A review on microbiological cause of periodontal disease: disease and treatment

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The human oral cavity presents as an exceptional microbiological environment from other surfaces of the body. This environment allows for the extended colonization and development of microbial communities. It is essential to gain a complete understanding of all the microbes within the oral flora to better define the role of plaque as the primary cause of periodontitis. This review will provide an insight into effective methods to evaluate the cause and pathogenesis of periodontal diseases. The following information provides a summary of the primary research techniques that have been employed in the effort to describe the natural and pathologic flora of human gingival sites. Discovery and refinement of these methods have led to the development of our current research methods.

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Introduction

Early research of the oral microflora can be traced to Antoni van Leeuwenhoek (1632–1723), who illustrated findings observed from his own dental plaque [1]. In his notebook, he wrote 'I did not clean my teeth for three days and then took the material that had lodged in small amounts on the gums above my front teeth. I then most always saw, with great wonder, that in the said matter there were many very little living animalcules' [2,3]. Substantial advancements in research methods over the last century have significantly improved microbial findings since this report. Despite such progress, only associations between specific pathogens and periodontitis have been noted. In fact, a precise spectrum of the microbial flora within the gingival biofilm that is responsible for eliciting periodontitis has not been established [4]. The main impediments to this goal have stemmed from technical research limitations, and the uniqueness of the pathogenesis of periodontal diseases.

It is generally accepted that the primary cause for periodontal disease is dental plaque including the bacteria, bacterial products, and the resulting inflammatory cascade. However, the human oral cavity presents a unique microbiological environment from other surfaces of the body. Teeth provide a solid and nonshedding surface that remains in close proximity to epithelial cells and tissues of the periodontium [5]. This environment allows for the extended colonization and development of microbial communities.

Biofilms are natural communal aggregations of microorganisms that form on liquid–air and liquid–solid interfaces [6,7]. The establishment of these systems involves a sequential process by which early colonizing microbes such as *Streptococci gordonii* adhere to, and begin to condition, the tooth surface and gingival sulcus. Other cells attach and organize by means of auto aggregation and co-aggregation. The local environment begins to change

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(e.g., from aerobic to facultative anaerobic) as extracellular matrix products are produced by the various flora at each stage [8,9]. These ubiquitous aggregations occur in health, but can also alter their environments to promote disease, as is the case in periodontal diseases. In fact, it is estimated that 65–80% of all physiological infections are biofilm-related [10,11].

Supragingival and subgingival dental plaques are classic examples of liquid–solid surface biofilms. Highlighting the diversity of these biofilms, studies have identified more than 700 species in the oral cavity [12,13] and over 400 bacterial species in subgingival sites [14]. Additionally, recent studies have illustrated the complexity of such biofilm communities by identifying the process of quorum sensing [15]. 'Quorum sensing' bacteria produce and release chemical signal molecules that enable them to communicate with one another to coordinate gene expression, metabolic functions, and behavior of the entire community. These behaviors include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation. Research suggests that this process can also be used by biofilms to elicit specific responses from their corresponding host, thereby altering or controlling their local environment [16]. The capacity of biofilms to coordinate these behaviors is thought to be a significant reason for the failure of antimicrobial therapies to infections [11]. These complex interactions also present a challenge in illustrating a complete description of the subgingival environment.

In light of these discoveries, it is essential to gain a complete understanding of all the microbes within the oral flora to better define the role of plaque as the primary cause of periodontitis. This will better inform researchers in their quest for more effective methods to evaluate the cause and pathogenesis of periodontal diseases. We summarize the primary research techniques to describe the natural and pathological flora of human gingival sites.

Microscopy

Early investigations of the periodontal flora began during the 'golden age of microbiology' (~1857–1914) when the understanding of the association between microbes and diseases led to many medical discoveries of etiologic pathogens. These studies were primarily based on observations from wet mount or stained smear microscopy and limited bacterial culturing. Investigators from this period identified amoebae, spirochaetes, fusiforms, and streptococci as the four possible etiologic causes of periodontal lesions [5,17].

It is now abundantly clear that these observations were heavily influenced by the methods employed in each investigation. Those suggesting amoebae and spirochaetes were using wet mounts or specific stains that selectively identified these microbes within samples [18–20]. The implication of fusiforms was based on the frequency of observation noted in microscopic analyses of subgingival plaque, and their association with Vincent's infection [5,21,22].

Successive observations progressed with the development of the microscope. At a time when culture studies still experienced limitations, Listgarten [23] was able to report a clear differential composition between the microflora of the periodontium in health and disease based on observations from light and electron microscopy. This report indicated more spirochetes, Gram-negative, and flagellated species in disease.

Another development was the use of dark-field microscopy. Many of the studies involving this method were able to reveal more dramatic differences than were previously reported from culture data [24,25].

Bacterial culturing

Streptococci were initially identified as prevalent periodontal pathogens based on methods of microbial culturing. The ease of growing these microbes in artificial laboratory conditions led to their frequent observation. Unfortunately, culturing techniques inherently limit observational findings to those microbes that can be cultivated by the in-vitro methods employed. These limitations result from the variable growth or inhibited growth among the sampled species on the selected media [26]. In fact, it was estimated that only ~0.5% of microbes could be counted based on the techniques available during the early 20th century [27].

Limited clinical applications from such findings led to a decrease in the enthusiasm to search for etiologic microbes. By the 1930s, research in this area virtually ceased [5,28]. Pathogenesis of the disease was attributed to several factors including a constitutional defect of the patient or trauma from occlusion.

A resurgence of interest in identifying a specific microbial cause for periodontal disease was renewed by the studies of Keyes & Jordan in the 1960s [5]. These researchers demonstrated the transmissibility of periodontal disease to healthy/nondiseased hamsters by housing the animals in single cages, as well as by swabs from plaque and feces [29]. Studies illustrating the invasive potential of spirochaetes into the connective tissue and epithelium of ANUG lesions also emphasized the possibility of a specific microbial cause [30].

The subsequent cultural studies undertaken during the 1960s, like that from Socranski *et al.* [31], attempted to

analyze the microbiota of both healthy and diseased sites. Unfortunately, these studies were still affected by many of the limitations from earlier reports and, therefore, were not able to identify significant differences between sites.

These studies continued to be limited by growth media selection, challenges in recreating the subgingival atmosphere (anaerobic and so on), and difficulty in maintaining this atmosphere following sampling [26]. Studies have also illustrated that plaque dispersion techniques employed during this time preferentially killed Gram-negative anaerobic organisms [32].

Necessary advances in culturing techniques were made following this period including the development of balanced anaerobic transport mediums such as reduced transport fluid [33], more effective growth media such as tryptic soy-serum-bacitracin-vancomycin [34] for *Aggregatibacter actinomycetemcomitans*, and refinements of anaerobic incubators. Owing to these advances, a report in 1977 estimated that up to 70% of the enumerated species identified microscopically could be cultivated [35]. However, this estimate did not approximate the number of species that had yet to be identified. One recent study confirms that approximately 50% of oral microbes do not grow on conventional in-vitro culture media/environments [36].

During the 1980s, sufficient studies were available for comparison whereby researchers noted associations of microbes with inflammatory periodontal diseases, the so-called 'Perio-pathogens.' By 1994, Haffaje and Socranski [37] proposed a list of microbes ranked according to their likely involvement in the cause and progression of periodontal diseases. In reviewing the literature, evidence for each microbe was organized based on a modified version of the classic postulates of Robert Koch. The following periodontal pathogens were listed as having a 'Very Strong' or 'Strong' relationship to periodontitis: *A. actinomycetemcomitans*, spirochetes (in ANUG), *Porphyromonas gingivalis, Bacteriodes forsythus, Prevotella intermedia, Campylobacter rectus, Eubacterium nodatum*, and *Treponema* species [37].

This list provided direction as to which microbes would be selected for analysis by future culture-independent techniques such as immunological assays, bacterial enzyme assays, DNA probes, and PCR.

Immunological assays

Based on these findings, techniques were developed to improve the sensitivity in the identification of the 'Periodontal Pathogens' from subgingival plaque samples. Immunofluorescence is a method based on the development of rabbit antisera against whole cells and/or monoclonal antibodies against a specific antigen [38]. In 1989, Seida [39] confirmed immunofluorescence as comparable with culture methods for microscopic counting. In 1997, Ellwood *et al.* found *P. gingivalis* to be associated with sites having a deep probing depth of more than 3 mm, using enzyme-linked immunosorbent assay (ELISA). However, these techniques require a thorough knowledge of the serology behind the periodontal pathogen(s) in question. Furthermore, antigenic variability of cell surface markers can lead to cross-reactivity of polyclonal antibodies [40]. This type of error produces false-positive results, thereby affecting the accuracy of the test.

Bacterial enzyme assays

Bacterial enzyme assays provide another method for testing the presence of periodontal pathogens within gingival sites. These tests [ex. *N*-benzoyl-DL-arginine-2-naphthylamide (BANA) and *N*-benzoyl-L-arginine-p-nitroanilide (BAPNA)] are based on the ability of *Treponema denticola, P. gingivalis, B. forsythus*, and unspeciated *Capnocytophaga* to hydrolyze β-napthylamide derivatives. Evidence shows a good correlation between the detection of the three BANA periodontal pathogens and the results from ELISA tests [41]. A common drawback to both the immunological assays and these enzymatic assays is their requirement for a detection level of at least 10^4 cells. Another limitation from this system is that the BANA test does not provide any qualitative or quantitative information on which of the three test species is present in a given site. Additionally, false-positive reactions may occur by other enzymatic activity produced by the host [42].

PCR

The development of PCR methods to amplify genetic material has created an especially powerful molecular research tool. These techniques have illustrated such extreme sensitivity as detecting a single *Treponema pallidum* cell, and as few as 50 *A. actinomycetemcomitans* and *P. gingivalis* cells in clinical samples [41]. This technology is the basis for culture-independent research methods. Single-target PCR, multiplex PCR, and quantitative or 'real-time' PCR are the three predominant applications of this method in microbial analyses. PCR has been coupled with DNA probe research, but, in recent years, it has also been applied to studies involving sequencing of 16S rRNA.

Nucleic acid probes

With the advancements in the understanding and manipulation of genomic material, DNA probe methods

became useful for identifying pathogens that are difficult to grow, present in low numbers, and exist in mixed samples [43]. This method is based on DNA hybridization, or the ability of a portion of DNA to bind to complementary strands of DNA. This allows for more specific analysis with subspecies differentiation, and the ability to reveal associations of microbes within plaque samples. For example, such studies have been able to identify that patients, as well as individual sites, are more likely to harbor single clonal types of *P. gingivalis* and *A. actinomycetemcomitans* [44].

Additionally, Socransky *et al.* (1998) [15] analyzed 261 plaque samples using whole genomic DNA probes to 40 culturable bacterial species using checkerboard hybridization assays to define bacterial complexes, rather than individual species that were associated with periodontal disease and health [45].

Although all of these highly sensitive methods have been useful in research, they are not ideal for completely describing the microbiology of an environment because their scope is limited to those known microbes whose genomic information is already catalogued. Traditionally, these studies focused on the search for the species that have been identified from culture-based studies. It is possible to detect uncultured species only when the genome for these microbes, or their near relatives, have been characterized.

This allows for the preparation of specific primers that will selectively detect them. It is for these reasons that PCR, DNA hybridization, and microarray assays are considered 'closed-ended' culture-independent approaches.

16S rRNA

A tremendous advancement in the development of an 'open-ended,' culture-independent research technique resulted from the analysis of the nucleotide sequence of ribosomal RNA (rRNA). This approach allows for the identification of nearly all the bacteria in a sample population including uncultivated or previously unknown species.

Fox *et al.* [46] described this innovation as an impending 'revolution' in bacterial taxonomy promising to change the existing 'uncertain discipline'. Evaluation of the rRNA sequence was quickly applied in research to estimate the evolutionary relationships among species because it is one of the most conserved units of genetic material, and it is present in all free-living organisms [47]. It is now possible to analyze this genetic sequence and identify unknown bacterium to a given genus or species by comparing the results to large databases of known sequences such as GenBank [48]. This method has led to the discovery of many previously unrecognized species.

This culture-independent, 16S rRNA technique has recently been employed in intraoral microbiology studies. In 2001, Paster *et al.* performed a comprehensive study of 31 subjects with a variety of periodontal diseases. The researchers reported 347 phylotypes within the subgingival plaque samples, 40% of which were novel [12]. Later, Jorn *et al.* sampled nine intraoral sites of five clinically healthy patients with this new technique. Over 700 bacterial species or phylotypes were detected and more than 50% of the bacterial flora from the samples taken represented phylotypes, which had not yet been cultivated [13]. Faveri *et al.* examined subgingival samples from 10 generalized aggressive periodontitis subjects and found that 57% of the phylotypes were previously uncultivated species and that the species *Selenomonas* may be more associated with this form of periodontitis than previously expected [49].

Technological advances in this high-throughput sequencing technique have continued to improve our insight into microbial communities. Previous studies were based on methods whereby the 16S ribosomal sequences were isolated, amplified by PCR, cloned into *Escherichia coli*, and then sequenced [50]. Next-generation sequence analysis involves partial sequencing of variable 16S rRNA gene regions. There are nine different variable gene regions surrounded by conserved stretches that can be targeted by selected PCR primers [51]. At this time, there is no consensus on a single best region, although V2 and V4 have been reported to be suitable for community analysis given their low error rates when assigning taxonomy [52]. Researchers also combine analysis to include these moderately conserved regions with analysis of variable regions such as V6 [53]. These selected amplicons are typically quantified by pyrosequencing. The shorter sequence reads may be less discriminatory than full-length 16SrRNA genes. However, pyrosequencing offers the significant advantages of higher coverage per sample, much greater resolution of the community composition, cheaper, faster, and eliminating the need for preparing clone libraries [54].

These results provide encouragement for the discovery of additional novel species, as well as gaining a further understanding of the subgingival microflora. It is evident from several research studies that previous findings have been influenced by the research design and methods employed. The heterogeneous nature of periodontal infections requires a comprehensive understanding of the complete gingival microflora associated with health and disease. The recent findings illustrate the complexities and host-modifying ability of biofilms, and emphasize the importance of attaining this information. The new open-ended culture-independent techniques offer a method to explore and identify the phylotypes of the oral biofilm

completely. The purpose of this study is to describe the subgingival bacterial biodiversity in untreated chronic periodontitis patients through the use of 16S rRNA molecular analysis, and to determine similarities or differences between deep and shallow pockets within the same patients.

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Conflicts of interest

The authors certify that there is no conflict of interest with any financial organization regarding the materials discussed in the article.

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