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Molecular tools for the diagnosis of periodontitis

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Abstract

Periodontitis is a multifactorial chronic inflammatory disease associated with dysbiotic biofilms and aberrant host inflammatory response. It is characterized by destruction of the tissues that support the teeth. Periodontitis is the major cause of tooth loss in adults significantly affecting the quality of life and is associated with many chronic non communicable diseases by contributing to systemic inflammatory burden. Early and accurate diagnosis is the key to the successful management of periodontitis as the entire treatment plan, prognosis, and maintenance directly depend on the quality of periodontal diagnosis. Traditionally the diagnosis of Periodontitis is based on recording medical and dental history, periodontal examination and radiographic findings. The current periodontal diagnostic process reveals only historical tissue destruction and does not provide any information regarding current disease activity, future progression or for monitoring response to therapy. For these reasons, new molecular diagnostic aids are being developed that allow an early detection of disease, determine the presence of current disease activity, predict sites at risk for future breakdown and monitor the response to periodontal therapy. Advanced molecular diagnostic techniques are a class of diagnostic tests that are used to detect and measure nucleic acids, proteins or metabolites in clinical samples to identify risk factors, screen asymptomatic patients, provide more accurate diagnosis and guide the process of development of an ideal therapeutic intervention. This paper provides a review of the molecular diagnostic tools that have the potential to be utilized for diagnosis and management of periodontitis.

Keywords: Periodontitis, diagnosis, molecular aids, screening, prognosis, PCR.

INTRODUCTION

Periodontitis is a chronic multifactorial inflammatory disease which affects the supporting structures of the teeth (periodontium).[1] It results in an irreversible loss of periodontal ligament, destruction of alveolar bone and, if left untreated, can lead to loss of tooth.[2] The tissue destruction in periodontitis is mainly caused by dysregulated host immunoinflammatory response induced by dysbiosis of periodontal microbiota and is determined by genetic factors, epigenetic influences, systemic diseases (such as diabetes mellitus) and environmental factors (such as smoking, diet and stress).[2-5] The clinical features of periodontitis include gingival bleeding, formation of periodontal pockets, gingival recession, tooth mobility and halitosis. However, the disease is usually asymptomatic in its early stages and many patients do not seek dental care.until significant disease progression has taken place. [1,6,7]

The disease progresses in a nonlinear fashion and is characterized by periods of activity and quiescence.[2,8] Periodontits is highly prevalent, with prevalence rates around 50% and severe periodontitis is the sixth most common human disease worldwide affecting 11.2% of the global population.[9,10] Periodontitis is one of the major cause of tooth loss leading to masticatory disability, compromised speech, poor aesthetics and reduced quality of life.[7] It is a significant burden on global health care system with annual spending of $54 billion in direct treatment costs and a further $25 billion in indirect costs.[11]

Moreover, periodontitis contributes to the chronic, systemic inflammatory burden via multiple mechanisms[12] and is associated with higher mortality and increased risk of several noncommunicable diseases such as diabetes mellitus, cardiovascular diseases, chronic kidney diseases and chronic obstructive pulmonary disease.[13,14]

DIAGNOSIS

Because periodontitis is usually “silent” initially and the ability to regenerate the periodontium is limited after alveolar bone loss, early diagnosis and prompt treatment should be the main goals of periodontal therapy.[15, 16] Early diagnosis followed by effective periodontal treatment can improve oral as well as general health and reduce the social and financial burden associated with periodontal care.[7,15]

Periodontal diagnosis involves:

1. Public health campaigns to increase public awareness regarding periodontal disease so that patients can self-detect signs and symptoms of periodontal disease and seek professional care.
2. Periodic professional periodontal screening to categorize individuals into health, gingivitis and periodontitis.
3. Complete periodontal examination and diagnosis to organize effective treatment of periodontitis.[7]

Traditional methods of periodontal diagnosis include taking medical and dental histories from the patient, utilizing diagnostic imaging like digital photographs, comprehensive periodontal examination and radiographic assessment of alveolar bone loss.[17,18] Complete periodontal examination includes visual inspection for signs of inflammation like redness and swelling, assessment of plaque and plaque retention factors and assessment of the degree of existing periodontal destruction by measuring probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP), gingival recession and tooth mobility with calibrated periodontal probes.[17]

Bleeding on probing is used to diagnose gingival inflammation and as a predictor of future periodontal breakdown.[19, 20] Absence of BOP is a good indicator of periodontal stability (negative predictive value of 98.1%) [21] and continuous presence of BOP is a predictor of future periodontal breakdown(positive predictive value of 30%).[22,23]

Periodontal diagnosis should also include an assessment of risk factors for periodontitis such as smoking and diabetes as heavy cigarette smokers are at high risk for periodontal disease progression.[7, 24] Moreover periodontitis shares many risk factors with other chronic diseases like cardiovascular disease and diabetes mellitus and assessment of periodontal risk factors is a part of the Common Risk Factor Approach strongly advocated by the WHO for improving human health.[7]

However, traditional diagnostic methods only reveal previous periodontal disease and do not provide information about current disease activity, risk of future tissue destruction and response to periodontal therapy.[25, 26]

Moreover, periodontal probing is painful, invasive as well as laborious and development of a more acceptable diagnostic tool for early diagnosis of Periodontitis is the most investigated field of periodontal research.[25, 27]

The ideal periodontal diagnostic method should be able firstly to screen susceptible subjects in the general population, secondly to differentiate active and inactive sites, thirdly to predict future tissue destruction in particular individuals and sites, and finally to monitor the response to periodontal therapy The ideal periodontal diagnostic method should have the ability to screen susceptible individuals in the general population, diagnose active periodontal disease, predict future tissue destruction, monitor the response to treatment and reveal disease progression.[24, 25]

MOLECULAR DIAGNOSTIC AIDS

As already mentioned, periodontitis is a complex, multifactorial, chronic infalmmatory disease which arises as a result of interplay between multiple casual componenets including dysbiotic microbial biofilms and host's immune- inflammatory response as well as environmental and behavioral factors, genetic and epigenetic factors, systemic diseases and miscellaneous factors (such as dentition and tooth-related).[28,29]

The oral microbiome inhabiting the human oral cavity consists of over 700 species and about 400 species are found in the subgingival plaque.[30]

The periodontal microbiota from a healthy state to Periodontitis demonstrate an ecological change, with emergence of dominant (anaerobic gram negative) species in periodontitis without replacement of health-associated (gram positive) species and an increase in bacterial biomass with count of intraoral bacteria in periodontal health being around 1_x10^9 whereas in case of periodontitis, these amounts exceed counts of 1_x10^{87}.[29,31,32]

Evaluation of subgingival biofilm by traditional microbiological approaches associate periodontits with the rise of key periodontal pathogens[33] namely Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia (red complex bacteria) as well as Actinobacillus actinomycetemcomitans (Aa), Prevotella intermedia (Pi), Fusobacterium nucleatum (Fn), and Filifactor alocis (Fa). These bacteria are located in periodontal pockets in increased numbers suggesting that these can be potential biomarkers for the disease diagnosis.[30,31]

However recent advances in molecular diagnostic techniques have changed this traditional view on the role of bacteria in Periodontitis.[34, 35]

According to this new model periodontitis is caused by complex alteration (dysbiosis) of entire microbial community rather than by appearance of few key periopathogens.[34, 36]

Dental plaque is now regarded as "whole organism" (meta organism) where each bacterium is dependent on the presence of other species and studies utilizing culture independent diagnostic techniques have revealed high diversity of plaque bacteria as well as complex interactions within the subgingival microbiota and between the microbes and the host.[33,35,37]. Hence, diagnostic tools deciphering microbial function in oral biofilms rather than microbial composition can provide a better understanding of the role of bacteria in periodontitis.[37]

Heritability plays an important role in the etiology of periodontitis by controlling the structural integrity of the periodontium as well as affecting the host response to periopathogens.[38] In younger patients the contribution of genetic factors may be around 50% of all causal factors, while in older patients the genetic contribution to the total of all causes is at most 25%.

Like other chronic inflammatory diseases, Periodontitis is polygenic and variants in at least 65 genes has been suggested to be associated with this disease.[29]

By analyzing allelic variants of genes associated with Periodontitis, patients with enhanced disease susceptibility could be identified and this genetic information can be used to develop strategies aimed at diagnosis and treatment of Periodontitis.[39,40]

Plaque microorganisms account for only 20% of the risk for Periodontitis and the remaining 80% is contributed by environmental and genetic factors that modify inflammation and its resolution.[32]

Since Periodontitis is a multifactorial disease and current diagnosis of the disease is based on clinical rather etiologic criteria, a more sensitive and specific molecular diagnostic tool is required which could facilitate an earlier diagnosis before clinical manifestations, generate an accurate prognosis, and provide therapeutic guidance.[28,41,42]

In order to address the limitations associated with current diagnostic methods, molecular diagnostic aids are used.[35, 43]

Molecular diagnostics are class of widely used and highly sensitive diagnostic aids that assess a patient's health at a molecular level by examining the alterations of DNA, RNA, proteins or the metabolites in saliva, gingival crevicular fluid (GCF), periodontal tissues and dental plaque.[44-47] These tests detect and quantify the presence of specific

viruses, bacteria, or types of cells and examine patient's genes to determine the presence of disease associated mutations.[35]

Thus, molecular techniques provide human gene information as well as microbial genetic and functional information which can help in better understanding of periodontal disease pathogenesis.[37]

Since molecular diagnostic tests analyze samples at DNA, RNA, protein, or metabolite levels, the studies utilizing these methods are termed as genomics/epigenomics (the study of the genome/epigenome), transcriptomics(the study of mRNA), proteomics(, the study of functional proteins) and metabolomics (the study of cellular metabolism).[48,49]

Similarly metagenomics, meta-transcriptomics, meta-proteomics, and meta-metabolomics are the studies used to understand the composition and function of microorganisms within the biofilm.[37,50]

Molecular diagnostics therefore can assess a person's risk of developing a disease, screen for diseases that are present but asymptomatic, provide a diagnosis of existing symptoms and guide in therapy selection/monitoring.[44]

Traditional culture based methods of microbial detection, once considered as a golden standard, have several shortcomings like requirement of specific growth conditions(as most periopathogens are anaerobic), issues with sampling and transportation, difficulties in maintaining bacterial vitality, extensive waiting periods before diagnosis, inability to discriminate between closely related taxa, and low detection values of bacterial cells.[31]

Molecular techniques have many advantages over traditional culture methods like providing results rapidly and more accurately, ability to detect and identify all micro-organisms in oral biofilms including the uncultivables, unraveling true complexity of oral microbiome and detecting emergence of antibiotic resistance in periodontal pathogens.[33,37,50]

This article reviews the current status of these culture-independent molecular diagnostic techniques for use or potential use in diagnosis of periodontitis.

Molecular DNA-based diagnostic aids have been used in the assessment of the qualitative and quantitative composition of periodontal pathogens directly from clinical samples eliminating the need for in vitro cultivation. Molecular diagnostic techniques used for the purpose of periodontal diagnosis are classified into three broad categories based on the (1) amplification technologies which include single target polymerase chain reaction(PCR), multiplex PCR, quantitative PCR reverse transcription-PCR (RT-PCR), and loop-mediated isothermal amplification (LAMP) technique; (2) analysis of nucleic acids by hybridization such as in situ hybridization, checkerboard hybridization, and 16S rRNA-based microarrays; and(3) sequencing technology including the latest, next-generation sequencing (NGS) techniques, such as pyrosequencing, real-time single-molecule DNA sequencing, and nanopore-based sequencing.[31,45,51]

Other molecular diagnostic techniques like mass spectrometry, nuclear magnetic resonance (NMR) spectroscopy, SDS-Polyacrylamide Gel Electrophoresis, Two-Dimensional Gel Electrophoresis and Liquid chromatography(Gel filtration chromatography, ion-exchange chromatography, affinity chromatography) are used to study proteins, nucleic acids, and other molecules in various samples**.** [52]

MOLECULAR DIAGNOSTIC TECHNIQUES BASED ON AMPLIFICATION TECHNOLOGIES

Polymerase chain reaction (PCR)-based methods

Polymerase chain reaction, developed by chemist Dr. Kara Mullis in 1983, is the most widely used amplification method that reflects the natural process of replication of genetic material and enables to amplify a single copy of a particular DNA sequence to generate thousands to millions of copies thus facilitating ready detection.[35,45,51]

PCR-based molecular diagnostic techniques are capable of qualitatitive and quantitative assessment of bacterial DNA or RNA, which can aid determination of pathogenic organisms that are not culturable or have a low virulence.[35,45]

Various studies utilizing PCR for detection of periodontal pathogens have demonstrated that PCR is rapid, efficient and easiest in comparison to traditional culture based methods and has excellent detection limits with few cross-reactions under ideal conditions. These PCR based studies not only permit the detection of known pathogens, but also contribute toward the identification of new pathogens involved in periodontitis.[53]

PCR is also used for the detection of viruses that may be associated with periodontal disease as it carries minimal risk of contamination and poses fewer problems associated with transport of the samples to the laboratory.[35]

Single target PCR applications

Single target PCR is used to detect a single specific species directly from the plaque samples of healthy subjects and diseased subjects. Studies utilizing the sequence analysis of 16S rRNA genes from the oral cavity have identified a number of bacterial species as candidates as putative pathogens for periodontitis, including the more traditional pathogenic species, such as P. gingivalis, T. denticola and T. forsythia as well as several additional species, including those that have not yet been cultivated in vitro.[51]

Multiplex PCR

This PCR based technique is used for the simultaneous detection of multiple species by incorporating more than one pair of species-specific primers in a single PCR assay. These assays have high sensitivity with detection limits of 10 to 100 cells per PCR reaction and are used for simultaneous assessment of A. actinomycetemcomitans, T. forsythia, and P. gingivalis.[51]

Commercially available diagnostic test based on multiplex PCR such as the MicroDent® Test(Hain Lifescience GmbH (Nehren, Germany) has been used to compare the microbiota between subgingival plaque samples to support the clinician in the diagnosis of periodontitis.[37,51]

Real-time PCR

The standard PCR process (single or multiplex) described above is a qualitative method which can detect the presence of a given pathogen, but does not quantify it.[35]

Real-time PCR also known as quantitative PCR(qPCR), qRT-PCR, and kinetic PCR is used for the qualitative assessment and quantification of plaque periopathogens. Real time PCR allows better visualization of the dominant bacteria or theirs complexes in oral biofilms which makes possible the use of effective targeted therapy against detected microorganisms.[35, 51]

PCR based molecular diagnostic tests like MyPerioPath test® and oralDNA® (OralDNA Labs, Brentwood, TN, USA) and the Integrated Periodontal® test (Biomolecular Diagnostic, Florence, Italy) are commercially available services provided by some clinicians and private companies for determination of the microbial profiles of putative periodontal pathogens from oral specimen.[37,51]

Reverse transcription-PCR (RT-PCR)

In this PCR based molecular technique, RNA instead of DNA is used as a starting template for PCR reaction. Reverse transcription reaction is carried out first to produce complementary DNA (cDNA) and a part of this cDNA is amplified by PCR. RT-PCR provides a rapid and sensitive method for analyzing gene expression in cells and tissues.[54] RT-PCR can detect only living bacteria because RNA rapidly degrades upon cell death, and is thus able to estimate the viable bacterial load.[45] RT-PCR has been used to assess the expression of mRNA for proteins involved in the development of Periodontitis.[54]

Shelburne *et al*. conducted a study utilizing quantitative reverse transcription PCR (QRT-PCR) to examine gene expression of a variety of putative virulence factors of P. gingivalis in human dental plaque from periodontitis subjects. PCR primers and probes for six target genes(groEL, dnaK, htpG, sodA, PG1431 and rgp-1)representing putative virulence factors were chosen and evaluated in vitro for specificity.The study revealed correlations between disease status(probing depth and attachment loss) and increased transcription of dnaK and htpG.[55]

In order to study the role of chemokines in periodontitis, Garlet, *et al*.11, in 2003, conducted a study to examine the expression of chemokines, chemokine receptors and cytokines in gingival biopsies from periodontitis patients by means of reverse transcription-polymerase chain reaction (RT-PCR) techniques. The study revealed that the expression of the chemokines macrophage inflammatory protein-1 alpha and interferon-gamma inducible protein 10 and of their respective receptors, CCR5 and CXCR3, were more prevalent and elevated in periodontitis. In addition, these patients also showed an elevated interferon-gamma expression.[56]

Loop-mediated isothermal amplification (LAMP) technique

The loop-mediated isothermal amplification (LAMP) technique is a novel molecular diagnostic tool developed by Notomi *et al*. and is utilized for bacterial identification.

LAMP is considered a powerful molecular diagnostic tool due to its properties such as the elimination of the DNA denaturation step, DNA polymerase exhibiting displacement ability, high specificity due to four primers and enhancement of efficiency due to isothermal conditions by eliminating time loss.

The advantages of the loop-mediated isothermal amplification (LAMP) technique in relation to PCR or qPCR include higher sensitivity, superior specificity, efficiency, ease of management and the ability to determine multiple pathogens simultaneously.

Many research groups have utilized LAMP technology for the detection of periodontal pathogens and have demonstrated high efficacy and specificity for the LAMP, which could be suitable for rapid oral bacteria screening and chairside diagnosis.

LAMP colorimetric method makes possible to identify individual strains of perio-pathogens from both extracted DNA and directly from whole cells, in a highly specific and rapid manner, through visual interpretation of the results.

A new isothermal detection method called MB-LAMP (molecular isothermal loop amplification) that combines the advantages of LAMP and qPCR has a higher specificity and sensitivity than the traditional LAMP.

The small equipment, ease of handling and rapid use could make LAMP technology an ideal apparatus for in-office periodontal pathogen screening and monitoring.[31]

DNA-DNA HYBRIDIZATION METHODS

Nucleic acid hybridization is a molecular diagnostic method employed for the detection and quantitation of several species of microorganisms in DNA samples. Hybridization techniques utilize nucleic acids of known sequence of nucleotides known as probes that are complementary to defined sequences of the pathogen genome in a sample. The probe hybridizes to target complementary sequences of the pathogenic genome thereby allowing detection of the microbe. Probes are obtained by the process of chemical synthesis or cloning[35, 57,58]

Fluorescence in situ hybridization (FISH)

Fluorescent in situ Hybridization is a hybridization based technique in which fluorescent labeled probe is used and the hybridization process is visualized using fluorescence or confocal fluorescence microscopy. FISH technique has been employed for the detection of periodontal pathogens like A. actinomycetemcomitans, P. gingivalis, Actinomyces spp. and Streptococcus spp. In addition, FISH in combination with flow cytometery can be used for the analysis of mixed microbial populations.[51]

Checkerboard hybridization

Checkerboard hybridization is a semi-quantitative technique which enables the testing of DNA, RNA, tissue, bacterial and viral samples with multiple probes at the same time. This method allows the rapid and simultaneous identification of several microbial species in a large number of oral samples and the number of individual microorganisms in the sample can be detected by the intensity of the fluorescent or chemiluminescent light from the molecular probes.[35,57-59]

The Checkerboard hybridization technique was developed by Socransky *et al*. in 1994 to permit the simultaneous detection of the presence of several bacterial species in single or multiple dental plaque samples. The method enabled the hybridization of 43 DNA samples against 43 DNA probes and permitted up to 1849 hybridization reactions on a single support membrane.[59]

In 1998 Socransky *et al* applied Checkerboard DNA-DNA hybridization to analyze 13000 plaque samples from 185 subjects using whole genomic DNA probes to 40 bacterial cultivable species. The study was conducted to understand the nature of the microbial complexes that exist in the subgingival plaque.[60]

Xime´nez-Fyvie *et al*. conducted a study to examine and relate the microbial composition of supra and subgingival plaque using whole genomic DNA probes and checkerboard DNA-DNA hybridization. 1,170 samples of supra and subgingival plaque from 23 adult Periodontitis subjects were analyzed for the presence and levels of 40 bacterial species.[61]

Using a checkerboard DNA-DNA hybridization technique A.P. Vieira Colombo *et al* conducted a study to determine the prevalence and levels of pathogenic species of medical importance in the microbiota of individuals with different periodontal clinical status. Subgingival biofilm obtained from patients with periodontal health, gingivitis and generalized aggressive periodontitis was analyzed for 39 microbial taxa. The study revealed that enterobacteria, C. albicans, Neisseria spp., P. aeruginosa, O. uli, Hafnia alvei, Serratia marcescens and Filifactor alocis were associated with periodontal inflammation where as Fusobacterium

necrophorum, Lactobacillus acidophilus, Staphylococcus aureus and Streptococcus pneumoniae were associated with periodontal health.[62]

Reverse-capture checkerboard hybridization is a PCR based checkerboard hybridization technique. It utilizes Labeled 16S rRNA amplicons that are hybridized to 16S rRNA-based probes that are on the Membrane.[35, 51]

This molecular method is used to identify uncultivable as well as cultivable species associated with periodontitis.[51]

Microarray technology

Microarrays are devices that consist of thousands of DNA probes bound on a solid support and are used for identification of microorganisms and determining gene expression by simultaneously measuring the relative concentrations of many different DNA or RNA sequences in a sample via hybridization and subsequent detection of the hybridization events.[35, 63, 64]

The Human Oral Microbe Identification Microarray, or HOMIM is a high sample-throughput, 16S rRNA-based technology developed for oral bacterial profiling and it allows the simultaneous detection of about 300 bacterial species, including the uncultivable ones in a single hybridization on glass slides.[37,51]

HOMIM has been used to compare the microbial profiles of bacterial species from subjects with Periodontitis, successfully treated Periodontitis and periodontal healthy.[51]

Several studies have employed microarrays in their investigation to study the transcriptional responses caused by microorganisms present in the periodontal pocket.[65,66]

ParoCheck® DNA chip is a commercially available 16S rRNA-based microarray. It is used in clinical periodontal diagnostics and enables the detection of 10 or 20 periodontal pathogens.[35,51] Phylochip (Affymetrix Corporation ® and Lawrence Berkeley Labs), which can detect up to 32,000 16S rRNA phylotypes, is a high density microarray used to study complex bacterial communities.[51]

Using pooled microarray gene expression (transcriptomic studies) data sets Suzuki *et al.*, identified key genes related to periodontal pathogenesis such as CSF3, CXCL12, IL1B, TAGLN, CD19, IL8, and CD79A as well as genes of biomarker candidates such as TNF and FGF2, which could provide potential targets for periodontal diagnosis.[67]

In an animal model of experimental Periodontitis, Ebersole *et al*., analyzed phase specific gene expression profiles by using microarray technology during different phases of Periodontitis lesion (initiation, progression, resolution) to delineate underlying biological processes that occur during the transition from periodontal health to disease. The study demonstrated a significant gene expression variations across the various phases of Periodontitis and a subset of these gene products could be used for early diagnosis as well as development of targeted therapies for Periodontitis.[68]

Sequencing methods

Nucleic acid sequencing is a process of determining the exact order of nucleotides present in a given DNA or RNA molecule.[69]

Next-generation sequencing (NGS) technique, also known as high-throughput sequencing, differs from first generation Sanger sequencing in that it enables scientists to sequence hundreds of genes at one time and has a deeper coverage of microbial community.[36]

NGS has increased our understanding of the diversity of oral bacteria through two commonly used approaches: sequencing of conserved 16S ribosomal RNA genes and untargeted (shotgun) sequencing of whole genomes (metagenomics).[70]

This deep sequencing technique has revealed an unexpectedly high diversity of the human oral microbiome comprising of about 10,000 microbial phylotypes which is significantly higher than the previously reported 700 oral microbial phylotypes as identified by culturing methods and culture independent methods.[33]

In a pilot study Liu *et al* demonstrated the role of oral microbial system in periodontitis by using 16S rDNA analysis and high-throughput deep sequencing. The study revealed genetic, metabolic and ecological differences between the healthy and diseased periodontal microbiome.

The 16S rRNA sequencing and whole-metagenome sequencing was used to characterize the genomes of key oral microbes, including an unculturable TM7 organism. The authors observed a shift from a gram-positive dominated community in the healthy samples to a gram-negative dominated community in Periodontitis. In addition, the study revealed differences in metabolic profiles of healthy and diseased samples with diseased microbiome being enriched in metabolic pathways that are consistent with a parasitic lifestyle like functions for fatty acid metabolism and acetyl-coenzyme A degradation, aromatic amino acid degradation, ferrodoxin oxidation, and energy-coupling factor (ECF) class transporters. Also a number of virulence factors such as the presence of conjugative transposons, type IV secretion systems, and the biosynthesis of toxic factors (e.g. acetone, butanol, and ethanol biosynthesis), as well as the Lipid-A of lipopolysaccharide (LPS) biosynthesis were enriched in disease microbiome. Moreover, in one of the samples microbiota typical of periodontits was observed just prior to clinical features of disease suggesting that microbial dysbiosis precedes the clinical signs of disease, and that the oral microbiota could be a potential tool for the early diagnosis of Periodontitis.[33]

By applying a comparative metagenomics approach Torres *et al*., investigated the relationship of the uncultivated Candidatus Bacteroides periocalifornicus (CBP) bacterium, a recently discovered member of the Bacteriodetes phylum to periodontal disease and to members of key periodontal pathogens in the orange and red complex. The study revealed that CBP genome harbors several genes and pathways similar to the other known oral pathogens involved in Periodontitis and it co-exists with F. nucleatum, T. denticola, and P. gingivalis. The authors suggested that CBP is a novel candidate member of the symbiotic and pathogenic red complex.[70]

Using a metagenomic/metatranscriptomic approach Yost *et al* conducted a study to characterize functional differences between the subgingival microbiota of periodontal health and severe Periodontitis. By utilizing Metatranscriptome analysis conducted on samples from subgingival biofilms from progressing and stable sites from periodontitis patients using Next Generation Sequencing (Illumina) the authors identified a distinct molecular signatures of periodontitis progression.The study revealed physiological changes in the microbial community that are associated with initiation of periodontitis including that of citrate transport, iron transport, potassium transport, amino-acid transport, isoprenoid biosynthesis, and ciliary and flagellar motility.The authors concluded that the whole microbial community, and not just few oral pathogens, is responsible for an increase in virulence that is responsible forperiodontits progression.[71]

Since Periodontitis is caused by dysbiosis of entire microbiome rather than a few dominant pathogens, analyzing the entire disturbed microbial community using NGS technique might be the key in understanding Periodontitis. In addition studies have revealed that detection of specific pathogens by using targeted microbial techniques (microarrays) is poorly predictive of the prognosis of Periodontitis and metagenomic studies utilizing NGS are more sensitive than clinical

parameters in determination of the effectiveness of different treatment modalities for Periodontitis.[36]

Califf *et al* used high-throughput sequencing of bacterial community 16S rRNA, shotgun metagenomics, and a metabolomic analysis using tandem mass spectrometry (MS/MS) to analyze sub- and supragingival biofilms in adults with periodontitis pre- and posttreatment with 0.25% sodium hypochlorite. The results revealed a significant correlation between phylogenetic diversity and pocket depth at the baseline as well a strong correlation between metabolite diversity and maximum pocket depth (MPD). The study demonstrated the high sensitivity of the Mass spectrometery data to temporal changes in the microbial community(different than the taxonomy-based analyses) indicating that the MS/MS approach could be a powerful additional diagnostic aid for studying periodontal disease. The authors suggested that molecular tools could enhance treatment prediction and reveal patients most likely to improve posttreatment.[50]

In a similar study, H. S. Na *et al*., employed 16S rRNA sequencing for metagenomic analysis and nuclear magnetic resonance (NMR) for metabolome analysis to assess the oral microbiome and its metabolome in periodontitis patients.The findings of the study provided evidence of a close oral microbiome-metabolome associations which may help in diagnosis and management of Periodontitis at molecular level.[72]

Various metagenomic studies have demonstrated that the deeper pockets tend to be richer in metabolic pathways, have greater concentration of virulence factors, and have few biosynthesis pathways than healthy shallower pockets.[50]

OTHER MOLECULAR DIAGNOSTIC TECHNIQUES

MASS SPECTROMETERY

Mass spectrometry is a powerful, simple, inexpensive and fast technique technique for measuring the mass of molecules like proteins and peptides from clinical samples.The technique requires a method for ionizing the sample, accelerating the molecular ions, and then detecting the ions creating a characteristic spectrum for analytes in the sample.[46, 52]

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is also used for the identification of bacteria by analyzing the relative frequency of peaks within the same spectrum.[46]

To explore the diagnostic potential of protein profiling. Antezack *et al*., analyzed saliva, GCF and dental plaque samples from periodontitis and healthy subjects using Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). MALDI-TOF MS differentiate periodontitis from healthy periodontium by generating specific patterns in mass signals from protein profiles in saliva, GCF and dental plaque. The authors concluded that specific protein profiles could represent the molecular signature of Periodontitis and can be used to develop easy, non-invasive and fast routine diagnostic tests.[46]

Proteomic and peptidomic technologies as well as improvement of protein databases could allow a more comprehensive analysis of inflammatory status and host responses which can reveal more specific biomarkers to be used for the risk assessment, diagnosis, and monitoring of periodontitis.[73]

Various studies utilizing mass spectrometery have demonstrated differences in protein expression in saliva and GCF samples of periodontitis patients and healthy controls.

Also, Proteomic studies of plaque bacteria have revealed alterations in global protein expression of microbes during growth in a biofilm. The data from these studies could be used to develop diagnostics and therapeutics for Periodontitis.[74]

Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical method for the detection and analysis of organic acids, amino acids, fatty acids, amines, and alcohol in the provided biofluid sample (such as saliva).[75]

NMR has numerous applications like localization and characterization of salivary metabolites for disease detection as well as understanding the periodontal diseases by gingival crevicular fluid biomarkers analysis.[76,77]

NMR has been used in various clinical studies to establish a metabolomic profile for periodontal disease. These studies have demonstrated that the metabolomic profile could distinguish between healthy periodontal sites and active periodontal lesions. In addition NMR analysis could be used to monitoring effectiveness of periodontal therapy as well as correlating the metabolic changes after tissue repair.[76]

Gawron *et al*. conducted a study using proton NMR-based metabolomics analysis of mouth washouts and tongue swabs to demonstrate the pathologic changes in the oral cavity during transition from health to Periodontitis. The findings in the study revealed that Periodontitis associated bacteria have higher metabolic activity in comparison to microbiota associated with periodontal health as revealed by changes of lactate, acetone, isopropanol, glycerol and methanol levels. The authors suggested that these metabolites could serve as potential candidate biomarkers for periodontal diagnosis and management.[78]

CONCLUSION

Periodontitis is a complex, chronic inflammatory disease that affects the supporting structures of the teeth and is associated with microbiological, genetic and environmental factors. Currently the diagnosis of Periodontitis is based on traditional clinical and radiological assessments which reveal only past disease activity but do not provide any information about current disease status or predict future activity. As Periodontitis is a multifactorial disease, and the current diagnostic tests do not reveal information about its etiology additional assessments using diagnostic aids with high sensitivity, specificity and predictive value are required to provide an early and better diagnosis, therapeutic guidance and patient monitoring. The diagnostic methods utilized for Periodontitis have continuously advanced with the inclusion of various technologies like molecular diagnostic tools.

These culture-independent molecular methods enable us to study periodontal disease at the molecular level (DNA, RNA, proteins, metabolites) which is valuable for identification of the disease at a very early stage much before the appearance of clinical and radiological manifestations allowing for the utilization of preventative strategies prior to disease onset rather than treatment options that attempts to repair the tissue when significant damage has already occurred.

Such molecular diagnostic tests are capable of identifying pathogenic organisms including those that are not cultivable, assess viable bacterial load, identify periodontal disease signatures by assessing protein profiles and metabolite analysis and determine an individual's susceptibility to Periodontitis by the detection of human genetic variations.

The data generated by these high-throughput methods provides a greater understanding of the pathophysiology of Periodontitis which can be used to develop new methods of disease diagnosis, risk assessment, prognosis, prevention and therapeutic guidance.

Most of the molecular diagnostic tools described in this review are used for research purposes and as scientific knowledge and technology improves, many of these aids will be available for periodontal diagnosis.

Conflict of interest

The auther reports no conflicts of interest.

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