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Review Article

Molecular Biology for better basic medical science

Molecular Diagnostics In Periodontics - A Review

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Abstract: Although over 700 bacterial species make up the oral flora, it is thought that only a handful of species contribute to the initiation and progression of periodontitis. For over 20 years, culture and microscopy techniques have been the primary method of identifying and studying putative pathogens. The very fact that each organism has a unique 16s rRNA sequence is the basis for the evolution of the Molecular Science Approximately 35% of the oral bacterial species have not yet been cultured in vitro. The drawbacks of these various techniques have unraveled the scope of molecular diagnostic techniques in periodontal diagnostics. With the application of these methods significant improvements have been made in the field of periodontal diagnostics. These tests are based on the identification of the inflammatory mediators, decomposed tissue products or detection of bacteria or its antigens. Herein our focus is to describe these DNA based, culture independent methods for their use or potential use in molecular microbial diagnosis and particularly highlight the methods that are prominent in the field of Periodontics.

Keywords: Molecular diagnostics, PCR, DNA Hybridization, Sequencing.

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1. INTRODUCTION

The microbiology of Periodontal disease has been subjected to intense exploration for years together and still remains to be a wonder as more organisms are being identified to be associated with the disease. Various types of microbiological tests can be used for evaluation of etiological factors, evaluation of disease activity, determination of treatment effects and monitoring the recall interval. Numerous tests can be used for detection of defense mechanisms of the host, subgingival microorganisms and its products, such as phase-contrast microscopy, dark-field microscopy, bacterial culture, immunological tests, nucleic acid tests, enzyme tests, etc,. A significant proportion of the total oral microbiota remains unculturable, and hence non-culture methods are required to describe the overall species richness of the oral microbiome, leading to the inclination of the field of science to a more molecular level. Molecular techniques open up gates to understanding the microbial life in a much deeper plane i.e., the structure and functions of the macromolecules proteins and nucleic acids that are essential for all living organisms. The very fact that each organism has a unique 16s rRNA sequence is the basis for the evolution of this branch. The 16S rRNA is a tool commonly used for identifying bacteria for several reasons.[1] First, the gene is relatively short (approximately 1,500 bp). Second, there are ten regions in the 16S rRNA gene sequence that are common among most bacteria (conserved region) and are separated into nine diverse regions (hypervariable regions). Therefore, some universal primers are established in the conserved regions. Third, the gene sequences registered in public databases are increasing substantially, because the gene sequence is important information for identification and classification in bacterial taxonomic studies. These molecular technologies thus have allowed us, for instance, to examine the presence of the unculturable segment of the microbiota in greater detail by targeting this 16S rRNA region, expanding our knowledge of the diversity of the supra-gingival and the subgingival microbiota. These techniques support the analysis of large samples in shorter duration circumventing some of the limitations of the culture techniques.The various molecular microbial analysis methods that are employed in the field of Periodontics can be categorized under the following headings -

- Polymerase Chain Reaction (PCR) based methods
- DNA - DNA hybridization based methods
- Sequencing methods

This review will provide an overview of the various techniques available with each of the above mentioned molecular analysis methods.

1.1 Polymerase chain reaction based methods

PCR, Polymerase Chain Reaction is one of the most potent diagnostic tool available in the field of medical science since its invention by Dr. Kary Mullis in 1987.[2]It is used to amplify a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded (ds) DNA molecule. The polymerase chain reaction can be used to amplify both double and single stranded DNA. Generally, PCR amplifies small DNA targets 100-1000 base pairs (bp) long. It is technically difficult to amplify targets >5000 bp long. A pair of single stranded oligonucleotide primers, which have DNA sequences complementary to the flanking regions of the target sequence, must be synthesized. The primers are complementary to either end of the target sequence but lie on opposite strands. The primers are usually 20-30 nucleotides long and bind to complementary flanking region at 3' end.[2,3]

1.1.1 Requirements

- Thermal cycler (thermocycler)
- PCR amplification mix typically containing :-
- Sample dsDNA with a target sequence
- Thermostable DNA polymerase
- Two oligonucleotide primers
- Deoxynucleotide triphosphates (dNTPs)
- Reaction buffer containing magnesium ions and other components

1.1.2 A typical thermal cycle might be as follows

- Heat denaturation at 94°C for 20 seconds
- Primer annealing at 55°C for 20 seconds
- Primer extension at 72°C for 30 seconds

Average time for each cycle is approximately 4-5 minutes, considering the fact that heating and cooling between each stage also have to be considered. Initially these steps at three different temperatures were carried out in separate water baths but nowadays a thermal cycler is used (a machine that automatically changes the temperature at the correct time for each of the stages and can be programmed to carry out a set number of cycles). After the introduction of thermocycler, each cycle of replication can be completed in less than 5 minutes. After 30 cycles, a single molecule of DNA is amplified into more than a billion copies ($230 = 1.02 \times 10^9$).[2]

1.1.3 Post amplification detection

Following PCR, the amplification product can be detected using gel electrophoresis followed by ethidium bromide staining and visualization with UV transillumination.[2]

1.1.4 Modifications and different types of PCR

- Real-time PCR
- Inverse PCR
- Reverse Transcriptase PCR (RT-PCR)
- Multiplex PCR
- Nested PCR
- Long-range PCR
- Single target PCR
- Fast-cycling PCR
- Methylation-specific PCR (MSP)
- Hot start PCR
- In situ PCR
- Asymmetric PCR
- Assemble PCR
- Inter Sequence-specific PCR(ISSR)
- Ligation-mediated PCR
- Methylation –specific PCR
- Mini Primer PCR
- Solid phase PCR
- Touchdown PCR
- Allele specific PCR

Of these extensive types of PCR available, the most frequently or widely reviewed type of PCR in the field of Periodontology are the Real time PCR, Reverse transcriptase

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PCR, Single Target PCR, Multiplex PCR. However, the other types are still widely used in the field of Medicine and is yet to find its path in Periodontology.

1.1.5 Single Target PCR

A very great specificity is achieved for amplification of the desired sequence of DNA in this method. It has been devised with such remarkable sensitivity that it can detect a single molecule of a specific sequence. The amplification is accomplished by first denaturing the DNA sample, hybridizing two oligonucleotide primers to the DNA, elongating with DNA polymerase, and repeating this cycle up to 40 times. As a result, each round of synthesis doubles the number of product DNA molecules present.[2,4]

1.1.6 Multiplex PCR

This technique is an expansion of single target PCR methodology. It was first described in 1988 by Chamberlain. Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more target sequences can be amplified by including more than one pair of primers in the same reaction. Multiplex PCR has the potential to produce considerable savings of time and effort in the laboratory. However, challenge would be in designing the PCR primers compatible with one another.[2,4,5]

1.1.7 Real Time PCR

Real-Time PCR is identical to a simple PCR except that the progress of the reaction in monitored by a camera or detector in "real-time". There are a number of techniques that are used to allow the progress of a PCR to be monitored. Each technique uses some kind of fluorescent marker which binds to the DNA. Hence as the number of gene copies increases during the reaction so the fluorescence increases. This is advantageous because the efficiency and rate of the reaction can be seen. There is also no need to run the PCR product out on a gel after the reaction. There are two types of real-time PCR, namely an intercalator-based method and a probe-based method. The intercalator-based method, also known as the SYBR Green method, intercalates SYBR Green, which binds to newly synthesized double-stranded DNA producing a fluorescently labeled PCR amplicon. The probe-based method, or TaqMan PCR, is more specific in that it utilizes a fluorogenic-labeled probe that binds only to its complementary sequence in the internal portion of the generated PCR amplicon.[2,6,7,8]

1.1.8 Reverse Transcriptase PCR

Reverse transcription (RT) followed by polymerase chain reaction (PCR) represents a powerful tool for the detection and quantification of mRNA. RNA can be better than genomic DNA for detecting structural changes in long genes, since amplifying the spliced RNA transcript instead of the genomic sequence greatly reduces the length of DNA to be handled without losing any of the coding regions where clinically significant deletions may be expected. Real-time RT-PCR (or kinetic RT PCR) is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range.[9,10,11,12]

1.2 DNA-DNA hybridization based methods

DNA-DNA hybridization based methods are highly specific in nature and doesn't require cell viability. They are majorly used for epidemiological or ecological study purposes. It measures the similarity of the genomes by estimating the amount of heat required to melt the hydrogen bonds between the base pairs of the double helix of the DNA. The comparison may be between the two DNA strands of an individual or of different individuals representing different levels of genetic and taxonomic divergence. The melting temperature of a DNA duplex molecule is a function of the number of correctly base-paired nucleotides. There are two types of DNA - DNA Hybridization techniques that are most commonly employed in Periodontology, they are

a. Checkerboard DNA hybridization
b. In situ hybridization

1.2.1 Checkerboard DNA Hybridization

It was introduced by Socransky et al. in 1994 to be used in studies of the oral microbiota. Cassio do Nascimento et al in the year 2006 proposed a modified methodology for the Checkerboard DNA hybridization. They suggested to employ fluorescein as a labeling reagent as an alternative to the widely used DNA labeling reagent, digoxigenin.[13,14] The same author in the year 2010 again suggested a modified protocol which employs rapid direct labeling and detection of whole genomic DNA probes. This was given to overcome the technical difficulties associated with the original method proposed by Socransky et al in 1994, the material costs and mainly the processing time. The whole genomic DNA probes were directly labeled with thermostable alkaline phosphatase enzyme. Briefly, the denatured DNA was mixed with the labeling buffer and alkaline phosphatase enzyme. Formaldehyde was then covalently cross linked to the probe. These labeled probed are optimized to distinctively detect cells with the lowest possible background like for example even at 10^4.[14]

1.2.2 In situ Hybridization

In situ hybridization is a technique that is used for localization and detection of specific DNA and RNA sequences by hybridizing the complementary strand of a nucleotide probe to a particular sequence. Particular sequences are identified by their ability to specifically anneal to each other to form hybrids. After a labeled probe is annealed to matching sequences in fixed cells or tissue, the hybridized probe is visualized. There are two main approaches for labeling a probe; radioisotope labeling and non-isotope labeling.[15] Radioisotope labeling is considered the most sensitive method of labeling by many researchers, but others believe that non-isotopic methods are just as sensitive. The results of radioisotope labeling are easily quantified or semi-quantified. Drawbacks of radioisotope labeling include along exposure time, poor spatial resolution, risk of exposure to radioactivity and difficulty in disposal of radioactive waste. For non-isotopic labeling, compounds including biotin, fluorescein, digoxigenin, alkaline phosphatase, or bromodeoxyuridine are used and are visualized by histochemistry or immunohistochemistry (Jin and Llyod, 1997). Drawbacks of non-isotopic labeling are that it is generally considered not as sensitive as radioactive labeling, and the hybridization results are difficult to quantify.[15]

1.3 Sequencing based methods

Sequencing has made identification of the slow and fast growing bacteria easy by reducing the time required for the analysis as well as in reduction of the lab equipment. However the cost of the test for processing, still remains to be a challenge that is yet to be met in the field so as to make the test available for everyday use. There are various types of sequencing methods available in the field of medicine like

- Sanger's sequencing
- Pyrosequencing
- Single molecule sequencing
- Nanopore based sequencing

Of these types, few of the sequencing methods have found its way into periodontology and is being used for molecular analysis of the samples (for example - dental plaque) are discussed in the following headings.

1.3.1 Sanger's Sequencing

Sanger and his co-workers described a new breakthrough method for sequencing oligonucleotides via enzymatic polymerization (Sanger *et al*. 1977). This was initially known as the chain termination method or the dideoxynucleotide method. There are two different approaches on this type of sequencing described in the literature - the random approach and the direct approach.[16]

1.3.2 Random approach

Also known as the shotgun sequencing. It is a random process because there is no control of the region that is going to be sequenced. The steps are represented below in the flowchart (figure 1).

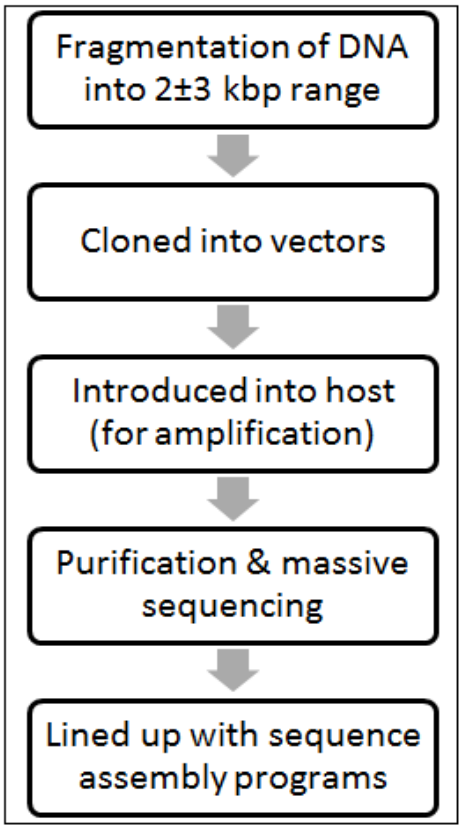

Fig 1.Steps in Sanger sequencing - random approach

1.3.3 Direct approach

In this method the unknown DNA is directly sequenced within sites in which the sequence is known.[16] The unknown sequence of DNA is inserted into a vector and amplified. The first sequencing reaction is performed using primers that hybridize to the vector sequence and polymerize the strand complementary to the template. Later a second priming site is chosen inside the newly generated sequence, following the same direction as the previous one. This approach is known as primer walking.[17,18]

1.3.4 Pyrosequencing

Pyrosequencing technique is based on the principle of sequencing - by - synthesis given by Hyman and Melamede (1985 and 1988) and on the detection of released Pyrophosphate (PPi) during DNA synthesis.[19] It employs a series of four enzymes to accurately detect nucleic acid sequences during the synthesis - DNA polymerase, ATP sulfurylase, luciferase and apyrase (Gharizadeh *et al*., 2007).[20]The four different nucleotides are added stepwise to the immobilized primed DNA template and the incorporation event is followed using the enzyme ATP sulfurylase and luciferase. The intensity of the light corresponds to the number of nucleotides of the same type that have been incorporated. For example, if there are three consecutive As in the fragment, the amount of light generated would be three times that of a single A in the fragment. By plotting this pattern of light intensity on a graph, the sequence of the original piece of DNA can be decoded. After each nucleotide addition, a washing step is performed to allow iterative addition. The multiple other techniques available is yet to form a more strong affiliation to the field of

Periodontics and lay down new avenues for the scope of a molecular level assessment.

2. DISCUSSION

The inability of current periodontal variables to provide high-confidence information on present or future disease progression may result in diagnostic misjudgement and over-treatment or under-treatment.[21] Rapid and cost-effective methods to predict active periodontitis in clinical practice remain an important research goal. Periodontal research has explored integrative biology to gain better insights into the pathogenesis of periodontitis. The diversity in genetic, infectious and immunologic subtypes of periodontal disease argues for personalized therapy. Periodontics of the past did not assess specific etiologies, probably because of cumbersome and expensive testing methodologies, but molecular technologies have advanced the concept of precision Periodontics . Multiplex nucleic acid-based tests are rapid methods used to quantify markers in biological samples and are particularly well suited for development of user-friendly diagnostics. Early disease detection and targeted treatments are expected to improve periodontal health status and yield significant cost savings, and become an important part of future dentistry.Modern molecular technologies have significantly improved studies on periodontal pathogenic agents, virulence genes and haplotype aberrations, and genomic data analysis is becoming user-friendly and scalable. These techniques have the potential for rapid identification of collective communities of periodontal viruses, bacteria, fungi and parasites, and is cost- efficient when processing higher numbers of samples. Metagenomic and metabolomic functional profiling of periodontal microbial communities are used in longitudinal comparisons of patient cases and control groups.[22] Disease-related shifts in the composition of the periodontal microbiome parallel changes in immune responses, and studies that use omics technologies have begun to provide a molecular-level understanding of the successional dynamics, stability and perturbation of the periodontal ecosystem. Such knowledge might foster innovative methods of ecosystem management to prevent and control periodontal disease. Computerized algorithms may enhance periodontal diagnosis, therapy and prognosis and structure clinical decision making. In the long term, novel diagnostic and therapeutic methodologies may permit patients to self-detect and arrest mild- moderate Periodontitis.

2.1 PCR based methods

Multiple studies have utilized Single target PCR method to detect specific species directly from oral clinical samples. Multiplex PCR technique is an expansion of single target PCR methodology in which more than one pair of species specific primers are used in a single PCR assay, that permits multiple species to be detected simultaneously. Such assays have been used to detect A. actinomycetemcomitans, T. forsythia and P. gingivalis all at the same time.[23,24] Although the optimization of multiplex PCR is laborious, these assays are quite sensitive, with detection limits of 10–100 cells per PCR reaction. Real-time PCR or quantitative PCR is a method that quantifies the number of DNA copies in a clinical sample. There are two types of real-time PCR, namely an intercalator-based method and a probe-based method of which the latter is more specific, in that it utilizes a fluorogenic-labeled TaqMan probe that binds only to its complementary sequence in the internal portion of the generated PCR amplicon.[25,26]There are several studies in literature that quotes the accuracy of the quantitative PCR method by comparing them with the traditional culture based methods in periodontitis conditions.[25,27-31-] All of these studies are conclusive of the fact that quantitative PCR technique is extremely sensitive and specific with a high positive predictive value for the targeted bacteria.[32]PCR assay can be preceded by a reverse transcription when RNA is the available genetic material (RT-PCR). RT-PCR is more sensitive and easier to perform than other RNA analysis techniques. RT-PCR has also been used in dental research in order to assess the expression of mRNA for proteins involved in the development of periodontal disease.[33,34] High sensitivity (getting enough product from small samples) and high specificity (selective amplification of only the desired product) are the hallmarks of successful PCR. Optimal RT-PCR and PCR can be obtained through careful experimental design, including selecting the appropriate enzymes, designing optimal primers, using different buffers and additives, establishing cycling parameters, and preparing high quality templates.[33]

2.2 DNA-DNA Hybridization methods

In a landmark paper, Socransky et al. analyzed about 13,000 plaque samples using whole genomic DNA probes in checkerboard hybridization assays to define bacterial complexes, that were involved in oral health and periodontal disease.[35,36]Lately, checkerboard hybridization has also been used for the quantification of multiple inflammatory mediators in gingival crevicular fluid samples.[37]In situ hybridization can be used to quantify, determine the spatial configuration and demonstrate the morphology of individual bacterial cells in complex natural communities, such as dental plaque.[38] In FISH, fluorescently labeled, ribosomal RNA-targeted oligonucleotides are hybridized to partially fixed, whole cells on microscope slides and are visualized using fluorescence or confocal fluorescence microscopy. [39,40]Bhat KG et al in his study suggest the use of FISH technique as a diagnostic tool not only for its ability to rapidly detect and quantify the organisms, for its high sensitivity to detect even a single microbial cell in a mixed bacground.[41] The only limitation of this technique is the need for fluorescent microscope which is pretty expensive and further research is under process to improve the specificity of the method by increasing the length of the probes used and the usage of peptide nucleic acids as probes.[42]

2.3 Sequencing methods

Next-generation sequencing is the more recent technology for high-throughput genomic analysis using a pyrosequencing platform. Most next-generation sequencing technologies eliminate the need for cloning and sequencing by amplifying a single DNA molecule.[43,44] The three main next-generation sequencing technologies are the 454 pyrosequencing, SOLiD, Illumina methodology. 454 pyrosequencing methodology allows reads of 400,000 DNAs that are each about 250 bases in length, although newer technology allows for longer reads of up to 500 bp. SOLiD, which is similar to 454 pyrosequencing in that fragmented DNA is amplified on an agarose bead. Illumina / Solexa methodology also utilizes fragmented DNA and specialized adaptors, but attaches to a slide rather than a bead. SOLid and Illumina allow for millions of reads, albeit only 35–50 bp in length.[44] The transition from

Sanger sequencing to 454 sequencing has made it possible to collect over hundreds of thousands of sequences spanning hundreds of samples.[45] Also there are studies that suggest by using a variant of barcoding strategy for 454 with Ilumina platform could analyze thousands of sample in a single run with remarkable depth.[46,47]The sequence accuracy of the Next Generation Sequencing (NGS) technologies depends on the alignment depth with enormous sequence reads which are comparatively short and low quality rather than on the individual sequence reads. Therefore each sequence read of the NGS technologies is more irregular than the Sanger method in length and accuracy. On the contrary, the Sanger method could be applied in studies to clarify presence of the predominant bacteria in a community at the genus or species level.[48]Molecular diagnosis may soon become the standard detection method for the various periodontal pathogen complexes owing to the inherent capacity of sensitivity and specificity of the various molecular methods described. As the scientific questions are answered and the technology improves, then many of these techniques will probably be utilized as diagnostic tools.

3. CONCLUSION

The competitiveness of the various molecular techniques is promising in producing greater insight, starting from identification of periodontal pathogens to effective therapeutic approach. Hopefully, all of these techniques would soon evolve to be an even more user - friendly tool in clinical microbiology laboratories in the coming years.

4. AUTHORS CONTRIBUTION STATEMENT

All the authors contributed equally from the conception and design of the work, data collection and analysis, drafting of the final article and its critical revision.

5. CONFLICT OF INTEREST

Conflict of interest declared none.

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