

ORIGINAL ARTICLES

Identification of the microorganisms responsible for periodontopathy by Multiplex RT-PCR

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Key words

Subgingival biofilm • Multiplex RT-PCR • Periodontopathies

Summary

The aim of our research was to identify by bacterial genomic DNA analysis the prevalence of five different species of periodontopathogenic bacteria present in the subgingival biofilm, specifically: *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Prevotella intermedia* (Pi), *Bacterioides forsythus* (Bf), *Treponema denticola* (Td).

For the analysis we used the systematic Multiplex-PCR-microdent kit with species-specific primers. We studied a group of 48 subjects, 18 males and 30 females, from 18 to 78 years of age. The initial clinical screening enabled us to select, among the group analysed, 24 subjects with signs of active periodontopathy (Group A) and 24 patients without identifiable clinical evidence of the

disease used as the control group (Group B). Within the two experimental groups (A and B), the test was found to be positive in 75% of subjects from group A, whereas the test was found to be negative in all the subjects from group B. Our research shows that the Multiplex-PCR system is reliable. Furthermore, the sensitivity and simplicity of this technique, as well as the decrease in working times and the possibility of identifying non-culturable bacteria, since the presence of viable organisms is not essential, make this technique indicated for the simultaneous identification of periodontopathogenic bacteria and might, in perspective, provide a more effective clinical alternative to the techniques of bacterial typing of the subgingival plaque.

Introduction

It is by now unanimously acknowledged that the most common diseases of the oral cavity (periodontal disease and caries) are to be considered as infectious diseases caused by the bacteria commonly present in the mouth's ecosystem.

The pathogenicity of these microbial species cannot be entirely attributed to the detection of their presence but it is rather connected with the interaction that the bacteria, organised in the plaque, develop with the host organism. The caries, according to this etiopathogenetic interpretation, is, for instance, considered to be an infection whose more evident symptom is represented by the caries lesion that consequently entails a more complex pathological condition [1, 2].

Lately, even the concept of plaque has been subjected to a critical revision by identifying the active intervention of specific bacteria (*Streptococcus mutans*, *Lactobacilli*) in its formation and maturation and by evaluating how bacteria are organized in a biofilm [3].

In actual fact, the bacteria in plaque colonize a substrate by adhering and associating in micro-colonies and forming the biofilm composed of bacteria, products of bacterial metabolism and organic liquids (blood and saliva) [4-6].

Even the periodontal disease (PD) appears to be the effect of the pathogenic action of specific species of the subgingival bacterial biofilm although there are many species that can be identified and their variability is considerable [7, 8].

Epidemiological studies on PD confirm that the percentage of individuals with healthy periodont decreases with increasing age and does not represent more than 10% of the adult population. It has been reported that in Europe and North America the indices of prevalence vary between 9% and 95% in children and between 70% and 95% in adults [9].

Other studies emphasize that in the United States approximately 48% of the adult population has chronic periodontitis, while in Canada there is an incidence of 50-75% in young adults [10, 11].

It has been estimated that periodontal diseases are responsible for 30-35% of all tooth extractions, a percentage that tends to increase considerably (around 50%) when considering cohorts more than 45 years old. Chronic periodontitis is in fact responsible for an average loss of tooth elements that varies from 3.5 to 16 depending on the age cohort examined [12].

Although it is not possible to correlate with absolute certainty the periodontal disease to specific pathogens, because of the difficulty in identifying all the microbial species of the oral cavity, and their interactions, the etiopathogenetic role of these bacteria cannot be ignored.

Using the criteria proposed by Socransky in 1970 it has been demonstrated that there is a significant statistical correlation between the detection of bacterial species such as *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg) and *Tannerella forsythensis* (Tf) and the triggering and progression of periodontopathy [12, 14].

As a matter of fact, Aa has been related to aggressive periodontitis; Pg is believed to be associated with the chronic course of the disease, while there seems to be close correlation between the number of *P. gingivalis*, the depth of the pockets and the periodontal status [15-20]. Furthermore, numerous studies have shown that the identification of these pathogens is a reliable index of risk that is clinically useful, especially in patients with aggressive and recurrent periodontitis, and enables to implement a targeted antibiotic therapy, further reducing the need for periodontal surgery [21-24]. Nonetheless, the actual efficacy and clinical importance of characterizing the gingival microflora in periodontal diagnosis and therapy seems to be still controversial. Although traditional microbiological culture techniques are still the gold standard in periodontal microbiology, because they represent a reliable system for characterizing subgingival bacteria, recently these methods have been supported by systems using immunoanalysis or the analysis of nucleic acids to identify and qualify the oral bacteria [25, 26]. These systems show a high degree of sensitivity and specificity and intend to overcome the limitations of bacterial culture techniques, such as the difficulty in culturing some bacteria and in isolating small quantities of bacterial samples, as well as the high costs involved [26].

Methods

The aim of our research was to identify by bacterial genomic DNA analysis the prevalence of five different species of periodontopathogenic bacteria present in the subgingival biofilm, specifically: *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Prevotella intermedia* (Pi), *Bacterioides forsythus* (Bf), *Treponema denticola* (Td).

For the analysis we used the systematic Multiplex-PCR-MICRODENT kit with species-specific primers that, through recent studies, have been shown to possess, within this type of techniques, high specificity for subgingival bacteria [27].

The study was carried out on a group of 48 subjects, 18 males and 30 females, from 18 to 78 years of age. The exclusion criteria included the presence of systemic diseases (diabetes), nicotine use and antibiotic therapy during the month prior to the test.

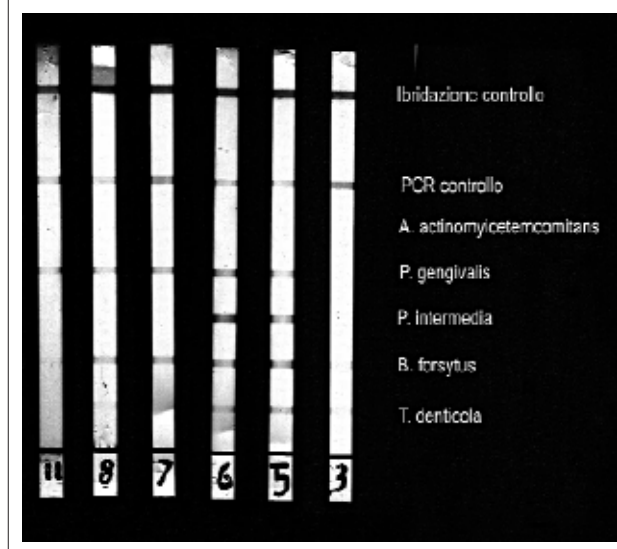
Each subject was subjected to a preliminary periodontal visit aimed at detecting the presence of periodontopathy by probing (pocket depth), routine periodontal indices (Loe-Silness gingival index and gingival bleeding index) and xographic tests (bone reabsorption) [29]. The patients, in line with other studies, were subdivided into two groups [26, 28]:

Group A: patients with active periodontal disease;

Group B: patients with no signs of periodontopathy.

A sample of subgingival plaque was then taken by inserting in the sulcus a tip of sterile paper for 20 seconds. In all patients the sampling sulcus was the vestibular gingival sulcus 4.6.

Fig. 1. Examples of inverse hybridisation with Microdent.



We then analysed these samples by Multiplex-PCR, inverse hybridisation for the identification of *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Prevotella intermedia* (Pi), *Bacterioides forsythus* (Bf), *Treponema denticola* (Td), which includes the following steps as detailed below.

The isolated DNA (DNA extraction from the small cone) is amplified (multiplex PCR) in the 16SrRNA region with biotin-labelled primers. The fragments of the amplified DNA were then used for the inverse hybridisation test, which starts by denaturing the amplified DNA.

Subsequently, the hybridisation buffer is added to the strip coaptated with 2 control lines and the specific probes for the 5 different species.

In the event that the amplified DNA belongs to an identified strain, it will bind to the corresponding and complementary probe during incubation in a shaking water-bath. After the addition of streptavidine, conjugated with alkaline phosphatase, to the hybrid formed by the probe and the biotin-labelled amplified DNA, the complex becomes visible upon addition of NBT/BCIP (Fig. 1).

Results

The results of the observations, the means for the overall sample and for the two groups examined, are reported in the following tables (Tabs. I, II).

The initial clinical screening, in line with other studies, enabled us to select in the group analysed 24 subjects with signs of active periodontopathy (Group A); the remaining 24 patients with no identifiable clinical evidence of the disease were used as the control group (Group B). In the overall sample examined, the PCR analysis was positive in 18 subjects evenly distributed between the two sexes.

Within the two experimental groups (A and B) examined, the test was positive in 75% of the subjects in group A, whereas the test was negative for all the subjects in group B. The distribution per age group as

Tab. I. Positive subjects in group A.

Age	Sex	Tooth	Pocket	Positive	<i>Actinobacillus actinomycetemcomitans</i>	<i>Porphyromonas gingivalis</i>	<i>Prevotella intermedia</i>	<i>Bacteroides Forsythus</i>	<i>Treponema denticola</i>
52	M	4-8	8 mm	+	+	+	+	+	+
64	M	4-7	7 mm	+	-	+	+	+	+
75	F	3-6	6 mm	+	-	+	-	+	-
63	M	4-6	6 mm	+	-	+	-	+	-
60	F	3-8	4 mm	+	+	+	-	-	-
37	F	4-6	6 mm	+	-	-	+	-	-
50	F	4-6	4 mm	+	-	+	+	-	-
47	M	4-6	6 mm	+	-	+	-	+	-
63	F	4-4	4 mm	+	+	+	-	-	-
18	F	4-4	5 mm	+	-	+	-	+	-
48	M	1-6	6 mm	+	-	+	-	-	-
27	M	4-6	4 mm	+	-	-	+	-	-
46	F	4-6	5 mm	+		+	+		
41	M	4-6	4 mm	+	+				
78	F	4-6	8 mm	+		+			+
41	M	4-6	6 mm	+	+				
22	F	3-5	6 mm	+	+				
48	M	3-5	7 mm	+			+	+	

detailed in Table II shows and confirms an increase in positive subjects with advancing age.

By observing the results obtained in the positive samples of group A (Tab. I) we can deduce that in one individual (5.5%) all the bacteria to which the test is sensitive were detected in the subgingival plaque whereas in another person (5.5%) we detected four species, in most of the cases (50%) we detected an association between two microbial species and only in 39% we detected the presence of only one bacterial species.

By a detailed evaluation of the group of periodontopathic patients positive to the test, in terms of the rate of detection of the individual species searched for, we observed in the group of periodontopathic patients a high rate of isolation of *Porphyromonas gingivalis* (66.6%); in most of the cases (91%) this bacterium was associated with another microbial species. In addition, we observed that *Prevotella intermedia* and *Bacteroides forsythus* are present individually in 38.86% whereas they coexist in 42.86% of the cases, on the other hand the presence of *Actinobacillus actinomycetemcomitans* (33.3%) and *Treponema denticola* (16.6%) is less frequent.

As reported in other studies, *Treponema denticola* was detected exclusively in periodontopathic patients with gingival pockets seven-eight mm deep [29].

Conclusions

Our study shows the reliability of the DNA-based Multiplex-PCR (Microdent) system, which allows the simultaneous identification of five different bacterial species that are potentially pathogenic for the periodont.

Although it is not possible to correlate with certainty the detection of specific bacterial species with the presence and progression of the periodontal pathology, in accordance with other preliminary studies that have tested similar systems, some conclusions can still be drawn [29, 30].

The isolation of the microorganisms analysed is always accompanied by periodontal lesions, whereas the test was always negative in the group of healthy patients.

Porphyromonas gingivalis, singularly and in association, is the most frequently identified bacterium in the subjects with periodontal disease [29].

Treponema denticola, absent in all the subjects with pockets less than five mm deep, is present in subjects with more serious periodontal situations [29].

The methods based on the analysis of nucleic acids for the identification of bacterial species seem, also in the light of the results of our study, valid substitutes for the traditional culture techniques [31].

The sensitivity and simplicity of this technique, as well

Tab. II. Distribution per age group in the subjects examined.

Age group	Positives	Positives	Negatives	Negatives	Totals	Totals
	M	F		F	M	F
15-40	1	3	5	15	6	18
> 41	8	6	4	6	12	12
Total	9	9	9	21	18	30

as the decrease in working times and the possibility of identifying nonculturable bacteria, since the presence of viable bacteria is not essential, make this technique indicated for the simultaneous identification of periodontopathogenic bacteria and may, in perspective, provide a more effective clinical alternative to the techniques of bacterial typing of the subgingival plaque.

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Improvement of these techniques and the identification of a definite etiopathogenetic correlation between bacteria and the activation and maintenance of periodontopathy may, consequently, allow more effective and timely preventive measures and, where necessary, a more specific pharmacological therapy, which would reduce the need for periodontal surgical techniques.

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■ Received on March 1, 2006. Accepted on September 7, 2006.

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