dental plaque revisited

oral biofilms in health and disease

proceedings of a conference held at
the Royal College of Physicians, London,
3-5 November, 1999

edited by
Hubert N Newman and Michael Wilson

Eastman Dental Institute
University College London

ISBN 0-9520432-7-0

BioLine

Cardiff School of Biosciences, Cardiff University
PO Box 915, Cardiff CF10 3TL, UK

Printed and bound in the UK by Antony Rowe Ltd,
Bumper's Farm, Chippenham, Wiltshire

PLAQUE MICROBIOLOGY IN HEALTH AND DISEASE

A. D. Haffajee, S. S. Socransky, M. Feres and
L. A. Ximenez-Fyvie

In order to understand the role of plaque species in maintaining periodontal health or initiating disease, it is necessary to determine plaque composition in different clinical states. Studies using whole genomic DNA probes and checkerboard DNA-DNA hybridization have indicated some of the associations that occur among subgingival species. For example, species such as ***Bacteroides forsythus***, ***Porphyromonas gingivalis*** *and* ***T. denticola*** *(red complex) frequently occur together and are more prevalent in periodontally diseased subjects than healthy controls. These species relate strongly to measures of gingival inflammation and probing pocket depth. The checkerboard technique has also allowed the comparison of supra and subgingival plaque composition on the same tooth surfaces in both periodontal health and disease.* ***Actinomyces*** *species predominated in healthy supragingival plaque, but declined in proportions in diseased and subgingival sites. In contrast,* ***Fusobacterium***, ***Prevotella***, ***Campylobacter*** *and red complex species increased in counts and proportions in disease and subgingivally. All 40 species examined could be detected in both health and disease and in supra or subgingival sites, although proportions were significantly different among sample locations. The effect of different periodontal therapies including SRP, systemically administered antibiotics and professional supragingival plaque removal, on the composition of the subgingival microbiota was examined. Metronidazole had a profound clinical and microbiological effect. Levels and proportions of red complex species were significantly reduced by 3 days and remained at low levels up to 180 days post-therapy. Disruption of plaque by weekly professional supragingival plaque removal led to decreases in many subgingival species. At 3 months post therapy the microbial profile was similar to that found in periodontal health. Improved microbiological techniques provide a clearer picture of the levels and proportions of species in supra and subgingival plaque in health and disease, their ecological relationships and the effects of periodontal therapies on this ecosystem.*

Introduction

Oral biofilms consist of complex communities of bacterial species that reside on tooth surfaces or soft tissues. These biofilms are important, because some resident species have the potential to cause local or systemic disease, and because other species contribute to the maintenance of oral health. The study of oral biofilms has been slowed by a number of difficulties. One of the most important has been the complexity of the microbial communities. It has been estimated that between 400 and 1,000 species may, at some time, colonize oral biofilms. Many of these species are difficult to cultivate or even uncultivable. Once isolated, strains are frequently difficult to identify using conventional

phenotypic tests. Detection of 20 to 30 species in a sample from a single tooth surface is common and one suspects that many more species may reside in individual biofilm samples. Early studies of biofilm composition employed light microscopy, but this method was incapable of distinguishing the wide array of resident species and has been largely discarded. Cultural techniques, while useful for many investigations, had serious limitations. Difficulty in recovering all or the majority of species in samples and failure to adequately recover even cultivable species were major concerns. On a practical level, the enormous labor and cost involved in evaluating hundreds or thousands of samples precluded major studies of biofilm ecology or its relationship to local and systemic diseases. Comprehensive study of the complex communities of oral biofilms required a major technological shift. One example of this frame shift has been the use of the checkerboard DNA-DNA hybridization technique employed in all the studies described in this manuscript. This method overcomes many of the limitations of culture and is more rapid and cost-effective than antibody-based techniques.

In this meeting, plaque is being revisited, in part, because of technical improvements in our ability to examine plaque, and in part, because of cognitive changes in our understanding of biofilm development, biology and ecology. The examination of the composition of dental plaque in periodontal health serves as a logical point of departure to understand the changes that occur leading to periodontal disease. Comparison of the microbiota found in diseased and non diseased subjects should indicate the species that initiate or contribute to disease progression. In addition, the composition of the microbiota found in health provides a desired endpoint for periodontal disease prevention and treatment procedures. Thus, in this manuscript, data will be presented that indicate the nature of plaque composition in health, the nature of changes in disease, outline some of the factors that affect plaque composition and examine the effects of treatments attempting to change plaque composition.

Checkerboard DNA-DNA Hybridization

Microbiologists were slowed in earlier investigations by the need to culture microorganisms in order to provide identification to a species level. For example, Figure 1 indicates the number of subgingival plaque samples enumerated at Forsyth Dental Center between the years 1969 to 1999. From 1969 to 1979, 135 samples were examined culturally to evaluate the microbiota in localized juvenile periodontitis and adult periodontitis lesions. Cultural techniques were also employed between 1982 and 1988 to examine 300 plaque samples that included actively progressing lesions and the microbiota after treatment of these lesions. From 1988 to 1993, a colony lift method that utilized DNA probes, was employed to evaluate 9,600 plaque samples. The checkerboard DNA-DNA hybridization technique was introduced in 1993 and was used to evaluate 34,400 plaque samples, to date, for their content of 40 bacterial species.

The technique involves the deposition of the bacterial DNA from 28 plaque samples and sets of standards in parallel lanes on a nylon membrane (Socransky

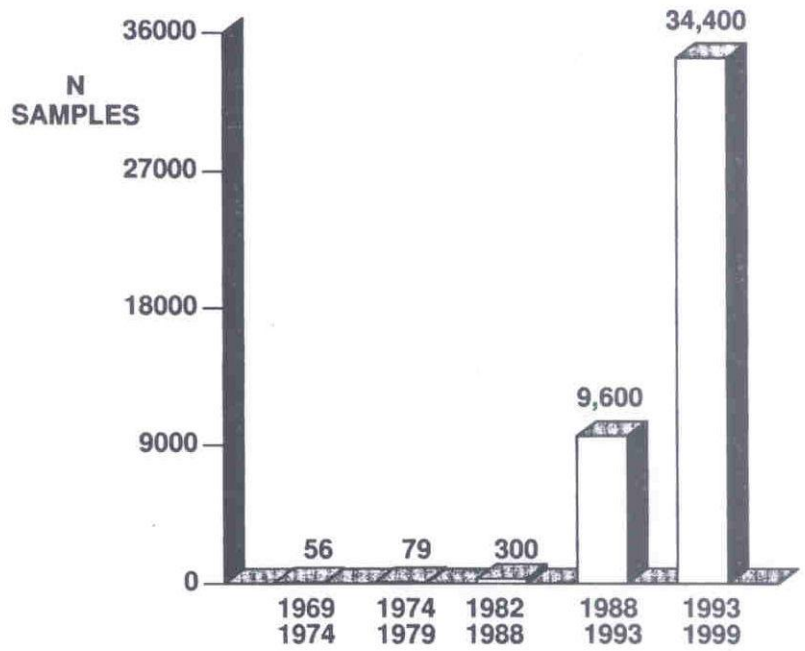

Figure 1 Bar chart of the number of plaque samples examined using different microbiological identification techniques at different time periods.

et al., 1994). Forty digoxigenin-labelled whole genomic DNA probes are run at right angles to the lanes of the samples. After stringency washing, signals are detected by using antibody to digoxigenin conjugated with alkaline phosphatase and substrates that provide either chemiluminescent or fluorescent signals. The images can be captured by film or computer linked detection systems. Signals can be converted to ranks or counts by comparison with the 10^5 and 10^6 standards on the same membrane. Figure 2 is an example of a checkerboard from one subject for one visit. The intensity of the signals relates to the number of cells of a given species in the samples from the individual sites. This single membrane provides 1,120 bacterial counts. The advantages of this technique include the speed of identification of bacteria, the low cost per sample and the ability to identify uncultivable or difficult to grow species. Disadvantages include the fact that detection is limited to species for which probes are available, the need for precise quality control and the possibility of cross reactions. The last concern can be virtually eliminated by appropriate control of stringency of hybridization and washing and the use of competitive hybridization or improved probes.

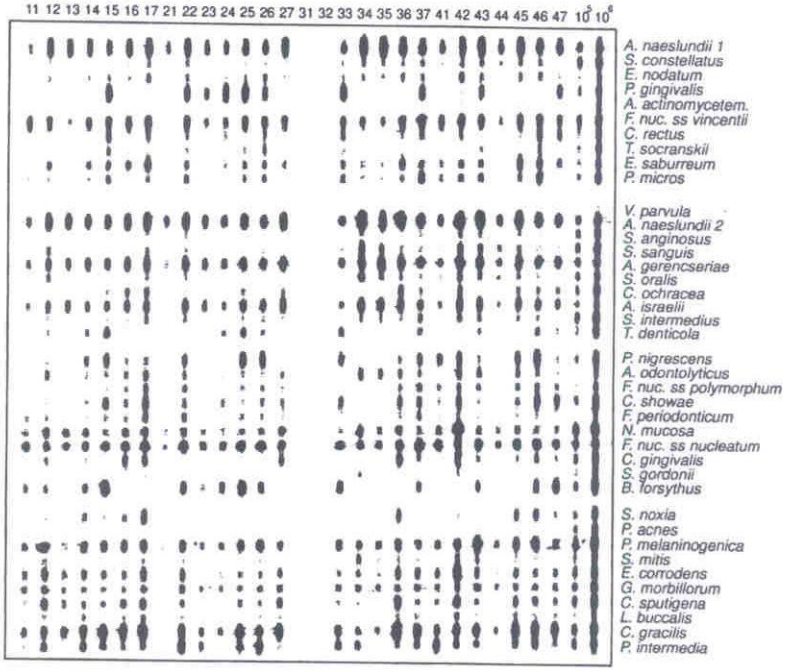

Figure 2 Example of checkerboard DNA-DNA hybridization being used to detect 40 bacterial species in 28 subgingival plaque samples from a single patient. The vertical lanes are the plaque samples numbered from 11 (upper right central incisor) to 47 (lower right second molar). The 2 vertical lanes on the right are standards containing either 10^5 or 10^6 cells of each test species. The horizontal lanes contained the indicated DNA probes in hybridization buffer. A signal at the intersection of the vertical and horizontal lanes indicates the presence of a species. The intensity of the signal is related to the number of organisms of that species in the sample. In brief, samples of plaque were placed into individual Eppendorf tubes and the DNA released from the microorganisms by boiling in NaOH. After neutralization, the released DNA was transferred to the surface of a nylon membrane using the 30 channels of a Minislot device (Immunetics, Cambridge MA). The DNA was fixed to the membrane by UV light and baking and placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at right angles to the 45 channels of the Miniblotter device. Whole genomic DNA probes labelled with digoxigenin were placed in hybridization buffer into 40 of the lanes and hybridized overnight. After stringency washing, the signals were detected using phosphatase-conjugated antibody to digoxigenin and chemifluorescent substrates. Signals were read using a Storm Fluorimager (Molecular Dynamics). The empty vertical lanes, 31 and 32 were due to missing teeth.

Microbial complexes in dental plaque

Bacterial species rarely exist in pure culture in supra or subgingival plaque samples. Indeed, in most microbial communities, there are observable associations between specific species due in part to synergistic or antagonistic relationships and in part due to the nature of available surfaces for colonization or nutrient availability. The nature of the complexes that occur within dental plaque are poorly understood. Knowledge of these bacterial associations may be

Table 1 2 x 2 contingency table of the association between *P. gingivalis* and *B. forsythus* in subgingival plaque samples.

		P. gingivalis -	*P. gingivalis* +	
B. forsythus	-	9024	311	9335
	+	1,991	1,727	2,146
		11,015	2,038	13,053

important in determining optimal (to the host) ecosystems for long-term periodontal health.

The associations between species in subgingival plaque samples from subjects with different levels of periodontal disease were examined. 13,261 subgingival plaque samples from 185 subjects were evaluated for the presence of 40 subgingival species using the checkerboard DNA-DNA hybridization technique (Socransky *et al.*, 1998). The associations between pairs of microbial species in plaque samples were assessed by preparing 2 X 2 contingency tables for all possible pairs of the 40 species. An example of such a table for *B. forsythus* and *P. gingivalis* is presented in Table 1. The computed phi coefficient was 0.536. The strength of the associations between all possible pairs was assessed using this coefficient which was scaled to range from 0 to 100% similarity. The similarities were used in a cluster analysis using an average unweighted linkage sort. In the analyses to follow, species that were detected in < 5% of sites were omitted, providing a total of 32 taxa.

The dendrogram in Figure 3 depicts the five clusters that were formed with > 60% similarity. The red complex was comprised of 3 suspected periodontal pathogens, *Bacteroides forsythus, Porphyromonas gingivalis* and *Treponema denticola*. This combination was found more frequently at deeper periodontal pocket depths. The orange complex was comprised of a tightly related central group consisting of *Fusobacterium* species and subspecies, *Peptostreptococcus micros, Prevotella nigrescens* and *Prevotella intermedia.* These were joined by *Campylobacter rectus, Campylobacter gracilis, Campylobacter showae, Eubacterium nodatum* and *Streptococcus constellatus*. Many of the species in this complex are suspected periodontal pathogens and like the red complex species, this complex was detected more often at deeper periodontal pockets, possibly reflecting the selective pressures of such pockets. The yellow complex was comprised solely of members of the genus *Streptococcus*, while the green complex consisted of members of the genus *Capnocytophaga, Actinobacillus actinomycetemcomitans* serotype a, *Campylobacter concisus* and *Eikenella corrodens. Actinomyces odontolyticus* and *Veillonella parvula* made up the purple complex. Three species did not fall into any of the cluster groups. Species of the yellow, green and purple complexes together with *Actinomyces*

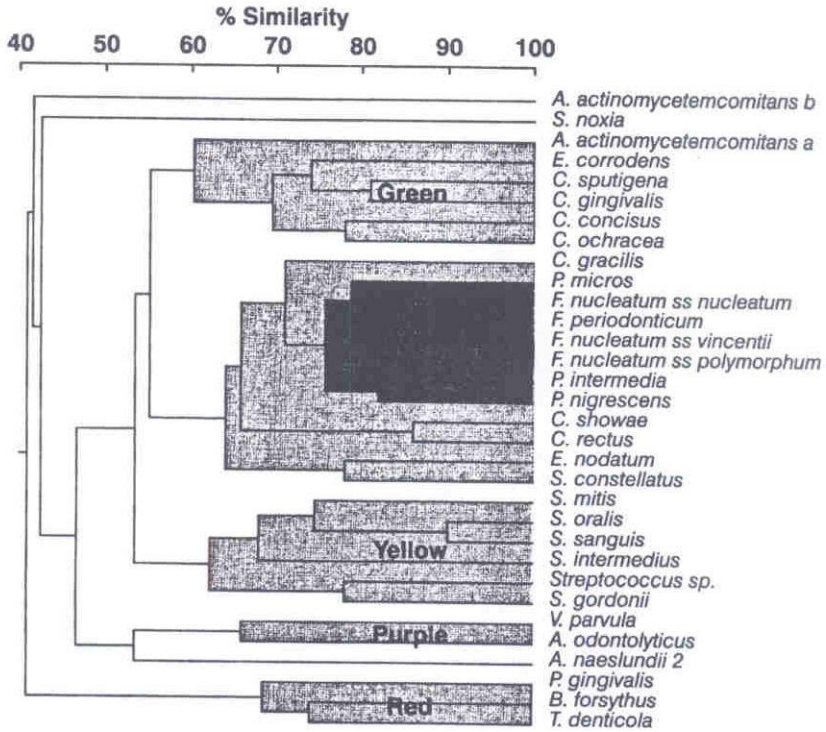

Figure 3 Dendrogram of a cluster analysis of 32 subgingival taxa. The similarity between pairs of species was computed using a phi coefficient, the coefficients were scaled and then sorted using an average unweighted linkage sort. 29 of the taxa fit into 5 clusters that were formed using a threshold level of 60% similarity. *A. naeslundii* genospecies 2, *A. actinomycetemcomitans* serotype b and *S. noxia* were not part of any complex. Colors are used to designate different microbial complexes. (Adapted from Socransky *et al.*, 1998).

naeslundii genospecies 2, are for the most part considered to be host compatible or beneficial species. *A. naeslundii* genospecies 2, for example, was detected more frequently at sites with shallow pockets.

Community ordination was used as a second approach to examine the data. This procedure attempted to indicate closely related species within a community and then demonstrate the relatedness among different communities of species within the ecosystem of interest. Figure 4 is a correspondence analysis of the data from the 13,261 samples from 185 subjects. The x and y axes indicate the first and second components respectively. A third component was plotted in 3-dimensional plots (data not shown). The Figure reinforces groupings observed using the cluster analyses and also relates communities of species to each other. For example, the red complex of *B. forsythus, P. gingivalis* and *T. denticola* is most closely related to the orange complex consisting of species of the genera

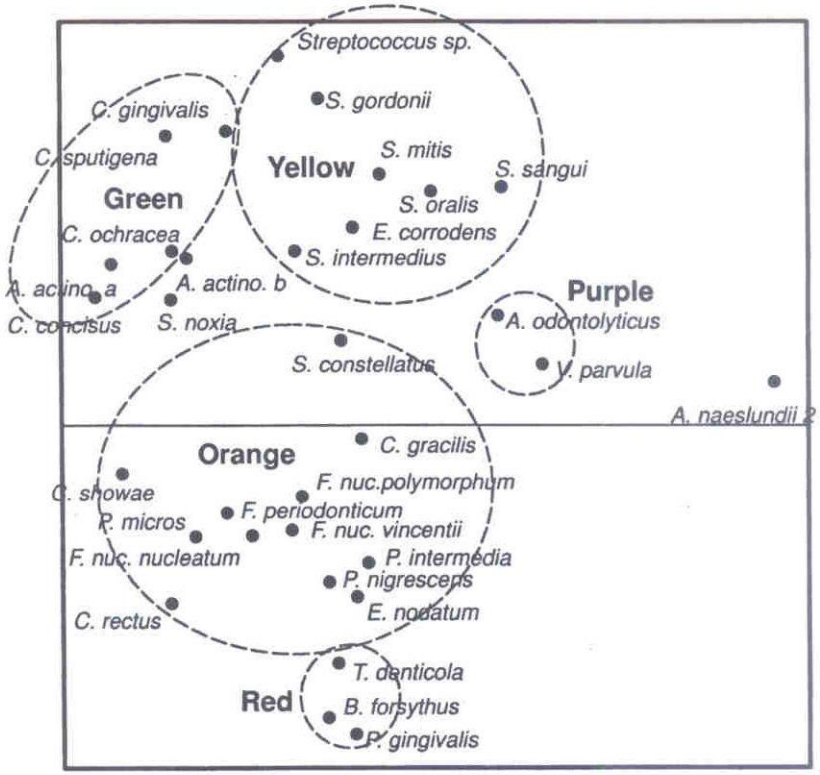

Figure 4 Community ordination of 32 subgingival taxa using correspondence analysis. The relationships among species were evaluated using the levels of the species at each of the sampled sites. Correspondence analysis was performed as described by Ludwig & Reynolds (1988) and the species were plotted along the first (x-axis) and second (y-axis) axes. The dotted circles represent the different microbial complexes indicated in Figure 3.

Fusobacterium, Prevotella, Campylobacter, P. micros, E. nodatum and *S. constellatus*. This complex in turn related to 3 other groups observed in the cluster analysis. The data indicated that bacterial species were not randomly distributed but occurred in distinct complexes. The large number of plaque samples from 185 subjects used in this analysis suggested that these groupings occur in more than a handful of subjects. Cluster analysis using different similarity coefficients (e.g. Bray-Curtis, Mahalanobis d^2) and different subsets of the data as well as community ordination using correspondence analysis and principal components analysis showed quite consistent grouping of species and relationships among the groups. These are depicted in Figure 5.

These data suggest the nature of some of the microbial complexes in subgingival plaque. Although the number of plaque samples was large and the number of species evaluated was reasonably extensive, the results represent an initial