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Methods of detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review

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Abstract

Background: Certain specific bacterial species from the subgingival biofilm have demonstrated aetiological relevance in the initiation and progression of periodontitis. Among all the bacteria studied, three have shown the highest association with destructive periodontal diseases: *Actinobacillus actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*) and *Tannerella forsythensis* (*Tf*). Therefore, the relevance of having accurate microbiological diagnostic techniques for their identification and quantification is clearly justified.

Aim: To evaluate critically all scientific information on the currently available microbial diagnostic techniques aimed for the identification and quantification of *Aa*, *Pg* and *Tf*.

Summary: Bacterial culturing has been the reference diagnostic technique for many years and, in fact, most of our current knowledge on periodontal microbiology derives from cultural data. However, the advent of new microbial diagnostics, mostly based on immune and molecular technologies, has not only highlighted some of the shortcomings of cultural techniques but has also allowed their introduction as easy and available adjunct diagnostic tools to be used in clinical research and practice. These technologies, mostly polymerase chain reaction (PCR), represent a field of continuous development; however, we still lack the ideal diagnostic to study the subgingival microflora. Qualitative PCR is still hampered by the limited information provided. Quantitative PCR is still in development; however, the promising early results

reported are still hampered by the high cost and the equipment necessary for the processing.

Conclusion: Quantitative PCR technology may have a major role in the near future as an adjunctive diagnostic tool in both epidemiological and clinical studies in periodontology. However, culture techniques still hold some inherent capabilities, which makes this diagnostic tool the current reference standard in periodontal microbiology.

Key words: *Actinobacillus actinomycetemcomitans*; culture; diagnostic methods; microbiology; *Porphyromonas gingivalis*; polymerase chain reaction; periodontitis; *Tannerella forsythensis*

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Destructive periodontal diseases are chronic inflammatory conditions characterised by connective tissue and alveolar bone destruction, eventually leading to tooth loss. A substantial number of publications have reported that certain microorganisms from the subgingival microbiota, particularly Gram-negative anaerobes, are the major aetiological factors of chronic and aggressive periodontitis (Dzink et al. 1985, Slots 1986, Newman 1990). Although the subgingival microenvironment in the periodontal pocket is characterised by a wide diversity, with over 300 species having been isolated from different individuals and as many as 40 from a single site, only a few species have been associated with disease (Moore & Moore 1994). Despite the difficulty in identifying all the members of the oral microbiota and understanding how they interact with each other and with the host, a limited number have demonstrated a clear aetiological role and these have been identified as periodontal pathogens (Genco 1996). Evidence for aetiology is based on the fulfilment of several criteria described by Socransky (1970). Using these criteria, strong evidence has been demonstrated for *Actinobacillus actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*) and *Tannerella forsythensis* (*Tf*) (formerly *Bacteroides forsythus*), as concluded at the World Workshop in 1996 (Genco 1996).

Aa is a member from the genus *Actinobacillus*, belonging to the family of *Pasterurellaceae*. It is a Gram-negative, small rod (1–1.5–0.5 mm), capnophilic facultative anaerobe. In culture, colonies grow in small flat, circular colonies that have slightly blurred borders and a translucent outlook in solid media, sometimes with a typical inner star-like morphology (Zambon 1985, Alsina et al. 2001) (Fig. 1). Several lines of evidence support its aetiological role as a true periodontal pathogen, mostly in relation to aggressive periodontitis (Fives-Taylor et al. 1999, Slots & Ting 1999, Socransky et al. 1999). This bacterial species has a wide intra-specific diversity defined by its six serotypes (Henderson et al. 2002). In the oral cavity, serotypes a and b are the most frequent in Caucasians, serotype b being most frequently associated with localised aggressive periodontitis and therefore, the most virulent and pathogenic (Zambon 1985, Zambon et al. 1988, Albandar et al. 1991, Preus et al. 1994, Haraszthy et al. 2000a, b, Henderson et al. 2002).

Pg belongs to the genera *Porphyromonas* from the family *Bacteroidaceae*. These bacteria are Gram-negative strict anaerobic coco-bacilli. They grow in culture media forming convex, smooth glossy colonies of 1–2-mm diameter, which demonstrate a progressive darkening in the centre, because of the production of protoheme, the substance responsible for the typical colour of these colonies (Fig. 2) (White & Mayrand 1981, Sha & Collins 1988, Ohta et al. 1991, Haraszthy et al. 2000b). Several lines of evidence support its aetiological role as a true periodontal pathogen, more likely associated with chronic periodontitis (Holt et al. 1999, Slots & Ting 1999, Socransky et al. 1999). Its importance as a periodontal pathogen is also highlighted by the research efforts aimed at developing a vaccine aimed at immunisation against this bacterial species and thus prevent chronic periodontitis (Gibson & Genco 2001, Nakagawa et al. 2001, Rajapakse et al. 2002, Yang et al. 2002).

Tf is a non-pigmenting saccharolytic anaerobic Gram-negative rod. For a long time, it was denominated *Bacteroides fusiformes*. It was then renamed *Bacteroides forsythus* by Tanner et al. (1986), and very recently a new name has been proposed and accepted, *Tf* (Sakamoto et al. 2002). Its habitat is the gingival sulcus, and it is usually isolated in periodontal pockets, although its presence has also been detected in the tonsils, dorsum of the tongue and saliva. This bacterium was not considered a true periodontal pathogen until recently, mostly because of its fastidious growth in culture media. These difficulties in culturing are related to the lack of capacity of *Tf* to synthesise *N*-acetylmuramic acid (NAM), an essential component of cell wall peptidoglycan. Therefore, it grows poorly in pure culture, unless the medium is supplemented with NAM, or by growing together with other microorganisms (Fig. 3).

Moderately strong evidence has been demonstrated for other bacteria isolated from the subgingival microbiota such as *Campylobacter rectus* (*Cr*), *Eubacterium nodatum*, *Fusobacterium nucleatum* (*Fn*), *Peptostreptococcus micros* (*Pm*), *Prevotella intermedia* (*Pi*) and *P. nigrescens* (*Pn*), *Streptococcus intermedius* and various spirochetes, such as *Treponema denticola* (*Td*) (Genco 1996). Most of these pathogens are

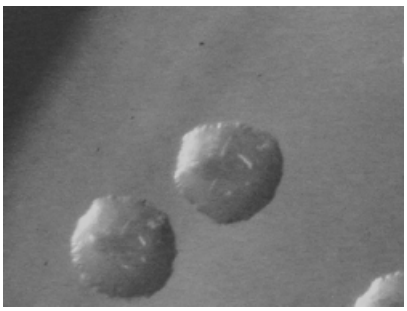

Fig. 1. Colonies of *Actinobacillus actinomycetemcomitans* growing on Dentaid-1 medium.

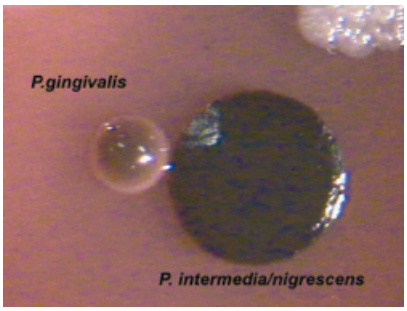

Fig. 2. Colony of *Porphyromonas gingivalis*, close to another colony of *Prevotella intermedia/nigrescens*, growing on blood agar medium, supplemented with haemin and menadion.

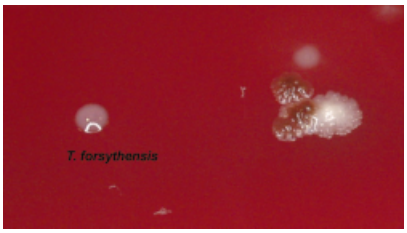

Fig. 3. Colony of *Tannerella forstythesis*, growing on blood agar medium.

members of the resident oral microbiota and are usually present in healthy or gingivitis subjects. Therefore, their aetiologic role is less evident. Preliminary evidence of periodontal pathogenicity has been considered for *Eikenella corrodens* (*Ec*), enteric rods, *Pseudomonas* species, *Staphylococcus* species and yeasts (Genco 1996). Some of these species, such as *Pseudomonas* species or enteric rods species, are rarely found, and in relatively low numbers, in populations from Western countries; therefore, their aetiologic role also remains unclear.

More recently, viruses including cytomegaloviruses, Epstein–Barr virus, papillomavirus and herpes simplex virus have been proposed to play a role in causing periodontal diseases (for a review, see Contreras & Slots 2000, Slots & Contreras 2000). The presence of these viral genomes has been isolated from periodontitis lesions in cross-sectional studies. These genomes have been found in chronic periodontitis (Contreras & Slots 1996), aggressive periodontitis (Michalowicz et al. 2000) and periodontitis associated with systemic diseased patients (Contreras et al. 2001). These authors hypothesise that environmental or systemic factors would allow for the activation of these viruses, whose inhibitory impact on various host factors would allow for the accumulation of periodontal pathogens and development of destructive inflammatory disease.

Even though the aetiological role of *Aa*, *Pg* and *Tf* in periodontitis seems uncontentious, the use and utility of diagnostic tests aimed at identifying and quantifying the presence of these bacteria in periodontitits patients remain very controversial. The purpose of this review is, firstly, to analyse critically the available evidence on the rationale of the use of microbial diagnosis in the management of periodontitis patients, and secondly, to review the different diagnostic methodologies used to identify and quantify these putative periodontal pathogens, with special emphasis on those methods aimed at clinical use and based on molecular technologies.

Rationale for microbial diagnosis in the management of patients with periodontitis

For microbial diagnosis to be of value, it needs to have an impact on the overall diagnosis and/or treatment planning, resulting in a superior treatment outcome, and/or providing a clear benefit to the patient.

The utility of microbiological testing for diagnosing the different forms of destructive periodontal diseases remains controversial. The limitations of testing for the presence or absence of *Pg* and *Aa*, aimed at distinguishing subjects with aggressive periodontitis from subjects with chronic periodontitis, was clearly shown in the systematic review recently published by Mombelli et al. (2002). Although the diagnosis of aggressive periodontitis may be less likely in a subject with no detection of *Aa*, the sensitivity and specificity for a positive clinical diagnosis of aggressive periodontitis in the presence of these bacteria are low and heterogeneous, and therefore, the mere presence or absence of these putative pathogens is not able to discriminate subjects with aggressive periodontitis from those with chronic periodontitis.

The utility of microbial identification as an aid in the treatment planning of patients with periodontitis has been tested in a limited number of studies, mostly case reports dealing with aggressive or non-responding periodontitis patients (Levy et al. 1993, Rosenberg et al. 1993, Renvert et al. 1996, Eickholz et al. 2001, Ishikawa et al. 2002). The aim of most of these studies was to guide in the selection of adjunctive antimicrobial therapy based on the microbial data. Only one of these publications reported a controlled study (Levy et al. 1993), where two different groups of periodontists developed their treatment plans based on the results of adjunctive diagnostic microbiology (test), or just on standard clinical diagnosis (controls). The use of microbial diagnosis resulted in the use of more systemic antibiotics and less periodontal surgery. However, the long-term outcome of these patients was not reported. The rest of the publications involved case reports, where the use of microbial diagnosis guided periodontal therapy. In most of these studies, the patients improved following treatment, some even showing dramatic improvements. The lack of appropriate controls, however, makes the interpretation of these results difficult and therefore, the utility of microbial testing in developing specific treatment plans cannot be ascertained.

The utility of microbiological testing as an indicator of healing or disease progression has been suggested by several prospective studies, where the detection or lack of detection of putative periodontal pathogens was significantly associated with a different clinical response (Wennström et al. 1987, Haffajee et al. 1991, 1995, 1996, Rams et al. 1996, Dahlen & Rosling 1998, Buchmann et al. 2000, Chaves et al. 2000, Tran et al. 2001). In most of these studies, the absence of these pathogens was a better predictor of periodontal health than their presence as a predictor of periodontal disease (Wennström et al. 1987, Dahlen & Rosling 1998). Some studies showed that the presence of these pathogens above certain critical levels increased the risk for periodontitis recurrence. Rams et al. (1996) showed a 2.5 times increased risk of disease recurrence when *Aa*, *Pg*, *Pi*, *Cr* or *Pm* were detected at baseline. Similarly, Haffajee et al. (1995, 1996) showed a significant increased risk of attachment loss when *Aa* was present at threshold levels above 10^4, and *Pg* above 10^5. Chaves et al. (2000) showed that the presence of *Pg* was predictive of disease progression and bone loss, and Tran et al. (2001) demonstrated that patients with persistent *Tf* were 5.3 times more likely to develop loss of attachment than those without *Tf*. Although these prospective studies monitoring patients after therapy would indicate that the use of microbial testing could aid in the selection of a more targeted therapy, mostly in patients with aggressive or recurrent periodontitis, the lack of clinical trials with adequate controls prevents from demonstrating the real value of microbial diagnosis. Therefore, the available evidence does not fully prove the utility of microbiological testing in periodontitis patients.

Microbiological diagnostic methods

Different methods have been used for the detection of putative periodontal pathogens in subgingival samples. Some

of these methods have been strictly used for research purposes, while others have been adapted or modified for clinical use.

Bacterial culturing

Historically, culture methods have been widely used in studies aimed at characterising the composition of the subgingival microflora and are still considered the reference method (gold standard) when determining the utility of a new microbial diagnostics in periodontics. Generally, subgingival plaque samples are cultivated anaerobically and by using selective and non-selective media, together with several biochemical and physical tests, the different putative pathogens can be identified. The main advantage of this method is the possibility to obtain relative and absolute counts of the cultured species. Moreover, it is the only method able to characterise properly new species and to assess the antibiotic susceptibility of the grown bacteria (Socransky et al. 1987, Greenstein 1988, Marchal et al. 1991, Lamster et al. 1993). However, culture techniques have significant shortcomings. Culture methods can only grow viable bacteria; therefore, strict sampling and transport conditions are essential. Moreover, some of the putative pathogens, such as *Treponema* sp. and *Tf*, are very fastidious and difficult to culture (Sakamoto et al. 2002). The sensitivity of bacterial culturing can be rather low, especially for non-selective media, with detection limits averaging 10^3–10^4 bacterial cells, and therefore, low numbers of a specific pathogen in a subgingival sample will be undetected. However, the most important drawback is that culture requires specific laboratory equipment and experienced personnel, besides being relatively time-consuming and expensive.

Methods based on immune diagnosis

Immunological assays use antibodies that recognise specific bacterial antigens, and the identification of these specific antigen–antibody reactions allows the detection of target microorganisms. This reaction can be visualised using a variety of techniques and reactions, including direct (DFA) and indirect (IFA) immunofluorescent microscopy assays, flow cytometry, enzyme-linked immunosorbent assay (ELISA), membrane assays and latex agglutination (Greenstein 1988, Lamster et al. 1993). Both direct (DFA) and IFA are able to identify the selected pathogen and quantify its percentage in the flora by using a plaque smear. IFA has been used mainly to detect *Aa*, *Pg* and *Tf*. Zambon et al. (1985) showed that this technique was comparable with bacterial culture in its ability to identify *Aa* and *Pg* in subgingival plaque samples. In fact, IFA demonstrated a higher sensitivity when compared with culture, probably because of a lower detection limit. Comparative studies indicated that the sensitivity of these assays ranged from 82% to 100% for detection of *Aa* and from 91% to 100% for detection of *Pg*, with specificity values of 88% to 92% and 87% to 89%, respectively (Zambon 1985, Zambon et al. 1985, 1986). Listgarten et al. (1995) compared the diagnostic utility of IFA and culture for the detection of *Aa*. They showed a higher sensitivity of IFA (41.8%) and a significantly higher detection rate (39.4 % for culture versus 81.8% for IFA). These authors also compared the diagnostic utility of IFA for the detection of *Pg* and *Tf* when compared with oligonucleotide probes complementary to the hypervariable region of the 16S rRNA of the target bacteria. They also demonstrated significantly higher detection rates and higher sensitivity than DNA probes for both bacteria.

Cytofluorography or flow cytometry for the rapid identification of oral bacteria involves labelling bacterial cells from a patient plaque sample with both species-specific antibodies and a second fluorescein-conjugated antibody. The suspension is then introduced into the flow cytometer, which separates the bacterial cells into an almost single-cell suspension by means of a laminar flow through a narrow tube (Kamiya et al. 1994). The sophistication and cost involved in this procedure precludes its wide usage

ELISA is similar in principle to other radioimmunoassays, but instead of the radioisotope, an enzymatically derived colour reaction is substituted as the label. The intensity of the colour depends on the concentration of the antigen and it is usually read photometrically for optimal quantification. ELISA has been used primarily to detect serum antibodies to periodontal pathogens; however, it has also been used in research studies to quantify specific pathogens in subgingival samples using specific monoclonal antibodies. A membrane immunoassay has been adapted for chair-side clinical diagnostic use and has been marketed (Evalusite®, Eastman Kodak, Rochester, NY, USA). It involves linkage between the antigen and a membrane-bound antibody to form an immunocomplex that is later revealed through a colorimetric reaction. Evalusite® has been designed to detect *Aa*, *Pg* and *Pi* (Boyer et al. 1996, Chaves et al. 2000). Snyder et al. (1996) found a detection limit of 10^5 for *Aa* and 10^6 for *Pg*.

In summary, immunological assays for oral bacteria provide a quantitative or semi-quantitative estimate of target microorganisms. These methods have shown a higher sensitivity and specificity than bacterial culturing for the detection of target microorganisms (*Aa*, *Pg* and *Tf*); however, they require the use of monoclonal antibodies to assure high specificity and the detection limits are not significantly lower than bacterial culturing (10^3–10^4). These tests also have the advantage of not requiring stringent sampling and transport methodology to assure bacterial viability. However, they are limited to the number of antibodies tested, they are not amenable for studying antibiotic susceptibility and they lack the validity of well-controlled clinical studies.

Enzymatic methods of bacterial identification

Tf, *Pg*, the small spirochete *Td* and *Capnocytophaga* species share a common enzymatic profile, since they all have a trypsin-like enzyme in common. The activity of this enzyme can be measured with the hydrolysis of the colourless substrate *N*-benzoyl-DL-arginine-2-naphthylamide (BANA). A diagnostic kit has been developed using this reaction for the identification of this bacterial profile in plaque samples (Perioscan®, Oral B Laboratories, Belmont, CA, USA). Loesche (1992) proposed the use of this BANA reaction in subgingival plaque samples to detect the presence of any of these periodontal pathogens and thus serve as a marker of disease activity. Using probing depths as a measure of periodontal morbidity, they showed that shallow pockets exhibited only 10% positive BANA reactions, whereas deep pockets (7 mm) exhibited 80–90% positive BANA reactions. These authors (Loesche et al.

1992) tested the diagnostic utility of the BANA test compared with culture, IFA, ELISA and DNA probes, rendering similar results with regard to sensitivity and accuracy (above 90% to detect combination of these organisms). However, a clear BANA reaction was indicative of more than 10^5 BANA-positive organisms, which indicates a rather high limit of detection. Beck et al. (1990) also used the BANA test as a risk indicator for periodontal attachment loss; however, the obtained odds ratios were not statistically significant. In summary, this chair-side test, although very easy and fast to use, has significant limitations. Mainly, since it only detects a combined and limited number of pathogens, its negative result does not rule out the presence of other important periodontal pathogens or even the tested pathogens when present in low number.

The simplicity, quick response and easy-to-read use made these enzymatic and immune-based diagnostic methods ideal for chair-side use. In the 1990s they were commercially available (Evalusite® and Perioscan®). However, the lack of appropriate clinical trials to validate their diagnostic utility and their intrinsic problems regarding low sensitivity (Evalusite®) and low specificity (Perioscan®) made them disappear from the market soon.

Molecular biology techniques

The development of techniques in molecular biology, aimed at the detection of bacterial pathogens, has allowed not only the acquisition of knowledge in microbial genetics but has also set the bases for the development of improved diagnostic techniques (Holt & Progulske 1988, Saiki et al. 1988, Gibbs 1990). The principles of molecular biology techniques reside in the analysis of DNA, RNA or the protein structure or function (Lewin 1993, Dawson et al. 1996). The genetic material of a bacterium is composed of a chromosomal DNA and transferring, ribosomal and messenger RNA. Chromosomal DNA is dispersed in the bacterial cell without any membrane envelope (Holt & Progulske 1988). Diagnostic assays using molecular biology techniques require specific DNA fragments that recognise complementary-specific bacterial DNA sequences from target microorganisms. For the development of a microbiological diagnostic test using this technology, it is therefore fundamental to be able to extract the bacterial DNA from the plaque sample and to be able to amplify the specific DNA sequence of the target periodontal pathogens.

Different chemical, enzymatic or physical methods have been used to obtain DNA of sufficient quantity and quality for its subsequent analysis by means of either DNA probes or the polymerase chain reaction (PCR). Among them, organic chemicals or detergents have been used to destroy the cellular components (membranes and proteins) and thus avoid interference with the subsequent DNA assays (Smith et al. 1989a, b). As enzymes, lysozyme is used to cleave the bacterial wall and proteinase K is used to destroy the proteinic components of the cell (Leys et al. 1994). Physical methods such as heat allow disruption of the cell and denaturation of the proteins. Subsequently, the use of centrifugation and chromatographic columns enables the separation and purification of DNA. For periodontal microbial diagnosis, most of the tests developed have used proteinase K or boiling and centrifugation (Ting & Slots 1997, Umeda et al. 1998b). Once the DNA from the subgingival plaque samples have been extracted and purified, different diagnostic methods have been developed to specifically detect and in some cases quantify the target periodontal pathogens.

Nucleic acid probes

A probe is a known nucleic acid molecule (DNA or RNA) from a specific microorganism artificially synthesised and labelled for its detection when placed together with a plaque sample. DNA probes entail segments of a single-stranded nucleic acid, labelled with an enzyme or radioisotope that is able to hybridise to their complementary nucleic acid sequence and thus detect the presence of target microorganism. Hybridisation refers to the pairing of complementary DNA strands to produce a double-stranded nucleic acid. The nucleotide base-pair relationship is so specific that strands cannot anneal unless the respective nucleotide strand sequences are complementary. All hybridisation methods use radiolabelled or fluorescence-labelled DNA probes that bind to the target DNA of interest, thus allowing its visualisation (Lawer et al. 1990, Loesche 1992, Nicholl 1994, Dawson et al. 1996, Tanner et al. 1998, Crockett & Wittwer 2001). DNA probes may target whole-genomic DNA or individual genes. Whole-genomic DNA is more likely to cross-react with non-target microorganisms because of the presence of homologous sequences between different bacterial species. Currently, most of the probes used are oligonucleotides ranging from 20 to 30 nucleotides (Nicholl 1994, Dawson et al. 1996).

Whole-genomic probes for the detection of *Aa*, *Pg*, *Pi* and *Td* have been developed and tested, being the bases of commercially available diagnostic methods (DMDx®, Omnigene, Cambridge, MA, USA). When compared with culture, van Steenberghe et al. (1999) reported a sensitivity of 96% and specificity of 86% for *Aa*, and 60% and 82%, respectively, for *Pg* in pure laboratory isolates. However, when tested in clinical specimens, both the sensitivity and specificity were reduced significantly, suggesting cross-reactivity with unknown bacteria in subgingival plaque samples. In order to overcome this drawback, oligonucleotide probes complementary to variable regions of the 16S rRNA bacterial genes have been developed for the detection of various periodontal pathogens. These bacterial 16S rRNA genes contain both regions shared by different bacteria and short stretches of variable regions shared only by specific organisms of the same species or genus (Moncla et al. 1990). When these oligonuclotide probes were compared with culture in clinical samples for the detection of *Aa*, *Pg* and *Pi* (Savitt et al. 1988), an effectiveness of 100% in detecting *Aa* and *Pi* and of 91% in detecting *Pg* was calculated at culture-positive levels ($\geq 10^3$ cells). However, DNA probes were more sensitive than culture in detecting these pathogens in samples from periodontitis patients (for example, *Aa* was detected by probe analysis in 70% of localised juvenile periodontitis samples, but only detected in 10% by culture analysis). Conversely, when these probes were compared with IFA for the detection of *Pg* and *Tf* (Listgarten et al. 1995), IFA showed significantly higher detection rates and higher sensitivity.

Checkerboard DNA–DNA hybridisation technology

Socransky et al. (1994) developed this technique by for the detection and levels of 40 bacterial species commonly found

in the oral cavity. The assay uses whole genomic, digoxigenin-labelled DNA probes and facilitates rapid processing of large numbers of plaque samples with respect to a multiple hybridisation for up to 40 oral species in one single test. The DNA probes used in this technology are commonly be adjusted to permit detection of 10^4 cells of each species, but can adjusted to detect 10^3 cells. The method requires sophisticated laboratory equipment and expertise, and it is highly specific. These factors have not led to generalisation of this assay for diagnostic purposes. It is particularly applicable, however, for epidemiological research and ecological studies, since it does not require viable bacteria and allows for the assessment of a large number of plaque samples and multitude of species (Haffajee et al. 1997, 2001, Papapanou et al. 1997a, Levy et al. 1999, Ximenez-Fyvie et al. 2000a, b, Feres et al. 2001, Haffajee & Socransky 2001). Papapanou et al. (1997b) made a comparison of this method with culture for the identification of subgingival bacteria. The checkerboard technology resulted in higher prevalence figures for half of the species tested (*Pg*, *Pi*, *Pn*, *Fn* and *Tf*) and statistically significant higher bacterial counts for the majority of the species. Both techniques rendered a reasonable degree of agreement.

PCR methodology

PCR has emerged as the most powerful tool for the amplification of genes and their RNA transcripts. This technique, developed in 1985, is the single technique used almost universally to study DNA and RNA obtained from a variety of tissue sources. PCR allows for obtaining high quantities of DNA in a simplified and automated fashion (Schochetman et al. 1988, Shibata 1992, Dawson et al. 1996, Jankowski & Polak 1996). PCR typically begins with the isolation of DNA from a fresh tissue specimen. By heating, the complementary double strands of DNA split into single-stranded forms intended to act as the template dictating the nucleotide sequence in vitro. Then, the amplification is followed using a DNA polymerase that requires a primer, or known short oligonucleotide sequence corresponding to the border of the region that is amplified. For obtaining amplified fragments of constant length and in high quantities, a second primer, complementary to the opposed chain, must be used to anneal (bind) the template and flank the region of interest. This amplification can be performed several times, known as cycles. In each cycle, the processes of complementary chains denaturation, primer hybridisation and primer extension by means of the polymerase take place (Fig. 4). With each cycle, there is an exponential increase in the quantity of DNA. During all these processes, the temperature during the cycle is critical in order to control the double chain denaturation and the stability of the hybridisation between the model fragment and the primer. In 1988, a thermo-stable DNA polymerase, isolated from the organism *Thermus aquaticus*, known as Taq polymerase, was developed (Saiki et al. 1988). This Taq polymerase has allowed the automatisation of the reaction using specific appliances named thermo-cyclers. This sequenced DNA is then detected and visualised through electrophoresis in agarose gel and ethidium bromure, obtaining a qualitative signal (Jankowski & Polak 1996, Neumaier et al. 1998).

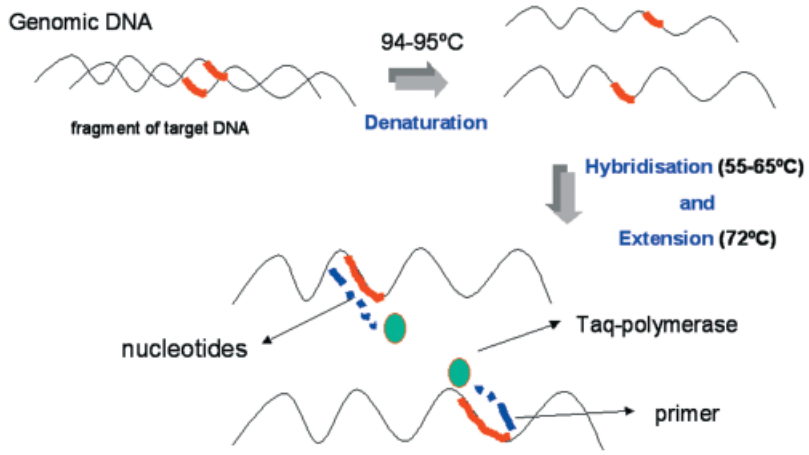

Fig. 4. Diagram depicting the polymerase chain reaction process defined by the three stages that comprise this technique: amplification is carried out by a DNA polymerase, once in each cycle. Each cycle includes the denaturalisation or separation of complementary chains, hybridisation of primers with the original chains and the extension of the primer by the polymerase. Between 30 and 40 cycles are necessary to obtain a significant amount of the studied sequence.

Although PCR is an extremely sensitive technique, being able to detect even one copy of the searched DNA fragment (Greenstein 1988), it has a number of important limitations. Difficulties can be encountered when studying small quantities of DNA, since the ingredients necessary for PCR (oligonucleotide primers, dNTPs, Taq polymerase) may be exhausted before sufficient target is produced. The specificity of the reaction depends on many complex, interrelated factors, including oligonucleotide primer size, annealing temperature and buffer salt concentration. A major limitation of PCR is the susceptibility of the process to contamination, particularly in experiments intended to detect rare DNA sequences (Gibbs 1990, Neumaier et al. 1998). In order to overcome some of these drawbacks, different varieties of PCR have been developed. Multiplex PCR allows the amplification of several target regions, placing all the necessary primers in one single reaction (Dangtuan & Rudney 1996, Henegariu et al. 1997, García et al. 1998). Quantitative PCR allows the quantification of all DNA fragments detected by PCR using specific controls of known quantity (Doungudomdacha et al. 2001). Quantification of DNA by PCR has tremendous potential in periodontal microbial diagnosis since it allows the analysis of a large number of samples relatively easily, providing a measure of flexibility not permitted by other conventional laborious and time-consuming methods. In practice, however, a number of technical challenges had to be overcome to develop reliable and reproducibility quantitative PCR. One of the principal challenges in this technique is the nature of PCR product accumulation. During the reaction, there are two defined growth phases. At low cycles, PCR product accumulates exponentially (exponential phase); however, at higher cycles, as template DNA and primer are being consumed, the rate of

product formation progressively decreases until it ends (saturation phase). To obtain reliable quantitative PCR, the measurement of the obtained product must be made during the exponential phase of the reaction, since during the saturated phase the results will be inaccurate. At present, the method of choice for quantitative PCR is continuous monitoring of the amount of product at the end of each cycle. This is accomplished by real-time quantitative PCR. It uses the 5′–3′-endonuclease activity of Taq DNA polymerase to detect target sequences during PCR and included in the mixture is a short fluorescent oligonucleotide probe as a labelling system. During PCR, the probe hybridises to the target DNA, and Taq polymerase cleaves the probe into shorter fragments, thereby releasing fluorescence that is directly proportional to the amount of PCR product generated. By measuring the amount of PCR produced at the end of each single cycle, PCR growth curves can be plotted and measurements can be taken from the exponentially expanding region of the reaction (Crockett & Wittwer 2001, Meuer et al. 2001).

PCR in the detection of periodontal pathogens

Since the advent of PCR technology, different microbiological tests have been developed for the detection of *Aa*, *Pg* and *Tf* using a variety of DNA extraction methods and primers (see Tables 1–3). Riggio et al. (1996) compared the detection of *Aa* and *Pg* in subgingival plaque samples in patients with periodontitis using either PCR or culture. PCR was more accurate than conventional culture methods for identification of these periodontal pathogens in subgingival plaque samples and demonstrated a higher frequency of detection of these target microorganisms. Ashimoto et al. (1996) developed a 16S rRNA-based PCR detection method to determine the prevalence of *Aa*, *Tf*, *Cr*, *Ec*, *Pg*, *Pi*, *Pn* and *Td*. Matched results between PCR and culture occurred in 28% (*Tf*) and 71% (*Aa*) of the samples; the major discrepancy occurred in the PCR-positive/culture-negative category. This is probably because of the PCR lower detection limit, ranging from 25 to 100 cells, in comparison with culture (10^4–10^5 cells). Eick & Pfister (2002) recently compared a commercial multiplex PCR of 16S rDNA for *Aa*, *Pg*, *Pi*, *Tf* and *Td* with standard culturing. The PCR test was able to detect *Pg* and *Tf* more often than cultivation. *Aa* was detected in similar numbers with both techniques. Most of the available PCR tests developed to detect *Aa*, *Pg* or *Tf* have used, as primers, the 16S rRNA genes (Umeda et al. 1998a, b, Takamatsu et al. 1999, Choi et al. 2000, Contreras et al. 2000, Darby et al. 2000, Mullally et al. 2000, Okada et al. 2000, 2001, Kamma et al. 2001, Takeuchi et al. 2001, Tan et al. 2001a, Avila-Campos & Velásquez-Meléndez 2002). Only a small number of PCR assays have used oligonucleotides derived from single copy genes as primers, such as the *Pg* collagenase prtC gene or the fimbrillin fimA gene, the *Aa* leukotoxin lktA gene or the *Tf* protease prtH gene. 16S rRNA genes as primers, although very specific (cross-reactivity is rare), may not be appropriate for quantitative analysis since there are a variable number of these molecules per cell, which prevents a reproducible quantification. These PCR tests provide only qualitative information (prevalence) and therefore, their use for diagnostic and prognostic purposes in clinical use is limited. Most of these diagnostic tests have been used in ecological studies assessing the prevalence of the target microorganisms in different populations, both in health or disease. Results from these studies are very heterogeneous showing different prevalences (ranging from 2% to 95% for *Aa*) of the target bacteria in different populations both in health and disease (Tables 1–3). This heterogeneity might indicate that the high sensitivity of this technique is able to detect low numbers of bacteria that might be irrelevant in terms of pathogenicity or the possibility of different serotypes from the same species being highly prevalent in some populations without leading to pathogenicity.

The importance of a quantitative assessment of the target bacteria has led to the recent development of quantitative PCR methods. The first assays used end-point PCR (Fujise et al. 1995, Doungudomdacha et al. 2001). However, this technique obtains PCR product in the saturated phase, disregarding the early exponential phase; therefore, the amount of PCR product obtained shows a weak correlation with the initial DNA quantity. To overcome this limitation, real-time PCR assays have been developed. With this technology and by using a single copy of these genes per cell, a good correlation between the fluorescent signal measured and the number of cells was obtained (Lyons et al. 2000, Shelbourne et al. 2000). Morillo et al. (2003) tested a real-time PCR assay, based on single copy gene sequence and on the SYBR Green I chemistry, aimed at the quantification of *Aa* and *Pg* in subgingival plaque samples. This assay demonstrated a high degree of specificity and a very reproducible and consistent method to quantify these pathogenic species. Real-time PCR, although demonstrating a high degree of sensitivity, specificity and reproducible quantification, requires expensive laboratory equipment, which makes this method very expensive for routine diagnostic clinical microbiology. Recently, Rudney et al. (2003) also reported a quantitative PCR assay for *Aa*, *Pg* and *Tf*. This proposed technique, although cheaper than real-time PCR, may be associated with a higher variability, since it lacks detection during the exponential phase. Moreover, the primers used are 16S rRNA genes, which makes a reproducible quantification difficult.

This review clearly shows that standard PCR technology, although demonstrating high sensitivity and specificity for the identification of target periodontal pathogens, is unable to accurately quantify them in clinical samples, and therefore its role as a routine clinical diagnostic tool is limited. However, the advent of quantitative PCR technology may circumvent this limitation and at the same time may clearly improve some of the shortcomings of standard cultural techniques. These quantitative PCR assays must be validated in clinical studies in order to demonstrate their diagnostic utility, and actual cost–benefit evaluation of their use in routine clinical diagnosis must also be carried out since they require expensive and sophisticated technology. Moreover, it is important to keep in mind that although quantitative PCR technology may have a major role as an adjunctive diagnostic tool in both epidemiological and clinical studies in periodontology, culture techniques still hold some inherent capabilities, such as its ability to detect multiple bacterial species coincidentally, to detect unexpected bacteria or to allow the determination of antibiotic resistance, which still makes this diagnostic tool the current

Table 1. Prevalence of *Actinobacillus actinomycetemcomitans* using PCR technique

Reference	Study group	Sampling method	DNA extraction	Primers	Prevalence
Yuan et al. (2002)	328 subjects, 7–12 years old, Taiwan	Curette and dilution in 200 μl distilled water	Dilution in 250 μl Tris-EDTA/Triton X-100; boiling, 10 min; centrifuge, 10,000 × *g*, 3 min; store supernatant	lkt A gene	7 years old, 1.3%; 8 years old, 3.5%; 9 years old, 2.8%; 10 years old, 8.3%; 11 years old, 7.7%; 12 years old, 9.5%
Yuan et al. (2001)	246 periodontal subjects: 105 non-insulin-dependent diabetes, 141 no diabetes, Taiwan	Curette and 200 μl Tris-EDTA/Triton X-100	Boiling, 10 min; centrifuge, 10,000 r.p.m., 3 min; store supernatant	kt A	Prevalence: 5.7% sites, no difference between groups
Okada et al. (2000)	104 subjects, 12 years old: 21 periodontitis, 73 gingivitis, 10 healthy, Japan	Dental brush and dilution in distilled water	Centrifuge, 1600 × *g*, 20 min; chemical purification of pellet	16S rRNA	Total, 7.7%; healthy, 4.8%; gingivitis, 6.8%; periodontitis, 20%
Takamatsu et al. (1999)	26 patients: 11 adult periodontitis, 15 EOP, Japan	Paper point and dilution in 100 μl distilled water	Boiling, 10 min	16S rRNA	Before treatment, 30.8%; post-treatment, 19.2%
Tan et al. (2001b)	92 adult subjects, 25–65 years old: 50 healthy, 15 moderate periodontitis, 27 severe periodontitis, China	Curette and dilution in 500 μl reduced transport medium RTF	Centrifuge and dilution of pellet in 200 μl RTF, with small glass beads; incubation, 94°C, 10 min; cooling, 5 min	kt A	Total, 74%; periodontitis, 69%; healthy, 78%
Choi et al. (2000)	29 severe periodontitis, 20 healthy, Korea	Paper point and 1 ml RTF	Dilution in 100 μl and centrifuge 13,000 r.p.m., 10 min; dilution in Tris-EDTA/SDS	Consensus bacterial 16S-rRNA and hybridization with specific oligos	Periodontitis, 89.7%; healthy, 5%
Tan et al. (2002)	146 patients: 113 chronic periodontitis, 33 aggressive periodontitis, Singapore	Curette and dilution in 500 μl RTF	Centrifuge and dilution of pellet in 200 μl RTF and glass beads; incubation, 94°C, 10 min	tx A cdt, ABC	xt A: Ch.P., 67%; Ag.P., 91%; genotype cdt: Ch.P., 4%; Ag.P, 33%
Riggio et al. (1996)	43 patients: United Kingdom	Curette and dilution in 500 μl RTF	Mix 90 μl sample and 10 μl Tris-EDTA/Triton X-100; boiling, 5 min	lkt A	Prevalence, 40%
Doungudomdacha et al. (2001)	50 adult periodontitis, United Kingdom	Curette and 700 μl RTF	Centrifuge and dilution of pellet in 250 μl distilled water	lkt A	Diseased sites, 91.8%
Darby et al. (2000)	57 periodontitis: 33 severe periodontitis, 24 generalized EOP, United Kingdom	Curette and dilution in 500 μl Tris-EDTA	Mix with Tris-EDTA/Triton X-100; boiling, 5 min	lkt A	Severe periodontitis, 3%; EOP, 20.8%
Mullally et al. (2000)	42 EOP: 25 localized, 17 generalized, Ireland	Curette and dilution in 500 μl PBS	Centrifuge, 14,000 r.p.m., 5 min; dilution of pellet in 500 μl PBS two more times	16S rRNA	Prevalence, 19%; 28% localized periodontitis; 5.9% generalized periodontitis
Kamma et al. (2001)	16 post-treatment (mechanical and antimicrobial therapy) patients in supportive phase, Greece	Paper point	Dilution in 500 μl Tris-EDTA and disperse in vortex. Mix 250 μl suspension and 250 μl water; centrifuge and resuspend pellet two more times; boiling, 10 min; centrifuge and store supernatant	16S rRNA	Prevalence: active sites, 18.8%; non-active sites, 6.3%
Umeda et al. (1998a)	199 subjects: 52 Caucasian, 49 Afroamerican, 48 Asian, 50 Hispanic, USA	Paper point	Dilution in 500 μl distilled water and disperse in vortex; centrifuge and resuspension of pellet twice; store supernatant	16S rRNA	Prevalences: Caucasian, Afroamerican, Asian, Hispanic healthy/ging: 23.1%, 50%, 29.4%, 50% initial perio.: 7.7%, 27.8%, 36.4%, 50%; mod. perio.: 8.3%, 21.4%, 75%, 56.3%; sev. perio.: 35.7%, 30.8%, 50%, 58.3%
Contreras et al. (2000)	218 patients: EOP, USA	Paper point	Dilution in 500 μl Tris/Tween-20/proteinase K; incubation, 60°C, 1 h; incubation, 97°C, 15 min; boiling, 10 min; centrifuge and store supernatant	16S rRNA	Caucasian, 36%; Hispanic, 49%; Asian, 26%; Afroamerican, 38%
Avila-Campos & Velásquez-Meléndez (2002)	100 subjects: 50 periodontitis, 50 healthy, Brazil	Paper point and dilution in VMGA III medium	Mix sample with 500 μl water; centrifuge and resuspend pellet twice; boiling, 10 min	16S rRNA	Prevalence: healthy, 70%; periodontitis, 90%
Fujise et al. (2002)	104 subjects, 1149 sites, Japan	Paper point	Dilution in distilled water and boiling 10 min	lkt A	Sites ⩾6 mm, 40%; sites <6 mm, 40%; healthy sites, 20%

EOP, early-onset periodontitis; Ch.P, chronic periodontitis; Ag.P., aggressive periodontitis; ging., gingivitis; mod. perio., moderate periodontitis; sev. perio., severe periodontitis; lkt, leukotoxin; cdt, cytolethal distending toxin.

Table 2. Prevalence of *Porphyromonas gingivalis* using PCR technique

Reference	Study group	Sampling method	DNA extraction	Primers	Prevalence
Okada et al. (2001)	104 subjects, 2–12 years old: 21 periodontitis, 73 gingivitis, 10 healthy, Japan	Dental brush and dilution in distilled water	Centrifuge, 1600 × *g*, 20 min; chemical purification of pellet	16S rRNA	Total, 9.6%; healthy, 4.8%; gingivitis, 9.6%; periodontitis, 20%
Nozaki et al. (2001)	31 periodontitis, Japan	Paper point and dilution in 200 μl distilled water	Mix with Tris/salts/Tween-20/ proteinase K; incubation, 55°C; 1 h; extraction with phenol/ chloroform/ethanol; resuspend in Tris-EDTA	Outer membrane protein gene OMP 40 kDa and hybridization	Pre-treatment, 80%; post-treatment, 48.4%
Takeuchi et al. (2001)	123 subjects: 20 healthy, 103 periodontitis: 38 aggressive, 65 chronic, Japan	Paper point and dilution 1 ml distilled water	Centrifuge, 10,000 r.p.m., 5 min and resuspend pellet in 200 μl water; boiling, 10 min	16S rRNA	Healthy, 10%; chronic periodontitis, 95.3%; aggressive periodontitis, 84.2%
Amano et al. (1999)	93 periodontitis, Japan	Curette and dilution in 1 ml PBS	Centrifuge, 12,000 r.p.m., 1 min; extraction with kit Puregene; resuspended in 100 μl distilled water	Fim A genotype-specific and species16S rRNA	Prevalence, 78.5%; predominance of genotypes II and IV
Amano et al. (2000)	519 subjects: 380 healthy, 139 periodontitis, Japan	Curette and dilution 1 ml PBS	Centrifuge, 12,000 r.p.m., 1 min; extraction with kit Puregene; resuspended in 100 μl distilled water	Fim A genotype-specific and species16S rRNA	Prevalence: 36.3%, healthy (genotypes I and V); 87.1%, periodontitis (genotypes II and IV)
Yuan et al. (2001)	246 periodontitis subjects: 105 non-insulin-dependent diabetes, 141 no diabetes, Taiwan	Curette and dilution in 200 μl TE/Triton X-100	Boiling, 10 min; centrifuge, 10,000 r.p.m., 3 min; store supernatant	Fim A	Prevalence: 46.7–66.7%, site; no differences between groups
Choi et al. (2000)	29 severe periodontitis, 20 healthy, Korea	Paper point and 1 ml RTF	Dilution, 100 μl and centrifuge, 13,000 r.p.m., 10 min; resuspended in TE/SDS	Consensus bacterial 16S rRNA and hybridization with specific oligos	Periodontitis, 100%; healthy, 30%
McClellan et al. (1996)	198 subjects, 0–18 years old, USA	Paper point	Extraction with commercial kit	Intermediate region rRNAs (ISR); nested PCR	Prevalence, 37%; no racial or gender differences
Tuite-McDonnell et al. (1997)	101 families, 564 subjects, USA	Paper point	Extraction with commercial kit	Intermediate region rRNAs (ISR); nested PCR	Prevalence, 37.1%
Griffen et al. (1999)	311 subjects: 181 healthy, 130 periodontitis, USA	Paper point	Extraction with commercial kit	Intermediate region rRNAs (ISR); nested PCR	Healthy, 25%; periodontitis, 79%
Griffen et al. (1998)	311 subjects: 181 healthy, 130 periodontitis, USA	Paper point	Extraction with commercial kit	Intermediate region rRNAs (ISR); nested PCR	More virulent strains in the periodontitis patients, according to differences in ISR sequences
Umeda et al. (1998a)	199 subjects: 52 Caucasian, 49 Afroamerican, 48 Asian, 50 Hispanic, USA	Paper point	Dilution in 500 μl distilled water and disperse in vortex; centrifuge and resuspended pellet twice; store supernatant	16S rRNA	Prevalence: Caucasian, Afroamerican, Asian, Hispanic healthy/ging.: 15.4%, 0%, 41.2%, 42.9%; initial perio.: 23.1%, 61.1%, 54.5%, 75%; mod. perio.: 50%, 4.3%, 83.3%, 87.5%; sev. perio.: 50%, 69.2%, 75%, 83.3%
Darby et al. (2000)	57 periodontitis patients: 33 severe periodontitis, 24 generalized EOP, United Kingdom	Curette and dilution 500 μl TE	Mix with TE/Triton X-100 boiling, 5 min	Fim A	Severe periodontitis, 54.5%; generalized EOP, 2.5%
Doungudomdacha et al. (2001)	50 adult periodontitis, United Kingdom	Curette and dilution 700 μl RTF	Centrifuge and resuspended pellet in 250 μl distilled water	Fim A	Affected sites, 93.9%
Mullally et al. (2000)	42 EOP patients: 25 localized, 17 generalized, Ireland	Curette and dilution 500 μl PBS	Centrifuge, 14,000 r.p.m., 5 min and resuspended pellet in 500 μl, PBS three times	16S rRNA	Prevalence, 16.7%; 16% localized EOP; 17.6% generalized EOP
Kamma et al. (2001)	16 post-treatment (mechanical and antimicrobial therapy) patients in supportive phase, Greece	Paper point	Dilution in 500 μl TE and disperse in vortex; mix 250 μl suspended in 250 μl water; centrifuge and resuspended pellet twice; boiling, 10 min; centrifuge and store supernatant	16S rRNA	Prevalence: active sites, 1.9%; non-active sites, 37.5%
Avila-Campos & Velásquez-Meléndez (2002)	100 subjects: 50 periodontitis, 50 healthy, Brazil	Paper point and dilution in VMGA III medium	Mix sample with 500 μl water; centrifuge and resuspended pellet twice; boiling, 10 min	16S rRNA	Prevalence: healthy, 66%; periodontitis, 78%
Fujise et al. (2002)	104 subjects: (1149 sites) Japan	Paper point	Dilution in water and boiling 10 min	Gen PrtC	Sites ⩾6 mm, 87%; Sites <6 mm, 79%; healthy sites, 38%

EOP, early-onset periodontitis; ging., gingivitis; mod. perio, moderate periodontitis; sev. perio, severe periodontitis.

Table 3. Prevalence of *Tannerella forsythensis* using PCR technique

Reference	Study group	Sampling method	DNA extraction	Primers	Prevalence
Okada et al. (2001)	104 subjects, 2–12 years old: 21 periodontitis, 73 gingivitis, 10 healthy, Japan	Dental brush and dilution in distilled water	Centrifuge, 1600 × *g*, 20 min; chemical purification of pellet	16S rRNA	Healthy, 33.3%; gingivitis, 63.9%; periodontitis, 58.3%
Choi et al. (2000)	29 sev. perio., 20 healthy, Korea	Paper point and 1 ml RTF	Dilution, 100 μl and centrifuge, 13,000 r.p.m., 10 min; resuspended in TE/SDS	Bacterial common16S rRNA	Periodontitis, 96.6%; healthy, 55%
Tan et al. (2001a)	160 subjects: 74 healthy, 86 adult periodontitis, Singapore	Curette and dilution, 500 μl RTF	Centrifuge and resuspended pellet in 200 μl RTF and glass beads; incubation, 94°C, 10 min	Specifical 16S rRNA, prtH gene	Prevalence: periodontitis, 91%; healthy, 45%; prevalence of genotype prtH: periodontitis, 85%; healthy, 10%
Umeda et al. (1998a)	199 subjects: 52 Caucasian, 49 Afroamerican, 48 Asian, 50 Hispanic, USA	Paper point	Dilution in 500 μl distilled water and disperse in vortex; centrifuge and resuspended pellet two times; store supernatant	16S rRNA	Prevalence: Caucasian, Afroamerican, Asian, Hispanic: healthy/ging.: 46.2%, 50%, 35.3%, 50%; initial perio.: 46.2%, 44.4%, 45.5%, 37.5%; mod. perio.: 83.3%, 78.6%, 66.7%, 75%; sev. perio.: 85.7%, 84.6%, 87.5%, 100%
Leys et al. (2002)	293 subjects: 121 periodontitis, USA	Paper point		Intergenic rRNA region	34% healthy, 83% periodontitis
Darby et al. (2000)	57 periodontitis: 33 severe/ periodontitis, 24 generalized EOP, United Kingdom	Curette and dilution, 500 μl TE	Mix with TE/Triton X-100; boiling, 5 min	16S rRNA	Periodontitis severe, 21%; generalized early onset, 22%
Mullally et al. (2000)	42 EOP: 25 localized, 17 generalized, Ireland	Curette and dilution, 500 μl PBS	Centrifuge, 14,000 r.p.m., 5 min; resuspended pellet in 500 μl PBS three times	16S rRNA	Prevalence, 78.6%; localized periodontitis, 76%; generalized periodontitis, 82.4%
Kamma et al. (2001)	16 post-treatment (mechanical and antimicrobial therapy) patients in supportive phase, Greece	Paper point	Dilution in 500 μl TE and disperse in vortex; mix 250 μl suspended and 250 μl water; centrifuge and resuspended pellet twice; boiling, 10 min; centrifuge and store supernatant	16S rRNA	Prevalence: active sites, 87.5%; non-active sites, 6 8.8%
Avila-Campos & Velásquez-Meléndez (2002)	100 subjects: 50 periodontitis, 50 healthy, Brazil	Paper point and dilution in VMGA III medium	Mix sample with 500 μl water; centrifuge and resuspended pellet twice; boiling, 10 min	16S rRNA	Prevalence: healthy, 30%; periodontitis, 82%
Fujise et al. (2002)	104 subjects (1149 sites), Japan	Paper point	Dilution in distilled water and boiling 10 min	16S rRNA	Sites ⩾6 mm, 96%; sites <6 mm, 86%; healthy sites, 70%

Ging., gingivitis; mod. perio., moderate periodontitis; sev. perio., severe periodontitis.

reference standard in periodontal microbiology.

Summary

Although different methods of microbial diagnosis have been used for the detection and quantification of putative periodontal pathogens in subgingival samples, there is not a single one that has demonstrated ideal characteristics. The choice of the optimal microbial test for adjunctive diagnostic aid in periodontics should be based on the following parameters:

1. Sensitivity and specificity of the test. Real-time PCR technology has demonstrated a high degree of sensitivity and specificity, mostly when compared with standard culturing. Qualitative PCR tests, although highly sensible and specific, do not provide accurate information on the number of bacteria identified, and this information might be crucial in clinical diagnosis. Research evidence, although limited, clearly shows an association between a higher bacterial count and disease occurrence and severity. The mere presence of a bacterial pathogen has, therefore, limited value as an adjunct to clinical diagnosis and treatment planning.
2. Availability of use and cost. Culture, checkerboard analysis and real-time quantitative PCR require sophisticated laboratory facilities and are labour intensive, but makes them expensive for routine clinical diagnostic use. Standard PCR tests and other easy-to-use periodontal microbiological methods based on immune or molecular technologies have limited capability for accurate quantification and are limited to the target bacteria, which limits their diagnostic validity for clinical use.
3. Information provided. Only bacterial culturing enables the study of antibacterial susceptibilities and detection of unexpected bacteria, while PCR and other molecular techniques would allow the detection of non-cultivable microorganisms.
4. To date, there is no ideal microbial diagnostic for adjunctive clinical use in periodontics. Bacterial culturing still remains the gold standard. However, improved quantitative PCR technology may have an important role in the future, once it has been fully validated with well-designed clinical trials and their costs have been reduced with more available technology.

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