

Periimplantitis: Checkerboard DNA-DNA Hybridization for Bacterial Pathogen Identification

Periimplantitis: Checkerboard DNA-DNA Hybridization Método para la Identificación de Bacterias Patógenas

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ABSTRACT: A recent innovation in medical field is the use of DNA probes in microbiological diagnosis of the oral cavity. Thus, this study has the objective to present the mainly characteristics of Checkerboard DNA-DNA Hybridization method for bacterial pathogens identification related to periimplantitis, commonly disease found in the oral cavity, as wells as, to show the uses and applications of this technique.

KEY WORDS: checkerboard DNA-DNA hybridization, periimplantitis, DNA probe, bacterial pathogen.

INTRODUCTION

Dental plaque is involved in the etiology of the most common diseases of the oral cavity, i.e., dental caries and periodontal disease. A less common oral pathology, periimplantitis, is also caused by the presence of dental plaque. As its etiology and clinical behavior resemble periodontitis in many ways, it cannot be neglected in the present context. Analyzing the mechanisms underlying dental plaque formation and development can help to understand better the emergence and progression of these pathologies, as well as to define the most effective treatments. Dental plaque is a microbial biofilm formed by organisms tightly bound one to the other and to the solid substratum by means of an exopolymer matrix into which they are embedded. Such a state brings about profound changes in the behavior of bacteria, their relation to the host and their response to environmental conditions. Health and disease, therefore, depend on both host factors and various phenomena caused by growth of the biofilm: shifts in the composition of the predominant species, induced by mechanisms of synergy/antagonism among the microorganisms as well as by nutrient and atmospheric gradients, alter the balance

with the host and may lead to the onset of disease (Newman, 1998; 1998; Socransky & Haffajee, 1992; Socransky *et al.*, 1998). The issues involved have been under study for many years, and new technologies employing molecular diagnosis such as checkerboard DNA-DNA hybridization and DNA probes have lead to great improvements in the identification of strict anaerobes. Nevertheless, due to the complexity of the factors involved, determining the biological events leading to pathology and the best way to prevent and treat it is still difficult. The aim of the present review is to present the infectious risks and to present the use of the DNA Checkerboard method for bacterial pathogens identification in oral diseases.

Part I: Biofilm and development of dental plaque.

Healthy gingival have been associated with a very simple supragingival plaque composition: few layers of predominantly Gram positive cocci (*Streptococcus* spp: *S. mutans*, *S. mitis*, *S. sanguis*, *S. oralis*; *Rothia dentocariosa*; *Staphylococcus epidermidis*), followed by some Gram positive rods and filaments (*Actinomyces* spp: *A. viscosus*, *A. israelis*, *A. gerencseriae*;

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Corynebacterium spp.) and very few Gram negative cocci (*Veillonella parvula*; *Neisseria* spp.). These latter are aerobic or facultative aerobic bacteria, able to adhere to the non-exfoliating hard surfaces; initial adhesion is promoted by surface free energy, roughness and hydrophilia, and is mediated by long- and short-range forces (Dzink *et al.*, 1987; Listgarten, 1976; Olsson *et al.*, 1992; Quirynen & Bollen, 1995; Socransky, 1977; Slots, 1977). The early colonizers are organisms able to withstand the high oxygen concentrations and to resist the various removal mechanisms of the oral cavity such as swallowing, chewing, nose blowing and salivary, nasal and crevicular fluid outflow (Quirynen *et al.*, 2001). Their replication enables the subsequent adhesion of other bacterial species, which though unable to stick to tooth hard surfaces, are quite capable of attaching themselves to already present microorganisms. This is so-called "secondary colonization." As the number of plaque layers increases, nutritional and atmospheric gradients are created, the oxygen level decreases and the anaerobes can survive (Bradshaw *et al.*, 1998; Cook *et al.*, 1998; Lamont & Jenkinson, 1998). Clinical gingivitis is associated with the development of a more organized dental plaque. Such biofilms are characterized by several cell layers (100–300), with bacteria stratification arranged by metabolism and aero tolerance; besides the Gram positive cocci, rods and filaments associated with healthy gingival, the number of Gram negative cocci, rods and filaments increases and anaerobic bacteria appear (*F. nucleatum*, *C. gracilis*, *B. forsythus*, *Capnocytophaga* spp.) [Listgarten, 1976; Moore *et al.*, 1982; Mouton *et al.*, 1980; Slots *et al.*, 1978; Tanner *et al.*, 1998]. The species involved vary depending on local environmental characteristics, but the colonization pattern is always the same (Marsh, 1999).

The shift from gingivitis to periodontitis or periimplantitis does not come about automatically, either in every patient or every site, but depends on three main factors: host susceptibility, pathogenic bacteria and "protective bacteria" (Quirynen *et al.*, 2001). Pathogenic bacteria possess virulence features that decrease the effectiveness of the host response by causing tissue breakdown and hindering tissue healing. Pili, fimbriae and blebs allow adhesion and colonization, and host defenses are impaired through a number of mechanisms: proteases that inhibit polymorphonuclear leukocyte (PMN) chemotaxis; capsules that mask LPS or increase resistance to phagocytosis; inhibition of PMN superoxide production, proteases against IgA1 and IgG immunoglobulins; and the production of volatile

acid fats, which inhibit B and T lymphocyte proliferation. *A. actinomycetemcomitans* (anaerobic gram negative), in addition, produces a leucotoxin that alters the cell membranes of PMNs and monocytes. Moreover, tissue destruction and impaired healing are caused by epitheliotoxins, exotoxins against macrophages, acid and alkaline phosphatase, collagenase and fibroblast inhibiting factors, osseo-destructive toxins, mitogenic factors, the induction of monocyte cytokine production, phospholipase A, volatile sulphurs, ammonia and butyric acid, which inhibit gingival fibroblast proliferation (Bartold *et al.*, 1991; Bartold *et al.*, 1998; Garrison & Nichols, 1989; Grenier *et al.*, 1989; Kurita-Ochiai *et al.*, 1995; Sbordone *et al.*, 1990; Sbordone *et al.*, 2000; Slots & Genco, 1984; Zambon, 1983). The biofilm associated to periodontitis or periimplantitis is complex and formed by many cell layers. The composition of the bacterial population in the active, destructive phase differs slightly from that during the remission period, adding support to the theory of the high specificity of pathogenic plaque; a preponderance of *B. forsythus*, *P. gingivalis*, *T. denticola*, *C. rectus*, *P. intermedia* is associated with increasing probing depth and bleeding on probing (Dzink *et al.*, 1987; Grossi *et al.*, 1994; Moore *et al.*, 1991; Socransky *et al.*, 1988; Socransky *et al.*, 1998; Slots & Genco, 1984; Tanner *et al.*, 1987; Tanner *et al.*, 1998; Wennstrom *et al.*, 1987).

Communication among the different species within biofilms appears to be the key to understanding how plaque can act as a single unit, and how specific bacteria emerge and impair the balance with the host. Physical (co-aggregation and co-adhesion), metabolic and physiological (gene expression and cell-cell signaling) interactions yield a positive cooperation among different species within the biofilm: the metabolic products of some organisms may promote the further growth of other bacteria or prevent the survival of others (Kolenbrander, 2000; Quirynen & Bollen, 1995; Quirynen *et al.*, 2001). A key role in the cooperative processes is played by *F. nucleatum*, able to form the needed "bridge" between early, i.e., *Streptococci* spp., and late colonizers, especially obligate anaerobes. In the absence of *F. nucleatum*, *P. gingivalis* cannot aggregate with the microbiota already present such as the facultative aerobes *A. naeslundii*, *N. subflava*, *S. mutans*, *S. oralis* and *S. sanguis*. The presence of *F. nucleatum*, on the other hand, enables anaerobes to grow, even in the aerated environment of the oral cavity (Bradshaw *et al.*, 1998; Pratten *et al.*, 1998). Other microorganisms are also able to link otherwise non-communicating bacteria (i.e., *S. sanguis* forms a complex together with *B. matruchotii* and *F. nucleatum*),

and this may represent the basic event leading to biofilm initiation and development (Bowden, 1999; Palmer *et al.*, 2001; Socransky *et al.*, 1998; Wilson, 1999). The pattern of colonization and coaggregation is often unidirectional, proof that some bacteria need to have the environment prepared by other microbiota in order to colonize. *P. gingivalis* can adhere to oral *Streptococcus* spp. and *A. naeslundii*, forming small co-aggregates resistant to removal, if that is, the substratum has been previously exposed to *S. gordonii*. Lacking *S. gordonii*, only few *P. gingivalis* cells manage to attach and are moreover easily removed (Cook *et al.*, 1998; Lamont & Jenkinson, 1998; Quirynen & Bollen, 1995; Rosan & Lamont, 2000).

Part II: Periodontitis and periimplantitis.

Periodontitis. Periodontal disease affects the great majority of the adult population and can thus be rightly considered a public health problem. Teeth are the ideal substrate for plaque formation because of their non-exfoliating surfaces. They also link the infected "open space" of the mouth to the deep periodontal space, offering microorganisms an easy route of entry: dentinal tubules, enamel fissures or irregularities are easily colonized by microbes, but difficult to reach for the host defense mechanisms. Plaque accumulation leads to gingivitis, but the shift to periodontitis depends on both host factors and the selection of virulent bacteria. Periodontitis is not a single disease, but rather a collection of pathologies with similar patterns and symptoms (Socransky *et al.*, 1982). Though many classifications have been proposed, during the 1999 International Workshop for Classification of Periodontal Diseases and Conditions, the previously accepted terms "Early-Onset Periodontitis" and "Adult Periodontitis" were replaced by "Aggressive Periodontitis" and "Chronic Periodontitis". Thus, age and microbiological features no longer represent the primary classification criteria, but rather, clinical behavior and laboratory findings are used to distinguish the two forms (Armitage, 1999). Chronic Periodontitis is defined as an infectious pathology leading to slow or moderately slow, progressive loss of attachment and bone. It occurs mainly in adult patients in either a localized or generalized form, but can nonetheless affect children and adolescents. Predisposing factors such as cigarette smoke, stress and local (e.g., tooth-related or iatrogenic) or systemic (e.g., diabetes mellitus or HIV infection) conditions can enhance the destructive effects of the microbiota (Lindhe *et al.*, 1999). Aggressive Periodontitis, on the other hand, is characterized by rapid loss of attachment and bone

destruction in otherwise clinically healthy patients and presents familial aggregation.

Periimplantitis. Periimplantitis is very similar to the periodontitis, even though some important differences between natural teeth and dental implants must clearly be borne in mind, the most important being that implants are not surrounded by a periodontal ligament and therefore present different biomechanics and defensive cell recruitment (Fig. 1). Rosenberg *et al.* (1991) once categorized dental implant failure as either traumatic or infectious, depending on whether or not pathogenic bacteria could be detected around the failed implant, a distinction no longer accepted. The term periimplantitis is now used only to refer to the destruction of supporting periimplant tissue due to microbial infection. It is commonly believed that early failures are associated with surgical errors, while late failures are more often linked to the presence of plaque or improper prosthetic design or maintenance protocol (Piattelli *et al.*, 1998). Lee *et al.* (1999) used the cluster method developed by Socransky *et al.* (1998) to analyze the microbiota associated with healthy implants and study the implant and host-related factors able to influence the microbial biofilm. Prosthesis characteristics, surgical technique or age of the fixture revealed to have little impact on bacterial composition, while years of function and a history of periodontal or peri-implant infections were the most relevant parameters, associated with an increased number of orange and red cluster microorganisms, though still compatible with functioning fixtures (Lee *et al.*, 1999; Ong *et al.*, 1992). The periimplant microbial population is influenced by the surrounding environment, showing differing characteristics in totally or partially edentulous patient and in those with or without a history of periodontal disease or implant loss. Residual teeth or failing implants may act as bacterial "reservoirs," leading to a colonization of the periimplant sites (Fig. 2). Many researchers have underscored the similarity of the microorganisms found around teeth affected by periodontal disease or failing implants and fixtures placed in the same mouth: the healthy implants are colonized by the same pathogens (*P. gingivalis*, *P. intermedia*, *B. forsythus*, *E. corrodens*, *F. nucleatum*, *C. rectus*, *P. micros*, *Spirochetes* spp.), causing attachment and bone loss in the involved sites, though the bacterial count is definitely low (Bauman *et al.*, 1992; Becker *et al.*, 1990; George *et al.*, 1994; Listgarten & Lai, 1999; Rosenquist & Grenthe, 1996; Salcetti *et al.*, 1997; Sanz *et al.*, 1990; Sbordone *et al.*, 1995). Such findings have relevance for the planning of immediate post-extraction implants, especially if tooth loss is



Fig. 1. Clinical photograph illustrating periimplantitis lesions at implants after loading.



Fig. 2. Residual teeth may act as bacterial reservoirs, leading to a colonization of the periimplant sites.

determined by periodontal disease. Danser *et al.* (1997) suggest waiting at least 1 month after extraction to allow for elimination of *A. actinomycetemcomitans* and *P. gingivalis* from the extraction socket. The same rules apply when Guided Tissue Regeneration (GTR) and Guided Bone Regeneration (GBR) procedures are performed: membrane exposure and bacterial colonization impair the outcome in terms of tissue regeneration. Exposure is more likely in patients presenting periodontitis, periimplantitis or residual deep pockets: the smallest degree of attachment and bone gain occur when *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *Capnocytophaga* spp. are detected on the infected barriers (Haas *et al.*, 2000; Leghissa & Botticelli, 1996; Nowzari & Slots, 1995; Nowzari & Smith, 1996). It can be concluded that implant and GTR/GBR procedures achieve the best results in those subjects that comply with domestic plaque control routines and maintenance protocol schedules.

Part III: Dental implants and oral microbiota.

Longitudinal studies have reported high survival and success rates for osseointegrated titanium implants (Esposito *et al.* 1998). In connection with successful treatment, low amounts of and low levels of marginal inflammation have been identified at the implants (Adell *et al.*, 1981; Lekholm *et al.*, 1986; Leonhardt *et al.*, 1992). The microbiota at well-maintained fixtures resembles the microbiota associated with healthy dental conditions (Mombelli *et al.*, 1987; Apse *et al.*, 1989). After the first year of function, only minor bone loss has been observed (Adell *et al.*, 1990; van Steenberghe *et al.*, 1999) and, as a result, it has been possible to maintain peri-implant health over long-term follow-up periods. However, both early and late failures occur, and it is assumed that multiple factors may contribute (Esposito *et al.*, 1998). Several studies have noted the detrimental effect of anaerobic plaque bacteria on periimplant health. Distinct qualitative and quantitative differences in the microbiota, associated with successful and failing implants, have also been shown (Leonhardt *et al.*, 1992). In order to understand the role of plaque in maintaining periodontal health or initiating disease, it is necessary to determine plaque composition in different clinical states.

Part IV: DNA-DNA hybridization focusing oral microbiota of dental implants.

The early studies on biofilm composition employed light microscopy, but this method was incapable of distinguishing the wide array of resident species and has been discarded. Culture techniques are very time consuming, especially when all species have to be identified, and sometimes inadequate to recover some difficult to culture species (Sakamoto *et al.*, 2005). The DNA-Checkerboard technique overcomes many of the limitations of culture, is faster and more cost-effective (Socransky *et al.*, 1994).

DNA Checkerboard is a recently established technique that gives a simultaneous and quantitative analysis of up to 28 plaque samples against 40 microbial species (Socransky *et al.*, 1994). It was developed initially to study the predominantly Gram-negative subgingival microorganisms involved in periodontitis (Socransky *et al.*, 1998). DNA Checkerboard method offers the ability to include more potential periodontal pathogens in large-scale studies with a single analysis than is usually practicable with cultural analysis. These new probe-target format permits enumerate a large numbers of species in very large numbers of samples. This will enable a more detailed evaluation of the clinical and epidemiological relationships of the complex plaque microbiota with

respect to the development of oral diseases. For this analysis, 28 dental sample plaques, and DNA standards representing 10^5 and 10^6 cells for target species are fixed on a membrane in thin lanes, then simultaneously cross-hybridized with 40 labeled, whole genome probes (Fig. 3). Using this technology, Socransky *et al.*, 1998, 2002, 2004, showed that a set of clusters of species related to the development of periodontitis existed in dental plaque. Nascimento *et al.* (2007) presented an alternative protocol for labeling and detecting whole

genomic DNA probes in the Checkerboard DNA-DNA hybridization method. Whole genomic DNA was labeled with fluorescein. The results reveal that the sensitivity of fluorescein is comparable to digoxigenin and that it constitutes a an adequate labeling reagent to be employed in the Checkerboard DNA-DNA hybridization method (Fig. 4). Some of these clusters enabled the establishment of a pathogen complex, which in turn allowed the establishment of the major pathogens causing periodontitis (Table I).

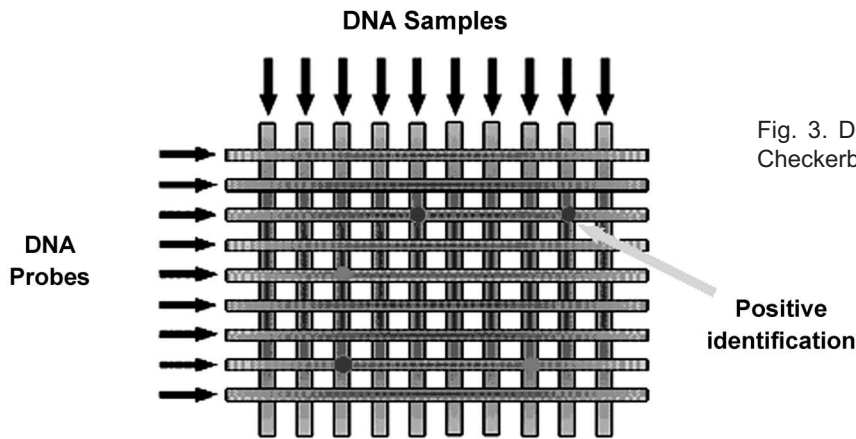


Fig. 3. Diagrammatic representation of DNA Checkerboard hybridization format.

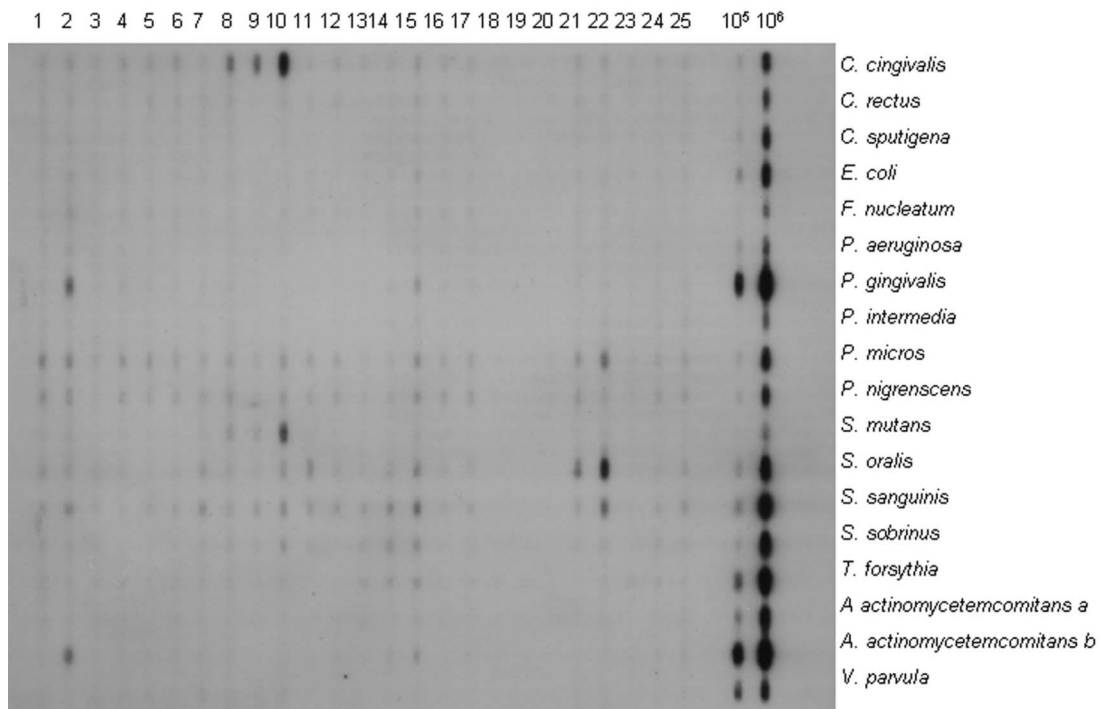


Figure 4. Example of DNA Checkerboard hybridization to detect bacterial species tested against dental plaque samples. The horizontal lanes numbered 1 to 28 are the plaque samples, and the two vertical lanes on the right are standards containing either 10^5 or 10^6 cells in each tested species. The vertical lanes contain the indicated DNA probes in hybridization buffer.

Table I. Microbial complexes in subgingival plaque according to Socransky *et al.* 1998.

Complexes	Bacterial species
Purple	<i>V.parvula, A.odontolyticus</i>
Yellow	<i>Streptococci spp.: S.mitis, S.oralis, S.sanguis, S.gordonii, S.intermedius</i>
Green	<i>E.corrodens, C.gingivalis, C.sputigena, C.ochracea, C.conciscus, A.actinomycescomitans serotype a</i>
Orange	<i>P.intermedia, P.nigrescens, P.micros, C.gragilis, C.rectus, F.periodonticum, F.nuc.nucleatum, F.nuc.vincentii, S.constellatus, E.nodatum, C.showae, F.nuc.polymorphum</i>
Red	<i>P.gingivalis, B.forsythus, T.denticola, A. viscosus, Selenomonas noxia, A.actinomycescomitans serotype b</i>

The DNA Checkerboard hybridization technique has been used to comprehensively examine the microbial composition of supra and subgingival plaque in subjects in health and periodontitis, the salivary microbiota levels in relation to periodontal status, the relationship of cigarette smoking to the composition of the subgingival microbiota (Ximenez-Fyvie *et al.*, 2000; Sakamoto *et al.*, 2005), the differences between the subgingival microbiota in subjects from different geographic locations, the relationship of ethnic/racial group, occupational and periodontal disease status, and effects of different periodontal therapies (Colombo *et al.*, 1998; Ximenez-Fyvie *et al.*, 2000). Recently, it was reported that this hybridization technique is useful for the enumeration of bacterial species in microbiologically complex systems. This technique is rapid, sensitive, and relatively inexpensive (Socransky *et al.*, 1994).

Whole genomic DNA probes have been used extensively in studies evaluating the composition of subgingival plaque and the microbiota associated with endodontic lesions (Haffajee *et al.*, 1998; Ximenez-Fyvie *et al.*, 2000; Siqueira *et al.*, 2002). Whole genomic probes are constructed using the entire genome of a bacterial species as the target. One of the criticisms of these probes is that the use of the entire genome may increase the probability of cross-reactions between species because of common regions of DNA among closely related species. Other concerns have been that the whole genomic DNA probes might not detect all strains of a given species and that the probes would

have a low sensitivity in terms of the numbers of cells that they detect. Investigations at the Forsyth Institute, however, using whole genomic DNA probes have indicated that many of the concerns regarding their use are unjustified or can be overcome. DNA probes can be very effective for the detection of bacterial species, but when employed in the typical format, only limited numbers of probes can be employed to enumerate relatively large numbers of samples. Checkerboard format procedures, whether employing direct or reverse hybridization procedures, can extend markedly the number of samples evaluated for a wide range of bacterial species.

The DNA Checkerboard hybridization technique outlined in this manuscript offers a number of advantages for the study of multiple species of bacteria in large numbers of samples containing complex mixtures of microorganisms. The technique is rapid, sensitive, and relatively inexpensive. It overcomes many of the limitations of cultural microbiology including loss of viability of organisms during transport, the problem of enumerating difficult to cultivate species, and the difficulty encountered in speciating certain taxa that are difficult to grow or which exhibit few positive phenotypic traits. Another advantage is that the entire sample may be employed without dilution or amplification, overcoming problems in quantification imposed by either serial dilution or PCR amplification procedures. Finally, the technique provides quantitative data which may be important in treatment studies of biofilm infections where species levels and proportions

may be markedly decreased but the species not eliminated. Another advantage of the technique is that membranes may be stripped and re-probed with a new set of 40 different DNA probes. The DNA checkerboard technique does have limitations. The technique can detect only species for which DNA probes have been prepared. Thus, novel pathogens or environmentally important species which might be detected in culture or by other molecular techniques would not be detected by this method. The technique must be optimized for a given biological or environmental site. The use of probes developed for subgingival plaque samples is unlikely to be optimum for samples for other body sites or other sites in nature. The probes must be used to detect organisms in samples of the appropriate size. Probes optimized to detect species in the 10^4 and 10^7 range often will provide cross-reactions if much larger samples are employed. When properly employed, DNA Checkerboard hybridization and other rapid microbiological techniques permit investigation of etiologic, therapeutic, and environmental problems which could not be approached by other means.

CONCLUSIONS

Plaque-related diseases are amongst the most common ailments of the oral cavity. Clinicians should therefore know and understand the complex mechanisms involved in the shift from oral health to pathology. Microorganisms in a biofilm exhibit different properties from when they are in isolation, a fact that can explain why coping with a community-related

disease differs so radically from treating a single-species infection. Antagonism, synergy and commensalisms among the species select the population, and knowledge of these relations should guide therapeutic choices.

Basic research and long-term clinical trials are needed to obtain a better differential diagnosis of the cause of marginal bone loss. Implants in partially edentulous patients, in contrast to fully edentulous subjects, will easily be colonized by putative periodontal pathogens.

With the advancement of molecular biology in recent years, the initiation and progression mechanisms of periodontitis are becoming clearer gradually. As the culture-independent approaches have revealed the diversity of human oral microbiota and the existence of a large number of as-yet-to-be-cultured organisms which are presumed as periodontal pathogens, the researches on periodontal disease and human oral microbiota are coming to a new turn.

DNA Checkerboard hybridization is a powerful and fast identification method in the clinical laboratory. This technique is applicable for routine identification of several groups of bacteria as well as for identification of novel isolates. As the technical resources for bacterial identification become more abundant and less expensive, more microbiologists will consider using this method in their studies.

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RESUMEN: Una innovación reciente en medicina es la utilización de sondas de DNA para diagnóstico microbiológico. Este trabajo tiene como objetivo, presentar las principales características del método Checkerboard DNA-DNA Hybridization para la identificación de bacterias patógenas asociadas a periimplantite en la cavidad oral, mostrando las diferentes utilizaciones y aplicaciones de esta técnica.

PALABRAS CLAVE: checkerboard DNA-DNA hybridization, periimplantitis, sonda de DNA, bacteria patógena.

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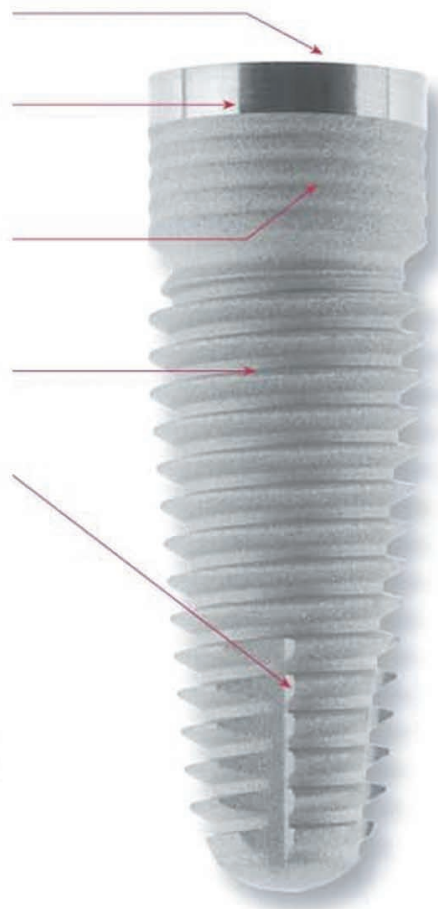
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