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Molecular Analysis of Oral Microbiota of Periodontal Diseases: Review Article

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Abstract

Molecular analysis is application of molecular approaches which help to bypass cultivation step to researchers. It has proven its usefulness in studying the microbial composition in a variety of ecosystems, including the human oral cavity, as different hypothesis has been proposed for the bacterial etiology in periodontal diseases. Microbiota associated with periodontal diseases had impact by changing the paradigm regarding etiology and pathogenesis of dental diseases over the years. Periodontal diseases, gingivitis and periodontitis, are inflammatory processes in tooth supporting tissues, caused by host defense factors induced by bacterial infection. These diseases involve an inflammatory response to dental plaque biofilms. Oral microbiota is found in complex biofilms or on teeth (dental plaque). This review article aims to describe the role of oral microbiota in periodontitis involving identification of periodontitis associated species which determine their possible association with other dental diseases.

Keywords: Microbiota, Periodontal Diseases, Biofilms, Molecular Analysis

Introduction

There is a diverse environment in the human oral cavity consisting of near about 215 cm^2 surface, soaked in the saliva.[1] Optimal for microorganisms to grow as biofilms. Plaque is basically an aggregated microbial community which is very organized by nature. This plaque is the main aetiology behind the most common diseases like: carious lesions as well as other inflammatory diseases of the periodontium. Bacterial cells in oral biofilms interact by special manner, these are: exchange of the metabolic substances, by forming structures such as co-aggregation, communicating by "cell to cell" method, also by exchanging the genetic material of the bacterial cell.[2] All these techniques are essential for surviving the organism inside the host as well as producing further in making the other dental and periodontal diseases.[3]

Historical Perspective

All these bacteria were isolated by certain culture methods in the past & was categorized according to the bodily characteristics such as cellular features, metabolic characteristics. Cultivation of bacterial cells had made it achievable for working to live with other organisms, resistance offered by the other antimicrobial substances. In the 1970s, hybridization of DNA- DNA components had been introduced to differentiate between different species of the bacteria.[4] The initiation of r-RNA of 16S variant to determine of relationship of phylogenetic origin, marked a new genomic era for microbiologists. Appreciating the prokaryotic-diversity, was one of the major promotions in the field of molecular microbiology.[5]

While studying about the subject of molecular genetics, to pick out the involved genes as well as the associated regulatory pathways were necessary for the development of the biofilm .[6] Expressing the genes which were associated for motility, communicating between the intra & inter-species, antibiotic resistance, vary in bacterial growth as biofilms compared with those in planktonic lifeform.[6-9] it is ver crucial to know bacteria forming biofilm and regulating for clinical approach, as chronic infections, such as osteomyelitis, endocarditis, cystic fibrosis pneumonia, otitis media, periodontitis, and dental caries, acquire from biofilms.[10]

Dental Plaque

Plaque biofilm attaches to the hard surfaces which is composed of microorganisms. With reference as gingival margin, it is classified as Supra gingival and Sub gingival plaque. Plaque found with the gingival margin is known to as Marginal plaque. Material Alba is a term that delineates aggregations of bacteria and cells derived from host, but without organized internal structure of biofilm. It can be easily ousted with water-spray. An organic film deposited on the tooth surface found to be pellicle mainly gleaned from the saliva with few or no bacteria. A biofilm, defined as a clique of microorganisms irredeemably attached to a surface, with increased resistance to host cellular and chemical reactions.

Complex biofilm consists of bacterial aggregations that attaches on the hard in the oral cavity. Plaque colonizes following a pattern with initial colonizers adhesion to the enamel salivary-pellicle, ensuingly secondary colonization through inter bacterial adhesion. These steps induce the happening of diseases such as caries and inflammatory diseases of periodontium.[11]

Dental Diseases

A well-maintained oral cavity was shown by culture mainly involving Actinomyces, Rothiadentocariosa as well as Streptococcus species.[12] Biofilm which is supragingivally attached has been enormously evaluated from very first colonization of bacteria on surfaces of tooth to initiation of caries. Streptococcus species are the initial colonizers.[13-16] Gram-positive Gemella, Granulicatella, Actinomyces, and Rothia species, as well as Gram-negative Neisseria, Prevotella, and Veillonella species are other pioneering genera. Gram-positive organisms with coccoid cells were extracted from non-diseased volunteers' biofilm. .[17]

Subgingival plaque forms from apical movement of the plaque growing on the surface of tooth,[18] Gram-negative and Spirochete bacteria are predominating in the subgingival biofilm.[17]

Using immunohistochemical and DNA hybridization methods bacteria are discovered in the profound sections of the biofilms on epithelial surface include A-saccharolytic obligate anaerobes, P. gingivalis, T. forsythia and T. denticola species that are pathogens of periodontium are acknowledged.[19-21] Genomic DNA probes are utilized to analyze bacteria's of soft tissue surfaces, distinct differences in composition and proportions of bacteria in the sampled soft tissues, and similarity between tongue microbiota with salivary bacteria was evinced .[22]

In tooth supporting tissues inflammatory processes as periodontal diseases, gingivitis and periodontitis are noticed. Host defense factors are the very often cause which is produced by bacterial infection.[23,24]

Gingivitis

Gingival inflammation growing adjacent to the gingival margin includes an inflammatory response to plaque biofilms. In reversible type of plaque-induced gingival inflammation, if exquisite oral hygiene is pulled off, and homeostatic state of the gingiva can re-achieve without lasting damage.[25] In adults' gingivitis is invariably present and life-experience of periodontal attachment loss of >3 mm in dentate adults is observed in 53% of dentate adults.[26]

Inflammation of Periodontium

Periodontal inflammation is driven by microbes which causes destruction of the supporting structures of tooth. Connective tissue attachment is adrift in periodontitis, as conflicted to gingivitis. Junctional epithelium shifts apically that is at the base of gingival crevice because of the host defense against bacterial insult. Deepened periodontal pockets is the ultimate result for this scenario as there is conjunction of gingival edema, due to the inflammatory activity. Because of the host defense there is destruction of bone and connective tissue in

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periodontitis in order to control the inflammation. Periodontal probe is the instrument to clinically evaluate the presence and extent of periodontal fiber loss to evident the exact tissue destruction. Radiographical examination is required for marginal alveolar bone loss.[27]

Analysis Of Microbes

Hybridization of DNA-DNA

DNA Probe Assay from whole genome

Analysis of plaque samples by whole genomic DNA probes.[28] Thoroughly, specimens were denatured, neutralized, and fixed by ultraviolet (UV) irradiation on a membrane. For 105 and 106 equivalents of bacterial cell quantitative reference was involved by using two last lanes from each membrane which engulfed species mixed as a DNA standard. Hybridization of membraned samples and standards was carried out with digoxigenin probes in the species of interest. Chemiluminescence where anti-digoxigenin antibodies, bound with covalent bonds to alkaline phosphatase was used to visualize the end product. Storm Fluor-imager system detected signals and subsequently converted to absolute bacterial counts, comparing the standards on the membrane. Nylon membrane cross-linked oligonucleotide DNA probes (17-22 bases) by UV irradiation. Two standard probes were applied for each membrane. Amplification of DNA from samples was done with two universal primers, one of them was labeled with digoxigenin. Storm Fluor-imager system was used to investigate signals and responses were collected as species detected or not.[29]

Pcr - Polymerase Chain Reaction

Streptococcus mutans and Aggregatibacter actinomycetemcomitans PCR

To detect specific pathogens from samples PCR for S. mutans and of A. actinomycetemcomitans was used. First study, to inactivate proteases samples were treated by Proteinase K[30] and resulted as a template for the PCR. Second study, removal of genomic DNA and purified from samples.[31] A nested format A. actinomycetemcomitans PCR was performed, by running a universal PCR first and then using the PCR template. All A. actinomycetemcomitans positive reactions were sequenced to ensure correct amplification products.[32]

Genotyping PCR A. actinomycetemcomitans type

Using the random oligonucleotide sequence OPA-13 (5'-CAG CAC CCC AC-3') arbitrarily primed PCR was performed to separate A. actinomycetemcomitans strains into distinct genotypes.[33-35]

Serotype-specific PCR A. actinomycetemcomitans type

Serotypes a were assessed by gene sequences specific for A. actinomycetemcomitans. For serotype a, c, and f were analyzed for the entire serotype-specific gene clusters.[36-38]

Multiplex PCR P. gingivalis and T. forsythia type

Tran and Rudney gave a PCR assay based on which a multiplex PCR was to improve detection of the two major periodontitis-associated pathogens P. gingivalis and T. forsythia.[39]

To Clone And To Sequence

With formerly expressed concordat genomic DNA was drawn out from the samples.[31] Under standard conditions universal PCR primers outlined PCR amplification of the 16S rRNA gene, and how to clone & sequence.[40] Following manufacturer's instructions PCR-amplified DNA duplication was carried out with TOPO TA Cloning kit, Invitrogen, Carlsbad, CA, a trading. One-Shot TOP 10 E was used to bring out transformation .[41] Potent conversion was established with an X-galactose screen.

48-96 modified colonies were used as templates of PCR with vector primers M13F and M13R. Big Dye Terminator cycle sequencing kit with ampli-Taq DNA polymerase FS, Perkin-Elmer, Waltham, MA was involved in cycle-sequencing the 1600 bases having right insert colonies with 533 reverse primers. ABI 3100 DNA Sequencer arranged reactions after last cleanup.[42]

Sequencher® (Gene Codes Corporation, Ann Arbor, MI) edited sequences, arranged them, and made surety individually for every chimeric inserts. BLAST (Basic Local Alignment Search Tool) compared sequences to a reference set in the Human Oral Microbiome Database, having >98% identity threshold for strains distinctness. CLUSTAL V alignment method was used in DNASTAR Meg-Align software to design adjacent phylogenetic trees

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from whole 16S rRNA Human Oral Microbiome Database reference sequences.[43]

Antigen Recognition

SDS-Polyacrylamide gel ionophoresis and Western Blot Test

SDS-Polyacrylamide gel ionophoresis and Western Blot Test were performed for serotypes antigen detection of Aa.[44] Using chemiluminescence immunoreactions were detected. Proteinase K treatment was done on some preparations from the whole cell preparations done with phosphate buffer solution (PBS). Aa serotypes a-f and human sera are rabbit antisera involved here.

Outcherlony Test

A through E serotypes with serotype F of specific rabbit antisera against Aa were used as antibodies. Whole cell suspensions were autoclaved with supernatant in outcherlony test where antigens were prepared from each Aa strain.[45]

Multifactorial analysis

Clinical measurements were evaluated at baseline by using Logistic regression, P. gingivalis and T. forsythia PCR detection and subsequent loss of attachment using SAS® software.

Oral Microbiota Of Early Adult Periodontitis

Studies existed with oral microbiota showing its association with early adult periodontitis. 141 individuals participated comprising of 28 periodontally fit subjects, 71 had Early Periodontitis

Mean attachment level ≤1.5 mm and minimal one site ≥ 2 mm attachment loss, & 42 with Early Periodontitis Mean attachment level >1.5 mm and mean attachment loss ≥ 2 mm represented in Table 1.

STUDY	DESIGN	POPULATION (N OF SUBJECTS)	AGE (yrs.)	DENTAL DISEASE	LABORATORY METHOD
I	Cross-sectional Descriptive	(N=141)	20-40	Early periodontitis	Oligonucleotide probes, multiplex PCR
II	Longitudinal cohort	(N=117)	20-40	Progressing periodontitis	oligonucleotide probes, Whole genomic probes, multiplex PCR
III	Cross-sectional Experimental	(N=152) (N=37)	13-74	Aggressive and chronic periodontitis	Outcherlony test, SDS-PAGE, AP-PCR Western blot, PCR

Table 1: Summary of study design, origin of study populations, number and age of participants, dental disease category, and laboratory methods used for bacterial identification. The studies are presented in the order attached and discussed in the thesis.

117 individuals were followed over eighteen months and 19% that is 22 from 117, exhibited positive periodontal damage by losing >1.5 mm CAL in one or multiple interproximal sites.

Oligonucleotide DNA probes proved to be markers for phylotypes having no, mentioned as Obsidian Pool phylotypes and TM7, fastidious species, including Treponemes and F. alocis.[46]

Microbial Environment Of Periodontally Inflamed With Periodontally Healthy Subjects

Most detected species, >60% of the subjects included Strep., Fusob., and Granulicatella species. Investigations showed, most of the uncultivated phylotypes were detected in >20% of subjects.

T. denticola, F. alocis, P. endodontalis, Bacteroides sp. HOT-274 oral clone (AU126), F. nucleatum, and

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A. odontolyticus species were detected more regularly in early periodontal inflammation than in periodontally fit subjects ($p<0.01$) by oligonucleotide DNA probes and P. gingivalis ($p<0.001$) and T. forsythia ($p=0.03$) by PCR. Above species with P. endodontalis were associated with periodontitis by means of oligonucleotide DNA probes.[47] Due to fastidious growth requirements F. alocis and P. endodontalis, both strict anaerobes were underrepresented by culture of periodontium inflammation samples. These two species in the panel of species are proposed for periodontitis diagnostic purposes.[48]

Notable significant association was seen for P. gingivalis with early periodontitis when analyzed with oral health ($p<0.001$) and progressing periodontal disease when compared to stability ($p<0.001$). No association of P. gingivalis with periodontitis was found, Incipient periodontitis in adults were examined in few studies. By culture and molecular methods advanced and chronic periodontitis were recognized as depicted in the literature.[49-51]

Only association seen was of T. forsythia by PCR with early periodontitis at baseline ($p=0.03$) and not at follow-up. T. forsythia in relation with initial periodontitis by culture was investigated by Tanner and co-workers.[52] T. forsythia was predictive of advancement of periodontal attachment loss in a longitudinal prospective study of adults using the same PCR method.[53] Aa was detected at 24% from subjects involving and at 21% from subjects not involving attachment loss. In 18–25-year-old men with minimal attachment loss detection frequency was same at baseline in comparison to detection frequency by culture (27%). Methods used did not allow for specific serotype identification of Aa, as the strains could have been of serotypes prior associated with periodontal health.[33] Clones or serotypes of bacteria linked to periodontal health or disease.[54]

Discussion

Biofilm can be controlled with comprehensive mechanical and chemotherapeutic oral hygiene practices; it cannot be eradicated. Likelihood of periodontal disease prevention and reduction can be increased by encouraging patients to use interdental cleaning, everyday brushing, and antimicrobial mouth rinses having ADA Seal of Acceptance. Minimization of periodontal diseases on specific systemic conditions can be carried out by cost-effective, preventive strategies.

Extensive molecular methods and examination of numerous children showed here the assessment of person's oral microbiota. 16S rDNA cloning and sequencing and whole genomic DNA probes complemented each other to result in species recognition. Diverse microbiota of 138 taxa enabled identification by Clonal analysis. 107 of 3909 clones remained unidentified turning to be novel phylotypes. By DNA probe assay 74 cultured species were identified and quantified. Out of which, 37 species were unidentified by clonal analysis and certain species, such as Actinomyces species, were not efficiently represented.

With the use of different molecular methods used, association of bacterial species with dental diseases in patients also differed. The microbiota of the early phase of adult periodontitis has been minimally studied. To study initial microbial changes incipient periodontal disease could be considered as an informative model, from health and during periodontitis progression.

Generally, similarities were observed in the microbiota of initial periodontitis and previously well characterized advanced periodontitis, with regards to detection of fastidious species such as T. denticola, F. alocis, and P. endodontalis and a strong association with P. gingivalis and T. forsythia. Complexity of early adult periodontitis was revealed by detection of uncultivated Treponema, Actinobaculum, and Bacteroidetes phylotypes, as well as environmental species with no cultured representatives and warrants further investigation. "Hidden" associations with early periodontitis and increased sensitivity detection was seen with the help of PCR, this was not observed with the other methods employed. Previously cultured species and the oligonucleotide DNA probes allowed screening of cultured and uncultivated phylotypes by genomic DNA probes as they provided quantification.

Conclusion

Present study on Aa concluded that besides bacterial diversity at phylotype level, various genotypes and phenotypes of a species complex the oral microbiota. The cause for non-serotype ability for number of Aa

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strains was detection of lack of serotype antigen expression, instead of that that the strains carried serotype as specific genes. A competitive advantage against humoral immune response over strains expressing this antigen. That the strains were mainly found in chronic periodontitis suggests that the serotype antigen deficient phenotype may provide Various molecular methods used in this thesis shed light on different subsets of oral bacterial populations, giving a variable perspective on their members. Upto, short while ago, identification of oral pathogens and their association was based on the sporadic focus on specific species. More rapid methods like microarray analysis in the near future can result in recognition of diagnostic bacterial profiles of dental diseases. Through screening methods such as 16S rDNA cloning and sequencing, complete array of bacteria found in dental disease or health can be easily located.

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