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Review Article

A comprehensive review of molecular insights in endodontic microbiology

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ABSTRACT:
Molecular methods of detection in endodontics have revolutionized the way clinicians diagnose and manage root canal infections. Traditional diagnostic approaches, such as culture-based techniques, often fail to identify low-abundance or difficult-to-culture pathogens, leading to incomplete treatment and persistent infections. Recent advancements in molecular techniques, including polymerase chain reaction (PCR), real-time PCR, and DNA sequencing, offer superior sensitivity, specificity, and the ability to detect a wide range of microorganisms at the species and even strain level. These methods allow for a more accurate understanding of the microbial composition within the root canal system, providing valuable insights into infection persistence, resistance patterns, and treatment outcomes. This article explores the various molecular detection methods used in endodontics, their clinical applications, and their potential to improve treatment strategies and outcomes. The integration of these advanced techniques into routine practice could significantly enhance diagnostic accuracy, optimize therapeutic approaches, and ultimately improve patient care in endodontics.
Keywords- Molecular methods, Endodontic microbes, PCR, Hybridisation, Bacterial Cultivation and Identification, Culture methods

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INTRODUCTION

The Principles of Bacterial Cultivation and Identification is called as Phenotypic methods which form the foundation of diagnostic bacteriology by analyzing observable bacterial traits and behaviors.[1] As we advance into the 21st century, there has been a noticeable transition from traditional culture-based methods to molecular approaches for understanding infections, including endodontic infections. This shift from phenotype-based to genotype-based methods reflects the advancements in molecular biology over the past two decades.[1-4]

Molecular techniques now allow direct examination of microbial DNA and RNA, bypassing the need to analyze their enzymatic or phenotypic products. Leveraging these technologies provides valuable insights into the composition of endodontic microbiota, improving the identification of causative pathogens and enhancing success rates in endodontic treatments.[5-6]Among molecular diagnostic tools, polymerase chain reaction (PCR) stands as a gold standard.

TRADITIONAL AND MOLECULAR DIAGNOSTIC METHODS

1. CULTURE-BASED APPROACHES

For over a century, cultivating microorganisms on artificial media has been the cornerstone of diagnosing infectious diseases. This method, however, requires understanding the growth requirements of the target organisms. Many microorganisms remain uncultivable due to unknown growth factors. Of the 36 bacterial divisions identified by Hugenholtz et al., 13 consist solely of uncultivable bacteria. Recent

analyses have identified 52 bacterial phyla, of which 26 are candidate phyla, known only through gene sequencing.[7-8]

Advantages of Cultivation-Based Methods:
1. Broad-spectrum detection, including unexpected species.
2. Quantification of viable microorganisms in samples.
3. Determination of antimicrobial susceptibilities.
4. Enables physiological and pathogenicity studies.
5. Widely accessible.

Limitations of Cultivation-Based Methods:
1. Expensive and time-intensive, especially for fastidious anaerobes.
2. Limited sensitivity and specificity, relying on microbiologist expertise.
3. Dependence on strict sample transport conditions.
4. Laborious and unable to cultivate numerous bacterial species.

Challenges in Cultivation [9]
1. Nutritional Deficiency - Lack of essential nutrients or growth factors in artificial media.
2. Toxicity-Culture media may contain inhibitory substances.
3. Inhibitory Substances-Other species in a mixed culture may produce compounds that hinder target bacteria.
4. Metabolic Dependence-Certain bacteria rely on other species for growth.
5. Disrupted Communication-Separation on solid media may interfere with bacterial signaling.
6. Bacterial Dormancy-Dormant cells with low metabolic activity may require a resuscitation phase before cultivation.

Challenges in Identification [9-10]

Accurate microbial identification is crucial in clinical microbiology but hinges on successful cultivation. Even when growth is achieved, slow-growing or fastidious organisms pose significant challenges. Phenotypic identification methods are time-consuming, subjective, and rely heavily on expertise.

Key Challenges:[9-10]
1. Phenotypic Divergence-Genetically similar strains exhibit different phenotypes, complicating identification.
2. Phenotypic Convergence- Distinct strains evolve similar traits, leading to potential misidentification.

These challenges highlight the limitations of relying solely on phenotypic methods and underscore the need for integrating molecular diagnostic techniques.

2. MICROSCOPY

Microscopy can provide clues about a potential etiological agent, but it rarely offers conclusive evidence for identifying a specific species. Observations of bacterial morphology under a microscope may be misleading, as many species share similar appearances, and interpretations are often influenced by the investigator's subjective judgment. Additionally, microscopy has inherent limitations in sensitivity and specificity when detecting microorganisms in clinical samples. Its sensitivity is restricted because a significant number of microbial cells must be present to be visualized. Moreover, certain microorganisms require specialized stains or techniques to become detectable. The lack of specificity arises from the inability to differentiate species based solely on morphology and staining characteristics.[11-12-13]

3. IMMUNOLOGICAL METHODS

Immunological techniques utilize antibodies that specifically recognize microbial antigens to detect target species directly. Alternatively, antibodies that bind to host immunoglobulins specific to the target species can be employed for indirect detection. These reactions can be visualized using methods such as direct and indirect immunofluorescence, flow cytometry, and enzyme-linked immunosorbent assay (ELISA). Monoclonal antibodies are often necessary to ensure high specificity. However, the sensitivity of these methods is generally comparable to culture-dependent approaches.[11-12]

Advantages of Immunological Methods
1. Rapid identification within a few hours.
2. Capability to detect dead microorganisms.
3. Easy standardization.
4. Cost-effective.

Limitations of Immunological Methods
1. Limited to detecting only target species.
2. Low sensitivity, requiring approximately 10^4 cells for detection.
3. Specificity depends on the type and quality of antibodies used.

Advantages of Molecular Genetic Methods Over Traditional Techniques
1. Identification of both cultivable and uncultivable microbial species or strains.
2. High specificity and precise identification of strains, even with ambiguous phenotypic characteristics.
3. Direct detection of microbial species in clinical samples, bypassing the need for cultivation.
4. Greater sensitivity than traditional methods.
5. Faster processing times, facilitating rapid diagnosis.
6. Particularly useful for diagnosing life-threatening conditions caused by slow-growing microorganisms.
7. No requirement for strictly controlled anaerobic conditions during sample collection and transport,

preserving the viability of fastidious or fragile microorganisms. [10-11]
8. Effective even during antimicrobial treatment.
9. Useful for large-scale epidemiological studies, as samples can be stored and analyzed collectively.

GENOTYPIC IDENTIFICATION OF MICROORGANISMS

Genotypic identification involves analyzing specific portions of a microorganism's genome using molecular techniques for DNA or RNA detection. This process often focuses on identifying a gene or a portion of a gene, or an RNA product unique to the target organism. The detection of a specific nucleic acid sequence serves as a definitive indicator of the organism's presence. Genotypic methods offer high specificity and sensitivity, making them reliable for accurate microbial identification. [14-15]

Gene Targets For Microbial Identification

Molecular techniques for microbial identification are based on the premise that specific genes contain critical information about microbial identity. Ideally, the target gene for identification should have unique regions specific to each species. [11]

Ribosomes, intracellular particles made of rRNA and proteins, play a central role in these techniques. Ribosome sizes are measured in svedberg (S) units, which reflect how quickly particles sediment during ultracentrifugation. In bacterial and archaeal cells, ribosomes are 70S, comprising a 30S subunit with a 16S rRNA molecule (~1,540 nucleotides) and a 50S subunit containing a 23S rRNA molecule (~2,900 nucleotides) and a smaller 5S rRNA (~120 nucleotides). Fungal cells, in contrast, have 80S ribosomes composed of a 40S subunit with 18S rRNA and a 60S subunit containing 25S rRNA and 5.8S rRNA. [14]

Genes encoding large ribosomal subunits (23S and 25S rDNA) and small subunits (16S and 18S rDNA) are widely used in microbial identification, classification, and phylogenetic studies. Among these, small subunit rDNA is one of the most highly conserved macromolecules across all life forms. Its advantages include its universal presence, optimal length for analysis, and sequence regions that combine conserved segments—identical across domains—with variable regions that differ between species. These variable regions hold critical information for genus- and species-level identification through unique genetic signatures. [15]

The 16S rRNA gene for bacteria and archaea and the 18S rDNA for fungi and eukaryotes have been extensively sequenced to elucidate evolutionary relationships. Moreover, rDNA sequence data enable rapid and precise identification of known species without the need for cultivation. Currently, databases contain over 90,000 bacterial 16S rDNA sequences, whereas 23S rDNA sequences, though fewer in number (~1,400), are steadily growing. As a result, the 16S rDNA remains the most widely used target for bacterial identification in molecular studies, with the 23S rDNA emerging as a promising alternative.[14-15]

OVERVIEW OF MOLECULAR METHODS:

The molecular methods to be discussed are classified into one of three categories:
1. Hybridization
2. Amplification (PCR)
3. Sequencing
4. Enzyme digestion of nucleic acid

HYBRIDIZATION METHODS

The hybridization technique utilizes a DNA probe, which is a single-stranded DNA molecule labeled with an enzyme, radioactive isotope, or chemiluminescent marker. This probe binds to a complementary nucleic acid sequence of known identity, forming a double-stranded molecule, often referred to as a duplex or hybrid. Hybridization methods performed on macroscopic matrices, such as nylon membranes, are sometimes called "microarrays." Because hybridization relies on sequence homology, a positive reaction between nucleic acid strands from two different sources indicates genetic similarity between the organisms from which the strands originated. [16-17]

In hybridization assays, one strand (the probe) is derived from an organism of known identity, while the other strand (the target) comes from an unknown organism that needs to be detected or identified. A positive result confirms that the unknown organism is genetically similar to the probe source. Conversely, a negative result means the organism remains unidentified. Hybridization can involve DNA-DNA, DNA-RNA, or RNA-RNA interactions, depending on the assay design, as the single-stranded nucleic acid components used can be either RNA or DNA.[16]

Hybridization steps and components:

The basic steps in a hybridization assay include -
1. Production and labeling of single stranded probe nucleic acid
2. Preparation of single stranded target nucleic acid
3. Mixture and hybridization of target and probe nucleic acid
4. Detection of hybridization.

The checkerboard DNA-DNA hybridization

Socransky and colleagues developed the checkerboard DNA-DNA hybridization method, which enables the hybridization of numerous DNA samples against multiple digoxigenin-labeled whole genomic DNA or 16S rDNA-based oligonucleotide probes on a single support membrane. In this technique, denatured DNA from clinical samples is applied to lanes on a nylon membrane using a Mini slot device. Once the DNA samples are fixed to the membrane, it is positioned in a Mini blotter 45, with the sample lanes oriented at a 90° angle to the blotter’s lanes. Digoxigenin-labeled

DNA probes are then introduced into individual lanes of the blotter. After hybridization, the membrane is washed under high-stringency conditions, and the probes are detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase, followed by chemiluminescent detection. [16]
This method allows the simultaneous identification of numerous bacterial species in single or multiple clinical samples, making it particularly useful for large-scale epidemiological research. Unlike other molecular approaches, DNA-DNA hybridization offers the added advantage of not requiring microbial cultivation or DNA amplification, furtherstreamlining the process.[17]

Amplification and PCR
While hybridization methods are highly specific for detecting and identifying organisms, they often lack sufficient sensitivity. This limitation can lead to false-negative results when the target nucleic acid is present in low quantities.
The advent of molecular amplification techniques, which do not rely on organism multiplication, has addressed these limitations by significantly improving both speed and sensitivity while maintaining specificity. Polymerase Chain Reaction (PCR), developed by Kary Mullis in 1983, has revolutionized molecular biology. It enables the amplification of a single gene copy into millions or even billions. Today, PCR is a cornerstone technology in genome sequencing, allowing researchers to isolate virtually any gene from any organism.[16]
PCR involves the in vitro replication of DNA through repetitive cycles that include:

1. **Denaturation**-Separation of the DNA strands.
2. **Primer Annealing**-Binding of primers to specific target sequences.
3. **Extension/Polymerization**-Synthesis of new DNA strands by DNA polymerase.

Variants of PCR
Several derivatives of the PCR technique have been developed to meet specific research and diagnostic needs, including:

1. Multiplex PCR
2. Nested PCR
3. Arbitrary Primed PCR
4. Quantitative PCR (qPCR)
5. Reverse Transcriptase PCR (RT-PCR)
6. Real-Time PCR

Multiplex PCR
Multiplex PCR enables the simultaneous detection of multiple bacterial species in a single reaction. It uses several primer pairs, each targeting a specific bacterial species, allowing for efficient use of time, reagents, and DNA templates. To ensure successful amplification, primers in multiplex assays must be carefully designed to have similar annealing temperatures and avoid complementary sequences that could lead to primer-dimer formation. [18]

Nested PCR
Nested PCR is a technique that increases sensitivity by using the product of a primary PCR amplification as the template for a second PCR reaction. The second round employs a distinct set of primers that anneal internally to the initial PCR products, enhancing specificity and sensitivity. This approach allows for the detection of target DNA at levels significantly lower than conventional PCR, owing to the large number of amplification cycles and the dilution of non-target DNA and inhibitors during the first round. Additionally, the second primer set ensures specificity by reducing the background interference of eukaryotic DNA and non-target bacterial DNA. Even if nonspecific amplification occurs during the first round, these products are unlikely to serve as templates in the second reaction, as they typically lack complementary regions for the second primer set. However, the major drawback of nested PCR is the high risk of contamination when transferring products from the first round to the second reaction tube, necessitating stringent precautions.[19]

Reverse Transcriptase PCR (RT-PCR)
RT-PCR is designed to amplify RNA targets by leveraging the enzyme reverse transcriptase, which synthesizes complementary DNA (cDNA) from an RNA template. Most RT-PCR protocols follow a two-step process: first, reverse transcriptase converts RNA into single-stranded cDNA. Second, PCR primers, DNA polymerase, and nucleotides are used to synthesize the complementary strand, forming a double-stranded DNA template for amplification. This process can also be streamlined into a one-step approach, where an enzyme with both reverse transcriptase and DNA polymerase activities, such as that from Thermus thermophilus, is utilized. [17]

Arbitrary Primed PCR (AP-PCR)
AP-PCR, also known as Random Amplified Polymorphic DNA (RAPD), is a genomic fingerprinting technique used for clonal analysis of microorganisms. This method employs a random sequence primer of 10-20 bases, which binds to unspecified DNA targets under low stringency conditions, allowing for mismatched base-pairing. The genetic variations between DNA templates produce unique DNA fingerprints visible on electrophoretic gels, which can indicate strain specificity. AP-PCR offers the advantage of generating highly specific DNA profiles without requiring prior knowledge of the DNA sequence. It is particularly useful for identifying epidemiological relationships between isolates and tracing the origins of microorganisms in disease settings. [10-11]

Real-Time PCR
Real-time PCR combines traditional amplification

with the ability to detect targets in real time using fluorescently labeled probes. This technique allows for rapid detection, often within 30-120 minutes, and continuous measurement of the amplification process, providing quantitative results. Unlike conventional PCR, which is generally qualitative or semi-quantitative, real-time PCR monitors the accumulation of the amplification product throughout the reaction, offering unparalleled speed and precision[6-7]

Advantages of PCR

1. High sensitivity, detecting as few as 1-10 cells.
2. Excellent specificity under optimized conditions.
3. Rapid results, typically within hours.

Disadvantages of PCR

1. Limited quantitative capabilities, except in real-time PCR.
2. Most assays target only one or a few species, though broad-range PCR can identify diverse species.
3. Laborious and costly processes in some cases (e.g., broad-range PCR).
4. Challenges with microorganisms having thick cell walls, requiring additional lysis steps.
5. Risk of false positives due to contamination and false negatives from inhibitors or degraded DNA.

Advantages of Molecular Biology Methods

1. Detect both cultivable and uncultivable species.
2. Provide high specificity and accuracy, even for ambiguous strains.
3. Allow direct detection in clinical samples.
4. Rapid results, often within minutes to hours.
5. Effective during antimicrobial treatment.
6. Samples can be stored frozen and DNA transported easily.
7. Detect dead microorganisms.

Limitations of Molecular Biology Methods

1. Many assays are qualitative or semi-quantitative.
2. Most methods detect a limited number of species at a time.
3. Some techniques are labor-intensive and expensive.
4. Risk of bias in broad-range PCR due to homogenization and DNA extraction steps.
5. Hybridization assays using whole genome probes are restricted to cultivable species.
6. Detection of dead microorganisms may lead to misinterpretation.

CONCLUSION

In conclusion, molecular methods of detection in endodontics represent a significant advancement in the diagnosis and management of endodontic infections. Techniques such as polymerase chain reaction (PCR), real-time PCR, and DNA sequencing offer unparalleled sensitivity and specificity in detecting microbial pathogens at the species level, even in cases of low microbial load. These methods enable clinicians to identify resistant strains of bacteria, assess the microbial diversity within root canal systems, and guide more effective treatment strategies. Moreover, molecular diagnostics can improve outcomes by facilitating targeted therapy, reducing the reliance on traditional culture methods, and offering insights into the dynamics of microbial colonization in the root canal. While the integration of these technologies into clinical practice may require specialized equipment and expertise, their potential to enhance diagnostic precision and therapeutic success makes them an invaluable tool in modern endodontic care.

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