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CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

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Diversity of Endodontic Microbiota Revisited

ABSTRACT

Although fungi, archaea, and viruses contribute to the microbial diversity in endodontic infections, bacteria are the most common micro-organisms occurring in these infections. Datasets from culture and molecular studies, integrated here for the first time, showed that over 460 unique bacterial taxa belonging to 100 genera and 9 phyla have been identified in different types of endodontic infections. The phyla with the highest species richness were *Firmicutes*, *Bacteroidetes*, *Actinobacteria,* and *Proteobacteria*. Diversity varies significantly according to the type of infection. Overall, more taxa have been disclosed by molecular studies than by culture. Many cultivable and as-yet-uncultivated phylotypes have emerged as candidate pathogens based on detection in several studies and/or high prevalence. Now that a comprehensive inventory of the endodontic microbial taxa has been established, future research should focus on the association with different disease conditions, functional roles in the community, and susceptibility to antimicrobial treatment procedures.

KEY WORDS: endodontic microbiology, molecular biology methods, culture, taxonomy.

INTRODUCTION

Essentially, endodontic infection is the infection of the dental root canal system and the major etiologic agent of apical periodontitis (Siqueira, 2008). Although chemical and physical factors can induce periradicular inflammation, a large body of scientific evidence clearly indicates that micro-organisms are essential to the progression and perpetuation of the different forms of apical periodontitis (Kakehashi *et al.*, 1965; Sundqvist, 1976; Möller *et al.*, 1981). For endodontic infection to develop, the root canal must be devoid of vital pulp tissue and its defenses, as a consequence of either pulp necrosis (as a sequel to caries, trauma, periodontal disease, or iatrogenic operative procedures) or pulp removal for treatment. The borderline between the infecting microbiota and the host defenses is often located intraradicularly, *i.e.,* short of or at the apical foramen. In some cases, however, micro-organisms may reach the periradicular tissues, and the borderline is then situated extraradicularly, *i.e*., beyond the boundaries of the apical foramen.

Because apical periodontitis is an infectious disease, the rationale for endodontic treatment is inarguably to eradicate the occurring infection and/or to prevent micro-organisms from infecting or re-infecting the root canal or the periradicular tissues. The cardinal principle of any healthcare profession is the thorough understanding of the disease etiology and pathogenesis, which provides a framework for effective prevention and treatment. In this context, understanding and defining the endodontic microbiota associated with different forms of disease are the basis for an endodontic practice of high quality and founded on a solid scientific basis. Such knowledge has the potential to contribute to the development of more effective preventive and therapeutic protocols. Furthermore, establishing an inventory of endodontic bacteria may also help future investigations of potential sources of pathogenic species for diseases in other human sites.

Traditionally, endodontic infections have been studied by means of culture approaches. Such studies have resulted in the establishment of a set of species thought to play an important role in the pathogenesis of apical periodontitis. More recently, not only have findings from culture-based methods been confirmed, but they have also been significantly supplemented with those from culture-independent molecular diagnostic techniques. Molecular methods have confirmed and strengthened the association of many cultivable bacterial species with apical periodontitis and have also revealed suspected new endodontic pathogens (Siqueira and Rôças, 2005b). The list of candidate pathogens has expanded to include culture-difficult species or even as-yet-uncultivated bacteria that had never been previously found in endodontic infections by culturing approaches. As a consequence, the endodontic microbiota has been refined and redefined by molecular methods (Siqueira and Rôças, 2005b).

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Because knowledge of the endodontic infection associated with different clinical conditions originates from culture-dependent and culture-independent molecular biology studies, the most comprehensive view of the diversity of the endodontic microbiota requires integration of the data from both types of studies. This review focuses on the diversity of the microbiota in different types of endodontic infections and is mostly based on cataloguing the microbial taxa already identified in culture and molecular studies. Overall, data from 128 studies published up to March 2008, were used for compilation (listed in the Appendix). Culture studies included in this compilation are restricted to those performed from the mid-1970s to 2008, when reliable methods for the cultivation and identification of fastidious anaerobic bacteria were introduced and adopted in endodontic microbiology research. Species names were considered valid when listed on the DSMZ Bacterial Nomenclature Web site (http://www.dsmz.de/microorganisms/bacterial_nomenclature.php) or mentioned in recent articles published in the *International Journal of Systematic and Evolutionary Microbiology*.

MOLECULAR STUDIES AND THE UNCULTIVATED MAJORITY

Culture methods have been highly successful over the past 100 years, nurturing the expectation that most micro-organisms can be grown in the laboratory. It is now widely recognized that culture techniques can strongly bias and underestimate the diversity of microbial populations (Hugenholtz, 2002; Relman, 2002). Only a small fraction of the bacteria present in most microbial ecosystems is amenable to propagation *ex vivo* (Handelsman, 2004; Fredricks and Marrazzo, 2005). Less than 1% of bacteria have been estimated to be cultivated from environments such as sea water, lakes, and soil (Amann *et al.*, 1995; Gewin, 2006). To sidestep the limitations of culture, tools and procedures based on molecular biology have become available and have been substantially improved to achieve a more realistic description of the microbial world without the need for cultivation. The most significant contributions of culture-independent molecular biology methods to medical microbiology relate to the identification of previously unknown human pathogens (Fredricks and Relman, 1996; Relman, 1999; Kellam and Weiss, 2001; Lawson, 2004) and the discovery of a far broader diversity of the human microbiota associated with different human sites. Studies based on the 16S rRNA gene approach have re-vealed that about 60% of the bacterial species in the oral cavity (Paster *et al.*, 2001; Aas *et al.*, 2005; Kumar *et al.*, 2005; de Lillo *et al.*, 2006), 50% on the skin (Dekio *et al.*, 2005), 38% in the esophagus (Pei *et al.*, 2004), 50% in the stomach (Bik *et al.*, 2006), 45% in the vagina (Fredricks *et al.*, 2005), and about 80% in the gut (Suau *et al.*, 1999; Eckburg *et al.*, 2005) represent as-yet-uncultivated and uncharacterized bacteria.

The study of the human gastrointestinal microbiota is a formidable example of the revolution fomented by molecular studies with regard to the study of the human microbiota in health and disease (Furrie, 2006). Culture-dependent studies of the gastrointestinal microbiota have resulted in the characterization of over 400 species (Rajilić-Stojanović *et al.*, 2007). Application of molecular biology techniques revealed that the microbiota in the gut is significantly more complex than previously anticipated, since only a fraction of the bacteria encountered can currently be cultivated (Suau *et al.*, 1999; Eckburg *et al.*, 2005). Another major outcome of the revolution brought about by molecular methods was the recognition that the composition of the microbiota is subject-specific and dominated by as-yet-uncultivated and uncharacterized phylotypes (Hold *et al.*, 2002; Eckburg *et al.*, 2005; Hayashi *et al.*, 2005). The integration of data from culture and molecular studies reveals that more than 1000 different taxa compose the microbial diversity in the human gastrointestinal tract (Rajilić-Stojanović *et al.*, 2007).

The oral cavity is another good example of the impact of culture-independent molecular biology methods on the knowledge of the microbial diversity. Data from culture and molecular studies have collectively revealed that almost 800 distinct bacterial taxa may be able to live in the human oral cavity, though not all of them are present in the same individual at the same time (Paster *et al.*, 2006). Indeed, any particular individual can harbor about 100-200 of these 800 taxa of oral bacteria, indicating that there is a substantial diversity among different people (Paster *et al.*, 2006). Taken as a whole, bacteria detected from the oral cavity fall into 13 separate phyla, namely, *Firmicutes*, *Bacteroidetes, Actinobacteria*, *Proteobacteria*, *Spirochaetes*, *Fusobacteria*, *Synergistes* (including phylotypes previously assigned to the phylum *Deferribacteres*), SR1 (originating from the OP11 division), TM7, *Chloroflexi*, *Deinococcus*, *Acidobacteria,* and *Cyanobacteria* (Paster *et al.*, 2001, 2002; Kazor *et al.*, 2003; de Lillo *et al.*, 2006; Aas *et al.*, 2007). This number may be even higher, since a recent study with DNA microarray technology suggested that members of 4 other phyla (*Aquificae, Nitrospira, Planctomycetes,* and *Thermomicrobia*) may have oral representatives, even though none has been identified (Huyghe *et al.*, 2008). A recent study with pyrosequencing, a high-throughput molecular approach that allows for extensive sequencing of microbial populations, revealed about 5600 and 10,000 species-level phylotypes representing 22 phyla in saliva and plaque, respectively (Keijser *et al.*, 2008). The estimated number of oral phylotypes is about 20,000, which is considerably higher when compared with that reported in previous culture and clone library studies.

It is worth pointing out that both culture and molecular approaches have their own limitations in portrayals of microbial diversity. Limitations of culture have been extensively recognized, and many of them have been successfully overcome by molecular technology (Fredricks and Relman, 1999; Hugenholtz, 2002; Siqueira and Rôças, 2005a). However, molecular methods can also give a biased picture of bacterial diversity, and their limitations in this regard are mostly related to different levels of effectiveness of cell lysis for DNA extraction and PCR-related biases (von Wintzingerode *et al.*, 1997; Siqueira and Rôças, 2005a; Rajilić-Stojanović *et al.*, 2007).

It has been claimed that the high sensitivity of molecular methods allows for the detection of species present in very low

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numbers and, consequently, with possibly no clinical importance. However, in microbial ecology, it has been demonstrated that some of these low-abundant bacteria might still serve as keystone species within complex mixed consortia (Sogin *et al.*, 2006). Moreover, some of the low-abundant species may be a result of historical ecological changes, in the sense that they may have been dominant in the past or have the potential to become dominant in the future in response to shifts in environmental conditions that favor their growth. Thus, low-abundant populations may eventually become dominant in response to environmental changes (Sogin *et al.*, 2006). Furthermore, species that are low-abundant in one individual can dominate the community in another individual. Therefore, at least from an ecological perspective, all species in a mixed community should be successfully detected and identified (Siqueira and Rôças, 2009b).

A broad range of molecular biology techniques has been used for diverse purposes in microbial ecology research (Siqueira and Rôças, 2005a; Spiegelman *et al.*, 2005). In endodontic microbiology research, the vast majority of molecular studies have addressed the issue of species composition in different types of endodontic infections. In spite of the great advantages of these methods, at this time, there is no available technique that can provide a comprehensive list of all bacterial species in a sample. As a consequence, a variety of techniques has been used to offer a better picture of the endodontic bacterial communities.

The chronology of the study of the diversity of the endodontic microbiology can be didactly divided into phases based on different strategic approaches. Early studies of the endodontic microbiota were conducted with broad-range culture methods (Bergenholtz, 1974; Kantz and Henry, 1974; Wittgow and Sabiston, 1975; Sundqvist, 1976; Baumgartner and Falkler, 1991; Sundqvist, 1992). These were followed by a generation of studies that used molecular detection methods, such as species-specific PCR and the original checkerboard DNA-DNA hybridization assay, to target cultivable bacteria previously isolated from infected canals or from other diseased oral sites (Conrads *et al.*, 1997; Jung *et al.*, 2000; Machado de Oliveira *et al.*, 2000; Rupf *et al.*, 2000; Siqueira *et al.*, 2000a,b, 2001b). These methods allowed for the inclusion of some culture-difficult species in the set of putative endodontic pathogens. The adoption of 16S rRNA gene clone library analysis facilitated an even more comprehensive broad-range investigation of bacterial communities in endodontic infections (Munson *et al.*, 2002; Saito *et al.*, 2006; Sakamoto *et al.*, 2006, 2008). By this approach, not only cultivable species but also as-yet-uncultivated and uncharacterized bacteria have been identified. Studies with 16S rRNA gene clone library analysis have revealed that 40-55% of the bacterial taxa found in primary endodontic infections have not been cultivated and validly named (Munson *et al.*, 2002; Sakamoto *et al.*, 2006, 2007). While technical difficulties and high cost can make it difficult to analyze a large number of samples by the clone library method, cataloguing bacterial species in the oral cavity by clone libraries provides 16S rRNA gene sequence data that can be used to design primers or oligonucleotide probes to target both cultivable and as-yet-uncultivated bacteria in infected root canals. Primers and probes can be used in PCR and DNA-DNA hybridization assays (*e.g.,* reverse-capture checkerboard, microarrays) in large-scale clinical studies to investigate prevalence and association with disease (Rôças and Siqueira, 2005b, 2008; Siqueira and Rôças, 2005c, 2009a).

DIVERSITY OF THE ENDODONTIC MICROBIOTA—OVERALL FINDINGS

'Microbiota' is arguably the best collective term for micro-organisms, since widely used terms such as 'microbial flora' and 'microflora' perpetuate an outdated classification of micro-organisms as plants (Dethlefsen *et al.*, 2006). 'Diversity' is a function of both the number of species present in a community (species richness) and the relative abundance of those species in the system (species evenness). The highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (evenness) (Huston, 1994). The richness and evenness of microbial communities are the result of selective pressures that shape diversity within communities. This review discusses the diversity of the endodontic microbiota, with the main focus on the aspect of species richness or composition. The integration of data from culture-dependent and culture-independent methods for microbial identification in endodontic infections is presented here for the first time.

Representatives of the domains *Archaea* and *Eukarya* have been occasionally reported to occur in endodontic infections. *Archaea* diversity is restricted to an as-yet-uncultivated *Methanobravibacter oralis*-like phylotype (Vianna *et al.*, 2006a; Vickerman *et al.*, 2007). Except for a couple of molecular studies targeting *Candida albicans* (Baumgartner *et al.*, 2000; Siqueira and Rôças, 2004a), the diversity of *Eukarya* in infected canals has been assessed exclusively by culture-dependent methods. Six *Candida*, one *Geotrichum*, one *Rhodotorula,* and one *Saccharomyces* species have been isolated from root canals (see Appendix for references).

Although a few representatives of the domains *Eukarya* and *Archaea* have been found in endodontic infections, and reports indicate the occurrence of herpesviruses (cytomegalovirus and Epstein-Barr virus) in apical periodontitis lesions (Sabeti *et al.*, 2003), the domain *Bacteria* is far more dominant and diverse in endodontic infections. Integrated datasets from culture and molecular studies published as of March 2008, showed that 468 unique bacterial taxa have been found in endodontic infections associated with different clinical conditions. The vast majority of these bacterial taxa fall into 100 genera, while 22 taxa remained undefined and were assigned to family or even to phylum level. *Prevotella* (39 taxa), *Eubacterium* (27 taxa), *Streptococcus* (26 taxa), and *Lactobacillus* (21 taxa) are the most represented genera. All taxa already reported in endodontic infections and the respective studies that detected them are listed in the Appendix.

At the broader phylogenetic level, all the detected bacterial taxa have been found to belong to 9 of the 13 phyla that have oral representatives, namely, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Spirochaetes*, *Synergistes*, TM7, and SR1 (Table 1). The most common representatives from each of these phyla are shown in Fig. 1. Members of *Acidobacteria*, a phylum sporadically found in the oral cavity, were not detected by group-specific PCR (unpublished data from the authors' laboratory).

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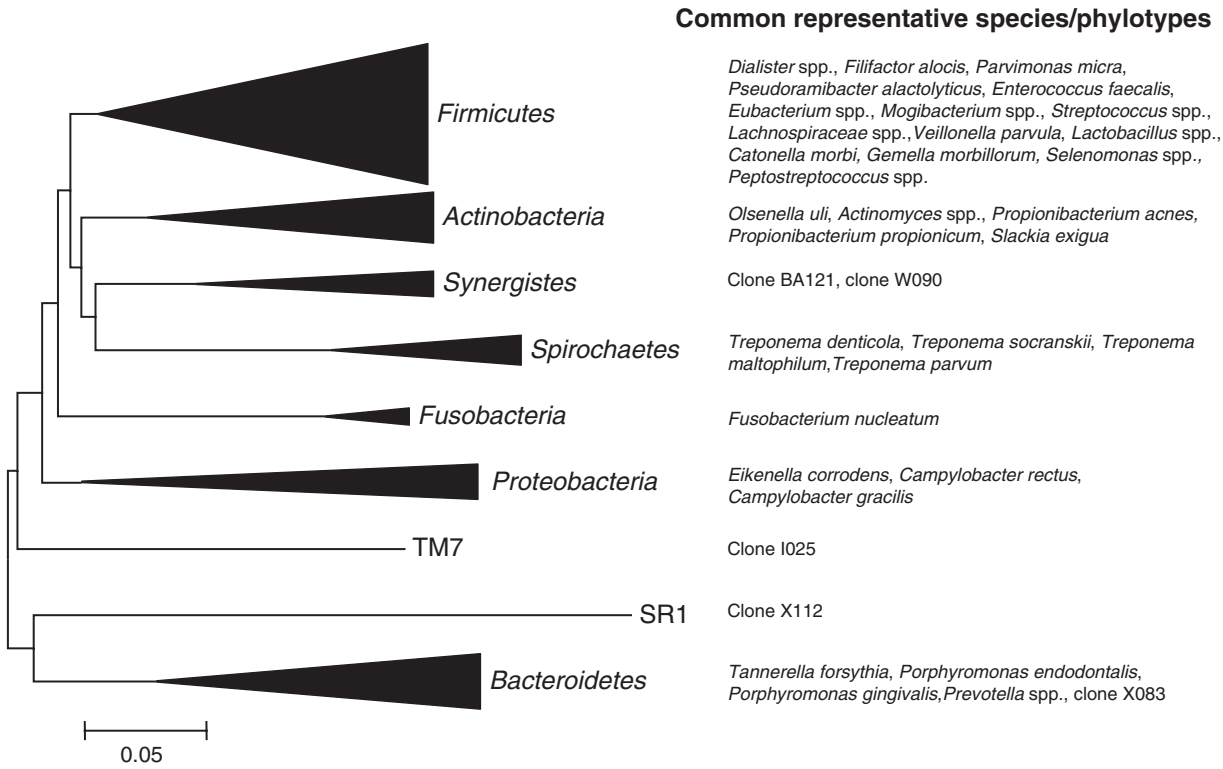

Figure 1. Bacterial phyla that have representatives in endodontic infections. On the right, example species or phylotypes for each phylum are presented.

It is important to point out that the numbers of bacterial taxa reported refer exclusively to richness within each phylum and have no relationship to prevalence and importance in disease causation. For instance, the phyla *Fusobacteria*, *Spirochaetes,* and *Synergistes* have few representative species/phylotypes in endodontic infections, but some of them, including *Fusobacterium nucleatum*, *Treponema denticola,* and *Synergistes* clone BA121, respectively, are among the most commonly detected taxa in primary intraradicular infections.

Of the recovered taxa, 204 (44%) were isolated or detected in only one study. This may indicate that they are subject-specific and then contribute to the high level of interindividual variation in bacterial community profiles, or they may be contaminants or species poorly identified. Contaminants may be introduced in the clinical setting during sample collection (usually oral contaminants) or in the laboratory during specimen handling (non-oral contaminants). Also, "single-study" taxa were rarely very prevalent in the individual study that reported their occurrence. In contrast, as-yet-uncultivated phylotypes or culture-difficult species detected in only one molecular study may not necessarily be interpreted as irrelevant because their occurrence in a single study may reflect the fact that, thus far, only a few broad-range molecular studies have been published, and the microbiota have been investigated in only a limited number of individuals.

Table 1. Overall Findings of Bacterial Species/Phylotype (taxa) Richness in the Different Types of Endodontic Infections

Phyla	Taxa	As-Yet-Uncultivated Phylotypes	Taxa Detected by Molecular Studies	Taxa Detected by Culture Studies
Firmicutes	220	81	146	124
Bacteroidetes	73	28	47	48
Actinobacteria	69	21	43	42
Proteobacteria	65	17	44	34
Fusobacteria	15	6	11	9
Spirochaetes	14	4	14	0
Synergistes	10	10	10	1
TM7	1	1	1	0
SR1	1	1	1	0

The analysis of the bacteria detected in endodontic infections revealed that 210 distinct bacterial taxa were reported in molecular biology studies alone. This figure corresponds to 45% of all taxa already found in endodontic infections, as opposed to 151 taxa (32%) detected by culture studies alone. One hundred seven taxa, 23% of the total bacterial species richness, were detected by the application of both culture-dependent and culture-independent techniques (Fig. 2).

Culture-dependent and culture-independent molecular studies provide a somewhat different description of the diversity of the endodontic microbiota, as indicated by the degree of overlapping findings (Fig. 2). However, both approaches show unequivocally that *Firmicutes* are by far the most diverse group, even though the community structure of this group has been largely underestimated in the reports from culture-dependent studies. Representatives of 4 phyla—*Spirochaetes*, *Synergistes*, TM7, and SR1—have been found almost exclusively in endodontic infections by molecular

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biology methods. While the latter two have only one representative each, both of which have been detected in low prevalence, members of the two former have been as frequent as, or even more frequent than, most representatives of the other, more diverse, phyla.

Molecular studies have allowed for the detection of 169 phylotypes representing bacteria that have not yet been cultivated and/or fully characterized. Most of these phylotypes belong to the phyla *Firmicutes*, *Bacteroidetes,* and *Actinobacteria*. As-yet-uncultivated and/or uncharacterized phylotypes can be important pathogens that have been overlooked by culture-dependent studies.

Molecular methods have succeeded over culture methods in the detection of not only as-yet-uncultivated and uncharacterized phylotypes, but also of cultivable and validly named species, since about 40 taxa included in this category have been exclusively detected by molecular approaches. These bacteria either represent fastidious species that were not recovered (probably because specific culture media were not used), or they are species that were successfully cultivated but inadequately identified, because methods improper for definite identification were used or the isolate presented ambiguous phenotypic behavior (Siqueira *et al.*, 2007c). Indeed, application of the 16S rRNA gene-sequencing approach for the identification of culture isolates has demonstrated that some phylotypes previously supposed to be "uncultivable" can actually be cultivated by conventional anaerobic techniques with ordinary growth media (Munson *et al.*, 2002; Siqueira *et al.*, 2007c). Most of these previously "uncultivable" bacteria remain uncharacterized, and a valid species name is pending.

The strength of molecular methods to unravel the bacterial diversity and its superiority over culture approaches in this regard is in clear agreement with studies in other areas (Amann *et al.*, 1995; Rappe and Giovannoni, 2003; Janssen, 2006; Rajilić-Stojanović *et al.*, 2007). In endodontic microbiology research, it is quite remarkable that molecular studies performed over the past 10 years have revealed 317 distinct bacterial taxa in infected canals, while culture studies over the past 30 years revealed 258 taxa.

Bacterial taxa for which there is moderate-to-strong evidence of involvement with causation of apical periodontitis are shown in Fig. 3. Selection of these taxa was based on their detection in several independent studies and moderate-to-high prevalence in more than one study. Thus, the taxa shown in Fig. 3 can be regarded as the main candidate endodontic pathogens associated with different types of infection. One will notice that there are some ubiquitous endodontic taxa that have been found by at least one study in every type of endodontic infection. Most of these "cosmopolitan" species have been found to be very prevalent in association with some specific types of infection, mainly primary infections. In spite of having been found in samples from every type of infection, *Enterococcus faecalis* is encountered more frequently in treated root canals of teeth evincing post-treatment disease (Appendix). All of these ubiquitous bacteria are cultivable and validly named species. However, it is worth pointing out that this list is biased by the fact that, for some types of infection, there is no or only one molecular study using broad-range analysis. Thus, some taxa found in primary infections in even high prevalence may have been deprived of this "status" because of the lack of a body of molecular studies investigating other disease categories.

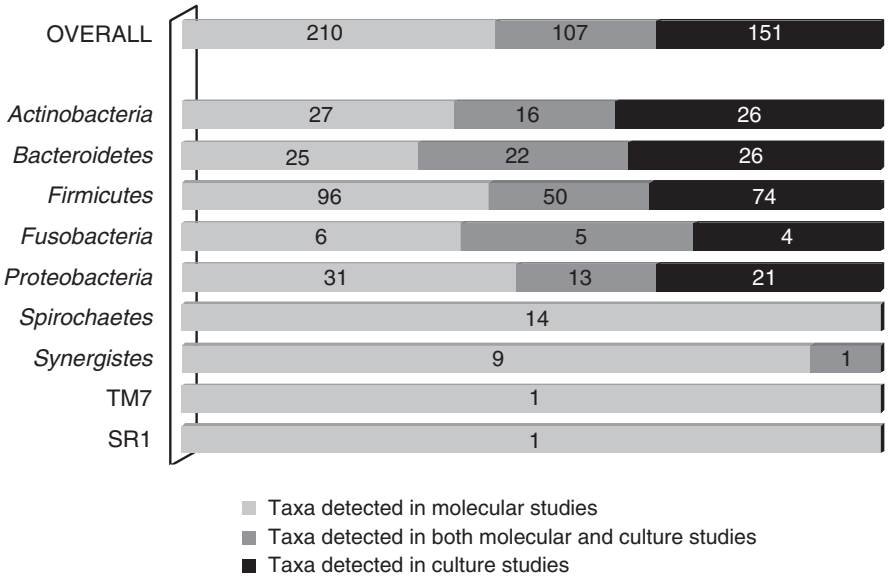

Figure 2. Distribution of bacterial species/phylotypes found in endodontic infections according to the detection method. Data are given overall and for the 9 phyla that have endodontic representatives.

PRIMARY INTRARADICULAR INFECTIONS

Primary intraradicular infection is caused by micro-organisms that initially invade and colonize the necrotic pulp tissue. It is characterized by a mixed consortium conspicuously dominated by anaerobic bacteria and composed of 10 to 30 species *per* canal (Munson *et al.*, 2002; Siqueira *et al.*, 2004b; Siqueira and Rôças, 2005b). Total bacterial counts vary from 10^3 to 10^8 cells *per* infected canal (Sundqvist, 1976; Vianna *et al.*, 2006b; Sakamoto *et al.*, 2007; Siqueira *et al.*, 2007d).

Bacterial profiles of the endodontic microbiota vary from individual to individual (Siqueira *et al.*, 2004b; Sakamoto *et al.*, 2006), *i.e.*, each individual harbors a unique endodontic microbiota in terms of species richness and abundance. This indicates that primary apical periodontitis has a heterogeneous etiology, where no single species can be considered as the main endodontic pathogen, and multiple bacterial combinations play a role in disease causation. This is consistent with the high numbers of different taxa that have been detected in primary infections.

Collectively, culture and molecular methods have allowed 391 bacterial, 4 fungal, and one archaeal taxa to be detected in primary

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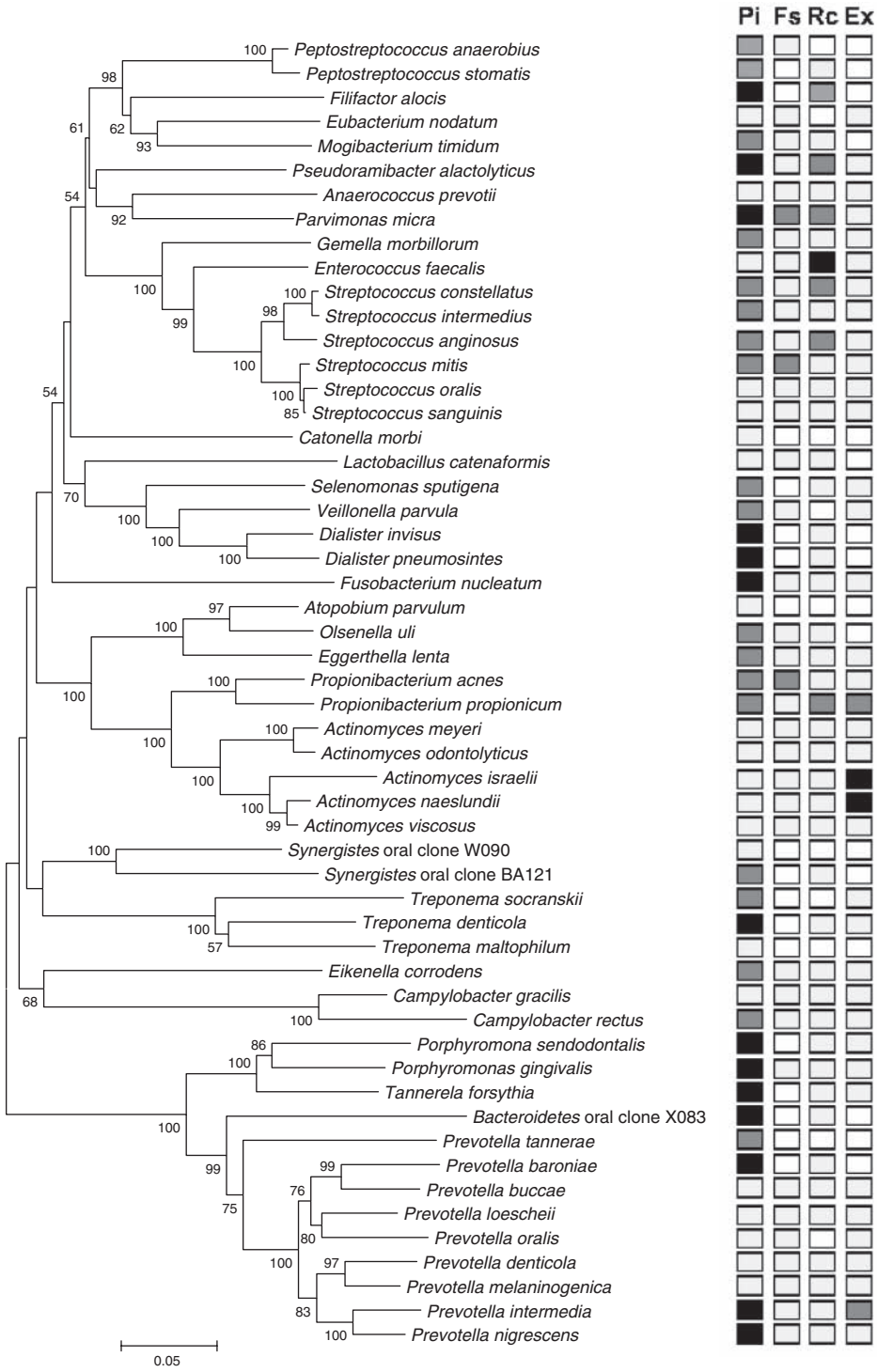

Figure 3. Phylogenetic tree based on 16S rRNA gene comparisons, showing several candidate endodontic pathogens and the clinical conditions with which they have been associated. Distribution of bacterial species/phylotypes among the different types of infections is shown by the columns of boxes to the right of the tree. Different darknesses of the box indicate strength of association based on prevalence data and the number of studies in which the species was detected, *i.e.*, clear box means not detected, while black box means detected by several studies and/or in high prevalence. The reference bar indicates 5% sequence divergence. *Pi*, primary infections; *Fs*, filling stage; *Rc*, retreatment cases; *Ex*, extraradicular infections.

infections. As-yet-uncultivated and/or uncharacterized bacteria have been represented by 136 taxa. Molecular methods have identified 271 taxa, while 216 taxa have been isolated by culture.

Bacterial species/phylotypes detected in primary infections fall into the 9 phyla mentioned above and 82 genera. Seventeen taxa have not been assigned to a genus, only to family or phylum. The highest species richness was observed for the *Firmicutes*, followed by *Bacteroidetes* and *Actinobacteria* (Table 2). Of the 391 taxa detected, 261 were unique to primary infections, *i.e.*, they have not been detected in samples from other types of infections.

Diverse groups of Gram-negative and Gram-positive bacteria have been identified in primary infections. Black-pigmented Gram-negative anaerobic rods are named after their ability to form brown to black colonies on blood agar plates and have been classified into two genera—*Prevotella* (containing saccharolytic species) and *Porphyromonas* (containing asaccharolytic species). The genus *Prevotella* also includes some bile-sensitive non-pigmented species. *Prevotella* species, especially *P. intermedia, P. nigrescens, P. tannerae, P. baroniae*, and *P. denticola*, have been among the most frequently detected taxa in primary infections (see Appendix for references). Of the *Porphyromonas* species, *P. endodontalis* and *P. gingivalis* have been consistently encountered in endodontic infections, and a role in the etiology of acute abscesses is suspected (van Winkelhoff *et al.*, 1985; Haapasalo *et al.*, 1986; Sundqvist *et al.*, 1989; Siqueira *et al.*, 2001a; Seol *et al.*, 2006).

An important periodontal pathogen, *Tannerella forsythia* (formerly *Bacteroides forsythus*), a fastidious Gram-negative obligate anaerobe that had never been detected in root canals by culture, was for the first time reported to occur in primary endodontic infections in a study with species-specific PCR (Conrads *et al.*, 1997). Subsequent studies with different molecular biology approaches have confirmed that *T. forsythia* is a common member of the microbiota

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associated with different types of primary infections, including abscesses (Jung *et al.*, 2000; Siqueira *et al.*, 2000b, 2001b; Fouad *et al.*, 2002; Siqueira and Rôças, 2003c).

Dialister species are asaccharolytic obligately anaerobic Gram-negative coccobacilli that represent another example of bacteria that have been consistently detected in endodontic infections only after the advent of molecular biology techniques. *D. pneumosintes* and the recently described *D. invisus* have been frequently present in the microbiota associated with asymptomatic and symptomatic primary infections (Appendix).

Fusobacterium nucleatum is an anaerobic spindle-shaped rod that is one of the most commonly encountered Gram-negative species in endodontic infections. *F. nucleatum* has been frequently isolated from or detected in primarily infected root canals as well as in endodontic abscesses (Appendix).

Spirochetes are highly motile spiral-shaped bacteria that have been frequently observed by microscopy in samples taken from endodontic infections. Nonetheless, they had never been identified to the species level. The application of molecular diagnostic methods to the identification of spirochetes has demonstrated that occurrence of these spiral bacteria in endodontic infections has been overlooked by technical hurdles of culture techniques. All of the currently cultivable and named oral *Treponema* species have been disclosed by molecular studies of primary endodontic infections (Appendix). The most predominant species in primary infections are *T. denticola* and *T. socranskii* (Siqueira *et al.*, 2000b; Baumgartner *et al.*, 2003; Rôças *et al.*, 2003; Siqueira and Rôças, 2004b).

Even though anaerobic Gram-negative bacteria have been found to be the most common micro-organisms in primary infections, several Gram-positive rods have also been frequent members of the endodontic microbial consortium. Of these, *Pseudoramibacter alactolyticus* has been frequently isolated from or detected in samples from endodontic infections, in prevalence values as high as those of the most commonly found Gram-negative species (Sundqvist, 1992; Siqueira and Rôças, 2003d; Siqueira *et al.*, 2004a). *Filifactor alocis* is an obligately anaerobic rod that has been detected by molecular studies in about one-half of the samples from primary infections (Siqueira and Rôças, 2003a; Gomes *et al.*, 2006). *Actinomyces* species, which have been associated with failure of the endodontic treatment by causing apical actinomycosis, have been found in about 10% of infected canals (Sundqvist, 1992; Siqueira *et al.*, 2002). *Propionibacterium propionicum*, another species that can participate in apical actinomycosis, has also been commonly detected in samples from primary infections (Siqueira and Rôças, 2003e). *Olsenella* species consist of small non-motile Gram-positive obligately anaerobic rods, which represent another example of bacteria that have been detected in endodontic infections only after the introduction of molecular biology methods (Fouad *et al.*, 2002; Munson *et al.*, 2002). Among the *Olsenella* clade, *O. uli* has been the most commonly found species in endodontic infections (Chávez de Paz *et al.*, 2004; Rôças and Siqueira, 2005a).

Gram-positive cocci, specifically peptostreptococci and streptococci, are frequently present in primary endodontic infections. *Parvimonas* (formerly *Peptostreptococcus* or *Micromonas*) *micra* is an asaccharolytic anaerobic Gram-positive small coccus that has been isolated from or detected in about one-third of the primarily infected canals, and its prevalence in symptomatic infections has also been relatively high (Sundqvist, 1992; Weiger *et al.*, 1995; Gomes *et al.*, 1996; Khemaleelakul *et al.*, 2002; Siqueira *et al.*, 2003; Chu *et al.*, 2005). Members of the *Streptococcus anginosus* group have been reported to be the most prevalent streptococci, but *S. oralis, S. mitis,* and *S. sanguinis* can also often be recovered/detected (Sundqvist, 1992; Siqueira *et al.*, 2002).

Table 2. Bacterial Species/Phylotype (taxa) Richness in Primary Endodontic Infections

Phyla	Taxa	As-Yet-Uncultivated Phylotypes	Taxa Detected by Molecular Studies	Taxa Detected by Culture Studies
Firmicutes	184	69	131	98
Bacteroidetes	69	24	42	48
Actinobacteria	54	11	31	39
Proteobacteria	44	11	32	21
Fusobacteria	14	5	9	9
Spirochaetes	14	4	14	0
Synergistes	10	10	10	1
TM7	1	1	1	0
SR1	1	1	1	0

Campylobacter species, including *C. rectus* and *C. gracilis*, are Gram-negative anaerobic rods that have been detected in primary endodontic infections, but in low-to-moderate prevalence values (Sundqvist, 1992; Le Goff *et al.*, 1997; Siqueira and Rôças, 2003b). *Catonella morbi*, a saccharolytic obligately anaerobic Gram-negative rod associated with marginal periodontitis, has been found in about one-fourth of the cases of primary endodontic infections (Siqueira and Rôças, 2006). Other bacteria detected in low-to-moderate frequencies in primary infections include: *Veillonella parvula, Eikenella corrodens, Neisseria mucosa, Centipeda periodontii, Granulicatella adiacens, Gemella morbillorum, Capnocytophaga* species, and anaerobic lactobacilli (Appendix).

Investigations of the diversity of the microbiota of primary infections by broad-range PCR and 16S rRNA gene clone library analysis have demonstrated that it is far more complex than previously reported by culture studies. Noteworthy is the common occurrence of as-yet-uncultivated bacteria—about 40-55% of the endodontic microbiota is composed of as-yet-uncultivated phylotypes (Munson *et al.*, 2002; Sakamoto *et al.*, 2006, 2007). A molecular study investigating primary infections revealed that 55% and 40% of the taxa detected in association with chronic apical periodontitis and acute apical abscesses were as-yet-uncultivated phylotypes, respectively (Sakamoto *et al.*, 2006). As for their abundance in these infections, as-yet-uncultivated phylotypes corresponded to 38% and 30% of the clones sequenced from samples of chronic apical periodontitis and abscesses, respectively (Sakamoto *et al.*, 2006).

As mentioned above, uncultivated phylotypes have been reported for all phyla with endodontic representatives. Several of these phylotypes can be candidate endodontic pathogens based on association data. For instance, oral *Synergistes* clones BA121, E3_33, BH017, and W090, which had been originally assigned to the *Flexistipes* or *Deferribacteres* group (Paster *et al.*, 2001; Godon

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et al., 2005), have been commonly detected in samples from asymptomatic and symptomatic endodontic infections (Rôças and Siqueira, 2005b; Siqueira and Rôças, 2005c; Siqueira *et al.*, 2005; Vianna *et al.*, 2007). The great majority of *Synergistes* bacteria remain uncultivated, and this can be the primary reason for the fact that their presence in endodontic infections has been overlooked by culture studies. Similarly, clones I025 and X112 from the TM7 and SR1 candidate phyla, respectively, which so far have no cultivable representatives, have also been detected in primary infections (Rôças and Siqueira, 2005a, 2008; Siqueira and Rôças, 2005c). Uncultivated phylotypes related to several genera have been disclosed, mainly *Dialister, Prevotella, Peptostreptococcus*, *Solobacterium, Olsenella, Selenomonas, Eubacterium*, *Megasphaera,* and *Veillonella,* as well as phylotypes related to the family *Lachnospiraceae* (Rolph *et al.*, 2001; Munson *et al.*, 2002; Saito *et al.*, 2006; Sakamoto *et al.*, 2006; Vickerman *et al.*, 2007). One study (Sakamoto *et al.*, 2006) found some uncultivated phylotypes among the most prevalent bacteria in primary infections, including *Lachnospiraceae* oral clone 55A-34, *Megasphaera* oral clone CS025, and *Veillonella* oral clone BP1-85. *Prevotella* oral clone PUS9.180, *Eubacterium* oral clone BP1-89, and *Lachnospiraceae* oral clone MCE7_60 were exclusively detected in symptomatic samples (Sakamoto *et al.*, 2006). Uncultivated phylotypes are previously unrecognized and overlooked bacteria that may play a role in the pathogenesis of different forms of apical periodontitis.

PERSISTENT/SECONDARY INTRARADICULAR INFECTIONS

Secondary infection is caused by micro-organisms that were not present in the primary infection, but were introduced in the root canal at some time after professional intervention (so called because it is secondary to intervention) (Siqueira, 2002). Persistent infection is caused by micro-organisms that were members of a primary or secondary infection and that, in some way, resisted intracanal antimicrobial procedures and endured periods of nutrient deprivation in treated canals (Siqueira, 2002).

Persistent or secondary intraradicular infections are the major causes of several clinical problems, including endodontic treatment failure, which is characterized by persistence or appearance of apical periodontitis after treatment (Siqueira, 2008). Studies have attempted to identify the micro-organisms found at the root-canal-filling stage, which may jeopardize the treatment outcome, and the micro-organisms in root-canal-treated teeth with apical periodontitis, which are arguably the cause of failure.

Analysis of integrated data from culture and molecular studies revealed that 51 bacterial and 2 fungal taxa have been detected in samples taken at the time of filling and also at the time of re-treatment. The following taxa have been detected by several studies: *Propionibacterium acnes, P. propionicum, Actinomyces naeslundii, Actinomyces odontolyticus, P. intermedia, Anaerococcus prevotii, Eggerthela lenta, E. faecalis, G. morbillorum, P. micra, P. alactolyticus, S. anginosus* group, *S. mitis, F. nucleatum,* and *C. albicans.* Theoretically, taxa found at both the time of filling and during re-treatment may be involved in persistent infections. Likewise, taxa that were found only at the time of re-treatment, but not at the time of filling, may represent secondary infections that developed by lack of a bacteria-tight coronal seal. Taxa detected at the filling stage, but not at the time of re-treatment, may not be able to endure the conditions within obturated canals. Although all this discussion sounds logical and interesting, it is largely speculative, because the data belong to separate cross-sectional studies, and no strong evidence can be taken in this regard.

BACTERIA AT THE ROOT-CANAL-FILLING STAGE (POST-TREATMENT SAMPLES)

Root canal samples can be taken at the time of treatment to assess the antimicrobial efficacy of treatment protocols and/or check the bacteriologic conditions of the canal before filling procedures. Samples positive for bacterial growth after chemomechanical preparation, followed (or not) by intracanal medication, have been shown to harbor an average of 1 to 5 bacterial species, at counts reaching up to 10^2 to 10^5 cells *per* canal (Byström and Sundqvist, 1985; Sjögren *et al.*, 1997; Vianna *et al.*, 2006b; Sakamoto *et al.*, 2007; Siqueira *et al.*, 2007a,b). These numbers indicate that, even if total bacterial elimination does not occur, at least a substantial reduction in species diversity is attained after treatment.

Culture and molecular methods have allowed 103 bacterial and 6 fungal taxa to be detected in samples taken after chemomechanical preparation and/or intracanal medication. Molecular methods have detected 26 taxa, while 88 taxa were isolated in culture studies. Forty-one taxa were found in only one study.

Bacterial species/phylotypes detected in post-treatment samples belong to 5 phyla and 41 genera. Three taxa have been assigned to taxonomic levels above genus. The highest species richness was observed for the *Firmicutes*, followed by *Proteobacteria* and *Actinobacteria* (Table 3).

Gram-negative bacteria, which are common members of primary infections, are usually eliminated after treatment procedures (Chávez de Paz, 2005). Exceptions may include some anaerobic rods, such as *F. nucleatum*, *Prevotella* species, and *C. rectus*, which are among the species found in post-instrumentation/medication samples (Appendix). However, most studies have clearly revealed that, when bacteria resist treatment procedures, Gram-positive bacteria are more frequently present. They include streptococci (*S. mitis*, *S. gordonii*, *S. anginosus*, *S. sanguinis,* and *S. oralis*), *P. micra*, *Actinomyces* species (*A. israelii* and *A. odontolyticus*), *Propionibacterium* species (*P. acnes* and *P. propionicum*), *P. alactolyticus,* lactobacilli (*L. paracasei* and *L. acidophilus*), *E. faecalis*, and *O. uli* (Appendix). Other Gram-positive bacteria, including *Bifidobacterium* species, *Eubacterium* species, and staphylococci, can also be found, but in lower frequencies (Sjögren *et al.*, 1997; Chávez de Paz, 2004; Chu *et al.*, 2006).

With the recent findings showing as-yet-uncultivated bacteria as constituents of a significant proportion of the endodontic microbiota (Munson *et al.*, 2002; Sakamoto *et al.*, 2006), studies on the effects of intracanal antimicrobial procedures should also focus on these bacteria. Thus far, the only broad-range molecular study to investigate samples from this condition used a 16S rRNA gene clone library analysis to identify the bacteria persisting after chemomechanical preparation, with 2.5% NaOCl as irrigant and intracanal medication with a calcium hydroxide paste (Sakamoto

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Table 3. Bacterial Species/Phylotype (taxa) Richness in Samples Taken at the Time of Root Canal Filling

Phyla	Taxa	As-Yet-Uncultivated Phylotypes	Taxa Detected by Molecular Studies	Taxa Detected by Culture Studies
Firmicutes	45	2	10	40
Proteobacteria	20	3	3	17
Actinobacteria	19	2	9	15
Bacteroidetes	16	3	3	13
Fusobacteria	3	0	1	3

et al., 2007). Eleven, 4, and 5 taxa were detected in initial (S1), post-instrumentation (S2), and post-medication (S3) samples, respectively. No taxon found in post-treatment samples was shown to dominate the initial (primary) infection (baseline). *Streptococcus* species were detected in all post-treatment samples and were also the most dominant taxa in these samples, except for a S2 sample in which *Solobacterium* clone K010 corresponded to 56% of the clones sequenced. Forty-two percent of the taxa found in post-treatment samples were as-yet-uncultivated bacteria, indicating that previously uncharacterized bacteria may also persist after treatment, and their role in the long-term treatment outcome requires elucidation.

MICROBIOTA IN ROOT-CANAL-TREATED TEETH (RE-TREATMENT SAMPLES)

Root canal samples can be taken from teeth that were treated months to years previously and need endodontic re-treatment because of the emergence or persistence of disease. The microbiota in these cases also exhibit a decreased diversity in comparison with that of primary infections. Canals apparently well-treated contain from 1 to 5 species, while the number of species in canals with inadequate treatment can reach 30, which is very similar to that in untreated canals (Sundqvist *et al.*, 1998; Pinheiro *et al.*, 2003; Rôças *et al.*, 2004c; Siqueira and Rôças, 2004a). A single treated canal associated with post-treatment disease can harbor a density of 10^3 to 10^7 bacterial cells (Peciuliene *et al.*, 2001; Sedgley *et al.*, 2006).

Integrated datasets from culture and molecular methods have revealed 158 bacterial and 3 fungal taxa in samples taken at the time of root canal re-treatment of teeth evincing apical periodontitis lesions. Forty-six as-yet-uncultivated phylotypes have been detected by molecular studies. Molecular methods have detected 109 taxa, while culture studies isolated 72 taxa. A great many taxa (106) have been reported by only one study.

Bacterial species/phylotypes detected in re-treatment samples belong to 7 phyla and 58 genera. Nine taxa have been assigned to the family or phylum level. The highest species richness was again observed for the *Firmicutes*, followed by *Actinobacteria* and *Proteobacteria* (Table 4). Molecular studies have allowed members of the *Spirochaetes* and *Synergistes* phyla to be detected in these cases.

Culture-dependent and culture-independent studies have revealed that *E. faecalis* is the most frequent species in root-canal-treated teeth, with prevalence values reaching up to 90% of the cases (Molander *et al.*, 1998; Sundqvist *et al.*, 1998; Pinheiro *et al.*, 2003; Rôças *et al.*, 2004a,b; Siqueira and Rôças, 2004a; Sedgley *et al.*, 2006; Zoletti *et al.*, 2006). Root-canal-treated teeth are about 9 times more likely to harbor *E. faecalis* than cases of primary infections (Rôças *et al.*, 2004a). Other bacteria found in re-treatment cases include streptococci and some fastidious anaerobic bacterial species—*P. alactolyticus*, *P. propionicum*, *F. alocis*, *D. pneumosintes,* and *D. invisus* (see Appendix).

Table 4. Bacterial Species/Phylotype (taxa) Richness in Persistent/Secondary Infections Related to Treatment Failure

Phyla	Taxa	As-Yet-Uncultivated Phylotypes	Taxa Detected by Molecular Studies	Taxa Detected by Culture Studies
Firmicutes	76	21	50	40
Actinobacteria	28	11	21	10
Proteobacteria	26	5	17	11
Bacteroidetes	22	6	16	9
Fusobacteria	4	2	3	2
Spirochaetes	1	0	1	0
Synergistes	1	1	1	0

In a study using 16S rRNA gene clone library analysis, Sakamoto *et al.* (2008) found a mean of 10 taxa *per* treated root canal, ranging from 1 to 26. As-yet-uncultivated phylotypes corresponded to 55% of the taxa detected. Only 11 taxa were found in more than one case: *Bacteroidetes* oral clone X083, an uncultured *Saprospiraceae* clone, *Prevotella oris*, *P. baroniae*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Synergistes* clone BA121, *Dialister* clone 9N-1, *E. faecalis*, *Flavobacteriaceae* genomospecies C1, and *Peptostreptococcus* clone FG014. A high interindividual variability in the composition of the microbiota was clearly evident, and, along with the findings from a bacterial community profiling technique (Rôças *et al.*, 2004c), suggests that distinct bacterial combinations can play a role in treatment failures. Bacterial abundance also varied from sample to sample, and some uncultivated phylotypes were the most dominant taxa in about one-half of the cases examined. In fact, uncultivated phylotypes made up a significant fraction of the microbiota in root-canal-treated teeth, since they comprised about 50% of the total number of clones sequenced (Sakamoto *et al.*, 2008).

Fungi are only occasionally found in primary infections, but *Candida* species have been detected in root-canal-treated teeth in up to 18% of the cases (Cheung and Ho, 2001; Peciuliene *et al.*, 2001; Egan *et al.*, 2002). *C. albicans* is by far the most commonly detected fungal species in re-treatment cases.

EXTRARADICULAR INFECTIONS

Extraradicular infection is characterized by microbial invasion of and proliferation in the inflamed periradicular tissues, and is derived from intraradicular infection (Tronstad *et al.*, 1987; Siqueira, 2002; Tronstad and Sunde, 2003). Extraradicular infection in cases of chronic apical periodontitis represents one of the greatest controversies in endodontics (Bergenholtz and Spangberg, 2004). The infection is conceivably limited to a small proportion of cases, and, when present, it can be dependent on or independent of intraradicular infection (Siqueira,

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Table 5. Bacterial Species/Phylotype (taxa) Richness in Extraradicular Infections

Phyla	Taxa	Taxa Detected by Molecular Studies	Taxa Detected by Culture Studies
Firmicutes	47	16	41
Bacteroidetes	18	10	12
Proteobacteria	12	6	8
Actinobacteria	10	7	8
Fusobacteria	3	3	2
Spirochaetes	2	2	0

2008). A study revealed that the number of species *per* infected lesion ranged from 11 to 34 (Sunde *et al.*, 2000).

Culture and molecular methods have revealed 92 bacterial taxa and one fungal species in samples taken from apical periodontitis lesions associated with treated canals. No reliable broad-range molecular study has thus far been conducted to survey these infections. For instance, results from samples taken from extracted teeth were not considered herein because of the obvious risks of contamination of the lesion. Therefore, molecular methods to investigate the bacteria in extraradicular infections associated with treated teeth evaluated only cultivable taxa. These molecular methods alone have detected 44 taxa, while 71 species have been isolated in culture studies. Forty-six taxa were found in a single study.

Bacterial taxa detected in extraradicular infections belong to 6 phyla and 38 genera. The *Firmicutes* was the phylum with the largest number of representatives, followed by *Bacteroidetes* and *Proteobacteria* (Table 5). Species reported by many studies include: *Actinomyces* species (*A. israelii, A. naeslundii, A. odontolyticus, A. viscosus*), *P. acnes, P. propionicum, P. gingivalis, P. intermedia, Prevotella oralis, P. micra,* and *F. nucleatum*.

CONCLUDING REMARKS

The integration of datasets from culture-dependent and culture-independent molecular biology studies has shown that 468 unique bacterial, one archaeal, and 9 fungal taxa have been, as of then, reported to occur in endodontic infections associated with different clinical conditions. Of the bacterial taxa, 264 have been found in more than one study.

It is important to point out that these figures might be somewhat reduced had only studies with stringent culture (*e.g.*, biochemical tests and gas-liquid chromatography) and/or molecular (*e.g.*, 16S rRNA gene sequencing) identification approaches been surveyed. However, this would have significantly reduced the number of studies evaluated, with the inevitable risk of excluding many important species that can be easily identified by less stringent approaches. Moreover, several other factors may have influenced the number of species/phylotypes compiled in this study, including the protocol for disinfection of the operative field, as well as the care, level of training, and experience during sample collection, sample handling in the laboratory, and interpretation of identification data. All of these factors may have influenced the species/phylotype numbers in our list. However, we could not remediate this by ruling out some studies, since most of these factors cannot be measured based on a printed paper.

While there was an apparent difference in the prevalence of some of the bacterial species found in primary infection *vs*. persistent or secondary infections, many species were present in only one specific study but not in others, making it difficult to determine their association with any specific form of endodontic infection. Several of these species are culture-difficult fastidious species, newly named taxa, or as-yet-uncultivated phylotypes, for which studies are very recent, with current molecular technology, and no further corroborative findings have become available. Further prevalence studies should target such species, especially those very prevalent in a single isolated study, to check their actual prevalence and look for associations with different forms of disease.

Now that a comprehensive inventory of the microbial species present in these infections has been established by culture and molecular studies, further efforts should be directed toward finding associations of the most prevalent taxa with symptoms and other clinical conditions, unraveling the functional role of the detected species in the mixed endodontic consortium, and disclosing their susceptibility to antimicrobial substances and treatment procedures. Extensive review of the endodontic microbiology literature leads to the conclusion that apical periodontitis has a polymicrobial etiology, and that the different types of endodontic infections are usually represented by a mixed consortium, whose diversity varies according to the type of infection. The bacterial community profile exhibits a high interindividual variability, and differences are even more pronounced when samples are taken from different geographical locations.

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