

PRELIMINARY RESULTS OF THE USE OF AN ULTRASONIC EQUIPMENT IN THE TREATMENT OF MODERATE PERIODONTITIS: clinical and microbiological real time polimerase chain reacion-based (PCR) study

*Resultados preliminares da utilização de um equipamento ultrassônico no
tratamento da periodontite moderada: estudo clínico e microbiológico com
reação da cadeia de polimerase em tempo real (PCR)*

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Abstract

OBJETIVE: This preliminary study aimed to evaluate clinically and microbiologically the treatment of patients with light to moderate periodontal disease by using an ultrasonic device. **MATERIAL AND METHODS:** Sixteen adults with moderate periodontitis were studied. A Real-time PCR protocol was optimised using specific primers for the three microorganisms. Clinical and microbiological testing was performed prior and following treatment. **RESULTS:** Despite clinical improvement, bacteria quantification didn't change. All the patients were positive for *P. gingivalis* and *P. intermedia* and nine for *A. actinomycetemcomitans*. **CONCLUSION:** VectorTM-system treatment showed clinical improvement. A Real-time PCR protocol is now available for periodontal diagnostics and monitorization. However, further studies with larger populations are indicated.

Keywords: Periodontitis. Piezo electric devices. Anaerobic bacteria. Microbiology. Real time PCR protocol.

Resumo

OBJETIVOS: Este estudo preliminar teve a finalidade de avaliar clinicamente microbiologicamente o tratamento de pacientes portadores de periodontite leve a moderada, utilizando-se um novo aparelho piezo-elétrico disponível no mercado (Vector™ system). **MATERIAL E MÉTODO:** Dezesesseis pacientes adultos, portadores de periodontite leve a moderada, foram submetidos ao tratamento. Utilizou-se um protocolo de PCR em tempo real utilizando primers específicos para três microrganismos. Realizaram-se avaliação clínica e um estudo microbiológico simultâneo antes e após os tratamentos. **RESULTADOS:** A avaliação clínica foi positiva, embora não tendo ocorrido alterações na quantidade de bactérias. Todos os pacientes apresentaram amplificação específica para *P. intermedia* e *P. gingivalis* e nove pacientes para *A. Actinomycescomitans*. **CONCLUSÃO:** Considerando-se os resultados deste estudo preliminar, tratamento com o aparelho testado mostrou eficácia clínica. O protocolo utilizado neste estudo está agora disponível para diagnóstico periodontal e monitoração de resultados de tratamento. Entretanto, indica-se estudos clínicos e microbiológicos mais aprofundados, com amostras maiores, para conclusões mais consistentes.

Palavras-chave: Periodontite. Aparelhos piezo elétricos. Bactérias anaeróbicas. Microbiologia. Protocolo PCR.

INTRODUCTION

Periodontitis is a chronic inflammatory disease characterized by connective tissue and alveolar bone destruction, eventually leading to tooth loss (1). The aetiology of periodontitis is thought to be multifactorial. Previous studies have established a connection between periodontitis and cardiovascular disease (2, 3) obesity (4) or rheumatoid arthritis (5). However, it is widely accepted to be primarily a result of infection by a group of specific bacteria from the subgingival flora, particularly Gram-negative anaerobes (1, 6, 7). Infectious disease is always an interaction between the microorganisms, the host and his risk factors (6). Different microorganisms have been implicated in periodontitis but all of them could also be present in healthy people (1, 6). Why, in some patients, those microorganisms cause disease, may have several explanations.

Without considering the variability of host response and only considering the microbiological aspect, two hypotheses can be made: one being that the quantity of microorganisms is imperative, as it is likely that irreversible host destruction occurs only when bacterial levels reach a critical threshold; or secondly that the type of microorganisms be the

leading cause of disease progression, i.e. different serotypes could have different pathogenicity patterns (8-11) According to the microbiological methods used in periodontitis diagnosis, different results could be obtained (7). Classic methods are based on culture and, especially in the case of anaerobic bacteria; they present low sensitivity and are not, in most cases, quantitative (12). Real time PCR, with species-specific primers, can provide a precise and sensitive method for more accurate quantification of individual species (7 - 15). *Porphyromonas gingivalis*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*, all of them anaerobic gram-negative rods have previously been implicated in periodontitis (1, 13, 16, 17).

In this study we optimized the PCR conditions for the detection and quantification of these bacteria. Samples were taken prior to and following a mechanical treatment Vector™, a new piezo-driven ultrasonic device that generates longitudinal oscillations. This method is only recommended on moderate periodontitis and its main effect seems to be the disorganization of the dental plaque (18). The relationship between the quantity of the bacterium and the periodontal status was investigated performing the optimised real time PCR protocol.

MATERIAL AND METHODS

Clinical evaluation and sample collection

Sixteen adult patients with moderate periodontitis, i.e., with destruction of connective tissue of 2 to 4 mm, dental mobility degree 1 or 2 and with dental pockets of 3-6 mm were selected. Relevant data such as age and sex were registered; smokers were excluded as well as patients with systemic diseases. None of the patients were under antimicrobial therapy at the time of this study.

Clinical evaluation was performed according to three parameters: bleeding index and plaque index, calculated as the percentage of affected zone relatively to the total mouth, and pocket depth, the medium value of the pockets depth. A sterile medium paper point was inserted into the 4 deepest pockets and removed 10 seconds after placement. The paper points of a single patient were pooled and stored frozen at -70°C in a sterile 2 ml microcentrifuge tube until posterior analysis.

Clinical and microbiology evaluation was done before mechanical treatment with Vector™ and after 2 and 6 weeks.

Bacterial strains used as controls

Bacterial cultures of *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* were kindly supplied by the Odontology School, Complutense University, Madrid. *Actinobacillus actinomycetemcomitans* was cultured in Dentaid medium (Dentaid® Barcelona) in microaerofilia atmosphere (Oxoid®) and *Porphyromonas gingivalis* and *Prevotella intermedia* were cultured in SCS® medium (BioMerieux®) in anaerobic conditions (Oxoid®). Concentrations of 6×10^7 CFU/ml of each bacterium were resuspended in PBS for DNA extraction.

DNA extraction

The Qiagen® method was performed according to QIAamp DNA Mini Kit protocol (from Qiagen®) from paper points samples and from bacterial cultures. These were previously incubated in 400 µl PBS, at 25°C , 150 rpm, and overnight. To

calculate the concentration of DNA extracted, the absorbance was measured by spectrophotometry (Biophotometer 6131, Eppendorf) at 260 nm (A260). A ratio between A260 and A280 was obtained to determine the DNA quality. DNA solutions were stored at -20°C for posterior analysis.

Primers for real-time PCR

Primers published by Morillo et al. (14) were used for *A. actinomycetemcomitans* and were designed for *P. gingivalis* and *P. intermedia* using the programme "Primer Select 3.10" (Dnastar Inc). For *A. actinomycetemcomitans* the primers were designed from leukotoxin C gene promoter region (LktC) (National Center for Biotechnology Information – NCBI, GenBank, accession number: U51862) (Table 1); for *P. gingivalis* from the specific arginin-gene (Arg-gingipain) (NCBI accession number: D64081); for *P. intermedia* the gene Dipeptidil-peptidase IV (NCBI accession number: AB127116). All the primers were confirmed using the BLAST programme (Basic Local Alignment Search Tool) (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the "MegAlign 3.16" programme to confirm the respective gene sequences. All the primers were resuspended in TE solution (Tris HCl 10 mM, TrisBase, 1mM Na₂EDTA, pH 8.0) according to manufacturer instructions (Operon).

Real-time PCR conditions

All the PCR reactions were performed using Light Cycler from Roche Molecular Biochemicals and the parameters were determined according to the melting point of the primers, size and the enzyme used in the reaction. Each experiment was analyzed using Version 3.5 of Light Cycler software. The parameters analysed and used to evaluate each experiment were: cycle threshold (C_T), melting temperatures (T_M) of amplified products, the coefficient of regression and the error. The slope of the standard curve was used to determine the efficiency of the reaction. Using bacterial cultures, different final concentrations of the primers were assayed (0.8 µM, 0.6 µM and 0.4 µM) and to confirm the specificity of the primers they were tested with the three bacteria. To design the standard curve for further quantification, serial dilutions of each

bacterial DNA were prepared from a pre-determined concentration, which were used in the PCR experiments.

For *A. actinomycetemcomitans* ten different concentrations were used: 1.4×10^5 UFC/ml, 6.9×10^4 UFC/ml, 3.4×10^4 UFC/ml, 1.7×10^4 UFC/ml, 8.6×10^3 UFC/ml, 4.3×10^3 UFC/ml, 2.1×10^3 UFC/ml, 5×10^2 UFC/ml and 2.5×10^2 UFC/ml. The total volume of the reaction was 20 μ l, being 10 μ l of SYBR Green (QuantiTect SYBR[®] Green PCR kit Qiagen), 1 μ l of forward primer 0.8 μ M (Operon[®]), 1 μ l of reverse primer 0.8 μ M, 7 μ l from each of the DNA dilutions and 1 μ l of water (Qiagen[®]). The PCR program used: 1 cycle of 10 minutes at 95°C to activate the enzyme Taq Polymerase Hot Start; 40 cycles of 15 seconds at 95°C, 30 seconds at 55°C and 10 seconds at 72°C.

For *P. gingivalis* eight different concentrations were used to design the standard curve: 2.4×10^5 UFC/ml, 1.2×10^5 UFC/ml, 5.9×10^4 UFC/ml, 2.9×10^4 UFC/ml, 1.5×10^4 UFC/ml, 7.4×10^3 UFC/ml, 3.7×10^3 UFC/ml and 1.8×10^2 UFC/ml. The total volume of the reaction was 20 μ l being 10 μ l of SYBR Green, 1 μ l of forward primer 0.6 μ M, 1 μ l of reverse primer 0.6 μ M, 5 μ l of each dilution of DNA and 3 μ l of water. The PCR program used: 10 minutes to 95°C; 40 cycles of 15 seconds, 30 seconds at 60°C and 15 seconds at 72°C.

For *P. intermedia* eight different concentrations were used: 3×10^5 UFC/ml, 1.8×10^5 UFC/ml, 8.3×10^4 UFC/ml, 4.1×10^4 UFC/ml, 2×10^4 UFC/ml, 1×10^4 UFC/ml, 5×10^3 UFC/ml and 3×10^2 UFC/ml. The total volume of the

reaction was 20 μ l, being 10 μ l of SYBR Green, 1 μ l of forward primer 0.6 μ M, 1 μ l of reverse primer 0.6 μ M, 8 μ l of bacteria DNA. The PCR program used: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 25 seconds at 60°C and 20 seconds at 72°C.

For all three bacteria to determine the melting curve a final step, post amplification, was included, with continuous fluorescence acquisition, for 1.30 min, ranging the temperature from 65°C to 95°C.

For each experiment, three known concentrations of specific bacterial DNA were run in duplicated to quantify clinical samples.

Statistic analysis

Clinical and microbiology results, before and after treatment, were correlated using T-student software version SPSS 13.0 for Windows.

RESULTS

The patient's ages were between 43 and 53 years (11 male and 5 female). All the patients presented a bleeding index superior to 25% and in two patients more than 90%. Relatively to plaque index, most patients presented values superior to 35%. The pocket depths were between 2.8 and 5.1 mm. After treatment, the bleeding index and the plaque index presented significant improve ($p < 0.001$). However the pocket depth didn't show a significant reduction ($p = 0.202$) (Table 2).

TABLE 1 - Primers used for identification of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia*

Bactéria	Primer	Sequência 5' → 3'	Gene
<i>Aggregatibacter actinomycetemcomitans</i>	Forward	ACG CAG ACG ATT GAC TGA ATT TAA	Leucotoxin C gene promotor (LktC)
<i>Aggregatibacter actinomycetemcomitans</i>	Reverse	GAT CTT CAA GCT ATA TGG CAG CTA	Leucotoxin C gene promotor (LktC)
<i>Porphyromonas gingivalis</i>	Forward	CCT ACG TGT ACG GAC AGA GCT ATA	Arginin specific cystein proteinase (Arg gingipain)
<i>Porphyromonas gingivalis</i>	Reverse	AGC ATC GCT CAG CGT AGC ATT	Arginin specific cystein proteinase (Arg gingipain)
<i>Porphyromonas gingivalis</i>	Forward	CCT ACG TGT ACG GAC AGA GCT ATA	Arginin specific cystein proteinase (Arg gingipain)
<i>Porphyromonas gingivalis</i>	Reverse	GCG GAG CAA AGT TTA CTA CCT GAC	Arginin specific cystein proteinase (Arg gingipain)
<i>Prevotella intemedia</i>	Forward	GCT CAA TGG GTG CAG GTG GTA TCT	Dipeptidil peptidase IV
<i>Prevotella intemedia</i>	Reverse	CGA AAG ACC AAG TTG AGG CTG CC	Dipeptidil peptidase IV

TABLE 2 - Clinical data of the patients before and after treatment

Patient	Age	Sex	Plaque Index		Bleeding Index		Pocket depth	
			Before Treatment	After Treatment	Before Treatment	After Treatment	Before Treatment	After Treatment
1	41	Male	82,14	22,75	27,26	4,21	2,70	2,80
2	64	Male	77,13	24,82	77,28	21,83	3,60	3,90
3	54	Male	69,21	27,94	67,19	12,50	4,00	3,20
4	64	Female	69,77	31,77	69,27	19,87	3,70	3,20
5	52	Male	95,14	18,13	70,86	14,01	3,50	2,90
6	43	Male	24,98	16,77	28,13	6,76	3,00	2,70
7	52	Female	55,21	23,05	28,02	13,08	3,20	3,00
8	48	Female	68,75	30,73	93,75	11,98	3,50	2,90
9	41	Male	64,04	25,60	71,80	19,79	3,40	2,90
10	45	Male	29,69	21,81	31,08	8,36	3,10	3,02
11	43	Female	100,00	26,76	100,00	7,19	3,40	2,10
12	53	Female	69,79	20,99	61,46	8,33	4,30	3,50
13	44	Male	22,00	16,95	62,50	11,46	3,50	2,80
14	44	Male	69,70	13,00	78,13	8,33	5,10	3,60
15	43	Male	40,10	12,50	28,65	8,33	3,10	2,65
16	45	Male	70,83	21,95	60,42	9,90	3,70	3,04

For most of the clinical samples the concentration of DNA extracted was not very high, being the ratio A260 and 280 between 1.7 and 2.0 (Data not shown). Real Time PCR reactions presented values of slope, error and regression coefficient allowed. There was no cross-reaction between primers of different bacteria.

For each patient the results of amount of bacteria were expressed according to the bacterial

standard curves used in each experiment, being the range of efficiency accepted between 80%-100%, corresponding to a slope of 3.9 - 3.3, respectively.

Relatively to *A. actinomycetemcomitans* nine of the 16 patients (56%) showed amplification (Figure 1). In all that patients the two other bacteria were present. All of the patients showed amplification for *P. gingivalis* and *P. intermedia* and, in some patients, with values superior to 10^5 CFU/ml (Figure 2 and 3).

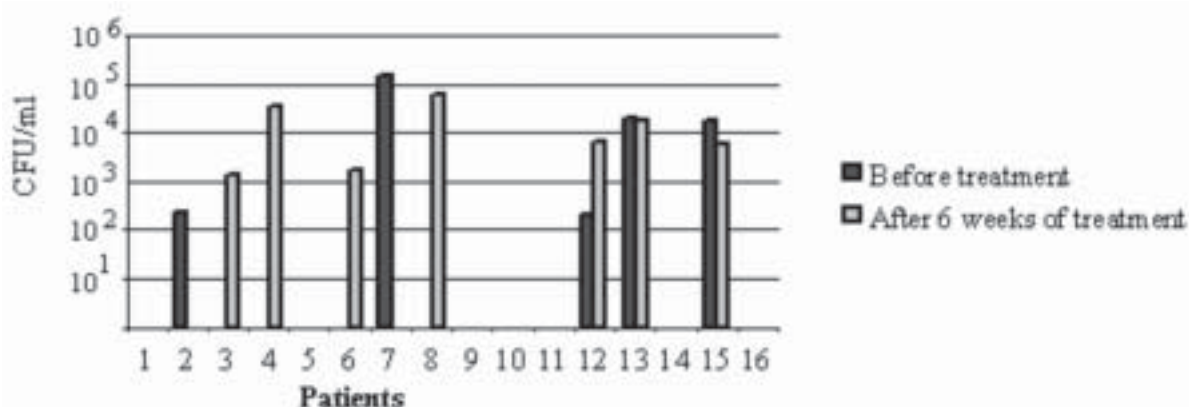


FIGURE 1 - Results of the colony forming units (CFU/ml) of the different clinical samples related to *Aggregatibacter actinomycetemcomitans* obtained by Real Time PCR

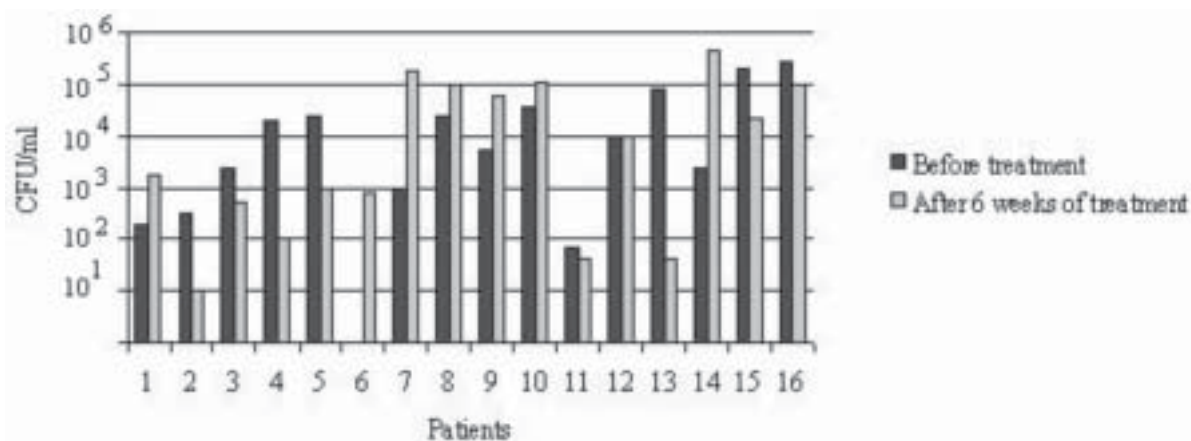


FIGURE 2 - Results of the colony forming units (CFU/ml) of the different clinical samples related to *Porphyromonas gingivalis* obtained by Real Time PCR

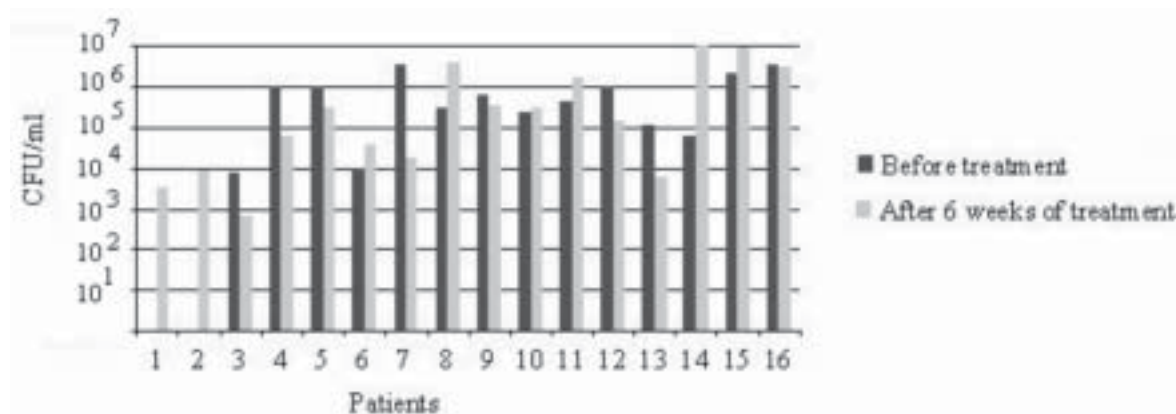


FIGURE 3 - Results of the colony forming units (CFU/ml) of the different clinical samples related to *Prevotella intermedia* obtained by Real Time PCR

Despite an expected reduction in the amount of bacteria, this was not constant. There was no significant correlation between clinical and laboratory results.

DISCUSSION AND CONCLUSION

To better understand periodontitis, it is important to examine the relationship between the periodontopathic bacterial population and periodontal status. Many investigations have established a correlation between the presence of some bacteria and pocket depth (14, 19, 20) but an optimised method for the detection and quantification of bacteria urged. In this study, the detection and quantification of three bacteria

was optimised and used in clinical samples for evaluation of Vector™ treatment.

The QIAmp DNA minikit showed to be a practical and good method for the extraction of DNA. The great prevalence of *P. gingivalis* and *P. intermedia* showing values superior to 10⁵ CFU/ml confirms findings by other authors especially in the case of *P. gingivalis* (15, 21, 22). There are several studies showing that, patients with periodontic disease have an increase in the amount of bacteria compared with healthy subjects (13, 22). Others correlated the depth of the pocket with the amount of bacteria (23). The result showing that few patients presented *A. actinomycetemcomitans* also agrees with most authors as this bacteria is more prevalent in juvenile periodontitis (8).

As most patients showed a clinical improvement, a reduction on the amount of bacteria would be expected. There was a reduction in some cases but this was not consistent in all of them. The depth of the pocket, the clinical parameter that better correlates with the bacterial amount, did not reduce significantly in these patients. The Vector™ treatment seems to have a great effect on reducing bleeding and inflammation but not on reducing the amount of bacteria or the depth of the pockets. Sample collection is a crucial aspect that interferes with the results and should be standardized. Control testing was performed 6 weeks after treatment, which could be present to be a short period for PCR evaluation.

Real time PCR with SYBR® Green showed to be sensitive and reliable without being a very expensive method. This study demonstrates an optimised protocol for detecting and quantifying anaerobic pathogens associated with periodontitis.

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