Anaerobe 48 (2017) 12–18

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Anaerobes in human infections (dental/oral infections)

Microbial analysis of root canal and periradicular lesion associated to teeth with endodontic failure

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ARTICLE INFO

Article history:
Received 20 December 2016
Received in revised form
21 April 2017
Accepted 26 June 2017
Available online 27 June 2017

Handling Editor: Elisabeth Nagy

Keywords:
Endodontic infection
Apical root canal
Periradicular lesion
Polymicrobial infection
Real-time PCR

ABSTRACT

The quantification of ten microorganisms at the root ends and in the surrounding periradicular lesions was performed. Thirty 3 mm samples root ends and 30 samples of the surrounding chronic periapical infection were collected during apical microsurgery. Samples were triturated, and the bacterial DNA was obtained. The bacterial quantification was performed by using the SYBR Green system. At least one microorganism was detected in all patients. In both the root end and periapical samples, *Fusobacterium nucleatum* (71.6%), *Dialister pneumosintes* (58.3%) and *Tannerella forsythia* (48.3%) were the most prevalent species. *Dialister pneumosintes* showed statistically significant values in the root end, and *F. nucleatum* was also significant in the apical periodontitis samples. A statistically significant association between *T. forsythia* and *Porphyromonas gingivalis* in the root ends was observed. Bacterial associations from 2 to 7 species were observed in most samples. Extra-radicular and/or intra-radicular infections were present in all teeth with failed endodontic treatment, and showed polymicrobial infection in most cases, with a predominance of *F. nucleatum*, *D. pneumosintes* and *T. forsythia*. When present, *Enterococcus faecalis* was never found to be the most prevalent species. The presence of a microbial diversity in post-treatment apical periodontitis confirms the polymicrobial and synergistic characteristic of this process. Our results show that the bacterial array associated with the 3 mm root ends and periradicular lesions in post-treatment apical periodontitis are complex and with a high inter-individual variability. These results might be useful to delineate treatment strategies for microbial elimination in apical periodontitis. Further studies are necessary to elucidate the role of these microorganisms in endodontic treatment failures.

1. Introduction

Endodontic therapy aims to eliminate infection from the inner root canal system and prevent re-infection by obturation [1]. However, several authors have recognized that one of the main causes of root canal treatment failure leading to post-treatment apical periodontitis is the presence of residual microorganisms after endodontic therapy (persistent infection). The reinfection of a previously disinfected root canal environment (secondary infection) can also lead to endodontic failure [2,3].

Over the years, the majority of authors have stated that the major cause of endodontic treatment failure is the presence of microorganisms within the root canal system [4,5]. Studies using molecular methods have shown contamination on the external root surface of treated teeth [6,7] and within soft-tissue lesions in the periapical region [8–10].

The endodontic therapy should treat the infected root canal as a complex system. The main canal includes a system of lateral canals, apical ramifications and an isthmus, all of which can be challenging to reach with endodontic therapy, as bacteria can spread and remain unaffected by treatment procedures in these areas [11]. The dental community is in agreement that the elimination of microorganisms from the root canal system is critical in preventing and treating apical periodontitis [12].

Traditionally, bacterial identification has been accomplished through biochemical methods, but these can be laborious, expensive, and time-consuming and have limitations in terms of the

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http://dx.doi.org/10.1016/j.anaerobe.2017.06.016
1075-9964/

Table 1
Target microorganism and species-specific primers used in the bacterial quantification.

Microorganisms	Oligonucleotides (5′- 3′)	PCR conditions	Amplicon (bp)	References
Aggregatibacter actinomycetemcomitans	CCC ATC GCT GGT TGG TTA GGC ACG TAG GCG GAC C	1 Hold: 95 °C, 2 min 40 cycles: 95 °C, 45 s 60 °C, 2 min	696	Kuboniwa et al. [32]
Fusobacterium nucleatum	CTT AGG AAT GAG ACA GAG ATG TGA TGG TAA CAT ACG AAA GG	1 Hold: 95 °C, 2 min 40 cycles: 95 °C, 45 s 60 °C,1 min	140	Periasamy & Kolenbrander [34]
Porphyromonas gingivalis	ACC TTA CCC GGG ATT GAA ATG CAA CCA TGC AGC ACC TAC ATA GAA	1 Hold: 95 °C, 2 min 40 cycles: 95 °C, 45 s 60 °C,1 min	83	Kuboniwa et al. [32]
Porphyromonas endodontalis	GCT GCA GCT CAA CTG TAG TCT TG TCA GTG TCA GAC GGA GCC TAG TAC	1 Hold: 95 °C, 10 min 40 cycles: 95 °C, 15 s 60 °C, 1 min	110	Nonnenmacher et al. [33]
Prevotella intermedia	TCCACCGATGAATCTTTGGTC ATCCAACCTTCCCTCCACTC	1 Hold: 95 °C, 2 min 40 cycles: 95 °C, 45 s 60 °C,1 min	98	Kuboniwa et al. [32]
Prevotella nigrescens	CCG TTG AAA GAC GGC CTAA CCC ATC CCT TAC CGG RA	1 Hold: 95 °C, 10 min 40 cycles: 95 °C, 15 s 57 °C,1 min	82	Kuboniwa et al. [32]
Dialister pneumosintes	GAG GGG TTT GCG ACT GAT TA CCG TCA GAC TTT CGT CCA TT	1 Hold: 95 °C, 10 min 40 cycles: 95 °C, 15 s 55 °C,1 min	166	Nonnenmacher et al. [33]
Tannerella forsythia	AGC GAT GGT AGC AAT ACC TGT C TTC GCC GGG TTA TCC CTC	1 Hold: 95 °C, 10 min 40 cycles: 95 °C, 15 s 57 °C,1 min	88	Kuboniwa et al. [32]
Treponema denticola	CCGAATGTGCTCATTTACATAAAGGT GATACCCATCGTTGCCTTGGT	1 Hold: 95 °C, 10 min 40 cycles: 95 °C, 15 s 57 °C,1 min	122	Kuboniwa et al. [32]
Enterococcus faecalis	CGC TTC TTT CCT CCC GAGT GCC ATG CGG CAT AAA CTG	1 Hold: 95 °C, 10 min 40 cycles: 95 °C, 15 s 60 °C, 1 min	143	Williams et al. [35]
Universal primers 16S rDNA	AGA GTT TGA TCC TGG CTC AG GGC TAC CTT GTT ACG ACT T	1 Hold: 95 °C, 10 min 30 cycles: 95 °C, 30 s 58 °C, 30 s		Amano et al. [36]

Table 2
Bacterial prevalence in the root end and periradicular lesion samples.

Microorganisms	Root end		Periradicular lesion		Samples total	
	N°	%	N°	%	N°	%
Aggregatibacter actinomycetemcomitans	7	23.3	8	26.6	15	25
Fusobacterium nucleatum	22	73.3	21	70	43	71.6
Porphyromonas gingivalis	5	16.6	4	13.3	9	15
Porphyromonas endodontalis	3	10	3	10	6	10
Prevotella intermedia	4	13.3	5	16.6	9	15
Prevotella nigrescens	1	3.3	0	0	1	1.6
Dialister pneumosintes	22	73.3	13	43.3	35	58.3
Tannerella forsythia	16	53.3	13	43.3	29	48.3
Treponema denticola	4	13.3	6	20	10	16.6

microbiological diagnosis. Approximately 50% of oral bacteria are not cultivable; therefore, unknown bacteria are always present in such infections. Molecular analysis has revealed a more diverse array of bacteria associated with endodontic infections than culture methods alone [13].

The qualitative and quantitative polymerase chain reaction (PCR) has been used for bacterial detection from endodontic infections due to its great sensitivity [14–16]. The current findings, based on molecular methods, suggest that new candidates for endodontic pathogens may be responsible for post-treatment apical periodontitis and also suggest that it is a complex and polymicrobial disease, with a high level of interspecies variability [17]. Since the elimination of microorganisms from root canal is necessary for preventing the apical periodontitis, the detection of a specific microbiota involved in this process could collaborate with dentists to delineate a better treatment in cases of root canal failure or persistence of apical periodontitis. Thus, the aim of this study was to investigate and compare the presence and quantity of ten microorganisms from root ends and the associated periradicular tissues collected from cases of failed endodontic therapy.

2. Materials and methods

2.1. Patients

Thirty patients (17 female and 13 males) between the ages of 16 and 58 years old (mean 41 years) were selected. All patients had at least one tooth with a performed satisfactory endodontic treatment, between 1 and 15 years prior to enrollment (mean 4 years). The characteristic radiographic evidence of periradicular bone destruction of post-treatment apical periodontitis was observed in all selected asymptomatic first molar (anterior, posterior, inferior or superior). All treated teeth were coronally restored, and no evidence of root canal filling material exposure to the oral cavity was

Table 3
Values in $\log_{10}$ of the bacterial distribution in root end and its periradicular lesion from patients with post-treatment endodontic apical periodontitis.

Patients	*F. nucleatum*		*P. gingivalis*		*D. pneumosintes*		*A. actinomycetemcomitans*	
	Lesion	Apex	Lesion	Apex	Lesion	Apex	Lesion	Apex
P1	0	0	0	0	0	0	0	1.5
P2	0	0	0	0	0	0	4.5	3.3
P3	0	1.0	0	0	0	3.5	3.4	2.4
P4	0.9	0	0[a]	0[a]	3.5	3.3	4.5	0
P5	0	0	0	0	0	2.2	3.0	3.7
P6	0	0	0	0	0	5.5	2.8	0
P7	0.6	0	0	0	3.6	4.2	0	0
P8	0	2.2	0	0	2.3	0	0	0[a]
P9	3.1	4.8	0	0	0	0	0	0
P10	7.2	4.1	0	0	0	4.8	0	0
P11	2.7	5.1	0	0[a]	0	6.8	0	0
P12	6.4	2.7	0[a]	0[a]	3.9	5.9	0	0
P13	3.4	0	0[a]	0	5.2	0	0	0
P14	2.4	1.4	0	2.8	0	4.5	0[a]	0[a]
P15	2.3	3.8	0[a]	0[a]	4.9	3.8	0	0[a]
P16	0	2.8	0.1	0.2	0	2.9	0[a]	0.5
P17	2.6	4.3	0	0	3.7	4.2	1.7	0
P18	5.3	4.8	0	0[a]	0	0	0	0
P19	6.2	2.7	0	0.4	0	0	0	0.4
P20	2.2	2.5	0[a]	0	5.3	4.5	0[a]	0
P21	2.5	1.2	0[a]	0	5.5	4.8	0	0[a]
P22	0	2.7	0	0.2	0	4.1	0	0
P23	6.1	1.7	0	0	0	0	3.6	0[a]
P24	2.2	3.0	0	0	2.9	4.7	0[a]	0[a]
P25	5.4	2.3	0	0	0	3.6	0[a]	0
P26	7.7	1.4	3.7	1.3	5.7	4.1	0	0
P27	0	1.0	0	0	4.5	5.3	0	0
P28	3.4	2.3	0	0	5.9	4.4	0	0
P29	4.9	2.4	1.5	0	0	4.5	0	0
P30	4.2	0	0	1.9	0	4.6	0	0

[a] From 0 to 1.

observed. The patients selection was performed by a dentist specialized in endodontology. This study was approved by the Research Ethics Committee of the Federal University of Espirito Santo (Process no. 056/11) and all procedures performed in this study were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All enrolled patients signed an informed consent form. Patients who received antibiotics three months prior to the sample collection, displaying primary chronic apical periodontitis, endoperiodontal lesions, periodontal pockets (>3 mm of depth), or teeth with longitudinal root fractures, were excluded.

2.2. Sample collection

Initially, teeth with endodontic treatment previously performed were selected. Radiographic images showed periradicular lesion and it was considered as failure of endodontic treatment. A para-endodontic surgery was performed instead of retreatment due to the correct parameters that could indicate a persistent apical periodontitis. Thus, thirty samples of 3 mm of root ends sectioned during apical surgery and 30 samples of the surrounding chronic periradicular infections were collected with curettes. Samples were taken from April 2012 to January 2013. After surgical site disinfection with a 0.12% chlorhexidine solution and local anesthesia administration (2% lidocaine with 1:50,000 epinephrine), a marginal incision was made, and a full-thickness mucoperiosteal flap was lifted. Saliva was collected using a sterile foam pellet into 200 μl TE buffer in order to verify the effectiveness of disinfection. Access to the apices was achieved with a low rotation using a spherical carbide drill applying under-tungsten phosphate buffered saline (PBS, pH 7.4). After exposure, curettage of the periradicular lesion surrounding of the root end was performed, and 3 mm of the root ends were removed, sectioned perpendicular to the long axis with a tapered diamond bur at a high speed with sterile PBS. To avoid cross-contamination, each sample from the root end and the surrounding chronic periradicular infections was transferred to separate tubes containing 200 μl TE buffer and stored at -80 °C until use. All of the procedures were performed under high magnification with a dental operating microscope (DF Vasconcellos Ltd, Sao Paulo, SP, Brazil). Salivary bacterial contamination and protracted exposure to air were prevented.

2.3. Bacterial quantification

Initially, root end samples were triturated in sterile Petri dishes using sterilized orthodontic pliers. Samples of the triturated root ends and periradicular lesions were used to obtain bacterial DNA with an Easy-DNA Kit (Invitrogen do Brasil Ltd, Sao Paulo, SP, Brazil) in accordance with the manufacturer's instructions. Samples were then stored at -80 °C until use. The DNA concentration and purity were determined using a NanoDrop-2000c Spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The presence of DNA in all obtained samples was observed by 16S rDNA PCR (Table 1).

The bacterial quantification was determined with a Real-time PCR using SYBR Green system. Amplification reactions were performed in final volumes of 20 μL, containing 2X μL of SYBR Green PCR Master Mix (Promega, Madison, USA), 100 mM species-specific primers for each microorganism and 2 ng of DNA. As a negative control, sterile ultrapure water was used instead of DNA. Amplifications were performed in a thermocycler Rotor Gene (Life Science Corbett, Mort Lake, New South Wales, Australia) using the Rotor Gene 6000 analytical software. The primer sequences and qPCR conditions are shown in Table 1. DNA obtained from *Aggregatibacter actinomycetemcomitans* ATCC 29523, *Fusobacterium nucleatum*

P. intermedia		*P. nigrescens*		*T. forsythia*		*T. denticola*		*E. faecalis*		*P. endodontalis*	
Lesion	Apex	Lesion	Apex	Lesion	Apex	Lesion	Apex	Lesion	Apex	Lesion	Apex
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	3.6	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0[a]	0
0	0	0	0	0	0	0	0	0	0	0	0
9.45	0	0	0	0	2.1	0	0	2.4	4.5	0	0
0	0	0	0	3.6	0	1.67	0	0	0	0[a]	0
0	0	0	0	0	2.9	0	0	0	0	0	0
0	0	0	0	1.9	5.4	0	0	0	0	0	0
0	0	0	1.7	3.6	6.0	0	0	0	4.4	0[a]	0.4
0	0	0	0	0	5.2	0	0	0	0	0	0[a]
0	0	0	0	6.2	0	0	0	0	0	1.0	0
0	0	0	0	4.1	6.6	2.1	4.8	0[a]	0[a]	3.5	0
0	0	0	0	5.3	4.2	3.7	0	0	0	0[a]	0[a]
0	0	0	0	2.4	0.5	0	0	0	0	0	0
0	0	0	0	0	3.7	3.0	2.1	0	0[a]	0	0
11.2	0	0	0	0	2.4	0	0	0	0	0	0
0	2.0	0	0	1.8	2.4	0	0	0[a]	0[a]	0	0
0	0	0	0	0	2.5	0	0	0	0	0[a]	0[a]
0	0	0[a]	0	2.3	0	0	0	0	0[a]	0	0[a]
0	0	0	0[a]	0	2.7	0	0	0	0[a]	0	3.0
0	0	0	0	0	2.5	0	0	0	0[a]	0	0
0	0	0	0	0	0	0	0	0	0	0	0
11.5	0	0	0	3.1	0	0	0	0	0	3.0	2.8
0	1.2	0	0	5.1	1.6	0	2.6	1.8	2.4	0	0[a]
3.4	0	0[a]	0	0	0	0	2.2	0	0	0[a]	0[a]
0	1.0	0	0	2.3	0	0	0	0	0	0	0
1.1	0	0	0	0	0	1.2	0	0	4.6	0	0[a]
0	0	0	0	3.7	3.3	2.7	0	0	0	0[a]	0[a]

ATCC 25586, *Porphyromonas gingivalis* ATCC 33277, *Porphyromonas endodontalis* ATCC 35406, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* ATCC 33563, *Dialister pneumosintes* ATCC 33048, *Tannerella forsythia* ATCC 43037, *Treponema denticola* ATCC 33520, and *Enterococcus faecalis* ATCC 29212, were used as positive controls and to construct the respective standard curves. A dissociation curve was obtained to determine the primer specificity, and melting analysis showed only a single peak of amplification for all primer pairs. A standard curve was also derived using 10-fold DNA dilutions from the reference strains with their respective primer pairs. Amplifications were adjusted to R^2 values > 0.900. A *P* value of <0.05 was considered statistically significant.

2.4. Data analysis

Descriptive statistical analyses using the absolute and relative frequencies for each microorganism and the bacterial average rate from root end and apical lesions were conducted. A significance level of 5% was used to compare the presence and quantity of microorganisms in root ends and apical periodontitis using the Kruskal-Wallis test, Chi-square test with Yates' correction, and Fisher Exact test.

3. Results

The bacterial prevalence in the root ends and their surrounding periradicular tissues is shown in Table 2. The bacterial population in both the root ends and the periradicular tissues consisted of, in decreasing order of prevalence: *F. nucleatum* (71.6%) > *D. pneumosintes* (58.3%) > *T. forsythia* (48.3%) > *A. actinomycetemcomitans* (25%) > *T. denticola* (16.6%) > *P. intermedia* (15%) > *P. gingivalis* (15%) > *E. faecalis* (11.6%) > *P. endodontalis* (10%) > *Prevotella nigrescens* (1.6%). By using the Chi-square test with a Yates' correction, statistically significant differences ($P = 0.036$) were detected for prevalence of *D. pneumosintes* in the root ends. Saliva was used to verify the effectiveness of disinfection and no evaluated microorganism was detected, proven the good action of 0.12% chlorhexidine.

The bacterial distribution (Table 3) and their associations (Table 4) in the root end and periradicular lesion samples are shown. Root ends showed statistically significant differences ($P < 0.001$, Kruskal-Wallis test) between the evaluated bacteria. *Dialister pneumosintes* showed a statistically significant concentration ($P < 0.001$), followed by *F. nucleatum* and *T. forsythia,* which also showed significantly higher values regarding the other bacteria.

In apical periodontitis, statistically significant differences ($P < 0.001$, Kruskal-Wallis test) were observed in the number of bacterial copies between the evaluated bacteria. *Fusobacterium nucleatum* showed statistically significant values ($P < 0.001$), followed by *D. pneumosintes* and *T. forsythia,* which also had significantly higher values relative to other bacteria.

Prevotella intermedia was detected in 3 mm root end samples varying from $\log_{10}$ 1.0 to 2.0. This bacterium was detected in high values only in five periradicular lesion samples, varying from $\log_{10}$ 1.1 to 11.5 (Table 3). In the clinical samples of the root ends and periradicular lesions, the following value ranges, in $\log_{10}$, were 0–2.8 and 0 to 3.7 for *P. gingivalis*, 0 to 3.7 and 0 to 4.5 for *A. actinomycetemcomitans*, from 0 to 1.7 and 0 for *P. nigrescens*, 2.1 to 4.8 and 1.2 to 3.7 for *T. denticola*, 0 to 3.0 and 0 to 3.5 for *P. endodontalis,* and from 0 to 4.6 and 0 to 2.4 for *E. faecalis,* respectively (Table 3). A statistically significant association ($P = 0.045$) between the levels of *T. forsythia* and *P. gingivalis* in the root end samples was observed.

Most clinical samples harbored 2 to 7 microorganisms. The presence of a unique bacterium was also observed in five cases from both types of clinical samples, and the unique species were

Table 4
Bacterial presence and association in root end and periradicular lesion samples.

Patients	Root apex	Periapical lesion
P1	*A. actinomycetemcomitans*	None
P2	*A. actinomycetemcomitans*	*A. actinomycetemcomitans*
P3	*A. actinomycetemcomitans, F. nucleatum, D. pneumosintes, E. faecalis*	*A. actinomycetemcomitans*
P4	*D. pneumosintes*	*A. actinomycetemcomitans, F. nucleatum, D. pneumosintes*
P5	*A. actinomycetemcomitans, D. pneumosintes*	*A. actinomycetemcomitans*
P6	*D. pneumosintes*	*A. actinomycetemcomitans*
P7	*D. pneumosintes, T. forsythia, E. faecalis*	*F. nucleatum, D. pneumosintes, P. intermedia, E. faecalis*
P8	*F. nucleatum*	*D. pneumosintes, T. forsythia, T. denticola*
P9	*F. nucleatum, T. forsythia*	*F. nucleatum*
P10	*F. nucleatum, D. pneumosintes, T. forsythia*	*F. nucleatum, T. forsythia*
P11	*F. nucleatum, D. pneumosintes, P. nigrescens, T. forsythia, E. faecalis, P. endodontalis*	*F. nucleatum, T. forsythia*
P12	*F. nucleatum, D. pneumosintes, P. intermedia, T. forsythia*	*F. nucleatum, D. pneumosintes*
P13	None	*F. nucleatum, D. pneumosintes, T. forsythia, P. endodontalis*
P14	*F. nucleatum, P. gingivalis, D. pneumosintes, T. forsythia, T. denticola*	*F. nucleatum, T. forsythia, T. denticola, P. endodontalis*
P15	*F. nucleatum, D. pneumosintes, T. forsythia*	*F. nucleatum, D. pneumosintes, T. forsythia, T. denticola*
P16	*F. nucleatum, A. actinomycetemcomitans, P. gingivalis, D. pneumosintes, T. forsythia*	*P. gingivalis, T. forsythia*
P17	*F. nucleatum, D. pneumosintes, T. forsythia, T. denticola*	*F. nucleatum, A. actinomycetemcomitans, P. gingivalis, D. pneumosintes, T. denticola*
P18	*F. nucleatum, T. forsythia*	*F. nucleatum, P. intermedia*
P19	*F. nucleatum, A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia*	*F. nucleatum, T. forsythia*
P20	*F. nucleatum, D. pneumosintes, T. forsythia*	*F. nucleatum, D. pneumosintes*
P21	*F. nucleatum, D. pneumosintes*	*F. nucleatum, D. pneumosintes, T. forsythia*
P22	*F. nucleatum, P. gingivalis, D. pneumosintes, T. forsythia, P. endodontalis*	None
P23	*F. nucleatum, T. forsythia*	*F. nucleatum, A. actinomycetemcomitans*
P24	*F. nucleatum, D. pneumosintes*	*F. nucleatum, D. pneumosintes*
P25	*F. nucleatum, D. pneumosintes, P. endodontalis*	*F. nucleatum, P. intermedia, T. forsythia, P. endodontalis*
P26	*F. nucleatum, P. gingivalis, D. pneumosintes, P. intermedia, T. forsythia, T. denticola, E. faecalis*	*F. nucleatum, P. gingivalis, D. pneumosintes, A. actinomycetemcomitans T. forsythia, E. faecalis*
P27	*F. nucleatum, D. pneumosintes, T. denticola*	*D. pneumosintes, P. intermedia*
P28	*F. nucleatum, D. pneumosintes, A. actinomycetemcomitans, P. intermedia*	*F. nucleatum, D. pneumosintes, T. forsythia*
P29	*F. nucleatum, D. pneumosintes, E. faecalis*	*F. nucleatum, P. gingivalis, P. intermedia, T. denticola,*
P30	*D. pneumosintes, T. forsythia*	*F. nucleatum, T. forsythia, T. denticola*

A. actinomycetemcomitans, *F. nucleatum* and *D. pneumosintes* (Table 4). The absence of the evaluated microorganisms was observed in 1/30 and 2/30 samples from the root ends and periapical lesions, respectively. However, all post-treatment apical periodontitis cases were considered infected because at least one microorganism was always detected in the root end or periapical lesion samples of the same patient.

4. Discussion

Recent advances in diagnostic methods using molecular biology techniques have overcome many culturing limitations, and there has been a significant increase in the knowledge of the microbial diversity in failed endodontically treated teeth [17,18]. Studies have shown that secondary and persistent infections are predominantly polymicrobial. This may be explained because in these cases, the endodontic infection requires more time to accumulate microorganisms and to manifest clinical and radiographic signs [19].

PCR is used for the microbial DNA detection, as it is a highly sensitive method with the ability to detect as-yet-uncultivated bacteria. Using this technique, we showed a great microbial diversity in post-treatment apical periodontitis. However, because bacterial DNA may remain detectable by PCR for a long period after cell death, the PCR-based examination of genetic material can potentially overestimate the true bacterial load [20]. In addition, a simple microbiological examination cannot determine whether specific bacteria are the primary pathogens, or transient/permanent species that are unrelated to the disease [21].

In this study, a bacterial quantitative evaluation by Real-time PCR using the SYBR green system was performed to analyze root end and periradicular tissue samples from teeth with post-treatment apical periodontitis. This method was previously used to investigate the presence, assess the levels and quantify the microorganisms present in endodontic infections [22,23]. In a recent study, Siqueira Jr. et al. [24] using a next generation sequencing approach identified several bacterial DNA from cryopulverized apical root samples from root canal-treated teeth with post-treatment disease. These authors showed a highly complex bacterial community in the apical root canal system of adequately treated teeth with persistent apical periodontitis. Similarly, in our study multispecies bacterial communities were also detected.

It is known teeth with sealed canal display low or no nutrients and it makes difficult the bacterial survival in post-treatment endodontic infections when compared to the primary infections. In this study, using the orthodontic pliers a large amount of DNA was obtained from both root apex and periradicular samples.

It is established that patients with intra-radicular infection commonly present symptomatic teeth [25]. The main cause of persistent apical periodontitis is the permanence of residual bacteria in the complex canal system that remained unaffected by the treatment procedures [5]. Saber et al. [21] analyzed periradicular lesions using pyrosequencing technique and observed a high level of bacterial diversity in 53.8% of symptomatic endodontic treatment failure cases. The presence of different bacterial combinations observed in this study, confirm those data, and the microbial variety and heterogeneity suggest their ability to survive in unsuitable environment or any protection among them.

Other studies have also found evidence of bacterial contamination in extra-radicular sites of endodontically treated teeth with persistent apical periodontitis lesions, as well as the apical third, apical delta, and outer surface biofilm [6,26]. The host' immune response inflammatory is the first line of microbial elimination in the periapical region. However, in long-standing infections, the host's defenses appear to be less effective, as microorganisms can survive outside the root canal, on the root surface, or in the core of the periradicular lesions from endodontically filled asymptomatic

teeth [27]. Such microorganisms have the ability to sequester nutrients and overcome host defense mechanisms [7].

Previous methodologies have used absorbent paper points placed in the main canal until the apical portion to collect a pool of bacteria; however, this strategy cannot differentiate between the coronal, middle and apical thirds. It is known that the apical microbiota is highly diverse, differing in composition from that of the coronal/middle thirds of the same tooth [28]. These differences can be explained by different ecological conditions. The apical third of the canal has a low oxygen tension and generous availability of proteins and glycoproteins, which are inducers for establishing of oral anaerobic bacteria [4]. In this study, most of microorganisms were detected in root apex samples, and it may be explained by their ability to adhere to rough and/or mucosa surfaces.

Sample collection with absorbent paper points could likely not reach bacteria located in distant sites in the main canal, including those within the dentinal tubules, lateral canals, apical ramifications and isthmuses, and it leading to an underestimation of the number and diversity of microorganisms from the root canal due to these technical restrictions, in accordance with Tennert et al. [19]. In this study, was provided a more representative sample of the apical third of the root canal system as orthodontic pliers were used to crush 3 mm of the root ends. Furthermore, high bacterial DNA values were obtained by trituration of the root ends, suggesting that microorganisms are indeed colonizing the dentinal tubules. Following this line of reasoning, studies have been completed using techniques such as cryogenic grinding [4] and pulverization [29].

In addition, bacteria with the ability to adapt to these poor conditions could become established in the canal space and play a role in the etiology of post-treatment diseases [3]. In our study, most of the collected samples from the root end or periradicular lesions harbored at least one bacterial species, and the samples on average contained four (range 1–8) bacterial species. Since the use of qPCR to detect viable and non-viable microorganisms does not determine the action of the infection, the clinical relevance of the presence of bacterial DNA in periapical lesions is uncertain because it might reflect an active or inactive injury due to the dead bacteria or source of detected bacterial DNA, as suggested by Tennert et al. [19].

Discrepancies in the literature regarding the bacterial prevalence in endodontic infections may be explained by differences in the detection methods, sample collection, and patient's clinical conditions. Specific microorganisms have been found to have similar prevalence in primary and secondary infections, showing that they are not totally eradicated during endodontic treatment, such as *F. nucleatum* [15]. *Prevotella nigrescens* and *Treponema denticola* can also be found in both cases [16]. *Prevotella intermedia* and *P. gingivalis* have been found in asymptomatic teeth with persistent endodontic infection [19]. *Dialister pneumosintes* was included as member of the microbiota of primary endodontic infections and periradicular infection due to its high prevalence and pathogenicity [30]. Furthermore, it has been found to be one of the most prevalent bacteria in root filled teeth with periradicular lesions [2], and it was also observed in this study showing a total prevalence of 8.3% (Table 2).

The presence of *P. endodontalis* (65%), *F. nucleatum* (53%) and *T. forsythia* (47%) was reported in teeth with primarily infected root canal systems [5]. In teeth with persistent endodontic infections, *F. nucleatum* (27%), *P. gingivalis* (20%) and *T. denticola* (13%) were previously reported [17]. This study was performed in teeth with endodontic treatment failure, and the presence of *F. nucleatum* (73.3%), *T. forsythia* (53.3%), *P. gingivalis* (16.6%), *T. denticola* (13.3%) and *P. endodontalis* (10%) was observed in the 3 mm root end samples, suggesting the composition of a complex microbiota with different bacterial counts.

The quantitative analysis of root end samples showed a significant association between *T. forsythia* and *P. gingivalis*. Bacterial associations are commonly observed in oral infections, and a possible synergistic relationship between these bacteria in root end and periradicular diseases is suggested, as reported by Siqueira & Roças [12].

Studies have shown the presence of different bacteria in extraradicular samples. Noguchi et al. [31] reported that fourteen out of twenty extra-radicular biofilm samples exhibited *F. nucleatum*, *P. gingivalis* and *T. forsythia* as the most frequent species. Li et al. [10] also showed the presence of *T. forsythia*, *T. denticola*, *P. gingivalis*, *P. intermedia* in 24 persistent periapical lesions; and Saber et al. [21] showed that seven of the thirteen evaluated periradicular lesions contained of the most abundant genera (*Fusobacterium*, *Prevotella* and *Porphyromonas*). In the present study, from 30 periradicular lesions 21 *F. nucleatum*, 13 *T. forsythia* and 4 *P. gingivalis* were detected. Our results agree with Lin et al. [10], Saber et al. [21] and Noguchi et al. [31].

In addition, our findings are consistent with previous reports [2,30], in that *F. nucleatum*, *D. pneumosintes* and *T. forsythia* were found to be the most common pathogens, as well as a lower proportion of *A. actinomycetemcomitans*, *P. gingivalis*, *P. endodontalis, T. denticola, P. intermedia, P. nigescenes* and *E. faecalis* was found. However, the precise bacterial role, alone or in association within the filled root canal system and periradicular tissues could not be determined. Interestingly, the presence of *P. intermedia* in 3 out of 5 periradicular lesions showed high values, in $\log_{10}$ (9.45, 11.2, and 11.5), and to our knowledge it has not been reported even for periodontitis samples.

Few reports have shown the presence of *A. actinomycetemcomitans* in periapical lesions. Lin et al. [10] detected this microorganism in 12.5% of persistent periapical lesions, while we found it in 23.3% of the root ends and in 26.6% of the periapical lesion samples. It was interesting to note that the evaluated teeth did not display pocket depths of more than 3 mm. Since *A. actinomycetemcomitans* is a resident component of the oral microbiota and commonly found in periodontal infections, its presence in periapical and root end samples is possible, and it must be in consideration in persistent endodontic infections.

The failure of secondary/persistent endodontic infection treatment has been related to the presence of *E. faecalis* [19]; however, recent study has questioned the idea that this microorganism is the main pathogen in teeth with post-treatment apical periodontitis [23]. As expected, we detected *E. faecalis* in low numbers, in accordance with Siqueira Jr. et al. [28]. Secondary/persistent apical periodontitis presents a high level of inter-individual variability in the composition of microbiota. Thus, apical periodontitis appears to have a heterogeneous microbiota, in which multiple bacterial communities including uncultivated phenotypes may produce similar diseases [28,32]. These data suggest an evaluation of different methods of bacterial detection in apical periodontitis cases.

Our results supply additional information regarding the microbial diversity of post-treatment apical periodontitis. However, the presence of bacteria does not definitively mean that periapical disease will follow. For this reason, further research focusing on bacterial pathogenicity and virulence factors will be important to elucidate the role of these species in endodontic treatment failures as well as to evaluate the susceptibility to antimicrobial drugs.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors thank Mrs. Marcia Harumi for her technical support. This study was supported by grants from CAPES-PNPD (No. 2009), FAPESP (No. 2013/13652-6).

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