Diversity of spirochetes in endodontic infections

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Running headline: Spirochetes in endodontic infections

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ABSTRACT

The diversity of spirochetes in primary endodontic infections of teeth with chronic apical periodontitis or acute apical abscesses was investigated using 16S rRNA gene clone library analysis. Prevalence of 3 common cultivable oral Treponema species was also determined using species-specific nested polymerase chain reaction (nPCR). All detected spirochetes belonged to the genus Treponema. Overall, 28 different taxa were identified from the 431 clones sequenced: 9 cultivable and validly named species, 1 cultivable as-yet-uncharacterized strain and 18 as-yet-uncultivated phylotypes, 17 of which were novel. The large majority of clones (94%) were from cultivable named species. The number of Treponema species/phylotypes per selected positive sample ranged from 2 to 12. Species-specific nPCR detected T. denticola, T. socranskii and T. maltophilum in 59 (66%), 33 (37%) and 26 (29%) of the 90 cases of primary endodontic infections, respectively. Clone library analysis revealed diverse Treponema species/phylotypes as part of the microbiota associated with asymptomatic and symptomatic (abscess) endodontic infections. Although several as-yet-uncultivated Treponema phylotypes were disclosed, including novel taxa, cultivable named species were more abundant and frequently detected.

Key words: Spirochetes; endodontic infections; apical periodontitis; acute apical abscess; 16S rRNA gene clone library
INTRODUCTION

In his milestone study published in 1894, Willoughby Dayton Miller first suggested that spirochetes could play a role in the pathogenesis of apical periodontitis, particularly in cases of acute apical abscesses (21). However, despite several studies revealing their occurrence in endodontic infections by microscopy (49, 50), it was not until approximately 100 years after Miller’s study that spirochetes were consistently detected and identified by culture-independent molecular techniques in association with diverse forms of apical periodontitis and a pathogenetic involvement was supported (30, 43, 46).

Phylogenetic analyses of 16S rRNA gene clone libraries suggest that all oral spirochetes belong to the genus *Treponema* (7), and so far, ten oral species have been cultivated and validly named. They include four asaccharolytic species (*T. denticola*, *T. medium*, *T. putidum*, and “*T. vincentii*”) and six saccharolytic species (*T. socranskii*, *T. pectinovorum*, *T. maltophilum*, *T. amylovorum*, *T. lecithinolyticum*, and *T. parvum*) (36). All these species have been recently targeted and disclosed in primary endodontic infections by studies using molecular methods (4, 11, 12, 15, 27, 38-40, 42-44, 51). The most predominant treponemes in these infections are usually *T. denticola* and *T. socranskii* (4, 27, 40, 43), while *T. parvum*, *T. maltophilum* and *T. lecithinolyticum* have been moderately prevalent (4, 15, 25, 38, 40). Among several other taxa, a recent study targeted all 10 cultivable and four as-yet-uncharacterized oral treponemes (26). Of the 9 treponemes detected, *T. denticola* and *T. socranskii* were the most prevalent species, which is consistent with other studies (4, 27, 40, 43). The other most frequently found *Treponema*
species/phylotypes included *Treponema* sp. VI:G:G47 (16%), *T. putidum* (16%), *Treponema* sp. II:10:D12 (14%) and *T. parvum* (12%)(26).

To the best of our knowledge, virtually all studies of the occurrence of spirochetes in endodontic infections have evaluated the occurrence of target species. An exception was a study using denaturing gradient gel electrophoresis with group-specific primers, which after sequencing of some DGGE bands revealed the occurrence of two novel as-yet-uncultivated phylotypes (41). Thus, the diversity of spirochetes in endodontic infections remain to be fully appreciated. Because there are near 60 oral *Treponema* species, of which approximately 80% remain uncultivated (7), a comprehensive analysis of the diversity of spirochetes associated with oral diseases, including apical periodontitis, is necessary and requires the utilization of culture-independent molecular approaches. The present study was undertaken to decipher the diversity of oral spirochetes in infections of endodontic origin using 16S rRNA gene clone library analysis. Additionally, the prevalence of 3 common cultivable oral *Treponema* species was assessed.
MATERIAL AND METHODS

Case description, sample taking and DNA extraction

Samples were taken from patients who had been referred for root canal treatment or emergency treatment to the Department of Endodontics, Estácio de Sá University, Rio de Janeiro, RJ, Brazil. Abscess samples were also taken from patients who were seeking emergency treatment in 3 hospitals in Rio de Janeiro. Only single-rooted teeth from adult patients (ages ranging from 18 to 74 years), all of them having carious lesions, necrotic pulps and radiographic evidence of apical periodontitis, were included in this study. Selected teeth showed an absence of periodontal pockets deeper than 4 mm. In general, 90 samples of infections of endodontic origin were obtained. Teeth were grouped as follows:

a) 32 asymptomatic cases diagnosed as chronic apical periodontitis;

b) 10 cases diagnosed as acute apical periodontitis, which showed symptoms such as tenderness to percussion and/or palpation, induced or spontaneous pain exacerbated by mastication, absence of pus in the canals, no swelling; and

c) 48 cases diagnosed as acute apical abscesses, which showed pain and localized or diffuse swellings along with fever, lymphadenopathy, and/or malaise. No apparent communication from the abscess to the oral cavity or the skin surface was observed.

In cases of chronic or acute apical periodontitis, samples were obtained from the root canals using sterile paper points. Abscesses were sampled by aspiration of purulent exudate from the swollen mucosa using a sterile syringe.
Sampling procedures were as described earlier (45). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University.

**Amplification of 16S rRNA gene**

Whole-genomic DNA extracts from clinical samples were used as templates in a 16S rRNA gene-based nested PCR method for detection of spirochetes in endodontic samples. In the first PCR reaction, a practically full-length 16S rRNA gene fragment was amplified using a universal 16S rRNA gene primer pair (Table 1). Aliquots of 5 µl of the DNA extracts from clinical samples were used as target in the first PCR reaction. Next, 2 µl of the resulting PCR product from each sample was used in the second round of amplification, which was specific for detection of spirochetes as a group or for *T. denticola, T. socranskii* and *T. maltophilum*. A nested PCR protocol was used to improve the performance of the method in samples with low DNA concentration and to provide increased specificity (20, 37). Primer sequences are shown in Table 1.

All PCR amplifications were performed in a 50 µl of reaction mixture containing 1 µM concentration of each primer, 5 µl of 10× PCR buffer, 2 mM MgCl₂, 2 U of *Tth* DNA polymerase and 0.2 mM of each deoxyribonucleoside triphosphate (all reagents from Biotools, Madrid, Spain). Positive controls used DNA extracted from *T. denticola* B1 (Forsyth Dental Institute, Boston, Mass.), *T. socranskii* S1 (Forsyth Dental Institute), and *T. maltophilum* ATCC 51939. One negative control consisting of sterile milliQ water instead of the sample was included for every 5 samples in all batches of samples analyzed.
Preparations were amplified in a DNA thermocycler (Mastercycler personal, Eppendorff, Hamburg, Germany). The PCR temperature profile for the first reaction using universal primers included an initial denaturation step at 95°C/1 min, 26 cycles at 94°C/45 s, 55°C/45 s, and 72°C/1.5 min, and a final step at 72°C/15 min. PCR cycling conditions for the second round of amplification using primers specific for spirochetes included an initial denaturation step at 95°C/4 min, 30 cycles at 94°C/1 min, 52°C/1 min, 72°C/2 min, and final extension at 72°C/15 min. Amplicons were separated by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and viewed under ultraviolet transillumination. Temperature profiles for *T. denticola*, *T. socranskii* and *T. maltophilum* were as described previously (27, 38, 40). PCR amplicons were identified in agarose electrophoretic gel and visualized for size using ultraviolet transillumination. Positive reactions were assigned based on the presence of clearly visible bands of the expected molecular size (Table 1). Representative amplicons were sequenced to check for specificity.

Prevalence of the three treponemes was recorded as the percentage of cases examined. The chi-square test with Yates correction was used to analyze the association between these species and abscesses or the overall occurrence of symptoms (joint cases of acute apical periodontitis and acute apical abscesses). Significance was set at 5% (p<0.05).

**Clone library analysis**

Nine samples positive for the spirochete-specific primers and exhibiting the strongest bands in the agarose gel were selected for further 16S rRNA gene clone library analysis (32). PCR products were purified using an UltraClean
PCR Clean-up DNA purification kit (Mo Bio Laboratories, Inc., CA). Purified amplicon was ligated into the plasmid vector pCR\textsuperscript{®}2.1, and then transformed into One Shot\textsuperscript{®} INV\alpha F\textsuperscript{'} competent cells using the Original TA Cloning Kit (Invitrogen, San Diego, CA). Plasmid DNAs were prepared using the Illustra TempliPhi DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK) from recombinants and used as templates for sequencing. Attempts were made to sequence all clones (white colonies). Clones not showing the inserts were excluded. Sequencing was conducted using the spirochete-specific primers, a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). All sequences were checked by the Chimera Check program of the Ribosomal Database Project-II (RDP-II) (6) to eliminate chimeras which could result from the amplification of an accidental mixture of bacterial genes - no chimeric sequences were detected. Nucleotide sequences were analyzed by a BLASTN search (1) for the nearest matches. Database sequences with the highest similarities and scorebits to our sequences were chosen for their identification. The criterion to define a novel phylotype was set at sequences that differed from the closest GenBank entry by more than 2\% (17). The sequences were aligned with the CLUSTAL W program (48) and corrected by manual inspection. Neighbor-joining phylogenetic tree was constructed from the alignment using Molecular Evolutionary Genetics Analysis package (MEGA version 2.1) (18). Distances were calculated with the Jukes-Cantor algorithm. Robustness of the phylogeny was tested by bootstrap analysis with 500 iterations. Sequences for
the novel phylotypes were deposited in the GenBank database under accession numbers AB465691 to AB465707.
RESULTS

Spirochetal diversity by clone library analysis

Of the nine samples selected for clone library analysis of spirochetal diversity, 3 were from root canals associated with chronic apical periodontitis and the other 6 were from pus aspirates from acute apical abscesses. The total number of clones sequenced from the nine samples was 431 (mean, 48; ranging from 10 to 78) (Table 2). No non-treponemal clones were found, indicating a good specificity for the primers and protocol used. Overall, 28 different Treponema taxa were identified from the 431 clones sequenced: 9 cultivable and validly named species (not including the T. socranskii subspecies division), one as-yet-uncultivated phylotype (Treponema sp. IV:18:C9), one cultivated but not-yet-validly named strain (Treponema sp. V:19:D36) and 17 novel as-yet-uncultivated Treponema phylotypes, i.e., taxa that have never been previously detected (Figure 1). Of the novel as-yet-uncultivated taxa, 3 were disclosed in chronic cases and the other 14 were from abscesses.

The huge majority of clones (404 or 94%) were from cultivable named species. Four clones were from Treponema sp. V:19:D36, two from Treponema sp. IV:18:C9 and the other 21 clones were from novel phylotypes. Therefore, as-yet-uncultivated and/or uncharacterized phylotypes made up only a minor fraction of the Treponema diversity in endodontic infections, as they represented only 6% of the total number of clones sequenced.

Overall, the mean number of Treponema species/phylotypes per selected case was 6. Chronic cases harbored a mean number of 5 taxa (range, 4 to 6), while abscesses presented a mean number of 6 taxa (range, 2 to
However, these figures are only illustrative, because the examined cases were selected based on the strength of amplicons. 

*T. maltophilum* was found in 8 samples (3 chronic cases and 5 acute abscesses), followed by *T. socranskii* subspecies *buccale* (6 samples – 2 chronic and 4 acute), *T. socranskii* subspecies *socranskii* (4 samples – 3 chronic and 1 acute), *T. medium* (4 acute samples) and *T. denticola* (3 samples – 2 chronic and 1 acute). *T. amylovorum, T. putidum, “T. vincentii”, Treponema sp. IV:18:C9 and Treponema sp. V:19:D36* were found in two cases each. *T. parvum, T. lecithinolyticum* and each of the novel phylotypes were found in only one sample. 

The most dominant species/phylotypes in the 3 root canals associated with chronic apical periodontitis were: *T. maltophilum* (68% of the clones sequenced from case 1), *T. socranskii* subspecies *socranskii* (41% from case 2) and *T. denticola* (53% from case 3). Dominant *Treponema* taxa in the 6 abscess samples were: *T. medium* (50% of the clones sequenced in one sample, 50% of another sample, and 60% of a third sample), *T. maltophilum* (77% and 80%), and *T. socranskii* subsp. *buccale* (95%)

**Prevalence of three named Treponema species**

In general, species-specific nested PCR detected *T. denticola, T. socranskii* and *T. maltophilum* in 59, 33 and 26 of the 90 cases of primary endodontic infections, respectively. No association with symptoms or abscesses was found for any of the test species (p>0.05). Findings of the species-specific nested PCR are detailed in Table 3.
DISCUSSION

Treponema species are examples of culture-difficult bacteria that have been identified in endodontic infections only by culture-independent molecular methods (4, 15, 27, 46). Most of the previous molecular studies focused on the detection of selected Treponema species or phylotypes (4, 11, 13, 15, 25-27, 38, 40, 43, 46). Of the several studies that have performed broad-range analyses for overall bacteria in primary endodontic infections (14, 22, 29, 31-33, 52, 53), only a couple reported the occurrence of spirochetes, even so of a few species in a few cases (31, 32). These studies using universal bacterial primers may underestimate spirochetal populations mainly if they are in low numbers in the environment (5, 16). Indeed, a study revealed that no Treponema species were found in infected root canals associated with chronic apical periodontitis at levels >10^5 (26).

To the best of our knowledge, this is the first attempt to examine the diversity of spirochetes in infections of endodontic origin using group-specific primers followed by cloning and 16S rRNA gene sequencing. Nine cases that presented strong amplicons after PCR amplification were chosen for this analysis. Up to 12 Treponema species/phylotypes were found per positive sample. Because of the obvious biases introduced by the criterion to select samples, clone library analysis did not serve the purpose of revealing the mean number of treponemes per canal or the prevalence of the detected Treponema species, even though this information is provided herein for illustrative reasons. Spirochetal diversity in endodontic infections was the main focus of this study.
Clone library analysis corroborated previous findings from species-specific PCR (4, 11, 15, 25, 27, 38, 40) or checkerboard studies (26, 35, 46, 47) in that virtually all cultivable *Treponema* species can be found in infections of endodontic origin. Of the 10 cultivable species, only *T. pectinovorum* was not detected herein. As-yet-uncultivated and/or uncharacterized *Treponema* phylotypes detected in a previous study (26) were not found in the present one. However, it was noteworthy that 18 as-yet-uncultivated phylotypes were detected, 17 of which were novel in the sense that they have never been previously found in other sites. Therefore, 64% of the *Treponema* species found in endodontic infections in the present study have yet to be cultivated and phenotypically characterized. These findings also indicate that spirochetal diversity in the oral cavity is still broader than previously reported (7). Regarding abundance, these phylotypes comprised only a minor proportion of the total number of clones sequenced (6%). This means that they are not dominant components of the spirochetal endodontic communities. This finding however does not imply lack of relevance, as even low-dominant species may exert important ecological functions.

Cultivable *Treponema* species found to dominate the spirochetal community in most samples included *T. maltophilum*, *T. socranskii*, *T. denticola*, and *T. medium*. Of these, only the latter has not been found at high prevalence values in previous studies (4, 38). It is salient to point out that the fact that these species corresponded to most of the clones sequenced in this study actually means that they dominated the spirochetal community, but their proportion in relation to the whole bacterial community was not established. Hence, any
inferences with regard to pathogenicity must be exercised with caution, even though these species have been suggested to play a role in other oral diseases, including marginal periodontitis (2, 8, 10, 34, 54).

Of the 3 species targeted in the species-specific nested PCR assay, *T. denticola* was the most prevalent, which is in consonance with our previous findings (27, 40). However, data from clone library analysis revealed that this species was not the most prevalent. Differences may have been because species-specific PCR assays arguably display higher sensitivity when compared to group-specific or broad-range primers (19, 28). Nonetheless, because cases for clone library analysis were selected on the basis of strong amplicon signal, the possibility exists that most cases positive for *T. denticola* were left out of the analysis. Indeed, of the 6 cases subjected to clone library analysis that were negative for *T. denticola*, only one was positive in species-specific nested PCR. Biases resultant of sample selection for analysis might also explain the opposite situation, *i.e.*, why *T. maltophilum* was encountered more frequently in clone libraries than in species-specific nested PCR.

None of the 3 target species was positively associated with symptoms and/or abscessed cases. Although the 3 species were detected in several of the acute cases, they were also very frequent in cases of chronic apical periodontitis. However, the results presented in this study were only qualitative (presence/absence data). Further studies should quantify oral treponemes present in both asymptomatic and symptomatic endodontic infections so as to determine whether these bacteria are found in larger numbers in any particular clinical situation.
In conclusion, 16S rRNA gene group-specific PCR followed by cloning and sequencing revealed that diverse Treponema species/phylotypes can take part in the microbiota associated with asymptomatic and symptomatic (abscess) endodontic infections. Although several as-yet-uncultivated Treponema phylotypes were disclosed, including novel taxa, cultivable named species were more frequently detected and abundant. Species-specific nested PCR demonstrated that T. denticola, T. socranskii and T. maltophilum, in decreasing order of prevalence, are frequently found in different forms of apical periodontitis.
This study was supported by grants from the RIKEN BioResource Center and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), a Brazilian Governmental Institution.
REFERENCES


FIGURE LEGEND

Figure 1. Phylogenetic tree of Treponema species and phylotypes identified in samples from primary endodontic infections. Sequences were aligned with CLUSTAL W, and distances were calculated with the Jukes-Cantor algorithm. Bootstrap values (based on 500 replicates) are represented at each node when >50%. The scale bar represents a 2% difference in nucleotide sequences.
TABLE 1. PCR primers used for identification of *Treponema* species in primary endodontic infections

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequences (5'-3')</th>
<th>Position (bp)</th>
<th>Amplicon length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spirochete-specific</td>
<td>CAC ATT GGG ACT GAG ATA C TAC CTG TTA GTA ACY GGC AGT AG</td>
<td>312-1,138</td>
<td>827</td>
<td>(23)</td>
</tr>
<tr>
<td><em>Treponema denticola</em></td>
<td>TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA</td>
<td>193-508</td>
<td>316</td>
<td>(3)</td>
</tr>
<tr>
<td><em>Treponema maltophilum</em></td>
<td>AGA GTT TGA TYM TGG CTC AG* CCT ATT GTG CTT ATT CAT CAG GC</td>
<td>8-443</td>
<td>436</td>
<td>(54)</td>
</tr>
<tr>
<td><em>Treponema socranskii</em></td>
<td>GAT CAC TGT ATA CGG AAG GTA GAC A TAC ACT TAT TCC TCG GAC AG</td>
<td>148-435</td>
<td>288</td>
<td>(54)</td>
</tr>
<tr>
<td>Universal 16S rRNA gene</td>
<td>AGA GTT TGA TYM TGG CTC AG GAA GGA GGT GWT CCA RCC GCA</td>
<td>8-1,541</td>
<td>1,534</td>
<td>(9, 24)</td>
</tr>
</tbody>
</table>

* Forward universal primer

Y = T or C; M = A or C; W = A or T; R = G or A
Table 2. *Treponema* taxa found in samples from primary endodontic infections.

Clone library analysis

<table>
<thead>
<tr>
<th>Taxa</th>
<th>No. of clones (%)</th>
<th>Clinical samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1chr</td>
<td>2chr</td>
</tr>
<tr>
<td><em>Treponema amylovorum</em></td>
<td>2(3)</td>
<td></td>
</tr>
<tr>
<td><em>Treponema denticola</em></td>
<td></td>
<td>5(15)</td>
</tr>
<tr>
<td><em>Treponema lecithinolyticum</em></td>
<td></td>
<td>3(9)</td>
</tr>
<tr>
<td><em>Treponema maltophilum</em></td>
<td>52(68)</td>
<td>12(35)</td>
</tr>
<tr>
<td><em>Treponema medium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Treponema parvum</em></td>
<td>5(7)</td>
<td></td>
</tr>
<tr>
<td><em>Treponema putidum</em></td>
<td>1(1)</td>
<td>1(1)</td>
</tr>
<tr>
<td><em>Treponema socranskii ss buccale</em></td>
<td>11(14)</td>
<td>4(12)</td>
</tr>
<tr>
<td><em>Treponema socranskii ss socranskii</em></td>
<td>6(8)</td>
<td>14(41)</td>
</tr>
<tr>
<td><em>Treponema vincentii</em></td>
<td>2(6)</td>
<td></td>
</tr>
<tr>
<td><em>Treponema sp. IV:18:C9</em></td>
<td>1(10)</td>
<td></td>
</tr>
<tr>
<td><em>Treponema sp. V:19:D36</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Treponema clone 8T-43</em></td>
<td>1(3)</td>
<td></td>
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<td><em>Treponema clone 9T-42</em></td>
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<td><em>Treponema clone 9T-87</em></td>
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<td><em>Treponema clone 142-10</em></td>
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<td><em>Treponema clone 142-21</em></td>
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<td><em>Treponema clone 142-82</em></td>
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<tr>
<td><em>Treponema clone 18f-1</em></td>
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<tr>
<td><em>Treponema clone 18f-6</em></td>
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<td><em>Treponema clone 18f-7</em></td>
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<td><em>Treponema clone 18f-22</em></td>
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<td><em>Treponema clone 18f-33</em></td>
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<td><em>Treponema clone 18f-35</em></td>
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<td><em>Treponema clone 18f-48</em></td>
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<td><em>Treponema clone 94A-72</em></td>
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<td><em>Treponema clone 94A-89</em></td>
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<tr>
<td><em>Treponema clone 94A-92</em></td>
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<td></td>
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<tr>
<td><em>Treponema clone 94A-94</em></td>
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<td></td>
</tr>
</tbody>
</table>

**Total number of *Treponema* taxa** | 4 | 5 | 6 | 2 | 3 | 8 | 8 | 3 | 12 |

**Total number of *Treponema* clones** | 76 | 34 | 34 | 45 | 10 | 74 | 78 | 42 | 38 |

* Novel phylotypes identified in this study
** For calculation of total number of taxa in each sample, *T. socranskii* subspecies occurring together were considered as one taxon

chr, chronic apical periodontitis; abs, acute apical abscess
Table 3. Frequency of detection of three *Treponema* species in primary endodontic infections. 16S rRNA gene-based nested PCR analysis

<table>
<thead>
<tr>
<th>Species</th>
<th>Chronic apical periodontitis</th>
<th>Acute apical periodontitis</th>
<th>Acute apical abscess</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Treponema denticola</em></td>
<td>22/32 (69%)</td>
<td>8/10 (80%)</td>
<td>29/48 (60%)</td>
<td>59/90 (66%)</td>
</tr>
<tr>
<td><em>Treponema maltophilum</em></td>
<td>9/32 (28%)</td>
<td>5/10 (50%)</td>
<td>12/48 (25%)</td>
<td>26/90 (29%)</td>
</tr>
<tr>
<td><em>Treponema socranskii</em></td>
<td>12/32 (37.5%)</td>
<td>4/10 (40%)</td>
<td>17/48 (35%)</td>
<td>33/90 (37%)</td>
</tr>
</tbody>
</table>

* number of positive cases/number of samples examined (percentage)
Figure 1