

Treponema diversity in root canals with endodontic failure

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ABSTRACT

Objective: This study sought to investigate the prevalence of eight oral *Treponemas* (*Treponema denticola*, *T. amylovorum*, *T. maltophilum*, *T. medium*, *T. pectinovorum*, *T. socranskii*, *T. vicentii* and *T. lecithinolyticum*) in teeth with endodontic treatment failure and periapical lesion.

Methods: Samples were taken from 40 root canals presenting endodontic failure and periapical lesion. DNA extraction was performed and Nested-PCR technique was used for the detection of *Treponema* species using specific primers.

Results: *Treponemas* was detected in 56.5% of the samples analyzed (22/39). Individual root canals yielded a maximum of 6 target *Treponema* species. *T. denticola* (30.8%) and *T. maltophilum* (30.8%) were the most frequently detected species followed by *T. medium* (20.5%), *T. socranskii* (20.5%), *T. pectinovorum* (17.9%) and *T. vicentii* (17.9%). Positive association was verified between *T. denticola* and *T. maltophilum* such as *T. medium* ($P < .05$). *T. lecithinolyticum* was positively associated with intraradicular post ($P < .05$).

Conclusion: The present study revealed that a wide variety of *Treponema* species plays a role in persistent/secondary infection turning the root canal microbiota even more complex than previously described by endodontic literature. (Eur J Dent 2013;7:61-68)

Key words: Endodontic failure; microbiology; nested-PCR; bacteria; root canal

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INTRODUCTION

The persistence of symptoms or the presence of periapical lesion which remains unchanged, increased or appeared after endodontic treatment suggest that endodontic retreatment is necessary.¹⁻⁴ It has long been known that microorganisms resistant to instrumentation or medication (persistent infection) and those contaminating the root canal through coronary leakage (secondary

infection) after endodontic treatment are one of the main responsible for endodontic failures.⁵⁻⁹

Culture methods revealed that the bacterial etiology of post-treatment apical periodontitis is a Gram-positive bacterial infection.^{2,5,10} However, molecular methods have indicated a more complex microbiota with the involvement of Gram-negative bacteria, such as *Prevotella* spp., *Porphyromonas* spp. and *Treponema* spp.^{7-8,11-12}

Treponema spp., a very fastidious, Gram-negative, motile spirochetes, is known as an important periodontal pathogen isolated from the subgingival plaque.¹³⁻¹⁶ The 16S rRNA-based analysis revealed an unexpected diversity of oral *Treponema* species in the subgingival pocket. Among these, 8 species have been identified and named: *T. denticola*, *T. Vincentii*, *T. socranskii*, *T. pectinovorum*, *T. maltophilum*, *T. medium*, *T. amylovorum* and *T. lecithinolyticum*.¹³⁻²¹

Due to the microbial similarity between periodontal pockets and root canal microbiota, some species have been detected in primary endodontic infection.²²⁻²⁴ Therefore, they might participate in the pathogenesis of periradicular lesions in unsuccessful endodontic treatment.^{8,25-26} Currently, no clinical study has focused on the investigation of different *Treponema* species in failed root canals treatment.

The aim of this study was to detect by nested-PCR the presence of eight species of *Treponema* (*T. denticola*, *T. amylovorum*, *T. maltophilum*, *T. medium*, *T. socranskii*, *T. pectinovorum*, *T. Vincentii*, and *T. lecithinolyticum*) in those cases needing endodontic retreatment, in order to investigate the possible great diversity of *Treponema* spp. in persistence/secondary endodontic infection,

MATERIAL AND METHODS

The present study was approved by the Research Ethics Committee of the Piracicaba Dental School (State University of Campinas, Piracicaba, São Paulo, Brazil), and informed consent was obtained from all subjects.

Patient Selection

Patients in need of endodontic retreatment were selected on the basis of clinical and radiographic examination. Patients who had received antibiotic treatment during the preceding 3 months or who had systemic disease were not included in this study.

Sampling Procedure

The methods followed for the microbiologic procedures performed in this study have been previously described.^{27,28} Clinical features were recorded and samples were collected from 40 teeth with endodontic failure and periapical lesion. The teeth were isolated from the oral cavity with a rubber dam, and the disinfection of their external surfaces and the surrounding field was carried out by using 30% hydrogen peroxide followed by 2.5% NaOCl. The solutions were inactivated with 5% sodium thiosulfate to avoid interference with bacteriologic sampling. The sterility of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates, which was incubated aerobically and anaerobically. A 2-stage access preparation was performed. The access cavity was made without the use of water spray but under manual irrigation with sterile saline solution and by using sterile high-speed diamond bur. This first stage was performed to promote a major removal of the contaminants (microorganisms and endotoxins). In the second stage before entering the pulp chamber, the access cavity was disinfected following the decontamination protocol described above. Its sterility was checked by taking swab samples of the cavity surface and streaking on to blood agar plates, with subsequent incubation at 37°C under both aerobic and anaerobic conditions. A new sterile bur was used, accomplished by irrigation with sterile/ endotoxin-free saline, to access the canal. Root-filling materials were removed by rotary instrumentation [Gates-glidden drills #5, 4, 3, 2 (Dentsply-Maillefer, Ballaigues, Switzerland) and Hero-file #20.06 (MicroMega, Besançon, France)] and K-files in a crown-down technique without the use of chemical solvent, accomplished by irrigation with sterile/ endotoxin-free solution.

For microbial sampling, a sterile paper point was introduced into the full length of the canal (as determined with a preoperative radiograph), and kept in place for 60 s. In the cases that had been previously irrigated with saline, as many paper points as possible were used to absorb all liquid or fluid inside the canal. The paper point samples from the root canal were transferred immediately to a transport medium VMGA III and were

kept at -20°C . Afterwards, the tubes containing the samples were shaken during 60 seconds and then 300 μL of the transport medium were used to perform DNA extraction with QIAamp DNA Minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After extraction, DNA was kept at -20°C .

Detection of *Treponema* species by Nested-PCR

The isolated DNA was first amplified with universal prokaryotic ribosomal 16S primer. This universal reaction were performed in a total volume of 50 μL containing 10 μL of extracted DNA; 5 μL of 10X PCR buffer; 1.5 μL of 25 mmol/L MgCl_2 ; 4 μL of a mixture of each deoxynucleoside triphosphate (100mmol/L solution in a 10-fold dilution); 1 μL of 25 pmol forward-universal primer (5' GAGAGTTT-GATYMTGGCTCAG 3') and 1 μL of 25 pmol of reverse-universal primer (5' GAAGGAGGTGWTCCARCCGCA 3'); 0,5 μL of 5U/mL Platinum Taq DNA Polymerase. The reagents were synthesized and provided by Invitrogen (Carlsbad, CA, USA). Samples were previously subjected to 4-minute denaturation at 94°C , followed by 30 cycles of 45-second denaturation at 94°C , 45-second annealing at 60°C s, 1.5-minute extension at 72°C , and a final extension at 72°C for 15 minutes in automated thermal cycler (GenePro Bioer, China).²⁸ Positive controls were performed with standard stain, whereas negative controls corresponded to the reaction mixture without DNA.

Treponema spp. were identified by using a second nested amplification with species-specific 16S rRNA primers for *T. denticola*, *T. amylovorum*, *T. maltophilum*, *T. medium*, *T. socranskii*, *T. pectinovorum*, *T. vicentii*, and *T. lecithinolyticum*. Primer sequence and cycles are shown in Table 1, as previously described by Willis et al²⁹ The reactions were performed in a total volume of 25 μL containing 1.5 μL of universal PCR product; 2.5 μL of 10X PCR buffer; 1.25 μL of 25 mmol/L MgCl_2 ; 0.25 μL of a mixture of each deoxynucleoside triphosphate (100 mmol/L solution in a 10-fold dilution); 0.25 μL of each species-specific primer (25 pmol); 0.125 μL of 5U/mL Platinum Taq DNA Polymerase.

The PCR species-specific primer pairs used for detection of eight *Treponema* species in failed root canals as well as the amplicons size and PCR cycles are shown in Table 1.

The PCR products were electrophoresed on 1% agarose gel and tri-acetate-EDTA buffer stained with 0.5 $\mu\text{L}/\text{ml}$ ethidium bromide and visualized under ultraviolet light. Positive reactions were determined by the presence of bands of the appropriate size. A 1kb DNA ladder (Invitrogen) was used as size marker for universal PCR and a 100 bp DNA ladder was used for the second amplification.

Statistical Analysis

Data collected from each patient (clinical features) were entered into a spreadsheet and statistically analysed by using SPSS for Windows (SPSS, Chicago, IL, USA). Pearson's chi-square or Fisher's exact tests were chosen to determine whether there were significant statistical correlations between specific species and endodontic signs/symptoms and between lesion size and number of bacteria, including positive and negative association between the species.

RESULTS

All samples were positive for bacterial DNA as determined by the use of ubiquitous primer except for one negative sample, which was discarded. On the other hand, no positive results were observed in the negative-control sample regarding the presence of bacterial DNA.

The following radiographic/clinical features were observed in the 39 root canals analyzed: radiolucent area (39/39), inadequate root filling or restoration (30/39); presence of spontaneous pain (5/39), tenderness to percussion (11/39), and sinus tract (6/39). Eighteen out of the 39 teeth analyzed presented intra-radicular post (Table 2).

Treponema species were detected in 56.5% of the root canal samples analyzed (22/39). Individual root canals yielded a maximum of 6 target *Treponema* species, which was detected in 2.56% of the root canal samples analyzed (1/39) (Table 2). The most frequently detected species were *T. denticola* (30.8% - 12/39), *T. maltophilum* (30.8% - 12/39), *T. medium* (20.5% - 8/39) and *T. socranskii* (20.5% - 8/39), followed by *T. pectinovorum* (17.9% - 7/39) and *T. vicentii* (17.9% - 7/39) (Table 2). Low detection levels were observed for *T. lecithinolyticum* (10.2% - 4/39) and *T. amylovorum* (7.6% - 3/39) (Table 2). In addition, *T. lecithinolyticum* was positively associated with intra-radicular post ($P < .05$).

A combination of two or more *Treponema* species was detected in 18 out of the 39 root canals investigated (Table 2). Positive associations were found between *T. denticola* and *T. maltophilum* ($p = 0.002$, odds ratio [OR] = 11.500, confidence bound [CB] = 2.316 - 57.101) such as with *T. medium* ($p = 0.006$, OR = 12.500, CB = 2.002 - 78.051). No correlation was found between presence of any *Treponema* species and development of clinical or radiographic findings ($P > .05$).

DISCUSSION

Analysis of our data demonstrated that a wide variety of *Treponema* species do play a role in failed root canal treatment, particularly showing a predominance of *T. denticola* and *T. maltophilum* species.

Researches have shown a predominance of Gram-positive bacteria and the important role of *E. faecalis* in persistent/secondary infection mainly due to the resistance of this bacteria.^{2,5,10,37} Data concerning the detection of this species in endodontic treatment failures vary widely. Pinheiro et al,² using culture technique revealed that *E. faecalis* were the most commonly isolated microorganism in root-filled teeth with periapical lesions. On the other hand, Foschi et al³⁷ and Fouad et al,³⁸ using PCR, detected *E. faecalis* in 72% and 22%

of the cases, respectively. Gomes et al³⁹ isolated *E. faecalis* in 42% and 76% of root-filled teeth by culture and PCR respectively. Preliminary studies of our samples showed an occurrence of 30% of *E. faecalis* by culture and 40% by nested-PCR.

Culture is the only method that detects microbial viability (ability to reproduce) and therefore, what grows on culture media is directly related to the number of microbial cells present in the infection. For this reason, *E. faecalis* has been found in great number in root-filled canals investigated by this method. However, culture depends on the viability of the bacteria and requires methodology for bacterial isolation, growth and detection. Moreover, some of the bacteria involved in endodontic infections are nutritionally fastidious and extremely sensitive to oxygen. Consequently, culture can fail to detect some microorganisms present in endodontic infections, such as *Treponema* spp.⁴⁰ In retreatment cases the number of microorganisms is even lower and/or the number of microbial cells can be lost during the procedure to remove the previous root filling. As a consequence, the number of cells sampled can be lower than the detection rate of the culture method.³⁹ Therefore, molecular methods that are more sensitive may be necessary to better describe the infection composition and have indicated a more complex microbiota.^{7-8,11-12,37,39}

Table 1. PCR primer pairs used for detection of 8 *Treponema* species in teeth with endodontic failure by Nested-PCR.

Microorganism	Primer pairs [5'-3']	Amplicon size	Cycles
<i>T. denticola</i>	F: TAA TAC CGA ATG TGC TCA TTT ACA T R: TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp	Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min, 72°C for 2min and a final step 72°C for 10min.
<i>T. amylovorum</i>	F: AGA GTT TGA TCC TGG CTC AG R: CTC ACG CCT TTA TTC CGT GAG	193 bp	Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min, 72°C for 2min and a final step 72°C for 10min.
<i>T. maltophilum</i>	F: AGA GTT TGA TCC TGG CTC AG R: CCT ATT GTG CTT ATT CAT CAG GC	438 bp	Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min, 72°C for 2min and a final step 72°C for 10min.
<i>T. medium</i>	F: AGA GTT TGA TCC TGG CTC AG R: CCT TAT GAA GCA CTG AGT GTA TTC	192 bp	Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min, 72°C for 2min and a final step 72°C for 10min.
<i>T. socranskii</i>	F: GAT CAC TGT ATA CGG AAG GTA GAC A R: TAC ACT TAT TCC TCG GAC AG	285 bp	Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min, 72°C for 2min and a final step 72°C for 10min.
<i>T. pectinovorum</i>	F: AGA GTT TGA TCC TGG CTC AG R: ATA TAT CTC CAA CTT ATA TGA CCT	194 bp	Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min, 72°C for 2min and a final step 72°C for 10min.
<i>T. vincentii</i>	F: AGA GTT TGA TCC TGG CTC AG R: AAT ACT TCT TAT GAA CAT TGA GAC	193 bp	Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min, 72°C for 2min and a final step 72°C for 10min.
<i>T. lecithinolyticum</i>	F: CTT GCT CCT TTC TGA GAG TGG CGG R: ACG CAT CCG TAT CTC TAC GAA CTT	950 bp	Initial denaturation at 95°C for 2min and 36 cycles of: 94°C for 30s, 60°C for 1min, 72°C for 2min and a final step 72°C for 10min.

Treponema spp. play an important role in periodontal disease.^{13-16,21} and have been investigated in endodontic infections by molecular methods.^{22-24,31-32,37} However, little is known about this microorganism in persistent/secondary infection. Yet,

no clinical study has focused on the investigation of different *Treponema* species in root canals after endodontic treatment failure.

Species of *Treponema* have different virulence factors that can contribute to their pathogenicity.

Table 2. Occurrence of 8 *Treponema* species, clinical and radiographic features.

Cases	Treponema species								Signs and Symptoms					Nº Species detected
	Td	Tma	Tme	Ts	Tp	Tv	Tl	Ta	SP	TP	ST	IRFR	P	
1	+	+							+	+	+	+		2
2										+		+	+	0
3	+		+										+	2
4														0
5		+	+	+										3
6	+				+	+		+				+		4
7	+				+					+		+	+	2
8	+	+					+					+	+	3
9	+	+	+	+	+			+				+		6
10												+	+	0
11	+	+	+	+			+						+	5
12	+	+		+								+		3
13	+	+					+				+	+		3
14												+	+	0
15	+		+	+	+							+	+	4
16			+	+										2
17											+	+		0
18														0
19										+		+		0
20							+			+		+	+	1
21	+	+	+	+			+			+		+	+	5
22									+	+		+	+	0
23									+	+	+	+		0
24										+		+		0
25										+		+		0
26		+					+					+		2
27											+	+	+	0
28		+							+	+		+		1
29											+			0
30					+	+						+	+	2
31	+	+	+	+				+				+		5
32					+	+							+	2
33									+			+	+	0
34		+					+					+	+	2
35							+					+		1
36												+	+	0
37					+									1
38												+		0
39												+	+	0

Td – *Treponema denticola*; Tma – *Treponema maltophilum*; Tme – *Treponema medium*; Ts – *Treponema socranskii*; Tp – *Treponema pectinovorum*; Tv – *Treponema vicentii*; Tl – *Treponema lecitinoliticum*; Ta – *Treponema amylovorum*; SP – Spontaneous Pain; TP – Tenderness to Percussion; ST – Sinus Tract; IRFR – Inadequate Root Filling or Restoration; P – Intra-radicular post.

They can produce proteolytic enzymes, adhere to and invade diverse host cells. *Treponema* species are endowed with motility, which allows them to penetrate the tissues, thus increasing their pathogenicity. They are also able to inhibit both lymphocyte function and polymorphonuclear neutrophils. Moreover, they have lipopolysaccharide (LPS) in their membrane as they are Gram-negative microorganisms. Their endotoxin has a significant toxic effect on the host, thus exacerbating the inflammatory response and enhancing the damage caused.^{15,16,34}

In the current study, the positive detection of *Treponema* spp. in failed root canals is in agreement with the literature.^{8,25,26} Because of the difficulties in isolating and identifying *Treponema* spp., the use of nested-PCR protocol in this study was justified by the higher sensitivity and specificity of the assay when compared to the single PCR method.^{22,24}

The frequent isolation of *Treponema* spp. (in more than 50% of the root canals analyzed) supports the role of such microorganisms in persistent/secondary infection.^{8,25-26} Montagner et al²⁴ found *Treponema* species in 90% of the root canals associated with abscess.

Data obtained in the present study revealed combinations of two or more *Treponema* species found in 18 out of the 39 root canals analyzed. The pathogenicity enhanced by additive effects is an important feature of mixed infection,³⁵ as this may contribute to the maintenance of apical periodontitis.^{2,10,24}

T. denticola (30.8%) and *T. maltophilum* (30.8%) were the most frequently detected species found in the present study. Siqueira et al²⁵ found *T. denticola* in 11 out of the 21 primary infected root canals investigated (51.4%) by using 16S rDNA-based polymerase chain reaction (PCR). In addition, Montagner et al²⁴ used nested-PCR and found *T. denticola* in 8 out of the 20 symptomatic primary infection.

It is worth to point out that no previous study had reported the participation of *T. maltophilum* in secondary infection. This species have been detected in teeth with primary endodontic infection.²²⁻²⁴ However, Siqueira & Roças³² did not detect *T. maltophilum* in teeth with abscess of endodontic origin.

No correlation was found between the presence of a specific *Treponema* species and development of any clinical symptomatology in root canals with persistent/secondary infection. In contrast,

the highest incidence of *Treponema* spp. in acute cases indicates their high pathogenicity, which may suggest the association of these species with signs and symptoms.^{8,23,32,36,37}

CONCLUSION

The detection of a wide variety of *Treponema* species in persistent/secondary infection indicates that the root canal microbiota seems to be even more complex in teeth with endodontic treatment failure than that previously shown in the endodontic literature.

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