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Present status and future directions in endodontic microbiology

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Apical periodontitis and its different manifestations are caused primarily by bacterial infection of the root canal system. Bacteria are usually organized in biofilm communities and can colonize not only the main canal but also spread to other areas in the root canal system. In these regions, bacteria are more difficult to reach and eliminate during treatment. The knowledge of endodontic infections has substantially increased over the past 4 decades, including the main species involved in the different forms of disease, their virulence factors, interactions, ecology, organization and spatial distribution in the root canal system, patterns of antimicrobial resistance, and so on. However, there is a need for all of this information to be translated into improvements in clinical practice and treatment outcomes. This article reviews the present status of endodontic microbiology, discusses perspectives for future research and directions, and emphasizes the need for a call to action in the field of applied endodontic microbiology.

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Where we are now

Apical periodontitis is caused by bacterial infection

Evidence mounted over the last 50 years indicates that apical periodontitis is an inflammatory disease caused by microbial infection of the root canal system (1–4). Even though fungi and most recently archaea and viruses have been found in association with apical periodontitis (5–9), bacteria are regarded as the major microorganisms implicated in the etiology of this disease (10). Consequently, apical periodontitis can be considered as a disease of bacterial infection.

Apical periodontitis can be classified as either primary or post-treatment depending on whether it is associated with untreated or treated root canals, respectively. Although there is a consensus that the primary disease has a bacterial etiology, some authors have emphasized that the post-treatment disease may also be caused by non-microbial factors (11–13). Whereas the evidence for non-microbial factors such as epithelium from true cysts and cholesterol crystals serving as the cause of post-treatment disease is weak and comes from case reports or case series (13–15), bacterial involvement has been strongly supported by numerous studies using microscopy (4,15,16), culture (17–19), and molecular microbiology methods (20–25). Molecular studies have demonstrated that most, if not all, of the root canals of teeth with post-treatment apical periodontitis are associated with intraradicular or extraradicular infections (20,21,25,26). Based on these studies, post-treatment apical periodontitis can be regarded as a microbiological problem, even in teeth with root canal treatments that look adequate on radiographs. Infection is usually located within the root canal system (persistent or secondary intraradicular infection), but in a few cases it may extend to the periradicular tissues (extraradicular infection).

Apical periodontitis is a biofilm-associated disease

Biofilms are sessile multicellular microbial communities composed of microbial cells firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polymeric substances (EPS) (27,28). In bacterial biofilms, cells comprise about 10–15% and the EPS matrix over 85%–90% of the biofilm dry mass (29,30). A large number of human diseases, including caries and periodontal diseases, are caused by bacterial biofilms (27).

The first morphological description of bacterial structures resembling biofilms in infected root canals of teeth with apical periodontitis was made by Nair (31). Later, similar observations were also reported by *in situ* morphological investigations of teeth with primary or persistent/secondary root canal infections (16,32–35). Biofilms adhered to the apical root surface (extraradicular biofilms) have also been described in some teeth evincing post-treatment apical periodontitis (36–38).

These observations contributed to the assumption that apical periodontitis could be a disease caused by bacterial biofilms. However, the prevalence of biofilms and their association with diverse presentations of apical periodontitis were only revealed about 20 years after Nair's early reports. In a histobacteriological and histopathological study, Ricucci & Siqueira (4) evaluated the prevalence of biofilms in the apical root canals of teeth with primary or post-treatment apical periodontitis and observed that intraradicular biofilm arrangements were present in 80% of the untreated canals and 74% of the treated canals. Biofilms were more frequently observed in the apical canals of teeth with large lesions and those histopathologically diagnosed as cysts. Large lesions and cysts represent longstanding pathological processes, and the bacteria involved may have had enough time and conditions to establish a mature and organized biofilm community. Extraradicular biofilms were very infrequent as they occurred in only 6% of the cases, all of them symptomatic, and almost always associated with intraradicular biofilms.

Intraradicular bacterial biofilms are usually thick and composed of several layers of cells (4). Different bacterial morphotypes are commonly seen per biofilm, which is coherent with the multispecies nature of endodontic infections as reported by culture and molecular studies. The relative proportion between bacterial cells and the EPS matrix can vary significantly from case to case. Indeed, the morphology of endodontic biofilms differs consistently from individual to individual (4). It has been shown that the EPS matrix is not generally uniform but may vary spatially and temporally (39). Different species produce differing amounts of EPS, and the thickness of the matrix usually increase with the age of the biofilm (30,39,40). These factors help explain the large variations in morphology of endodontic biofilms and open another avenue for future research in the area.

Apical periodontitis seems to fulfill most of the established criteria used to determine whether a given infectious disease can be classified as a disease caused by biofilm communities (4,41,42). However, because biofilms are detected in the majority but not in all cases of apical periodontitis, it remains to be determined if biofilm formation is required for apical periodontitis development. Based on the knowledge available, it seems more prudent to state that apical periodontitis is a disease strongly associated with bacterial biofilms. This piece of knowledge assumes a great clinical relevance because biofilms can be very difficult to eliminate (43–45).

Apical periodontitis and the community-as-pathogen concept

In Nature and especially in the human body, biofilms usually comprise multispecies communities. Traditionally, microbiologists have studied bacterial pathogenicity on the basis of "guilt by association" as some classic diseases, such as tetanus, cholera, and syphilis, have a "single-species etiology." More recently, it has been established that several human diseases, especially those caused by endogenous infections (i.e. by members of the resident microbiota), have a polymicrobial etiology in which a set of species usually organized in multispecies biofilm communities is involved (27,46–48). Apical periodontitis is included in this role of diseases caused by mixed bacterial communities.

The concept of the community as the unit of pathogenicity is based on the principle that teamwork is what eventually counts. The community behavior and the outcome of the host–bacterial community interaction will depend on the types and numbers of

species present in the community biofilm and the network of resulting associations among them (48).

Studies using culture-independent community-profiling analyses have provided insights into the endodontic bacterial community structure, offering new perspectives related to the etiology and pathogenesis of apical periodontitis (49). These studies moved the focus from the "single-pathogen" to the "community-as-pathogen" concept. Bacterial community profiles seem to follow some patterns related to the different clinical presentations of apical periodontitis, e.g. the communities associated with symptomatic disease are significantly different in diversity from those occurring in asymptomatic cases (50–53). Likewise, the communities associated with post-treatment disease are different from those found in primary apical periodontitis (23,24). Therefore, disease severity or response to treatment may be related to the bacterial community profile.

Community-profiling molecular techniques have also demonstrated that the primary and persistent/secondary infections are usually composed of multispecies bacterial communities, with the former harboring a higher diversity (20,23–25,51,52,54–58). Moreover, the microbiota in the apical root canal is as diverse as that occurring in the more coronal aspects of the root canal, in spite of being significantly different in species composition (59–61).

Community-profiling studies showed that there is a great individual-to-individual variability in the composition of endodontic bacterial communities associated with the same clinical disease (23,51,54,55, 58,62), indicating that apical periodontitis has a heterogeneous etiology. However, despite the interindividual variability, these studies revealed a geography-related pattern in the endodontic bacterial community profiles (23,51,54,63).

Who's there?

Culture has been traditionally used to investigate the endodontic microbiota and permitted the establishment of a set of species thought to participate in the pathogenesis of apical periodontitis. Over the past 15 years, not only have findings from culture-based studies been confirmed but they have also been significantly supplemented by molecular microbiology studies (10). Molecular methods have confirmed and strengthened the association of many cultivable bacterial species with apical periodontitis and have also disclosed new candidate endodontic pathogens, including fastidious cultivable species and as-yet-uncultivated bacteria (10).

Investigations of the species occurring in endodontic infections have been divided into five generations on the basis of the principles of the methods used (10,64) (Fig. 1). The first generation of studies used open-ended culture methods, which revealed many cultivable species in association with apical periodontitis. Findings from this generation of studies were significantly refined after the introduction of anaerobic cultivation methods in endodontic research in the mid-1970s (2). The second generation comprises studies that employed closed-ended molecular detection methods, such as species-specific polymerase chain reaction (PCR) and its derivatives as well as the original checkerboard hybridization assay, to target cultivable bacteria. These methods are usually more sensitive than culture and allowed the inclusion of some difficult-to-grow species in the set of putative endodontic pathogens. The third generation is represented by studies adopting open-ended molecular methods, such as broad-range PCR followed by cloning and Sanger sequencing or terminal-restriction fragment length polymorphism (T-RFLP), which expanded the knowledge of the bacterial diversity in endodontic infections to include not only cultivable but also as-yet-uncultivated and uncharacterized bacteria. The fourth generation involved closed-ended molecular analyses with PCR and reverse-capture checkerboard hybridization in large-scale clinical studies to investigate the prevalence and association of cultivable and as-yet-uncultivated bacteria with endodontic infections. The fifth generation uses next-generation DNA sequencing (NGS) technologies, especially the pyrosequencing approach, for a deep-coverage open-ended analysis of endodontic infections.

Approximately 500 different microbial species (mostly bacteria) have been detected in different types of endodontic infections (10). Of these, about 45% were exclusively reported by molecular microbiology studies, compared to 32% detected by culture studies alone (10). The percentage of species detected by both approaches is 33% (10). It becomes evident that the endodontic microbiota has been refined and redefined by molecular methods, but a comprehensive analysis of

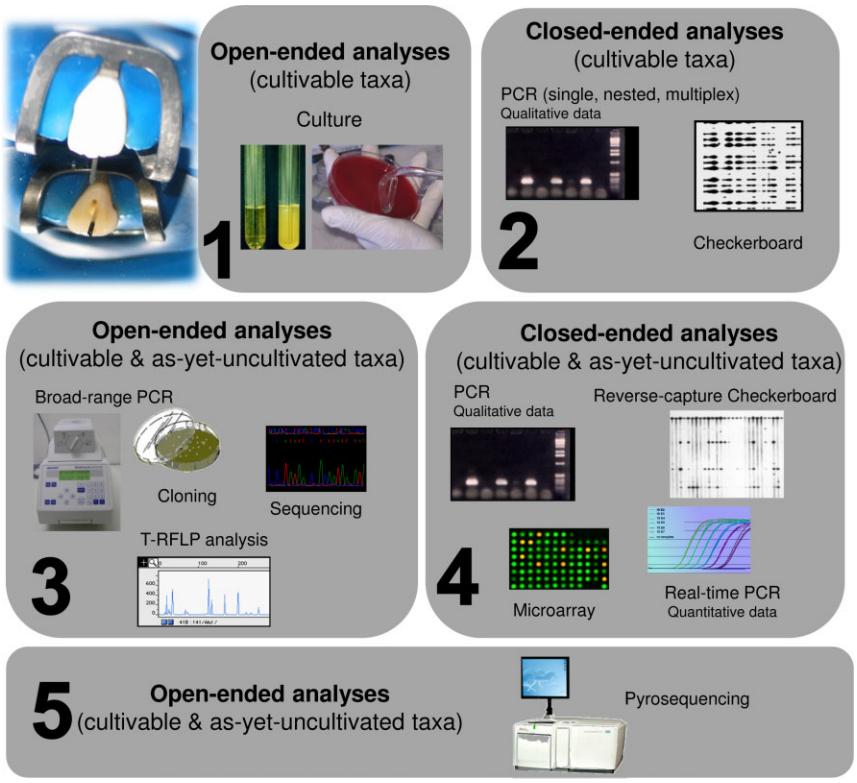

Fig. 1. The five generations of studies in endodontic microbiology.

the bacterial diversity in endodontic infections may require the association of culture and molecular data (65).

Primary intraradicular infections

Primary intraradicular infections are characterized by a mixed consortium composed of 10 to 30 species per canal (65), but these numbers may be still higher according to recent pyrosequencing studies (25,52). The bacterial load varies from 10^3 to 10^8 cells per infected canal (66–68). There is a significant difference in the bacterial community profiles associated with symptomatic and asymptomatic infections, and the former has been shown to harbor a significantly higher number of species (50–52).

Bacterial species/phylotypes detected in primary infections fall into 9 of the 13 phyla that have oral representatives, namely *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, *Proteobacteria*, *Spirochaetes*, *Synergistetes*, TM7, and SR1 (50,67,69–73). However, there may be representatives of at least nine other phyla in endodontic infections as revealed by pyrosequencing studies (25,52,58,61,62). These species belonging to uncommon phyla are likely low-abundance members of the endodontic community. The most prevalent and abundant bacterial taxa/groups occurring in primary infections include black-pigmented Gram-negative anaerobic species (*Prevotella* and *Porphyromonas* species), *Fusobacterium nucleatum*, streptococci, spirochetes (*Treponema* species), *Dialister* species, *Pseudoramibacter alactolyticus*, *Propionibacterium* species, *Parvimonas micra*, *Tannerella forsythia*, *Filifactor alocis*, *Eubacterium* species, and *Olsenella* species (2,50,67, 69–83).

Persistent/secondary intraradicular infections

Persistent or secondary intraradicular infections are the major causes of post-treatment apical periodontitis. This statement is supported by two strong evidence-based arguments. First, most (if not all) root canal-treated teeth evincing apical periodontitis lesions have been demonstrated to harbor an intraradicular infection (17–18,20,21,25,84–86). Second, it has been shown that there is an increased risk of adverse

treatment outcome when microorganisms are present in the canal at the time of filling (87–89).

Studies have intended to identify species resisting root canal procedures by taking samples immediately after chemomechanical preparation and intracanal medication. The bacterial species found in these studies have the potential to influence the treatment outcome. Most studies have revealed an overall higher occurrence of Gram-positive bacteria in both post-instrumentation and post-medication samples (88,90,91). Some as-yet-uncultivated phylotypes have also been found (67,68,92,93). Indeed, a study showed that 42% of the taxa found in post-treatment samples consisted of bacteria that remain to be cultivated and characterized (67).

Other studies identified the species occurring in root canal-treated teeth with post-treatment apical periodontitis. The species detected in this type of study are members of a persistent or secondary infection and represent the possible cause of the post-treatment disease. *Enterococcus faecalis* has been the most frequently detected species in root canal-treated teeth, with prevalence values reaching up to 90% of the cases (20,21,86,94–99). Canals of teeth with post-treatment disease are about nine times more likely to harbor *E. faecalis* than cases of primary disease (94).

Because *E. faecalis* is the most commonly found species in treated teeth and has attributes that may allow it to survive in treated canals, this species has been regarded as the main pathogen involved in treatment failure. Nevertheless, there are some findings from studies carried out in independent laboratories that put into question the role of *E. faecalis* as the most important causative agent of post-treatment disease. For instance, some studies have not detected enterococci in root canal-treated teeth with apical periodontitis (100,101). Furthermore, quantitative real-time PCR (qPCR) analyses revealed that this species usually comprise only approximately 1% of the total microbial community (21,95). This finding joins those from community-profiling studies to indicate that *E. faecalis* is not the dominant species in most re-treatment cases (21,23,24,56). Finally, some reports have demonstrated that *E. faecalis* can be as prevalent in root canal-treated teeth with no lesions as it is in diseased teeth (102,103).

Other species have been shown to participate in the bacterial communities associated with post-treatment disease, including streptococci, *Pseudoramibacter alactolyticus*, *Propionibacterium* species, *Parvimonas micra*, *Filifactor alocis*, and *Dialister* species (20,21,99). A qPCR study found streptococci to comprise approximately 76% of the total bacterial counts in root canal-treated teeth (21). Several as-yet-uncultivated bacteria have been identified in root canal-treated teeth, corresponding to approximately 55% of the detected taxa (24,100). In general, molecular microbiology studies have demonstrated that the microbiota of teeth with post-treatment apical periodontitis is usually mixed and more complex than previously anticipated by culture studies. Even so, bacterial diversity in adequately treated canals with post-treatment disease is less pronounced than in inadequately treated or untreated canals (17,18,20,24,50,79).

Extraradicular infections

Since Kronfeld's early observations (104), the commonly held opinion is that in asymptomatic (chronic) apical periodontitis, bacteria are usually confined to the root canal system, in an apparent balance between infection and host defense. Apical periodontitis lesions are formed in response to intraradicular infection and generally are an effective immunological barrier against the spread of the infection to the alveolar bone and other body sites. However, it has been suggested that, in certain circumstances, the balance can be broken and the inflamed periradicular tissues invaded by bacteria, resulting in extraradicular infection (105).

The most common form of extraradicular infection is the acute apical abscess. However, the controversy refers to asymptomatic cases. Some studies have found evidence of extraradicular bacterial infection forming either a biofilm adhered to the apical external root surface (36,106) or cohesive actinomycotic colonies within the body of the inflammatory lesion (107,108). In these cases, extraradicular bacteria have been discussed as one of the etiologies of post-treatment apical periodontitis (105,109).

A recent concept on this issue is that the extraradicular infection can be dependent on or independent of the intraradicular infection (109). Independent extraradicular infections are those no longer fostered by the intraradicular infection, and can persist even after successful eradication of the latter. So far, it has been suggested that the main bacterial

species implicated in independent extraradicular infections are *Actinomyces* species and *Propionibacterium propionicum*, in a pathologic entity named apical actinomycosis (107,110–112). The ability of these bacteria to form cohesive colonies within the lesion has been regarded as an important mechanism to evade phagocytosis (113). However, the existence of apical actinomycosis as a self-sustained pathological entity no longer nurtured by the intraradicular infection, and its involvement as the exclusive cause of treatment failure, still remain to be proven (114,115).

So far, there is no clear evidence that an extraradicular infection can exist as a self-sustained process independent of the intraradicular infection (114). A study (16) evaluated several treated teeth with post-treatment apical periodontitis and could not detect any case of independent extraradicular infection. In the few cases that bacteria were found invading the inflamed periradicular tissues, concomitant intraradicular infection was also observed. While not so common, extraradicular bacteria were more frequent in symptomatic teeth.

Except for acute and chronic apical abscesses, it is still controversial whether asymptomatic apical periodontitis lesions can harbor bacteria for very long beyond the initial tissue invasion (116). Studies using culture-dependent (117–121) or culture-independent molecular biology methods (26,122–125) have reported the extraradicular occurrence of a complex microbiota associated with apical periodontitis lesions that does not respond favorably to the root canal treatment. Apart from the discussion as to whether contamination can be effectively prevented during surgical sampling of apical periodontitis lesions, there are other issues that should be considered regarding studies of this nature. The frontline of intraradicular infection may sometimes be established at or slightly beyond the apical foramen/ina. When lesions are obtained by surgery, these bacteria may be displaced into the biopsy specimen. Except for the fluorescence *in situ* hybridization (FISH) approach, most of the methods used cannot distinguish those "contamination" or "displaced bacteria" cases from real tissue invasion. Moreover, these studies did not evaluate the bacteriological conditions of the apical part of the root canal, making it difficult to ascertain whether extraradicular infections were dependent on or independent of an intraradicular infection.

Symptoms are the result of a multitude of factors

A matter of great interest in endodontic microbiology is to find an explanation as to why only some infected teeth develop acute symptoms and complications. The desire to find a single or at least a group of major species that is associated with acute symptoms is an ever-recurrent topic in the field.

The cross-sectional nature of virtually all microbiological studies of acute endodontic infections in humans precludes strong conclusions about a cause-and-effect relationship between certain bacterial species and acute symptoms. Thus, only association can be inferred from these studies. Several bacterial species have been found to be very prevalent and associated with symptoms, but the very same species have also been found in asymptomatic cases, sometimes in the same prevalence (72,77,81,126–130).

Even though the etiology of acute infections is characterized by low specificity, certain species have been more frequently detected than others. These species may play a role in making the community more virulent (64,131). In addition to the presence of these species, a multitude of other factors can be regarded as influential to the development of acute infections (64), including (i) differences in the virulence ability amongst clonal types of the same species, (ii) bacterial interactions resulting in collective pathogenicity, (iii) overall and specific bacterial load, (iv) environment-regulated expression of virulence factors, and (v) host resistance and disease modifiers. Knowledge regarding the influence of all of these factors in the etiology of symptoms requires refinement by further research.

The issue of as-yet-uncultivated bacteria

The vast majority of microorganisms living in virtually all natural habitats cannot be cultivated under artificial conditions and this is mostly because their nutritional and physiological needs are unknown (132,133). Fortunately, advances in molecular microbiology technologies and techniques have allowed researchers to identify and phylogenetically classify several as-yet-uncultivated bacteria in diverse environments, including the human body (134).

As for primary endodontic infections, molecular studies have shown that as-yet-uncultivated bacteria comprise approximately 40%–60% of the detected

species-level taxa (50,67,70,73,135). A study (50) examined samples from primary infections and reported that uncultivated phylotypes accounted for approximately 55% of the taxa found (richness) and 38% of the clones sequenced (abundance). In pus aspirates from acute apical abscesses, as-yet-uncultivated phylotypes comprised 24%–46% of the taxa found (50,136), and 6%–30% of the clones sequenced (50,137). Uncultivated phylotypes found in endodontic infections belong to several genera, including *Dialister*, *Treponema,* *Prevotella*, *Solobacterium*, *Olsenella*, *Fusobacterium*, *Eubacterium*, *Megasphaera*, *Veillonella*, and *Selenomonas* as well as phylotypes related to the family *Lachnospiraceae* or the *Synergistetes* and TM7 phyla (50,69–71,80,100,138–140).

Herpesvirus and apical periodontitis

Studies published over the past decade have raised the possibility of herpesvirus participation in the pathogenesis of apical periodontitis, especially in symptomatic lesions (141,142). However, data related to the occurrence of herpesviruses in acute apical abscesses are rather inconclusive. Chen et al. (143) found herpersviruses in low prevalences and low copy numbers in abscess samples, and concluded that herpesviruses may be present but are not required for the development of abscesses of endodontic origin. Ferreira et al. (9) evaluated the presence of herpesviruses types 1 to 8 and human papillomavirus (HPV) in acute apical abscesses and reported that about 60% of the samples were positive for at least one target virus. The most prevalent was human herpesvirus (HHV)-8, followed by human papillomavirus, Varicella-Zoster virus, and HHV-6. Bacterial/viral co-infections are expected to occur and positive (while weak) associations between candidate endodontic bacterial pathogens and human viruses have been reported (144). Evidence of the herpesvirus infection has also been observed in large apical periodontitis lesions (142,145), and lesions from HIV-positive patients (5).

Although the association of herpesviruses with some forms of apical periodontitis has been suggested, a causative role remains to be proven. The possibility exists that the presence of viruses in the purulent exudate of abscesses or in the body of symptomatic lesions is merely a consequence of the inflammatory disease process induced by bacteria colonizing the apical root canal.

Geographical differences

Molecular studies comparing the endodontic microbiota of patients residing in different geographic locations have revealed significant differences in the prevalence of some candidate pathogens (146–148). Community-profiling analyses have confirmed that certain species are more prevalent in some locations and contributed still further by showing that bacterial community profiles may have a geography-related pattern. In other words, in spite of the interindividual varibility in the structure of bacterial communities, the endodontic microbiota of individuals residing in the same location are more similar among them when compared with individuals from distant locations (54,63). The implications of such differences in therapeutic terms are discussed later on in this article.

Looking into the future

Deeper look into diversity and genetic potential

Four generations of studies involving anaerobic culture and molecular microbiology techniques have provided a great contribution to the knowledge of microbial diversity in endodontic infections. Nonetheless, before we started to assume that the knowledge of "who is there" had been exhausted by those studies, high-throughput NGS technologies became available and their early application in endodontic microbiology revealed a much higher diversity than previously recognized. Some of these sequencing technologies include the 454 pyrosequencing (Roche Applied Science), Illumina/Solexa Genome Analyzer (Illumina), and SOLiD (Applied Biosystems). The former technology has been so far the most used NGS platform to study the human oral microbiome (149). One of the greatest advantages of the pyrosequencing approach over the conventional Sanger sequencing method is that hundreds of thousands of sequence reads can be obtained in a single run, generating sequence information data that are orders of magnitude larger (150). This means that the coverage of an individual

sample is much deeper than when using conventional sequencing techniques, increasing the chances of detecting low-abundance species.

Recent studies have investigated the diversity of the endodontic microbiome using the pyrosequencing approach. Analyses included samples from the canals of teeth with asymptomatic and symptomatic primary infections (52,58,151), the apical root canal of teeth with apical periodontitis (61,62), teeth with post-treatment disease (25), and extraradicular infections (152). In general, these studies have found a large bacterial richness, with numerous bacterial taxa, including representatives of phyla never previously reported to occur in the root canal. Also, the number of species-level taxa per case far exceeded what had been previously shown by culture and other molecular methods.

These next-generation sequencing technologies are also excellent for community analysis, permitting robust comparisons between bacterial communities from different sites, from the same site but in different periods of time, and before and after antimicrobial treatment. However, the accuracy of identification to the species level still requires refinement for most platforms. There is a great potential for these techniques to expand and further refine our knowledge regarding the species and communities associated with different clinical conditions.

Symptoms: an insight into bacterial associations and interactions

As discussed previously, evidence is growing that, similar to other polymicrobial infections in the body, the community is the unit of pathogenicity in apical periodontitis. Endodontic bacterial communities are composed of several different species that interact with each other to give rise to distinct individual features for each community. The community profiles associated with symptomatic infections are different from those occurring in asymptomatic cases (50,51,53), which has been confirmed by a pyrosequencing study (52). The fact that symptoms have not been strongly linked to any single pathogen does not preclude the possibility of some species being decisive in making the community more or less virulent. It remains to be clarified which species can increase the aggressiveness of the entire consortium. What they do and produce to cause acute infections should also be elucidated. In addition, there is a need to unravel the real role of herpesviral/bacterial interactions in the etiology of symptomatic diseases.

What are they doing there?

The two fundamental questions in any microbial ecology field are "who is there" and "what are they doing." "Who is there" has been answered by anaerobic culture and molecular microbiology methods, which have provided a great deal of information about the species composition in endodontic infections. Furthermore, this knowledge may still be expanded and refined by the fifth generation of studies using pyrosequencing technology. However, there is only very limited information on "what are they doing." This question refers to the role of the different species in the community, i.e. their physiological, functional, and pathogenic behavior.

The 16S rRNA gene has been widely used to identify bacteria in natural environments, but it has very little value in predicting physiology, function, and pathogenicity. Therefore, while the 16S rRNA gene often provides accurate identification, the other 99.95% of the bacterial genome provides the information for the vast array of metabolic, structural, and virulence abilities.

Metagenomics has been used to unravel the genetic potential of bacterial communities using an approach based on either gene expression or sequencing (153). Metagenomics treats the genomes of all microorganisms present in a specific habitat as an entity and provides valuable information on the genetic potential of the community. Metagenomics still remains to be used in endodontic microbiology research.

The physiology, function, and pathogenicity of a multispecies bacterial community can be mostly inferred by the substances produced and released by the community members. Methods such as metatranscriptomics (RNA), metaproteomics (proteins), and metabolomics (metabolites) can be used with the purpose of revealing these substances. In endodontic microbiology, only metaproteomic analyses have been performed so far (154,155). Nandakumar et al. (154) applied reverse-phase nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS) for the identification of bacterial

proteins in cases of primary or persistent infections and found bacterial proteins involved with adhesion, autolysins, proteases, virulence factors, conjugation, and antibiotic resistance. Provenzano et al. (155) evaluated the metaproteome of endodontic infections associated with acute apical abscesses and asymptomatic apical periodontitis lesions using two complementary mass spectrometry platforms (nanoflow liquid chromatography coupled with linear ion trap quadrupole Velos Orbitrap and liquid chromatography quadrupole time-of-flight). Human proteins associated with these infections were also identified. The number of proteins in abscesses was higher than in asymptomatic cases, which is possibly congruent with the higher species diversity in acute cases. The large majority of microbial proteins found in endodontic samples were related to metabolic and housekeeping processes, including protein synthesis, energy metabolism, and DNA processes. Moreover, several other proteins related to pathogenicity and resistance/survival were found, including proteins involved with adhesion, biofilm formation, and antibiotic resistance, as well as stress proteins, exotoxins, invasins, proteases, endopeptidases, and an archaeal protein linked to methane production. The majority of the human proteins detected were related to cellular processes and metabolism, as well as immune defense.

Future research applying metaproteomic, metatranscriptomic, and metabolomic analyses of clinical samples from endodontic infections as well as experimental multispecies infection models has the potential to bring an enormous contribution to our understanding of the pathogenesis of different forms of apical periodontitis. It is important that these analyses also include host products (155) in order to provide an insight into host–pathogen interactions. These "omic" approaches also have the potential to reveal biomarkers that can be used to improve therapy and predict prognosis.

Spatial distribution of the microbiota

Morphological studies using light (4,16,31,34), transmission electron (15,31,34), and scanning electron microscopy (32,33,156) have consistently demonstrated how bacteria are distributed in the root canal system in primary and persistent/secondary infections. They also showed that biofilm is the main form of bacterial organization in endodontic infections. Knowledge of the microbial location and organization within the root canal system assumes special importance in the understanding of the disease process and in the establishment of effective antimicrobial therapeutic strategies.

However, these studies are "broad-range" in nature in the sense that they only reveal morphology but not microbial identification. A technique that conciliates spatial distribution and identification and has a great potential to be used in endodontic microbiology is the fluorescence *in situ* hybridization (FISH) approach. FISH has been used to evaluate bacterial invasion of the pulp by caries bacteria (157) and to detect some specific species in extraradicular infections (124). Future research should focus on using FISH to evaluate the spatial distribution of some target species (including uncultivated phylotypes) in the root canal systems of teeth with primary or post-treatment apical periodontitis.

Bacterial interactions and ecological interference

How the bacterial species interact in the multispecies community is another possible target for future research. Different partnerships and associations between bacterial community members may influence the outcome of the disease. For instance, some species associations can result in a more virulent multispecies community, therefore giving rise to acute periradicular inflammation. A common form of bacterial association involves co-aggregation, which is an important phenomenon in polymicrobial infections; a few co-aggregating partners have been identified in the endodontic environment (158,159). Our knowledge of the species involved, as well as the nature and consequence of their associations, including co-aggregation partnerships, needs to be expanded. Other issues requiring attention include how the environment altered by disease influences the community virulence and how the clinician may interfere with the root canal ecology to enhance treatment results.

Actually, because sterilization of the root canal system is virtually impossible to obtain using contemporary instruments and irrigation systems, ecological interference may be an interesting approach to be explored in the future. Ecological interference

can be interpreted as any event that causes significant disturbances in the ecosystem, affecting communities and compromising their survival in some way. For instance, eradication of key elements in a community may lead to an ecological disaster for the entire community, which would ultimately result in the death of the remaining members (48). Examples of key members include species involved with cross-feeding and the acquisition of essential nutrients for less competent species, species that modify the environment and favor the establishment of others, and species producing protective enzymes that degrade antibiotics and host defense molecules.

Ecological interference is certainly much more complex and insightful than the current therapeutic paradigm, which stands essentially on attempts to eradicate the entire community using chemomechanical procedures and deny nutrient supply for persisting bacteria through sealing of the root canal space. Over the years, the main focus of research in applied endodontic microbiology has been to evaluate treatment protocols and techniques that promote total bacterial elimination or a perfect apical or coronal antibacterial seal. Even though a high success rate is expected for endodontic treatment following the existing paradigm under optimal clinical conditions (160–163), treatment outcomes for the general population are not so predictable and success rates decrease substantially to deplorable levels (164–170). In the large majority of cases, the low success rate is associated with inadequately treated teeth (170–172).

In the future, interference strategies focusing on the bacterial community ecology and virulence have the potential to serve as an interesting therapeutic alternative, especially if associated with less technically demanding clinical procedures.

Effectiveness of treatment and outcome predictors

The microbiological goals of endodontic treatment are to reduce bacterial counts to levels compatible with periradicular tissue healing, using chemomechanical preparation and intracanal medication, and keep these counts low by adequately filling the root canal system (173). There is a pathogenic threshold below which the host can cope with infection and healing can take place (174). However, given interindividual heterogeneities in bacterial community composition and virulence as well as in the host defense ability, the pathogenic threshold is expected to vary from case to case. Even so, it has been shown that root canal treatment approaches which predictably yield negative cultures offer an improved outcome in terms of the healing of apical periodontitis (17,88,175). Therefore, it is possible that the pathogenic threshold is somewhat related to the sensitivity of the culture technique. Because it usually takes one or more years to determine the outcome of endodontic treatment, culture results have also been considered as the surrogate endpoint for long-term treatment outcomes (174).

Nevertheless, limitations in culturing techniques, including low sensitivity and the inability to detect as-yet-uncultivated bacteria, put its validity as an outcome predictor into question. Quantitative open-ended molecular methods that are more sensitive than culture have the potential to establish more reliable standards to predict outcomes. An area of great interest for future research is to identify and quantify specific pathogens or molecular patterns present at the time of filling that somewhat affect the treatment outcome. Outcome predictors should be risk factors for post-treatment apical periodontitis and can be qualitative (types of species or virulence factors) or quantitative (total bacterial counts, or levels of specific species or virulence factors). This information would permit the establishment of tests to serve as a more accurate and reliable surrogate outcome.

Chairside tests and rapid antibiotic susceptibility tests

From the discussion above, it becomes apparent that an interesting area for future research is the development of rapid chairside tests to detect elements in the root canal that may predict treatment outcomes, which should include not only bacterial (species, virulence factors, or other markers) but also host-related factors (cytokines and other mediators of inflammation). As well, it would be useful to be able to detect risk factors for flare-ups. Challenges in the development of such tests include the need for more research to identify the risk factors, with results that are reproduced by different laboratories, and developing quick and affordable technologies.

A focus on host or bacterial products may be more productive than looking for bacterial species alone or in combinations. It is expected that the individual variability in species composition is higher than the types of products released, considering the possibility of functional redundancy in bacterial communities. As discussed above, host and bacterial products can be determined by global gene expression analyses (transcriptomics) or by comprehensive inventories of the released proteins (proteomics) and metabolites (metabolomics).

In cases where endodontic infections result in rapidly disseminating abscess/cellulitis with systemic involvement, systemic antibiotics are recommended and usually prescribed on an empirical basis. Highly sensitive molecular microbiology technologies now provide rapid and accurate bacterial identification in a matter of minutes to a few hours and these methods might be used for rapid microbiological diagnosis (64). Open-ended molecular approaches for the identification of bacteria in abscesses are still time-consuming and expensive but further technological advances have the potential to expedite bacterial identification and reduce costs. Alternatively, the detection of antibiotic resistance genes directly in abscess samples might guide the clinician's choice for drugs that have the potential to be more effective (176).

The role of as-yet-uncultivated bacteria in endodontic infections

Because many species associated with the human body are still uncultivated and have a potential clinical relevance, research efforts have been directed toward the development of specific approaches and culture media that allow cultivation of these bacteria (134). One of the most commonly used strategies to cultivate the so-called uncultivated bacteria relies on the application of conditions that are as close as possible to their natural environment (177).

Several species detected in endodontic infections that were recently referred to as uncultivated have been successfully cultivated, phenotypically characterized, and formally named. Curiously, some of these include bacteria that are relatively easy to cultivate, such as *Dialister invisus*, *Prevotella baroniae*, and *Peptostreptococcus stomatis.* However, other species, including *Fretibacterium fastidiosum* and *Pyramidobacter piscolens*, can be truly resistant to culture and require special strategies to grow (134).

One of the most prevalent as-yet-uncultivated phylotypes found in endodontic infections is *Bacteroidaceae* sp. HOT-272 (synonym, *Bacteroidetes* oral clone X083) (72,178). Given the high prevalence of this uncultivated phylotype in different forms of apical periodontitis, there is an urgent need to cultivate it so as to determine its pathogenic and antimicrobial resistance profiles.

Since more than one-half of the bacterial species found in infected root canals are uncultivated, it would be interesting to evaluate their susceptibility to treatment procedures and, in the case of persistence, to determine how these bacteria influence the outcome. In addition to evaluating the prevalence of selected as-yet-uncultivated and difficult-to-culture bacterial taxa in infected root canals, a recent molecular study addressed their susceptibility to chemomechanical procedures (93). *Bacteroidaceae* sp. HOT-272 and *Fretibacterium fastidiosum* were found in relatively high prevalence, but rarely as the dominant species. Chemomechanical procedures were highly effective in completely eliminating the target uncultivated taxa or at least substantially reducing their numbers in the large majority of cases. Further research should concentrate on evaluating the outcome of treatment in the cases where these and other as-yet-uncultivated bacteria endured the effects of treatment.

Translating biological knowledge into clinical solutions

Engineering has provided a tremendous breakthrough in technology, permitting the manufacture of endodontic instruments with new and improved alloys and instrument designs to prepare canals (179,180). However, in spite of these advances, there has been no tangible increase in the success rate of endodontic treatment. This is very likely to be related to the fact that technology has not been necessarily developed to deal with the advances in biological knowledge. Actually, endodontics as a healthcare specialty is related to biological issues and technology should be directed to provide tools for better diagnosis and treatment of these biological problems. In spite of the huge amount of scientific information about the etiology and pathogenesis of apical periodontitis generated over the past 4 decades, the translation of

this knowledge into better endodontic treatment outcomes has been subtle. The reason for this probably resides in the fact that the current treatment protocols have not been devised or even modified on the basis of this booming biological knowledge. Science has provided a lot of information on the nature of the problem, so the time has come for this knowledge to be used by scientists and clinicians to find a better way to treat patients. Actually, this is one of the foremost principles of basic sciences.

For instance, endodontic microbiology studies have provided a large amount of information about bacterial participation in primary and post-treatment disease. This includes the species involved, their virulence factors, interactions, ecology, organization and spatial distribution in the root canal system, patterns of antimicrobial resistance, and so on. These data have the potential to serve as the mainstay for the development of solutions to improve clinical practice.

Another example comes from studies on persistent endodontic infections, which are the main cause of endodontic treatment failure (174). These infections are caused by bacteria that resisted the effects of treatment and induced persistent periradicular inflammation. These bacteria are usually located in areas that are difficult for instruments and irrigants to access (181,182), and are often in direct contact with a source of nutrients from the periradicular tissues. Studies evaluating the cause of post-treatment disease in adequately treated teeth revealed bacteria persisting in the very apical part of the root canal (15,16), lateral canals (16,183,184), apical ramifications (15,16,114,185), isthmuses (16,34), and dentinal tubules (186,187). Intracanal medication may improve disinfection of these areas (182), but there is a need for developing clinical strategies to more effectively and predictably eliminate bacteria located in these difficult-to-reach areas, be it in single or multiple visits.

A great challenge for the endodontic specialty in the near future is to use the outstanding knowledge of the biological aspects of endodontic diseases to find the best way to treat them.

Geography and treatment effectiveness

The bacterial diversity associated with the same form of apical periodontitis significantly differs between individuals living in different geographical locations (54,63). This raises the inevitable question as to whether the same treatment protocols, especially systemic antibiotic therapy, are similarly effective in treating the same infection in different locations. In addition, the fact that geographical differences in the antibiotic susceptibility profiles of oral bacterial isolates have been reported (188) adds still more complexity to this issue. Future studies should further elaborate on the patterns related to geography and establish whether treatment should be customized for certain regions.

Systemic effects of endodontic infections

Whereas there is no solid scientific evidence so far indicating that an infected root canal may act as a focus of infection to distant body sites (except for systemically compromised patients), the opposite has not been proven either, i.e. there is no clear evidence showing that endodontic infections are a segregate event with no effect on the rest of the body (173). Actually, research over the past decade has suggested that apical periodontitis, especially as part of the total oral bacterial infectious burden along with caries and periodontal diseases, may have some systemic consequences for the host (189–195). The systemic involvement of endodontic bacteria as part of the total oral infectious burden or through bacteremia following treatment or acute disease remains to be investigated in light of current scientific concepts and technology. This is an important area for future research.

Concluding remarks

There have been great advancements in the field of endodontic microbiology over the past 4 decades. In spite of the large amount of refined information about endodontic infections and the pathogenesis of apical periodontitis, two points still need special attention and urgent upgrading: (i) treatment is still based on attempts to indiscriminately eliminate all of the bacteria in the root canal; and (ii) therapeutic procedures have their effectiveness restricted mostly to the main root canal. How the information about endodontic infections made available over the years can affect these points remains a challenge for the specialty. What is certain is that the time has come for

Table 1: Questions to be answered and suggestions of avenues for future research in endodontic microbiology

Issue	Questions/future research
Biofilm-related questions	• how bacterial interactions may influence the community's overall virulence and resistance to treatment • the extracellular matrix as a potential target for anti-biofilm treatment • how the different species are spatially distributed in the biofilm
Diversity of the endodontic microbiota	• refinement of the knowledge regarding the species involved and associated with different clinical conditions such as flare-ups, persistent exudation, and persistent symptoms
As-yet-uncultivated bacteria	• their role in the disease process and susceptibility to treatment
Post-treatment disease	• identification of the species involved, preferably associated with information on spatial distribution in the root canal system
Antibacterial treatment	• development of ecological interference approaches • development of strategies to reach infection in the entire root canal system, including ramifications, isthmuses, and dentinal tubules • development of less technically-demanding treatment procedures, while still being effective and affordable
Chairside and rapid susceptibility tests	• development of tests to predict treatment outcome based on markers such as type of bacterial species, virulence factors, or host molecules • how rapid antibiotic susceptibility tests may influence the treatment of spreading acute abscess/cellulitis
Extraradicular infections	• establish whether or not an extraradicular infection can be independent of the intraradicular infection • if independent extraradicular infections are proven to exist, how they can be clinically diagnosed
Herpesvirus infection	• establish whether or not herpesvirus infection influences the development and severity of apical periodontitis
Geographical differences in the microbiota	• establish proper antimicrobial protocols based on geographic differences, especially for specific antibacterial treatment such as antibiotic therapy in abscess cases
Symptomatic infections	• decipher the many possible factors involved with the development of symptoms (types of species, clonal types, host-predisposing factors, virulence factors, bacterial interactions, role of herpesviruses, etc.)
Physiology and pathogenicity	• reveal the products released in the bacterial community (proteins, RNA, and metabolites) and how they affect the community's physiology and the host–pathogen relationship • investigate the genetic potential of the bacterial community (metagenomics)
Systemic implications	• how primary and post-treatment apical periodontitis can influence the individual's overall health
Translational endodontics	• define how the vast biological information can permit therapeutic improvements

most of this knowledge to be translated into improvements in clinical practice and treatment outcomes.

Many issues related to the basic and applied science of endodontic microbiology still remain to be clarified (Table 1). Advances in molecular microbiology technologies and bioinformatics have the potential to answer many important questions and generate many others. Because the diseases that endodontists treat or prevent on a daily basis are of infectious origin, improvements in treatment outcomes will necessarily depend upon the practical application of the microbiological background and acquisition of more information in this area. That will certainly require a substantial increment in the field of applied endodontic microbiology. Thus, there is an urgent need for the spread of state-of-the-art technology usage and recruitment of qualified manpower to the field. There is so much to look for and learn, but so few engaged . . .

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