Identification of Dominant Pathogens in Periapical Lesions Associated with Persistent Apical Periodontitis

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Objective: *To identify dominant pathogens in the periapical lesions associated with persistent apical periodontitis.*

Methods: *Thirty-three root-filled teeth with persistent apical periodontitis referred for surgical treatment were selected. Microbial samples were collected from the periapical lesions during apical surgery. Microbial identification was performed with species-specific primers prepared according to the sequence analysis data using a 16S rRNA technique.*

Results: *Among the 33 cases, in 5 cases none of the target species were detected, 6 cases showed the presence of only one species, and 22 cases showed more than two species.* Porphyromonas endodontalis *(45% of sample) was the most commonly detected dominant microbial species in the study sample, followed by* Actinomyces viscosus *(42%),* Candida albicans *(36%) and* Porphyromonas gingivalis *(27%).* Fusobacterium, Actinomyces israelii *and* Enterococcus faecalis *were also detected in 27%, 21% and 15% of the sample, respectively. The most frequently isolated species,* P. endodontalis, *was in most cases detected together with* Actinomyces *(14 cases) and* P. gingivalis *(6 cases). None of the lesions analysed in the present study contained* Prevotella intermedia. *There was no correlation in relation to the presence of sinus tracts and the bacterial species.*

Conclusion: *A mixed population of pathogens was found in the endodontic lesions associated with persistent apical periodontitis.* P. endodontalis, A. viscosus, C. albicans *and* P. gingivalis *were the dominant species identified.*

Key words: *bacteria, microbiology, persistent apical periodontitis, 16S rRNA*

Root canal treatment is the most efficient therapy for infected root canals. It usually fails when the treatment is carried out inadequately[1-3]. Nevertheless, there are some cases in which the treatment has been performed to the highest technical standards and yet result in failures with poor outcome[4]. The persistence of periradicular lesions after satisfactory endodontic therapy has been attributed to an intra- or extraradicular infection, foreign body reaction to endodontic materials, cyst formation or healing by scar tissue[5]. Recent investigations have shown the presence of bacteria in the periapical region or existing in a biofilm form on the apical third of the root surface[6,7]. However, the identity of the specific species involved in lesions associated with persistent periapical periodontitis remains to be clarified.

Bacteriological sampling in most of the previous studies was performed by using paper points inserted into the root canals[8-10]. Bacteria established in the areas of isthmuses, ramifications, deltas, irregularities and dentinal tubules may sometimes be unaffected by endodontic instrumentation and disinfection procedures; thus might have been missed when the samples were taken[4,6]. This is particularly true in most of the retreat-

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Table 1 Primers used in the study

Species-specific pairs of primers	Sequence (5'-3')		Source
Bacterial ubiquitous primer	GATTAGATACCCTGGTAGTCCAC		
	CCCGGGAACGTATTCACCG	602bp	Siqueira et al[11]
Enterococcus faecalis	GTTTATGCCGCATGGCATAAGAG		
	CCGTCAGGGGACGTTCAG	310bp	Siqueira et al[11]
Prevotella intermedia	TTTGTTGGGGAGTAA AGCGGG		
	TCAACATCTCTGTATCCTGCGT	575bp	Siqueira et al[11]
Fusobacterium	GAGTTTGATCCTGGCTCAG		
	GTCATCGTGCACACAGAATTGCTG	360bp	Siqueira et al[11]
Actinomyces israelii	GCTTGTTGGTGGGGTGATGGGC		
	GCCTCCATCCGTCACGCGAC	172bp	Present study
Actinomyces viscosus	AAGGAGCCAGCTGCTGGTTCT		
	CAAACCTTTCCCAGGCCCACCATG	146bp	Present study
Porphyromonas gingivalis	GACATCTAAGTAGGTGCTGC		
	ACTGTTAGCAACTACCGATGT	404bp	Present study
Porphyromonas endodontalis	GACATCTAAGTAGGTGCTGC		
	CCGCTTCATGTCACCATGTC	245bp	Present study
Candida albicans	CTGATTTGCTTAATTGCACCACATG		
	TTGACTATTAAGTAATAATCTGGTGTGAC	141bp	Present study

ment cases, where difficulties arise when obtaining microbial samples from previously root-filled canals[10]. Pathogens may survive and continue to colonise at the periapical area associated with the non-vital tooth. Hence, collecting the surgically removed tissue would be the practical way to detect pathogens[7].

On the other hand, culture-based studies have shown that the microbial flora of cases associated with failed endodontic treatment is limited to a small number of predominantly facultative Gram-positive bacteria, such as *Enterococcus*, *Actinomyces* and *Propionibacterium*[7-11]. With the development of molecular technology, more and more anaerobic bacteria can be detected, especially some difficult-to-grow bacteria, such as *Prevotella intermedia*, *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Fusobacterium*[12-16]. Although anaerobic bacteria are the most commonly isolated species in endodontic lesions associated with persistent apical periodontitis, the dominant species of infective bacteria still remains to be identified[17].

Enterococcus faecalis and *Actinomyces*, for example, are commonly detected by culture techniques in retreatment cases[9,18]. In contrast, *P. intermedia*, *P. gingivalis* and *P. endodontalis* are the fastidious bacterial species not frequently detected by the culture method, but more commonly found by molecular methods[19]. On the other hand, *Fusobacterium*, a genus that is difficult to grow on agar plate, can be easily detected by polymerase chain reaction (PCR)[20]. These microorganisms may have a synergistic relationship in the pathogenesis of endodontic failure.

The aim of the present study was to detect the dominant microbial species present in the periapical lesions associated with persistent apical periodontitis, to examine the relationship between them using 16S rRNA PCR technology, and to evaluate the influence of sinus tracts on dominant microbial species.

Materials and methods

Microbiological sampling procedure

Thirty-three patients with radiographic evidence of persistent apical periodontitis referred for endodontic surgery were recruited. Conventional primary endo-

dontic treatment that had been completed for at least 2 years was the main inclusion criterion. Teeth that were found to have coronal leakage, deep periodontal pockets (> 4 mm), or perio–endo/endo–perio/combined lesions were excluded. Patients who were medically compromised or had received antibiotic therapy in the past 3 months were also excluded from the study. Patients were informed of the study protocol and aims, and gave their consent before recruitment. The following features were recorded for each patient: clinical symptoms, status of coronal restoration, presence/absence of sinus tract, swelling of periodontal tissues, tenderness to percussion, mobility and periodontal status.

Local anaesthesia (2 ml Primacaine; Pierre Rolland, Bordeaux, France) was delivered as local infiltration. The gingiva and mucosa were swabbed with 0.2% chlorhexidine gluconate solution prior to surgery. A submarginal incision with one vertical relieving incision was made, and a full-thickness mucoperiosteal flap was reflected. The tissue associated with the periapical lesion was removed using a sterile curette, and 3 mm of the root apex was resected. The lesion sample and the root apex were collected in a centrifugal tube containing 5 ml of sterile physiological saline solution and immediately frozen at -20°C.

Microbial identification

Samples were thawed to 37°C for 10 min and vortexed for 30 s. Afterwards, the microbial suspension was pelleted by centrifugation for 10 min at 5000 g, then the pellet was resuspended in 180 µl buffer ATL (a tissue lysis buffer). Subsequently, total bacterial genomic DNA was isolated according to the protocol of the TIANamp Bateria DNA Kit (Tiangen, Beijing, P.R. China). For PCR detection of the targeted species, DNA was first amplified with prokaryotic universal ribosomal 16S primer. Eight species-specific pairs of primers targeting 16S rRNA genes were used to detect the presence of pathogens (*E. faecalis*, *P. intermedia*, *Fusobacterium*, *Actinomyces israelii*, *Actinomyces viscosus*, *P. gingivalis*, *P. endodontalis* and *Candida albicans*). The species-specific PCR primers were prepared according to 16S rRNA sequence analysis data, and confirmed to be effective for PCR amplification (Table 1).

PCR was performed as described in previous studies[13-16], utilizing 25 µl of reaction mixture containing 0.5 µl of each primer, 2.5 µl of 10× PCR buffer, 0.5 µl Taq DNA polymerase (Gibco, Gaithersburg, MD, USA), and 0.5 µl deoxyribonucleotide triphosphates. PCRs were performed in a Mastercycler thermalcycler (Eppendorf, Hamburg, Germany) under opti-
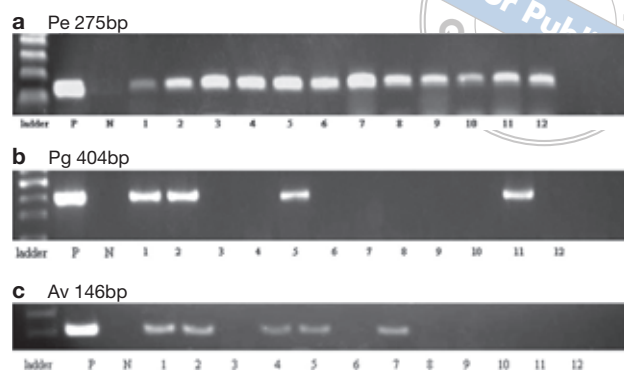

Fig 1a to c Identification of bacterial species with 16S rRNA technique. Lane P, positive control; lane N, negative control; lanes 1–12, transcription amplification of microbial samples.

mised conditions. For example, to detect *E. faecalis*, the PCR included an initial denaturation step at 95°C for 2 min, followed by 40 cycles of a denaturation step at 95°C for 30 s, a primer annealing step at 60°C for 30 s, an extension step at 72°C for 55 s, and a final step of 72°C for 5 min. The annealing temperature and the extension time were unique for each species.

Amplicons were analysed with 1.5% agarose gel electrophoresis, performed at 4 V/cm in Tris-borate ethylenediaminetetraacetic acid (EDTA) buffer. The gel was stained with 0.5 mg/ml ethidium bromide and photographed under ultraviolet light. One hundred bp and 1 kb DNA ladder digests were used as molecular weight markers.

DNA extractions of the following bacterial strains were used as the positive controls: *E. faecalis* ATCC29212, *P. intermedia* ATCC25611, *Fusobacterium* ATCC10953, *A. israelii* ATCC12836, *A. viscosus* ATCC15299, *P. gingivalis* ATCC33277, *P. endodontalis* ATCC35406 and *C. albicans* ATCC10231 (Fig 1).

Statistical analysis

Data collected for each sample were recorded in an electronic data spreadsheet and analysed with SPSS statistical software (v12.0; SPSS, Chicago, IL, USA). The Pearson chi-square test was chosen to examine the relationship between the presence of a sinus tract and the specific bacteria species.

Results

A total of 33 patients with 33 teeth which required surgical endodontic treatment were selected in the present study. There were 15 males and 18 females with a mean age of 37.2 ± 13.4 years (range from 20 to 69). Among
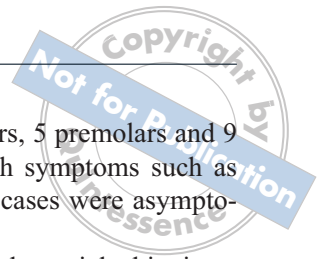
Table 2 Distribution of species in 33 apical lesions

Patient	Tooth	Sinus tract	Detected species
1	23	N	*Pg, Pe, Av*
2	21	N	*Pg, Pe, Av, Ef, Ca*
3	11	Y	*Pe, Av*
4	21	Y	*Pg, Pe, Av, Ca*
5	11	N	*Av*
6	11	N	*Pg, Pe, Av*
7	22	Y	*Av, Ai, Ca*
8	26	Y	*Pe, Av, Ai, Ca*
9	37	Y	*Pe, Ai, Ca*
10	36	N	*Pg, Pe, Av, Ef, Ca*
11	26	N	*Pe, Av, Ca*
12	32	Y	*Pe, Av, Ca*
13	15	N	*Ai, Fu, Ca*
14	46	Y	*Pg, Fu*
15	45	N	*Pe*
16	12	N	-
17	26	N	*Pg, Fu*
18	16	Y	*Fu*
19	24	Y	*Pg*
20	21	N	-
21	11	Y	-
22	22	Y	*Pe, Av, Ai, Ca*
23	35	N	-
24	21	N	*Pg, Pe, Ca*
25	46	Y	*Pe, Av, Ai*
26	16	Y	*Pe*
27	34	Y	-
28	42	N	*Fu*
29	21	Y	*Fu, Av*
30	21	N	*Ai, Ca*
31	11	N	*Fu, Ef*
32	11	N	*Fu, Ef*
33	21	Y	*Fu, Ef*

Ai, *Actinomyces israelii*; *Av*, *Actinomyces viscosus*; *Ca*, *Candida albicans*; *Ef*, *Enterococcus faecalis*; *Fu*, *Fusobacterium*; N, No; *Pe*, *Porphyromonas endodontalis*; *Pi*, *Prevotella intermedia*; *Pg*, *Porphyromonas gingivalis*; Y, Yes; -, none.

the 33 cases, there were 19 incisors, 5 premolars and 9 molars. There were 16 cases with symptoms such as sinus, pain or swelling, while 17 cases were asymptomatic.

All samples were positive for bacterial ubiquitous primer. Among the 33 cases, none of the targeted species were found in 5 cases, one species of pathogen was detected in 6 cases, and two or more species of pathogen were detected in 22 cases (Table 2).

P. endodontalis was detected in 15 cases (45%); *A. viscosus* was detected in 14 cases (42%); *C. albicans* was detected in 12 cases (36%); *P. gingivalis* was detected in 9 cases (27%); *Fusobacterium* was detected in 9 cases (27%); *A. israelii* was detected in 7 cases (21%); and *E. faecalis* was detected in 5 cases (15%). *P. intermedia* was not detected in any cases (Fig 2).

Bacterial detection was found to be positive in 28 cases. Single bacterial species were found in 6 cases, i.e. *Fusobacterium* (2), *P. endodontalis* (2), *A. viscosus* (1), and *P. gingivalis* (1). *P. endodontalis*, the most frequently isolated species, was mostly detected together with *Actinomyces* or *P. gingivalis*, in 12 and 6 of the cases, respectively.

Table 3 shows the microbial species found in periapical lesions, with and without sinus tracts. The main pathogens identified in the lesions associated with sinus tracts were *P. endodontalis* (50%) and *A. viscosus* (50%). The species *P. endodontalis* (41%) and *P. gingivalis* (35%) dominated the lesions without the presence of sinus tracts. However, a significant difference was not found between the species and sinus tracts ($P > 0.05$).

Discussion

Since microorganisms that are established in the periradicular tissues are inaccessible to endodontic instrumentation and disinfection procedures, extraradicular infection may be a critical factor leading to failure of endodontic therapy[4,5]. In addition, bacteria located in areas such as isthmuses, ramifications, deltas, irregularities and dentinal tubules may sometimes be unaffected by endodontic disinfection procedures. Characterising the microbial communities in the periradicular tissues and the root apex will advance the understanding of the pathogenesis of the disease and lead to important clinical applications.

Microbiological analysis was performed using the 16S rRNA sequence in the present study. The PCR-based identification is a highly sensitive method and allows detection of uncultivable strains of these species[11,13,14]. This method helps to overcome the inherent
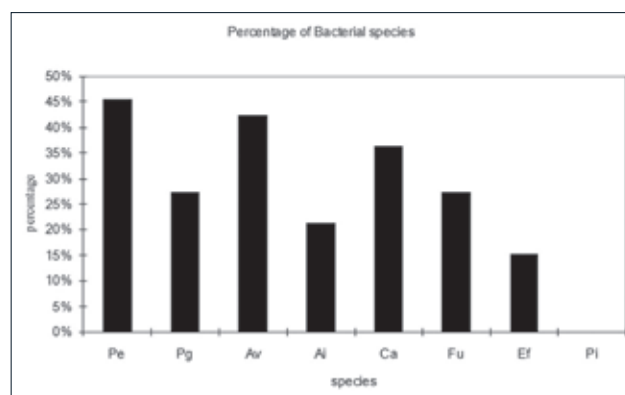
Fig 2 Percentage of bacterial species detected in periapical lesions.

Table 3 Microbial species in relation to the presence of sinus tracts

Bacteria species	Present	Absent
Porphyromonas endodontalis	50%	41%*
Actinomyces viscosus	50%	35%*
Porphyromonas gingivalis	19%	35%*
Candida albicans	38%	35%*
Fusobacterium	25%	29%*
Actinomyces israelii	31%	12%*
Enterococcus faecalis	6%	24%*

* $P > 0.05$

limitations of culture procedures such as the inability to culture certain bacteria and low sensitivity[10].

P. endodontalis and *P. gingivalis* are recognised pathogenic species within the group of black-pigmented anaerobic rods. *P. endodontalis* was detected in 39.5% of the infected root canals in a previous study[21], and it has been suggested that this bacterial species could serve as a potentially important endodontopathogen. *P. endodontalis* could release outer membrane blebs, probably containing lipopolysaccharide, which may be an important virulence factor in the pathogenesis of periradicular lesions[22]. In the present study, *P. endodontalis* and *P. gingivalis* were detected in 45% and 27% of the examined periapical lesions, respectively. This is in line with a culture study by van Winkelhoff et al[22], where *P. endodontalis* and *P. gingivalis* were isolated from 53% and 12% of the examined periradicular abscesses, respectively. Of particular interest was the finding that *P. gingivalis* was mostly found associated with *P. endodontalis*. Siqueira and co-workers observed the possible synergistic effect when associating *P. endodontalis* with either *P. intermedia* or *P. nigrescens*[4,23]. However, it has been reported that *Porphyromonas* species are able to inhibit *in vitro* other black-pigmented anaerobic rods, which might explain the infrequent isolation of black-pigmented rods in refractory endodontic lesions associated with polymicrobial infection[24].

A. viscosus and *A. israelii* were detected in 14 and 7 of the cases, respectively. Tang and co-workers have reported the detection of *Actinomyces* species in the infected root canals within the Chinese population using an oligonucleotide-DNA hybridization technique[25]. They found 16 of the 32 teeth were infected with one or more *Actinomyces* species including *A. odontolyticus*, *A. meyeri*, *A. naeslundii*, *A. israelii* and *A. gerencseriae*.

Actinomyces species have always existed in periapical lesions in the form of classic sulphur granules[25,26]. The characteristic light microscopic feature of an actinomycotic colony is the presence of an intensely dark staining, Gram- and periodic acid-Schiff (PAS)-positive, core with radiating peripheral filaments that gives the typical 'star burst' or 'ray fungus' appearance[5]. *Actinomyces* have been shown to possess a hydrophobic cell surface property, which may help the cells to aggregate into cohesive colonies and enable them to escape from host defence systems[25]. In the present study, *Actinomyces* was mostly detected together with *P. endodontalis* and *P. gingivalis*, which was rarely reported in previous studies. *Actinomyces* may play a significant role in the pathogenesis of root canal infections and its extension into the periapical regions, both by acting as a direct agent of sepsis and by supporting the colonisation of other co-pathogens[25,27].

E. faecalis is one of the main pathogens isolated from teeth associated with persistent periapical periodontitis. It has been reported in the literature that 24% to 77% prevalence of *E. faecalis* can be found in endodontically re-treated root canals[9,10,12,13,16]. However, the presence of *E. faecalis* in periapical lesions is not common. It varies from 0% to 17.8%[28,29]. Only those species that are capable of tissue adhesion and invasion, and have the ability to overcome host defences can persist in the soft tissues. Similarly, *E. faecalis* has been shown to adhere and invade epithelial cells to form a persistent biofilm associated with refractory infection[28]. The present data also suggested that *E. faecalis* may contribute to persistent periradicular infections by soft tissue adhesion and invasion.

We found 36% (12 cases) of *C. albicans* in the present study. The isolation frequency of *C. albicans*
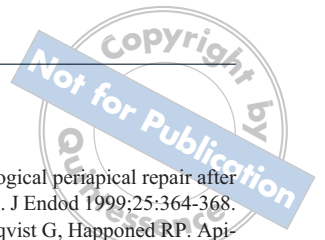
was less than 18% in previous studies using the culture technique[16,30,31]. Baumgartner et al[32] used a probe and PCR techniques to demonstrate yeasts in 21% of teeth with apical periodontitis. Furthermore, Waltimo et al[30] detected *C. albicans* 7% of the time in apical periodontitis using a pure culture technique, suggesting a pathogenic role for this organism. Miller and Kleinman[33] found that the presence of *C. albicans* could cause a significant increase in total plaque formation *in vitro*. In the present study, *C. albicans* was mostly isolated with *Actinomyces* and *E. faecalis*, which suggested that these three bacterial species may have co-aggregation reactions, which can play an important role in the colonisation of oral mucosal and hard tissues[34].

In the present study, *Fusobacterium* was found in 9 cases. *Fusobacterium* has several types of adhesins, which might play a central role in plaque formation[35]. Metzger et al[36] found that *F. nucleatum* can enhance the attachment of *P. gingivalis* to the host cells. They suggested that it might interact with many early colonisers of the dental plaque as well as with many of the late ones, thus serving as a bridge between the former and the latter. This process might enhance the perpetuating presence of several bacterial strains in the infected site, resulting in a complex formation of biofilm structure[36,37].

However, the types of periapical lesion were not identified in the present study. Wayman et al[38] found that bacteria could be found in periapical granulomas, radicular cysts and a periapical abscess. In the present study, the authors also failed to demonstrate a significant association between the presence of microbial species, clinical symptoms and sinus tracts. The correlation between the type of periapical lesion and microbial species, the particular relationships and the mechanism of synergistic impacts still need further study.

Conclusions

The bacteria species isolated from the periapical lesions were found to be mixed. *P. endodontalis*, *A. viscosus*, *C. albicans* and *P. gingivalis* were the dominant species. A significant relationship was not found in relation to the presence of sinus tracts and bacterial detection.

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