

Prevalence of *Treponema* spp. in endodontic retreatment-resistant periapical lesions

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Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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DOI: 10.1590/1807-3107BOR-2015.vol29.0031

Submitted: Jul 10, 2014
Accepted for publication: Oct 15, 2014
Last revision: Dec 15, 2014

Abstract: This study investigated the presence of the *Treponema* species in longstanding endodontic retreatment-resistant lesions of teeth with apical periodontitis, the association of this species with clinical/radiographic features, and the association among the different target species. Microbial samples of apical lesions were collected from twenty-five adult patients referred to endodontic surgery after unsuccessful root canal retreatment. Nested-PCR and conventional PCR were used for *Treponema* detection. Twenty-three periradicular tissue samples showed detectable levels of bacterial DNA. *Treponema* species were detected in 28% (7/25) of the cases. The most frequently detected species were *T. socranskii* (6/25), followed by *T. maltophilum* (3/25), *T. amylovorum* (3/25), *T. lecithinolyticum* (3/25), *T. denticola* (3/25), *T. pectinovorum* (2/25) and *T. medium* (2/25). *T. vicentii* was not detected in any sample. Positive statistical association was found between *T. socranskii* and *T. denticola*, and between *T. maltophilum* and *T. lecithinolyticum*. No association was detected between the presence of any target microorganism and the clinical or radiographic features. *Treponema* spp. are present, in a low percentage, in longstanding apical lesions from teeth with endodontic retreatment failure.

Keywords: Retreatment; Periapical Tissue; Polymerase Chain Reaction; *Treponema*.

Introduction

Conventional nonsurgical endodontic treatment and retreatment may be unsuccessful, in some circumstances, because of the persistence of microbial infection of the root canal system and/or in the periradicular area.¹ This failure has been reported even when nonsurgical procedures are adequately performed.²

Microbiological studies have demonstrated that microorganisms can cross the threshold of the apical foramen through the apical tissues, either by extending the infected area, or as a consequence of manipulation during root canal therapy.^{3,4} They have been detected in apical lesions⁴ or attached to the surface of the root-end in resorption gaps promoted by periradicular inflammatory infiltrate and bacterial toxins.⁵

Dark field interference microscopy and sensitive molecular techniques have detected the *Treponema* species in primary endodontic infections,^{6,7} failed endodontic treatment⁸ and acute apical abscesses.⁹ This suggests

their potential role in the etiology of chronic and acute endodontic diseases. *Treponema* spp. are strictly anaerobic, motile, helically shaped bacteria. They are a member of the *Spirochaetes* phylum, a clade now believed to be distinct from both Gram-positive and Gram-negative bacteria. Many *Spirochaetes* are fastidious and their demanding nutrient and environmental requirements are problematic for culture procedures.¹⁰ In addition, evidence suggests that species like *Treponema denticola* from infected root canals can stimulate bone resorption and disseminate to distant organs of the host.¹¹

Although the *Treponema* species has been related to periodontal and endodontic infections, no study has addressed their presence in periapical lesions of persistent apical periodontitis associated with failure of adequately retreated root canals. Therefore, the aim of this study was to investigate the presence of *T. socranskii*, *T. maltophilum*, *T. amylovorum*, *T. medium*, *T. lecithinolyticum*, *T. denticola*, *T. vicentii* and *T. pectinovorum* in the extraradicular environment of persistent apical lesions associated with failure of endodontic retreatment, and to determine possible associations among the species and between specific species and clinical and radiographic features.

Methodology

Subject Selection

Twenty-five adult patients referred to the Endodontic Department of the Piracicaba Dental School, Piracicaba, SP, Brazil, for root-end surgery were included in this research (17 females and 8 males, between the age of 18 and 65 years). Subjects having any contributory systemic diseases or treated with antibiotics over the last 3 months were excluded from the study. The endodontic status of each tooth was evaluated clinically for pain, percussion and palpation sensitivity, mobility, and both extraoral and intraoral swelling. The clinical crowns were examined for restorative integrity, open canals, cracks, and carious lesions. Radiographically, all the teeth had satisfactory previous root canal filling with an apical obturation limit at 2 mm or less from the apex, and persistent apical lesions after at least 1-year follow-up of the nonsurgical endodontic retreatment. No tooth presented a sinus tract, discernible broken

instruments, ledges, perforations, or blockages. The periodontal status was evaluated by radiographs, and periodontal probing; teeth with periodontal probing depths over 4 mm were not included.

The present study was approved by the Research Ethics Committee of the Piracicaba Dental School (Protocol no. 065/2010 – *Universidade Estadual de Campinas* – UNICAMP, Piracicaba, SP, Brazil). Twenty of the 25 samples included in this research had been previously investigated by culture-based methods.²

Sample Collection

The apical lesion samples were collected as described by Signoretti *et al.*¹² The surgical site was disinfected by oral rinse with 0.2% chlorhexidine gluconate and swabbing of the surgical area with 2% chlorhexidine gel (Endogel, Itapetininga, Brazil). The carryover effect was reduced by local rinse with 5% Tween 80 and 0.07% soy lecithin. Proper anesthesia was applied and a marginal incision was made with one vertical releasing incision. A full thickness mucoperiosteal flap was reflected and the root-end access ostectomy was performed, if required, using a surgical bur cooled with sterile water. The lesion tissue was curetted and part of the excised tissue was immediately placed in a 10% formalin solution and sent for histopathological analysis at the Oral Pathology Department of the *Universidade Estadual de Campinas* – UNICAMP. The other part was pooled into a sterile tube containing 1 mL VMGA III³ and frozen at 80°C for subsequent microbial analysis. In order to check for cross contamination after flap reflection, periosteal tissue samples were collected from areas adjacent to the surgical site using curettes and paper points. These samples were analyzed by universal PCR to ensure the absence of bacterial DNA.

Treponema Species Detection

DNA Amplification for Nested-PCR (nPCR)

Genomic DNA purification of the samples was performed by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The nested-PCR protocol used to identify the *Treponema* species in this study was followed as mentioned hereinabove.^{9,14}

In the first set of reactions, 1.5 µL (±50ng/mL) of the DNA isolated from persistent apical periodontitis tissue was amplified by universal prokaryotic ribosomal 16S primer (Table 1). The PCR mix reactions with total volume of 25µL were performed with 2.5 µL of 10 X PCR buffer (500 mmol/L KCl, 200 mmol/L Tris-HCl, pH 8.4; Invitrogen, Eugene, USA) plus 1.25 µL of 50 mmol/L MgCl₂, 0.125 µL of 5 U/µL Platinum Taq DNA Polymerase, 0.25 µL of a mixture of each deoxynucleoside triphosphate (100 mmol/L solution, in a 10-fold dilution) and 0.25 µL 25 mmol/L of each forward and reverse primer. Samples were submitted to initial denaturation at 97°C for 1min and to 26 cycles of denaturation for 45 s at 97°C, annealing for 45 s (temperatures listed in Table 1) and extending for 1min at 72°C, followed by a final extension at 72°C for 4 min in an automated thermal cycler (GenePro Thermal Cycler, Bioer Technology, Hangzhou, China). Universal PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide (Invitrogen, Eugene, USA). Inserts of ≈1500 bp

viewed under ultraviolet transillumination were considered positive (Table 1).

In the second set of reactions, *Treponema* primers were used to amplify a specific target in an internal region of a 16S amplified sequence. In this second group of reactions, 1.5 µL of universal reaction products were used as targets for specific primers in the same amplification conditions described for the universal set. Positive controls were performed with the DNA of previous positive clinical samples from the Nobrega *et al.*¹⁵ study. Negative controls corresponded to the reaction mixture without the DNA template. The total volume of each nPCR product was analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide. Positive detection was determined by the presence of bands of predicted size (Table 1). The second set of reactions was repeated with twice the initial universal product volume to confirm absence of the target bacterial DNA in all negative samples.

All primers were synthesized by Invitrogen (Eugene, USA) and tested for all control bacteria; no bands of the predicted size were produced with non-

Table 1. PCR primer pairs used to detect the *Treponema* species.

Target	Sequences (5'-3')	Position (bp)	*T _m (°C)	Reference
Universal 16S rRNA	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTACGACTT	8 - 1,513 (1,505)	55	# ¹⁴
<i>T. amylovorum</i>	AGAGTTTGATCCTGGCTCAG CTCACGCCTTATTCCGTGAG	8 - 211 (193)	54	# ¹⁴
<i>T. denticola</i>	TAATACCGTATGTGCTCATTTACAT TCAAAGAAGCATTCCCTCTTCTCTTA	193 - 508 (316)	60	# ¹⁴
<i>T. leciithinolyticum</i>	CTTGCTCCTTTCTGAGAGTGGCGG ACGCATCCGTATCTCTACGAACTT	54 - 1,003 (950)	65	# ⁷
<i>T. maltophilum</i>	AGAGTTTGATCCTGGCTCAG CCTATTGTGCTTATTCATCAGGC	8 - 446 (438)	54	# ¹⁴
<i>T. medium</i>	AGAGTTTGATCCTGGCTCAG CCTTATGAAGCACTGAGTGTATT	8 - 200 (192)	54	# ¹⁴
<i>T. pectinovorum</i>	AGAGTTTGATCCTGGCTCAG ATATATCTCCAACCTTATATGACCT	8 - 205 (194)	53	# ¹⁴
<i>T. socranskii</i>	GATCACTGTATACGGAAGGTAGACA TACACTTATTCCTCGGACAG	179 - 468 (285)	53	# ¹⁴
<i>T. vicentii</i>	AGAGTTTGATCCTGGCTCAG AATACTTCTTATGAACATTGAGAC	8 - 201 (193)	56	# ¹⁴

*T_m – Melting temperature

target DNA. Primer sequences and corresponding annealing temperatures are shown in Table 1.

DNA Amplification for PCR

The positive nPCR samples were also submitted to conventional PCR reactions in order to verify if any of the samples had sufficient *Treponema* spp. DNA to reach the detection threshold of conventional PCR. This set followed the same parameters of *Treponema* specific reactions, but without the previous amplification of the 16S sequence.

Tissue Processing

Samples stored in formalin were washed in running water for 5 minutes and processed for embedding in paraffin. Five-micrometer-thick sections were cut from the paraffin blocks, stained with hematoxylin and eosin (H&E), and examined under light microscopy for diagnostic confirmation of cyst or granuloma. The following features were observed: presence and distribution of acute (polymorphonuclear leukocytes) and chronic inflammatory cells, granulomatous tissue, lining epithelium and fibrous connective tissue capsule.

Data Analysis

The data collected (clinical and radiographic features) was analyzed statistically by SPSS for Windows (SPSS, Chicago, USA). The Pearson chi-square or the Fisher's Exact test was chosen, as appropriate, to test the null hypothesis that there was no statistical association between the presence of a specific species and clinical and radiographic features, and to determine if there was no statistical relationship among the *Treponema* species in the same environment. The significance level was set at 5%.

Results

All 25 subjects presented mild to moderate discomfort on palpation and/or percussion, mostly (92%) associated with intraoral swelling. Fifteen lesions exhibited more than 5 mm in their widest diameter. The histopathological analysis of the excised tissue showed that 68% of the lesions were compatible with apical cyst formation and 32% with apical granuloma (Table 2).

Samples from periosteal tissues collected subjacent to the surgical site did not show bacterial DNA, confirming that the surgical site was sterile.

Detectable levels of bacterial DNA were found in all but two periradicular tissue samples. The nPCR method detected the *Treponema* species in 28% (7/25) of the cases. The number of species per positive case ranged from 1 to 7 (mean 2.86). The most frequently detected specie was *T. socranskii* (6/25), followed by *T. maltophilum* (3/25), *T. amylovorum* (3/25), *T. lecithinolyticum* (3/25), *T. denticola* (3/25), *T. pectinovorum* (2/25) and *T. medium* (2/25). *T. vicentii* was not detected in any sample. *T. socranskii* was commonly recovered in association with *T. denticola*, *T. maltophilum* and *T. lecithinolyticum* ($p = 0.009$). No statistically significant relationship was detected between the presence of any target microorganism and the clinical or radiographic features. Conventional PCR did not detect any of the species investigated.

Discussion

All the subjects of this research received previous nonsurgical endodontic retreatment. Radiographic follow-up showed no signs of regression of the apical lesion, and retreatment presented with symptomatology, despite its adequate performance. To our knowledge, no previous studies have attempted to identify spirochetes at the species level in the tissue of retreatment-resistant apical periodontitis. In the present study, we detected seven out of eight target species. All of these species have already been detected in primary, secondary and acute root canal infections.^{7,8,9,16}

The majority of the retreatment-resistant apical lesions enclosed bacterial DNA, with the exception of two samples. Similar findings were reported by Subramanian and Mickel,¹⁷ who found that 18.2% of the excised lesion did not present bacterial DNA. Either these samples were bacteria free, or bacterial colonies were located in the specific part of the samples that had been sent for histopathological examination.

Contamination control during surgical procedures is critical when sampling from the apical tissues. In this study, the periosteal tissue samples collected subjacent to the surgical site showed absence of bacterial DNA,

Table 2. Clinical and radiographic features, *Treponema* detection and histopathological diagnosis found in 25 lesions associated with persistent apical periodontitis.

Cases	Univ.	<i>Treponema</i> species									TPe	TPa	IS	LS		HD		No. of species detected
		Ts	Tma	Ta	Tme	Tl	Td	Tv	Tp	<5 mm				≥5 mm	Gr	Cist		
1	+	-	-	+	-	-	-	-	-	+	-	-	-	+	+	-	1	
2	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	0	
3	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
4	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	+	1	
5	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
6	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
7	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
8	+	+	+	-	-	+	-	-	-	+	+	+	+	-	-	+	3	
9	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	0	
10	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
11	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
12	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	0	
13	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	0	
14	+	+	-	-	-	-	-	-	-	+	+	+	-	+	+	-	1	
15	+	-	-	-	-	-	-	-	-	+	+	-	-	+	-	+	0	
16	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	7	
17	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
18	+	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	0	
19	+	+	-	-	-	-	+	-	-	+	+	+	-	+	-	+	2	
20	+	+	+	+	-	+	+	-	-	+	+	+	+	-	+	-	5	
21	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
22	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	0	
23	-	*	*	*	*	*	*	*	*	+	+	+	+	-	+	-	0	
24	-	*	*	*	*	*	*	*	*	+	+	+	-	+	-	+	0	
25	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	0	

Univ.: Bacterial DNA detected by the Universal 16S rRNA primer; Ts: *T. socranskii*; Tma: *T. maltophilum*; Ta: *T. amylovorum*; Tme: *T. medium*; Tl: *T. lecithinolyticum*; Td: *T. denticola*; Tv: *T. vicentii*; Tp: *T. pectinovorum*; TPe: Tender to percussion; TPa: Tender to palpation; IS: Intraoral swelling; LS: Lesion size; HD: Histopathological diagnosis.

indicating that the surgical site was sterile. In addition, all clinical procedures were performed by a highly qualified surgeon, who collected the samples under magnification of a dental operating microscope, and who was extremely careful to avoid contamination during excision of the lesions.

Prevalence of the *Treponema* species, as reported in the literature, varies according to the type of infection.¹⁸ Root canal samples from acute apical abscesses present significantly higher prevalence of spirochetes (90%)⁹ than primary (37.4%)¹⁹ and secondary (56.5%) chronic root canal infections.¹⁵ This pattern also seems to be found in the extraradicular environment, insofar as the chronic lesions targeted in this study showed lower prevalence of the *Treponema* species (28% of

the cases) than purulent exudate of acute apical abscesses (89% to 95% of the cases).^{9,16} *T. socranskii* and *T. denticola* were the most frequent species found in this study, and were also the most common *Treponema* species encountered in studies that investigated acute apical abscesses.^{9,16} These microorganisms have several virulence determinants that enable them to interact with other pathogenic bacteria and with the host immune system in ways that are likely to promote disease progression.¹⁰ *T. denticola* is also a putative periodontal pathogen that, in association with *Porphyromonas gingivalis* and *Tannerella forsythia*, forms the "red complex" described by Socransky *et al.*²⁰ as a bacterial group strongly related to clinical parameters of periodontitis diagnosis.

T. socranskii was commonly recovered in association with *T. denticola*, *T. maltophilum* and *T. lecithinolyticum*. Interspecies relations of the *Treponema* species was also observed by Baumgartner *et al.*,¹⁹ who found a significant association between *T. maltophilum* and *T. socranskii*, and between *T. maltophilum* and *T. denticola*.

The features that enable bacteria to become established in the apical tissues are not fully understood. Chronic infections may also have their source in biofilms on the surface of the root-end, which is protected from host defenses and resistant to antibiotic therapy.²¹ In these cases planktonic bacteria are constantly released into the apical lesions. Virulence factors, such as motility and low immunogenicity, may allow the *Treponema* species to colonize new sites rapidly, penetrate into the tissue and escape from host defense systems.¹⁰ Their unique spiral shape, the periplasmic location of their flagellar filaments, and the presence of surface protease²² may also give these species an invasive potential. Although there is evidence that the *Treponema* species from infected root canals have the ability to stimulate bone resorption,¹¹ infected lesions in this study showed no distinct clinical or radiographic features.

Complex growing requirements have made molecular methods such as nested-PCR a frequent choice for detecting the *Treponema* species from other endodontic infections.^{9,14} The two-step nPCR assay used in the present study and described by Willis *et al.*¹⁴ has proven effective in detecting the *Treponema* species directly in clinical samples. Nested-PCR is a powerful variant of the PCR technique. It differs from the standard PCR protocol, mainly in regard to its conceptual primer design, which involves two sets of DNA amplification.²³ Whereas the sensitivity

limit of conventional PCR reaches 10³ of target cells, nPCR can reduce the detection limit to about 10 cells.²⁴ On the other hand, great care must be taken in performing this technique, to avoid contamination during transfer of the product from the first reaction to the new tube for reamplification.²⁵

In the present study, none of the species recognized by nested-PCR were detected by conventional PCR. Low-abundant *T.* species may exert ecological community functions or may simply be the product of historical ecological changes with the potential of becoming dominant in response to shifts in environmental conditions (*e.g.*, local environmental changes could favor their growth).²⁶

Conclusion

At the time of sampling, most of the endodontic retreatment-resistant apical periodontitis tissues contained bacterial DNA with a low predominance of *Treponema* spp. Seven of the eight target species were detected successfully. *T. socranskii* was the most frequently recovered species found, and was statistically associated with *T. denticola*, *T. maltophilum* and *T. lecithinolyticum*. None of the target species were associated with clinical and radiographic features. In conclusion, *Treponema* spp. could be found in a low percentage in longstanding apical lesions from teeth with endodontic retreatment failure.

Acknowledgments

The authors wish to thank Dr. Rafael Stipp and Dr. Leticia Nóbrega for their technical assistance, as well as CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brazil, process no. 302575/2009-0) for its financial support.

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