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The Microbial Aetiology of Periodontal Diseases

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1. Introduction

The study of the aetiology of periodontal diseases has continued for decades with much progress shown in the last two decades. Having moved through periods of "whole" plaque (with emphasis on mass) being attributed to the disease process, to "specific" species being implicated, we have finally returned to examining the oral microbiota as an ecological niche involving not only a selected few species but looking at plaque as a whole where all the players are invited to participate with their roles no longer individually defined but viewed as a team effort with recognition of their individual strengths and contributions. Recent findings using advanced technology, are confirming findings viewed by electron microscopy nearly half a century ago, but we now have the knowledge and expertise to interpret those findings with deeper understanding. This chapter will attempt to examine the microbial succession within the plaque biofilm from health to disease, bearing in mind the susceptibility of the host, the microbial heterogeneity and the expression of virulence by the putative pathogens.

2. Theories proposed by early pioneers

Microbial plaque has been implicated as the primary aetiologic factor in chronic inflammatory periodontal disease (CIPD, Listgarten, 1988). Studies of experimental gingivitis in man and in animal models have confirmed that a positive correlation exists between plaque accumulation and CIPD, and that plaque control reverses the inflammatory process (Lindhe *et al.,* 1973; Löe *et al.,* 1965; Page & Schroeder, 1976, 1982 Theilade *et al.,* 1966). It has also become evident, at least in relation to chronic gingivitis, that plaque mass rather than quality is the main correlate with disease severity (Abdellatif & Burt, 1987; Ramfjord *et al.,* 1968). It was initially postulated that CIPD occurred as the result of an overgrowth of indigenous plaque microorganisms (Gibbons *et al.,* 1963; Löe *et al.,* 1965; Socransky *et al.,* 1963; Theilade *et al.,* 1966). But, since many of the organisms observed in periodontal health were also observed at diseased sites (Slots, 1977), the results indicated that shifts in microbial populations rather than specific pathogens would play a role in initiating disease. Failure to demonstrate an overt pathogen gave rise to the non-specific plaque hypothesis (NSPH, Loesche, 1976), which generally assumes that all plaque is capable of causing disease. If the plaque mass is increased, irritants produced by the plaque microbes are increased until gingival inflammation ensues.

However, the NSPH failed to explain why certain individuals with longstanding plaque and gingivitis do not develop periodontitis, while others, with minimal plaque, had lower resistance to disease. Comparisons of health and diseased sites, demonstrated an increase in Gram-negative organisms in the latter (Hemmens & Harrison, 1942; Rosebury *et al.,* 1950; Scultz-Haudt *et al*., 1954). By 1977, the focus had shifted from supra to subgingival plaque and since sampling and cultural methods had improved, more sophisticated studies were possible in relation to the microbial aetiology of CIPD. It was shown that subgingival plaque composition differs, not only between subjects, but also between sites within the same mouth (Listgarten & Hellden, 1978; Socransky *et al.,* 1992). The culture of plaque samples from single diseased sites lead to the association of certain bacterial species with various forms of CIPD (Listgarten, 1992; Socransky & Haffajee, 1992,).

While the NSPH focuses on quantitative changes, the specific plaque hypothesis (SPH) focuses on qualitative changes. Evidence for the specific plaque hypothesis has been derived from studies of subgingival microflora associated with health and disease, from evaluations of the pathogenic potential of various members of the periodontal microbiota as well as selective suppression of the microflora by chemotherapy using both human and animal models. These criteria have been used in association studies, since no single pathogen has been isolated which fulfils the criteria for Koch's postulates, namely, that a specific organism should be isolated in pure culture in all lesions of the disease and a similar disease produced in animals when inoculated with the causative organism, resulting in the recovery of that same organism from the lesions of the infected animals. These postulates have proved inadequate for CIPD since cultural studies of CIPD have revealed over 700 bacterial species, many of which are extremely difficult to cultivate, creating problems with animal inoculations. Another factor is that the disease produced in experimental studies with animals need not necessarily be the same disease observed in humans (Socransky, 1979), nor does a bacterium which is known to be pathogenic always cause disease in selected hosts even though they may be of the same species (Socransky & Haffajee 1992). It is therefore impracticable to compare virulence in different host species, even though the same pathogen is used. Alternatives for Koch's postulates were suggested by Socransky (1979), namely, that there be association of the organism with disease followed by elimination after treatment, and that host response, animal pathogenicity and mechanisms of pathogenicity are considered.

Association of a given organism with disease is demonstrated by an increase in the proportion of that organism at the site of infection and a decrease or absence in health and after treatment. The marked differences between plaques seen in health and disease, and the establishment in the subgingival plaque of species such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (Aa), which are seldom, if ever, detected in health or gingivitis, led to the hypothesis that severe periodontitis was caused by exogenous microorganisms (Genco *et al.,* 1988). However, this hypothesis failed to define a specific means of host entry or colonisation. Nor was the acquisition or mode of transmission adequately explained. Although treatment resulted in suppression or elimination of these species, the authors failed to include the effect of treatment on many of the indigenous species as well. Acceptance of an exogenous infection hypothesis was considered by many as an over-simplification of a very complex situation. Re-evaluation of the different hypotheses indicated that they all contained contradictions. Overlaps often occurred regarding suspected "periodontopathogens" in active and inactive sites. This negated both the SPH and the NSPH. Eradication of "exogenous pathogens" resulted in a microbial shift

from a disease-related to a health-associated microbiota, incorporating both the SPH and the NSPH. To confuse the issue even further, miscroscopic (Africa *et al.,* 1985a; Reddy *et al.,* 1986) and cultural studies (Africa, unpublished data) of plaque from two groups of subjects with heavy plaque accumulations, showing no clinical evidence of associated loss of attachment, demonstrated disease-associated microbial species. High percentages of spirochaetes and motile rods were indicated in these darkfield studies, while the cultural studies demonstrated the presence of *Porphyromonas gingivalis* and *Prevotella intermedia* amongst their predominant cultivable species. However, distinct differences in the cultivable microflora accompanying these species were observed when the periodontitis group was compared with the two periodontitis-resistant groups. This would indicate that the host response, along with microbial interactions within the plaque microbiota, determined disease progression and that neither the SPH nor the NSPH *per se* could be applied in this case. Theilade (1986) proposed an acceptance of a compromise between the two, in order to accommodate the microbial succession from health to disease, when attempting to establish the association of specific species with CIPD.

The inability to explain why some individuals developed disease and others not, created difficulties in comparing data, especially since inter-individual as well as intra-individual variability was often demonstrated. With more than 700 microbial species inhabiting the periodontal pocket, many of which are uncultivable and/or difficult to identify, contradictions often occur regarding the association of specific species with a particular disease entity.

These research outcomes are complicated by differences in sampling and detection methods and inaccuracies in the classification and diagnosis of disease. Added to that, is the fact that animal models of disease are often used for *in vivo* investigations of monomicrobial infections. The disease is therefore induced and differs from natural pathogens in humans (Arnett and Viney, 2007) where the disease process is initiated by the normal microbiota overcoming the tolerance threshold of the host, resulting in a polymicrobial infection (Gemmell *et al.,* 2002; Kesavalu *et al.,* 1997).

Because a precise definition of disease activity has not been clearly established, earlier studies of the microbial aetiology of CIPD have failed to implicate any single plaque bacterial species as the definitive causative species. Many of the subgingival flora could not be classified by existing taxonomic schema at the time, with the result that oral microbiologists often forced their isolates into existing species descriptions, a process which was not only incorrect but which confused and hampered the process of implicating specific aetiological agents in periodontal disease. The bacteria discussed in the subsequent sections can only be implicated by association with disease and have not been proven as single pathogens fulfilling the criteria of Koch's postulates. The flora of sites sampled at a particular time may not relate to that present at a time of an episode of disease activity or quiescence. Results may reflect previous episodes of disease activity and may have no bearing on the current level of disease activity (Listgarten, 1992; Socransky & Haffajee, 1992).

3. The ecological plaque hypothesis

The finding of suspected pathogens in mouths free of disease could either be due to avirulent clonal types of the microbial species or due to low levels of bacterial species in an insusceptible or "carrier" host. With the advent of molecular biology, our understanding has been greatly improved and our approach to identifying the putative pathogens has gone full

circle. We are once again looking at bacterial succession and ecological changes but with improved knowledge where, with the assistance of modern technology, we are viewing bacterial plaque as a "biofilm" of microbes possessing the chromosomal and extra chromosomal genetic properties necessary to initiate disease in a susceptible host. In order to initiate disease, a potential pathogen has to colonise a susceptible host with an appropriate infectious dose in an environment conducive to optimal bacterial interactions which will favour the expression of its virulence properties (Socransky & Haffajee, 1992). This environmental activity results in patterns of bacterial succession favouring the ecological plaque hypothesis (Marsh, 1991). The ecological plaque hypothesis suggests that periodontal disease is an opportunistic endogenous infection brought about by an ecological shift in the plaque biofilm from a predominantly Gram-positive facultatively anaerobic microflora to a Gram-negative obligate anaerobic or micro-aerophilic flora, resulting from host-microbial and microbe-microbe interactions, creating an anaerobic environment which favours their growth (Konopka, 2006). Thus any bacterial species may be pathogenic since ecological changes in the environment may dictate the pathogenicity and virulence mechanisms for that particular organism (Marsh, 1991, 1994, 1998). Disease may thus be prevented by interruption of the environmental factors responsible for the ecological shifts as well as elimination of the putative pathogen.

4. The oral cavity as a microbial ecosystem

The oral cavity is home to a multitude of microbes colonising a variety of surfaces, namely the tooth, tonsils, tongue, hard and soft pellets, buccal cavity, lips and associated gingival tissue. (Kolenbrander & Landon, 1993; Paster *et al.,* 2001; Rosan & Lamont, 2000; Whittaker *et al.,* 1996). With specific microbial species demonstrating tropism for specific tissues (Aas *et al.,* 2005; Gibbons, 1996; Mager *et al.,* 2003; Van Houte *et al.,* 1970), all of which interact with each other as well as with the oral environment, the oral cavity meets the criteria for the definition of a microbial ecosystem (Konopka, 2006; Marsh, 1992; Raes & Bork, 2008).

Factors which determine the oral microflora include environmental factors (temperature, oxygen tension, pH, availability of nutrients), host factors (host tissues and fluids, genetics, diet) and microbial factors (adherence, retention and coaggregation, microbial intra- and interspecies interactions, clonal heterogeneity, virulence mechanisms) thus creating a dynamic and complex ecosystem (Kuramitsu *et al.,* 2007; Kolenbrander, 2006; Marsh, 2005; Overman, 2000; Rosan & Lamont, 2000; Sissons *et al.,* 2007; Socransky & Haffajee, 2002; Ten Carte, 2006).

Dental plaque is a dynamic biofilm formed by the ordered succession of > 700 bacterial species. The recognition of dental plaque as an oral biofilm has now become widely accepted. (Aas *et al.*,2005; Bowden, 2000; Filoche *et al.,* 2010; Haffajee *et al.* ,2008; Jenkinson & Lamont, 2005; Marsh, 1991, 2003, 2006; Marsh & Percival, 2006; Socransky & Haffajee, 2005). In health these endogenous species live in symbiosis with the host , but changes in the oral microbial ecology due to nutritional and atmospheric gradients, synergistic and/or antagonistic interactions between microbial species, may alter the balance of the host and render an organism pathogenic (Carlsson, 1997; Kolenbrander, 2000; Lamont & Jenkinson, 1998; Marsh, 1999, Newman, 1988; Pratten & Wilson 1999, Quireynen *et al.* 1995, 2001, Rosan & Lamont, 2000; Sbordone & Bortolaia, 2003; Socransky & Haffejee, 1992, 1995; Socransky *et al.,* 1998). Most periodontopathogens are commensals in the oral cavity and express their virulence only in a susceptible host or when changes occur in their ecosystem. Microbial

species exhibit different properties when they form communities in the plaque biofilm and work together rather than in isolation. With synergy prevailing over antagonism, they respond to changes in the environment as a single unit rather than as individual species (Caldwell *et al.,* 1997). Formation of the plaque biofilm and a discussion of ecological succession in the development of CIPD, is essential in understanding the changes which occur in the periodontium during the progression from health to disease. Ecological succession is the process whereby a microbial population (e.g. plaque microbiota) undergoes a continuous series of changes in composition as different species colonise and become established at the expense of others. The microbial population present at any given time will determine the subsequent successional changes.

4.1 Formation of the plaque biofilm

The tooth surface is a non-shedding surface which allows for the colonisation of microbial species and the establishment of a plaque biofilm. If a tooth surface is professionally cleaned, a deposit called the acquired pellicle develops within 15-30 minutes. It is a thin, clear cuticle composed of mainly glycoproteins and its source is generally considered to be precipitations of mucoids from saliva, containing molecules which are recognised by bacterial adhesins during the initial selective adsorption of Gram-positive cocci (streptococci) to the surface of the acquired pellicle.

Saliva not only provides substrates for bacterial growth by the secretion of proteins and glycoproteins (endogenous nutrients) but also serves as a mode of transport for carbohydrates and peptides (exogenous nutrients) of dietary origin (Homer *et al.,* 1996; Palmer *et al.,* 2001; Scannapieco, 1994). When a microorganism adsorbs to the acquired pellicle, growth and multiplication will occur, accompanied by accumulation of bacterial products. Attachment of microorganisms is further enhanced by the production of dextrans by the streptococci and by the ability of bacterial cells to coaggregate (Kolenbrander, 2000). Differences in microbial growth rates cause population shifts to occur quickly once the initial microbial population has been established.

The cleansing activities of the mouth such as saliva, abrasion and swallowing are limited to the colonisation of supragingival plaque only. The subgingival plaque, due to the anatomy of the gingival sulcus, is undisturbed by the cleansing activites of the mouth and because a relatively stagnant environment is formed, harbours many more motile bacteria than supragingival plaque. Because the oxidation-reduction potential (Eh) of the gingival sulcus is very low (Loesche, 1988), the subgingival environment would favour the growth of a more anaerobic microflora than would be found in supragingival areas where the environment selects for the growth of aerobic and facultative microflora. The indigenous anaerobic microflora includes members of the genera *Actinomyces, Bacteroides, Bifidobacterium, Campylobacter, Capnocytophaga, Fusobacterium, Leptotrichia, Peptococcus, Peptostreptococcus, Propionibacterium, Veillonella* and many motile organisms such as *Selenomonas,* a few spirochaetes and vibrios. Many of these species co-exist with facultative and capnophilic bacteria in periodontal health and disease.

4.2 Bacterial interactions during biofilm development

Pathogens do not exist in isolation in the oral cavity but as part of a microbial community which may display synergistic or antagonistic interactions. Microbial diversity is spatially structured, not only by geographic location, but also by environment (O'Malley, 2008).

Early plaque is composed of mainly Gram-positive cocci which are gradually replaced by more filamentous Gram-positive forms and finally, an abundance of Gram-negative forms which were not found initially (Kolenbrander *et al.,* 1985 ; Haffajee & Socransky, 1988). Gram-negative colonisation of the gingival sulcus occurs only after the lawn of Gram-positive organisms has been established, since Gram-negative organisms cannot adhere directly to the tooth surface (Slots, 1977). An increase in the thickness of the plaque biofilm results in the creation of nutritional and atmospheric gradients which alter the environment, reducing oxygen levels and allowing for the growth of anaerobes (Bradshaw *et al.,* 1998; Cook *et al*., 1998; Lamont & Jenkinson, 1998). Coaggregation enables the colonisation of organisms that do not have receptor sites. Their colonisation is therefore facilitated by the colonisation of a synergistic species. Coaggregation can be defined as intrageneric, intergeneric or multigeneric cell-to-cell recognition (Kolenbrander, 1989) in a biofilm community and was reported to occur between viable as well as dead cells, providing evidence that interactions are mediated by existing specific surface molecules rather than cells responding actively to each other (Kolenbrander, 1993). An important factor of plaque biofilm formation is the spatial relationship of the community members (Dawes 2008; Mager *et al.* 2003; Mineoka *et al.,* 2008). The proximity of phenotypes allows for their interactions and influences their ability to survive within the biofilm.

Among the early studies of spatial relationships in plaque biofilm formation are the studies by Nyvad and colleagues (Nyvad, 1993; Nyvad & Fejerskov, 1987a; Nyvad & Fejerskov, 1987b; Nyvad & Killian, 1987). Using a stent that holds enamel pieces (commonly used in supragingival oral film investigations), they placed it in the oral cavity and monitored the formation of plaque biofilms. Among the first species to colonise were streptococci and actinomyces, including *Streptococcus sanguinis, Streptococcus oralis, Streptococcus mitis, Streptococcus salivarius* and *Actinomyces viscosus.* Plaque biovars were seen to develop at exactly the same rate from individual to individual, reaching a plateau around 12 hours after stent insertion (Nyvad & Kilian, 1987). Electron microscopy confirmed a change in species composition over the next 12 hours with both Gram-positive and Gram-negative bacteria appearing, providing evidence for direct interaction between species in the biofilm (Nyvad & Fejerskov, 1987b).

Further studies confirmed the importance of cell-to-cell recognition in early plaque development and examination of undisturbed plaque. Palmer *et al,(*2003) used antibodies to detect adhesins or their complimentary receptors on bacteria known to coaggregate. They examined the reactions using immunofluorescence and confocal microscopy and found that many of the cells which reacted with the adhesin antibody were adjacent to cells reactive with the receptor antibody. Diaz *et al., (*2006) used ribosome-directed fluorescence in situ hybridisation (FISH) to examine spatial relationships and produced similar results.

Electron microscopy has demonstrated that where 2 or more species coaggregate with a common partner using the same mechanism, they are likely to compete for receptor sites e.g. "corncob" formations, where coccoid cells such as streptococci attach to a long rod such as *Fusobacterium nucleatum* (Jones, 1972; Listgarten *et al.,* 1973) or *S. sanguinis* and *Corynebacterium matruchotii* (Bowden, 1999; Palmer, 2001; Socransky *et al.,* 1998; Wilson, 1999). Another example is the " test-tube brush" arrangement formed by *Eubacterium yurii* (Margaret & Krywolap, 1986). If 2 or more bacteria coaggregate with a common partner using different mechanisms of adhesion, the common partner acts as the coaggregation bridge for the coaggregation of the other 2 species e.g. *Prevotella loescheii* PK 1295 provides the bridge linking *Streptococcus oralis 34* to *Actinomyces israelii* PK 14 (Weiss *et al.,* 1987).

Intergeneric coaggregations occur with *Fusobacterium* and other bacteria such as *Aggregatibacter actinomycetemcomitans* (Rosen *et al*., 2003), *Tannerella forsythia* (Sharma *et al*., 2005), and oral *Treponema* (Kolenbrander, 1995). Intrageneric coaggregations occur among different strains of oral fusobacteria (Kolenbrander, 1995), *P. gingivalis* (Lamont *et al*., 1992), oral streptococci, and *Actinomyces* (Kolenbrander *et al*., 1989). Coaggregation bonds between *P. gingivalis* and oral streptococci or *Actinomyces naeslundi* are rendered resistant to removal if *P. gingivalis* adheres directly *to Streptococcus gordonii* (Brooks *et al*., 1997; Cook et al., 1998; Demuth *et al*., 2001, Rosan & Lamont, 2000; Quirynen *et al*., 1995).

The production of metabolic products by plaque bacteria may promote or inhibit the growth of other species (Kolenbrander, 2000; Quirynen *et al.,* 1995, 2001). Examples of cross-feeding include but are not limited to, the production of lactic acid by *Streptococcus* and *Actinomyces*, needed for the metabolism of *Veillonella* which, in turn, produce menadione which favours the growth of *Porphyromonas* and *Prevotella*. *Fusobactrium* produces fatty acids needed for the growth and metabolism of *Treponema* and in synergy with *P. gingivalis*, produces metabolic products needed for the growth of *Mogibacterium (Eubacterium) timidum* (Miyakawa & Nakazawa, 2010). Other beneficial microbial interactions include the prevention of colonisation of a pathogenic species by using receptors which may be needed for the attachment of latecomers (Rosen *et al.,* 2003) or by the production of substances which affect the growth of, or prevent the production or expression of, virulence factors by the pathogen (Socransky & Haffajee, 1992).

4.3 Quorum sensing

Another mechanism by which bacteria are able to communicate is via quorum sensing molecules. Quorum sensing has been described in both Gram-positive and Gram-negative bacteria. It has been defined by Miller (2001) as "the regulation of gene expression in response to fluctuations in cell population density".As they grow, quorum sensing bacteria produce to the external environment a series of molecules called autoinducers. The autoinducers accumulate as the bacterial population increases and once they reach a certain threshold, different sets of target genes are activated, thus allowing the bacteria to survive environmental changes. Cell-cell communication may occur between and within bacterial species (Miller, 2001) and controls various functions reflecting the needs of a specific bacterial species to inhabit a particular niche such as the production of virulence factors, or by the transmission and acquisition of the generic information needed to produce virulence factors from other species in the biofilm development (Passador *et al*., 1993; Reading *et al*., 2006). Several strains of *P. intermedia, T. forsythia, F. nucleatum* and *P. gingivalis* were found to produce quorum sensing signal molecules (Frias *et al*., 2001; Sharma *et al*., 2005).

4.4 Host susceptibility and inter-individual variation

It was previously understood that plaque control was effective in preventing and treating periodontal diseases. Now it is clear that the plaque biofilm alone is not enough to initiate or control the disease process. A susceptible host is needed and the susceptibility is genetically determined with individuals responding differently to various stimuli (Relman, 2008; Tombelli & Tatakis, 2003).

The severity of periodontal diseases differs amongst populations of different race (Douglas *et al*., 1983), in different areas of the same country, (Teixeira *et al*., 2006; Viera *et al*, 2009,) as well as in different countries (Cortelli *et al*., 2005; Gajardo *et al*., 2005; Haffajee *et al*., 2004;

Sanz *et al*., 2000; Rylev & Kilian, 2008). Asian and African popoulations have on average more severe periodontal disease than Europeans and Americans (Glickman, 1972; Baelum *et al*., 1986; Botero *et al*, 2007). While this may largely be due to differences in oral hygiene habits, customs and traditions, confounding factors may affect the immune response which, in turn, will affect the level of disease activity (Table 1).

As previously mentioned, not all individuals are susceptible to periodontitis and the literature shows that some individuals present with gingivitis which appears to remain contained. A much quoted study of the plantation workers in Sri Lanka (Löe, 1986), who practised no oral hygiene and had no access to professional dental care, demonstrated that some, but not all, developed periodontitis, while others remained with minimal disease. Studies by Africa *et al*., (1985a) and Reddy *et al*.(1986) reported on a periodontitis resistant population in South Africa . Although one of the first studies to report on increased prevalence of suspected periodontopathogens in the absence of periodontitis, thus suggesting a variability in host susceptibility to periodontitis as well as 'carriers" of avirulent strains, no genetic studies were done to confirm this.

Plaque biofilm formation has been described as a highly ordered sequential attachment of specific species over time, a process found to occur at the same rate for everyone (Palmer 2003). However, the architecture and function is person-specific and even though the same bacterial species may often be found in the same site of many different individuals, each individual may have a unique microbial fingerprint (Dethlefsen *et al.,* 2007), which dictates the outcome of disease progression and response to treatment (Filoche *et al*., 2007, 2008; Haffajee *et al.,* 2006; Preza *et al*., 2008; Sissons *et al*., 2007; Teles *et al*., 2006). Not only do different persons harbour different oral microbiota, but different sites within the same mouth as well as different sites of the same tooth in the same mouth also differ in microbial composition due to environmental differences (Dawes *et al*., 2008; Haffajee *et al*., 2006, 2009; Mager *et al*., 2003; Mineoka *et al*., 2008).

The bacterial challenge presented by the bacteria of the plaque biofilm activates the host inflammatory response which is also influenced by the factors listed in Table 1. The severity of periodontal disease is modified by the expression of three elements of the host response, namely, interleukin-1 (IL-1), prostaglandin-E_2 (PGE_2) and matrix metalloproteinases (MMPs) that destroy both collagen and bone. Increased production of IL-1 appears to be hereditary with specific IL-1 gene variation associated with response to the bacterial challenge (Assuma *et al*., 1988; Cavanaugh *et al.,* 1998; Gemmell *et al.,* 1998; Ishihara *et al*., 1997; McGee *et al*., 1998; Okuda *et al.,* 1998; Roberts *et al*., 1997).

Factor	Selected References
Smoking	Bergström *et al*., 2000; Calsina *et al*., 2002; Feldman *et al*., 1983; Haber, 1994; Haber *et al.,* 1993; Stam, 1986;
Genetics	Engebretson *et al*., 1999; Genco, 1998; Gore *et al*., 1998; Grossi *et al*., 1998; McDevitt *et al*., 2000; Mark *et a*l., 2000; Michalowicz *et al*., 2000; Lang *et al*., 2000; Shirodaria *et al*., 2000;
Diabetes	Genco, 1988; Grossi *et al*., 1998
Hormones	Marcuschamer *et al.,* 2009
Stress	Armitage 1999; Bascones & Figuero 2006; Flemming, 1999; Genco, 1998; Newman, 1998.
Age	Genco, 1998; Horning *et al,* 1992

Table 1. Factors which may influence host susceptibility.

4.5 Gene expression

As mentioned above, host susceptibility may be genetically determined; so also, can many important virulence traits be ascribed to heterogeneity among subspecies of bacteria. Some strains are associated with health or "carrier" states while others are associated with disease. In order to confirm this, researchers have embarked on demonstrating multiple clonal types within the periodontopathogens and reported on their different virulence properties.

Gene expression is regulated in response to changes in the environment with either up- or down-regulation of the production of virulence factors (Finlay & Falkov, 1989; Maurelli *et a*l., 1989; Miller *et al*., 1989), or when the organism comes into direct contact with partner community bacteria (Sharma, 2010) thus acquiring their virulence through cell-cell interactions (Araki *et al*., 2004; Brook *et al*., 1984; Kuriyama et al, 2010; Van Dalen *et al*., 1998).

The persistence of clones appears to vary for different species, with many clones simultaneously inhabiting the oral cavity at different periods. Genomic polymorphisms within bacterial strains along with the response of the host will determine the disease situation and progression in the individual patient (Hohwy *et al*., 2001; Kononen *et al*., 1994; Tambo *et al*., 2010). Early colonising species showing wide clonal diversity (reflected in antigenic variety) elicit natural immunity which benefits the host, while frequent turnover of clones within a particular host may allow the species to overcome the host response and exert its pathogenicity (Smith, 1988).

Multiple genotypes have been demonstrated in *Prevotella (*Yanagiswa *et al*., 2006), *P. gingivalis* (Amano *et al*., 2000; Nakagawa *et al*., 2000), *F. nucleatum* (George *et al*., 1997; Haraldsson *et al.,* 2004; Thurnheer *et al*., 1999), *T. denticola* and other spirochaetes (Choi *et al*., 1994; Reviere *et a*l., 1995), and Aa (Preus *et al*., 1987a,b). Cross-sectional and longitudinal studies of *T. forsythia* in periodontal disease (Hamlet *et al*., 2002, 2008) found the prtH genotype to be significantly raised in subjects with disease and lowered in subjects showing no attachment loss. It is generally accepted that species involved in infection will display a high degree of genetic similarity (Perez-Chaparo *et al.,* 2008). In the case of *P. gingivalis*, many different individuals may be colonised by a single genotype, but their clonal types may differ. Based on their nucleotide sequences, *P. gingivalis fim*a gene has been classified into 5 genotypes (I-V). Types I and V are most prevalent in healthy adults (Amano *et al.,* 2000), with type I showing the most significant association (Amano *et al.,* 1999a; Nakagawa *et al*., 2000). Anamo *et al*.(1999, 2000) reported Type II to be significantly associated with periodontitis, followed by type IV while the converse was found by Griffen *et al.(*1999), using ribosomal intergenic spacer region (ISR) heteroduplex typing, and Teixeira *et al.* (2009). These differences may be attributed to differences in techniques used and/or study population. Another explanation may be that virulent alles may be distributed at several genetic loci throughout the clones with only certain combinations producing a strain which may be associated with disease (Loos *et al*., 1993). More than 100 genes were reported to be missing from the genome of a non-invasive strain of *P. gingivalis* (Dolgilevich *et al.,* 2011). Types III and IV of *P. gingivalis* are believed to be virulent, showing reduced ability to adhere to host proteins, while non-encapsulated strains of type I are recognised as avirulent and showed better adhesion to salivary proteins (Nakagawa *et al*., 2000).

A key virulence factor of Aa is the powerful leucotoxin which is able to disrupt and destroy cells of the immune system. Aa serotypes c and b have been associated with health and disease respectively (Asikainen *et al*., 1991). The leucotoxic clone JP2 is associated with serotype b and is characterised by enhanced leucotoxin expression

associated with the 530bp deletion in the promoter region of the *ltx* operon. It is speculated that the clone might have a distinct host tropism being found mostly in adolescents in Mediterranean regions of Africa (e.g. Morocco) and West Africa from where it was transferred to the Americas during the slave trade. Although frequently found in subjects with aggressive periodontitis, clonal types other than JP2 have been associated with disease and carrier states. Recent evidence of aggressive periodontitis amongst adolescents in Morocco who do not have the JP2 clone (Rylev *et al*. 2011), and the finding of the JP2 clone in a Caucasian mother and daughter in Sweden who have no disease (Claesson *et al*. (2011), indicate that carriers do exist in Caucasians and that other serotypes may be associated with disease in African populations. Table 2 shows some examples of different serotypes in different population groups.

Ethnicity	%Aa isolates	Serotype distribution					
		a	b	c	d	e	NT
Chinese (Mombelli *et al*., 1998)	61.6	15	0	38.3	0	8.3	0
Chinese (Mombelli *et a*l., 1999)	62.7	18	7.7	57.7	0	7.1	9.4
Vietnamese Finish (Holtta 1994)	78 13	36 6	27 6	63 0	0 0	0 0	0 0
Turkey (Dogan *et al*., 2003)	66	0	0	34	0	0	34
Germans Koreans (Kim *et al*., 2009)	27 22	20 0	33 0	25 61.9	0 19	0 0	0 0
Spanish (Blasi, 2009)	72.5	37.5	20	15	0	0	0
Brazilian (Roman-Torres *et al,* 2010)	80	31.8	<10	52.9	0	0	0

Table 2. Distribution of serotypes in different ethnic groups (NT = non typeable).

Serotypes a and b are prevalent in Europeans while serotype c is prevalent in Asian and Mediterranean groups (Table 2 and Sakellari *et al.,* 2011). Cortelli *et al.,* (2005) recommended that serotype b be used as a diagnostic marker for aggressive periodontitis since they found a high prevalence of the JP2 clone in a Brazilian population. These findings have been contradicted by other studies on Brazilians which showed very low, if any, serotype b strains (Vieira et al., 2009; Roman-Torres *et al*., 2010). Yet another study showed similar frequencies of serotypes b and c but associated serotype b with health and c with disease (Teixeira *et al*., (2006). The contradictions in these results may be due to the fact that Brazil has a multi-ethnic population of predominantly African and Mediterranean origin, while the native Brazilians, descending from almost extinct ethnic groups who live in cultural isolation with no mixing with other ethnic groups (Vieira *et al*., 2009), have not been exposed to the toxic strains of Aa.

5. Plaque bacteria associated with health and periodontal disease

5.1 Plaque in health

The tooth surface harbours a microbial population which not only lives in harmony with host tissues, but also serves a protective function by occupying an ecological niche which would otherwise be colonised by potentially pathogenic bacteria. Bacterial species belonging to the genera *Streptococcus* and *Actinomyces* rapidly colonise bacteria–free surfaces, thus explaining their prevalence in dentitions which are well maintained (Listgarten, 1988). The relatively aerobic environment of the healthy gingival sulcus tends to preclude the growth of obligate anaerobes and the predominant flora includes members of the genera *Actinomyces, Atopobium, Eubacterium, Micromonas, Peptococcus, Staphylococcus, Streptococcus, Veillonella* while phylotypes Bacteroidetes and Deferribacteres have also been reported. Vibrios and spirochaetes are present in low numbers if at all (Dalwai *et al*., 2006; Grossi *et al*., 1994; Kumar *et al*., 2003; Listgarten & Helldén, 1978; Loesche, 1980; Marsh, 1994; Rosan & Lamont, 2000).

Direct darkfield and phase contrast microscopic counts from healthy sites also indicate that spirochaetes (1-3%) and motile rods (1-6%) are present in low numbers, while coccoid cells (62-79%) predominate (Lindhe *et al*., 1980; Addy *et al*., 1983; Africa *et al*., 1985b; Adler *et al*., 1995; Stelzel *et al*., 2000). Studies of healthy sites following treatment also show similar low counts of these forms due to their reduction or complete elimination, with a concomitant increase in cocci (Listgarten et al 1978; Loesche et al 1987; Africa *et al*., 1985b; Adler *et al*., 1995; Stelzel *et al*., 2000).

In the section that follows, the association of microbial species with periodontal diseases will be discussed according to the classification outlined in the World Workshop Proceedings (Armitage, 1999) and will be restricted to a selection of the species most frequently associated with periodontal diseases.

5.2 Plaque in gingivitis

The new classification of periodontal diseases recognises that gingivitis is more prevalent than periodontitis and has thus included in the classification of "gingival diseases" all the previous sub-classifications of periodontitis related to endocrine and host immune disturbances, associations with therapeutic agents and malnutrition. In addition, plaque induced gingivitis has been classified separately from non-plaque induced gingivitis involving other aetiologic agents such as *Treponema pallidum*, *Neisseria gonorrhoeae*, streptococci, herpesviruses, and *Candida* which may also present in the oral cavity (Armitage, 1999). A detailed description of the classification is outside of the scope of this chapter and readers are advised to read the chapter on disease classification for details.

For ease of reading and association, this section will describe the microbiota under the broad headings of gingivitis, chronic periodontitis and aggressive periodontitis only, since many of the species overlap in the subclassifications of the three disease entities and may all be contained within the broad listing of putative pathogens in Table 3.

If the plaque biofilm remains undisturbed, demonstrable inflammation of the gingiva will occur in 2-4 days due to the production of various noxious bacterial metabolites such as endotoxins, mucopeptides, lipoteichoic acids, metabolic end-products and proteolytic agents, which may penetrate the gingival tissues. In addition, the increased production of gingival fluid contains growth-promoting factors for a wide range of bacteria. The initial phase of gingivitis is characterised by predominantly Gram-positive cocci, followed by

fusiform bacilli after 2-4 days. Neutrophil transmigration through junctional and pocket epithelium is enhanced, accompanied by perivascular collagen destruction. Thinning and ultimate ulceration of the cuff epithelium may occur, followed by infiltration of lymphocytes and other mononuclear cells. Further loss of collagen from the marginal gingiva will occur, accompanied by an increase in vibrios and spirochaetes (Table 3) with a predominantly polymorphonuclear (PMN) leucocyte and plasma cell infiltrate apparent in the connective tissue. Bleeding on probing may occur and a relatively shallow gingival pocket may be evident. At this stage, chronic gingivitis can either be induced or eliminated by plaque control.

Bacterial species	**Gingivitis**	**Chronic periodontitis**	**Aggressive periodontitis**	
			Localised	**Generalised**
Aggregatibacter actinomycetemcomitans (Aa)		+	+	+
Campylobacter rectus	+	+		+
Capnocytophaga	+		+	+
Cryptobacterium curtum		+		
Eikenella corrodens	+	+	+	+
Enterobacteriaceae		+	+	
Eubacterium saphenum		+		
Fusobacterium nucleatum	+	+	+	
Micromonas (Peptostreptococcus) micros		+	+	
Mogibacterium (Eubacterium) timidum		+		
Peptostreptococcus anaerobius	+	+		
Pophyromonas endodontalis		+		
Porphyromonas gingivalis	+	+		+
Prevotella intermedia	+	+	+	+
Slackia (Eubacterium) exigua		+		
Tannerella forsythia		+		+
Treponema amylovorum		+		+
Treponema denticola	+	+		+
Treponema lecithinolyticum				+
Treponema maltophilum		+		
Treponema medium	+	+		
Treponema pectinovorum	+	+		+
Treponema socranskii	+	+		+
Treponema vincentii	+	+		+
Veillonella parvula	+			

Table 3. Bacterial species most frequently detected in periodontal diseases.

5.3 Plaque in chronic periodontitis

Previously referred to as adult periodontitis, this disease affects many teeth with no evidence of rapid progression. The onset appears to be after 30 years, but the condition may also be found in children and adolescents. Amounts of microbial deposits are usually associated with the severity of disease. Although chronic periodontitis can occur in a localised and a generalised form, both forms appear to be identical in their aetiology and pathogenesis. The microbial pattern varies, with reports of unusual species appearing in the literature. The species listed in Table 3, date post 1999 only, following the reclassification of periodontal diseases, since studies before 1999 might now fall within a different disease category under the new classification and create confusion.

When periodontal disease becomes active or destructive, the numbers of the bacteria in the unattached zone increases and Gram-negative organisms, particularly the motile organisms, predominate. If this condition is allowed to persist, the periodontal tissues are rapidly destroyed. Direct microscopy studies using both darkfield and phase contrast have revealed significant differences between subgingival microbial floras of healthy and diseased subjects. Listgarten & Helldén (1978) demonstrated that in chronic periodontitis-affected subjects, spirochaetes constituted 37.7% and motile rods 12.7% of the total microscopic count, with coccoid cells as low as 22.3%. These microbiological changes may signal an increase in periodontal disease activity. Many cycles of exacerbation and remission may continue till the alveolar bone is destroyed and the teeth lost (Socransky *et al.,* 1984).

Table 3 lists some of the species most frequently associated with periodontal diseases (Botero *et al*., 2007; Casarin *et al*., 2010; Dogan *et a*l., 2003, Gajardo *et al*., 2005; Kumar *et al*., 2003; Teixeira *et al*., 2006; Riep *et al*., 2009). Species associated with chronic periodontitis are predominantly Gram-negative with few Gram-positive anaerobes. Spirochaetes predominate along with *P. gingivalis* and *T. forsythia*. Bacterial antagonism and synergism are indicated with Aa seldom reported along with *P. gingivalis* , while species like *F. nucleatum, P. intermedia* and other species of the "orange complex" (Socransky *et al*.,(1998) are necessary for the colonisation of the "red complex" consortium. Subjects with high proportions of *P. gingivalis* were found to have few or no *P. intermedia* and *vice versa* (Loesche *et al.,* 1985, Africa, unpublished data). Recent studies would indicate that this inhibition has been overcome, probably due to interactions of emerging species or due to clonal diversity within the two species, resulting in a mutual tolerance.

Recently, our attention has been drawn to the colonisation of the asaccharolytic anaerobic Gram-positive rods (AAGPRs) which have been associated with periodontitis (Miyakawa & Nakagawa, 2010). Although some of these species have been reported in the past, their role in disease has not received much attention. While they have an inability to form biofilms when cultured individually, they appear to be dependent on *P. gingivalis* and *F. nucleatum* for their colonisation of, and establishment in, the plaque biofilm. Their irregular finding in plaque cultural studies may be due to their fastidious growth requirements and difficulties in their colony recognition. Some of the AAGPR species may form part of the viable but not cultivable (VNC) species in the oral cavity, playing a role in prolonging and stabilising of biofilms formed by *P. gingivalis*. Because they are able to inhibit cytokine production by human gingival fibroblasts stimulated by other bacteria, it is possible that they may prolong inflammation, causing chronic disease (Miyakawa & Nakagawa, 2010).

The role of Enterobacteriaceae in chronic periodontitis is not clear and they are thought to indicate superinfection. It is speculated that they are opportunists which thrive after periodontal treatment. The drugs of choice for treating periodontal disease include

amoxicillin, doxycycline, tetracycline and metronidazole. The Enterobacteriaceae show resistance to these drugs and may therefore persist after administration of therapy (Botero *et al.,* 2007). More studies are needed to explain their presence in the plaque biofilm and to elucidate their role in infection.

Herpes viruses may contribute to the pathogenesis of chronic and aggressive periodontitis (Table 4). There is speculation that Epstein-Barr virus-1 (EBV-1) and cytomegalovirus (CMV) may be involved in synergistic mechanisms with Aa, *P. gingivalis* and *T. forsythia* (Chalabi *et al*., 2010; Dawson et al., 2009; Imbronito *et al*., 2008; Slots 2010, Fritschi *et al*., 2008).

Microbe	Chronic Periodontitis	Localised Aggressive Periodontitis	Generalised Aggressive Periodontitis
Herpes simplex virus-1	+	-	+
Cytomegalovirus	+	-	
Epstein-Barr virus	+	-	+
Dialister pneumosintes	+	-	-
Prevotella denticola	+	-	-
Staphylococcus aureus	-	-	+

Table 4. Species less frequently reported but also implicated in periodontal diseases.

5.4 Aggressive periodontitis

This form of periodontitis is less common than chronic periodontitis and mostly affects young patients. Localised and general forms of the disease differ in aetiology and pathogenesis. Localised aggressive periodontitis (LAP) mostly restricted to the first molars and incisors, is characterised by rapid loss of attachment and bone destruction in otherwise clinically healthy individuals while generalised aggressive periodontitis (GAP) presents a clinical picture similar to LAP but the bone loss is generalised. Aggressive periodontitis was previously called localised and generalised juvenile periodontitis. Plaque films are thinner than in chronic periodontitis and age is no longer a criterion for diagnosis (Armitage, 1999).

Comparison of the microbiology of chronic periodontitis with aggressive periodontitis shows major overlaps , with very few species showing unique specificity for either condition (Table 3). The organisms most strongly associated with LAP and GAP are Aa and *P. gingivalis* respectively. The prevalence of Aa in LAP and GAP is often contradictory with some reporting it only in LAP and others reporting it in both LAP and GAP. However, the prevalence appears to be higher in LAP. A positive correlation was found between a highly toxigenic group of Aa and deep pockets, young age and mean attachment loss (Cortelli *et al*., 2005). Aa was found to be present in very low numbers in a Colombian population (Botero *et al.,* 2007) when compared with Asian populations (Yang *et al*., 2005; Leung *et al*., 2005) and a Brazilian population (Cortelli *et al.,* 2005). The Colombian population harboured *E. corrodens, P. gingivalis* and *T. forsythia* along with Enterobacteriaceae. The latter may be associated with halitosis in humans (Goldberg et al., 1997). As with chronic periodontitis, very few studies make a distinction between LAP and GAP. Most studies report on "aggressive periodontitis" (Botero *et al*., 2007; Cortelli *et al*., 2005; Sakellari *et al*., 2004) which, in the context of this chapter is interpreted as GAP.

6. Virulence mechanisms of plaque bacteria

Although the terms pathogenicity and virulence relate to the ability of a microorganism to produce disease, pathogenicity refers to the species and virulence refers to degrees of pathogenicity of strains within species. Microbial virulence is investigated by comparing the properties of virulent and avirulent strains. *In vitro* studies of enzymes, antigens, metabolic and biological properties indicate virulence markers which may be responsible for inhibiting host defence mechanisms or tissue damage. These results could often be misleading since many bacteria from infected animals have been shown to differ chemically and biologically from tissue grown *in vitro*. This could be explained by differences in growth conditions and phenotypic changes. However, there are some bacterial virulence determinants which were originally examined *in vivo* and then reproduced *in vitro* by approximate changes in cultural conditions (Smith, 1976). In order for bacteria to be considered pathogenic, they should be examined for their ability to colonise the appropriate site and initiate infection, multiply within the host's tissues, resist and overcome the host's defences and cause damage to the host's tissues. This section is limited to the discussion of selected microbial species and is based purely on association studies and the demonstration *in vitro* of their pathogenic potential but bearing in mind that true virulence is expressed in a susceptible host, rather than *in vitro*, where nutritional and other environmental conditions differ. Tables 5-8 list the important virulence factors of four of the species most frequently associated with periodontal diseases namely, *T. denticola, P. gingivalis*, Aa and *T. forsythia* respectively.

6.1 Adhesion and colonisation

Many of the suspected periodontopathogens have surface structures necessary for attachment, including fimbriae, capsules and lipopolysaccharides.

6.1.1 Fimbriae

The interaction between bacterial fimbriae and host factors could be an important component of the disease process.

Fimbriae are extracellular appendages which facilitate the adhesion of a Gram-negative organism to a surface. Aa possesses fimbriae and amorphous material which assist in adhesion (Fives-Taylor *et al.,* 1999). Protein sequence homology of *P. gingivalis* fimbriae polymers of repeating fimbrillin monomer subunits with a molecular weight of about 43kDa (Yoshimura *et al*., 1984; Lee *et al*., 1991) show no homology with the fimbriae of other Gram-negative bacteria. The *fim*A gene of *P. gingivalis* appears to be involved in most of the adhesive mechanisms of the organism. *P. gingivalis* fimbriae also facilitate coaggregation with other plaque organisms such as *T. denticola*, oral streptococci, fusobacteria, actinomyces and oral epithelial cells, amongst others. Other reported functions of fimbriae include chemotaxis and cytokine induction (Goulbourne & Ellen, 1991; Hashimoto *et al*., 2003; Ishihara et al 1997; Rosen *et al*., 2008; Yao *et al.,* 1996).

6.1.2 Capsules and surface layers (S-layers)

The outer layer of bacteria is often referred to as a capsule (uniform consistency) or a slime layer (ill- defined and loosely formed). Because it is this outer layer that is in direct contact with the environment, it is largely responsible for the ultimate survival of the producer bacterial cell.

The composition of capsular polysaccharide may vary among strains and may be composed of either carbohydrate or protein, depending on the conditions under which they were grown (Hofstad, 1992). *In vitro* studies have demonstrated a capsule on *P. gingivalis* (Listgarten & Lai, 1979; Woo *et al*., 1979), fusobacteria and peptostreptococci (Brook and Walker, 1985, 1986). Besides having adhesive properties, capsules are known to provide immunologic specificity and protection against phagocytosis.

T. forsythia lacks fimbriae and possesses a surface layer of glycoproteins. These serve as ligands for lectin-like receptors on other bacteria e.g. *F. nucleatum* (Murray *et al*., 1988), epithelial cell adherence and invasion (Tanner *et al*., 1996; Sakakibara *et al*., 2007) and as an external protective layer (Sleytr & Messner, 1988), highly regulated to respond to environmental changes (Kato *et al*., 2002). S-layers have also been reported for *C. rectus* (Haapasalo *et al*., 1990), *Prevotella buccae* (Kornman & Holt, 1981) and *Eubacterium yunii* (Kerosuo *et al*., 1988).

The oral spirochaetes possess an outer sheath or slime layer which envelopes the complete cell. In *T. denticola*, this layer is composed of 50% protein and 31% total lipid, of which 95% and 11% are phospholipid and carbohydrate respectively (Masuda & Kawata, 1982; Weinberg & Holt, 1990). The adhesive properties of *T. denticola* to hydroxyapatite (Cimansoni *et al*., 1987), human gingival epithelial cells (Olsen, 1984; Reijntjens *et a*l., 1986), fibroblasts (Weinberg & Holt, 1990), fibronectin (Dawson & Ellen, 1990; Haapasalo *et al*., 1992) fibrinogen and laminin (Haapasalo *et al*., 1992) as well as erythrocytes (Mikx & Keulers, 1992), have been demonstrated. The putative *T. denticola* adhesin was characterised as being a surface-bound 53 kDa protein (Cockayne *et al*., 1989; Umemoto *et al*., 1989; Haapasalo *et al*., 1992), while Weinberg & Holt (1990) described outer sheath surface proteins of 64 kDa and 54-58 kDa depending on the strain examined. These proteins were considered to be major degradation components of high molecular mass oligomers (Haapasalo *et al*., 1992). *T. denticola* major sheath protein (Msp) is thought to be responsible for its binding *to F. nucleatum, Streptococcus crista, P. gingivalis* and *T. forsythia* (Kolenbrander *et al*., 2000).

6.1.3 Haemagglutinins

Haemagglutinins are known virulence factors for a number of bacteria of which *P. gingivalis* produces 5 haemagglutinating molecules. Their role in colonisation is to mediate the binding of bacteria to human cell receptors. Our understanding of the complexities of the genetics and functions of the haemagglutinin process has been greatly informed by the cloning of the first haemagglutinin gene (*hag*A) from *P. gingivalis* (Progulske-Fox *et al*., 1989). Because *P. gingivalis* requires haem for growth, the binding to erythrocytes may also serve as a nutrient source (Progulske-Fox *et al*., 1989). Co-expression of genes associated with haemagglutination and proteolytic activity of *P. gingivalis*, suggest that they function in complexes on the cell surface (Shah *et al*., 1992). Haemagglutinating activity has also been described for *T. forsythia* (Tables 5- 8).

6.2 Impairing host immune systems

For adhesion to lead to colonisation, bacteria must be able to resist the host defence mechanisms such as phagocytosis and the protective antimicrobial factors which would otherwise destroy them. The innate immune system is the host's first line of defence against bacterial infection. Immunomodulation by bacteria allows for their survival and subsequent invasion.

6.2.1 Interfering with PMN function

The ability of *T. denticola* to suppress the production of β-defensin 3 by human gingival epithelial cells (Table 5) has been reported (Shin *et al.,* 2010). By preventing binding of such antimicrobial peptides, *Treponema* can evade the host defences and survive. Neutrophil chemotaxis and phagocytic activity may be impaired by *Treponema* Msp interactions, leading to reorganisation of host cells.

Aa produces a leukotoxin that alters cell membranes of PMNs and monocytes and interferes with antibody production (Table 7) thus ensuring its own survival (Fives-Taylor et al., 1999). The leukotoxin is encoded by a ltx operon consisting of four known genes, namely, ltxA, ltxB, ltxC and ltxD, which appear to be present in all strains of Aa with varied levels of expression with the JP2 ltx promoter being associated with high levels of leukotoxin expression.

Virulence mechanism	References
Adhesion and colonisation	
Haemagglutinin	Grenier, 1991
Major sheath protein (Msp)	Batista de silva et al., 2004; Kaplan *et al*., 2009; Kolenbrander *et al*., 1995, Rosen *et al*., 2008, Yao *et al*., 1996
Outer sheath (S-layer), outer sheath vesicles (OSV)	Kuchn & Kesty, 2005
Impairment of host defences	
Methyl mercaptan	Johnson *et al*., 1992; Lancero *et al*., 1996
Lipoproteins	Dashper *et al*, 2011
Suppression of β-defensin production	Shin *et al*., 2010
Internalisation by epithelial cells	Colombo *et al*., 2007
Tissue invasion / bone resorption	
Motility	Li *et al*., 1999; Kataoka *et al*., 1997
Metabolic end products	Chu *et al*., 2002; Fiehn, 1989; Fukamachi *et al*., 2005; Kuramitsu *et al*., 2007; Yoshimura *et al*., 2000
Phosphatases	Ishihara *et al*., 1995; Laughon *et al*., 1982;
Trypsin-like protease	Loesche *et al*., 1987; Ohta *et al*., 1986
Tissue degrading enzymes	Fiehn 1986b; Mikx, 1991; Uitto *et al*., 1986

Table 5. Virulence factors of *T. denticola.*

Spirochaetes, including *T. denticola*, have been reported to inhibit lysosome release (Taichman *et al*., 1982) thereby inhibiting PMN degranulation and other immune reactions to spirochaetes and other plaque microrganisms in the periodontal pocket (Hurlen *et al*., 1984). Besides interfering with PMN function, spirochaetes are also able to suppress proliferation of fibroblasts (Boehringer *et al*., 1984), endothelial cells (Taichman *et al*., 1984) and lymphocyte responsiveness (Taichman *et a*l., 1982; Shenker *et al*., 1984). The ability of bacteria to overcome the host defence mechanisms may also place the host at risk for opportunistic infections and could be relevant to the progression of periodontitis.

Virulence mechanism	References
Adhesion and colonisation Haemin Fimbriae Outer membrane proteins	 Holt & Bramanti, 1991 Dickinson *et al.,* 1988; Lamont & Jenkinson, 1998
Impairment of host defences Induction of cytokines Ability to subvert host intracellular events and localise intracellularly Proteases	 Frandsen *et al.,* 1987; Hanazawa *et al*., 1992; Murakami *et al*., 2002; Schifferie *et al*., 1993; Shapira *et al*., 1997
Tissue invasion / bone resorption Hyaluronidase, heparin Chondroitin sulphatase Phopholypase A Acid and alkaline phosphatases	 Bulkacz *et al.,* 1981; Capestany *et al*., 2004; Frank, 1980; Frank & Vogel, 1978; Holt & Bramanti, 1991; Kawata *et al*., 1994; Lindemann *et al.,* 1988; Sismey-Durrant & Hopps, 1991;

Table 6. Virulence factors of *P. gingivalis.*

Oppa, a *T. denticola* lipoprotein has been proposed to act as an adhesin for the purpose of covering the surface of *T. denticola* with host proteins in order to avoid, or at least delay, immune recognition (Dashper *et al*., 2011), while surface proteins of *T. forsythia* activate host cells to release pro-inflammatory cytokines and induce cellular apoptosis (Hasebe *et al*., 2004).

6.2.2 Endotoxins

True endotoxins are derived only from Gram-negative bacteria and normally exist within the bacterium as integral components of the bacterial cell wall in the form of unique glycolipid, lipopolysaccharide (LPS). Endotoxin can be released from cells during active growth as well as by cell lysis. Normal macrophages are not cytotoxic but following exposure to LPS, can selectively release lysosomal enzymes. So also can PMNs and lymphocytes (Koga et al., 1985). Most of the LPS-related injury in tissues seems to be due to constituents of PMN lysosomes which, not only may digest connective tissue components, but also increase vascular permeability and activate other mediators of inflammation (kinins). LPS is thought to be able to induce B-lymphocyte differentiation, resulting in the production of immunoglobulin-synthesising cells, mainly IgG and IgM. It can also reduce adhesion of periodontal ligament fibroblasts and stimulate bone resorption *in vitro* (Koga *et al*., 1985; Wilson *et al*., 1986). Toll-like receptors (TLRs) bind to host epithelial cells and macrophages which sense LPS, thereby preventing triggering of intracellular signalling systems which lead to the production of inflammatory mediators and the migration of macrophages and PMNs to the site of infection (Dauphinee & Karsan, 2006).

Treponemes lack the genes encoding the enzymes for LPS synthesis. The treponemal outer sheath contains lipooligosaccharides (LOS) with a diacylglycerol lipid anchor and hexose-hexosamine-hexose core. Fragments in the lipid anchor resemble a glycolipid membrane anchor found in Gram-positive lipoteichoic acid (Dashper *et al*., 2010). The function of LOS

is similar to LPS, stimulating the expression of MMPs and fibroblasts thereby inducing the production of a variety of inflammatory mediators which could exacerbate the disease process (Choi *et al*., 2003).

Induction of cytokine production from macrophages has been demonstrated with LPS in *Bacteroides, Prevotella*, and *Porphyromonas* (Fujiwara *et al*., 1990; Yoshimura *et al*., 1997). Because of the immunologic and physiologic effects that LPS has on the host-parasite relationship in periodontal disease, it should be considered as highly significant.

Virulence mechanism	References
Adhesion and colonisation Fimbriae Vesicles Amorphous material	Fives-Taylor et al., 1994
Impairment of host defences Chemotaxis inhibitor Resistance to phagocytosis Capsular polysaccharide Surface antigens Inhibition of fibroblast cytokines Leukotoxin	Ebersole *et al.*, 1996; Fives-Taylor & Meyer, 1999; Mangan *et al*., 1991; Nakashima *et al.*, 1997; Wilson & Henderson, 1995
Tissue invasion / bone resorption Lipopolysaccharide (LPS) Haemolysin Proteinases Phospholipase C Extracellular vesicles Collagenase Acid and alkaline phosphatases Epithelial toxin	Kimizuku *et al*., 1996; Lai *et al*., 1981; Mayrand *et al*., 1996; Saglie et al., 1988; Wang *et al*., 2001; Wilson & Henderson, 1995; Zambon, 1983

Table 7. Virulence factors of *Aggregatibacter actinomycetemcomitans* (Aa).

6.2.3 Protease production

Porphyromonas, Prevotella and *Capnocytophaga* produce proteases against IgA and IgG (Grenier *et al*., 1989). Although all their virulence mechanisms have not been studied in great detail, bacterial species that produce these proteases are associated with invasion of mucous membranes where IgA may be found (Hofstad, 1992). *Prevotella* and *P. gingivalis* (Table 6) each produce different antigenic forms of IgAI protease (Frandsen *et al*., 1987).

6.3 Colonisation and multiplication in vivo

Having established themselves, the bacteria must be able to multiply within the host. Factors such as temperature, nutrients and atmospheric conditions should be supplied by the tissues or through bacterial interactions. In the gingival crevice, there is much evidence for symbiosis amongst plaque bacteria.

Virulence mechanism	References
Adhesion and colonisation	
Haemagglutinin	Murakami *et al.* 2002
S-layer	Sabet *et al*., 2003
Leucin rich proteins BspA	Sakakibara *et al.,* 2007
Glucosidases	Sharma *et al*., 1998, 2010
Impairment of host defences	
Proteolytic enzymes corrupt host immunity	Holt & Bramanti 1991
Surface lipoproteins induce apoptosis	Hasebe *et al*., 2004
Tissue invasion / bone resorption	
Trypsin-like protease	Grenier, 1995
α-D-glucosidase and N-acetyl-β-glucosaminidase	Hughes et al., 2003
PrtH proteinase (forsythe detachment factor)	Maiden et al., 2004
Methylglyoxal product	Saito et al., 1997

Table 8. Virulence factors of *T. Forsythia.*

6.3.1 Synergistic virulence expression

Many virulence genes in plaque bacteria are only expressed when the bacterial species comes into contact with the host or with other partner community bacteria, e.g. the virulence properties of *P. gingivalis* are enhanced by interaction with *F. nucleatum* (Frias *et al*., 2001; Kinder & Holt, 1989; Kolenbrander & Andersen, 1987), *T. denticola* (Grenier, 1992; Ikegami *et al*., 2004), and *T. forsythia* (Yao *et al*., 1996).

T. denticola and *P. gingivalis* display a symbiotic relationship in degrading proteins, utilisation of nutrients and growth promotion (Grenier, 1992; Grenier & Mayrand, 2001; Hollman & van der Hoeven, 1999; Kigure *et al*., 1995; Nilius *et al*., 1993; Yoneda *et al*., 2001).

Interactions between *T. forsythia* and other bacteria such as members of the "red complex" result in synergistic mechanisms in alveolar bone loss and immune-inflammatory responses in rats (Kesavalu *et al*., 2007). This bacterial consortium has frequently been associated with the clinical progression of chronic and aggressive periodontitis (Holt & Ebersole, 2005; Lamont & Jenkinson, 1998; Socransky *et al*., 1998). Because of its motility, *T. denticola* is able to respond chemotactically to environmental stimuli. It appears that *T. forsythia* may be a necessary precursor for the colonisation of *T. denticola* and *P. gingivalis*, since these species were rarely found in subgingival plaque without *T. forsythia* (Dashper *et al*., 2011). Studies of subcutaneous abscess showed that inoculation with *P. gingivalis* resulted in more severe, ulcerative lesions than monoinfection with *T. denticola, T. pectinovorum* or *T. vincentii* (Kesavalu *et al*., 1997, 2007). Low doses of *P. gingivalis* co-infected with *T. denticola* significantly enhanced tissue damage, showing that *P. gingivalis* was needed for invasion and tissue damage to occur.

6.3.2 Toxin-antitoxin systems

Toxin-antitoxin systems (TA) are composed of a stable toxin and a labile antitoxin which retard essential cell components and counteract the effects of the toxin respectively. They play a major role in biofilm formation in that they are involved in programmed cell death and reversible bacteriostasis (Kim *et al*., 2009; Makarova *et al*., 2009). *T. denticola* contains 33 predicted TA systems which, when they show an increase in expression, may demonstrate a role for them in biofilm persistence and resistance to environmental assaults (Jayaraman, 2008; Lewis, 2000).

6.4 Damage of the host's tissues

An increase in microorganisms results in high concentrations of endotoxin, mucopeptides, lipoteichoic acids, metabolic products and proteolytic activity in the subgingival area.

6.4.1 Outer membrane vesicles

Gram-negative bacteria produce outer membrane vesicles (OMV) previously thought to be random blebbing of the outer sheath resulting in the formation of spherical vesicles 50-100nm in diameter (Devoe & Gilchrist, 1977; Grenier & Mayrand, 1987b). We now know that their formation is a highly regulated response to strengthen the bacterium during environmental changes. Such blebs have been identified in *P. gingivalis* (Grenier & Mayrand, 1987b), Aa (Kato et al., 2002) and *Treponema. T. denticola* outer sheath vesicles have been reported to penetrate tissues more readily than the bacterium itself (Cimansoni & McBride, 1987).

6.4.2 Leucin-rich repeat proteins

Leucin-rich repeat proteins (LRR) are found in many eukaryotic and prokaryotic cells with a variety of cellular locations and functions. They belong to the CTD family of proteins involved in protein-protein interactions and signal transduction. Genes encoding LRR proteins have been identified in *P. gingivalis*, *T. denticola*, *P. intermedia* and *F. nucleatum. T. denticola* LrrA protein plays a role in coaggregation with *T. forsythia* but not *P. gingivalis* or *F. nucleatum*. lrrA also mediates binding to epithelial cells (Ikegami *et al*., 2004, Rosen *et al*., 2008). Six Lrr proteins are predicted in the *T. denticola* genome. Two Lrr proteins have been characterised from *P. gingivalis*. The InIJ protein of *P. gingivalis* (Capestany *et a*l., 2006) is secreted and attached to the surface of the cell. It is important in coaggregation and biofilm development as well as for epithelial cell invasion. OMV of *P. gingivalis* promote the BspA-mediated invasion of epithelial cells by *T. forsythia* (Inagaki *et al*., 2006, Lewis *et al*., 2008). *T. forsythia* BspA protein is also associated with alveolar bone loss (Capestany *et al*., 2006; Dashper *et al*., 2009; Inagaki *et al.,* 2006; Sharma *et al*., 1995, 2005). To date, one Lrr protein has been characterised and another five predicted. *P. intermedia* BspA protein (Lewis *et al.,* 2008) is associated with bacterial adherence and invasion, and triggers the release of bone-resorping proinflammatory cytokines from monocytes (Hajishenghallis *et al*., 2002).

6.4.3 Enzymes

Many Gram-negative bacteria contain proteolytic and hydrolytic enzymes in their periplasmic space and in addition, they produce extracellular enzymes. Plaque bacterial enzymes are many, with a resultant variety in capacity to damage the host tissues or modulate the behaviour of other strains; for example, they alter bacterial attachment and interfere with host defence systems by inactivating important proteinase inhibitors.

Spirochaetes are able to damage periodontal tissue directly by the production of surface components such as endotoxins and histolytic enzymes. Indirect damage may result from the initiation of excessive inflammation or tissue reaction in response to toxins, products of tissue breakdown, or specific hypersensitivity of the protective host inflammatory response to bacterial plaque antigens (Holt & Bramanti, 1991; Kontani *et al.,* 1996; Kuramitsu *et al*., 1995; Potempa & Pike, 2009; Travis *et a*l., 1997).

Certain plaque bacteria such as *Capnocytophaga, T. forsythia*, *T. denticola, T. vincentii* and *P. gingivalis* produce collagenolytic proteases referred to as trypsin-like enzyme (Laughon *et al*., 1982; Yoshimura *et al*., 1984). This enzyme is able to break down intrinsic protease inhibitors such as α-antitrypsin and could therefore interfere with the control of normal proteolytic processes on human mucosal surfaces (Travis *et al*., 1997). Trypsin-like enzymes also activate latent tissue collagenase (Uitto *et al*., 1986). The *P. gingivalis* trypsin-like enzyme differs from the *T. denticola* enzyme (Yoshimura *et al*., 1984) in that it is a true protease capable of degrading albumin, azocoll and gelatin and is stimulated by reducing agents such as dithiothreitol. Both enzymes are cell-bound and released by cell lysis (Loesche *et al*., 1987).

Mucopolysaccharidases (e.g. hyaluronidase and chondroitin sulphatase) are able to exert their effects by diffusing into the tissues and breaking down the intercellular acidic mucopolysaccharides of the epithelium without there being any direct bacterial penetration of the host tissues (Fiehn 1986b, Reijntjens *et al.,* 1986). Hyaluronidases are produced by the gingival tissues as well as by oral spirochaetes and *P. gingivalis* and are present in most salivas but increased in subjects with poor oral hygiene and periodontal disease (Holt & Bramanti, 1991). Both *P. gingivalis* and *T. denticola* demonstrate chondroitin sulphatase activity (Fiehn, 1986b; Holt & Bramanti, 1991).

Collagenolytic activity also requires gelatinase and other proteases (Uitto, 1987). Gelatinase may originate from both the plaque bacteria and human leucocytes and is potent in degrading basement membrane collagen (Uitto, 1987). Elastase participates in collagen degradation by solubilising the polymeric collagen fibres into individual tropocollagen molecules. Spirochaetes are known gelatinase and elastase producers (Uitto *et al.,* 1986). The ability of spirochaetes to degrade basement membrane collagen could well be related to their ability to penetrate host tissues (Ellen & Galimanas, 2005; Kigure *et al*., 1995). Dentilisin is a protease located on the surface of the cell which contributes to disease by disrupting intercellular adhesion proteins (Choi *et al*., 2003) allowing for *T. denticola* to penetrate epithelial cell layers.

The *T. forsythia* genome encodes several glycosidases which can hydrolyse terminal glycosidic linkages in oligosaccharides and proteoglycans from saliva, gingival crevicular fluid and periodontal tissue, thus promoting disease progression. They can also be involved in adherence, colonisation and cross-feeding of community bacteria (Sharma, 2010). Bacterial glycosidases may expose host cell-surface sugars which bind to haemagglutinins identified in *T. forsythia* (Murakami *et al*., 2002). Glycosidase activity was sometimes observed with *T. denticola* (Mikx, 1991) but not with *T. vincentii* nor *T. pectinovorum* (Fiehn, 1986b; Mikx, 1991).

P. gingivalis and oral spirochaetes show esterase activity (Lamont & Jenkinson, 1998; Mikx, 1991). In conjunction with phospholipase, esterases may play a role in tissue destruction. Phospholipase may provide prostaglandin precursors and help initiate prostaglandin-mediated bone resorption (Bulkacz *et al*., 1981).

A neutral phosphatase gene has been cloned and expressed from *T. denticola* (Ishihara & Kuramitsu, 1995). Bacterial acid and alkaline phosphatases cause alveolar bone breakdown, and have been demonstrated in small spirochaetes (Fiehn, 1986) and *P. gingivalis* (Frank & Voegal 1978, Slots, 1991), while peptidases contribute to the pathogenesis of periodontal disease by directly penetrating and degrading basement membrane collagen (Fiehn 1986b, Grenier *et al*., 1990).

The outer envelope of Gram-negative bacteria consists of 2 layers, namely, the outer membrane and the peptidoglycan layer. The purpose of the peptidoglycan layer is to maintain cell shape. Cell lysis will therefore not only yield membrane fragments but fragments of peptidoglycan as well which interact with host tissue, resulting in a range of biological activities, including activation of complement and immunosuppression. Peptidoglycan is also considered to be involved in stimulating bone resorption (Nissengard *et al*., 1988) and may therefore constitute an important virulence factor in periodontal disease.

6.4.4 Metabolic end-products

A variety of potentially cytotoxic metabolites are synthesised by oral bacteria including hydrogen sulphide, low molecular weight organic acids and ammonia. Hydrogen sulphide is a metabolic end product of cysteine fermentation and is cytotoxic for epithelial cells and gingival fibroblasts (Beauchamp *et al*., 1984), exerting both pro-and anti-inflammatory mediators which may disturb host defences (Chen *et al*., 2010). Both T. *denticola* and *P. gingivalis* produce hydrogen sulphide. *T. denticola* produces hydrogen sulphide from glutathione and thus glutathione metabolism plays an important role in pathogenicity mediated by *T. denticola* (Chu *et al*., 2002).

Volatile sulphur compounds may increase the permeability of the oral mucosa and reduce collagen and non-collagenous protein synthesiss. Methyl mercaptan, a volatile sulphur compound produced by *T. denticola* and *P. gingivalis* and derived from methionine, is known to reduce protein synthesis by human gingival fibroblasts, as well as inhibit cell migration in periodontal ligament cells (Johnson *et al*., 1992; Lancero *et al*., 1996).

T. forsythia releases metabolites which favour the growth of *P. gingivalis* which in turn, degrades host proteins releasing nutrients such as peptides and amino acids for *T. forsythia*. The synergy between these two species and with *T. denticola*, provide evidence for their combined virulence expression in periodontal disease.

Virulence is multifactorial, being influenced by microbial interactions (which often differ *in vivo* and *in vitro*) as well as host susceptibility. Molecular biology has contributed greatly to our understanding of virulence and disease progression but many questions still remain unanswered.

7. Conclusion

Certain subgingival plaque morphtypes predominate in different forms of periodontal disease and shifts in microbial proportions probably relate to health and disease. There is no proof of a causal relationship between the organisms described above and periodontal disease. One can only suggest an association. Because the oral microbiota contains around 700 species of microrganisms, it has been accepted that periodontal disease is a polymicrobial infection, with shifts in the proportions of some species relating to different forms of periodontal disease.

Identification and monitoring of specific bacteria could aid in management and treatment by determining the causative species, monitoring of treatment and deciding on recall intervals. Most methods currently employed in microbiological assessment have major shortcomings. Inconsistencies between cultural microbiological data from cases with similar clinical features are often encountered. These inconsistencies may be attributed to differences in detection methods as well as to different stages of the disease process. Differences in data from different research centres could indicate not only technical problems, but also problems related to the classification of a given site as active or inactive. However, major advances have occurred during the past decade and continued efforts are being made to facilitate and standardise the microbiological diagnosis of periodontal diseases. Although this chapter describes a role for many species with different forms of periodontal disease, the interaction and role of bacterial products is vast and complex. Therefore the association of a given organism with disease (even though it may be constantly present) could be considered as being the result rather than the cause of disease. However, in examining association studies, spirochaetes cannot be ignored since they have been considered amongst the most highly suspect of the plaque microbiota, being consistently observed in different forms of periodontal disease and demonstrating significant pathogenic potential.

The increased prevalence of Aa, *T. denticola, P. gingivalis* and *T. forsythia* in different forms of periodontal disease has earned them the recognition as diagnostic markers in the disease process. However, they should not be considered with the exclusion of other important contributers such as *F. nucleatum*. New and unusual species are emerging which may, in time, prove to be the real initiators of the disease process with the above species having to relinquish their position at the top of the list of suspected periodontopathogens. Many contradictions occur and while some advocate the use of microbial biomarkers, others find them misleading and suggest that microbiota should be examined for both pathogenic and protective flora and results interpreted as they pertain to the susceptibility of the host (Quirynen *et al*., 2001; Riep, 2007).

Treatment must be effected with the bacterial communities of the biofilm in mind and should concentrate on preventing biofilm formation, interfering with the process of bacterial succession and elimination of specific organisms in the biofilm. The recent isolation of an Aa serotype b bacteriophage, which is able to lyse bacteria within a biofilm, holds some promise in this area (Castillo-Ruiz *et al*., 2011). Until this can be put to practice, professional plaque control coupled with individual oral hygiene practices will continue to serve in maintaining a healthy oral ecosystem.

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9. Disclaimer

Any opinion, findings and conclusions or recommendations expressed in this material are those of the author and therefore the NRF does not accept any liability in regard thereto.

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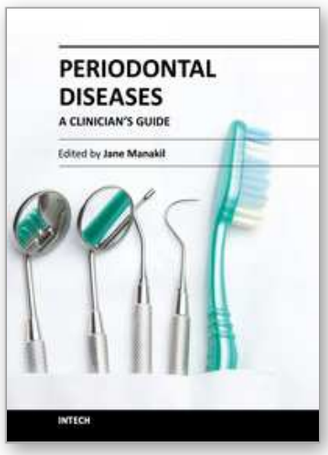

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"Periodontal diseases" is a web-based resource intended to reach the contemporary practitioners as well as educators and students in the field of periodontology. It is fully searchable and designed to enhance the learning experience. Within the book a description is presented of the current concepts presenting the complex interactions of microbial fingerprint, multiple genotypes, and host modulations. In addition, an overview is given of the clinical outcome of the disease's progression, as influenced by the epigenetic factors. Emerging concepts on periodontitis as a risk factor for various systemic diseases and as a bilateral modulating factor have been elucidated in detail as well.

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