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A NOVEL DIAGNOSTIC APPROACH IN RESTORATIVE DENTISTRY AND ENDODONTICS – AN UPDATE.

Endodontic

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ABSTRACT

Despite all the dental information acquired over centuries and the importance of proteome research, cross-link between these two areas only emerged around mid-nineties. Proteomic tools help dentistry in the identification of risk factors, early diagnosis, prevention, and systemic control that promotes the evolution of treatment in all dental specialties. It is a remarkable tool that can revolutionize the treatment of oral disease in all the context's leading to changes in treatment philosophy. A literature review is done using electronic databases, such as "PubMed," "Google Scholar," and "Scopus," using keywords "Proteomics," and "Mass Spectrometry". Furthermore, an advanced or refined search was carried out using the keywords "Proteins," "Enamel," "Dentin," "Cementum," "Periodontal ligament" and "pulp". The idea is to make dentistry a more individualized area, with the need for minimal interventions. Proteomics tools bring a drastic change in diagnosis, treatment, and prevention of dental diseases.

KEYWORDS

Proteomics, Mass Spectrometry(ms), Endodontics, Restorative Dentistry.

1. INTRODUCTION

Proteomics is the large-scale study of proteins. Proteins are referred to as the building blocks of the human body and are known as the "working horses" of a cell.[1] Proteins with many functions are vital parts of living organisms; In analogy with genomics, the study of the genome the term proteomics was coined in 1997. The word "Portmanteau" of protein and genome and was invented by Marc Wilkins[2] in 1994, at Macquarie University. Macquarie University also coined the first dedicated proteomics laboratory in 1995 (the Australian proteome analysis facility – APAF).

The study of proteins in particular concerned to an organism is called Proteomics. It is a non-invasive, accurate, dependable, and a correct way of diagnosing or identifying a cause. Proteins are present both in normal favoring physiologic and abnormal supporting pathological conditions. Proteins present in both clinical situations are different; physiologically proteins produced by healthy living cells are normal, unlike the ones provided tumor markers or bacteria that are responsible for the pathology. This varies with distinct requirements, time and stresses, that an organism or cell undergoes. An interdisciplinary domain that has greatly benefited from the genetic information of the human genome project is proteomics. It refers to the large-scale experimental analysis of proteins and is often mainly used to refer to protein purification and mass spectrometry.

In short, proteomics is the technology that rescues the understanding of the molecular behavior of protein activity and their response to the process of disease.[1] A literature review is done using electronic databases, such as "PubMed," "Google Scholar," and "Scopus," using keywords "Proteomics," and "Mass Spectrometry". Furthermore, an advanced or refined search was carried out using the keywords "Proteins," "Enamel," "Dentin," "Cementum," "Periodontal ligament" and "pulp".

2. PROTEOMICS IN DENTISTRY

Recently, proteomic-based techniques have gained tremendous significance. It leads to a consequential increase in the growth from laboratory development to clinical practice. [3]

By enlarge, proteomics is the grouping of techniques that highlights on multi-protein systems and the interaction of multiple and/or distinct proteins and their part within a general or specific biologic system. Proteomic tools are more frequently used to distinguish the behavior of an entire collection of expressed gene products rather than tracking a single expression.

The literature reports the use of various proteomic tools on a wide variety of organic samples, such as saliva, blood, microorganisms and different tissues (includes normal or pathologic enamel, dentin, pulp, gingiva, bone, ligament, cementum, and mucosa). It was seen that about 64% of dental samples came from a human source, while about 11% of dentistry proteome studies were obtained from different animals (e.g., mice, sheep, rats, pigs, cattle)[3]. Also, about 18% of dentistry proteomics involved the analysis of microorganisms, while, 3% involved the study of different cell lines involved in health and oral disease setting. Among such samples, saliva is a rich-proteome environment with great diagnostic potential and also easily obtained with noninvasive procedures[3,4,5].

Interestingly, Proteomics has spread its wings in the field of Restorative dentistry and endodontics making the diagnosis, prognosis, and treatment becomes more accurate and surer to the molecular level.

The focus is to discuss the evolution of proteomic based research about

restorative dentistry and endodontics and how these tools can improvise understanding in dentistry. Greater integration of these areas will help to understand what is still unknown in oral health and disease.

3. SAMPLE COLLECTION

The samples can be collected from various sites and the collected samples are prepared for protein quantification.

A. SAMPLE COLLECTION FROM HARD TISSUES:

ENAMEL SAMPLES

Coronal sections of 1.2 mm thickness are cut under a continuous, non-recycled stream using a low-speed saw with a, diamond blade. Sections passed through dentin horns and maximum cervical enamel extent on buccal and lingual sides.[6] After cutting, the tooth crown surface was lightly scraped with a scalpel blade to remove debris. Each section was washed three times under continuous 1% PBS rinse for 30s and mounted onto silica wafers with Quickstick 135 Mounting wax. The enamel from each section was diced in a grid pattern with zinc-aluminum blades under continuous flow of distilled water. The samples are prepared[6] and proteins are quantified.

DENTIN SAMPLES

The teeth are washed thoroughly with water, the cementum and soft tissues are mechanically scraped off with an iron spatula, and the teeth should be cleaned with brushes.[7] The crown is dissected out. The roots are crushed into smaller fragments. The dentin samples are demineralised with 0.5mM EDTA at a pH of 8 at 4°c for 16 days or else the smaller fragments of dentin can be frozen with liquid nitrogen and then pulverized to powder with a mortar and pestle. The prepared dentin samples are sequentially extracted, with guanidine buffer and then with disodium ethylenediaminetetraacetic acid (EDTA) buffer. The samples are centrifuged followed by protein quantification.

DENTAL CEMENTUM AND ALVEOLAR BONE

Following tooth extraction, soft connective tissues adhering to the tooth surfaces are carefully scraped off using a sterile curette, and discarded. Teeth are then rinsed in sterile phosphate buffered saline (PBS) several times and cementum samples are collected from the apical region of the root using a curette under stereomicroscope.[8] DC samples were extracted from the apical third of the root, the thickest area of DC, in order to avoid contamination by dentin. Alveolar bone fragments are collected from the tooth extraction sites when osteotomy is indicated. After several rinses in PBS to remove potential contaminants, DC and AB samples were ground using a chisel and stored in sterile PBS at −80 °C, then protein concentration is determined by Bradford's method.[8]

ACQUIRED ENAMEL PELLICLE(AEP)

Subjects are not allowed to eat or drink 2 hours before the sample collection and subjected to dental prophylaxis to remove the previous existing AEP.[9] Subsequently, with a waiting period for each time-points for AEP to form on the enamel surface. Four different time-points 5, 10, 60, and 120 min were used in a study. The collections were carried out on different days for each time-point using the same volunteers. After collecting, the samples were kept at –80°C.

AEP Evaluation from Collection Strips via Sonication.[9] All collection strips are pooled into a 15 ml Falcon tube. Pool samples from each time-point are kept separately. Three ml of 50 mM NH_4HCO_3, pH 7.8 are added into the tubes until all the strips got submerged. Subsequently, the samples are sonicated at room temperature for 1 min. The supernatants are collected and dried in a rotary evaporator. Micro-BCA, a biochemical assay was performed to measure the total protein concentration from each AEP time-point.

B. SAMPLE COLLECTION FROM SOFT TISSUES:

PULP

Followed by access cavity preparation, microbial specimens will be usually obtained from root canals using one sterile K file or H-file, used to disrupt canal wall biofilms, and three sterile paper points. The metallic portion of the file and the paper points should be placed in sterile, DNA-free, and RNA-free vials containing 1.5 ml sterile 10 mm Tris–HCl (pH 8.0) and 0.5 g sterile glass beads (0.71–1.18-mm diameter). The vials are to be frozen. The vials containing paper point specimens must be agitated using a vortex mixer to disperse microbial cellular material into suspension followed by centrifugation. The supernatant is then withdrawn and used for proteomic analysis.[10]

PERIODONTAL LIGAMENT(PDL)

From the literature, a sulcular incision shall be made around the tooth to a limited extent. Gingival tissue tags must be removed by through curetting, contamination should be minimized and the Pdl tissues attached to the root are to be curetted and removed for protein quantification.[11]

C. SAMPLE COLLECTION FROM FLUIDS:

GINGICAL CLEVICULAR FLUID

Methods for collecting GCF are the use of capillary tubes, paper strips, gingival washing, and paper cones. The limited amount of GCF compromises the biochemical and proteomic analysis, and the severity of inflammation in periodontium affects its collection. Its non-invasive sample collection technique helps in analyzing any age group of human subjects and the attraction of allowing multiple sites for sampling within the oral cavity. Many researchers have recently preferred to use paper strips.[12] GCF can also be collected via suction, lavage, or absorption. Suction methods usually employ microca pillaries/micropipettes. The method of collection via abso rpt ion is used in some studies as it is easy to perform, causes minimal bleeding, minimally invasive, and is usually the preferred method of choice. Absorbent paper points are used to collect subgingival plaque or other oral samples to analyze the presence of any microbes, particularly periodontal pathogenic bacteria. Furthermore, the extremely apical portions of the periodontal pocket are accessible, allowing sampling of this niche of front-line species.

WHOLE SALIVA (WS)

Patients should be refrained from eating, drinking, and oral hygiene procedures for at least an hour before saliva collection. The optimum collection time is 8–10 a.m. The sample collected should be processed in the laboratory within an hour. Generally, Spitting method is followed to collect salivary samples from the subjects. In this method, 14 times more bacterial contamination is introduced into the sample.

For the stimulation of glands, chewing different things like natural gum, citric acids, a piece of paraffin wax and powdered drink crystals have been used in the literature[5].

PAROTID GLAND

This method was introduced by Carlson and Crittenden in the year 1910. A double-chambered metallic cup with two outlet tubes is used in this method. One end of the tube holds the cup using vacuum suction. The second part works as a collection vehicle for saliva. Sample collection can be increased by smearing citric acid (10%; 1 mL) on the dorsum of the tongue every 30 s. The first 1.5 mL of saliva before sample collection should be discarded.

SUBMANDIBULAR/ SUBLINGUAL GLAND

This method is introduced by Truelove, Bixler, and Merrit in the year 1967. A "V"-shaped collector is used in this method. It is similar to that for parotid gland collection, but in this method, the initial 2 mL is discarded.

For collection from Minor Glands capillary tubes are used to collect saliva from the everted surface of the lower lips. Introduced by Kutscher et al. (1967).

4. PROCESSING OF SAMPLES

Commonly used means for separation of proteins are liquid chroma tog raphy and 2D gel electrophoresis. 2D gel electrophoresis is widely used to separate a mixture of proteins into single detectable protein spots. The 2D separation of proteins on the gel is achieved according to their isoelectric point and molecular weight. The insolubility of membrane proteins is still an obstacle for 2Delectrophoresis, the advantages being its consistency and high resolution. The ionic detergents used for solubilization of membrane proteins can interfere with the focusing process. Additionally, the mass range and the detection limits also represent the technical limitations of the 2Delectrophoresis method.[13]

The proteins are separated on 2D gels traditionally and stained by silver staining and Coomassie blue. Nevertheless, these detection methods remained problematic due to poor reproducibility or low sensitivity (for Commassie) and dynamic range (for silver). The recent advancements of fluorescent dyes, namely SYPRO™ Ruby, overcame these problems with its sensitive (1–2 ng) detection limits and linear dynamic range over three orders of magnitude. The resulting

2D map is verified by software designed for image analysis, which allows gel–to–gel comparison. Followed by 2D image analysis, the protein spot of interest is excised and treated with acetonitrile and ammonium bicarbonate to remove detergents. The excised spots are in-gel-digested with a protease (trypsin is commonly used) in an optimal buffer for its activity. The digested peptides are then easily evaded from the gel to undergo mass spectrometry analysis. Mass spectrometry (MS) in simpler terms, is a mass spectrum that measures the masses within a sample for characterization of the molecule of interest. It is an investigative technique that ionizes chemical species and sorts the ions based on their mass-to-charge ratio.Mass spectrometry is composed of an ionization source, a mass analyzer, a sample inlet and a detector. Mass spectrometry helps in identification and characterization of thousands of proteins rapidly in a sample. The development of electrospray ionization (ESI)and matrix-assisted laser desorption/ionization (MALDI), the two soft ionization techniques capable of ionizing peptides or proteins, revolutionized protein analysis using MS.

Databases are available for theoretical digests of all known proteins. Matching the peptide-mass data obtained from a sample of interest to theoretically digested protein database can be used to identify unknown proteins successfully. Protein identification via MS is usually carried out in the form of whole-protein analysis ("topdown" proteomics) or analysis of enzymatically or chemically produced peptides ("bottom-up" proteomics).

In top-down proteomics, intact proteins are analyzed using MALDI or ESI, and by gas-phase fragmentation method, the peptides are generated. The bottom-up analysis also called "shotgun proteomics" is the more conventional approach & is used for analyzing peptides produced through chemical or enzymatic cleavage of proteins with posttranslational modification as well as through liquid chromatography (LC) in conjunction with MS. Mrozik et al., evaluated proteomes between bone marrow, periodontal ligaments (PDL), and dental pulp using 2-DE, MS/MS.[14] Salmon et al., found a novel biomarker, 'superoxide dismutase-3' associated with cementum cells through his proteomics study of dental cementum using LC-MS/MS and also detected 231 proteins related to cementum.[15]

5. PROTEOMICS IN RESTORATIVE DENTISTRY

Proteomics is first in the line as a diagnostic tool to date. Nevertheless, it has its significance in treatment and prevention. Newer advancements in operative dentistry such as fluoride-releasing and remineralizing agents are introduced to prevent dental caries in an initial stage. Protein content or derivates will differ from a physiological condition with a pathological condition. These changes in proteins will help in early identification of enamel and dentinal defects such as Amelogenesis Imperfecta, Dentinogenesis Imperfecta and Dental caries[16,17] at an early stage which changes the treatment plan from repair to regeneration.

Ameloblasts secrete Amelogenin(AMELX), Ameloblastin(AMBN), and Enamalin(ENAM), which are structural proteins unique to enamel and derived from the secreted calcium-binding phosphoprotein (SCPP) gene family that evolved over 600 million years ago. Changes in AMELX, ENAM, and AMBN abundance and modification occur within a broader proteomic context that helps regulate mineralization. Proteomic studies of enamel, dentin, bone, and cementum have identified over 200 unique proteins with functions that include calcium-binding, cytoskeletal and cell adhesion, immune function, proteolysis, and protease inhibition.[6] Gel electrophoresis and nano-LC-MS/MS analyses identified structural molecules including 20 keratins, collagens, serpins, ubiquitin and serum albumin. Messenger RNA transcript profiling studies have identified as many as 1700 gene transcripts that are upregulated or downregulated in ameloblasts during either early or late phases of enamel formation (secretion or maturation stage) with annotations in the Gene Ontology (GO) database corresponding to ion transport, pH regulation, calcium interactions, and other functions.

Guo et al. [3] found differences in the proteome profile between S. mutans, from the same donor. It implies relative differences possibly between virulence factor and mechanisms of cariogenesis strains. Certain stressful conditions (oxidation, acid, starvation, salt, and heat) also led to differences in S. mutant protein expression by 2-DE which showed that in all terms, six proteins had enhanced levels by presence of complex and diverse protein alteration in S. mutans survival, considered general stress proteins. Increased synthesis of GroESL and DnaK under S. mutants stressed conditions were demonstrated by 2-DE.

Acquired enamel pellicle (AEP) is a protein biofilm that is formed on the surface of enamel by selective adsorption of peptides and proteins present in the mouth. This protein film builds the interaction with enamel and damages the oral biofilm, which regulates the attachment of bacteria found in oral biofilm. It is a known fact that AEP is formed by the deposition of successive protein layers, which consist of initial binding to enamel and subsequent protein-protein interactions. A study was conducted to observe the quantitative and qualitative changes in pellicle composition during the first two hours of AEP formation in the oral cavity.[9]

Dentin is first composed with the help of odontoblasts, which form the main structure of the tooth on which enamel and cementum are deposited. It has been hypothesized that dentin mineralization is initiated based on the proteins deposited.[4] Dentin has proteins in common with other mineralized tissues (e.g., α2-HS glycoprotein), as well as unique proteins with dentin-specific roles in mineralization.[18] Till date, organic matrix of dentin has been reported to contain collagen, noncollagenous proteins (proteoglycans, phosphophoryns, and glycoproteins), phospholipids, and growth factors.1Type I collagen is reportedly the major protein of the dentin matrix, which also contains lesser amounts of types III, V, and VI collagens. In association with collagen fibrils, Fibronectin has also been found in the predentin. Interestingly, the tissue inhibitor of matrix metallop ro teinase 1 (TIMP1), another secretory product of odontoblasts, is found in high concentrations in the predentin.[4] Evidence exists to show that first of all, the dentin extracellular protein matrix is formed and then based on the protein deposited, dentin mineralization is initiated. [19]

Furthermore, another study revealed identification by 2-DE and MALDI-TOF MS of 18 up-regulated proteins (phosphoglucomutase, cell division proteins, enolase, fructose bisphosphate aldolase, la ctoylglutathione lyase, superoxide dismutase, neutral endopeptidase, acetoin reductase, 60-kDa chaperonin, and lactate dehydrogenase) from S. mutans in pH 5.2 and 12 down-regulated proteins[20] (small-subunit ribosomal protein S1P, protein translation elongation factors G, Tu, and Ts, DnaK, protein phosphotransferase, large-subunit ribosomal protein L12P, and multiple sugar-binding transport systems) in the same pH.[3] Proteomic profile of s.mutans in studies revealed distinct protein expressions that help in better understanding, prevalence, and predicting of virulence and mechanism of action of s.mutans.

Dental cementum is a mineralized tissue covering the tooth root, critical for anchoring the tooth to the surrounding alveolar bone via the periodontal ligament. For both tissues, type I collagen is the primary ECM component, with the remaining organic matrix being composed of varying amounts of noncollagenous proteins (NCPs). These include proteoglycans (e.g., versican, decorin, and biglycan), glycoproteins that are often phosphorylated and sulfated (e.g., osteonectin and arginine– glycine–aspartic acid (RGD) integrin-binding proteins), and gamma-carboxyglutamic acid (gla)-containing proteins (e.g., matrix gla protein, protein S, and osteocalcin). Together, these proteins most likely participate in regulation of cell metabolism, matrix deposition and mineralization, and may contribute to determining the structure and biomechanical properties of the tissue. According to a study, the tissue distribution of 318 proteins was identified. A total of 235 proteins were identified in alveolar bone and 213 proteins were identified in cementum.[8] Of the total proteins identified, 105 were exclusive to alveolar bone samples, whereas 83 were exclusive to Dental cementum. The presence of differentially localized novel proteins in the dental cementum (SERFINF1 and SOD3) and alveolar bone (COL14A1) was identified.

6. PROTEOMICS IN ENDODONTICS

Dental pulp tissue plays an important role in tooth immune defense, formation, sensitivity, and nutrition. In this aspect, proteomic tools have been helping endodontics to understand pulp and periapical tissues in a better way, their reaction when stimulated and how mesenchymal stem cells can be used in tissue engineering. In this regard, proteomic techniques have been widely used in endodontic studies, mainly to analyze and interpret parameters such as the structural formation of these tissues and diagnosis/pathogenesis of the

endodontic disease.[3]

According to Noidorf's generalizations, endodontic infections are very much prevalent and have a polymicrobial etiology associated with complex interrelationships between endodontic microorganisms and the host defenses. Proteomic analysis of endodontic diseases can provide varying insights into the invasion, pathogenicity mechanisms, and multifactorial interactions existing between root canal bacteria and the host in the initiation and progression of periapical pathogenesis. Protein mainly of the cell wall or membrane origin, from endodontic bacteria, especially Enterococcus faecalis, Enterococcus faecium, Porphyromonas gingivalis, Fusobacterium nucleatum, and Treponema denticola. LC-MS/MS(liquid chromatography, mass spectrometry) offers a sensitive analytical platform to study the disease processes in the root canal environment. An array of proteins expressed in endodontic infections projects the complex microbial presence and highlights the bacterial species responsible for the inflammatory process.[10]

Even though DNA-based molecular microbiology methods have allowed to precisely specify and expand the list of microbial species responsible for endodontic infections and associated with different clinical conditions, it is arduous or even impossible to infer pathogenicity and physiology based on these identification methods.[21] Contrarily, Mass spectrometry-based peptide sequence analysis can provide global insights into the pathogenicity mechanisms, virulence, invasion, and multifactorial interactions present between the endodontic microorganisms and the host.[10] A combination of mass spectrometry (MS) with liquid chromatography (LC) has become a dynamic approach for the recognition of proteins occurring in complex mixtures. In methods based on LC, hundreds of proteins or peptides are separated by chromatographic columns, detected, identified, and quantified by mass spectrometry in a single operation.[20,21]

According to a study, Endodontic specimens were aseptically obtained from seven patients with root canal infections.[10] Proteins, mainly of the cell wall or membrane origin, from endodontic bacteria especially Enterococcus faecalis, Enterococcus faecium, Fusobacterium nucleatum, Treponema denticola, and Porphyromonas gingivalis were analyzed from all the samples tested. Analyzed proteins included autolysins, proteases, adhesins, antibiotic-resistance proteins, and virulence factors. The array of proteins expressed in endodontic infections resonates the complex microbial presence and highlights the bacterial species involved in the inflammatory process.

PROTEOMICS-IN CASE OF ABSCESS AND APICAL PERIODONTITIS:

A recent study by Fukushima and Associates in which root apex and periradicular region were evaluated for the presence of bacteria showed Eubacterium,Bacteroids,Peptococcus,Peptostreptococcus. A study, in which samples were collected from pulp chambers of teeth with asymptomatic apical periodontitis before and after chemome cha nical preparation using either NaOCl or chlorhexidine as the irrigant. A number of 308 proteins of microbial origin were identified.[21]

The number of proteins was higher in abscesses compared to asymptomatic teeth. Eight proteins with proteolytic activity were found in a study[21], most of them associated with abscesses. They included a collagenase, a metalloprotease, a serine protease, an extracellular protease, and endopetidases. These enzymes may play an important role in several ecological and pathogenic effects, including tissue invasion, acquisition of nutrients from proteins, destruction of the connective tissue matrix, and inactivation of host defense molecules. Streptopain (SPE B), a protein of streptococcal origin, was found in one pool of samples. The majority of human proteins identified were related to metabolism and, cellular processes, as well as immune defense. Investigation of the metaproteome of endodontic microbial communities provides information on the physiology and pathogenicity of the community at the time of sampling. There is a growing need for expanded and more curated protein databases that permit more accurate identifications of proteins in metaproteomic studies about endodontics.

A wide variety of proteolytic enzymes have been identified in GCF, such as elastase, collagenase, and cathepsin B, D, H, and L[11] as early detection of periodontitis can help clinician to limit the activity of lesion which if not treated early might lead to a perio-endo lesion or vice-versa. These proteolytic enzymes have the capability of degrading type-I collagen and glycoproteins hence named as the destructor of periodontal tissues. The most commonly reported identified proteins from GCF are actin, keratins, histones, annexins, proteins S100-A9, apolipoprotein A-1, albumin, salivary gland antimicrobial peptides (histatins, HNP- 1, -2 & -3, LL-37, statherin), and cystatin B. Some immune-related proteins present in GCF include lactoferroxin-C, Ig gamma-1 chain C region, Ig gamma-3 chain C region, leukocyte elastase inhibitor, alpha 1 antitrypsin, heat shock protein beta-1, and coronin1A.[21] Depiction of periodontal ligament (PDL) fibroblast proteome is a valuable tool for identifying disease-related protein markers and for understanding PDL physiology and regulation. PDL fibroblast protein expression has been studied using immunological methods. So far, 117 proteins have been identified from PDL fibroblasts which can serve as a reference map for futher clinical studies as well as basic research.[22]

7. FUTURE OF ORAL PROTEOMICS

Proteomic analysis of the human body is a significant recent scientific endeavor. The human body contains billions of proteins and peptides that perform various functions to maintain body homeostasis and a functional repair system. These proteins and peptides also assist in monitoring the normal and any diseased condition of the body.[21] Analyzing blood parameters for the detection of pathological lesions involves invasive techniques. The method to obtain blood is not suitable for use in immunocompromised and vulnerable patients, patients with a terminal illness, and patients with clotting disorders such as hemophilia.

Moreover, blood investigations do not detect all specific proteins or antigens related to pathological conditions. The oral cavity comprises the tongue, salivary glands, teeth, gingiva, and the surrounding tissues. These structures produce specific proteins which might be useful in the detection or monitoring of diseases in early stages via proteome analysis. For instance, human saliva contains millions of proteins and peptides that can allow the identification of different oral conditions such as dental caries, periodontitis, gingivitis, oral cancer, fungal diseases.[23] Also, it provides a microbe profiling and a virus screening in different pathological conditions.

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