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Short communication

Occurrence of two newly named oral treponemes – *Treponema parvum* and *Treponema putidum* – in primary endodontic infections

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Rôças IN, Siqueira JF Jr. Occurrence of two newly named oral treponemes – Treponema parvum *and* Treponema putidum – *in primary endodontic infections.*
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Background/aims: Recent evidence from molecular genetic studies has revealed that oral *Treponema* species are involved in infections of endodontic origin. This study assessed the occurrence of two newly named oral treponemes – *Treponema parvum* and *Treponema putidum* – in primary endodontic infections using a culture-independent identification technique.
Methods: Genomic DNA was isolated directly from clinical samples, and a 16S rRNA gene-based nested polymerase chain reaction (PCR) assay was used to determine the presence of *T. parvum* and *T. putidum*. Species-specific primer pairs were developed by aligning closely related 16S rRNA gene sequences. The specificity for each primer pair was validated by running PCR against a panel of oral bacteria and by sequence analysis of PCR products from positive clinical samples.
Results: *T. parvum* was detected in 52% of the root canals associated with chronic apical periodontitis, in 20% of the cases diagnosed as acute apical periodontitis, and in no abscessed case. In general, *T. parvum* was detected in 26% of the samples from primary endodontic infections. *T. putidum* was found in only one case of acute apical periodontitis (2% of the total number of cases investigated).
Conclusions: The devised nested PCR protocol was able to identify both *T. parvum* and *T. putidum* directly in clinical samples and demonstrated that these two treponemes can take part in endodontic infections.

Key words: endodontic infections; nested polymerase chain reaction; *Treponema parvum*; *Treponema putidum*

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Spirochetes are helical-shaped bacteria that have been implicated as etiologic agents of a variety of human and animal diseases (6, 11). In a study that became a cornerstone in the history of endodontic microbiology, W. D. Miller suggested that spirochetes could play a role in the causation of periradicular diseases (12). Over the ensuing years, the involvement of spirochetes with endodontic infections was underrated, in spite of several microscopy reports on the occurrence of these bacteria in endodontic samples (3, 21). The advent of molecular genetic technology for microbial detection and identification has sparked a better understanding of the roles that difficult-to-grow or not-yet-cultivated bacteria, including spirochetes, play in diverse polymicrobial infections, including periradicular diseases (4, 16). Based on very recent epidemiologic studies relying on molecular genetic evidence, different spirochetal species have been suggested to be candidate endodontic pathogens (2, 7, 9, 14, 15, 17–19). About 90% of the samples from different types of endodontic infections have been demonstrated to harbor at least one species of spirochetes (14, 17, 18).

Virtually all spirochetes so far identified in the oral cavity belong to the genus *Treponema* but only a few species have been cultured and validly named (4). Oral *Treponema* species can be classified into

two groups: the saccharolytic (*Treponema pectinovorum*, *Treponema socranskii*, *Treponema amylovorum*, *Treponema lecithinolyticum*, and *Treponema maltophilum*) and the asaccharolytic oral treponemes (*Treponema denticola*, *Treponema medium*, and *Treponema vincentii*). All these named species have been found in endodontic infections, with varying prevalences (2, 7, 9, 14, 15, 17–19). Recently, two new oral species have been proposed –*Treponema parvum* (22) and *Treponema putidum* (23). No study has reported on the occurrence of these two treponemes in endodontic infections.

T. parvum is a small obligate anaerobic, helically coiled, motile, saccharolytic treponeme (22). Cells are approximately 1 μm long and 0.18 μm wide, with a wavelength of 0.8 μm and amplitude of 0.3 μm. They show a 1 : 2 : 1 flagellar arrangement. On the basis of 16S rRNA gene sequence comparisons, the closest relatives of *T. parvum* are *T. pectinovorum* (88% similarity) and *T. amylovorum* (90% similarity).

T. putidum is an obligate anaerobic, helically coiled, motile, asaccharolytic and proteolytic treponeme (23). Cells are approximately 10 μm long and 0.25 μm wide, with a wavelength of approximately 3 μm and amplitude of approximately 1.5 μm. They have a 2 : 4 : 2 arrangement of the periplasmic flagella. The status of this novel species is supported by the 16S rRNA gene sequence, with 98.5% similarity to its closest cultured relative, *T. denticola*.

The purpose of this study was to assess for the first time the occurrence of two newly named oral treponemes – *T. parvum* and *T. putidum* – in primary endodontic infections using a devised culture-independent 16S rRNA gene-based nested polymerase chain reaction (PCR) assay.

Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University, Rio de Janeiro, RJ, Brazil. Samples collected for previous studies (17, 18) were stored and available for reanalysis in this study. Fifty samples of infections of endodontic origin were investigated. According to the forms of periradicular diseases, teeth were divided as follows:

- 21 asymptomatic cases diagnosed as chronic apical periodontitis;
- 10 cases diagnosed as acute apical periodontitis;
- 19 cases diagnosed as acute apical abscesses.

In cases of chronic apical periodontitis and in cases of acute apical periodontitis, samples were obtained from the root canals. Abscesses were sampled by aspiration of pus from the swollen mucosa. Sampling procedures and DNA extraction protocol were as described earlier (17, 18). Reference DNA from *T. putidum* ATCC 700334 and *T. parvum* ATCC 700770 was also extracted to serve as positive controls for the primers used. Negative controls included the PCR mixture without DNA template or extracted DNA from *Actinomyces radicidentis* CCUG 42377, *Enterococcus faecalis* ATCC 29212, *Filifactor alocis* ATCC 35896, *Fusobacterium nucleatum* ss. *nucleatum* ATCC 25586, *Porphyromonas endodontalis* ATCC 35406, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Prevotella pallens* ATCC 700821, *Propionibacterium propionicum* U13a-a, *Pseudoramibacter alactolyticus* C11b-d, *Streptococcus intermedius* ATCC 27335, *Tannerella forsythia* ATCC 43037, *T. denticola* B1 (Forsyth Institute), *T. lecithinolyticum* ATCC 700332, and *T. socranskii* S1 (Forsyth Institute).

16S rRNA gene species-specific PCR primers were used. Primers specific for *T. parvum* and *T. putidum* were designed for this study as follows. 16S rRNA gene sequences of these species were retrieved from the GenBank at the National Center for Biotechnology Information website and aligned with the sequences of their nearest neighbors in the phylogenetic tree using the CLUSTAL W program (20) to identify variable areas between species. Potential primers were designed from these areas and BLAST (1) was used to verify their specificity by comparing primer sequences with all available sequences in the GenBank database. Based on the BLAST search, two primer sequences for each species were selected and further tested for specificity against DNA from the bank of reference strains used as controls and by sequencing of PCR products obtained from positive clinical samples. Primers for *T. parvum* were 5′-TGCTTTGGCACCGCAGGG-3′ and 5′-GCCCTTCAGTGACTCCAGTCATC-3′ (base position 191–651 of the *T. parvum* 16S rRNA gene, GeneBank accession no. AF302938), producing a PCR amplicon of 461 bp. Primers for *T. putidum* were 5′-GTGCTTATACGGATAAAGCCGT-3′ and 5′-GAAGCATTCCCTCTTCCCC-3′ (base position 88–387 of the *T. putidum* 16S rRNA gene, GeneBank accession no. AJ543428), producing a PCR amplicon of 300 bp.

The whole-genomic DNA extracts from clinical samples were used as templates in a 16S rRNA gene-based nested PCR method devised to detect the two target species in endodontic samples. In the first PCR reaction, a practically full-length 16S rRNA gene fragment was amplified using universal 16S rRNA gene primers 27f and 1492r (10). Aliquots of 5 μl of the DNA extracts from clinical samples were used as targets in the first PCR reaction. The PCR reaction mixture and temperature profile for the first amplification were as presented previously (14, 17, 18).

Afterwards, 1 μl of the universal reaction was used as the template for the nested specific reaction. The second PCR reaction used to assess the occurrence of *T. parvum* or *T. putidum* was performed in a 50 μl reaction mixture containing 1 μM concentration of each primer, 5 μl of 10× PCR buffer (Biotools, Madrid, Spain), 2 mM $MgCl_2$, 1.25 U of *Tth* DNA polymerase (Biotools) and 0.2 mM of each deoxyribonucleoside triphosphate (Biotools). Negative and positive controls were included with each batch of samples analyzed. The positive control comprised genomic DNA extract from the reference strains, and the negative control contained sterile ultrapure water instead of sample.

Preparations were amplified in a DNA thermocycler (Mastercycler Personal, Eppendorff, Hamburg, Germany). The PCR temperature profile for the first PCR reaction using universal primers was as described previously (17, 18). PCR cycling conditions for the second round of amplification specific for both *T. parvum* and *T. putidum* consisted of an initial denaturation step at 95°C for 2 min, and a touchdown PCR performed as follows: denaturing temperature of each cycle at 95°C for 30 s, annealing temperature initially set at 65°C and then lowered 0.5°C every other cycle until it reached 62°C. Twenty-one additional cycles were carried out at 62°C. Primer annealing was performed using this scheme for 30 s, and primer extension was carried out at 72°C for 1 min. The final extension step was at 72°C for 5 min. Amplicon detection was carried out by agarose gel electrophoresis of 8 μl amplification product through horizontal 1.5% agarose gel in 1× Tris-borate-EDTA buffer. After electrophoresis, gels were stained with 0.5 μg/ml ethidium bromide and viewed under ultraviolet transillumination.

To confirm the specificity of the primers, amplicons were purified using a PCR purification system (Wizard PCR Preps, Promega, Madison, WI) and then

Table 1. Frequency of detection of two newly named treponemes in endodontic infections as revealed by 16S rRNA gene-based nested PCR

Species	Chronic apical periodontitis	Acute apical periodontitis	Acute apical abscess	Total
Treponema parvum	11/21 (52)*	2/10 (20)	0/19 (0)	13/50 (26)
Treponema putidum	0/21 (0)	1/10 (10)	0/19 (0)	1/50 (2)

*Number of positive cases/number of samples examined (percentage).

sequenced directly on the ABI 377 automated DNA sequencer using dye terminator chemistry (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Sequence data and electropherograms were inspected and edited by using BioEdit software (8). Sequences were then compared with those available in GenBank by using the BLAST algorithm (1).

Prevalence values of *T. parvum* and *T. putidum* were recorded as the percentage of the cases examined. The Chi-squared test with Yates' correction was used to analyze the association between these species and the occurrence of symptoms (joint cases of acute apical periodontitis and abscesses). Significance for the Chi-squared test was established at 5% ($P < 0.05$).

Utilization of a nested PCR protocol in this study is justified by the increased sensitivity and specificity of the assay when compared to single PCR (16). A touchdown procedure was also carried out to maximize the specificity of the assay. The annealing temperature in the initial nested PCR cycle was above the T_m of the primers and, in subsequent cycles, the annealing temperature was decreased in steps of 0.5°C per cycle until a high stringent annealing temperature was reached and maintained up to the final cycles. Touchdown protocols have been considered useful to avoid the amplification of spurious DNA fragments (nonr-RNA gene fragments and/or fragments without proper sizes) (5).

All sample extracts were positive by the first round of PCR amplification using broad-range 16S rRNA gene primers, indicating that the DNA extraction procedure was adequate, that bacteria were present in all examined samples, and that inhibitors of the PCR reaction were not present. Negative controls using sterile ultrapure water instead of sample yielded no amplicon.

The specificity of the primers was tested against a panel of representative oral bacteria. The use of each primer set resulted in no PCR product of the expected size from nontargeted species. Primers resulted in one band of the expected size when the respective reference DNA was used. To confirm the specificity of the primers, representative PCR products of the expected size obtained from clinical samples were sequenced and compared to the original sequences in GenBank database. For *T. parvum*, the levels of similarity between the sequences obtained from six positive samples and the sequences in GenBank ranged from 99.8% to 100%. For *T. putidum*, the sequence obtained from the only one positive clinical sample was 100% similar to the sequence in GenBank.

T. parvum was detected in 11/21 (52%) teeth showing chronic apical periodontitis, in 2/10(20%) teeth with acute apical periodontitis, and in no abscessed case. *T. parvum* was significantly associated with asymptomatic cases ($P = 0.001$). The overall prevalence of *T. parvum* in primary endodontic infections was 26% (13 of 50 cases). *T. putidum* was found in only one case (10%) of acute apical periodontitis (2% of the total number of cases investigated) (Table 1). Despite the high similarity in the 16S rRNA gene sequences between *T. putidum* and *T. denticola*, our assay succeeded in specifically detecting the former without cross-reacting to the latter. Indeed, there was a much lower prevalence of *T. putidum* in endodontic infections as compared to our previous findings for *T. denticola* using a similar protocol (14).

A large proportion of oral bacteria, notably most spirochetes, are fastidious and difficult or refractory to cultivation (4, 13). A small number of the cultivable oral bacteria have acquired the status of suspected pathogens based both on epidemiologic data and on the presence of potential virulence factors. To the best of our knowledge, this is the first study to report the occurrence of *T. parvum* and *T. putidum* in samples from primary endodontic infections. Our findings expand the list of bacteria involved with infections of endodontic origin to include these newly named *Treponema* species. Based on our current and previous findings (14, 15, 17–19), *T. parvum* can be regarded as one of the most prevalent treponemes found in endodontic infections, along with *T. denticola, T. socranskii*, and *T. maltophilum*.

In conclusion, our findings revealed that the nested PCR protocol used in this study was able to detect both *T. parvum and T. putidum* directly in clinical samples and demonstrated that these two species can take part in the microbiota associated with different types of periradicular diseases. Occurrence of these bacteria in a previously sterile site such as the root canal, particularly in the high prevalence reported for *T. parvum*, may suggest an involvement in the pathogenesis of periradicular diseases. Nonetheless, virtually nothing is known about the virulence traits and mechanisms of pathogenicity of these species. Further studies are thereby warranted in order to confirm involvement with periradicular disease causation and to look for specific virulence factors of these newly described treponemes.

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References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990: **215**: 403–410.
2. Baumgartner JC, Khemaleelakul S, Xia T. Identification of spirochetes (treponemes) in endodontic infections. J Endod 2003: **29**: 794–797.
3. Dahle UR, Tronstad L, Olsen I. Observation of an unusually large spirochete in endodontic infection. Oral Microbiol Immunol 1993: **8**: 251–253.
4. Dewhirst FE, Tamer MA, Ericson RE, Lau CN, Levanos VA, Boches SK, et al. The diversity of periodontal spirochetes by 16S rRNA analysis. Oral Microbiol Immunol 2000: **15**: 196–202.
5. Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 1991: **19**: 4008.
6. Edwards AM, Dymock D, Jenkinson HF. From tooth to hoof: treponemes in tissue-destructive diseases. J Appl Microbiol 2003: **94**: 767–780.
7. Fouad AF, Barry J, Caimano M, Clawson M, Zhu Q, Carver R, et al. PCR-based identification of bacteria associated with endodontic infections. J Clin Microbiol 2002: **40**: 3223–3231.
8. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Series 1999: **41**: 95–98.
9. Jung I-Y, Choi B-K, Kum K-Y, Yoo Y-J, Yoon T-C, Lee S-J, et al. Identification of oral spirochetes at the species level and their association with other bacteria in endodontic infections. Oral Surg Oral Med

Oral Pathol Oral Radiol Endod 2001: **92**: 329–334.
10. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. *Nucleic Acid Techniques in Bacterial Systematics*. New York: John Wiley and Sons, 1991, 115–175.
11. Loesche WJ. The role of spirochetes in periodontal disease. Adv Dent Res 1988: **2**: 275–283.
12. Miller WD. An introduction to the study of the bacterio-pathology of the dental pulp. Dent Cosmos 1894: **36**: 505–528.
13. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
14. Rôças IN, Siqueira JF Jr, Andrade AFB, Uzeda M. Oral treponemes in primary root canal infections as detected by nested PCR. Int Endod J 2003: **36**: 20–26.
15. Rôças IN, Siqueira JF Jr, Andrade AFB, Uzeda M. Identification of selected putative oral pathogens in primary root canal infections associated with symptoms. Anaerobe 2002: **8**: 200–208.
16. Siqueira JF Jr, Rôças IN. PCR methodology as a valuable tool for identification of endodontic pathogens. J Dent 2003: **31**: 333–339.
17. Siqueira JF Jr, Rôças IN. PCR-based identification of *Treponema maltophilum, T. amylovorum, T. medium*, and *T. lecithinolyticum*. in primary root canal infections. Arch Oral Biol 2003: **48**: 495–502.
18. Siqueira JF Jr, Rôças IN. *Treponema* species associated with abscesses of endodontic origin. Oral Microbiol Immunol 2004: **19**: 336–339.
19. Siqueira JF Jr, Rôças IN, Souto R, Uzeda M, Colombo AP. Microbiological evaluation of acute periradicular abscesses by DNA–DNA hybridization. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001: **92**: 451–457.
20. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994: **22**: 4673–4680.
21. van Winkelhoff AJ, Carlee AW, de Graaff J. *Bacteroides endodontalis* and other black-pigmented *Bacteroides* species in odontogenic abscesses. Infect Immun 1985: **49**: 494–498.
22. Wyss C, Dewhirst FE, Gmur R, Thurnheer T, Xue Y, Schupbach P, et al. *Treponema parvum* sp. nov., a small, glucuronic or galacturonic acid-dependent oral spirochaete from lesions of human periodontitis and acute necrotizing ulcerative gingivitis. Int J Syst Evol Microbiol 2001: **51**: 955–962.
23. Wyss C, Moter A, Choi B-K, Dewhirst FE, Xue Y, Schupbach P, et al. *Treponema putidum* sp. nov., a medium-sized proteolytic spirochaete isolated from lesions of human periodontitis and acute necrotizing ulcerative gingivitis. Int J Syst Evol Microbiol 2004: **54**: 1117–1122.