Deoxyribonucleic Acid Probes Analyses for the Detection of Periodontal Pathogens

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ABSTRACT

Background: In clinical microbiology several techniques have been used to identify bacteria. Recently, Deoxyribonucleic acid (DNA)-based techniques have been introduced to detect human microbial pathogens in periodontal diseases. Deoxyribonucleic acid probes can detect bacteria at a very low level if we compared with the culture methods. These probes have shown rapid and cost-effective microbial diagnosis, good sensitivity and specificity for some periodontal pathogens in cases of severe periodontitis.

Materials and methods: Eighty-five patients were recruited for the study. Twenty-one subjects ranging between 22 and 48 years of age fulfilled the inclusion and exclusion criteria. Seventy-eight samples became available for DNA probe analysis from the deepest pockets in each quadrant.

Results: All 21 patients showed positive results for *Prevotella intermedia*; also, *Prevotella gingivalis* was identified in 19 subjects, *Aggregatibacter actinomycetemcomitans* in 6 subjects. *P. intermedia* was diagnosed positive in 82% of the subgingival samples taken, 79% for *P. gingivalis*, and 23% for *A. actinomycetemcomitans*.

Conclusion: This study shows a high frequency of putative periodontal pathogens by using DNA probe technology, which is semi-quantitative in this study. Deoxyribonucleic acid probes can detect bacteria at very low level about 10^3 which is below the detection level of culture methods. The detection threshold of cultural methods.

Clinical significance: The three types of bacteria can be detected rapidly with high sensitivity by using the DNA probe by general practitioners, and thus can help in the diagnosis process and the treatment.

Keywords: *A. actinomycetemcomitans*, DNA probes, *P. intermedia*, *P. gingivalis*, Periodontal disease.

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INTRODUCTION

It is well known that periodontitis is an infectious disease caused by bacteria, notably Gram-negative species. The number of bacterial species that can inhabit periodontal lesions has been estimated at more than 500, of which about 50% can be cultured.¹ In recent years, only few bacteria have been associated with disease progression, the number of cultivable species is limited to less than 10 which include: *Aggregatibacter actinomycetemcomitans*, *Eikenella*, *Campylobacter*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Tannerella* and *Treponema*.^{2,3} The prevalence of periodontitis in human beings is about 30%, of whom 10 to 15% have a severe form of the disease.⁴⁻⁶

In oral microbiology several techniques have been used to identify bacteria. For the analysis of subgingival plaque, anaerobic bacteria culture has been the gold standard for many years.⁷ Recently, molecular microbial techniques have become available helping to identify quantity target organisms with high specificity and sensitivity. The technique of real-time polymerase chain reaction (RT-PCR) provides a new tool to detect oral pathogens,⁸ in addition to the DNA probe technique, which is also used as a detection method.

Microbiological tests are available to dentists in order to determine the presence of these microorganisms. There is a big discussion if these microbial tests can help the patients in clinical practices. Mombelli et al¹⁰ in a previous study, addressed the question of: Are the

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presence of these periodontal pathogens in a high level can be differentiated between subjects with chronic or aggressive periodontitis, and whether microbial testing could either indicate success or failure of conservative treatment earlier than a clinical assessment.¹¹ The most important aspect of any microbial test is its sensitivity and specificity in identifying these microorganisms; it is also important for it to be rapid and easy to use by dentists.

The aim of the present study is to describe the DNA technique in detecting three microorganisms *Porphyromonas gingivalis*, *Prevotella intermedia*, and *A. actinomycetemcomitans* in patients with minimal periodontal disease, defined by a mean probing depth <3.5 mm and the presence of less than 5% of pockets deeper than 5 mm.

MATERIALS AND METHODS

This was a descriptive and analytical cross-sectional study in which a quantitative DNA-probe data collection method was used. This study was conducted in accordance with the Helsinki agreement for research on humans; ethical clearance was obtained, and every participant signed an informed consent. Eighty-five patients in total (65 males and 20 females) ranging between 22 and 48 years of age were screened for participation in this study.

Inclusion criteria: Patients should have at least 20 teeth in their mouths with no history of dental care except extractions and fillings, the presence of minimal periodontal disease, defined by a mean probing depth <3.5 mm and the presence of less than 5% of pockets deeper than 5 mm.

Exclusion criteria: Patients with systemic diseases were excluded, as well as pregnant women. Patients with history of antibiotics or antiseptic treatments for the last 6 months were excluded also. Only 21 subjects who fulfilled the following clinical criteria, 16 males and 5 females (ranging between 29 and 33 years of age) were included in the study.

Plaque Sampling and Clinical Measurements

First, The plaque index (PI) Silness et al¹² was scored on four surfaces of every tooth. Then, subgingival microbiological samples were taken with paper-points from the deepest pocket in each quadrant as described in Mombelli et al^{7,13} Supragingival plaque was removed with sterile cotton pellets before sampling. The area was isolated and dried; one sterile paper point (Absorbent points 2715 fine, Johnson and Johnson, NJ, USA) was inserted to the bottom of a pocket for a period of 10 seconds.^{7,13} After sampling, probing of the pocket depth and recession were measured, bleeding on probing was determined on four surfaces of each tooth. One

single investigator (ZA) collected all clinical indices and samples. A standard periodontal probe (GF-w, Hu-Freddy, 11, USA) was used for periodontal probing.

DNA Probe Analysis of Bacteria

Commercially available DNA probes were used for specific identification and quantification of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* (Omni Gene, MA, USA; ANAWA Laboratories, Switzerland). The probes were used as described by French et al¹⁴ In brief, the processing of specimens of dental plaque for DNA probe analysis required disruption of the bacterial cells and the denaturing of the DNA molecules into two separate strands. This was accomplished by suspending the plaque specimens in a high pH solution (0.3% sodium dodecyl sulphate, 10 mM NaOH) and subsequent boiling. The samples were then equilibrated with loading buffer (1:1 [v/v] of 0.3 M NaOH, 3 M NaCl:2 M NH₄OAc), applied to nitrocellulose filters, rinsed in 0.5 M NaCl, air-dried and baked at 80°C for 2 hours. After prehybridization with Denhard's solution, containing 20 mg/ml of salmon sperm DNA, for 1 hour at 65°C, the filters were hybridized with the corresponding P 32-labeled DNA-probe for 4 hours with WASH I (0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4, 0.1% sodium dodecyl sulfate) and twice at 65°C for 15 minutes with WASH II (1 × SSPE = 0.18 M NaCl, 10 mM sodium phosphate buffer, 1 mM EDTA, pH 7.4, 0.1 per cent sodium dodecyl sulphate). The filters were then air-dried and exposed to Kodak X-OMAT AR film (Kodak, Switzerland) for 48 hours at -70°C using an intensifying screen. The developed X-ray films were recorded with a video camera and digitally processed using image analysis-software (Image 1.47, NIH, MD, USA). Each of the three filters for *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* contained 10⁵, 10⁴ and 10³ control-cells in triplicate, which were used as references for a standard curve. Each patient sample was measured and compared with the reference curve to give a numerical value representing the number of specific bacteria present in the plaque samples.

RESULTS

Clinical Parameters

Out of 85 subjects screened for participation in this study, 15 individuals were not included, because they were considered periodontally healthy, while 30 were excluded because they showed evidence of more than minimal periodontal disease. A further 19 subjects were not included because they missed one or more of the other criteria.

The age and sex of the subjects were recorded together with the use of antibiotics in the last 5 years, frequency of teeth brushing and smoking habits are listed in Table 1.

Table 1: Age, sex and origin, exposure to antibiotics in the last 5 years, frequency of tooth-brushing and smoking habits of the 21 study subjects

Patient	Age	Sex	Antibiotics last 5 years	Brushing	Smoking > 1 pack/day
1	23	M	No	1 × day	No
2	30	M	No	5 × week	Yes
3	25	M	No	1 × day	Yes
4	22	M	Yes	1 × day	Yes
5	31	F	Yes	2 × day	No
6	48	F	No	2 × day	Yes
7	29	M	No	2 × day	Yes
8	28	F	No	1 × day	Yes
9	30	M	No	4 × week	Yes
10	35	M	No	1 × day	Yes
11	26	F	No	2 × day	No
12	34	M	No	2 × day	No
13	29	M	Yes	1 × day	No
14	31	M	No	2 × day	No
15	29	M	No	1 × day	No
16	26	M	No	Never	No
17	30	M	No	3 × week	No
18	36	M	Yes	1 × day	Yes
19	21	F	No	3 × day	No
20	25	M	No	1 × day	No
21	28	M	No	2 × week	Yes

Table 2: Number of positive samples for *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg) and *P. intermedia* (Pi)

Patient	AA	PG	PI	Total number of samples
1	0	4	4	4
2	4	4	4	4
3	0	1	1	4
4	4	4	4	4
5	4	4	4	4
6	4	4	4	4
7	1	4	2	4
8	0	3	3	4
9	0	3	4	4
10	0	4	4	4
11	0	3	4	4
12	0	4	2	4
13	0	3	3	4
14	0	3	4	4
15	1	4	3	4
16	0	4	2	4
17	0	2	4	4
18	0	2	1	1 (3 sites)
19	0	0	3	4
20	0	2	1	1 (3 sites)
21	0	0	4	4
Total	18	62	64	78

The average plaque index was 1.17. The mean pocket depth was 2.70 mm, the mean recession amounted to 0.30 mm and the percentage of sites bleeding upon probing was 62% (Graph 1).

Microbiological Parameters

Table 2 showed the presence of the three periodontal pathogens *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*. 0.23% of all samples were *A. actinomycetemcomitans* positive, 79% were positive for *P. gingivalis* and 82% for *P. intermedia* (Graph 2). The amount and the association of the three marker organisms in the 78 sub-gingival samples from 21 patients are shown in Graph 3. Graph 3A shows the distribution of *P. gingivalis* and *P. intermedia* in *A. actinomycetemcomitans* negative samples (AA: nd, n = 60). Graph 3B shows the same relationship when *A. actinomycetemcomitans* is present at a level below 10^4 (AA: 10^3 Vn = 7). Graph 3C lists the 11 samples with *A. actinomycetemcomitans* at a level above 10^4 (Each square represents one sample and nd = not detected).

The statistic analysis showed highly significant Fischer's exact test). The majority of *P. gingivalis*—positive samples were also positive for *P. intermedia* (Table 3A); only 8% of all samples were positive for *P. gingivalis* but not for *P. intermedia* and only 6% were *P. intermedia*, but not *P. gingivalis* positive. Statistically, the association between these two species is highly significant Fischer's exact test). Table 3B shows the relationship between *P. gingivalis* and *A. actinomycetemcomitans*. This association was also statistically significant ($p = 0.016$) all *A. actinomycetemcomitans*-positive samples were also *P. gingivalis*—positive. No significant relationship was found between *P. intermedia* and *A. actinomycetemcomitans* (Table 3C).

Table 3: Presence of *P. gingivalis* in relation to presence of *P. intermedia* (A), *A. actinomycetemcomitans* in relation to *P. gingivalis* (B) and *A. actinomycetemcomitans* in relation to *P. intermedia* (C) in 78 samples evaluated

		<i>P. gingivalis</i>	
A		Found	Not found
<i>P. intermedia</i>	Found	56	6
	Not found	5	
		<i>P. gingivalis</i>	
B		Found	Not found
<i>A. actinomycetemcomitans</i>	Found	18	0
	Not found	44	16
		<i>P. intermedia</i>	
C		Found	Not found
<i>A. actinomycetemcomitans</i>	Found	17	13
	Not found	47

Pll	1.17	REC	0.3
PD	2.7	BOP	0.62

Graph 1: Mean values of clinical measurements in the 21 patients

DISCUSSION

Testing periodontal pathogens should be concentrated on a small group of patients. Subjects for whom microbial testing is indicated include:

- Patients with aggressive periodontitis.
- Patients ranging between 25 and 35 years of age, with rapid destruction of attachment and bone loss in relatively a short period of time.
- Patients who continue to lose attachment despite adequate treatment.

Before using any microbial tests we should complete the phase one of the therapy (mechanical treatment and oral hygiene control). This is an essential condition for using any tests, and to be useful these information since we believe that periodontal diseases are site specific disease, clinicians must get an overview on the first session before sampling. The sample should be collected in a second

Pg	79
1

Aa	23
1

Pi	82
1	

Graph 2: Frequency of *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg) and *P. intermedia* (Pi) in 78 subgingival samples

Graphs 3A to C: The amount and the association of the three marker organisms in 78 subgingival samples from 21 patients: (A) The distribution of *P. gingivalis* and *P. intermedia* in *A. actinomycetemcomitans* negative samples (AA: nd, n = 60). (B) The same relationship when *A. actinomycetemcomitans* is present at a level below 10^4 (AA: 10^3 , n = 7) and (C) The 11 samples with *A. actinomycetemcomitans* at a level above 10^4 . Each square represents one sample (nd: not detected)

session so as to avoid provocation of bleeding,¹⁶ from the deepest pocket. In this case, a diagnostic result can be representative of the entire mouth's condition.

Two different molecular genetics strategies have been introduced to detect periodontal pathogens in subgingival plaque, based on (1) genomic or oligonucleotide DNA Probes (2) PCR Test (Murray).

The aim of the study was to determine the associations between the three marker periodontopathogens (Aa, Pg, Pi) to evaluate their levels in subgingival plaque of patients with minimal periodontal disease and to describe our experience with commercial test systems for the detection of pathogens.

There is evidence linking *A. actinomycetemcomitans* and *P. gingivalis* with pathogenesis of periodontitis,^{17,18} also a positive correlation between both microbes indicates the existence of a synergistic association between *A. actinomycetemcomitans* and *P. gingivalis* as was previously described,¹⁹ the fact that two pathogens were present in health and disease indicates that the level of these bacteria is more significant with regard to the disease progression,²⁰ which supports the hypothesis that periodontal disease is

associated with a shift in the balance of the sub-gingival microbiota rather than the action of a single pathogen or a simple increase in diversity.²¹

There was a significant association between *P. gingivalis* and *A. actinomycetemcomitans* in the present study and also in several studies 8,13, and a highly association was observed between gingivalis and *P. intermedia* in 56 sites both species were found at the same time. In contrast,^{22,23} reported an inverse relationship between *P. gingivalis* and *P. intermedia*, with patients diagnosed with advanced periodontitis.

Culture, however, has several disadvantages. It is time consuming and has a low sensitivity this is because of the slow growth of some oral pathogens, and maybe for this reasons, several alternative techniques have been developed for the detection of periodontal pathogens, such as immunoassays,²⁴ DNA probes assays,²⁴⁻²⁶ and PCR assays.²⁷⁻³⁰

This study shows a high frequency of putative periodontal pathogens by using the DNA probe technology. The test is semi-quantitative. It has been reported that DNA probes can be detected at levels below the detection

threshold of cultural methods; it is specific, sensitive, rapid and easy to use by general practitioners. The present study demonstrates high prevalence of periodontal pathogens in a group of Middle-Eastern subjects with minimal periodontal disease. It may be assumed that the oral micro flora of these subjects developed over several years without any external disturbances since this group of patients has no exposure to dental care except extraction and fillings.

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