TITLE

Molecular Diagnostic and Exploratory Assays and Arrays- Periodontal uses and comparative applications

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ABSTRACT

Multiple biomarkers pave the way for diagnosis of periodontal disease and to determine the disease prognosis. Thus, biomarkers have substantial application in disease diagnosis, prognosis, and therapeutics. Quantification of levels of cytokines as well as other biomarkers in conditions like periodontitis and peri-implantitis help in identifying ongoing pathological processes because research has revealed that the intensity of periodontal disease is related to the level of the biomarker. Furthermore, identification of the status of ongoing disease activity maybe hastened using chairside diagnostic kits which utilize specific biomarkers as their basic analytical element. Multiplex arrays that identify multiple biomarkers from a single sample are useful to arrive at unambiguous diagnosis as compared to single biomarker assays. Understanding and comparing the various assays and arrays available, along with the overall knowledge of their working principles and characteristics, help in categorically choosing specific assays or arrays. This review gives an overview of the diagnostic assays and arrays used in periodontics with a focus on application of multiplex arrays in periodontology.

Keywords

Biomarker, Chairside diagnostics, Cytokine, Multiplex array, Periodontitis, Peri-implantitis

Introduction

A biomarker is a material that can be factually measured and evaluated as the indicator of a biologic activity which is physiologic, pathologic or occurring as a response to a therapeutic interference.[1] Biomarkers that are of significant use in diagnosis and assessment of periodontal diseases include microbial factors including microbial genetic elements, host response factors such as the cytokines like IL-1β, TNF-α, products of connective tissue breakdown such as osteocalcin.[1]

The predominant category of biomarker utilized in periodontal disease diagnosis is the cytokine. Cytokines, the cell signalling molecules, as well as cytokine receptors and signal cascades, maintain a delicate balance to preserve the health of periodontal and peri-implant tissues. The cytokines can be found in the Gingival Crevicular Fluid (GCF), PICF, tissues, body fluids, serum, and blood and may have proinflammatory or anti-inflammatory properties which help in manipulating the progression of periodontal and peri implant disease. The extent of inflammatory response is intricately linked to the levels of cytokines produced. Cytokines exhibit pleiotrophism and they are regulated through autocrine, paracrine and juxtacrine pathways. In peri implant tissues, early stages of inflammation are led by proinflammatory cytokines and chemokines. The peri-implant crevicular fluid (PICF) at peri-implantitis sites have demonstrated higher quantities of pro-inflammatory cytokines and chemokines as compared to healthy sites.[2] New therapeutic strategies are being developed that help in modulating the inflammatory process, causing major changes in cytokine interplay.

Identifying patients with periodontal diseases require accurate quantification of the biomarkers associated with inflammation.[3] Research has shown that combined assessment of four key biomarkers IL-1β, IL-6, MMP-8 and MIP-1α can facilitate in differentiating gingivitis from

periodontitis.[4] Mesoscale Discovery (MSD) Human Pro-Inflammatory kit has been utilized to assess levels of multiple cytokines to determine the regulatory role of phytocannabinoids in inflammation.[5]Another study involving identification of overexpression of KLF-2 protein which enhanced transcription of IL-10 and TGF-β helped understand that enhancing the levels of anti- inflammatory cytokines help in inhibiting bone loss.[6] Thus, it can be inferred that the assessment of biomarkers from PICF, GCF, cells or tissues enables the clinician to understand the disease process and to design treatment strategies.

Assessment of cytokine levels, combined with microbial-based tests for identification of microbial factors in initiation and progression of periodontal disease along with tests for other biomarkers that help in determining the host response as well as tissue breakdown factors, can be used to improve the diagnostic efficiency of biomarker assays and arrays.

A variety of biomarker assays have been developed for diagnostic purposes and the chairside biomarker detection kits make it easier to detect and treat cytokine and other biomarker-mediated diseases in the medical as well as the dental field.

Conventional biomarker detection methods like traditional ELISA kits, immunoprecipitations, or western blots help in identifying a single biomarker. Besides being expensive, time consuming and requiring large sample volumes, the lesser sensitivity of these kits limit their application. Assessing multiple biomarkers concurrently provide more comprehensive information regarding the tissues due to the simultaneous cytokine as well as other molecular interactions at play in the biological systems. Thus, multiplex-type formats (DNA and protein microarrays) which can assess multiple biomarkers at a single time-point are gradually coming to the forefront of diagnostic technologies.[7,8] A complete analysis of biological responses and functions can be obtained through multiplex assays, and they offer a rapid, sensitive, and cost-effective alternative to detecting multiple proteins in a single liquid sample.[9] ELISAs and newer

multiparametric technologies/multiplex assays/cytokine arrays share the same general principles with difference in specific methodologies.

Assessment of levels of molecular biomarkers has diagnostic and prognostic potential to facilitate therapeutic decisions and monitoring disease progression. This literature review focuses on the molecular and cellular biomarker detection assays and available multiplex assay kits and will facilitate selection of a specific biomarker assay or array kit for specific diagnostic purposes.

Method

A literature search was performed in Pubmed/Medline, SCOPUS, Web of Science using the search terms 'cytokine OR biomarker AND assay OR array AND periodontal disease'. The search terms were adapted for each of the databases. All articles published in English language till 31st of January 2023 were included for initial screening. The references in the included papers were checked for additional records. Following the search, all relevant articles were collected and uploaded into Mendeley reference manager (version 1.19.4) and duplicates were removed. Titles and abstracts were screened by two independent reviewers (MR, LP). Articles that were possibly related to the topic were identified and retrieved in full and were assessed in detail by the reviewers (MR, LP). After screening the full text, articles pertaining specifically to the multiplex arrays used in periodontal research and comparison of single plex and multiplex assays were evaluated, and the data obtained was summarised as a narrative review.

Results

5702 articles were obtained at the end of the initial search. After removal of duplicates, 3528 articles were subjected to title and abstract screening, of which 128 were included for full text screening. 55 papers were included in the final review. The information obtained from the

reviewed papers has been divided into: i) categories of assay or array, ii) comparison of the kits iii) chairside diagnostic kits.

i)Classification of biomarker assays

The Biomarkers, EndpointS and other Tools (BEST) glossary[10] have classified biomarkers into i) *susceptibility/risk biomarker* for which C-reactive protein is an example, ii) *diagnostic biomarker* which includes gene expression profiling, assessment of the salivary levels of alkaline phosphatase (ALP) aspartate aminotransferase (AST) lactate dehydrogenase(LDH), iii) *monitoring biomarkers* for assessing the status of a disease or effect of exposure to a medical product; example: International normalized ratio (INR) for assessing the desired effect of anticoagulation, iv) *prognostic biomarker* for periodontal disease such as ALP v) *predictive biomarker* that may be used for prediction and early diagnosis of periodontal disease include MMP-8 (Metalloproteinase-8), MIP-1α (Macrophage inflammatory protein-1 alpha), IL-1 β (Interleukin-1 beta), IL-6 (Interleukin-6), vi) *response biomarker* that assesses the response to a therapy or medical product, v) *safety biomarker* which may be used to assess the extent of toxicity.

Majority of biomarker assays in periodontics are based on assessment of cytokines. Cytokine levels can be assessed through *ex vivo* assays or *in vivo* assays. *In vivo* assay measures biological activity of cytokines in humans. However, its use is restricted, and this has paved the way for in vitro/ex-vivo bioassays and immunoassays. Bioassays measure biological activity of cytokines in tissues and body fluids while immunoassays evaluate cytokine levels but not the cytokine activity.[8]

Bioassays confirm the identity of the measured cytokine through neutralization with cytokine specific antibodies. They are highly sensitive but lacks specificity and exhibit cytokine

redundancy, that is, same biologic activity of different cytokines. Bioassays may be classified based on the type of target cell function measured as: i) proliferation or growth inhibition assays; ii) cytotoxicity assay; iii) assays that depend on induction of particular cell function; iv) target cell protein induction estimation assays. [8]

Immunoassay, measuring the levels of cytokines, depend on the quality of capture and detection antibodies and it has greater advantage than bioassays as its specificity and sensitivity are acceptable, it has a rapid turnaround time, it is convenient to use, and has relatively low cost.[8] Immune assays have been studied extensively for their use in determining the diagnosis and prognosis of periodontal diseases. Teles et al utilized multiplexed bead immunoassay (Luminex) to detect multiple cytokines in whole saliva and concluded that mean salivary cytokine levels could not differentiate between periodontal health and disease.[11] On the other hand, Thunell et al utilized 22-multiplex fluorescent bead-based immunoassay and found that several proinflammatory cytokines decreased significantly at diseased sites after initial periodontal therapy.[12]

Cytokine array kits use antibody pair-based assay which uses membrane rather than a plate. It can simultaneously measure multiple cytokines at the same time and has high sensitivity. It requires less volume of samples and saves time as well as eliminates the need for running multiple Enzyme Linked Immunosorbent Assays (ELISAs) required for the same number of analytes.[13] In cytokine arrays, capture antibodies are arranged on a membrane and each pair of spots represent a non-identical analyte. Factors to consider when selecting a suitable assay format to detect and analyse cytokines include the type of sample material, the number of analytes, and the sensitivity and throughput required.[13]

The methods for cytokine assessment have been classified by Whiteside in 2002 into assays to identify i) cytokines that present in body or tissue fluids or cell supernatants, ii) cytokine that

are produced by different cell population, iii) cytokine expression assays for single-cell cytokine secretion iv) assays for detection of tissue cytokines, v) assays for Cytokine profiling.[8] Another classification of biomarker assays depending on the type of information required has been given by Sachdeva and Asthana in 2007.[7]

Understanding the various types of biomarker assay/array kits will enable researchers and clinicians identify the assay most suitable for a particular condition.

ii) Biomarker assays and arrays

The following section gives a brief description of the various types of biomarker assays and arrays that may be utilized for diagnostic purposes.

ii.a) Enzyme Linked Immunosorbent Assay (ELISA)

It is based on antigen-antibody interactions and accordingly four types of ELISAs are there. In direct ELISA, the immobilized antigen is coated on with detection antibody. In indirect ELISA antigen is bound by primary antibody which is specific to it and a secondary detection antibody is added to it. In sandwich ELISA, multi-well plate coated with capture antibody facilitates antigen immobilization and then secondary detection antibody is coated over it. In competitive ELISA, a multi-well plate is pre-coated with the reference antigen and sample is added to the well after it is pre-incubated with labelled antibody.[14]

Singleplex assays measure the level of single cytokine. These singleplex assays have limited application in research involving detection of multiple cytokines. Parallel performance of several workflows may result in wastage of time with an increased risk of errors. An increase in sample volume required in proportion with number of analytes measured is another disadvantage of singleplex assays.[15]

Hence there is a shift towards use of multiplex ELISA which requires small sample volume and gives data on large number of analytes. Electrochemiluminescence **(**ECL)-based multiplex assays are more responsive with a significant dynamic range and are more time-efficient than standard ELISAs. Commonly used multiplex techniques are bead based which also works on sandwich ELISA principle but vary in detection methodology. It uses fluorescence detection systems whereas calorimetric substrate amplification is performed in ELISA and its capture antibodies are immobilized on suspended spherical beads.[15]

ii.b) Radioimmunoassay

Usually, binding specificity of antibody is given importance in immunoassays for detection of an antigen and so it is labelled using a radioactive isotope. When this is applied, the term radioimmunoassay is given. In radioimmunoassay, the unknown samples containing antigen are incubated with the Antibody (Ab) and radiolabelled antigen (Ag*) along with the control in which the Ab and Ag* are incubated in the absence of the unknown samples containing Ag. Either free or antibody-bound Ag* are quantified at the end of the reaction, through radioactive counting. By estimating the reduction in Ag* binding, the concentration of antigen in the unknown sample is calculated.[16]

Immunoradiometric assay (IRMA) uses radiolabelled antibody in excess concentration as reagent. In this technique, the radiolabelled antibody binds to the free epitopes of antigens and form the antigen-antibody complex, and the unbound radiolabelled antibody are removed using another reaction with solid phase antigen. Concentration of analyte is given by radioactivity detection in solid phase after washing. It has increased sensitivity and specificity than radioimmunoassay.[17]

ii.c) Chemiluminescence Assays

In this, luminescent molecule is used to indicate analytic reaction and it is termed chemiluminescence assay because an exorgenic chemical reaction is considered an effective source of energy to produce the electronic excited state. Direct method uses luminophore markers and indirect method uses enzyme markers. It uses enhancer which makes it highly advantageous over radioimmuoassay, immunoenzymatic (ELISA), fluoroimmunoenzymatic (FEIA) methods, etc., because it involves electronic activation and has elevated analytic sensitivity.[18]

ii.d) Cytokine Bioassays

Basis of cytokine bioassays is the detection of specific biologic activity of cytokines on the different cell lines. Immunoassay measures only immunologically reactive material while, bioassays can be precise and accurate and can give information on what all cytokines are biologically inactive and what all are in bound form to receptors. Thus, it assesses cytotoxicity, cell proliferation, secretion and inhibition of cytokine activity.[19]

ii.e) Multi-Parametric Flow Cytometry

Fluorescent detection of analytes using beads of known fluorescence saves time by simultaneous detection of multiple cytokines and it uses fewer sample dilutions compared to conventional ELISA. Fluorescent labelled antibody helps in analysing intracellular expression of cytokine also. Carrio et al in 2018 applied multiparametric flow cytometry to enumerate antigen-specific $CD4^+$ T cell precursor frequency accurately in which LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA) was utilized in the assessment and analysis was done using FlowJo software (Tree Star, Ashland, OR, USA).[20] Ayman Rezk et al in 2020 utilized cytokine secreting assay (CSA) combined with multiparametric flow-cytometry (CSA-

Flow). It enabled simultaneous isolation of multiple, low-frequency, cytokine-secreting cells and they found that multi-parametric FACS panels can be integrated to CSA-flow in complex cell-sorting strategies. The flow cytometry isolated IL-10^{+}, GM-CSF^{+} and TNF^{+} B cell fractions which exhibited excellent purities and viabilities.[21]

ii.f) Magnetic Bead Based Quantification

Bead based immunoassays are that which are performed on external surface of beads.[22] The concept of magnetic beads and flow cytometry combine to give an analytical method of magnetic bead-based quantitation. It eliminates matrix interference and generates low limit of detection. It is called suspension arrays as suspended beads are employed. Flexibility of this assay type and requirement of smaller sample volume, detection of multiple targets simultaneously and less cost makes it advantageous.[23] Multiplexing up to 500 targets is achieved by this assay by the use of three different dyed fluorophores coated on polystyrene beads thus multiple biomolecular interactions could be detected in a single well. [22]

ii.g) mRNA Based Assays

Cytokine transcripts can be detected in tissues as well as cells using real time polymerase chain reaction (RT-PCR), RNase protection assay, in situ hybridization. The high-quality nucleic acid requirement is a disadvantage in mRNA-based methods. This may be subjected to degradation while tissue/cell handling.[7]

ii.g.1) Quantitative reverse transcriptase linked-polymerase chain reaction (RT-PCR)

Zhou W et al in 2016 studied mRNA expression of virulence and iron/heme utilization with the help of RT-PCR.[24] Iwai et al in 2018 also used RT-PCR in testing the effect of Interleukin-1 beta (IL-1β) on *follicular dendritic cell-secreted protein (FDC-SP*) gene expression in

immortalized human periodontal ligament (HPL) cells, FDC-SP mRNA and protein levels in HPL cells.[25] In the study by Wang et al in 2019, RT-PCR was used in the detection of the influence of IL-18 on expression of mRNA and release of matrix metalloproteinase (MMP)1, MMP2, MMP3 and MMP9 in HPL fibroblasts.[26] Kim et al in 2020, assessed the mRNA expression of proinflammatory cytokines interleukin (IL)1β and IL6 using RT-PCR.[27] In a study by Pei Ying et al in 2020, the process of miR-181b-5p controlled proliferation as well as osteogenic differentiation using PTEN/AKT pathway was assessed using real-time PCR. Thus, these assays help in the monitoring the complex processes involved in the delicate balance between periodontal health and disease progression.[28]

ii.g.2) Northern Blotting

It is a hybridization technique utilizing DNA/RNA probe on isolated RNA. Several transcript characteristics could be identified through this technique.[29] Zhang et al in 2003 did a study to find whether TGF-β stimulates mineralization. For that, osteoblast cultures were obtained and RNA extraction was done. Northern blot analysis measured type 1 collagen, osteocalcin thus determining the role of exogenous TGF-β1 in human osteoblast/ implant system mineralization.[30]

ii.g.3) In-Situ Hybridization

Detection and localization of specific nucleic acid sequence is done through the in-situ hybridization technique.[31] In situ hybridization allows complementary base pairing using radiolabelled or chemically labelled DNA/ RNA probe.[32] Digoxigenin labelled RNA probe was used for in situ hybridization in the study by Harune et al to analyse the disease related genes in patients with periapical periodontitis.[33]

ii.g.4) Ribonuclease Protection Assay

Ribonuclease protection assay is a method to detect RNA expression. It is more sensitive than northern analysis as it needs less than 105 copies of mRNA sequence unlike northern blotting which requires more than 106 mRNA copies to be detected. However, it is less sensitive than RT-PCR.[34] Qingde et al used multiprobe RNase-protection assay system (Pharmingen, San Diego, Calif.) to detect *Porphyromonas gingivalis* (*P.gingivalis*) induced cytokine profile using cytokine mRNA and their results indicated that host immune cells are able to detect expression of different inflammatory profiles.[35]

ii.h) Intracellular Cytoplasmic Staining

This method gives information on the type of cells which produce cytokine of interest. Flow cytometer is used in this method to detect the frequency of cytokine producing cells and their phenotypes.[36] Verronesse et al used intra-cytoplasmic staining and multi-parametric flow-cytometry to find whether the short term stimulated natural killer cells, dendritic cells, monocytes produce specific cytokines.[37]

ii.i) Enzyme Linked Immunospot Assays

The frequency of cytokine secreting cells at single cell level can be detected using highly sensitive immunoassay- Enzyme linked immunospot assays. The cells are coated with capture antibodies and when these cells secrete proteins such as cytokines, they are captured, detected by detection antibodies and individual cytokine secreting cells are identified by each spot. It is highly sensitive, easy to perform and able to analyse large number of samples.[38] Katoka et al used enzyme-linked immunospot (ELISPOT) assay to enumerate the numbers of rFimA-specific IgA or IgG Ab-forming cells (AFCs) utilizing *r*FimA and FL/CpG of *P.gingivalis* as potential candidates in the production of mucosal vaccines for periodontal patients.[39]

ii.j) DNA Microarrays

Gene expression levels are measured using DNA microarrays. Specific DNA sequences are used as well as measured in this method. Location and sequence of each spot is analysed using a computer database. Nucleic acid hybridization is the basic principle of DNA microarray. Relative concentration of nucleic acid species in a solution is determined using a target or a probe.[40] Petra et al used DNA-microarray kit (Protean Ltd., Ceske Budejovice, Czech Republic) and found that the development of aggressive periodontitis is due to Th1/Th2 balance disruption by periodontal bacteria and genetic predisposition for altered expression of IL-10.[41]

ii.k) Protein Microarrays

Biochemical activities of proteins are studied using protein microarrays. This can be analytical microarray which makes a profile using complex mixture of proteins or functional microarray uses functional proteins. They let the study of numerous protein interactions detect altered protein levels causing disease.[42] In Yudong et al study, they employed protein microarray which revealed differentially expressed mRNA in peri-implantitis and also an upregulated cyclooxygenase-2 pathway and high *RANKL*/*OPG* ratio in peri-implantitis than periodontitis.[43]

iii) Comparison of biomarker arrays and assays

Among the different commercial kits available, each multiplex proteomic platform has unique strength, performance characteristics and limitations. Thus, selection of the appropriate multiplex biomarker profiling method depends on the consideration of sensitivity, reproducibility, volume of sample to run the assay, time and then cost of the assay.[44] The comparison of the different biomarker assays and arrays are given in table 1.

Table 1: Comparison of different biomarker array/assay kits

	Examples of Available kits	Principle	Sensitivity	Cost	Benefits
Multiplex assay kits	Luminex Multiplex Immunoassay	Bead based technology	Less sensitivity in obtaining quantitative data. Better sensitivity in qualitative data.[45]	Expensive as compared to traditional ELISA	Detection and quantification of multiple secreted proteins.
Multiplex assay kits	Invitrogen ProcartaPlex immunoassays	Magnetic bead reagent kits and panels for multiplex protein quantification which is antibody based. It uses the Luminex instrument platform.	Optimal performance and reproducibility [46]	Expensive	Detection of over 300 protein targets
Multiplex assay kits	FirePlex® particle technology	Immunoassays which are bead based and can be used for flow cytometers	Efficient in quantification of proteins	Cost-effective	Multiplex panels which are customizable. May be used with standard bench-top flow cytometers Efficiently quantifies multiple proteins
Multiplex assay kits	Bio-Plex® multiplex immunoassay system- Bio-Plex 200 and Bio-Plex 3D systems	Flow cytometry based analysis	Higher sensitivity as compared to simple ELISA	Cost effective	Less time, less sample volume
Multiplex assay kits	Bio-Plex® MAGPIX™ multiplex reader	LED/image based analysis	Higher sensitivity as compared to simple ELISA [47]	Cost effective [47]	Less time, less sample volume [47]
Multiplex assay kits	Becton Dickinson Immuno-cytometry Systems	The Cytometric Bead Array (CBA) system from BD Biosciences (San Jose, CA, USA) uses a single fluorophore to accomplish multiplexing	Sensitivity is excellent. Reduced number of assays than in array defined by two fluorochromes	Expensive	Flow cytometer used for clinical applications which is present in the laboratory may be used [48]
Multiplex assay kits	Copalis multiplex technology	Latex monomeric microspheres are differentiated from cells other latex aggregates based on the light scatter properties by flow cytometry	compared to fluorescent techniques, they have reduced sensitivity	Expensive	Useful when antibodies to infectious agents or autoantigens, which are coated onto latex microparticles are to be detected. [48]

Radio-immunoassay	Multiple RIA antibodies	In this, radio labelled target antigen is bound to the specific antibodies. A competitive antigen-antibody reaction of the labeled antigen from the preparation and unlabeled antigen from serum is initiated by adding the sample	High sensitivity which can be further improved by reducing the quantity of radiolabelled antigen or antibody and also by disequilibrium incubation.[49]	More expensive than ELISA	Simplicity, sensitivity.
Chemiluminescence	Lumigen kits	quantitative detection using the ligand-binder assay	High detection sensitivity	Cost effective	It is capable of measuring very low and very high light intensities using simple instrumentation. It has the ability to provide a large dynamic range of measurement[50]
mRNA based assays	QuantiGene Gene Expression Assays for Biomarker Verification	Branched DNA technology is used for profiling gene expression accurately. Signal amplification is used for direct measurement of RNA transcripts	Highly sensitivity	Expensive	Demonstrated concordance[51] whole-transcriptome microarrays (Hall, Linton) Measure up to 80 genes simultaneously, minimal sample preparation required
RNAase protection (RPA) assays	RiboQuant RPA kits	Target mRNA is hybridized to the probe in a liquid matrix	In comparison to RT-PCR, RPA is less sensitive	Not cost effective	Relatively high throughput[52]
DNA microarrays	GeneID DamID	Hybridization between two DNA strands is the basic principle of the microarrays.	high specificity, sensitivity, and reproducibility.[53] DNA microarrays are limited in its ability in determining gene expression levels in proteomics	The expense is less than that of DNA sequencing.[54]	Comparative genomic hybridization may be used to detect DNA and RNA detection is possible mostly through cDNA after reverse transcription
Protein microarrays	PISA (protein in situ array), NAPPA (nucleic acid programmable protein	High-throughput method which is useful for tracking protein activities, interactions and functions	Protein microarrays are use minimal amounts of samples and reagents and is rapid, sensitive and automated. [55]	Economical, [55]	Large number of proteins will be tracked parallelly.

	array) and DAPA (DNA array to protein array).				

iv) Chairside biomarker detection kits

Chairside tests help dentists in the assessment of present disease activity, prediction of progression of the disease and evaluation of efficacy of the treatment which complements the traditional probing and radiographic methods.[56] Periodontitis related biomarkers assist in determining the current disease status as well as risk evaluations

A subgroup of micro electromechanical system (MEMS) known as the Lab-on-a-chip (LOC), consists of a small chip with miniaturised laboratory function integrated into it and has the advantage of providing quicker diagnostic results. It works on chemically sensitized beads and its microfluidic LOC technology can detect as low as picolitres with the help of microchannels in LOC. This enables enabling complex assays with the manipulation of biochemical reactions at very small volumes, with short analysis times with reduced reagent costs.[57]

These LOC platforms have the benefits such as consumption of less reagent and sample volume with a fast turnaround time to obtain results. However, need for complex accompanying facilities and high fabrication costs limits the widespread utilization of these platforms.[56]

Point of care testing (POCT) can be used onsite for the detection of periodontitis biomarkers which gives results within 20 minutes, thus enabling the people to pay more attention for their oral health. It also has ease of use and low cost as its advantages.[56]Prognosis of periodontitis can also be evaluated using POCT platforms including fluorescence and electrochemical methods which gives more accurate results.[56]

POCTs have been used in periodontal diagnosis have been categorised into three: Lab-on-a-chip (LOC), paper-based platforms, and chairside tests. LOC platforms primarily utilize the principle of immunoassays for periodontal disease diagnosis. An integrated microfluidic platform for oral diagnostics (IMPOD) which is an immunoassay-based LOC platform, can identify salivary proteins in a sample volume which is as small as 10 µl with a high degree of sensitivity.[56,58]

Paper-based platforms such as lateral flow strips and vertical flow strips have been developed to aid in periodontal disease diagnosis.[58]Detection of periodontal micro-organisms have been performed using tests such as Omnigene Diagnostics test which depends on nucleic acid hybridization, the Evalusite kit that utilizes sandwich enzyme immunoassay and the PerioScan kit in which the reaction involving hydrolysis of N-benzoyl-DL-arginine-2 naphthylamide (BANA) is used. PerioCheck, a chairside test kit which is approved by the FDA, has a high sensitivity for detection of periodontitis. It also detects neutral protease and possesses 61% specificity for polymorphonuclear leukocyte (PMN) collagenase. PerioGard and PocketWatch, are other chairside test that are capable of measuring GCF AST concentration by a colorimetric reaction but are not of significant use in clinical practice because of the low sensitivity and complex procedure involved. Dento Analyzer is capable of analysing and quantifying, within 20 minutes, dentition-related bacteria and marker enzymes (e.g., MMP-8) in saliva or GCF. It is preferable to use easy and accessible qualitative or semi-quantitative readout POCT methods such as methods using colorimetric assays for diagnosis of periodontal disease.[59]

Conclusion

Analysis of biological responses and functions in a pathological condition mediated by cytokines and other biomolecules is of paramount importance as it helps in arriving at quicker

decision on diagnosis and establishment of relevant therapeutic strategy. Rather than going for a single biomarker detection, multiple biomarker analysis allows usage of minimal sample volumes and gives data on large number of analytes. Higher sensitivity was found in bioplex assays, radioimmunoassays, mRNA-based assays, protein microarrays. Higher specificity was found in DNA microarray. Quick assessment of existing disease activity is enabled with the help of chairside test kits. LOC technology detects sample volumes as low as picolitres with shorter analysis time and reduced reagent costs. Thus, the selection and application of each biomarker assay has immense diagnostic and prognostic potentials and will facilitate appropriate therapeutic decisions and monitoring of the disease progression.

References

1. Taba M, Kinney J, Kim AS, Giannobile W V. Diagnostic Biomarkers for Oral and Periodontal Diseases. *Dent Clin North Am*. 2005;49(3):551-571. doi:10.1016/j.cden.2005.03.009

2. Petkovic-Curcin A, Matic S, Vojvodic D, Stamatovic N, Todorovic T. Cytokines in pathogenesis of peri-implantitis. *Vojnosanit Pregl*. 2011;68(5). doi:10.2298/vsp1105435p

3. de Lima CL, Acevedo AC, Grisi DC, Taba M, Guerra E, de Luca Canto G. Host-derived salivary biomarkers in diagnosing periodontal disease: systematic review and meta-analysis. *J Clin Periodontol*. 2016;43(6). doi:10.1111/jcpe.12538

4. KC S, Wang XZ, Gallagher JE. Diagnostic sensitivity and specificity of host-derived salivary biomarkers in periodontal disease amongst adults: Systematic review. *J Clin Periodontol*. 2020;47(3). doi:10.1111/jcpe.13218

5. Abidi AH, Abhyankar V, Alghamdi SS, Tipton DA, Dabbous M. Phytocannabinoids regulate inflammation in IL -1β-stimulated human gingival fibroblasts. *J Periodontal Res*. 2022;57(6):1127-1138. doi:10.1111/jre.13050

6. Tanaka U, Kajioka S, Finoti LS, Palioto DB, Kinane DF, Benakanakere MR. Decitabine Inhibits Bone Resorption in Periodontitis by Upregulating Anti-Inflammatory Cytokines and Suppressing Osteoclastogenesis. *Biomedicines*. 2021;9(2). doi:10.3390/biomedicines9020199

7. Sachdeva N, Asthana D. Cytokine quantitation: Technologies and applications. *Frontiers in Bioscience*. 2007;12(12). doi:10.2741/2418

8. Whiteside TL. Cytokine assays. *Biotechniques*. 2002;33(4 SUPPL.). doi:10.2144/oct0202

9. Elshal MF, McCoy JP. Multiplex bead array assays: Performance evaluation and comparison of sensitivity to ELISA. *Methods*. 2006;38(4):317-323. doi:10.1016/j.ymeth.2005.11.010

10. FDA-NIH Biomarker Working Group. BEST (Biomarkers, EndpointS, and other Tools) Resource [Internet]. Silver Spring (MD): Food and Drug Administration (US); 2016-. Glossary. 2016 Jan 28 [Updated 2021 Nov 29]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK338448/ Co-published by National Institutes of Health (US), Bethesda (MD).

11. Teles RP, Likhari V, Socransky SS, Haffajee AD. Salivary cytokine levels in subjects with chronic periodontitis and in periodontally healthy individuals: A cross-sectional study. *J Periodontal Res*. 2009;44(3). doi:10.1111/j.1600-0765.2008.01119.x

12. Thunell DH, Tymkiw KD, Johnson GK, et al. A multiplex immunoassay demonstrates reductions in gingival crevicular fluid cytokines following initial periodontal therapy. *J Periodontal Res*. 2010;45(1). doi:10.1111/j.1600-0765.2009.01204.x

13. Easthope E. Methods for Detecting and Analyzing Cytokines. Biocompare, The buyer's guide for life scientists.

14. Basic principles and types of ELISA | Abcam. Published online October 2021. https://www.abcam.com/kits/elisa-principle#Choosing%20the%20right%20type%20of%20ELISA

15. Multiplex Immunoassays. https://www.bio-rad.com/en-nl/applications-technologies/multiplex-immunoassays?ID=LUSM0E8UU

16. Parker CW. Radioimmunoassay. *Annu Rev Pharmacol Toxicol*. 1981;21. doi:10.1146/annurev.pa.21.040181.000553

17. Sharma A, Pillai MRA, Gautam S, Hajare SN. MYCOTOXINS | Immunological Techniques for Detection and Analysis. In: *Encyclopedia of Food Microbiology*. Elsevier; 2014:869-879. doi:10.1016/B978-0-12-384730-0.00233-0

18. Cinquanta L, Fontana DE, Bizzaro N. Chemiluminescent immunoassay technology: what does it change in autoantibody detection? *Auto Immun Highlights*. 2017;8(1). doi:10.1007/s13317-017-0097-2

19. Liabakk NB, Waage A, Lien E, Stenvik J, Espevik T. Cytokine Bioassays. In: *Meningococcal Disease*. ; 2003. doi:10.1385/1-59259-149-3:487

20. Carrio R, Zhang G, Drake DR, Schanen BC. A novel dendritic cell-based direct ex vivo assay for detection and enumeration of circulating antigen-specific human T cells. *Cytotechnology*. 2018;70(5):1325-1335. doi:10.1007/s10616-018-0222-8

21. Rezk A, Li R, Bar-Or A. Multiplexed detection and isolation of viable low-frequency cytokine-secreting human B cells using cytokine secretion assay and flow cytometry (CSA-Flow). *Sci Rep*. 2020;10(1):14823. doi:10.1038/s41598-020-71750-z

22. van der Wal FJ, Bergervoet JH, Achterberg RP, Haasnoot W. Bead-based immunoassays. In: *Novel Approaches in Immunoassays*. Future Medicine Ltd; 2014. doi:10.2217/ebo.13.560

23. Su R, Tang X, Feng L, Yao G long, Chen J. Development of quantitative magnetic beads-based flow cytometry fluorescence immunoassay for aflatoxin B1. *Microchemical Journal*. 2020;155. doi:10.1016/j.microc.2020.104715

24. Zhou W, Zhang X, Zhu CL, He ZY, Liang JP, Song ZC. Melatonin receptor agonists as the "perioceutics" agents for periodontal disease through modulation of Porphyromonas gingivalis virulence and inflammatory response. *PLoS One*. 2016;11(11). doi:10.1371/journal.pone.0166442

25. Iwai Y, Noda K, Yamazaki M, et al. Effects of interleukin-1β on human follicular dendritic cell-secreted protein gene expression in periodontal ligament cells. *J Oral Sci*. 2018;60(4). doi:10.2334/josnusd.17-0473

26. Wang F, Guan M, Wei L, Yan H. IL-18 promotes the secretion of matrix metalloproteinases in human periodontal ligament fibroblasts by activating NF-κB signaling. *Mol Med Rep*. 2019;19(1). doi:10.3892/mmr.2018.9697

27. KIM MJ, YOU YO, KANG JY, KIM HJ, KANG MS. Weissella cibaria CMU exerts an anti-inflammatory effect by inhibiting Aggregatibacter actinomycetemcomitans-induced NF-κB activation in macrophages. *Mol Med Rep*. 2020;22(5). doi:10.3892/mmr.2020.11512

28. Lv PY, Gao PF, Tian GJ, et al. Osteocyte-derived exosomes induced by mechanical strain promote human periodontal ligament stem cell proliferation and osteogenic differentiation via

the miR-181b-5p/PTEN/AKT signaling pathway. *Stem Cell Res Ther*. 2020;11(1). doi:10.1186/s13287-020-01815-3

29. *Handbook of Glycomics*. Elsevier; 2010. doi:10.1016/B978-0-12-373600-0.X0001-7

30. Zhang H, Ahmad M, Gronowicz G. Effects of transforming growth factor-beta 1 (TGF-β1) on in vitro mineralization of human osteoblasts on implant materials. *Biomaterials*. 2003;24(12). doi:10.1016/S0142-9612(02)00616-6

31. Laurent-Huck FM, Felix JM. Measurement of Oxytocin and Vasopressin Gene Expression by in Situ Hybridization. In: ; 1991:159-182. doi:10.1016/B978-0-12-185259-7.50015-6

32. Shakoori AR. Fluorescence In Situ Hybridization (FISH) and Its Applications. In: *Chromosome Structure and Aberrations*. Springer India; 2017:343-367. doi:10.1007/978-81-322-3673-3_16

33. Naruse H, Itoh S, Itoh Y, Kagioka T, Abe M, Hayashi M. The Wnt/β-catenin signaling pathway has a healing ability for periapical periodontitis. *Sci Rep*. 2021;11(1). doi:10.1038/s41598-021-99231-x

34. Rottman JB. The Ribonuclease Protection Assay: A Powerful Tool for the Veterinary Pathologist. *Vet Pathol*. 2002;39(1):2-9. doi:10.1354/vp.39-1-2

35. Zhou Q, Desta T, Fenton M, Graves DT, Amar S. Cytokine Profiling of Macrophages Exposed to *Porphyromonas gingivalis* , Its Lipopolysaccharide, or Its FimA Protein. *Infect Immun*. 2005;73(2). doi:10.1128/IAI.73.2.935-943.2005

36. Kunisawa J, Kiyono H. Analysis of Intestinal T Cell Populations and Cytokine Productions. In: ; 2010:183-193. doi:10.1016/S0580-9517(10)37009-7

37. Verronèse E, Delgado A, Valladeau-Guilemond J, et al. Immune cell dysfunctions in breast cancer patients detected through whole blood multi-parametric flow cytometry assay. *Oncoimmunology*. 2016;5(3). doi:10.1080/2162402X.2015.1100791

38. Axelsson B. Detection of Cytokine-Secreting Cells by. In: ; 2022:61-79. doi:10.1007/978-1-0716-1771-7_5

39. Kataoka K, Kawabata S, Koyanagi K, Hashimoto Y, Miyake T, Fujihashi K. Respiratory FimA-Specific Secretory IgA Antibodies Upregulated by DC-Targeting Nasal Double DNA Adjuvant Are Essential for Elimination of Porphyromonas gingivalis. *Front Immunol*. 2021;12. doi:10.3389/fimmu.2021.634923

40. Bumgarner R. Overview of DNA microarrays: types, applications, and their future. *Curr Protoc Mol Biol*. 2013;Chapter 22. doi:10.1002/0471142727.mb2201s101

41. Borilova Linhartova P, Danek Z, Deissova T, et al. Interleukin Gene Variability and Periodontal Bacteria in Patients with Generalized Aggressive Form of Periodontitis. *Int J Mol Sci*. 2020;21(13). doi:10.3390/ijms21134728

42. Hall DA, Ptacek J, Snyder M. Protein microarray technology. *Mech Ageing Dev*. 2007;128(1). doi:10.1016/j.mad.2006.11.021

43. Liu Y, Liu Q, Li Z, et al. Long non-coding RNA and mRNA expression profiles in peri-implantitis vs periodontitis. *J Periodontal Res*. 2020;55(3). doi:10.1111/jre.12718

44. Günther A, Becker M, Göpfert J, Joos T, Schneiderhan-Marra N. Comparison of Bead-Based Fluorescence Versus Planar Electrochemiluminescence Multiplex Immunoassays for Measuring Cytokines in Human Plasma. *Front Immunol*. 2020;11. doi:10.3389/fimmu.2020.572634

45. Vitl Life Science Solutions. Multiplex Immunoassays vs Gold Standard Assay Techniques. Published online October 2018. https://www.news-medical.net/whitepaper/20170926/Multiplex-Immunoassays-vs-Gold-Standard-Assay-Techniques.aspx

46. ProcartaPlex Immunoassays | Thermo Fisher Scientific - NL. https://www.thermofisher.com/nl/en/home/life-science/antibodies/immunoassays/procartaplex-assays-luminex/procartaplex-immunoassays.html

47. Multiplex Immunoassays. https://www.bio-rad.com/en-nl/applications-technologies/multiplex-immunoassays?ID=LUSM0E8UU

48. Elshal MF, McCoy JP. Multiplex bead array assays: Performance evaluation and comparison of sensitivity to ELISA. *Methods*. 2006;38(4). doi:10.1016/j.ymeth.2005.11.010

49. Kramer DK. Radioimmunoassay (RIA) | www.antibodies-online.com. https://www.antibodies-online.com/resources/17/1215/radioimmunoassay-ria/

50. Chemiluminescence | Lumigen. http://www.lumigen.com/detection-technologies/chemiluminescence/assay-development

51. Gene Expression Analysis & Genotyping | Thermo Fisher Scientific - NL. https://www.thermofisher.com/nl/en/home/life-science/gene-expression-analysis-genotyping.html

52. Cummings R, Pierce M. *Handbook of Glycomics*. Vol 5. Elsevier Gezondheidszorg; 2009. doi:10.1016/B978-0-12-373600-0.00005-6

53. Song Y, Dou F, He S, Zhou Y, Liu Q. Laboratory and Clinical Evaluation of DNA Microarray for the Detection of Carbapenemase Genes in Gram-Negative Bacteria from Hospitalized Patients. *Biomed Res Int*. 2019;2019. doi:10.1155/2019/8219748

54. NHGRI. DNA Microarray Technology Fact Sheet. https://www.genome.gov/about-genomics/fact-sheets/DNA-Microarray-Technology

55. Mitchell P. A perspective on protein microarrays. *Nat Biotechnol*. 2002;20(3). doi:10.1038/nbt0302-225

56. He W, You M, Wan W, Xu F, Li F, Li A. Point-of-Care Periodontitis Testing: Biomarkers, Current Technologies, and Perspectives. *Trends Biotechnol*. 2018;36(11). doi:10.1016/j.tibtech.2018.05.013

57. Steigmann L, Maekawa S, Sima C, Travan S, Wang CW, Giannobile W v. Biosensor and Lab-on-a-chip Biomarker-identifying Technologies for Oral and Periodontal Diseases. *Front Pharmacol*. 2020;11. doi:10.3389/fphar.2020.588480

58. He W, You M, Li Z, et al. Upconversion nanoparticles-based lateral flow immunoassay for point-of-care diagnosis of periodontitis. *Sens Actuators B Chem*. 2021;334:129673. doi:10.1016/j.snb.2021.129673

59. Srivastava N. Point of Care- A Novel Approach to Periodontal Diagnosis-A Review. *JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH*. Published online 2017. doi:10.7860/JCDR/2017/26626.10411