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Chapter 5

Discriminating Life Forms in Oral Biofilms

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75746

Abstract

The bacteria colonizing the hard and soft tissues of the oral cavity are known to significantly influence oral health and disease. Recent studies of subgingival dental plaque, based on different identification methods, provide direct evidence of substantial diversity of plaque microbiota. Till date only about 280 bacterial species have been isolated by cultivable methods, characterized and formally named out of this enormous microbial diversity of oral biofilms. As a consequence, there is a complete lack of information about the properties of a substantial proportion of the plaque microbiota, apart from their position in the taxonomic hierarchy of bacteria. This limited knowledge about the behavior and properties, combined with recognition of the considerable diversity that exists within individual species, raises serious questions to the foundations on which previous conclusions, concerning the etiology of periodontal diseases, rest. The emerging realization is it is impossible to fully understand oral health and disease without identifying and understanding the pathogenic potential of all of the bacteria that colonize the oral cavity. The current chapter shall provide an update on current status of oral microbiota, ecological significance of their biofilm life style and various methods to study microbes residing in oral biofilms.

Keywords: biofilm, dental plaque, microbes, methods, identification

1. Introduction

Upon formation of earth about 3.5 billion years ago life began under anaerobic conditions, which resulted in current form as a result of evolution that is continued with the time. Initially, earth was colonized by unicellular prokaryotic bacteria that could survive under anaerobic conditions and eventually facilitated aerobic conditions that turned into evolution. However, till date we can find these microbes in various anaerobic environments. The estimated microbial diversity on earth constitutes 1.2×10^{29} in oceans, 2.6×10^{29} in terrestrial environment [1].

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Remarkably, much more diversity was observed in subsurface environment with an estimate of 2.5×10^{30}, suggesting the adaptation of these microbes to such conditions at the formation of earth. Thus, microbial diversity constitutes a significant mass on earth among living organisms. However, most of the microbial diversity remained undisclosed due to limited knowledge on their adaption strategies and functions under diverse environments. In fact, their association has been observed with higher forms of living organisms including plants, animals and humans. There were various projects dealt with understanding the role of these microbes in host. Among those human microbiome project is considered to be important that helped in understanding the ecology of microbes in human including their disease causing abilities. Various habitats on human body are composed of vast microbial flora which include both autochthonous and allochthonous populations. Among those the oral microbiome is known to contain more than 700 different prokaryotic species with distinct subsets prevailing at different habitats of oral niche including periodontic and endodontic environments. Attempts were made for extensive characterization of this microbiome using both cultivation and culture-independent molecular methods. Unfortunately, most of the culture-independent methods revealed vast majority of oral taxa as uncultured clone and referenced by their 16S rRNA GenBank accession numbers [2, 3]. Application of recent advances in technology provided new insights in understanding the oral microbiome complexity and their role in both health and disease. In this chapter we have made an attempt to compile all updated information and current status of oral microbiota their biofilms, ecological significance and various methods to study microbes residing in oral biofilms. In 1978, Costerton invented the word "biofilm", referring to the matrix-enclosed bacterial community [4]. However, the first biofilm described by Antonie van Leeuwenhoek.

2. Oral microflora – general aspects

The "oral microbiome" represents a group of microorganisms that includes mutualistic, symbiotic, commensal and pathogenic microorganisms which determine oral health and disease1. Though babies are protected inside the amniotic sac during pregnancy and born with germ free oral cavity, various microbes of the vaginal environment of the mother comes into contact at the time of birth and subsequently establish their niche in oral cavity. Thus, the initial microbial flora of oral cavity resembles the mother's vaginal flora. Despite the possibility of contamination from the environment and surrounding personnel, the mouth of a newborn baby is usually sterile and microbes start invading with residential flora during feeding process. The natural history of oral bacteria acquisition and potential determinants of oral microbial composition are beyond the scope of this chapter. With direct exposure to the environment, oral cavity possesses a complex microbial ecosystem where wide variety of microbes including bacteria and fungi are continually involved in their establishment upon attachment to the surfaces like teeth, tongue, restorations and soft tissues. These varying colonizers primarily cause polymicrobial infection in the form of biofilm i.e., dental plaque with ecologic succession and inter-bacterial interactions between commensals, opportunistic pathogens and pathogenic microbes leading toward homeostasis in oral microflora [5]. Microbial studies of

human dental plaque carried out by Socransky clearly showed that the oral health depends on the type of microorganisms present [6], however, interspecies interactions among these microbes determines healthy or diseased condition [7]. In fact, dysbiosis of microbial communities leads to dental caries or periodontitis [8, 9]. Commensal bacteria persist in oral habitat for long duration upon colonization and thus, they co-evolve with host and prevent access to pathogenic microorganisms by stimulating the immune response [10]. Dental caries are actually result of disequilibrium between acid and alkali producing microorganisms or acid producers and utilizers [11].Thus, paradigm of microbial dysbiosis revealed significance of autochthonous or resident microflora in maintaining healthy oral environment [11, 12].

3. Dental plaque

Dental plaque is a sticky film comprising multiple bacteria assembled as biofilm on surface or periphery of teeth. It consists of highly structured complex that allows sequential bacterial/microbial succession. Dental plaque development studies under in vitro and in vivo investigations revealed occurrence of early and late colonizers. While early colonizers with ability to produce biochemical components that adhere to target tissue initiates biofilm formation on tooth surface including periodontal tissue. Subsequently, they allow the adhesion of late colonizers that are capable to adhere with early colonizers to impart metabolic and competitive advantages to biofilm. Usually, early colonizers include species of *Streptococcus, Lactobacillus, Lactococcus, Eikenella, Veillonella, Provetella*, *Propionobacterium* and *Hemophilus*. Late colonizers represented by members of the genera like *Actinomycetes, Eubacterium, Treponema* and *Porphyromonas*. A mature dental plaque biofilm contain bacterial species that are well bound to bacterial strains located adjacent to form a unique structure that improves their adherence ability and provides protection from adverse conditions. Previous comprehensive reviews by Kolenbrander et al. should be consulted for assessment of these important properties [13–15].

3.1. Microbial composition of dental plaque

Dental plaque represents a microbial community with high genetic diversity. Moreover, it maintains a stable structural complexity, despite the continuous exposure to external environment and various stress factors. The microbial composition largely remains constant as a result of balanced antagonistic and synergistic associations [16, 17]. This indicates specific contribution of physiological functions by individual participating microorganisms in biofilms. In addition, their physiological functions contribute to facilitate growth of other organisms such as anaerobic microbes. The biofilms formed on tooth are divided into supra- and subgingival biofilms. While supragingival biofilm is formed above the gum, subgingival biofilm formed under the gum. Most of the bacterial strains described from oral environment were isolated from these biofilms. With more than 700 Gram-positive and Gram-negative bacterial species oral ecosystem represents a complex ecosystem after gut environment [18, 19]. It is often observed that supragingival plaque contained Gram-positive bacteria, including members belonging to genera *Streptococcus Lactococcus*, *Lactobacillus*, *Veillonella*

and the subgingival plaque revealed primarily gram negative anaerobic bacteria such as *Actinobacteria, Tannerella, Campylobacter*, *Treponema, Fusobacterium, Porphyromonas, Prevotella*,. Majority of these microbes belongs to the phyla like *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, *Fusobacteria* as well as uncharacterized phyla like SR1and TM7 [18, 20]. Despite such huge diversity, only very limited number of bacterial species have been isolated and characterized by cultivable methods till date and this may be due to lack of understanding of microenvironment associated with these microbes [3, 19].

3.2. Dental plaque – a highly specialized host associated biofilm

As mentioned earlier, dental plaque is a biofilm attached not only to tooth surface but also under gums. Diverse community of microbes exists in the form of biofilm where all microbial strains bound tightly between them as well as to the tooth surface. Dental plaque is a form of biofilms, which engulf diverse bacterial populations adherent to each other and primarily results in formation of dental caries. Their structure is influenced with high and low bacterial biomass interlaced with aqueous channels formed to provide nutrients to the bacterial strains [21, 22]. Biofilms permit association of diverse species with increased metabolic efficiency, enhanced virulence and higher resistance to stress and antimicrobials as a result of entirely different expression of genes in comparison to planktonic form. Ability to adhere on surface, strong binding between cells gene regulation and genetic transfer are some of the important properties that define biofilm formation. In fact, extensive metabolites exchanges, signaling trafficking and different levels of interactions among different species were usually observed in biofilms [16, 23, 24]. However, introduction of biofilm theory into oral microbiology provided insights to understand the roles of different bacterial species at different time intervals. Most important is these biofilms proven to provide protection by increasing the antibiotic and acid tolerance, a property indicating it as a marker for caries production. Though biofilms consist of millions of cells of multiple species in thousands of layers, they behave like a single organism. These microbial cells are also encompassed in polysaccharide complex to stay together and acquire resistant properties to survive under stress environment.

4. Clinical relevance of biofilms in disease etiology

Planktonic microbes existing in dental ecosystem often involved in acute infections that can be diagnosed and treated appropriately before the establishment of disease. In contrast, bacteria existing in biofilms demonstrate an infectious course in disease establishment as observed in dental caries, where large quantities of acid formed as a result of increased acid tolerance. Dental plaque biofilm also increase the expression of virulence factors as differential expression of participants lead to formation of noxious products that initiates inflammation and development of periodontal disease. Biofilm exhibit all genetic network required for these activities as evident in global analysis of gene expression during biofilm formation. This state also imparts global adaptation to stress condition i.e. crowded environment. Thus, this lifestyle appears most important adaptation to any form of environmental stress and gain increased tolerance. This cooperative behavior among the participating species in a biofilm

covered by extracellular matrix with coordinated management between cells using quorum sensing signal molecules for communication mimics an integrated multicellular organism. Additionally, the virulence was increased in multispecies participating biofilms in comparison to their mono-species counterparts [24]. Most of the bacterial cells exhibit attachment sites on their surface for an effective attachment to abiotic and/or neighbor microbial cells and thereby multiplies inside the extracellular matrix. This amplification in biofilms results in formation of aggregates that play important role in virulence in establishment of diseases like endocarditis, dental caries, middle ear infections, osteomyelitis, chronic lung infections in cystic fibrosis patients [25–27]. Remarkably about 80% of all microbial infections are found to develop biofilms on host tissues associated with different organs. Cells residing in biofilms termed as persister cells that are mostly exist in dormant stage with minimal active metabolism to cause chronic infections. These infections include production of exo- and endotoxins, metabolites like acids and other products involved in inflammation of dental tissue. However, the intensity of infection is directly related to their antibiotic resistance and ability to modulate host immune system [28, 29].

5. Biofilm characterization methods

5.1. Methods to discriminate oral microbial flora

Several attempts made to discriminate oral microbial flora by cultivable and non-cultivable methods have provided limited information. Though several taxa have been reported to present, only few microbes could grow in pure culture. Cultivation of individual strains in pure culture through the perspective of Koch's postulates. Further, identification of these microbial isolates helps in understanding infection process and disease establishment. To achieve this numerical taxonomy was practiced earlier, however, it has been replaced with molecular taxonomy and polyphasic taxonomy (**Table 1**).

However, in the recent past microbiologists have refocused on microbial communities' identification instead of planktonic form as they developed disease in the form of biofilm. In fact, oral diseases like caries and periodontitis are reported to be outcome of a consortia of organisms in a biofilm. Therefore, detailed analysis of a microbial community is essential to understand their pathogenicity. It is pertinent to mention that our understanding of the microbial world is very limited due to the intrinsic limitation of the culture-dependent methods. Thus, only less than 1% organisms could be revived in pure culture form under in vitro conditions. Considering the fact that several microbial species involved in biofilm formation, comprehensive understanding on complexity and genetic diversity of these communities are severely hampered due to non-availability of cultivation techniques [30]. Furthermore, uncultured status of these microbes also intervening in completion of understanding human microbiome and thereby effects on human health and disease [31]. Various culture-independent techniques such as cloning and amplification of total DNA obtained from samples can be used to understand the total microbial taxa. For which various housekeeping genes like 16S rRNA gene have been employed in molecular cloning and sequence methods to reveal their exact

Phenotypic methods	Genotypic methods
Expressed, Characteristics	Amplification of housekeeping genes like 16S rRNA and rpoB genes
Colony morphology Size Shape Color	Phylogenetic analysis of gene sequences
Cell morphology Gram Staining Shape	Analysis of fragments obtained from random amplification of polymorphic DNA (RAPD)
Motility	DNA separation by pulsed-field gel electrophoresis (PFGE)
Biochemical, enzymology acid-gas Production, Oxidation – Fermentation	Multilocus sequence typing (MLST)
Whole cell protein analysis	Restriction fragment length polymorphism (RFLP) of DNA
Utilization of Carbon compounds	Nucleic acid base composition Mole % G + C
Antibiotic sensitivity	DNA–DNA hybridization
Susceptibility to phages	
Susceptibility to bacteriocins	
Chemotaxonomic (Lipids, Fatty acid methyl esters (FAMEs), Isoprenoid quinones, Mycolic acids, Pigments, Peptidoglycan, Cell wall sugars	

Table 1. Phenotypic and genetic methods used for identification of bacterial strains.

identity [3]. However, cultivation of individual strains in pure form is essential to fully understand their role in health and disease thereby to carry out meaningful clinical research.

The development of 16S rRNA gene as molecular chronometer by Woese and co-workers has transformed the microbial taxonomy as the alignment of these sequences and construction of their phylogenetic trees have allowed cataloging of microbial strains and establishment of novel species [32]. The 16S rRNA gene exhibits clocklike behavior, broad phylogenetic range and appropriate size and accuracy, and these properties made this gene to the best molecular chronometer. Moreover, rRNAs are essential for protein synthesis and readily isolated from all forms of life, they are structurally and functionally conserved. They display highly variable and conserved regions to distinguish into distinct. They appear to incorporate changes in sequence very slow and do not exhibit horizontal gene transfer. This finding in combination with various PCR methods opened the door for culture-independent analyses for exact identification of microbial strains present in different microbial communities, including the uncultured bacterial species. They allowed understanding of total number of species, their richness and distribution. During the past two decades, development of high throughput tools for microbial community analysis has further improved identification process. Most of these methods include nucleic acids isolated from samples being investigated. These techniques include both nucleic acids and their PCR products. While techniques like fluorescent in situ hybridization (FISH) with fluorescently-labeled taxon-specific oligonucleotide probes and checkerboard DNA–DNA hybridization method [33] used nucleic acid, others such as random amplified polymorphic DNA (RAPD), denaturing gradient gel electrophoresis (DGGE) [34] or temperature gradient gel electrophoresis [35], terminal restriction fragment length polymorphism (T-RFLP) [36] and automated ribosomal intergenic spacer analysis [20] were carried out using PCR amplified products to analyze environmental microbial communities. Application of these techniques has revealed large number of microbial species within dental plaque with

great genetic diversity. However, these techniques also showed limitations like cell lysis efficiency, nucleic acid extraction, differential amplification of target genes and differences in copy number of target genes, primer specificity and hybridization efficiency. Therefore, combining imaging tools such as the scanning electron microscope [37] and confocal laser scanning microscope [38], with molecular techniques can provide most effective identification [39].

5.2. Specific methods to discriminate oral microflora in biofilms – detection and quantification

Formation of biofilm containing multiple pathogens embedded in an extracellular polysaccharide matrix is a big threat to human health. Though biofilm formation is regulated by expression of various genes, there are multiple systems such as extracellular polysaccharides, lactones, pilin- or flagellin-like proteins, adhesins and other small molecules involved in quorum sensing and biofilm formation. Thus, considering the complexity of biofilm structure they are discriminated in qualitative and quantitative methods. The amount of EPS, types and total number of bacterial cells in biofilm must be considered as different "methods" requiring different experimental approaches. The biofilms are largely quantified using spectrophotometric and microscopic methods. The crystal violet (CV) staining method [40] is among the mostly used and also achieved by cangored method. CV staining can be performed as tube method or using microtitre plate.

5.2.1. Microtitre plate method

The microtitre plate method is most widely used method for detection of biofilm formation. It was initially developed as tissue culture plate method by Christensen et al. [40]. This method is used to test the influence of different media and addition of various sugars in media on biofilm production. Individual wells of sterile, polystyrene, 96 well-flat bottom microtitre plates were filled with 200 μl of diluted cultures in respective sterile media. They were incubated under optimal conditions required for the growth of microbes being tested. After incubation contents of wells were removed by gently tapping the plates and washed with sterile distilled water or buffer to remove free-floating bacteria. The biofilms formed by adherent mechanisms were stained with CV (0.1% w/v). Excess stain was removed by washing with deionized water and subsequently wells were air-dried. Adherent cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. The crystal violet was solubilized using absolute ethanol and the quantity of biofilm quantified by measuring the OD at 595 nm. Sterile uninoculated medium is usually used as a control.

5.2.2. Tube method (TM)

This method allows qualitative assessment of biofilm formation as described by Christensen et al. [41] The medium is inoculated with loopful of culture from plates that are overnight incubated at optimal conditions. Upon incubation these tubes are decanted, washed with distilled water or PBS (pH 7.3) and air-dried. They are stained with CV (0.1%). Excess stain removed as mentioned in microtitre plate method and observed for biofilm formation. Biofilm formation was detected by a visible film lined the wall and/or bottom of the tube.

5.2.3. Congo red agar method (CRA)

This is an alternative method of screening biofilm formation by microbes [42]. Microbes being screened are grown on solid medium supplemented with 5% sucrose and Congo red. Congo red usually added as concentrated aqueous solution. Plates were inoculated and incubated under optimal conditions. While positive result was indicated by black colonies slime producers showed pink colonies.

5.3. Other qualitative straining methods to detect biofilm

5.3.1. LIVE/DEAD BacLight assay

This method is performed using a bacterial viability kit for microscopy based on the use of two different nucleic acid binding stains. Two dyes employed are green fluorescent (SYTO 9) and propidium-iodide that should be used with appropriate care. While intact cells fluoresced green with Syto9, damaged or dead cells in biofilm stains red. These stained samples are usually observed under a fluorescent microscope. The main limitation to apply this method for quantification is low quantities of the representative sample used for the total population and it does not allow tracking of individual bacteria.

5.3.2. Immunofluorescence staining

Immunofluroscent staining is used to observe biofilms under optical fluorescence microscopes and is commonly used to stain biofilms under in vivo conditions. This method employs specificity displayed by antibodies toward antigens. Usually fluorescent dye-labeled antibodies are used to fluoresce specific target molecules within a cell. This method is often used in experiments that use cell lines or tissue culture studies. Immunofluorescence is also used with other non-antibody methods by using stains like DAPI and analyzed on epifluorescence or confocal microscope. Diverse florophore molecules are used to link with antibodies. Biofilms used for image analysis using electron microscope are treated with various staining and fixing protocols using fixative or stain like glutaraldehyde, osmium tetroxide, ruthenium red etc., and observed under electron microscope. A variety of fluorescent molecules like lipophilic styryl compounds (ThermoFisher) involving plasma membrane and vesiculation was also used for biofilm detection. These water soluble and exhibit fluorescence when interact with surface of microbial cell membrane.

5.4. Metabolic assays

Biofilms can be measured by different vital or non-vital dyes that interact with metabolic products.

5.4.1. Resazurin assay

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is a blue non-fluorescent biological dye that also known as Alamar Blue. It is used to quantify biofilms in microtiter plates as it gets converted to the pink-fluorescent resorufin upon reduction as a result of cellular metabolic

activity. The resorufin can be measured spectrophotometrically and intensity of fluorescence is directly proportional to number of cells or biofilm concentration [43, 44]. However, the test is highly susceptible to bacterial respiratory efficiency and calibration of curves established with planktonic cells is much lower than signal detected in biofilm [45]. Further, this assay also reveals the presence and efficiency of antimicrobial and antibiofilm compounds [46].

5.4.2. XTT and TTC assay

Tetrazolium dyes also can be used as resazurin assay to quantify metabolically active cells in biofilm by spectrophotometric method. Tetrazolium slats like 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) has been used to detect biofilm [47, 48] and another salt 2,3,5-triphenyl-tetrazolium chloride (TTC) also sued for the detection of biofilm [49] in microtitre plates by measuring absorbance. In fact, this method can be used to determine minimum biofilm inhibitory concentration (MBIC). Though these assays are highly sensitive and economical, the complexity and heterogeneity of biofilm structure of mature biofilm reduces the release of final products.

5.4.3. BioTimer assay

Bio Timer assay (BTA) is a biological method used to count adherent viable bacteria in biofilm life-style on any abiotic surface without manipulation of sample. BTA employs a specific reagent, phenol red that changes color from red-to-yellow based on microbial metabolism. This is specifically considering the microbes that produce diverse organic acids as their metabolic end products of fermenting bacteria. The time required for color change is determines the number of microbes as higher number of the organisms performs faster metabolism. Time required for initiation of color switch is correlated to the number of bacteria at time zero (N0) through a genus specific correlation described by equation $t^* = \log(1 + a/N0)/k$, where a represents metabolic product involved in color change and k is growth rate [50]. Though this technique is applied in microbiological quality analysis of foods and to evaluate antibiotic susceptibility of biofilm, is not applicable for the evaluation of multispecies biofilm.

5.5. Genetic assays to determine biofilms

Genetic assays have been used to assess the biofilm formation with focus on molecular mechanisms involved in biofilm formation. In particular, early stage of biofilm formation including attachment to surface, which is driven by expression of various genes in different microorganisms. Therefore, biofilms are associated with proteins and amplification or quantification of various genes including chaperone-usher fimbriae, outer membrane proteins, poly-N acetyl glucosamine, adherent proteins and pili proteins [51–53].

5.5.1. Polymerase chain reaction (PCR)

The most important diagnostic method used in genetic techniques is Polymerase chain reaction (PCR). PCR screening is often employed to detect the genes involved in biofilm formation. The amplified products are sequenced and analyzed using various bioinformatics tools such as BLASTp (NCBI) to align with homologous sequences. This method

allows to identify specific genetic sequences based on primer sequences used for individual bacterial species. The extracted DNA of the biofilm can be used for RAPD analysis by using specific oligonucleotide primers [54]. Amplification of genes like icaA, icaD, aap. The reaction mixture contains in general Taq polymerase enzyme, deoxynucleotides, primers, template DNA and MgCl2 in PCR buffer. The amplification is carried out in a gradient mastercycler with a program that includes initial denaturation of DNA at 95°C for 5 min. It is followed by 40 cycles of program at 94°C for 1 min, optimal temperature required for the binding of primers for 1 min, 72°C for 2 min (optimal enzyme activity and amplification) with a final extension at 72C for 5 min. Primers used in amplification of gens are as follows: icaA, 5′-AACAAGTTGAAGGCATCTCC and 5′-GATGCTTGTTTGATTCCCT [55]. Forr icaD, 5′-CCGGAGTATTTTGGATGTATTG (forward primer) and 5′-TTGAAACGCGAGACTAAATGTA (reverse primer). According to Vandecasteele et al. [56], for the detection of the aap gene, following primers were used: 5′-ATACAACTGGTGCAGATGGTTG (forward primer) and 5′-GTAGCCG TCCAAGTTTTACCAG (reverse primer). Nevertheless, PCR as such is not a suitable to quantify biofilm as it amplifies the DNA of both viable and dead cells, as well as any contamination leading to false positive results.

5.5.2. Fluorescence in situ hybridization and confocal laser scanning microscopy

Fluorescence in situ Hybridization (FISH) is a cytogenetic techniques that use fluorescent labeled oligonucleotide probes (like rRNA gene fragments) to detect microbes by hybridization of DNA with highly identical complementarity. This method allows direct visualization of species specific bacteria in a multispecies biofilm. These bacterial strains can be observed using confocal laser scanning microscopy. The technique can be modified with the samples to be observed, for example a modified version of the technique developed to identify based on peptides and termed as peptide nucleic acid-fluorescent in situ hybridization (PNA-FISH). Similarly, Flow-FISH employees flow cytometry to identify the microbes. Histo-FISH was developed to detect probiotic bacteria in gastrointestinal tract [57]. Interestingly, FISH can detect not cultivable bacteria and persister or dormant bacteria in biofilm. FISH technique is usually combined with confocal microscopy to visualize different species in a multispecies biofilm.

The aforementioned high throughput tools for microbial community analysis are largely based on PCR amplification of 16S rRNA gene sequences from microbial communities, which are relatively short, often conserved but varied enough to differentiate bacteria at species level. Although these approaches can provide us with the microbial composition within the community, unless we have genomic or other research data on those identified species, it reveals very limited information regarding what functions they might carry out within the flora.

5.6. Physical assays – biofilm imaging

5.6.1. Confocal laser scanning microscopy (CLSM)

It is a microscopy technique used in biology to study thick samples such as microbial biofilm, by processing images. Samples under investigation are stained with fluorescent dyes as mentioned in FISH so that the object can be illuminated and transformed by a photodiode

in electrical signal processed by a computer. Some systems use motorized computer assisted device control for adjustment or sectioning of the biofilm and automated image acquisition. This technique often used to understand the role of EPS components, live biofilms and their in situ gene expression studies [58, 59]. The main disadvantages are semi-quantitative investigation, limited fluorescent dye usage for few stains and expensive method.

5.6.2. Mass spectrometry (MS)

A powerful analytical technique used for detection of various molecules. MALDI-TOF showed to be a strong tool for proper identification bacterial strains in biofilms. This technique utilizes the protein profile of bacterial strains for identification with a reference database. In fact, it is used for accurate identification of clinical strains in biofilms with high resistance to antibiotics [60]. In this method the object under investigation is exposed to a beam of electrons to form ions that are separated based on mass that are detected by a spectrometer and identified by their mass/charge ratios. It fulfills both qualitative and quantitative analysis of the unknown compounds. However, many steps in MS are highly invasive for the sample: high vacuum environment, aggressive chemical solvent etc. To overcome this problem,

5.6.3. Desorption-electro-spray-ionization (DESI)

This method has been proposed to overcome the disadvantages of MS like chemical solvent exposure and vacuum environment. It is carried out at atmospheric pressure and the sample is maintained under ambient conditions and can be used to for the analysis of mixed biofilms [61, 62].

5.6.4. Electron microscopy (EM) techniques

Electron microscopic technique was used to understand microbial flora in dental environment [63]. This method provide high resolution and technique is used for both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). While SEM used to visualize biofilm surface TEM is used to image inner of biofilm [64]. For SEM analysis objects prepared on coverslips are washed (2–3 times) with buffer (pH 7.2) and fixed in 1% osmium tetroxide in the absence of light. Later, washed with distilled water and dehydrated in crescent concentrations of acetone baths. Upon drying, samples were mounted and analyzed on a scanning electron microscope. For TEM, the sample to be prepared as ultra-thin slices to acquire accurate images of bacterial cells and biofilms. Atomic force microscopy (AFM) is another technique used for morphological characterization. This method is used to check microbial cells in both planktonic and biofilm forms. The objective is fixed using 1 ml of modified Karnovsky fixative (containing 2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose, and 0.1 M cacodylate buffer, pH 7.2) at room temperature. The analyses were performed at ambient temperature on an atomic force microscope equipped with a scanner. All images obtained were processed on specific softwares.

5.6.5. Micro-scale biogeography

Micro-scale biogeography is upcoming technique to understand the microenvironment of microbes by biofilm imaging [65]. This method also includes mimicking microenvironment including chemical ingredients and oxygen. It provides insights in understanding physiology and

ecology of community their attachment with other microbes and spatial structure. Neighboring strains in physical contact play significant role in physiology such as protecting from stress conditions and secretion of metabolic end products as substrate for subsequent colonizers [66–68].

6. Metagenomics to understand complex microbial communities

The introduction and application of "metagenomics" by Jo Handelsman [69] has greatly enhanced our ability to study microbial communities including dental plaque. It includes understanding the microbial communities directly in their natural habitat using genomics approach The method do not require isolation and cultivation of any microbial strains. The basic components involved in metagenomics are PCR amplification of DNA, sequencing, bioinformatics with enhanced computational power to analyze large datasets obtained in sequencing [70]. The approach is simple and involves isolation of total DNA from sample, which is subsequently used for amplification of various genes and their subsequent analysis to gain functional and metabolic understanding [71]. Further, comparative genetics with expression microarrays and proteomics provides insights on network life style of microbes within the community such as dental plaque. Such studies provide information on potential pathogens that remained unidentified due to cultivation limitations [72].

7. Adjunctive novel technologies for biofilm study methods to complement microbial identification

7.1. Microfluidics

Miniaturization approaches to biofilm cultivation by using techniques like microfluidics studies are used to understand the natural habitat in laboratory conditions. It is performed in micro-scale channels by allowing fluid flow of growth media or chemicals with remarkable degree of control over the physical and chemical environment of microorganisms. Thus, allows manipulation of microenvironments of bacteria as these devices are made with microscopic compatible materials. It is developed as a new approach to understand cultivation method and dynamics of biofilms. It is also used as high throughput system to determine bacterial antibiotic resistance [73], cell variability in bacterial persistence, quorum sensing and chemotaxis in bacteria [74].

8. Concluding remarks

Since the initial observations of bacteria within dental plaque by Antonie van Leeuwenhoek using his primitive microscopes in 1680, our ability to identify the resident organisms in dental plaque and decipher the interactions between key components has rapidly increased. It is further increased significantly with the advent of imaging and molecular techniques

during the past decade. These new techniques will have a great impact on oral and periodontal microbiology. We envision that in the future, new diagnostic tools developed with metagenomics methods would allow early detection and effective methods to combat the diseases. It also provides insights to prevent the cariogenic, endo and periodontic diseases.

Acknowledgements

We acknowledge Dr. Suresh Korpole, CSIR-Institute of Microbial Technology for valuable information on identification techniques and for useful discussions.

Conflict of interest

Authors declare there is no conflict of interest.

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