

MICROBIAL IDENTIFICATION IN ENDODONTIC INFECTIONS WITH AN EMPHASIS ON MOLECULAR DIAGNOSTIC METHODS: A REVIEW

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ABSTRACT

Accurate and definitive microorganism identification, including bacterial identification and pathogen detection, is essential for correct disease diagnosis, treatment of infection and trace-back of disease outbreaks associated with microbial infections. Bacterial identification is used in a wide variety of applications including microbial forensics, criminal investigations, bio-terrorism threats and environmental studies. Overwhelming evidence indicates that periradicular diseases are infectious disorders. The question now is no longer whether microorganisms are involved in the pathogenesis of such diseases, but which specific microbial species are. The list of microorganisms involved in periradicular diseases keeps expanding and has the potential to become increasingly more accurate during the next few years. Molecular methods have contributed significantly to the knowledge about the microbial species involved. Undoubtedly, a great deal of additional research is needed to define the specific role played by suspected endodontic pathogens in the etiology of each form of periradicular disease and to determine the best therapeutic measures for the pathogen's eradication. In addition, there is an emergent need to define markers that permit the clinician to know when he or she should conclude the treatment and to predict the outcome of the treatment. This paper will discuss briefly the methods of microbial identification with special reference to the molecular diagnostic technologies and their potential to explore the diverse microbiota associated with endodontic infections.

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INTRODUCTION

There is no greater association between a basic science and the practice of endodontics, than that of microbiology. Microorganisms cause virtually all pathoses of the pulp and the peri-radicular tissues. To effectively treat endodontic infections, clinicians must recognize the cause and effect of microbial invasion of the dental pulp space and the surrounding peri-radicular tissues. Knowledge of the microorganisms associated with endodontic disease is necessary to develop a basic understanding of the disease process and a sound rationale for effective management of patients with endodontic infections. Accurate and definitive microorganism identification, including bacterial identification and pathogen detection, is essential for correct disease diagnosis, treatment of infection and trace-back of disease outbreaks associated with microbial infections. Bacterial identification is used in a wide variety of applications including microbial forensics, criminal investigations, bio-terrorism threats and environmental studies. Epidemiological studies using sophisticated culture and molecular biology techniques have collectively shown that approximately 300 different microbial species can be found in infected root canals usually in combinations of 10-30 species. [1] Theoretically any one of these species would have the potential to be an endodontic pathogen. The question now is no longer whether microorganisms are involved with causation of endodontic infection, but which species are. [2] This article describes the molecular biological techniques with the potential to be applied decipher the diversity of microbiota associated with endodontic infections.

CHALLENGES IN BACTERIAL IDENTIFICATION

Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using gram staining, culture and biochemical methods. However, these methods of bacterial identification suffer from two major drawbacks. First, they can be used only for organisms that can be cultivated in vitro. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic

of any known genus and species.

TRADITIONAL IDENTIFICATION METHODS

Culture

For more than a century, cultivation using artificial growth media has been the standard diagnostic test in infectious diseases. 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.[3] 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 innumerable micro-organisms to survive in virtually all habitats, including within the human body. [4] 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. [5] 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.[6] 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,[7-9] 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. [10]

Advantages and Limitations of culture

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.[3]

Difficulties in Cultivation

Lack of essential nutrients or growth factors in the artificial culture medium, toxicity of the culture medium itself, production of substances inhibitory to the target microorganism by other species present in a mixed consortium, metabolic dependence on other species for growth, disruption of bacterial intercommunication systems induced by separation of bacteria on solid culture media and bacterial dormancy, These are some possible reasons for bacterial unculturability:[5,11,12] Obviously, if micro-organisms cannot be cultivated, they cannot be identified by phenotype-based methods. Hence 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. [3] There have been developments in 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. [13, 14]

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. [3] 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. In both situations, phenotypically based diagnostic tests would result in misidentification. [3]

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 interpretations of the investigator. In addition, microscopy has limited sensitivity and specificity to detect microorganisms in clinical samples. [3] The knowledge of the endodontic microbiota is based mostly on culture studies. This is simply because of lack of real alternatives in the past. Microscopic studies have been used; however, they have severe limitations when it comes to deeper identification, to evaluate the composition, to characterize various micro-organisms, and to do further experimental studies on isolated species. [15] Microscopy of smears from the root canal is limited to main morphotypes. Microbial staining of histological sections has advantage of localizing the microbes in situ. Electron-microscopic pictures (transmission or scanning) have been valuable to distinguish main morphotypes in various locations. [3]

The scanning electron microscope (SEM) is an invaluable tool for describing biofilms because of its ability to provide an indiscriminate view on the surface topography at high-resolution and magnification. However, even high-resolution SEM examinations of biofilms are often compromised by the fact that matrix embedded bacteria cannot be easily visualized. Furthermore it is known that biofilm bacteria often lose their characteristic shape and size making them difficult, if not impossible, to identify. These limitations pose a problem when indisputable proof of the existence of bacterial biofilms growing in natural environments is required. In general, the presence of bacteria in a matrix is a sine qua non for the presence of a biofilm. If bacteria cannot be demonstrated to be present, the proposed existence of a bacterial biofilm remains questionable.

The use of confocal laser scanning microscopy on the other hand in combination with fluorescence in situ hybridization enables the visualization of matrix embedded bacteria in multi-layered biofilms. In a study by Schaudin, Carr et al., fluorescence in situ hybridization/confocal laser scanning microscopy and scanning electron microscopy were applied to visualize bacterial biofilm in endodontic root canals. The resulting fluorescence in situ hybridization/confocal laser scanning microscopy and scanning electron microscopy pictures were subsequently combined into one single image to provide high-resolution information on the location of hidden bacteria. The researchers concluded that combined use of scanning electron microscopy and fluorescence in situ hybridization/confocal laser scanning microscopy as the potential to overcome the limits of each single technique. [16]

Immunological Methods

Immunological methods are based on the specificity of antigen-antibody reaction. It can detect micro-organisms directly or indirectly, the latter by detecting host immunoglobulins specific to the target micro-organism. The enzyme-linked immunosorbent assay (ELISA) and the direct or indirect immunofluorescence tests are the most commonly used immunological methods for microbial identification. [3] Advantages of immunological methods for identification of micro-organisms include: (a) they take no more than a few hours to identify a microbial species; (b) they can detect dead micro-organisms; (c) they can be easily standardized; and (d) they have low cost. [17] However, they have also important limitations as they can detect only target species, they have low sensitivity (about 10⁴ cells), their specificity is variable and depends on types of antibodies used, and they can detect dead micro-organisms. [17, 18]

MOLECULAR GENETIC METHODS

Molecular biological methods have been recently used to decipher the diversity of the endodontic microbiota and many fastidious species and even uncultivated phylotypes have been disclosed. [19] During the last decade, numerous studies using various types of molecular biology techniques have been used to characterize more closely the microbial composition of the root canal microbiota. These methods have definitely showed that the root canal microbiota is much more complex than previously thought. This has made the clinical interpretations, diagnosis, and treatment strategies more difficult. Still, culture is a “gold standard” to identify specific targets for treatment

and to evaluate treatment strategies due to its easier accessibility than the new techniques. Culture is also used in experimental models, which have disclosed the dynamics of the infections and the nature of micro-organisms. [15] Molecular diagnostic methods have several advantages over other methods with regard to microbial identification. [3]

- Detection of not only cultivable species but also of uncultivable microbial species or strains.
- Higher specificity and accurate identification of microbial strains with ambiguous phenotypic behavior, including divergent or convergent strains.
- Detection of microbial species directly in clinical samples, without the need for cultivation.
- Higher sensitivity.
- Faster and less time-consuming.
- They offer a rapid diagnosis, which is particularly advantageous in cases of life-threatening diseases or diseases caused by slow growing micro-organisms.
- They do not require carefully controlled anaerobic conditions during sampling and transportation, which is advantageous since fastidious anaerobic bacteria and other fragile micro-organisms can lose viability during transit.
- They can be used during antimicrobial treatment.
- When a large number of samples are to be surveyed in epidemiological studies, samples can be stored and analyzed all at once.

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. [Figure- 1]

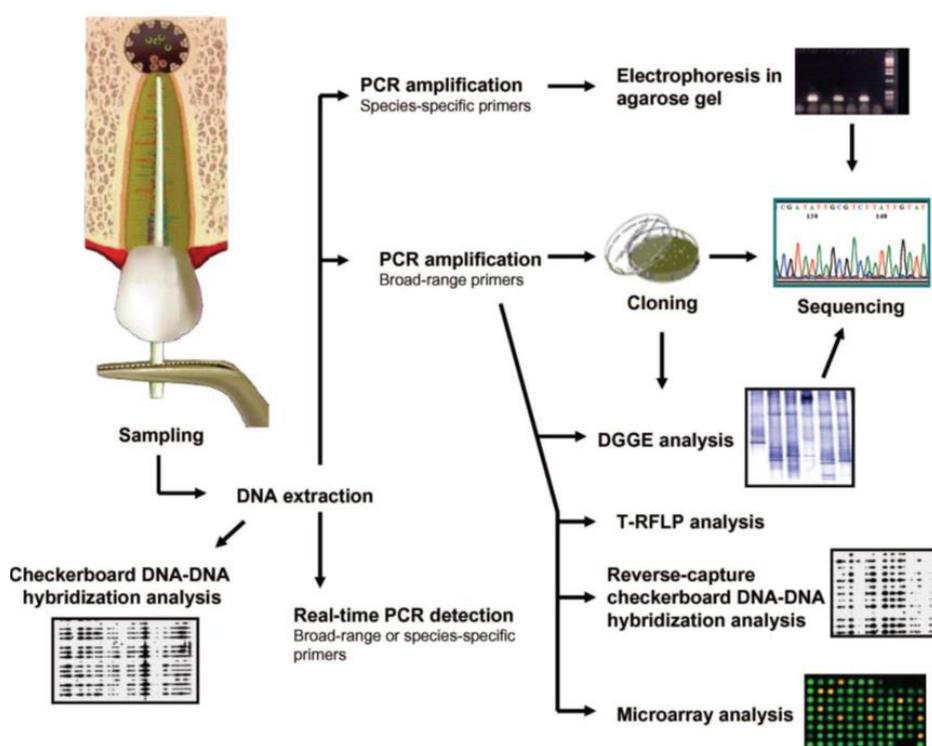


Fig: 1. Different molecular techniques that can be used to identify the diverse microbiota associated with endodontic infections

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. [3]

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 [20], 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. 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. [21] Large subunit genes (23S and 25S rDNA) and small subunit genes (16S and 18S rDNA) 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. 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. [20] 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). [3] 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 rDNA 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. [3]

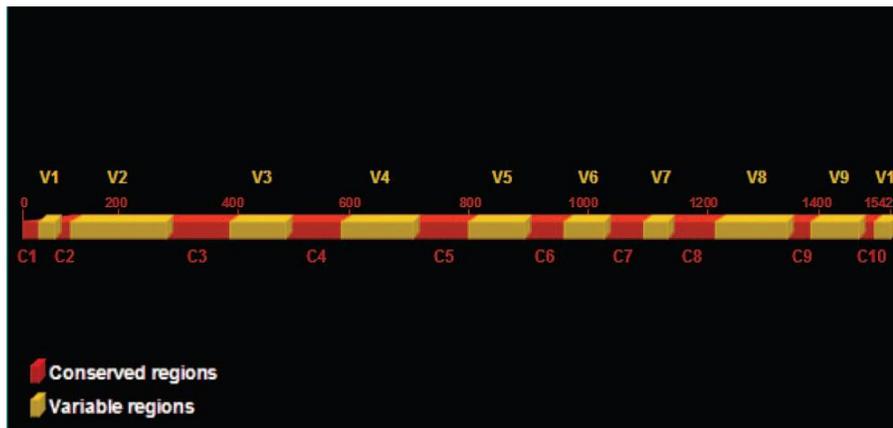


Fig: 2. Schematic drawing of the 16S rRNA gene (rDNA). Orange areas correspond to variable regions, which contain information about the genus and the species. Red areas correspond to conserved regions of the gene.

Polymerase Chain Reaction (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. 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. [22] Nowadays, it is possible to isolate essentially any gene from any organism using PCR, which made this technique a cornerstone of genome sequencing projects. The most

widespread advance in clinical diagnostic technology has come from the application of PCR for detection of microbial pathogens.[23] The PCR method is based on the in vitro replication of DNA through repetitive cycles of denaturation, primer annealing and extension steps. 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 bacterial, defining the two ends of the amplified stretch of DNA. In sequence, a complementary second strand of new DNA is synthesized through the extension of each annealed primer by a thermostable DNA polymerase in the presence of excess deoxyribonucleoside triphosphates. All previously synthesized products act as templates for new primer-extension reactions in each ensuing cycle. The result is the exponential amplification of new DNA products, which confers extraordinary sensitivity in detecting the target DNA. In fact, PCR has unrivaled sensitivity—it is at least 10 to 100 times more sensitive than the other more sensitive identification method. [18, 24] There are several methods to check if the intended PCR product was generated. The most commonly used method for detecting PCR products is electrophoresis in an agarose gel. Aliquots of the PCR reaction 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. The gel is usually visualized using ethidium bromidestaining and ultraviolet transillumination.[3] Numerous derivatives in PCR technology have been developed since its inception. For ex., nested PCR, Reverse Transcriptase PCR, Multiplex PCR, Broad-Range PCR and Real-Time PCR. These are discussed briefly in the following paragraphs.

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. [25] 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. [3] 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. [24] 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. [26] 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.[3]

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. [27] 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*. [3]

Multiplex PCR

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 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.[3]

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. [28] 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. [29,30] 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. [3]

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. [31] 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 uncultivable bacterial pathogens directly from diverse human sites. [11, 32, 33] 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. 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, uses of high-quality reagents and adequate sampling techniques and vials for clinical specimens. [3]

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 like infected root canals 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. [34, 35] 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). [3] 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 overtime, 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 by Siqueira JF Jr, Roças IN, Rosado AS. [36] Temperature gradient gel electrophoresis (TGGE) uses the same principle as DGGE, except for the fact that the gradient is temperature rather than chemical denaturants. [3]

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. [37] 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'-tetrachloro-6-carboxyfluorescein (TET) or phosphoramidite fluorochrome 5-carboxyfluorescein (6-FAM). [38] 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. [38] 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 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. [3]

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 non-target 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 non-target 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. [3] Socransky et al. [39] 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. 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. [40] 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 10³ to 10⁴ cells. [39, 41]

DNA Microarrays

DNA microarray methods were first described in 1995 and essentially consist of many probes that are discretely located on a nonporous solid support, such as a glass slide. 90, 92 Printed arrays and high-density oligonucleotide arrays are the most commonly used types of microarrays. 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 detection sensitivity similar to conventional molecular methods with the added ability to discriminate several species at a time. [3]

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes has been developed for in situ identification of individual microbial cells. [42] 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, morphology, number, organization and spatial distribution of micro-organisms. FISH not only allows the detection of cultivable microbial species, but also of as-yet uncultivable micro-organisms. [43] 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. [20] A typical FISH protocol includes four steps: the fixation and permeabilization 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. [44]

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 main limitations of PCR-derived technologies are: [3]

- Most PCR assays used for identification purposes qualitatively detect the target microorganism but not its levels in the sample. Quantitative results can however be obtained in real-time PCR assays.
- Most PCR assays only detect one species or a few different species (multiplex PCR) at a time. However, broad-range PCR analysis can provide information about the identity of virtually all species in a community.
- Like DNA-DNA hybridization, most PCR assays only detect target species and consequently fail to detect unexpected species. This can be overcome by broad-range PCR assays.

- In addition to being laborious and costly, broad-range PCR analyses can be affected by some factors, such as biases in homogenization procedures, preferential DNA amplification and differential DNA extraction.
- Microorganisms with thick cell walls, such as fungi, may be difficult to break open and may require additional steps for lysis and consequent DNA release to occur.
- False positive results have the potential to occur because of PCR amplification of contaminant DNA. The most important means of contamination is through carryover of amplification product and special precautions should be taken to avoid this.
- False negatives may occur because of enzyme inhibitors or nucleases present in clinical samples, which may abort the amplification reaction and degrade the DNA template, respectively. Analysis of small sample volumes may also lead to false negative results, particularly if the target species is present in low numbers.

METAGENOMICS

The two fundamental questions in microbial ecology are who is there and what are they doing. Molecular biology methods have provided a great deal of information about the species composition in diverse environments. Now the important question to be answered refers to the role of different species in the consortium, what they are doing there. Data from 16S rRNA gene cloning libraries is astonishing as far as the identification of bacterial diversity is concerned. The challenge now is to develop methods to move beyond cataloging 16S rRNA gene sequences toward an understanding of the physiology and functional roles of bacteria in different environments. While the 16S rRNA gene often provides accurate identification, the other 99.95% of the genome provides the blueprint for the vast array of metabolic, structural, and virulence abilities of each bacterium. Because as-yet-uncultivated bacteria make up a large proportion of most environments, studies of the physiological and functional roles of the community members should also rely on culture independent approaches. Metagenomics is the culture-independent analysis of the collective microbial genomes (metagenome) in an environmental community, using an approach based either on expression or on sequencing. Metagenomics treats the genomes of all microorganisms present in a specific habitat as an entity. Theoretically, a metagenomic library will contain DNA sequences for all the genes in the microbial community. Metagenomic libraries permit analyses of species diversity based on a PCR-independent approach as well as a comprehensive description of functionalities of the whole ecosystem. Metagenomic analysis involves one of the three approaches; functional approach, sequence based approach and whole-genome shotgun sequencing. In the near future, metagenomic analysis of the oral microbiome will provide invaluable information about the physiological and functional roles of the oral microbiota, including bacteria that have not yet been cultivated. [45]

CONCLUSION

The oral cavity harbors one of the highest accumulations of micro-organisms in the body. Even though viruses, archaea, fungi 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 billion bacterial cells in the mouth. [46] A high diversity of bacterial species is evident from culture studies, but application of molecular biology methods to the analysis of the bacterial diversity has revealed a still broader and more diverse spectrum of extant oral bacteria. Taken as a whole, bacteria detected from the oral cavity fall into 12 separate phyla that comprised over 700 different species or phylotypes. [47, 48, 49] Data based on culture-dependent and culture-independent approaches have revealed that there are presently 771 bacterial taxa in the oral cavity: 273 are named bacterial species, 412 phylotypes are known by 16S rRNA gene sequence only, and 86 are unnamed, partially characterized strains. Thus over 50% of the bacteria remain to be cultivated and fully characterized. This raises the interesting possibility that uncultivated and as-yet-uncharacterized species can play an important ecological role as well as participate in the etiology of oral diseases. [19] The introduction of molecular approaches in the oral microbiology research has brought about a significant body of new knowledge with regard to oral microbiota in health and disease. Despite of the great advances, endodontic microbiology is still undergoing a shift from a culture era to a molecular era. It obviously does not imply that culture has become obsolete. In fact efforts should be directed towards the cultivation of as-yet uncultivable species in an attempt to specify their role in the pathogenesis of periradicular diseases. Undoubtedly, the well-directed use of molecular methods will provide additional valuable information regarding the identification and understanding of the causative factors associated with endodontic diseases. PCR and other molecular biology techniques hold the hope of making the knowledge of endodontic infectious processes more accurate. Additionally, molecular methods have the potential to make diagnosis more rapid and directed evidence-based antimicrobial therapy a reality. The development of high density DNA

microarrays, with hundreds of rDNA sequence probes specific to oral bacterial species and phenotypes will significantly enhance our ability to rapidly determine the composition of the endodontic infection and to establish association with particular signs and symptoms of the disease, thus enhancing the success rate of the endodontic therapy .[50]

CONFLICT OF INTERESTS

There is no conflict of interest amongst the authors.

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