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Cultivable bacteria in infected root canals as identified by 16S rRNA gene sequencing

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Introduction: Traditionally, cultivable bacteria isolated from infected root canals have been identified by phenotype-based methods. Because 16S ribosomal RNA (rRNA) gene sequencing has emerged as a more accurate and reliable tool for bacterial identification, the present study applied this approach to identify bacterial isolates recovered from the root canals of teeth with chronic apical periodontitis.
Methods: Anaerobic techniques were used for culturing; identification of the isolates was carried out by polymerase chain reaction amplification and sequencing of the V5–V8 region of the 16S rRNA gene. Bacteria were found in all samples. The mean number of taxa per canal was 3.1, ranging from 2 to 8. The median number of cultivable bacterial cells in the root canals was 4.2×10^5, ranging from 2.8×10^3 to 3.3×10^7. Eighty-seven strains belonging to 52 bacterial taxa were identified. The most prevalent taxa were *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Pseudoramibacter alactolyticus*, *Micromonas micros* and streptococci. The following bacterial phyla were represented in this study: *Firmicutes* (22 taxa, 46% of the identified isolates), *Actinobacteria* (14 taxa, 25.3% of the isolates), *Bacteroidetes* (eight taxa, 13.8% of the isolates), *Fusobacteria* (three taxa, 9.2% of the isolates) and *Proteobacteria* (five taxa, 5.7% of the isolates). Some of the isolates represented unnamed species not previously cultivated and characterized. In conclusion, our findings using a combined anaerobic culture–molecular identification approach confirmed the polymicrobial nature of primary endodontic infections with dominance of anaerobic bacteria. Notably several bacteria that are difficult or impossible to identify by phenotypic means were identified, including previously uncultivated taxa, cultivated-but-not-yet-characterized taxa and newly named species.

Key words: apical periodontitis; culture; endodontic infection; 16S rRNA gene sequencing

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Apical periodontitis is an infectious disease caused by microorganisms infecting the dental root canal. Participating microorganisms can have been involved in the early stages of pulp tissue invasion, which culminated in inflammation and further necrosis, or they can be latecomers that took advantage of the environmental conditions in the root canal after pulp necrosis. Studies using culture-dependent and culture-independent techniques indicated that primary root canal infections are characterized by a mixed population conspicuously dominated by anaerobic bacteria, with the number of bacterial cells per canal varying from less than 10^2 to about 10^8 (27, 29, 36, 41).

Traditionally, bacteria involved in endodontic infections have been studied by means of culture-dependent techniques, which rely on isolation, growth and identification by physical and biochemical tests. Interpretation of results from culturing methods is based on characteristics observed in known and reference strains, with predictable biochemical and physical properties under optimal growth conditions. However, phenotypic characteristics are not static and can change under some circumstances, including stress (21, 23). Thus, when common microorganisms with uncommon phenotypes are present, reliance on phenotypes can compromise accurate identification. Technologist bias or inexperience may similarly compromise identification when the results of biochemical tests are interpreted to fit expectations. Therefore, one should be mindful that in some circumstances even the

successful culturing of a given microorganism does not necessarily mean that this microorganism can be successfully identified (28).

The 16S ribosomal RNA (rRNA) gene sequencing approach has emerged as a more effective, precise and reliable means for the identification of cultivable bacteria that cannot be accurately identified by phenotype-based tests (3, 6, 23, 40). Unlike phenotypic identification, 16S rRNA gene sequencing provides unambiguous data for species with atypical phenotypes, rare isolates or poorly described bacteria (3, 9, 10, 33). Additionally, the technique can lead to the recognition of novel species and as-yet-uncultivated bacteria (6). Analysis based on the 16S rRNA gene has been widely adopted because of the universal distribution of this gene among bacteria, the presence of conserved and variable regions within the gene, and its applicability to the inference of phylogenetic relationships (44).

Most culturing studies of the endodontic microbiota have been based on phenotypic identification of the isolates. However, results from these studies may have been influenced by misidentification of some isolates for the reasons mentioned above or because recently described species have not yet been included in the databases and the tables of the enzymatic kits commonly used for bacterial identification. The possibility also exists that many of the so-called 'uncultivable' phylotypes detected by molecular studies are indeed cultivable species that may have been previously isolated but misidentified by phenotype-based approaches. An approach combining culture and molecular identification might shed some light on these issues. To the best of our knowledge, only one study has identified cultivable bacteria in endodontic infections by means of 16S rRNA gene sequencing analysis but it evaluated only five samples because the study also included a labor-intensive culture-independent 16S rRNA gene clone library approach (20). The present study was undertaken to identify cultivable bacteria isolated from infected root canals of teeth showing chronic apical periodontitis lesions by means of 16S rRNA gene sequencing analysis.

Materials and methods

Clinical material

Patients presenting to the endodontic clinic at the School of Dentistry, Estácio de Sá University, for evaluation and treatment of apical periodontitis participated in the study. Thirty-two single-rooted teeth from 29 patients (16 females and 13 males, aged 12–74 years, mean 45.9 years) were selected for analysis in this study according to rigid inclusion criteria. Only teeth with intact pulp-chamber walls, with necrotic pulps (as confirmed by a negative response to sensitivity pulp tests) and with clinical and radiographic evidence of chronic apical periodontitis lesions were included in this study. Teeth from patients who had received antibiotic therapy within the previous 3 months; teeth with gross carious lesions; teeth with fractures of the root or crown; teeth that had received previous endodontic treatment; and cases with periodontal pockets over 4 mm deep were excluded from the study. The size of each lesion was calculated by taking the average of the lesion's largest dimension and the extent in the direction perpendicular to the largest dimension (32). Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University.

Sampling procedures

Sampling procedures were carried out under strict aseptic conditions. Before isolation with a rubber dam, each tooth had the supragingival plaque removed by scaling and cleansing with pumice. Caries and/or coronal restorations were removed with sterile high-speed and low-speed burs. After application of the rubber dam, dental floss was securely tied around the neck of the tooth and the operative field, including the tooth crown, cavity, clamp and surroundings, were cleaned with 3% hydrogen peroxide until no further bubbling of the peroxide occurred. All surfaces were then disinfected by vigorous swabbing with a 2.5% sodium hypochlorite solution. After completing the access with another sterile bur under sterile saline irrigation, the operative field, including the pulp chamber, was cleaned and disinfected once again in the same way as above. Sodium hypochlorite was neutralized with 5% sodium thiosulfate, and then a bacteriological sample of the tooth surface was obtained with sterile paper points. For inclusion of the tooth in the study, bacteriological control samples taken from the working field after surface disinfection had to be negative. Based on this criterion, two teeth had to be excluded from the study.

Root canal samples were taken as follows. Three sterile paper points were consecutively placed in the canal to a level approximately 1-mm short of the tooth apex, based on diagnostic radiographs, and used to soak up the fluid in the canal. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal. Paper points were then transferred aseptically to tubes containing 500 μl reduced transport fluid (39).

Culturing procedures

Samples were transported to the laboratory within 15 min for microbiological processing. Samples in reduced transport fluid vials were dispersed with a vortex for 30 s and 10-fold serial dilutions to 10^{-3} were made in pre-reduced anaerobically sterilized buffered salt solution (14). Aliquots of 100 μl from the undiluted suspension or from the highest dilution were each spread onto *Brucella* agar plates (BBL Microbiology Systems, Cockeysville, MD) supplemented with 5% defibrinated sheep blood, hemin (5 mg/l) and menadione (1 mg/l) and onto *Mitis salivarius* agar plates (Difco, Detroit, MI). Plates were immediately placed in anaerobic jars (BBL GasPak system; Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated anaerobically at 37°C for up to 14 days (11, 35). Following incubation, the total colony-forming units (CFUs) were counted and actual counts were calculated based on the known dilution factors. One or two colonies of each different colony type were isolated and each one was individually placed in cryovials containing TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). All black-pigmented colonies were picked up and each individual colony was identified in isolation. Cryovials were stored at −20°C for further identification by 16S rRNA gene sequence analysis.

16S rRNA gene identification

Genomic DNA from each isolated colony was extracted by heating the suspension for 10 min at 97°C with a thermocycler. The vials were then stored for 5 min on ice and centrifuged, and 5-μl aliquots of the supernatant were further used as template in the polymerase chain reaction (PCR) assay.

Polymerase chain reaction amplification of 16S rRNA genes was used for bacterial identification. The pair of universal 16S rRNA gene primers used was 5′-GAT TAG ATA CCC TGG TAG TCC AC-3′ and 5′-CCC GGG AAC GTA TTC ACC G-3′, corresponding to base positions 786–808 and 1369–1387, respectively, and spanning the variable regions V5–V8 of the *Escherichia coli* 16S rRNA gene. PCR

amplification was performed in a reaction volume of 50 μl, consisting of 0.8 μM of each primer, 5 μl of 10X PCR buffer, 2 mM $MgCl_2$, 1.25 U *Tth* DNA polymerase, 0.2 mM concentration of each deoxyribonucleoside triphosphate (all reagents from Biotools, Madrid, Spain). Cycling parameters included an initial denaturation step at 95°C for 2 min, followed by 36 cycles of a denaturation step at 95°C for 30 s, a primer annealing step at 60°C for 1 min, an extension step at 72°C for 1 min and a final step of 72°C for 2 min. The results of PCR amplification were examined by electrophoresis in a 1.5% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength ultraviolet light.

Polymerase chain reaction products were purified using a PCR purification system (Wizard PCR Preps; Promega, Madison, WI) and then 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 using the BioEdit software (13). Sequences were corrected when obvious sequencing software errors were observed. Sequences generated were compared to the GenBank database to identify the closest relatives by using the BLAST algorithm (1). An identity ≥99% in the 16S rRNA gene sequence was the criterion used to identify an isolate to the species level. A 97–99% identity in 16S rRNA gene sequence was the criterion used to identify an isolate at the genus level, while <97% identity in 16S rRNA gene sequence was the criterion used to define a potentially new bacterial species (9, 10).

Results

Bacteria were found in all root canal samples. The mean number of bacterial taxa per canal was 3.1, ranging from 2 to 8. Distribution of the number of different taxa per case was as follows: two taxa in 13 cases, three taxa in 10 cases, four taxa in three cases, five taxa in one case, six taxa in two cases and eight taxa in one case. Root canals of teeth with lesions <5 mm in diameter harbored a mean number of 2.5 taxa (median 2, range 2–4), while the canals associated with lesions ≥5 mm harbored a mean number of 3.4 taxa (median 3, range 2–8).

The median number of CFUs in the root canal samples was 4.2×10^5 (range 2.8×10^3–3.3×10^7). In teeth with lesions <5 mm, the median number of CFUs was 3.75×10^5 (range 2.8×10^3–2.8×10^7). The corresponding figure for canals associated with lesions ≥5 mm was 4.2×10^5 (range 4.0×10^3–3.3×10^7).

Eighty-seven isolates belonging to 52 bacterial taxa were identified. The most prevalent taxa were *Fusobacterium nucleatum* (five isolates), *Porphyromonas gingivalis* (four isolates), *Pseudoramibacter alactolyticus* (four isolates), *Micromonas micros* (four isolates), *Streptococcus mitis* biovar 2 (four isolates) and *Streptococcus oralis*/*Streptococcus mitis*/*Streptococcus sanguinis* (four isolates). The genera containing more representatives were *Streptococcus* (20 isolates from nine taxa), *Actinomyces* (10 isolates from six taxa), *Fusobacterium* (eight isolates from three taxa), *Prevotella* (six isolates from five taxa) and *Propionibacterium* (six isolates from two taxa). At broad phylogenetic scales, the endodontic microbiota was dominated by members of the phylum *Firmicutes*, which accounted for 46% of the identified isolates (22 taxa, 40 isolates). The other bacterial phyla represented in this study included *Actinobacteria* (14 taxa, 22 isolates, 25.3% of the identified isolates), *Bacteroidetes* (eight taxa, 12 isolates, 13.8% of the isolates), *Fusobacteria* (three taxa, eight isolates, 9.2% of the isolates) and *Proteobacteria* (five taxa, five isolates, 5.7% of the isolates) (Figs 1 and 2). Based on the 16S rRNA gene sequence data, some of the isolates represented unnamed species not previously cultivated and characterized (Table 1). In general, sequencing of the region from V5 to V8 of the 16S rRNA gene provided useful information for identification of the great majority of the isolates. However, some taxa could not be reliably identified and distinguished from closely related species and might require sequencing of the entire 16S rRNA gene for a more precise identification; this was not performed in this study. Thus, the range of possible identities of some isolates is given in Table 1.

Identification of six isolates was not possible because of poor sequences with too many ambiguous characters (Ns) (four isolates) or sequences with low-scoring homologies (<97% similarity) to sequences deposited in the GenBank (two isolates). The latter two isolates may represent novel species and require further testing (including sequencing of the entire 16S rRNA gene) for adequate phylogenetic classification.

After complete chemomechanical preparation of the canals in the first appointment, two cases developed severe pain and swelling. The cultivable microbiota present in one of these cases before preparation comprised *P. gingivalis*, *S. mitis* biovar 2 and *Campylobacter curvus* while the other case harbored a previously uncultivated peptostreptococcus (rRN-A007), *Acinetobacter* sp., *Propionibacterium propionicum* and *Actinomyces urogenitalis*.

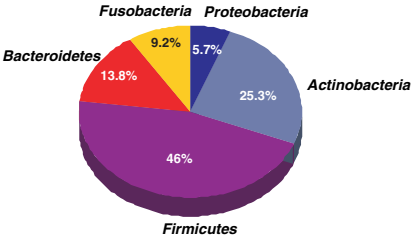

Fig. 1. Bacterial phyla with cultivable representatives in infected root canals of teeth with chronic apical periodontitis. Data refer to the number of identified isolates ($n = 87$) from each phylum.

Discussion

Virtually all the previous culture studies of the endodontic microbiota identified bacteria by their phenotypic features. In the present study, we used anaerobic culture procedures with ordinary culture media routinely employed by many clinical microbiology laboratories. The basic difference was that identification was carried out by 16S rRNA gene sequencing instead of by phenotype-based procedures. 16S rRNA gene sequencing analysis was used to achieve a more reliable identification of cultivable bacteria from infected root canals.

All of the five bacterial phyla previously reported to have cultivable representatives in the endodontic microbiota were found in this study. Most of the identified taxa (44/52) belonged to the *Firmicutes*, *Actinobacteria* and *Bacteroidetes* phyla, which corresponds with other culture studies of the root canal microbiota (5, 12, 37) (Fig. 2). The phylum *Firmicutes* had the largest number of representatives in the samples examined, confirming previous data from both culture-dependent (12, 38) and culture-independent studies (20, 24, 26). The *Actinobacteria* was the second most represented phylum in this study. In fact, a broader diversity of *Actinobacteria* is expected to be disclosed by culture-dependent methods when compared to 16S rRNA gene clone library analysis (19). Members of three other phyla (*Synergistes*, *Spirochaetes* and TM7) only found in endodontic infections by molecular approaches were not detected

Table 1. Cultivable bacterial taxa isolated from 30 infected root canals of teeth with chronic apical periodontitis and identified by 16S rRNA gene sequencing analysis

Bacterial taxa	No. strains
Actinobacteria	
Propionibacterium propionicum	3
Propionibacterium acnes	3
Actinomyces odontolyticus	2
Actinomyces israelii	2
Actinomyces naeslundii	2
Actinomyces oral clone GU009	2
Actinomyces urogenitalis	1
Actinomyces sp.	1
Rothia dentocariosa	1
Rothia mucilaginosa	1
Bifidobacterium dentium	1
Dietzia sp. E9_2 E1 oral isolate	1
Brachybacterium nesterenkovii	1
Rhodococcus rhodochrous	1
Bacteroidetes	
Porphyromonas gingivalis	4
Prevotella oral clone GU027	2
Prevotella oral clone FM005	1
Prevotella marshii	1
Prevotella oralis	1
Prevotella salivae	1
Capnocytophaga sputigena	1
Flavobacterium sp.	1
Firmicutes	
Micromonas micros	4
Pseudoramibacter alactolyticus	4
Streptococcus mitis biovar 2	4
Streptococcus oralis/*S. mitis*/*S. sanguinis*	4
Streptococcus anginosus	3
Streptococcus constellatus/*S. intermedius*	2
Streptococcus sanguinis	2
Streptococcus gordonii	2
Streptococcus parasanguinis	1
Streptococcus infantis	1
Streptococcus sp. oral strain T4-E3	1
Anaerococcus prevotii	1
Dialister invisus	1
Mogibacterium neglectum	1
Eubacterium yurii	1
Staphylococcus aureus	2
Staphylococcus saccharolyticus	1
Staphylococcus epidermidis	1
Staphylococcus pasteuri	1
Uncultured *Staphylococcus* sp. clone EarCan063	1
Uncultured bacterium clone rRNA007	1
Bacillus sp.	1
Fusobacteria	
Fusobacterium nucleatum	5
Fusobacterium oral clone BS019/*F. periodonticum*	2
Fusobacterium oral clone CZ006	1
Proteobacteria	
Campylobacter gracilis	1
Campylobacter rectus	1
Campylobacter curvus	1
Neisseria sicca	1
Acinetobacter sp.	1
Unidentified	6

in the present study, confirming their 'unculturability', at least under standard culturing conditions.

In the present study, samples were incubated exclusively in an anaerobic atmosphere. Over 70% of the isolates comprised obligate anaerobes, though it was not possible to determine the oxygen tolerance for some previously uncharacterized bacteria. The mean number of taxa per infected canal was 3.1, with a maximum of eight taxa in one canal. All cases harbored a mixed infection with at least two species per canal. These figures are in agreement with most previous culture studies, which have demonstrated that primary endodontic infections are characterized by a mixed consortium dominated by anaerobic bacteria and composed of a mean number of 2.6–5.4 taxa per canal (2, 15, 17, 18, 22, 38, 43). However, molecular biology methods have revealed a larger number of taxa per canal in teeth with asymptomatic apical periodontitis, with mean numbers ranging from 7 to 20 taxa (20, 26, 31). Therefore, data from culture studies tend to underestimate the number of bacterial taxa in infected canals, and this can be a result of difficulties with or the impossibility of culturing a significant proportion of the endodontic microbiota.

Most of the named bacterial species detected herein have already been found in endodontic infections by culture-dependent and/or culture-independent approaches, confirming their status as candidate endodontic pathogens. The most prevalent taxa were *F. nucleatum*, *P. gingivalis*, *P. alactolyticus*, *M. micros*, *S. mitis* biovar 2 and *S. oralis*/*S. mitis*/*S. sanguinis*. Collectively, the most prevalent genus was *Streptococcus*, which was also the genus containing most representatives. Nonetheless, prevalence figures for several anaerobic species were far lower when compared to previous molecular studies (29). Also, a number of fastidious bacteria frequently detected in endodontic infections by culture-independent studies and included in the set of putative endodontic pathogens, such as *Tannerella forsythia*, *Treponema* species, *Filifactor alocis*, *Dialister pneumosintes* and others (29), were not found in the present study, confirming the culture limitations in detecting these species.

Some previously uncultivated and uncharacterized taxa have already been isolated from endodontic infections and identified by the 16S rRNA gene sequencing approach (16, 20). In the present study, several species not-yet-identifiable by phenotypic means were isolated. They included previously uncultivated taxa (*Prevotella* oral clones GU027 and FM005, *Fusobacterium* oral clones BS019 and CZ006, *Actinomyces* oral clone GU009, an uncultured bacterial clone rRNA007 related to peptostreptococci and an uncultured *Staphylococcus* clone EarCan063) and previously cultivated but not-yet-characterized taxa (*Dietzia* sp. E9_2 E1 oral isolate and *Streptococcus* sp. oral strain T4-E3). Also, some newly characterized and named taxa were found, including *Dialister invisus* (7), *Prevotella marshii* (8) and *Prevotella salivae* (25). Prevalence of these taxa was low, but given the difficulties in growing and identifying these species, studies using more sensitive molecular methods are

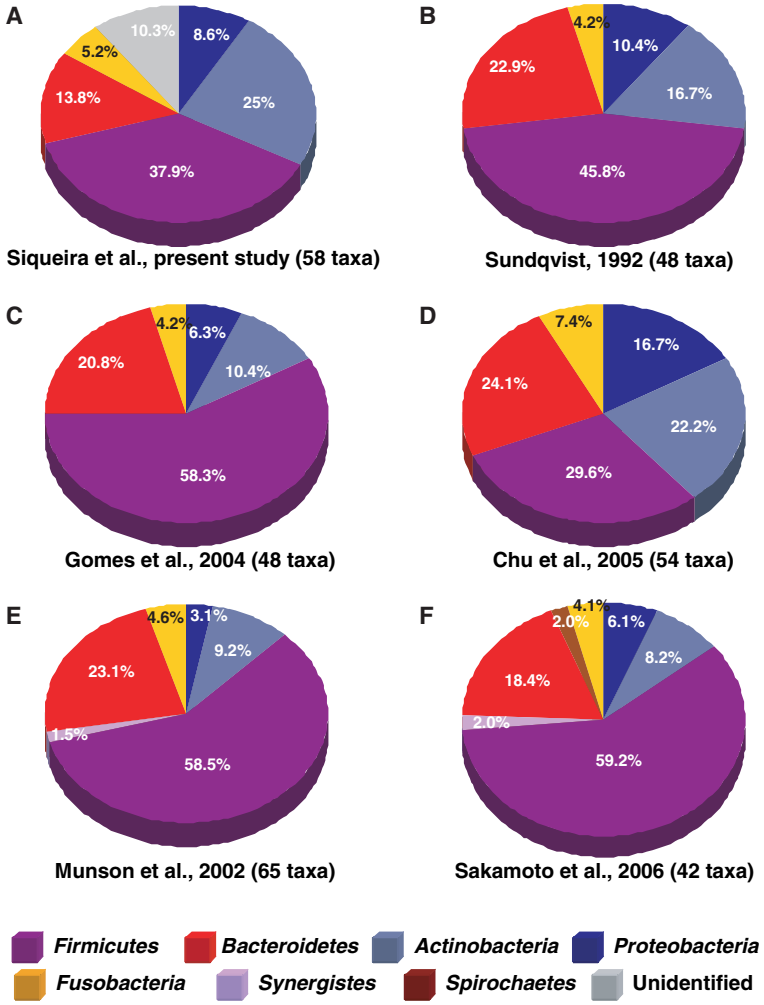

Fig. 2. Bacterial phyla with representatives in endodontic infections as revealed by the present study (A) and by others using culture-dependent (B–D) or culture-independent (E, F) approaches. Data refer to the number of different taxa found in each phylum.

required to investigate their prevalence in association with different forms of apical periodontitis, as was the case for *D. invisus*, a very frequent member of endodontic infections as revealed by molecular detection studies (20, 26, 30).

It has been suggested that the term non-cultured (or as-yet-uncultivated) bacteria be used instead of non-culturable (or uncultivable) bacteria, because conceptually all bacteria are able to grow under the proper conditions (6). The huge majority of the bacteria living in diverse environments have not yet been grown in artificial culture media for several reasons, including lack of essential nutrients or growth factors in the medium, conditions of overfeeding, toxicity of the culture medium itself, metabolic dependence on other species for growth; disruption of naturally occurring bacterial intercommunication systems and cells in a viable but non-cultivable state (28, 42). Special approaches and culture media to cultivate the large proportion of as-yet-uncultivated bacteria have been proposed and include the use of agar media with little or no added nutrients; relatively lengthy periods of incubation; and inclusion of substances that are typical of the natural environment in the artificial growth media (4, 34). However, it must be assumed that some of the as-yet-uncultivated bacteria revealed by molecular studies are indeed cultivable but as-yet-uncharacterized species that can grow in ordinary culture media and, if previously cultivated, may have been misidentified by phenotype-based approaches. Misidentification may be related to the fact that these not-yet-cultivated bacteria still remain to be phenotypically characterized and there are no described biochemical and physical attributes of a reference strain that can be used as parameters for their precise identification. It is possible that many of these species were cultivated in previous studies but may have been identified only at the genus level or may have been assigned to a species based on incomplete data.

In conclusion, our findings using a combined approach comprising anaerobic culturing and 16S rRNA gene-based identification confirmed the polymicrobial nature of primary endodontic infections with dominance of anaerobic bacteria. Several species previously reported in infected root canals were detected. Some newly named species were also found. Interestingly, some isolates showed significant sequence matches to existing sequences of unnamed taxa that have seemingly not been cultivated or characterized before. This combined approach has the potential to provide a more precise identification of bacterial isolates and to reveal the occurrence of previously uncharacterized taxa without the need for extensive and laborious phenotypic characterization. More extensive culture studies using 16S rRNA gene sequencing identification have the potential to disclose a more precise profile of the composition of the 'cultivable' endodontic microbiota, including identification of as-yet-uncharacterized species previously considered as 'uncultivable'.

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