"Assessing the Unseen"- Different detection methods for exploring Endodontic Microbes: A review

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ABSTRACT:
It is important that clinicians have a thorough understanding of the root canal microflora responsible for endodontic diseases in order to develop an effective rationale for treatment. A myriad of techniques have been introduced for the detection of bacterial diversity in the root canal system. Identification methods have evolved from cultural dependent methods to next generation sequencing technology. This review discusses different methods for detection of endodontic microbes that have been or have the potential to be used in field of endodontic microbiology.

Keywords: Detection methods, Endodontics, Endodontic microbiology.

INTRODUCTION

It is a well established fact that microorganisms are the main causative agents for the establishment of endodontic infections.[1] Microenvironment of endodontium is a polymicrobial flora which is dominated by obligate anaerobic bacteria.[2] It is crucial to identify these offending pathogenic microbes and study their characterization with pathogenic potential for successful endodontic therapy. Pathogen detection, identification and characterization are the gold standard for diagnosis.[3] Moreover, accurate detection and analysis of endodontic microbes will not only streamline the treatment plan but it also prevents the emergence of antimicrobial resistance.[3] In order to reveal these microorganisms, accurate and effective detection methods are required.

In the past, microbiological studies of endodontic infections primarily used microscopy, aerobic culturing techniques, and biochemical reactions to detect and identify microbes.[4] Bacterial detection was mainly based on conventional and culture-dependent approaches assessing only the phenotypic.[5] But microbial heterogeneity, its evolution and intra- species variations haveraised the need for newer methods.[4] Recent identification methods such as molecular biology methods and pyrosequencing have now greatly improved the bacterial detection and identification.[6]

VARIOUS DIAGNOSTIC METHODS

Many identification methods are available for the detection of endodontic pathogens. An ideal method must be sensitive, specific, accurate, rapid and easy to perform.[7] The resulting data must also be easy to interpret and cost effective with high-throughput. In general, comprehensive categorization of the various identification methods has been provided in Table 1.

I.Microscopic Methods

Microscopy is the traditional method for the identification of endodontic microbes.[8] These methods provide preliminary information regarding the morphological characterization of the microbes involved in an infection. Microscopic methods are based on the type of microscope used such as optical microscopy, phase contrast microscopy, darkfield microscopy, brightfield microscopy, fluorescence microscopy and electron microscopy.[8,9] Overall, microscopy does not give comprehensive information about the

microbial heterogeneity and variations within the species.[5]

METHODS FOR BACTERIAL DETECTION	
I. Microscopic methods	Optical microscopy, Bright field microscopy, Confocal microscopy, Darkfield microscopy, Phase contrast microscopy, Fluorescence microscopy and Electron microscopy.
II. Cultural methods	Aerobic and Anaerobic Cultural methods
III. Immunological methods	Based on detections of antibodies and antigens a) Enzyme-linked immunosorbent assay (ELISA) b) Radioimmunoassay (RIA) c) Immunochromatographic (ICG) assays d) Immunofluorescence e) Flow cytometry f) Immunoblotting
IV. Molecular biology methods	i. Hybridization a) Checkboard DNA-DNA hybridization b) DNA microarrays c) Fluorescence in-situ hybridization ii. Amplification a) PCR based methods b) Non-PCR based methods iii. Sequencing and enzymatic digestion method
V. Next Generation Sequence (NGS) technology	Pyrosequencing methods

Table 1: Various Identification methods for the bacterial detection

II.Cultural Methods

Culture-based diagnosis methods are based on the ability to grow microorganisms *in vitro*, i.e., in artificial conditions.[8,10] Culture-dependent analysis have been widely used in endodontics to study the composition of root canal microflora. These methods are still considered as the gold standard for detection and identification of microbes for dentists in the clinics.[11] Two types of basic culture methods include Aerobic and Anaerobic culture methods.[9] Culture based analysis in endodontics with adequate antiseptic measures, sampling procedures, transportation and laboratory procedures was given by Moller in 1966.[12] The main advantage of cultural methods is the possibility to obtain relative and absolute counts of the cultured species.[11] Main steps involved in culture based analysis in clinical practice are given below and shown in Figure 1.[10, 12,13]

i. Under aseptic conditions, microbial sampling is done using sterile paper points from the root canal system.
ii. Paper points are placed in the transport medium (VMGA III) followed by serial dilutions.
iii. Aliquots of the serial dilutions are plated on medium plates and incubated for 24–48 h under aerobic and 7–21 days under anaerobic conditions.
iv. In a plate with microbial growth, colonies are isolated according to their morphological characteristics and incubated again under aerobic and anaerobic conditions.
v. The pure colonies are stained and observed under microscope.

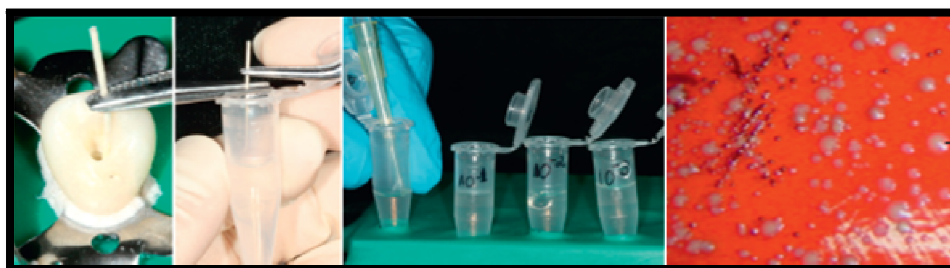

Figure 1: Culture based analysis in clinical practice

Cultural dependent studies have revealed only 10-12 species including anaerobic *Streptococcal* species, Gram-positive anaerobic rods, species of *Prevotella*, *Fusobacterium*, *Eubacterium* and *Campylobacter*.[10]

Main disadvantages of culture methods include growth of only viable bacteria, low sensitivity, strict sampling and transport conditions.[10]

III.Immunological Methods

Immunological methods are based on the identification of diagnostic antibodies and specific antigens. The presence of an antibody to a particular antigen in the serum can be determined by: Enzyme-linked immunosorbent assay (ELISA), Radioimmunoassay (RIA) and Radioimmunosorbent assay (RAST).[3] The techniques used for detection of antigens include immunochromatographic lateral flow assays (ICG), sandwich immunoassays, immunofluorescence assays (IF), flow cytometry and immunoblotting.[9] Sensitivity of immunological methods is low whereas specificity is variable and depends on the types of antibodies used.[14]

IV.Molecular Biology Techniques

Molecular biology methods have resulted in the identification of unidentified and uncultivated endodontic pathogens. Molecular methods are based on the sequence analysis of the 16S ribosomal RNA genes and have provided reliable alternatives to past cultural and microscopic methods for detection of microbes.[15] The Molecular biology methods have been classified into three categories.[9]

i. Hybridization include Checkboard DNA-DNA hybridization, DNA microarray technique and Fluorescence in-situ hybridization
ii. Amplification include PCR based and Non-PCR based methods
iii. Sequencing and enzymatic digestion of nucleic acid

i. Hybridization

These methods are based on the ability of nucleic acid strands with complementary base sequences to form a double-stranded molecules or duplex or hybrid.

Checkboard DNA DNA hybridization

It was introduced by Socransky et al. in 1994 for the hybridization of large number of DNA samples.[15] There are two types of checkerboard hybridization: (1) Whole genomic DNA probes are hybridized to sample DNA on the membrane. (2) Labeled 16S ribosomal RNA amplicons are hybridized to 16S ribosomal RNA-based probes on the membrane.[15] Reverse capture checkboard hybridization was proposed by Paster et al in 1998.[10] It is a modification of the checkboard method and consists of a PCR-based methodology. In thisassay, sample is fixed first to the membrane instead of probe.[10]

DNA microarray technique

This technique was first described in 1995 by Schena et al.to examine the complex oral microbial diversity in a single hybridization reaction on glass slides including species that has not yet been cultured.[15] Commercially available DNA and RNA microarray has been used to determine the microbial profiles of clinical samples from endodontic lesions and the normal microflora of gingival biopsies.[15]

Fluorescence in-situ hybridization

This technique utilizes fluorescently labeled rRNA-directed probes for hybridization.Intact microbial cells are detected directly in clinical specimens by using fluorescence microscope.[14] FISH technique is comprised of following steps: Fixation and permeabilization, hybridization, washing to remove unbound probe, detection of labeled cells by microscopy of flow cytometry.[10,13] Fluorescence in-situ hybridization of the samples allow the identification and provides information about morphology, number, community architectureand spatial relationship of microorganisms.[14]

ii. Amplification

These methods can be broadly divided into two categories: PCR based and Non-PCR based.

PCR based methods

The term PCR was coined by Kary Mullis in 1983.[6] This technique is based on the principle of nucleic acid hybridization and nucleic acid replication.[17] Main steps involved in PCR technique include Denaturation of target nucleic acid, Primer annealing, Extension of primer- target duplex and Detection of amplified PCR product (with Electrophoresis in Agarose gel, Cloning and sequencing, DGGE analysis, T- RFLP analysis) as shownin fig. 2.[9] Derivatives of PCR include touchdown PCR, nested PCR, multiplex PCR, Reverse transcriptase PCR (RT PCR), Quantitative PCR, PCR-based microbial typing, broad-range PCR , Real time PCR.[15] This technology allows for high throughput of samples, multiplexing reactions, quantitation of target, and on-line monitoring.[14]

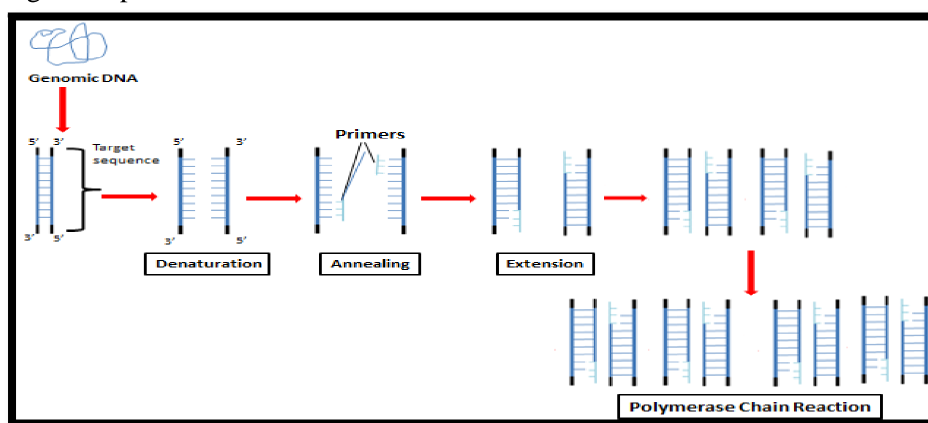

Figure 2: Polymerase chain reaction

Non-PCR based amplification methods

These methods do not require temperature cycling and annealing and operate at constant temperature.[9] Non-PCR based amplification methods act either by amplifying the signal used to detect the target nucleic acid or directly by amplifying the target nucleic acid. Major Non-PCR based amplification methods include Nucleic acid sequence based amplification (NASBA), Transcription based amplification (TMA), Standard displacement based amplification (SDA).[9]

iii. Sequencing and enzymatic digestion of nucleic acids

These methods include Nucleic acid sequencing and enzymatic digestion followed by electrophoresis of nucleic acids.[17,9] Nucleic acid sequencing determines the exact nucleotide sequence of a gene or gene fragment of the sample. Enzymatic digestion is accomplished by using enzymes like Restriction endonucleases which results in fragmented nucleic acid strand followed by electrophoresis of fragments.[15]

V.Next Generation Sequencing Technology

Next Generation DNA sequencing (NGS) technology is based on 'sequencing-by-synthesis' principle for the detection of nucleic acids sequences.[19] These NGS methods have resulted in-depth analysis of endodontic microbial communities.[20] The Sanger sequencing method is the gold standardof sequencing technique.[18] The automated Sanger approach involves DNA purification, DNA synthesis and labeling using the chain termination method withdye-labeled dideoxynucleotides (ddNTPs), capillary electrophoresis, and fluorescence detection.[18] Sanger sequencing approach has now been replaced by Pyrosequencing which is a novel DNA sequencing technology.[21] The five most currently used NGS technologies include the

454 pyrosequencing, ILLumina/Solexa Genome Analyzer, SOLiD, the HeliScope Single Molecule Sequencer and the Single Molecule Real Time technology.[18]

In 454 pyrosequencing, DNA is fragmented and amplified using special adaptors in an emulsion PCR that binds to an agarose bead.[22] This amplification results in the formation of 1 million copies around one bead. This methodology allows reads of 400,000 DNAs that are each about 250 bases in length.[15] Recently, the GS FLX + has been introduced which can read sequence up to 1 kb with a mode read length of 700 base pairs.[23] ILLumina /Solexa platformis based on the sequencing-by-synthesis principle. This methodology involves fragmentation of DNA and specialized adaptors. In this technology, emulsion PCR is attached to a slide rather than to a bead.[15] In the SOLiD (Sequencing by Oligonucleotide Ligation and Detection) system, sequencing is obtained by measuring serial ligation of an oligonucleotide to the sequencing primer by the utilization of a DNA ligase enzyme.[18] The HeliScope System by Helicos Biosciences is also based on the sequencing-by-synthesis methodology. In the HeliScope platform, single DNA molecules are sequenced directly, without clonal PCR amplification.[18] Single Molecule Real Time sequencing (SMRT) is a parallelized single molecule DNA sequencing method.[24] This method of sequencing employs a zero- mode waveguide (ZMW) and a single DNA polymerase enzyme for analysis of nucleic acids sequences.[24]

Sequencing methods also have limitations such as study of more samples rather than more sequences per sample, sequencing error while the detection of long homoplymers and availability of less phylogenetic information from short sequences.[25,26] Despite the limitations, NGS technologies have shown promising results to be established as the main identification means for microbial infections in clinical laboratories.

CONCLUSION

Detection of microorganisms involved in endodontic infections is essential not only for the accurate diagnosis and but also for the successful endodontic therapy. Methods for identification and detection of offending pathogens have undergone tremendous change over the years. A paradigm shift from the culture analysis to Next generation sequencing (NGS) technology has resulted in the detection of several as-yet-uncultivated species.All these identification methods in endodontics have enhanced the overall success rate of endodontic therapy.

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