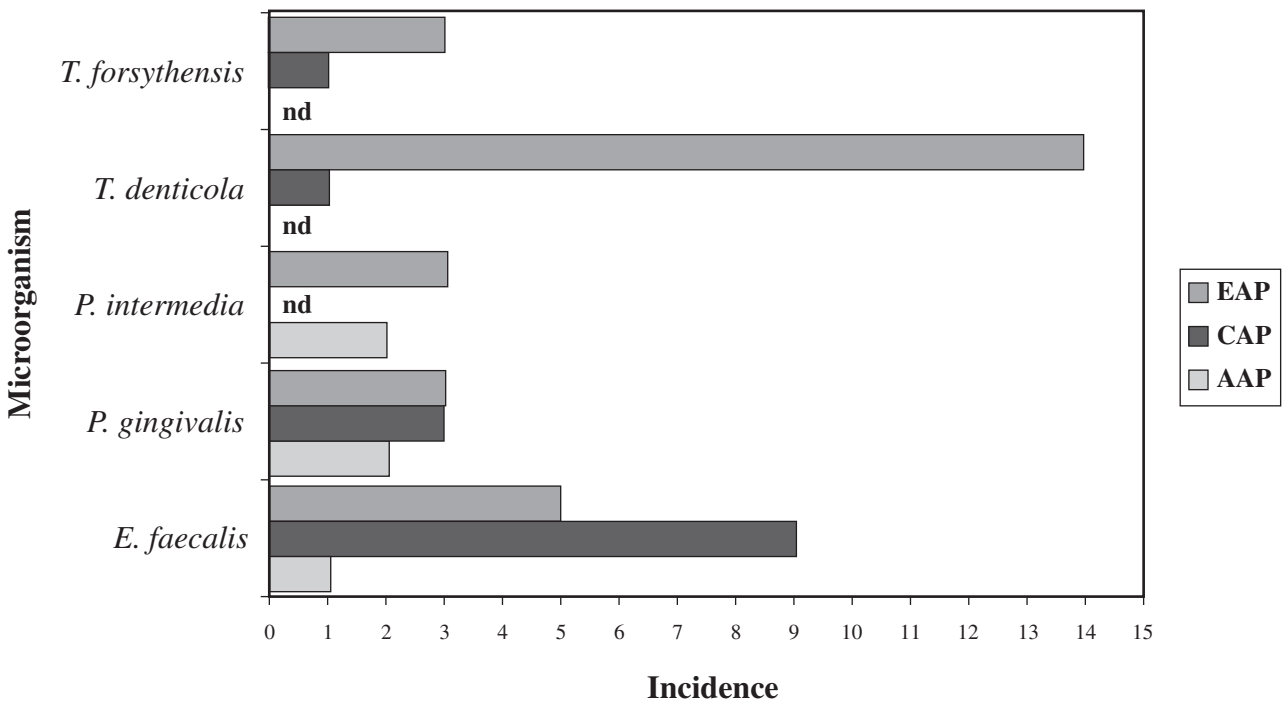

Fig. 1. Incidence of bacteria in different periapical disease syndromes. AAP: acute apical periodontitis. CAP: chronic apical periodontitis. EAP: exacerbated apical periodontitis. nd: not detected.

Table 3. Incidence of endodontic pathogens in symptomatic and asymptomatic cases

	E. faecalis	*P. gingivalis*	*P. intermedia*	*T. denticola*	*T. forsythensis*
Symptomatic (AAP, EAP) ($n = 47$)	12.8% ($n = 6$)	10.6% ($n = 5$)	10.6% ($n = 5$)	29.8% ($n = 14$)	6.4% ($n = 3$)
Asymptomatic (CAP) ($n = 15$)	60% ($n = 9$)	20% ($n = 3$)	0% ($n = 0$)	6.7% ($n = 1$)	6.7% ($n = 1$)

occlusion, tenderness to percussion and to palpation, swelling, and periapical radiolucency of teeth. A number of previous studies have examined associations between microorganisms and clinical symptoms, but without examining concurrent bone resorption and symptomatology (11, 12, 16). The presence of periapical bone resorption, detected radiographically, is indicative of a more complex pathogenic mechanism involving the presence of a large numbers of bacteria (6) for a sufficient period of time to stimulate periapical bone resorption induced by the innate immune response, which includes the cytokines IL-1 and possibly tumor necrosis factor α (49). Our results demonstrate that, of the microorganisms evaluated, *T. denticola* was highly associated with symptomatic endodontic infections and periapical bone resorption, whereas *E. faecalis* was associated with asymptomatic chronic apical periodontitis and secondary endodontic infections in failing cases.

T. denticola was detected in 24% of all samples and in 56% of samples associated with EAP. Siqueira et al. reported similar findings, with *T. denticola* in 26% in the apical third of teeth presenting with a periapical radiolucency (42). The detection of oral spirochetes has been demonstrated in association with abscesses of endodontic origin, with 79% positive for *T. denticola* (48). Very similar data were obtained in our study, with *T. denticola* present in 75% of samples with odontogenic swelling. It may be noteworthy that a similar incidence was seen despite the geographical differences of the populations analyzed (Italy vs. Brazil). This is in contrast to the results of Baumgartner et al. (1) who described significant geographical differences in the incidence of endodontic pathogens between subjects in the US and Brazil.

Our study confirms that *T. denticola* is involved in the pathogenesis of more severe periapical disease. Siqueira and coworkers also reported a significant association of *T. denticola* with periradicular lesions of endodontic origin (43). Jung et al., however, found no correlation between *T. denticola and* clinical symptoms (17). Previous studies also hypothesized that *T. denticola* may be involved in osteoclastogenesis during endodontic infections, perhaps due to the constituent virulence factors of this oral spirochete (4, 7). Due to its high prevalence in root canal infections, the hypothetical relationship between endodontic and periodontal infection by *T. denticola* and the development of atheromatous plaques deserves further assessment (2).

E. faecalis was detected in 60% of teeth with CAP and in 72% of secondary endodontic infections from teeth in which endodontic treatment was failing. A number of previous studies have also reported a strong association between the presence of *E. faecalis* and treatment failure (10, 12, 28, 29, 31, 41), although there are some exceptions (3). Using the DNA–DNA checkerboard technique, an 8% prevalence of *E. faecalis* was also reported in primary endodontic infections, which is in good agreement with our study (5%) in AAP (47). Other taxa, including Pseudomonas, Staphylococci and Streptococci, have also been associated with endodontic treatment failures (3, 32). Another molecular-based study revealed the concurrent presence of *E. faecalis* and other bacteria (Pseudoramibacter, Proprionibacterium, Dialister, and Filifactor) (41).

E. faecalis may be present in low numbers in root canals in primary infections, but its survival is favored during therapy, and it can also persist for a long time inside dentinal tubules before initiating

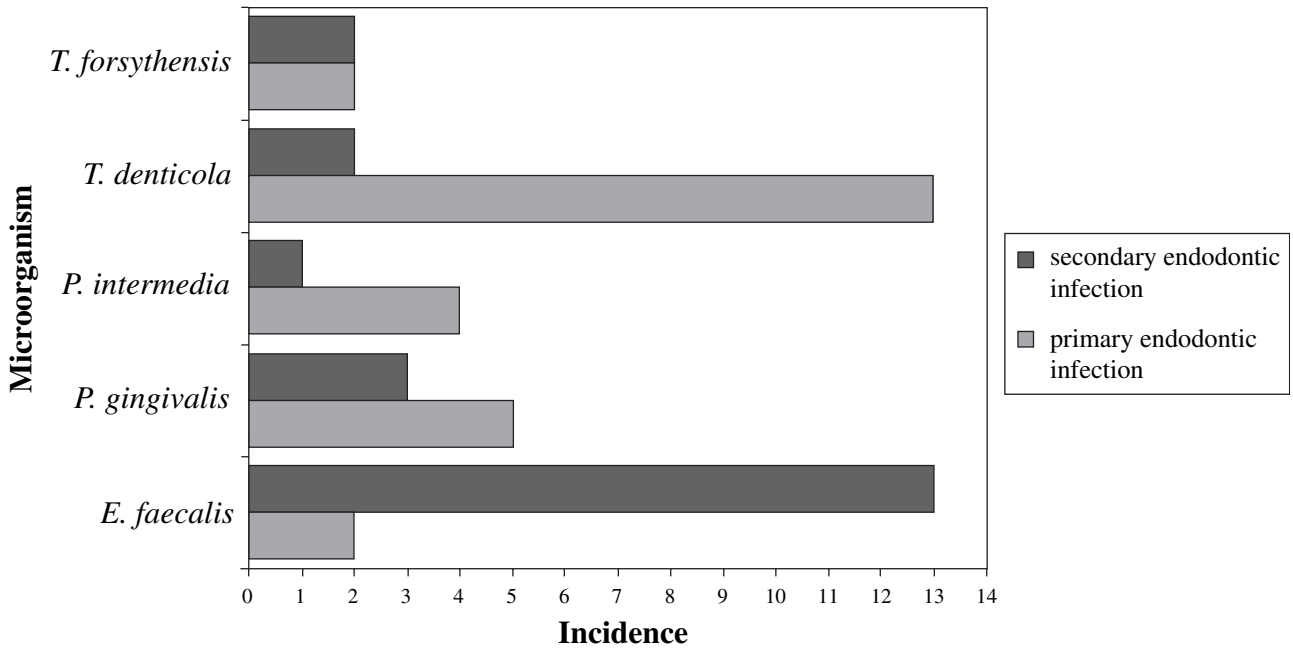

Fig. 2. Incidence of bacteria in primary and secondary endodontic infections (treatment failures).

secondary disease (29, 47). Furthermore, the survival and growth of *E. faecalis* are only marginally affected by endodontic medicaments such as $Ca(OH)_2$ dressings, due to alkali-resistance at pH 9.0–10.0 (23), as well as by antibiotic treatment, due to common resistance to erythromycin and azithromycin (29). Clearly, more effective methodologies for disinfection must be established to eradicate this pathogen in the course of endodontic treatment.

The role of *T. forsythensis* as a putative pathogenic agent of endodontic disease has been recently investigated (39). This anaerobic bacterium belongs to the group of periodontopathic pathogens known as the 'red complex', and is frequently identified in plaque obtained from deep gingival pockets (33). However, the identification of this microorganism in teeth with endodontic lesions has rarely been reported (39). In our study, the incidence of *T. forsythensis* was 7%, also in close agreement with the findings of a previous study (4%) in the apical third of teeth with periapical lesions (42). However, this microorganism was not associated with clinical symptoms, which concurs with the study of Jung et al. (17).

The presence of these pathogens inside the root canal may increase the risk for iatrogenic exacerbations (flare ups) when infected dentinal debris is transported into the apical region (47). These bacteria, in particular *E. faecalis*, may also survive in the smear layer and other debris inside the root canal, and may be extremely difficult to remove by irrigation and instrumentation (8).

PCR techniques have been increasingly used in investigations of the periodontal and root canal flora (13, 20, 22, 33, 38, 39), and are able to detect the presence of genomic DNA of bacteria present in the root canal space with a high degree of sensitivity and specificity. It should be emphasized that this technique only identifies the presence of specific DNA sequences in biological samples, and, consequently, the results obtained with this method do not guarantee in all the cases the presence of intact and metabolically active microorganisms. Nevertheless, all the patients evaluated in this study had a clinical presentation consistent with endodontic infection, suggesting that in a large proportion of the patients evaluated, PCR detected viable bacteria from an active infection.

In conclusion, the results of the present study confirm that certain species of bacteria are associated with clinical signs and symptoms of endodontic disease. In particular, *T. denticola* was strongly associated with symptomatic teeth and with apical bone resorption, and *E. faecalis* was correlated with treatment failures. Future investigations using animal models are required to further evaluate the mechanism(s) of *T. denticola* pathogenicity, including its ability to colonize the root canal and to induce alveolar bone resorption.

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