(International Print/Online Journal)

SJIF IMPACT FACTOR: 5.565
PUBMED-National Library of Medicine ID-101739732

ISSN (Print): 2209-2870
ISSN (Online): 2209-2862

International Journal of Medical Science and Current Research (IJMSCR)
Available online at: www.ijmscr.com
Volume 6, Issue 6 , Page No: 308-318
November-December 2023

IJMSCR

# Eyes Do Not See What Mind Does Not Know! - Microbial Tests In Periodontal Diagnosis

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Type of Publication: Review article
Conflicts of Interest: Nil

**Abstract**

For over a century, the medical profession has employed clinical microbiology as a tool in the diagnosis and treatment planning of infectious diseases. The identification of bacterial pathogens in periodontal diseases has been difficult because of number of factors and its complex community, and thus the identification of etiological agents helps in selecting the optimal drug therapy to support the patient in overcoming these diseases. Recently, microbiology a diagnostic tool in Periodontics has been evaluated, with the emphasis on sampling methods and different techniques to detect and quantify bacteria. Here we shall give more importance for the different types of microbial tests used currently for the identification of specific pathogens responsible for periodontal diseases.

**Keywords**: periodontitis, periodontopathogens, microbial tests, bacterial culture, immunoassay

## Introduction

The bacteria that inhabit the human oral cavity play a role in the etiology and pathogenesis of the different forms of periodontal disease. Diseases that affect periodontium are unique in that they occur in an unusually complex environment within a diverse group of specialized tissues. Clinicians need a test to facilitate the detection of periodontal pathogens, diagnosis of periodontal diseases and to monitor the efficacy of the treatment of periodontal diseases. Periodontal microbiota is a complex community of microorganisms many of which are difficult to isolate in the laboratory. The microbial tests may have the potential not only to diagnose various forms of periodontal disease depending on the bacterial profile, but also to determine patients at higher risk of undergoing active destruction. The microbial tests could also monitor periodontal therapy, directed at the suppression of periodontopathic organisms. The present review will discuss the role of microbial tests in diagnosis of periodontal diseases. It will focus on several questions related to the use of microbiologic diagnosis in the clinic such as

1. Can microbiological tests be used to identify subjects at risk?
2. Can it help determine treatment strategies and modalities, as well as assist in determining treatment end-point?

## Microflora Of The Oral Cavity

Bacteria inhabit the oral cavity from birth to death. The mouth consists of numerous distinct habitats, each of which has its own ecological conditions. However these conditions are susceptible to environmental factors such as dietary habits, oral hygiene and anti-microbial chemotherapy. The changes in the environment could affect the microbial community and produce shifts in the proportions of the resident species.

Beginning in the late 1920s investigators believed that periodontal disease was the result of mixed infection. By the end of 1960s, it was generally stated that dental plaque was in some way associated with periodontal disease [1]. In mid 1960 a study by Loe et al demonstrated that plaque accumulation directly preceded and initiated gingivitis [2].

Periodontal diseases are multi-factorial diseases in which the bacteria play an important role. They are essentially opportunistic infections caused by bacteria residing in the oral cavity. However, there is evidence that only a finite number of the 200 to 300 distinct bacterial species comprising the oral flora play any role in the initiation of these diseases (Tanner, 1991; Loesche, 1994; Haffajee and Socransky, 1994) [3,4,5]. The flora at sites with periodontal destruction differs from that at healthy gingiva. The flora from periodontal pockets is dominated by gram negative bacteria as well as a high proportion of spirochetes. Microbial tests may be a valuable tool to screen the status of oral ecology before and after treatment.

**Microbial Sampling Methods**

Two primary methods used to collect a patient's subgingival plaque [6]:

1. Removal using Curettes
2. Adsorption onto endodontic paper points

Both methods require careful removal of supragingival plaque at the site prior to sampling in order not to contaminate or dilute the subgingival sample. Information gathered from curette samples usually differs from that obtained from paper point samples because a curette collects plaque from the entire pocket, whereas the plaque absorbed onto a paper point is derived mostly from the outer layers of the biofilm, which may contain the more pathogenic microflora. Paper points are significantly less successful at collecting plaque in apical portions of a pocket than from areas near the gingival margin. Since bacteria in subgingival biofilms are not homogeneously distributed with the pocket, paper-point samples will not accurately represent the flora at the base of the pocket where the disease is progressing.

**Microorganism Content Varies According To The Pocket Anatomy**

Each pocket has a unique microbiological profile, and thus a sample of plaque from a diseased site may not be representative of the flora of the entire dentition or even of other diseased sites. This is especially true for infrequently detected organisms, such as *Aggregatibacter actinomycetemcomitans*, where its presence may not be detected in positive subjects even after sampling numerous diseased sites. Also the wide variations in genetic and clonal diversity amongst individual organisms may have an impact on microbiological analysis. Due to cross-reactivities with similar microorganisms, certain antibody based tests may fail to identify some strains. But some new techniques such as 16S rRNA-based PCR detection methods appear to have addressed these limitations by showing little or no cross-reactivity and the ability to detect wide range of clonal types [7].

**Tests For The Evaluation Of Periodontal Pathogens**

1. Direct microscopy
2. Bacterial culture
3. Enzymatic methods
4. Antibody based assays
    i. Immunofluorescence microscopy
    ii. Flow cytometry
    iii. Enzyme linked immunosorbent assay
    iv. Latex agglutination assays
5. DNA probes
6. Polymerase chain reaction

**Phase Contrast And Dark Field Microscopy**

These visual techniques determine the relative proportions of coccal and filamentous-shaped organisms. The size, shape and motility of bacteria are evaluated by this method. Sample of plaque in healthy condition have a small number of mainly immotile cocci and samples of plaque number in diseased condition have large number of different bacterial morphotypes.

Since individual species cannot be identified, the main usefulness of microscope identification comes from observing a shift in the appearance of flora with periodontal therapy. Organisms which are highly



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pathogenic, densely populated, motile and rod-like are replaced by healthy, sparsely populated coccal and non motile organisms upon successful treatment. However, these techniques have not been shown to help predict recurrence of disease for treated patients during maintenance phase.

**Bacterial Culture**

Culture methods are quantitative and are considered the gold standard against which the other microbiological methods have been compared. Anaerobic bacteria are considered responsible for periodontal diseases. Cultivation of anerobic bacteria is slow, complicated and expensive. Sensitivity tests are particularly problematic i.e. determination of resistance, particularly in the case of therapeutically resistant periodontitis. Approximately 70% of the species (*Prevotella species* and *Porphyromonas species*) are sensitive to penicillin, ampicillin and a group of anti-pseudomonas penicillin (ticarcillin, piperacillin) but only 5-20% in the group of *Bacteroides* [8]. The unique advantage of culturing over the other methods is that it permits the assessment of antibiotic sensitivity. Though all the microorganisms cannot be grown, through the use of selective and non-selective media, many putative periodontal pathogenic organisms can be identified. Selective media serves as isolation with the addition of an antibiotic. Tryptic Soy Serum Bacitracin Vancomycin Agar (TSBV), a selective nutrient media is used in this way, with the addition of antibiotic and horse serum for isolation of *A.actinomycetemcomitans*.

**Enzymatic Methods:**

An enzymatic assay has been developed that detects the bacteria by their enzymatic activity which is directed towards proteins and peptides. BANA (N-benzoyl-DL-arginine-2 naphthylamide) is a synthetic peptide, which is impregnated on a paper strip. When it reacts with the plaque sample, the colourless substrate BANA turns into blue-black colour [9]. The intensity of colour is proportional to the total amount of organisms in the plaque sample. Bacteria like Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola produce trypsin-like enzyme and causes hydrolysis of BANA (synthetic peptide) which results in blue-black colour. This reaction enables their detection. These tests indicate the presence of a group of periodontal pathogens by detecting enzymes, such as collagenase, peptidase and trypsin-like enzymes which destroy periodontal tissue. However, enzyme test cannot be able to detect pathogens which do not have a defined enzyme profile (eg: *A.actinomycetemcomitans*). Also its utility as a diagnostic method is however, uncertain due to its low reliability to predict clinical assessment of disease progression. Rapid and low cost method is an advantage; but the major disadvantage is that it cannot identify the bacterial species. Commercial kit is available.

**Immunoassays:**

They include

1. Immunofluorescence microscopy
2. Enzyme Linked Immunosorbent Assay
3. Latex Agglutination
4. Flow cytometry

**Immunofluorescence Assay (IFA)**

These tests are based on specific binding of monoclonal antibodies to the surface of the antigen of a particular organism. Immunofluorescent labeled tissue sections are studied using fluorescence microscope. Fluorescein is a dye which emits greenish florescence under UV light. This dye can be tagged to immunoglobulin molecules.

Methods

1. Indirect Immunofluorescence
2. Direct Immunofluorescence

Both direct and indirect IFA are able to identify pathogen and quantify the percentage of pathogens directly using plaque smear. These tests help in the detection of mainly *A.actinomycetemcomitans* and *P.gingivalis*. The advantage of immunofluorescence is that they do not require viable cells. Comparative studies indicate that sensitivity ranges from 82% to 100% for detection of *A.actinomycetemcomitans* and 91% to 100% of detection of *P.gingivalis* [10].

**Enzyme Linked Immunosorbent Assay (ELISA)**

ELISA is similar in principle to other radioimmunoassay, but an enzymatically derived colour reaction is substituted on the label in place of radioisotope. ELISA for bacterial species involves binding the species-specific serologic reagent to the walls of polystyrene plates or to membranes placed



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on an absorbent base. The bacterial plaque sample is dispersed in a buffer with the addition of a detergent or other extractant, and an aliquot of the bacterial extract is added to the antibody-coated wells. Binding of the target micro-organisms can then be detected by the addition a second antibody conjugated to an enzyme, followed the addition of the substrate. The substrate reacts with enzyme to produce product, usually coloured. The intensity of colour depends on the concentration of the antigen. ELISA is relatively inexpensive when compared to other tests.

**Flow Cytometry**

It is a technique for counting and examining microscopic particles such as cells and chromosomes by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It involves labelling bacterial cells with species-specific antibody and a second fluorescein-conjugated antibody. The immune complex suspension is then introduced into a flow cytometer which separates the bacterial cells into almost single cell suspension by means of laminar flow through a narrow tube. After incubation, the cells are passed though a focused beam of laser. The suspended particles passing through the beam scatters the ray and fluorescent chemicals found in particles excited into emitted light at a longer wavelength than the light source. These emitted and scattered light are picked up by the appropriate detectors and analyses the fluctuations in the brightness, that correlate with cell volume, and inner complexity of the cells.

**Advantages:**

1. It takes only a few minutes
2. Does not require any instrumentation and only minimal technical expertise.

**Disadvantages:**

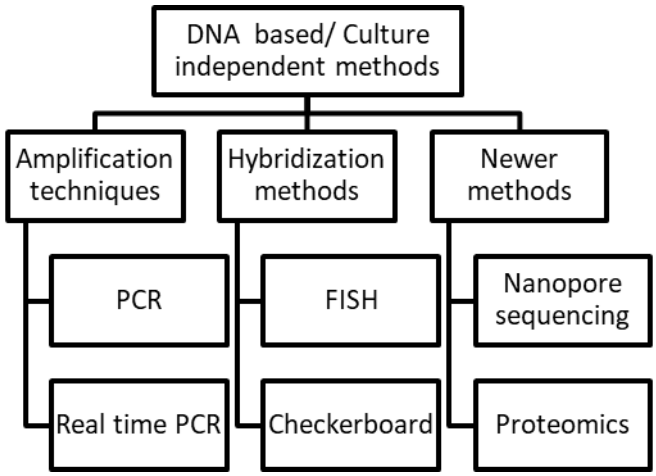
1. Expensive
2. This method cannot be demonstrated for use in dental plaque.

**Latex Agglutination**

It is an immunologic assay based on the binding of protein to latex. In this method, antibody (or antigen) is coated on the surface of latex particles which is known as sensitized latex. When a sample containing the specific antigen (or antibody) is mixed with the milky-appearing sensitized latex, it causes visible agglutination. Clumping of beads is usually visible in 2 to 5 minutes. A positive reaction causes the beads to clump together which can be seen with the naked eye. It is simple and quick to perform and requires least lab facilities. It has high sensitivity and specificity.

**Molecular Diagnostic Methods:**

**Figure 1 – Molecular Diagnostic Methods. Adapted from - Liesenfeld O et al [11].**




**Polymerase Chain Reaction (PCR)**

Dr. Kary Bank Mullis discovered PCR in 1985 for which he was awarded the noble prize. PCR is a molecular diagnostic technique for high-yield



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replication of DNA. It amplifies a single or few copies of a piece of DNA, generating millions or more copies of a particular DNA sequence. The mechanism is PCR specifically targets and amplifies a single sequence from within a complex mixture of DNA. PCR involves denaturation, annealing, extension steps and these steps are performed for 25 to 50 repetitive cycles thus resulting in DNA amplification.

PCR assays when used in combination with synthesized 16srRNA probes that are highly specific to individual species enable the detection of virtually any microorganism in a plaque sample. This technique is the most sensitive than any of the above.

**Real Time PCR**

Real-time PCR is a popular method for quantitative detection of periodontopathic bacteria. Also called quantitative real time polymerase chain reaction, allows the scientist to actually view and quantify the increase in the amount of DNA as it is amplified. The key feature of this reaction is that the amplified DNA is detected as the reaction progresses in real time. Traditional PCR is measured at End-point (plateau), while Real-Time PCR collects data in the exponential growth phase. Also, real time PCR quantifies periodontal pathogens accurately. Using this system we can demonstrate the correlation between the number of these microorganisms and periodontal pocket depth. After SRP the number of *A.actinomycetemcomitans* is significantly reduced. This system is useful in making it applicable for evaluation of therapeutic efficacy. The main disadvantage is one cannot discriminate between living and dead cells and it requires thermal cycler.

**Loop Mediated Isothermal Amplification (LAMP)**

LAMP technique is currently one of the most rapid bacterial diagnostic method. It is a modification of the PCR method and developed by Eiken Chemical CO, LTD, (Japan). Reactions are performed under isothermal conditions. In contrast to the thermal cycling necessary for PCR advantages of this method includes rapid analysis, time of about 1 hour and requires no special detection equipment, as the results can be observed by the naked eye [12].

**DNA-DNA Hybridization**

**Fluorescence In Situ Hybridization (FISH)**

This technique is used to quantify and determine the spatial configuration and demonstrate the morphology of individual bacterial cells. Fluorescently labeled r-RNA targeted oligonucleotides are hybridized to partially fixed, whole cells on microscope slides and are visualized using fluorescence microscopy. By this method, *A.actinomycetemcomitans* and *P.gingivalis* have been detected [13].

**Checkerboard DNA-DNA Hybridization Technique**

The technique was developed by Socransky et al in 1994 it for detection of 40 bacterial species often found in oral cavity [14]. The whole genomic, digoxigenin-labeled DNA probes are used. This probe permits detection of 104 cells of each species. The technique is used for diagnostic purposes. Also, high expertise and sophisticated lab equipment requirements makes this technique to be used very rarely. This technique is applicable for epidemiological research and ecological studies.

**Nucleic Acid Probes**

The application of DNA probes is based on specific reaction of segments of one chain of nucleic acids with a complementary sequence of nucleic acid of the bacteria. The principle for detection of hybridization products of a specific sequence of nucleic acid with a complementary sequence is mostly frequently radioactive or enzymatic. The DNA probe is most often used for identification of *A.actinomycetemcomitans, P.gingivalis, P.intermedia* and other periodontal pathogens [15]. The whole genomic probes are more likely to cross react with non-target micro-organisms because of presence of homologous sequences between different bacterial species. Nucleic acid probes have advantage of easy sampling and transport and can be used to accurately identify a wide spectrum of bacterial species along with non cultivable microorganisms. DNA extracts from the sample can be hybridized with so called antisense DNA probes.

**Next Generation Sequencing (NGS)**

This is the system which helps the researchers to quickly sequence the bacterial strains, enabling them to better track the origins and transmission of the outbreak as well as identify genetic mutation conferring the increased virulence. It enables



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population sequencing. People who do research can now analyze 1000 to 10000 samples in a single year [16].

**Nanopore Sequencing:**

Nanopore sequencing development is a method under development since 1995 for determining the order in which nucleotides occur on a strand of DNA [17]. A nanopore is simply a small hole of the order of 1 nanometer in internal diameter. Certain porous transmembrane cellular proteins act as nanopores. Graphene is also being explored as a synthetic substrate for solid-state nanopores. The principle behind the nanopore sequencing is that when a nanopore is immersed in a conducting fluid and a potential (voltage) is applied across it, an electric current due to conduction of ions through the nanopore can be observed. The amount of current is very sensitive to the size and shape of the nanopore. If single nucleotides (bases), strands of DNA or other molecules pass through or near the nanopore, this can create a characteristic change in the magnitude of the current through the nanopore. The change in the current through the nanopore as the DNA molecule passes through it, represents a direct reading of the DNA sequence. As of July 2010, information available to the public indicates that nanopore sequencing is still in the development stage, with some laboratory based data to back up the different components of the sequencing method. Despite these advancements, nanopore sequencing is not currently available, parallelized, routinized, nor cost effective enough to compete with "next generation sequencing" methods.

**Chairside Periodontal Test Kits**

- Microbial test kits
- Biochemical test kits
- Genetic kits

**Microbial Test Kits:**

These tests aid in the diagnosis of various forms of periodontal diseases, to serve as indicators of disease initiation and progression and to determine which sites are at higher risk for active destruction. The tests are mainly aimed at spirochetes, *A.actinomycetemcomitans, P.gingivalis, P.intermedia.*

Some of the in-office microbial tests used currently are:

- Omnigene
- Evalusite
- Perioscan

**Omnigene**

It is a DNA probe system for detecting known periodontopathogens. A paper point sample of subgingival plaque is placed in the container provided and mailed off to the company for assay. Probes are available for the detection of *A.actinomycetemcomitans, P.gingivalis, P.intermedia, F.nucleatum, C.rectus, T.denticola and E.corrodens*. Reports are provided within very short time periods.

**Evalusite**

It is a kit employing a novel membrane-based enzyme immunoassay for the detection of three putative periodontal organisms such as *A.actinomycetemcomitans, P.gingivalis and P.intermedia*. A subgingival sample is collected using paper points and added to a sample tube. The element is then added to the kit which employs a Sandwich type ELISA. A positive reaction is displayed as pink colour. The disadvantages of this test include:

- assumption that the three detected organisms are causing the disease
- multi-stage test
- it has a subjective calorimetric end point
- there is no permanent record the results.

**Perioscan**

Perioscan employs BANA (N-benzoyl-DL-arginine-2-naphthylamide) hydrolysis reaction to detect bacterial trypsin-like proteases in the dental plaque. In this test, subgingival plaque is collected and placed on a BANA-containing strip, which is then folded to contact a second strip containing "Fast Black" dye reagent. The folded card is placed inside an oven for 15 minutes at 55°C and the positive reaction is confirmed by the appearance of blue-black colour. The weakness of this test is that it assumes the detected organisms are only responsible for active



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disease. This is known not to be the case for all patients and sites. Potential difficulties of the test are that it may be positive at clinically healthy sites and might remain same even after the treatment.

**Biochemical Test Kits:**

They are utilized for the analysis of gingival crevicular fluid (GCF). GCF contains constituents such as host-derived enzymes, inflammatory mediators and extra-cellular components. By evaluating its constituents, biochemical test kits give the information about early signs of disease. Some of the in-office tests include:

- Perio 2000
- Prognos-stik
- Perio-check
- Periogard
- Pocket Watch

**Perio 2000**

A sulfide sensor, Perio 2000 developed by Diamond General Corp, can measure levels of these compounds [18]. Score ranges from 0 to 5 in increments of 0.5. It is exactly like a stainless steel periodontal probe, so the device requires little training and can be easily incorporated in routine clinical examination. It is a diagnostic kit that is employed that to detect sulfide producing organisms, like *P.gingivalis, P.intermedia and T.forsythia*. These organisms produce significant levels of volatile sulfide compounds (VSCs) by degrading the serum proteins like cysteine and methionine. These VSCs causes destruction of periodontal structures and may aggravate periodontitis. Perio 2000 has a probe which is hydrated using sterile wash solution and then inserted subgingivally at peak or hold operational mode. It is designed to show the sulfide level at each site.

**Prognos-Stik**

It detects elevated levels of MMPs in the GCF. The presence of elevated levels of elastase in GCF may thus be indicative of active disease sites. Elastase is released from the lysosomes of polymorphonuclear leukocytes which accumulate at sites of gingival inflammation. GCF collected in a filter paper which is impregnated with a known amount of substrate labeled with a fluorescent indicator. Elastase on the test strip cleaves the substrate during the reaction time of 4-6 minutes and releases the indicator, which is visible under fluorescent light [19].

**Perio-Check**

Perio-check is reported to measure neutral protease activity within GCF. The GCG sample is collected and placed on a gel containing insoluble dye labeled collagen fibrils and incubated. In the presence of neutral proteases which diffuse from the strip into the gel, the insoluble collagen-dye complex is digested to release soluble dye labelled fragments, which diffuse back into strip turning it blue. This test is not specific for PMNL collagenese, which is thought to be the dominant collagenase at active sites.

**Periogard**

It is used to detect an enzyme called aspartate amino transferase (AST). AST is released from within cell upon cell death. In periodontal pathogenesis, cell death is one of the features. So AST levels are markedly increased in GCF in periodontitis and they serve as marker of early periodontal disease. The test consists of a tray with two test wells for each tooth, and appropriate reagent for conducting the test. GCF is collected and placed on a filter paper impregnated with tromethamine hydrochloride buffer. A substrate which contains 1-aspartic acid and α-ketogluteric acid is added to the filter paper and made to react for 10 minutes. In the presence of AST, the aspartate and α-ketogluteric acid are catalyzed to oxaloacetate and glutamate. Then the addition of dye such as fast red results in a colour product. The intensity of colour is proportional to the AST activity in the GCF sample [20].

**Pocket Watch**

It was developed to detect to AST activity in pockets. In this test, AST catalyzes the transfer of an amino group of cysteine sulfuric acid by a keto glutaric acid to yield β-sulfinyl pyruvate. Glutamate β-sulfinyl pyruvate spontaneously and rapidly decomposes and releases inorganic sulfite. The sulfite ion reacts with malachite green (MG), causing MG to convert from a green dye to its colourless form, thereby allowing the pink-coloured rhodamine B dye to show through. The rate of conversion of MG is directly proportional to AST Concentration [21].



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### Toxicity Prescreening Assay (TOPAS)

By bacterial toxins and host proteins it detects the indirect presence of bacteria in gingival infection [22]. Toxic metabolites are produced by the periodontal pathogens continuously and are found in the GCF. This test includes two reagents one for measuring the concentration of bacterial toxins and the second for measuring the levels of total proteins in the GCF. First the paper points embedded in the crevicular fluid is introduced into the yellow cap vial. The reaction of the bacterial toxins from crevicular fluid with certain chemical colourless reagent contained in the yellow cap vial takes place and after 5 minutes colour will turn to yellow. Greater the concentration of toxins, brighter the yellow colour. The second reaction of TOPAS is based on the colour change produced by adding the reagent from the blue cap vial. Greater the proteins in the GCF brighter the blue colour.

### Genetic Test Kits

Gene polymorphisms are also considered as one of the risk factors for the initiation or progression of periodontal disease. Genome wide association studies (GWAS) has been the most common approach for identifying disease association in the whole genome. Genetic polymorphism is difficult to identify, but some new chair side kits are available for its detection.

### PST Genetic Susceptibility Test

Periodontal Susceptibility test (PST) is the first and only genetic test that analyzes two interleukins (IL-1α and IL-1β) genes for variations. The IL-1 genetic test can be used to differentiate certain IL1 genotypes associated with varying inflammatory responses to identify individuals at risk for severe periodontal disease before the age of 60 [23].

### Proteomics

The word proteome was coined by Mark Wilkins in 1996. They directly addresses the levels of gene products present in the given cell state and chacteristics of the protein activity. With advances in science and technology, proteomics can assist in finding new biomarkers in different diseases and discover new therapies [24].

### Conclusion

Periodontitis is a chronic inflammatory disease of complex multifactorial etiology. The presence of microbes especially gram negative anaerobes is essential in initiating and perpetuating periodontal diseases where periodontal destruction is a multistage process consisting of microbial infection, host immune response, tissue destruction and repair. Bacteria play a fundamental role in the development of periodontal diseases. Based on our knowledge of the periodontal microflora and its causal role, prevention and treatment of periodontal diseases can be directed to these microorganisms. Therefore the objective of periodontal treatment is to eliminate bacteria from the infected periodontal pockets. Failure to effectively eliminate them might result in persistent irritation, initiation and progression of periodontal disease.

The use of newer technologies and knowledge in the field of microbial diagnostics holds real time promise in terms of establishing a truly holistic picture of complex disease as well as providing targets for diagnostic and therapeutic strategies. No doubt new tests hold considerable promise and clinicians are on the threshold of treating patients with accuracy. No feat is final until we stop trying!

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