PREVALENCE OF PUTATIVE PERIODONTOPATHOGENS FROM PERIODONTAL PATIENTS AND HEALTHY SUBJECTS IN SÃO PAULO, SP, BRAZIL

Mario J. AVILA-CAMPOS(1) & Gustavo VELÁSQUEZ-MELÉNDEZ(2)

SUMMARY

A. actinomycetemcomitans, *B. forsythus, P. gingivalis, C. rectus, E. corrodens, P. intermedia, F. nucleatum,* and *T. denticola* were identified from subgingival plaque from 50 periodontal patients and 50 healthy subjects. PCR products from each species showed a specific band and could be used to identify periodontal organisms from clinical specimens. Identical negative or positive results between PCR and culture occurred in 66% (*A. actinomycetemcomitans*) to 93% (*F. nucleatum*) of the samples. PCR detection odds ratio values for *A. actinomycetemcomitans*, *B. forsythus*, *C. rectus*, *E. corrodens*, *P. intermedia*, and *T. denticola* were significantly associated with disease having a higher OR values for *B. forsythus* (2.97, 95% CI 1.88 – 4.70). Cultures showed that *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* were associated with periodontitis, however, *P. gingivalis*, *C. rectus*, *E. corrodens* and *F. nucleatum* were not significantly associated with the disease.

KEYWORDS: Periodontal pathogens; Periodontal disease; PCR.

INTRODUCTION

In oral microbiology it is essential to identify and characterize the various microorganisms involved in the different oral infections and such information will be useful for monitoring the disease progression[6].

It is known that periodontal pockets can harbor more than 300 different bacterial species[3, 9]. Organisms such as *Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia* or *Eikenella corrodens* have been considered to have a role in periodontal disease[9,10]. However, most of these organisms can also cause serious nonoral infections[13].

Several studies have demonstrated a strong correlation between the presence of putative periodontal organisms and the destruction of periodontal tissues[4,10].

Different methods of detecting phenotypic or genotypic variation have been used to characterize periodontal pathogens and differentiate them from commensal strains[4].

Molecular methods are currently available for the identification and subtyping of bacterial strains such as *A. actinomycetemcomitans* and *P. gingivalis*, but they vary in efficiency and the amount of required labor[5,7]. Techniques used to identify putative anaerobic pathogens from destructive periodontal disease such as bacterial culture are somewhat cumbersome and expensive[12].

Polymerase chain reaction (PCR) has been used for direct identification of periodontal pathogens in subgingival specimens[2,11] and also for elucidating the role of specific bacteria in the periodontal disease because of the ability to accurately detect specific species in mixed populations. In Brazil, the polymerase chain reaction (PCR) is not routinely used for the identification of periodontal microorganisms.

Specific primers are powerful molecular tools for the identification, typing or detection of clinically important microorganisms. 16S rRNA gene amplification has been used for detection of microorganisms that can not be cultivated, but these methods have given cross reactions with related organisms[11].

The goal of the present study was to detect putative periodontal microorganisms from clinical subgingival and gingival sulcus samples using conventional culture and 16S rRNA gene-based detection PCR methods.

MATERIAL AND METHODS

1. Patients and bacterial strains: Fifty periodontal patients (adult periodontitis) were selected from the School of Dentistry, at the University of São Paulo (São Paulo, SP, Brazil). All the patients showed clinical and radiographic evidence of alveolar loss confined to the molar and incisor teeth and periodontal pockets depth equal to or exceeding 5 mm. Fifty healthy subjects without clinical signs of periodontal disease were also selected. Characteristics of both of the studied groups are shown in

(1) Department of Microbiology, Institute of Biomedical Science, University of São Paulo, São Paulo, SP, Brazil.
(2) Public Health Department, School of Nursing, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.
Correspondence to: Dr. Mario J. Avila-Campos. Department of Microbiology, University of São Paulo, Av. Prof. Lineu Prestes 1374, 05508-900 São Paulo, SP, Brazil. Phone: +55-11-3818-7344; Fax: +55-11-3818-7354; E-mail: mariojac@icb.usp.br.

Table 1. None of the periodontal patients or controls had received antibiotics for the least three months prior sample collection. Briefly, supragingival plaque was removed from teeth with cotton rolls. Subgingival samples were obtained from one periodontal site by three fine sterile paper points (Dentsply Ind. and Co. Ltd., RJ, Brazil), inserted to the depth of the pocket or sulcus and left in place for 60s. The paper points were transported in VMGA III medium[8]. Samples were subcultured within 2h of collection. All samples were dispersed by vortex-mixing at maximal setting for 60s and 10-fold dilutions were prepared. Aliquots of 0.1 ml of undiluted and 10^{-3} diluted sample solution were plated onto trypticase soy (Difco Laboratories)-horse serum-bacitracin-vancomycin (TSBV) for *A. actinomycetemcomitans*, Omata & Disraely agar for *F. nucleatum*, Brucella blood agar (Difco Laboratories) (5% defibrinated sheep blood) supplemented with hemin (0.0001 mg/ml) and menadione (0.0001mg/ml) for *B. forsythus, C. rectus, P. gingivalis* and *P. intermedia*, and Brucella blood agar with 0.1% clindamycin for *E. corrodens*. No growth medium was used for *T. denticola*. The TSBV medium and Omata & Disraely agar were incubated for 3 days, and Brucella blood agar was incubated for seven days, at 37 ºC in atmosphere with 90% N_2 + 10% CO_2. Suspected colonies were selected from each sample and all the isolates were identified by colonial and cellular morphology (Gram) and by using conventional biochemical tests or ID 32-A kits (bioMérieux). Cultures were maintained on Brucella blood agar and stored at –70 ºC.

2. DNA extraction and analysis: From each clinical sample collected in VMGA III, 500 µl of sample was mixed with 500 µl of sterile Milli-Q water and washed twice at 12,000 rpm for 10 minutes. The pellet was resuspended in 500 µl of Milli-Q water and boiled for 10 minutes. After centrifugation (14,000 rpm, 10 minutes) the supernatant was saved and transferred to a new tube and used as template.

3. PCR detection: Fifty clinical samples obtained from patients with adult periodontitis and 50 samples from healthy subjects were included in the PCR analysis. Primers (Table 2) were designed in accordance with ASHIMOTO *et al*.[1] and were synthesized in the Biotechnology Core Facility Branch, Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). PCR amplification was performed in volumes of 25 µl containing 1X PCR/Mg++ buffer (Boehring Mannheim, Indianapolis, IN, USA), 0.2 mM each of dNTP (Pharmacia Biotech, Piscataway, NJ, USA), 0.5 U *Taq* DNA polymerase (Boehringer Mannheim), 0.4 µM of each primer pair and 10 ng of template. Amplification was performed in a DNA Thermal Cycler (Perkin Elmer, GeneAmp PCR System 2400, Norwalk, CT, USA) programmed for 94 ºC (5 min), followed by 30 cycles at 94 ºC for 30 seconds; annealing temperature adequate for each primer pair (Table 2) for 30 seconds; 72 ºC for 30 seconds, then 72 ºC (5 min) to allow the completion of DNA extension. A negative control without template DNA was included in each PCR run. Amplification products were compared by electrophoresis in 1% agarose gel in 1X TBE (1 M Tris, 0.9 M boric acid, 0.01 M EDTA, pH 8.4) buffer (Gibco BRL, Life Technologies, Ltd., Bethesda, MD, USA), stained with ethidium bromide (0.5 mg/ml), and photographed on a UV light transilluminator (Kodak Digital Science System 120). Molecular mass standard 1-Kb ladder (Gibco BRL) was included.

4. Statistical Analysis: All statistical analyses were performed using an Epi-info® 6.04 sofware (CDC/WHO). Standard methods to calculate descriptive statistics were used. The association between microbial detection by culture and PCR and periodontal disease was tested using a chi-square test (p values are two-sided and $\alpha = 0.05$) and the odd ratios were calculated (OR, 95% confidence interval). Cross tabulation to determine the matching results between culture and PCR for organisms detection using healthy subjects and periodontal patients ($n = 100$) was used.

RESULTS

Bacterial prevalence by PCR. Primer pairs were evaluated for identification of different putative periodontopathogens. Amplified products using bacterial DNA were compared and all primer pairs produced specific amplicons of predicted sizes (Fig. 1). Bacterial detection by PCR in the healthy and periodontitis subjects was 70% and 90% for *A. actinomycetemcomitans*, 92% and 96% for *F. nucleatum,* 66% and 78% for *P. gingivalis* and, 64% and 84% for *P. intermedia* (Table 3). When conventional culture was used for microbial detection from healthy subjects and patients we observed the following isolation rates: 48% and 80% for *A. actinomycetemcomitans*, 60% and 70% for *P. gingivalis*, 64% and 64% for *E. corrodens*, 84% and 90% for *F. nucleatum*, 50% and 74% for *P. intermedia*, 46% and 60% for *C. rectus*, 10% and 40% for *B. forsythus*. In addition the odds ratios (OR, 95% confidence interval-CI) for periodontal patients compared to control is also given in Table 3. Organisms detected by PCR: *A. actinomycetemcomitans, B. forsythus, C. rectus, E. corrodens, P. intermedia*, and *T. denticola* were significantly associated with periodontal disease with *B. forsythus* having the highest OR (2.97, 95% CI 1.88 – 4.70). Bacterial isolation by culture detection showed that *A. actinomycetemcomitans, B. forsythus* and *P. intermedia*, were associated with periodontal disease, but, *P. gingivalis, C. rectus, E. corrodens* and *F. nucleatum* were not significantly associated with the disease.

Table 1
Characteristics of periodontal patients and healthy subjects analyzed

	Periodontal patients ($n = 50$)		Healthy subject ($n = 50$)	
	Average (%)	Range	Average (%)	Range
Age (years)	45.5	18 – 65	32.3	18 – 51
Race:				
White	40	-	40	-
Black	9	-	10	-
Oriental	1	-	-	-
Periodontal pocket (mm)	8.4	6 – 13	-	-

Table 2

Nomenclature, composition and annealing temperature of the species-specific primer pairs for PCR

Primers	Oligonucleotide sequence 5′→3′	PCR annealing temperature (ºC)	Amplicon length (Kb)
A. actinomycetemcomitans			
	GCT AAT ACC GCG TAG AGT CGG		
	ATT TCA CAC CTC ACT TAA AGG T	50	0.5
B. forsythus			
	GCG TAT GTA ACC TGC CCG CA		
	TGC TTC AGT GTC AGT TAT ACC T	60	0.6
C. rectus			
	TTT CGG AGC GTA AAC TCC TTT TC		
	TTT CTG CAA GCA GAC ACT CTT	50	0.6
F. nucleatum			
	ATT GTG GCT AAA AAT TAT AGT T		
	ACC CTC ACT TTG AGG ATT ATA G	40	1.0
E. corrodens			
	CTA ATA CCG CAT ACG TCC TAA G		
	CTA CTA AGC AAT CAA GTT GCC C	45	0.7
P. gingivalis			
	AGG CAG CTT GCC ATA CTG CG		
	ACT GTT AGC AAC TAC CGA TGT	60	0.4
P. intermedia			
	TTT GTT GGG GAG TAA AGC GGG		
	TCA ACA TCT CTG TAT CCT GCG T	50	0.6
T. denticola			
	TAA TAC CGA ATG TGC TCA TTT ACA T		
	TCA AAG AAG CAT TCC CTC TTC TTC TTA	60	0.3

Kb: kilobases.

Table 3

Percentage of positive samples and odds ratios (confidence interval-CI 95%) for each test organism in subgingival plaque samples from periodontally healthy and periodontitis subjects

Microorganism	Culture detection				PCR detection			
	Healthy subjects	Periodontal disease	OR (CI 95%)	*P* Value	Healthy subjects	Periodontal disease	OR (CI 95%)	P Value
A. actinomycetemcomitans	48	80	1.93 (1.32-2.8)	0.00177	70	90	1.71 (1.20-2.44)	0.024
B. forsythus	10	40	3.0 (1.34-6.710)	0.001	30	82	2.97 (1.88-4.70)	0.00047
P. gingivalis	60	70	1.24 (0.84-1.83)	0.290	66	78	1.32 (0.90-1.96)	0.260
C. rectus	46	60	1.32 (0.89-1.96)	0.220	48	80	1.93 (1.32-2.80)	0.0017
E. corrodens	64	64	1.0 (0.66-1.50)	0.830	50	80	1.86 (1.28-2.69)	0.0033
P. intermedia	50	74	1.63 (1.12-2.39)	0.023	64	84	1.60 (1.11-2.31)	0.040
F. nucleatum	84	90	1.27 (0.79-2.06)	0.55	92	96	1.36 (0.75-2.49)	0.67
T. denticola	—	—	—	—	32	60	1.81 (1.16-2.83)	0.000

Comparison between PCR and culture. The percentage of identical results comparing PCR and culture (PCR-positive and culture-positive or PCR-negative and culture-negative) occurred from 93% for *F. nucleatum* to 66% for *A. actinomycetemcomitans* in healthy and periodontal subjects, respectively. The major discrepancy between PCR and culture (PCR-positive and culture-negative or PCR-negative and culture-positive) were several ranging from 55% for *A. actinomycetemcomitans* to 7.4% for *F. nucleatum* (Table 4).

DISCUSSION

The use of 16S rRNA gene-based PCR detection was able to identify unique regions suitable for PCR application. PCR was able to distinguish microorganisms with considerable phenotypic similarity such as *A. actinomycetemcomitans* and *H. aphrophilus,* species found in subgingival plaque. These organisms are inhabitants of periodontal pockets and are difficult to distinguish by conventional culture[1]. A series of PCR

Table 4

Microbial detection by culture and PCR in 50 gingival samples from healthy subjects and 50 periodontal disease patients

Microorganism	PCR (-)				PCR (+)				Identical results %
	Culture (-)		Culture (+)		Culture (-)		Culture (+)		
	n	%	n	%	n	%	n	%	
A. actinomycetemcomitans	11	55	9	45	25	31.3	55	68.8	66
B. forsythus	43	97.7	1	2.3	32	57.1	24	42.9	67
P. gingivalis	27	96.4	1	3.6	8	11.1	64	88.9	91
C. rectus	34	94.4	2	5.6	13	20.3	51	79.7	85
E. corrodens	21	60	14	40	15	23.1	50	76.9	71
P. intermedia	26	100	0	0	12	16.2	62	83.8	88
F. nucleatum	6	100	0	0	7	7.4	87	92.6	93
T. denticola	-	-	-	-	-	-	-	-	-

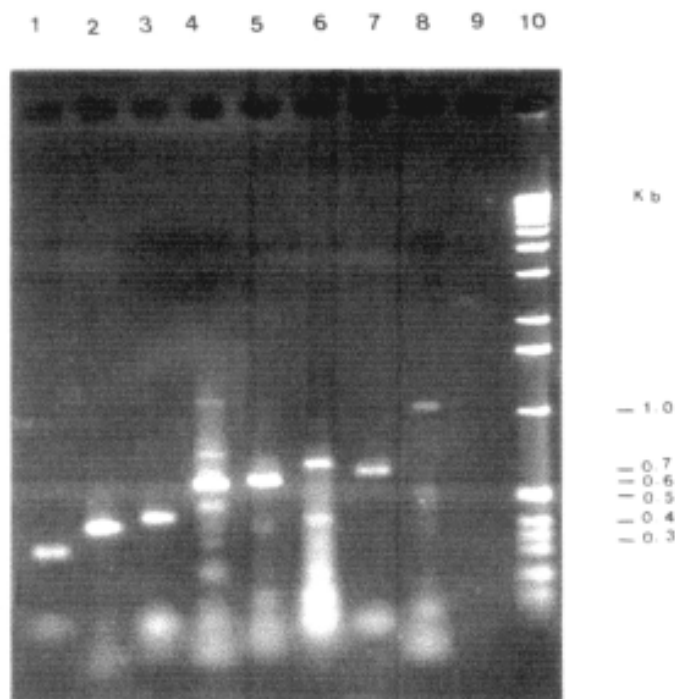(+): positive; (-) : negative; n: number of samples

Fig. 1 - PCR products to different periodontopathogenic microorganisms using various primer pairs. Lane 1, *T. denticola*; lane 2, *P. gingivalis*; lane 3, *A. actinomycetemcomitans*; lane 4, *P. intermedia*; lane 5, *C. rectus*; lane 6, *E. corrodens*; lane 7, *B. forsythus*; lane 8, *F. nucleatum*; lane 9, negative control; lane 10, 1-Kb DNA ladder.

amplification products with different sizes ranging between 0.3 to 1 Kb for every used primer pairs were obtained. PCR analysis was used sucessfully to identify different periodontopathogens associated with human periodontitis such as *A. actinomycetemcomitans*, *B. forsythus*, *P. gingivalis, P. intermedia*, *C. rectus, E. corrodens, F. nucleatum* or *T. denticola* (Fig. 1). It was valuable for identification of bacterial species with high degree of phenotypic and genotypic similarity. For example, *A. actinomycetemcomitans* and *H. aphrophilus*, share many characteristics and are both found in subgingival microbiota. On the other hand, further studies should be done to demonstrate the value of 16S PCR for species-specific detection of *A. actinomycetemcomitans* in clinical samples (in VMGA III medium) from patients with periodontitis.

Studies have shown that hemoglobin or other compounds in subgingival specimens do not significantly inhibit the DNA amplification reaction[2]. The presence of dead bacteria within the sample may have increased the detection using the PCR methodology, but it is unlikely that dead cells remain in the subgingival biofilm for very long, being degraded and disposed of by the other bacteria[4].

The prevalence of *A. actinomycetemcomitans*, *B. forsythus*, *C. rectus*, *E. corrodens*, *P. intermedia* and *T. denticola* was significantly higher by PCR detection ($p \leq 0.05$). *F. nucleatum* was the most commonly detected species in both the healthy and periodontal subjects by either method of detection. Also, *A. actinomycetemcomitans, P. gingivalis, C. rectus, E. corrodens* and *P. intermedia*, were prevalent in both of subject groups, and it may suggest that these six organisms (including *F. nucleatum*) may be part of the oral indigenous microbiota. Also, these organisms may be considered endogenous pathogens that occasionally contribute to the development of periodontitis. Our data, also revealed a lower prevalence of *B. forsythus* and *T. denticola* in healthy subjects suggesting that these pathogens may be increased in periodontal infectious processes.

Higher odds ratios observed for these detected periodontal organisms might suggest a symbiotic relationship in periodontal pockets producing a destructive disease without interacting with each other. Although, a suitable therapeutic to obtain a bacterial elimination may be of great clinical benefits[1]. In addition, the use of specific primers represent an appropriate tool to identify or detect periodontopathogens from mixed bacterial populations.

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RESUMO

Prevalência de periodontopatógenos em pacientes com doença periodontal e indivíduos sadios de São Paulo, SP, Brasil

Cepas de *A. actinomycetemcomitans, B. forsythus, P. gingivalis, C. rectus, E. corrodens, P. intermedia, F. nucleatum* e *T. denticola* foram identificadas da placa subgengival de 50 pacientes com doença periodontal e 50 indivíduos sadios. Os produtos de PCR de cada espécie microbiana mostraram bandas específicas, favorecendo a identificação direta de bactérias periodontais de espécimes clínicos. Resultados negativos ou positivos idênticos entre PCR e a cultura ocorreram de 66% (*A. actinomycetemcomitans*) a 93% (*F. nucleatum*) das amostras. A detecção de *A. actinomycetemcomitans, B. forsythus, C. rectus, E. corrodens, P. intermedia* e *T. denticola* por PCR foi significativamente associada com a doença, mostrando valores altos de odds ratio (OR) para *B. forsythus* (2,97, 95% CI 1,88 – 4,70). Através da cultura foi observada a associação de *A. actinomycetemcomitans, B. forsythus* e *P. intermedia* com a periodontite, entretanto, *P. gingivalis, C. rectus, E. corrodens* e *F. nucleatum* não foram significativamente relacionados à doença.

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