Exploiting Molecular Methods to Explore Endodontic Infections: Part 1—Current Molecular Technologies for Microbiological Diagnosis

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Abstract

Endodontic infections have been traditionally studied by culture-dependent methods. However, as with other areas of clinical microbiology, culture-based investigations are plagued by significant problems, including the probable involvement of viable but uncultivable microorganisms with disease causation and inaccurate microbial identification. Innumerous molecular technologies have been used for microbiological diagnosis in clinical microbiology, but only recently some of these techniques have been applied in endodontic microbiology research. This paper intended to review the main molecular methods that have been used or have the potential to be used in the study of endodontic infections. Moreover, advantages and limitations of current molecular techniques when compared to conventional methods for microbial identification are also discussed.

Key Words

Apical periodontitis, endodontic microbiology, molecular technology, microbiological diagnosis

In essence, endodontic infection is the infection of the root canal of the tooth and is the primary etiologic agent of the different forms of periradicular inflammatory diseases (1, 2). The pathological process is started when the dental pulp becomes necrotic, usually as a sequel to caries, and then infected by micro-organisms that are usually normal inhabitants of the oral cavity. The root canal containing a necrotic pulp affords micro-organisms a moist, warm, nutritious, and anaerobic environment, which is, by and large, protected from host defenses. Such conditions are highly conducive to microbial colonization and multiplication. After the endodontic infection is established, micro-organisms enter in close contact with the periradicular tissues via the apical foramen and occasional foraminas, inflict damage to these tissues, and give rise to inflammatory changes. Periradicular inflammatory diseases are arguably among the most common diseases that affect human beings (3, 4).

Traditionally, endodontic bacteria have been studied by means of cultivation-based techniques, which rely on isolation, growth, and laboratory identification by morphology and biochemical tests. However, cultivation and other traditional identification methods have been demonstrated to have several limitations when it comes to microbiological diagnosis (5). The past decade has brought many advances in microbial diagnostics, the most prolific being in DNA-DNA hybridization as well as in polymerase chain reaction (PCR) technology and its derivatives. Indeed, findings from cultivation-based methods with regard to the microbiota living in diverse ecosystems have been supplemented and significantly expanded with molecular biology techniques, and the impact of these methods on the knowledge about the oral microbiota in healthy and diseased conditions is astonishing and breathtaking.

The first part of this paper describes the state of the art of molecular methodologies already used or with potential to be applied to the study of the microbiota associated with endodontic infections. The significant contribution brought about by molecular technology and its impact on the redefinition of endodontic infections are discussed in the second part of this paper.

Traditional Identification Methods

Culture

For more than a century, cultivation using artificial growth media has been the standard diagnostic test in infectious diseases. The microbiota associated with different sites in the human body has been extensively and frequently scrutinized by studies using cultivation approaches. The success in cultivation of important pathogenic bacteria probably led microbiologists to feel satisfied with and optimistic about their results and to recognize that there is no dearth of known pathogens (6, 7). But should we be so complacent with what we know about human pathogens?

Making micro-organisms grow under laboratory conditions presupposes some knowledge of their growth requirements. Nevertheless, very little is known about the specific growth factors that are utilized by innumerous micro-organisms to survive in virtually all habitats, including within the human body (8). A huge proportion of the microbial species in nature are difficult to be tamed in the laboratory. Certain bacteria are fastidious or even impossible to cultivate. Some well-known human pathogens, such as Mycobacterium leprae and Treponema pallidum continue to defy scientists regarding their cultivation under laboratory conditions (9).
Of 36 bacterial divisions noted by Hugenholtz et al. (10), 13 divisions are exclusively comprised of as-yet uncultivated bacteria. Updated analyses have indicated that presently 52 phyla can be discerned, of which 26 are candidate phyla, that is, they are uncultivable and known only by gene sequences (11). In fact, there has been a pronounced bias towards the study of representatives of four bacterial phyla, namely Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, out of 52 bacterial phyla (12). This bias is arguably related to the fact that representatives of these phyla are easier to cultivate with the methods available nowadays. It is glaringly obvious that there is an urgent need for this bias to be rectified.

Infectious disease professionals have long been aware of the fact that when cultivation is applied to diagnose the causative agents of diseases of suspected microbial etiology, including pneumonias, encephalitis, meningitis, pericarditis, acute diarrhea, and sepsis, a large number of cases can not be explained microbiologically (13, 14). Moreover, the etiology of a number of chronic inflammatory diseases with features suggestive of infection, such as rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis, and diabetes mellitus, remain obscure (15). Taking into consideration that known bacterial pathogens fall within 7 out of the 52 candidate bacterial divisions and that cultivation-independent approaches have shown that 40 to 75% of the human microbiota in different sites are composed of as-yet uncultivated bacteria (16–18), it is fair to realize that there can be many pathogens which remain to be identified. Therefore, it is of concern that clinical microbiology continues to rely on cultivation-based identification procedures (6).

Advantages and Limitations

The main advantages of cultivation approaches are related to their broad-range nature, which makes it possible to identify a great variety of microbial species in a sample, including those that are not being sought after. Still, cultivation makes it possible to determine antimicrobial susceptibilities of the isolates and to study their physiology and pathogenicity.

However, cultivation-based identification approaches have several limitations: they are costly; they can take several days to weeks to identify some fastidious anaerobic bacteria (that can delay antimicrobial treatment); they have a very low sensitivity (particularly for fastidious anaerobic bacteria); their specificity may be also low and is dependent on the experience of the microbiologist; they have strict dependence on the mode of sample transport; they are time-consuming and laborious. Finally, the impossibility of cultivating a large number of bacterial species as well as the difficulties in identifying many cultivable species represent the major drawbacks of cultivation-based approaches.

Difficulties in Cultivation

There are many possible reasons for bacterial unculturability (7, 19, 20) (Table 1). Obviously, if micro-organisms can not be cultivated, they can not be identified by phenotype-based methods. While we stay relatively ignorant on the requirements of many bacteria to grow, identification methods that are not based on bacterial culturability are required. This would avoid that many pathogens pass unnoticed when one is microbiologically surveying clinical samples.

It is worth pointing out that the fact that a given species is uncultivable does not necessarily imply that the same species will remain indefinitely impossible to cultivate. For instance, a myriad of strict anaerobic bacteria were uncultivable 100 yr ago, but further developments in cultivation techniques have to a large extent helped solve this problem. There is a growing trend to develop approaches and culture media that allow cultivation of previously uncultivated bacteria. Strategies may rely on application of cultivation procedures that better mimic conditions existing in the natural habitat from which the samples were obtained. Recent efforts to accomplish this have met with some success by including the following: the use of agar media with little or no added nutrients; relatively lengthy periods of incubation (more than 30 days); and inclusion of substances that are typical of the natural environment in the artificial growth media (21, 22). However, it is not unreasonable to surmise that a huge number of species will remain uncultivable for years to come.

Difficulties in Identification

Accurate identification of microbial isolates is paramount in clinical microbiology. For a given microbial species to be identified by means of their phenotypic features, this species has to be cultivated. However, one should be mindful that in some circumstances even the successful cultivation of a given microorganism does not necessarily mean that this micro-organism can be successfully identified.

For slow-growing and fastidious bacteria, traditional phenotypic identification is difficult and time-consuming. In addition, interpretation of phenotypic test results can involve a substantial amount of subjective judgment and personnel’s expertise. Still, one major difficulty associated with microbial identification based on phenotypic features is that of divergence and convergence. Divergence occurs for strains of the same species, which are genetically similar, but have evolved to be different phenotypically. Convergence occurs for strains of different species, which are genetically different, but have evolved to have similar phenotypic behavior (23). In both situations, phenotypically based diagnostic tests would result in misidentification.

16S rRNA gene (rDNA) sequencing approach was first proposed to identify uncultivable bacteria (see section on Broad-Range PCR in this article), but it has also been recently used for identification of cultivable bacteria that shows atypical phenotypic behavior and cannot be accurately identified by culture. Unlike phenotypic identification, which can be modified by the variability of expression of characters, 16S rDNA sequencing provides unambiguous data even for rare isolates. Bosshard et al. (24) reported that 14% of all aerobic gram-positive rods isolated in their clinical microbiology laboratory required 16S rDNA sequence-based identification. Drancourt et al. (25) reported that about 0.5 to 1% of the bacterial isolates recovered in their laboratory were not possible to be identified by phenotypic criteria. Using 16S rDNA sequence analysis, they reported that several atypical isolates corresponded to 27 new
bacterial species associated with human diseases, of which 11 isolates were previously uncharacterized species and 16 isolates had never been reported in humans, being previously regarded as environmental. Dran-court et al. (26) evaluated the utility of 16S rDNA sequencing as a means to identify a collection of 177 isolates obtained from environmental, veterinary, and clinical sources. Conventional identification failed to produce accurate results because of inappropriate biochemical profile determination in 76 isolates (58.7%), Gram staining in 16 isolates (11.6%), oxidase and catalase activity determination in five isolates (3.6%) and growth requirement determination in two isolates (1.5%). They reported that the overall performance of 16S rDNA sequence analysis was excellent, because it succeeded in identifying 90% of phenotypically unidentifiable bacterial isolates. Song et al. (27) evaluated the utility of 16S rDNA sequencing as a means of identifying clinically important gram-positive anaerobic cocci. Based on the sequences of the 13 type strains obtained in their study, 84% (131 of 156) of the clinical isolates were accurately identified to species level, with the remaining 25 clinical strains revealing nine unique sequences that could represent eight novel species. This finding was in contrast to the phenotypic identification results, by which only 56% of isolates were correctly identified to species level. These studies clearly showed that sequence analysis of the 16S rDNA can be used to overcome difficulties in identifying cultivable species that are notoriously refractory to identification by phenotypic means.

Microscopy

Microscopy may suggest an etiologic agent, but it rarely provides definitive evidence of infection by a particular species. Microscopic findings regarding bacterial morphology may be misleading, because many species can be pleomorphic and conclusions can be influenced by subjective interpretation of the investigator. In addition, microscopy has limited sensitivity and specificity to detect microorganisms in clinical samples. Limited sensitivity is because a relatively large number of microbial cells are required before they are seen under microscopy (e.g. 10^4 bacterial cells/ml of fluid) (9). Some microorganisms can even require appropriate stains and/or approaches to become visible. Limited specificity is because our inability to speciate microorganisms based on their morphology and staining patterns.

Immunological Methods

Immunological methods are based on the specificity of antigen-antibody reaction. They can detect microorganisms directly or indirectly, the latter by detecting host immunoglobulins specific to the target microorganism. The enzyme-linked immunosorbent assay (ELISA) and the direct or indirect immunofluorescence tests are the most commonly used immunological methods for microbial identification. Advantages of immunological methods for identification of microorganisms include: (a) they take no more than a few hours to identify a microorganism species; (b) they can detect dead microorganisms; (c) they can be easily standardized; and (d) they have low cost (28). However, they have also important limitations as they can detect only target species, they have low sensitivity (about 10^4 cells), their specificity is variable and depends on types of antibodies used, and they can detect dead microorganisms (28, 29).

Molecular Genetic Methods

Investigations of many aquatic and terrestrial environments using cultivation-independent methods have revealed a great deal of previously unsuspected microbial diversity (30). In fact, the cultivable members of these living systems represent less than 1% of the total extant population (31, 32). Novel culture-independent methods for microbial identification that involve DNA amplification of 16S rDNA followed by cloning and sequencing have recently been used to determine the bacterial diversity within diverse environments, such as deep-sea sediments (33), hot springs (34), and human diseased and healthy sites (16–18, 35–39). Perhaps not surprisingly, the number of recognized bacterial phyla has exploded from the original estimate of 11 in 1987 to 36 in 1998 (10). The latest tally of bacterial phyla is now probably near 52, of which one-half has only uncultivable representatives (11).

A significant contribution of molecular methods to medical microbiology relates to the identification of previously unknown human pathogens (40–44). In addition, molecular studies have revealed that about 40 to 50% of the bacterial clones in the oral cavity (17) and about 70% of clones in the gut and colon (16, 35) represent unknown and as-yet uncultivable species. As a consequence, it is fair to realize that there can exist a number of uncharacterized pathogens in this uncultivable proportion of the human microbiota.

Molecular diagnostic methods have several advantages over other methods with regard to microbial identification (Table 2). Limitations of molecular approaches will be discussed further ahead in this paper.

There are a plethora of molecular methods for the study of microorganisms and the choice of a particular approach depends on the questions being addressed (Fig. 1). Broad-range polymerase chain reaction (PCR) followed by sequencing can be used to investigate the microbial diversity in a given environment. Microbial community structure can be analyzed via fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). Fluorescence in situ hybridization (FISH) can measure abundance of particular species and provide information on their spatial distribution in tissues. Among other applications, DNA-DNA hybridization macroarrays and microarrays, species-specific PCR, nested PCR, multiplex PCR, and real-time PCR can be used to survey a large number of clinical samples for the presence of target.
species. Variations in PCR technology can also be used to type microbial strains. This review will restrict discussion to the most commonly used approaches applied to the research of the endodontic microbiota and some with potential to be used with this intent.

**Gene Targets for Microbial Identification**

Molecular approaches for microbial identification rely on the fact that certain genes contain revealing information about the microbial identity. Ideally, a gene to be used as target for microbial identification should contain regions that are unique to each species. Following the pioneer studies by Woese (45), the genes encoding rRNA molecules, which are present in all cellular forms of life, namely, the domains *Bacteria*, *Archaea*, and *Eucarya*, have been widely used for comprehensive identification of virtually all living organisms and inference of their natural relationships.

Ribosomes are intracellular particles composed of rRNA and proteins. In *Escherichia coli*, about two-thirds of the ribosome is rRNA and the remainder is protein (46). The sizes of ribosomes are given in Svedburg (S) units, which represent a measure of how rapidly particles or molecules sediment in an ultracentrifuge. Bacterial and archaeal cells have 70S ribosomes composed of 30S and 50S subunits. The 30S subunit contains a 16S rRNA molecule, having approximately 1540 nucleotides. The 50S subunit contains a 23S rRNA molecule, having approximately 2900 nucleotides, and a small 5S rRNA molecule having only about 120 nucleotides. Fungi have 80S ribosomes composed of 40S and 60S subunits. The 40S subunit contains 18S rRNA and the larger 60S subunit has 25S rRNA and 5.8S rRNA (47).

Large subunit genes (23S and 25S rDNA) and small subunit genes (16S and 18S rRNA) have been widely used for microbial identification, characterization and classification. The small subunit rDNA is among the most evolutionary conserved macromolecules in all living systems (45). The advantages of using small subunit rDNA is that it is found in all organisms, is long enough to be highly informative and short enough to be easily sequenced, particularly with the advent of automated DNA sequencers (45). The small subunit rDNA contains some regions that are virtually identical in all representative of a given domain (conserved regions) and other regions that vary in sequence from one species to another (variable regions) (Fig. 2). Variable regions contain the most information about the genus and species of the bacterium, with unique signatures that allow specific identification. The 16S rRNA of bacteria and archaea and the 18S rRNA of fungi and other eukaryotes have been extensively examined and sequenced and have been used to determine phylogenetic relationships among living organisms. In addition, data from rDNA sequences can also be used for accurate and rapid identification of known bacterial species, using techniques that do not require microbial cultivation.
PCR

The PCR process was conceived by Kary Mullis in 1983 and ever since has revolutionized the field of molecular biology by being able to amplify as few as one copy of a gene into millions to billions of copies of that gene (52). The impact of PCR on biological and medical research has been remarkable, dramatically speeding the rate of progress of the study of genes and genomes. Nowadays, it is possible to isolate essentially any gene from any organism using PCR, which made this technique a cornerstone of genome sequencing projects (53). Since its introduction, PCR has spawned an increasing number of associated technologies for diverse applications. Perhaps the most widespread advance in clinical diagnostic technology has come from the application of PCR for detection of microbial pathogens (54–56).

The PCR method is based on the in vitro replication of DNA through repetitive cycles of denaturation, primer annealing and extension steps (Fig. 3). The target DNA serving as template melts at temperatures high enough to break the hydrogen bonds holding the strands together, thus liberating single strands of DNA. Two short oligonucleotides (primers) are annealed to complementary sequences on opposite strands of the target DNA. Primers are selected to encompass the desired genetic sequence but are not adequate to establish phylogenetic relationships (51).

Numerous derivatives in PCR technology have been developed since its inception. The most used PCR-derived assays are described in the following sections.

Nested PCR

Nested PCR uses the product of a primary PCR amplification as template in a second PCR reaction and was devised mainly to have increased sensitivity (58). The first PCR products are subjected to a second round of PCR amplification with a separate primer set, which anneals internally to the first products. This approach shows increased sensitivity allowing the detection of the target DNA several folds lower than conventional PCR. Increased sensitivity is because of the large total number of cycles. In addition, target DNA is amplified in the first round of amplification, with subsequent dilution of other DNA and inhibitors present in the sample. The set of primers used in the second round of PCR results in additional specificity. The second reaction is performed with reduced background of eukaryotic DNA and other regions of the bacterial DNA (57). Even if nonspecific DNA amplification occurs in the first round of amplification, the nonspecific PCR product does not serve as template in the second reaction, since it is highly unlikely to possess regions of DNA complementary to the second set of specific primers (59).

The major drawback of nested-PCR protocol is the high probability of contamination during transfer of the first-round amplification products to a second reaction tube and special precautions should be taken to avoid this.

Reverse Transcriptase PCR (RT-PCR)

RT-PCR was developed to amplify RNA targets and exploits the use of the enzyme reverse transcriptase, which can synthesize a strand of complementary DNA (cDNA) from an RNA template. Most RT-PCR assays employ a two-step approach. In the first step, reverse transcriptase converts RNA into single-stranded cDNA. In the second step, PCR primers, DNA polymerase, and nucleotides are added to create the second strand of cDNA. Once the double-stranded DNA template is formed, it can be used as template for amplification as in conventional PCR (60). The RT-PCR process may be modified into a one-step approach by using it directly with RNA as the template. In this approach, an enzyme with both reverse transcriptase and DNA polymerase activities is used, such as that from the bacteria *Thermus thermophilus*.

Multiplex PCR

Most PCR assays have concentrated on the detection of a single microbial species by means of individual reactions. In multiplex PCR, two or more sets of primers specific for different targets are introduced in the same reaction tube. Since more than one unique target sequence are loaded into the gel and an electrical gradient is applied through a buffer solution. The products migrate through the gel according to size, with larger products running a shorter distance in the gel because they experience more resistance in the gel matrix. DNA ladder digests represent DNA fragments of known size and are run in the same gel to serve as molecular size standard. This allows the size of the PCR products to be estimated. The gel is usually visualized using ethidium bromide staining and ultraviolet transillumination. Designed primers are expected to generate a PCR product of a given size and the observation of a PCR amplicon of the predicted size in the electrophoretic gel is consistent with a positive PCR result. Identity of PCR products can still be confirmed by sequencing of the PCR product, hybridization of a specific oligonucleotide probe to a region of the PCR product that is internal to the priming sites, or restriction enzyme cleavage of the PCR product using an enzyme that is known to cut a specific sequence within the product [restriction fragment length polymorphism (RFLP)].
in a clinical specimen can be amplified at the same time, multiplex PCR assays permit the simultaneous detection of different microbial species. Multiplex PCR assays have been used to minimize the time and expenditure needed for detection approaches. Primers used in multiplex assays must be designed carefully to have similar annealing temperatures and to lack complementarity (61, 62).

PCR-Based Microbial Typing

PCR technology can also be used for clonal analysis of microorganisms. An example of the PCR techniques used for this purpose includes the arbitrarily primed PCR (AP-PCR), also referred to as random amplified polymorphic DNA (RAPD) (63). AP-PCR is a relatively rapid PCR-based genomic fingerprinting technique that can be used as a tool to determine whether two isolates of the same species are epidemiologically related. The method utilizes a single 10- to 20-base random sequence primer that anneals to unspecified DNA target sites under conditions that allow for mismatched base-pairing. The use of a random-sequence primer at low stringency allows for priming at sites with imperfect matches. Genetic variations between two DNA templates result in discriminative DNA fingerprints because of the differences in the priming sites. The amplicons generated form a pattern in the electrophoretic gel that may be strain specific. The advantage of AP-PCR is its ability to furnish highly specific DNA profiles with no prerequisite for knowing the DNA sequences. Primers may also be designed to target known genetic elements, such as enterobacterial repetitive intergenic consensus sequences (ERIC-PCR) (64, 65) and repetitive extragenic palindromic sequences (REP-PCR) (66). Clonal analysis may help elucidate whether certain strains of a given species are more associated with signs or symptoms of a given disease. Clonal analysis also may help to track the origin of micro-organisms infecting a given site. For instance, by comparing bacterial strains isolated from the root canal and other oral sites, one can have information as to where micro-organisms present in the root canal system came from. Clonal analysis can also help to track the origin of the micro-organisms present in a suspected focal disease by comparing the isolates found in the infected site with others present in the suspected focus of infection, including the oral cavity (and infected root canals).

Real-Time PCR

Conventional PCR assays are qualitative or can be adjusted to be semi-quantitative. One exception is the real-time PCR, which is characterized by the continuous measurement of amplification products throughout the reaction (67).

There are several different real-time PCR approaches. The three general real-time PCR chemistries for amplifying and detecting DNA targets are SYBR-Green, TaqMan, and molecular beacon (68, 69). SYBR Green is the simplest and most affordable method, and consists of a fluorescent dye that binds to double-stranded DNA. During extension, increasing amounts of dye bind to the increasing amount of newly formed double-stranded DNA. Fluorescence is measured at the end of the extension step of every PCR cycle to monitor the increasing amount of amplified DNA. Dye that remains unbound exhibits little fluorescence in solution. The SYBR-Green assay is very sensitive but has diminished specificity, as the dye binds to all double-stranded DNA present, and primer dimers can result in a false reading (70).

The TaqMan method can be more specific than the SYBR-Green assay. Increased specificity of the TaqMan assay results from the utilization of a specific labeled oligonucleotide probe along with the primers (71, 72). The TaqMan probe is a 20- to 30-base-long oligonucleotide sequence that specifically anneals to a sequence flanked by the two primers. The TaqMan probe contains a reporter fluorescent dye at the 5′ end and a quencher dye at the 3′ end that quenches the emission spectrum of the reporter dye (Fig. 4). As long as the probe remains unbound, it is intact and no signal is generated. During the extension step of real-time PCR, the Taq DNA polymerase enzyme cleaves the TaqMan probe, resulting in separation of the reporter from the quencher (Fig. 4). This results in increased fluorescence emission.

Another real-time PCR assay uses molecular beacons, which are single-stranded nucleic acid molecules with a stem-and-loop structure (69). The loop portion is complementary to a sequence in the target

Figure 3. Scheme for PCR.
DNA. The stem is formed by the annealing of complementary arm sequences located on either side of the probe. A fluorescent marker is attached to the end of one arm, and a quencher is attached to the end of the other arm. Free molecular beacons acquire a hairpin structure when in solution, and the stem keeps the arm sequences in close proximity. This results in efficient quenching of the fluorescent dye (Fig. 4). When hybridizing to their complementary target, molecular beacons are forced to undergo a conformational change, with consequent formation of probe that hybridizes to the template. The conformational change forces the fluorescent dye and the quencher apart, generating fluorescence (73) (Fig. 4).

By monitoring the release of fluorescence with each PCR cycle, the progress of the reaction is recorded in real time and the amount of DNA in the sample is quantified. Thus, accumulation of PCR products is measured automatically during each cycle in a closed tube format using a thermocycler combined with a fluorimeter. Direct measurement of the accumulated PCR product allows the phases of the reaction to be monitored. The initial amount of target DNA in the reaction can be related to a cycle threshold defined as the cycle number at which there is a statistically significant increase in fluorescence. Target DNA can then be quantified by construction of a calibration curve that relates cycle threshold to known amounts of template DNA (74).

Real-Time PCR technology is commercially available as the TaqMan (Applied Biosystems, Perkin-Elmer Corp.) and LightCycler (Roche Diagnostics Corp.) systems. Real-time PCR assays allow the quantification of individual target species as well as total bacteria in clinical samples. The advantages of real-time PCR are the rapidity of the assay (30–40 min), the ability to quantify and identify PCR products directly without the use of agarose gels, and the fact that contamination of the nucleic acids can be limited because of avoidance of postamplification manipulation (75).

**Broad-Range PCR**

PCR technology can also be used to investigate the whole microbial diversity in a given environment. In broad-range PCR, primers are designed that are complementary to conserved regions of a particular gene that are shared by a group of micro-organisms. For instance, primers that are complementary to conserved regions of the 16S rDNA have been used with the intention of exploiting the variable internal regions of the amplified sequence for sequencing and further identification (76). The strength of broad-range PCR lies in the relative absence of selectivity, so that (in principle) any kind of bacteria present in a sample can be detected and identified. This aspect is in analogy to cultivation and in contrast to species-specific molecular approaches (48). Thus, broad-range PCR can detect the unexpected and in this regard it is far more effective and accurate than culture. Broad-range PCR has allowed the identification of several novel, fastidious or un cultivable bacterial pathogens directly from diverse human sites (5, 7, 77).

Initially, bulk nucleic acids are extracted directly from samples. Afterwards, the 16S rDNA is isolated from the bulk DNA via PCR with oligonucleotide primers specific for conserved regions of the gene (universa or broad-range primers). Longer distances between primer pairs generally result in less sensitivity, but this provides more variable sequence for more accurate identification and may reduce the risks of amplifying DNA from contaminants in PCR reagents, which appear to be fragmented into smaller sizes (48). Amplification with universal primers results in a mixture of all of the 16S rDNA amplified from the bacteria in the sample. In mixed infections, direct sequencing of the PCR products cannot be performed because there are mixed products from the different species composing the consortium. PCR products are then cloned into a plasmid vector, which is used to transform Escherichia coli cells, establishing a library of 16S rDNA from the sample.
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genes are then sequenced individually and submitted for identification to databases, usually via the World Wide Web. Preliminary identification can be done by using similarity searches in public databases. A >99% identity in 16S rDNA sequence has been the most accepted criterion used to identify a bacterium to the species level (25, 26). A 97 to 99% identity in 16S rDNA sequence has been the criterion used to define a potentially new bacterial species (25, 26). In addition to the similarity search, phylogenetic analysis should be accomplished since it provides a much more accurate assessment (48, 51).

The analytical sensitivity of most broad-range PCR assays is in practice above 10, if not 100, gene copies per PCR, which is significantly lower when compared to most species-specific PCR assays (48). Because broad-range primers are used, there is a high risk for DNA from microbial contaminants to be amplified. A wide range of precautions is necessary to avoid contamination, including separate room for pre- and post-PCR work, UV decontamination of surface areas, use of high-quality reagents, and adequate sampling techniques and vials for clinical specimens (48, 59, 78).

Denaturing Gradient Gel Electrophoresis

Techniques for genetic fingerprinting of microbial communities can be used to determine the diversity of different micro-organisms living in diverse ecosystems and to monitor microbial community behavior over time. A commonly used strategy for genetic fingerprinting of complex microbial communities encompasses the extraction of DNA, the amplification of the 16S rDNA using broad-range primers, and then the analysis of PCR products by denaturing gradient gel electrophoresis (DGGE).

In DGGE, DNA fragments of the same length but with different base-pair sequences can be separated (79, 80). The DGGE technique is based on electrophoresis of PCR-amplified 16S rDNA (or other genes) fragments in polyacrylamide gels containing a linearly increasing gradient of DNA denaturants (a mixture of urea and formamide). As the PCR product migrates in the gel, it encounters increasing concentrations of denaturants and, at some position in the gel, it will become partially or fully denatured. Partial denaturation causes a significant decrease in the electrophoretic mobility of the DNA molecule. Molecules with different sequences may have a different melting behavior and will therefore stop migrating at different positions in the gel. The position in the gel at which the DNA melts is determined by its nucleotide sequence and composition (81). A GC-rich sequence (GC-clamp) is added to the 5’-end of one of the PCR primers, which ensures that at least part of the DNA remains double stranded (82). DNA bands in DGGE can be visualized using ethidium bromide, SYBR Green I, or silver staining.

In DGGE, multiple samples can be analyzed concurrently, making it possible to compare the structure of the microbial community of different samples and to follow changes in microbial populations over time, including after antimicrobial treatment. Specific bands can also be excised from the gels, re-amplified and sequenced to allow microbial identification. The DGGE method and its application in endodontic microbiology research have been recently reviewed (83). Temperature gradient gel electrophoresis (TGGE) uses the same principle as DGGE, except for the fact that the gradient is temperature rather than chemical denaturants.

Terminal-RFLP

T-RFLP is a recent molecular approach that can assess subtle genetic differences between microbial strains as well as provide insight into the structure and function of microbial communities (84). T-RFLP analysis measures the size polymorphism of terminal restriction fragments from a PCR amplified marker. T-RFLP is a modification of the conventional RFLP approach. In T-RFLP, rDNA from different species in a community is PCR-amplified using one of the PCR primers labeled with a fluorescent dye, such as 4,7,2’,7’-tetra-chloro-6-carboxyfluorescein (TET) or phosphoramidite fluorochrome 5-carboxyfluorescein (6-FAM) (85). PCR products are then digested with restriction enzymes, generating different fragment lengths. Digestion of PCR products with judiciously selected restriction endonucleases produces terminal fragments appropriate for sizing on high resolution sequencing gels. The latter step is performed on automated systems such as the ABI gel or capillary electrophoresis systems that provide digital output (85).

The use of a fluorescently labeled primer limits the analysis to only the terminal fragments of the enzymatic digestion. This simplifies the banding pattern, thus allowing the analysis of complex communities as well as providing information on diversity as each visible band represents a single species. All terminal fragment sizes generated from digestion of a PCR product pool can be compared with the terminal fragments derived from sequence databases to derive phylogenetic inference. Through application of automated DNA sequencer technology, T-RFLP has considerably greater resolution than gel-based community profiling techniques, such as DGGE/TGGE (84, 85).

DNA-DNA Hybridization

DNA-DNA hybridization methodology employs DNA probes, which entail segments of single-stranded DNA, labeled with an enzyme, radioactive isotope or a chemiluminescence reporter, that can locate and bind to their complementary nucleic acid sequences. DNA-DNA hybridization arrays on macroscopic matrices, such as nylon membranes, have been often referred to as “macroarrays.” DNA probe may target whole genomic DNA or individual genes. Whole genomic probes are more likely to cross-react with nontarget micro-organisms because of the presence of homologous sequences between different microbial species. Oligonucleotide probes based on signature sequences of specific genes may display limited or no cross-reactivity with nontarget micro-organisms when under optimized conditions. In addition, oligonucleotide probes can differentiate between closely related species or even subspecies and can be designed to detect uncultivable bacteria.

Socransky et al. (86) introduced a method for hybridizing large numbers of DNA samples against large numbers of digoxigenin-labeled whole genomic DNA or 16S rDNA-based oligonucleotide probes on a single support membrane—the checkerboard DNA-DNA hybridization method. Denatured DNA from clinical samples is placed in lanes on a nylon membrane using a Minislot device. After fixation of the samples to the membrane, the membrane is placed in a MiniBlotter 45 with the lanes of samples at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes are then loaded in individual lanes of the MiniBlotter. After hybridization, the membranes are washed at high stringency and the DNA probes detected using antibody to digoxigenin conjugated with alkaline phosphatase and chemiluminescence detection.

The checkerboard method permits the simultaneous determination of the presence of a multitude of bacterial species in single or multiple clinical samples. Thus, it is particularly applicable in large-scale epidemiological research. In addition to the reported advantages of molecular methods, DNA-DNA hybridization technology has the additional feature that microbial contaminants are not cultivated, nor is their DNA amplified (87). Because the numbers of contaminating micro-organisms are not increased, one may assume that, if present, they would be in numbers below detection limits of the checkerboard DNA-DNA hybridization method, which have been reported to be by the order of 10^3 to 10^7 cells (86, 88).
A modification of the checkerboard method was proposed by Past-ter et al. (89) and consists of a PCR based, reverse-capture checker- board hybridization methodology. The procedure circumvents the need for in vitro bacterial cultivation, necessary for preparation of whole genomic probes. Up to 30 reverse-capture oligonucleotide probes that target regions of the 16S rDNA are deposited on a nylon membrane in separate horizontal lanes using a Minislot apparatus. Each probe is synthesized with a polythymidine tail, which is cross-linked to the mem- brane support via UV irradiation, leaving the probe available for hybrid- ization. The 16S rDNA from clinical samples are PCR amplified using a digoxigenin labeled primer. Hybridizations are performed in vertical channels in a Miniblotter apparatus with digoxigenin-labeled PCR am- plifications from up to 45 samples. Hybridization signals are detected using chemiluminescence procedures.

DNA Microarrays

Developing a high-density macroarray is a difficult task, as large membranes represent a practical limitation when it comes to the vol- ume of probes that must be used, and particularly when small amounts of samples are available. In addition, because of the slow hybridization kinetics involved, they usually require longer periods for analysis. Min- ilarization of these assays saves time while cutting costs in diagnostic applications and research (90). Low volumes reduce reagent consump- tion and increase sample concentration, thus improving reaction kinetic- ics. The use of nonporous supports allows faster hybridization and easier washing steps. As a consequence, hundreds to thousands of re- sults can be obtained in the time needed for a single macroarray exper- iment.

DNA microarray methods were first described in 1995 (91) and essentially consist of many probes that are discretely located on a non- porous solid support, such as a glass slide (90, 92). Printed arrays and high-density oligonucleotide arrays are the most commonly used types of microarrays. A printed array comprises probes that are spotted onto specific locations on the solid support platform and range in size from 500 to 2,000 base pairs in length. Probes can be DNA fragments such as library clones or PCR products. By means of a robotic arrayer and capillary printing tips, thousands of elements can be printed on a single microscope slide. A high-density oligonucleotide microarray has a large number of probes attached to a solid support substrate. In this method, arrays are constructed by synthesizing single-stranded oligonucleotides in situ by use of photolithographic techniques. These probes are typi- cally shorter gene sequences comprised of 15 to 30 nucleotides (93). Advantages of the former method include relatively low cost and sub- stantial flexibility. Moreover, sequence information is not needed to print a probe. Advantages of the latter method include higher density and elimination of the need to obtain and store cloned DNA or PCR products (94).

Once microarrays have been printed, targets are prepared for hybridization. Targets may be PCR products, genomic DNA, total RNA, cDNA and so on, and usually incorporate either a fluorescent label or some other moiety, such as biotin, that permits subsequent detection with a secondary label. Targets are applied to the array and those that hybridize to complementary probes are detected using some type of reporter molecule. Following hybridization, arrays are imaged using a high-resolution scanner. Spatial resolution of these systems can be as low as 3 to 5 μm, although 10 μm of resolution is sufficient. Sophis- ticated computer software programs can then be used to analyze the results.

DNA microarrays can be used to enhance PCR product detection and identification. When PCR is used to amplify microbial DNA from clinical specimens, microarrays can then be used to identify the PCR products by hybridization to an array that is composed of species-specific probes. Using broad-range primers, such as those that amplify the 16S rDNA, a single PCR can be used to detect hundreds of bacterial species simultaneously. When coupled to PCR, microarrays have detect- tion sensitivity similar to conventional molecular methods with the added ability to discriminate several species at a time.

The advent of DNA microarrays has already given a marked con- tribution to many fields, including cellular physiology (95, 96), cancer biology (97–99), and pharmacology (100). In clinical microbiology, in addition to being applied to microbial detection and identification using rDNA information, microarrays have been used to analyze the genetic polymorphisms of specific loci associated with resistance to antimicro- bial agents, to explore the distribution of genes among isolates from the same and similar species, to analyze quorum-sensing systems, to un- derstand the evolutionary relationship between closely related species and to study host-pathogen interactions, particularly by identifying genes from pathogens that may be involved in pathogenicity and by surveying the host response to infection (101–107).

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) with rRNA-targeted oli- gonucleotide probes has been developed for in situ identification of individual microbial cells (108). This technique detects nucleic acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell. In addition to provide identification, FISH gives information about presence, mor- phology, number, organization and spatial distribution of micro-organisms (109). FISH not only allows the detection of cultivable microbial species, but also of as-yet uncultivable micro-organisms (110).

rRNAs are the main target molecules for FISH. This is because they can be found in all living organisms; they are relatively stable and occur in high copy numbers (usually several thousands per cell); and they include both variable and highly conserved sequence domains (45). Signature sequences unique to a given group of micro-organisms, rang- ing from whole phyla to individual species, can be identified. In the vast majority of applications for identification of bacteria or archaea, FISH probes target 16S rRNA (109). The oligonucleotide probes used in FISH are generally between 15 and 30 nucleotides long and covalently linked at the 5’-end to a single fluorescent dye molecule. Common fluorophors include fluorescein, tetramethylrhodamine, Texas red and carbocya- nine dyes like Cy3 or Cy5 (108, 109).

A typical FISH protocol includes four steps: the fixation and perme- ability of the sample; hybridization with the respective probes for detecting the respective target sequences; washing steps to remove unbound probe; and the detection of labeled cells by microscopy or flow cytometry (111).

Limitations of Molecular Methods

Molecular techniques have been used to overcome the limitations of cultivation procedures. Nonetheless, as with all methods, they are not without their own limitations. The following discussion verses on the major shortcomings associated with the most used molecular tech- niques.

DNA-DNA Hybridization

As with most molecular assays, DNA-DNA hybridization assays, such as the checkerboard technique, have been used only to detect target microbial species and consequently fail to detect the unexpected. For whole genomic probe preparation, bacteria must be initially cul- tured. Consequently, DNA-DNA hybridization assays using whole ge- nome probes can only detect cultivable species. In addition, whole genomic probes used in the DNA-DNA hybridization assays may show cross-reactivity with nontarget micro-organisms because of the pres-
ence of homologous sequences between different bacterial species. This can give rise to false positive results. However, Socransky et al. (86, 88) reported that most probes tested in the checkerboard assay did not exhibit cross-reactions under the experimental conditions. Some probes, as those to Tannerella forsythia, Porphyromonas gingivalis, and Treponema denticola, did not exhibit cross-reactions with any heterologous species tested. When cross-reactions were observed they were always within genera and were quite limited (88).

Reverse-capture checkerboard assay using oligonucleotide probes may show higher specificity than the conventional checkerboard technique and probes can be devised to target and detect uncultivable species (89).

### PCR-Derived Techniques

Table 3 lists the main limitations of PCR technology. Many of them can be avoided around by variations in technology. The issues related to the ability of PCR to detect either an extremely low number of cells or dead cells are of special interest when one interprets the results of PCR identification procedures in endodontic microbiology research. Therefore, these issues are worth a separate detailed discussion.

### The “Too-High Sensitivity” Issue

The high detection rate of PCR may be a reason of concern, specifically when nonquantitative assays are employed. It has been commonly claimed that because PCR can detect a very low number of cells or dead cells are of special interest when one interprets the results of PCR identification procedures in endodontic microbiology research. Therefore, these issues are worth a separate detailed discussion.

When taking samples from endodontic infections, difficulties posed by the physical constraints of the root canal system and by the limitations of the sampling techniques can make it difficult the attainment of a representative sample from the main canal (116). If cells of a given species are sampled in a number below of the detection rate of the given species, the results obtained by this method may not have significance with regard to disease causation. Nevertheless, some other factors should be also addressed in this discussion, which represent great advantages of such highly sensitive identification procedure in endodontic research.

When taking samples from endodontic infections, difficulties posed by the physical constraints of the root canal system and by the limitations of the sampling techniques can make it difficult the attainment of a representative sample from the main canal (116). If cells of a given species are sampled in a number below of the detection rate of the diagnostic test, species prevalence will be underestimated.

It is also important to take into consideration the analytical sensitivity needed for the specific clinical sample. For example, a sensitivity of about 10^4 microbial cells per ml is required for urine, while a sensitivity of one cell may be of extreme relevance for samples from blood or cerebrospinal fluid (117). There is no clear evidence as to the microbial load necessary for a periradicular lesion to be induced. Endodontic infections are characterized by a mixed infection and individual species may play different roles in the consortium or dominate various stages of the infection. At least theoretically, all bacterial species established in the infected root canal have the potential to be considered endodontic pathogens (118). Based on this, it would be glaringly prudent to use the method with the highest sensitivity to detect all species colonizing the root canal.

In fact, detection of very low numbers of cells in clinical samples may not be as common as anticipated. There are numerous factors that can influence PCR reactions, sometimes dramatically reducing sensitivity for direct microbial detection in clinical samples. Thus, the analytical sensitivity of the method does not always correspond to its “clinical” sensitivity. It is well known that the effects of inhibitors are magnified in samples with low number of target DNA and therefore can significantly decrease the sensitivity of the method (62). Another impediment refers to the aliquots of the whole sample used in PCR reactions. Most of the PCR assays that have been used so far in endodontic research have an analytical sensitivity of about 10 to 100 cells. If one takes into account the dilution factor dictated by the use of small aliquots (5–10%) of the whole sample in each amplification reaction, the actual sensitivity of the assay is 200 to 400 to 2000 to 4000 cells in the whole sample, without discounting the effects of inhibitors (57). These numbers are still lower than the detection limits of other methods, but can represent more significance with regard to etiology.

Therefore, the use of highly sensitive techniques is welcome in the study of endodontic infections, decreasing the risks for potentially important species to pass unnoticed during sample analysis. Although qualitative results do not lack significance, the use of quantitative molecular assays, like the real-time PCR, can allow inference of the role of a given species in the infectious process while maintaining high sensitivity and the ability to detect fastidious or uncultivable microbial species.

### The “Dead-Cell” Issue

Detection of dead cells by a given identification method can be regarded as a double-edged sword. This can be at the same time an advantage and a limitation of the method. On the one hand, this ability can allow detection of uncultivable bacteria or fastidious bacteria that can die during sampling, transportation or isolation procedures (57, 119, 120). On the other hand, if the bacteria were already dead in the infected site, they may also be detected and this might give rise to a false assumption about their role in the infectious process (121, 122).

Several studies have addressed this issue. In a mouse model of Lyme disease, it was revealed by PCR that Borrelia burgdorferi DNA disappeared from tissues immediately after a 5-day antibiotic treatment course, and that the PCR results correlated well with culture results (123). Similarly, investigators using a chinchilla model of otitis media found a strong correlation between PCR results and culture results when animals were injected with viable Haemophilus influenza (124).
When animals were injected with purified bacterial DNA or with killed bacteria, the amplifiable DNA rapidly disappeared. The authors concluded that DNA, and DNA from intact but nonviable bacteria, does not persist in an amplifiable form for more than a day in the presence of an effusion. In a rabbit model of syphilis in which live or heat-killed *Treponema pallidum* was injected into the skin and testes, heat-killed treponemes were no longer detectable by PCR after 15 to 30 days, whereas viable micro-organisms were detected by PCR for months (125). A study of pulmonary tuberculosis treatment showed that spumt smear and cultures for *Mycobacterium tuberculosis* convert to negative before PCR results, but that PCR results do correlate with clinical response to antibiotics and also can predict relapse (126). It was not clear if the persistent PCR signal seen in some of these patients was a result of low levels of viable micro-organisms, or because of amplifiable DNA from nonviable micro-organisms. All these studies show that bacterial DNA can be cleared from the host sites after bacterial death and that DNA from different species may differ as to the elimination kinetics at different body sites. It remains to be clarified for how long bacterial DNA from dead cells can remain detectable in the infected root canal system.

Detection of microbial DNA sequences in clinical specimens does not indicate viability of the micro-organism. However, this issue should be addressed with common sense and without any sort of biases. The fact that some micro-organisms die during the course of an infectious process does not necessarily implicate that in a determined moment these micro-organisms did not participate in the pathogenesis of the disease. In addition, the fate of DNA from micro-organisms that have entered and not survived in root canals is unknown. DNA from dead cells might be adsorbed by dentine because of affinity of hydroxyapatite to this molecule (127). However, it remains to be shown if DNA from microbial dead cells can really be adsorbed in dentinal walls and, if even, it can be retrieved during sampling with paper points. In fact, it is highly unlikely that free microbial DNA can remain intact in an environment colonized by living micro-organisms. Dnases released by some living species as well as at cell death can degrade free DNA in the environment. It has been reported the presence of Dnase activity on whole bacterial cells and vesicles thereof that can degrade linear DNA (128). These bacteria include common putative endodontic pathogens, such as *Porphyromonas endodontalis*, *P. gingivalis*, *T. forsythia*, *Fusobacterium* species, *Prevotella intermedia*, and *Prevotella nigrescens*. Indeed, Dnases are of concern during sample storage, because they can be carried along with the sample and induce DNA degradation, with consequent false negative result after PCR amplification.

Therefore, although there is a possibility of detecting DNA from dead cells in endodontic infections, this possibility is conceivably low. In the event DNA from dead cells is detected, the results by no means lack significance with regard to participation in disease causation. However, the ability to detect DNA from dead cells poses a major problem when one is investigating the immediate effectiveness of antimicrobial treatment, because DNA from cells that have recently died can be detected. To circumvent or at least minimize this problem, one can use some adjustments in the PCR assay or take advantage of PCR technology derivatives, such as RT-PCR. McCarty and Atlas (129) investigated the effect of ampicillin size on the PCR detection of *Legionella pneumophila* after chlorine inactivation. Two amplicons specific to the *L. pneumophila* *mpg* gene were used for the PCR analyses. A 168-bp ampicillin was detectable longer after chlorine treatment than a 650-bp ampicillin. On the basis of their data, they concluded that larger amplicons appear to correet better with bacterial viability. Findings from their study showed that smaller fragments of DNA may persist for a longer time after cell death than larger sequences. Therefore, designing primers to generate large amplicons may reduce the risks of positive results because of DNA from dead cells. Moreover, because RNAs are more labile and have a shorter half-life when compared to DNA, they can be rapidly degraded after cell death (122). Thus, assays directed towards the detection of mRNA or microRNA through RT-PCR can be more reliable for detection of living cells.

**Unraveling the Oral Microbiota**

The oral cavity harbors one of the highest accumulations of micro-organisms in the body. Even though viruses, yeasts and protozoa can be found as constituents of the oral microbiota, bacteria are by far the most dominant inhabitants of the oral cavity. There are an estimated 10^10 bacteria in the mouth (130). A high diversity of bacterial species has been revealed in the oral cavity by cultivation and application of molecular methods to the analysis of the bacterial diversity has revealed a still more broad and diverse spectrum of extant oral bacteria. Overall, bacteria detected from the oral cavity fall into 11 phyla that comprised over 700 different species or phylotypes (17, 131, 132). About 40 to 50% of these bacteria are novel uncultivated species or phylotypes, which are known only by 16S rDNA sequences (17). This raises the interesting possibility that uncultivated and as-yet-uncharacterized species that have remained invisible to studies using traditional identification methods may make up a large fraction of the living oral microbiota, and may participate in the etiology of oral diseases.

In fact, the introduction of molecular approaches in oral microbiology research has brought about a significant body of new knowledge with regard to the oral microbiota in health and disease. The development of molecular bacterial identification methods has made it possible to study the role that uncultivable bacteria or even other micro-organisms such as archaea play in oral diseases (133–137). Consequently, a significant revolution in the knowledge of the oral microbiota in health and disease has taken place after the advent of molecular techniques for microbial identification. In this context, endodontic infections are far from being an exception. The second part of this review deals with the significant impact of molecular genetic methods for microbial identification on the knowledge of endodontic infections.

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**References**


