DETECTION OF *PORPHYROMONAS GINGIVALIS* AND *STREPTOCOCCUS INTERMEDIUS* IN CHRONIC PERIODONTITIS PATIENTS BY MULTIPLEX PCR

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ABSTRACT

A Multiplex PCR assay for the detection of Porphyromonas gingivalis and Streptococcus intermedius in chronic periodontitis is presented. A total of 180 samples from 65 adults with untreated periodontitis and 17 healthy volunteers were taken and processed in a simple boiling step. Cell lysates were used as DNA source for multiplex PCR assays. Primers were designed from 16S rRNA gene sequences from the GenBank-EMBL database showing specificity for target pathogens.

This multiplex PCR system could detect 8.2 P. gingivalis and S. intermedius cells. In untreated periodontitis patients, only 78.5% were positive for one or both bacteria; 37% were positive for P. gingivalis only, 17% for S. intermedius and 24.5% for both. P. gingivalis was detected in 23.5% of healthy volunteers, while S. intermedius was not detected in the same patients.

The distribution of these bacteria was related to the periodontal probing depth, while 95.23 % of patients with pockets wih 6 to 7 mm deep were positive for either or both, only 70.45 % of of them with 4 to 5 mm pockets were positive.

Key words: periodontitis, periodontal pocket, polymerase chain reaction, porphyromonas gingivalis, Streptococcus intermedius.

DETECCIÓN DE *PORPHYROMONAS GINGIVALIS* Y *STREPTOCOCCUS INTERMEDIUS* EN PACIENTES CON PERIODONTITIS CRÓNICA USANDO PCR MULTIPLEX

RESUMEN

Se muestra la utilización del ensayo de polimerasa en cadena (PCR) múltiplex para la detección de Porphyromonas gingivalis y Streptococcus intermedius en pacientes con periodontitis crónica. Se analizaron un total de 180 muestras de 65 adultos con periodontitis no tratada y 17 voluntarios sanos, las células se procesaron inicialmente colocándolas a baño María durante 10 min. El lisado celular fue usado como fuente de ADN para los ensayos del PCR múltiplex. Los primers fueron diseñados a partir de secuencias génicas de fracciones 16 rRNA obtenidas de la base de datos GenBank-EMBL y que mostraron especificidad para los patógenos mencionados. El sistema PCR múltiplex fue diseñado para identificar 8.2 células de P. gingivalis y S. intermedius. De los pacientes con periodontitis, sólo el 78.5 % fueron positivos para una o ambas bacterias. En el 37% se identificó únicamente P. gingivalis, en el 17% S. intermedius y en un 24.5% ambos. P. gingivalis fue detectada en el 23.5% de los voluntarios sanos, mientras que, S. intermedius no se detectó en ese grupo de pacientes.

La distribución de la identificación de estas bacterias está relacionada con la profundidad de las bolsas periodontales. Mientras que el 95.23 % de los pacientes con bolsas de 6 a 7 mm fueron positivas para ambas bacterias, mientras que sólo el 70.45 % de ellos fue positivo cuando las bolsas tenían de 4 a 5 mm de profundidad.

Palabras clave: periodontitis, bolsas periodontales, reacción en cadena de la polimerasa, Porphyromonas gingivalis y Streptococcus intermedius.

INTRODUCTION

Periodontitis represents the most common gingival disease. If infection and inflammation are not treated, gingivitis develops into periodontitis. This chronic disease is characterized by leukocyte infiltration, release of tissue-destructive collagenase and the activation of the bone-destroying cells, osteoclasts. This affects the supporting tissues of the teeth, causing loss of bone attachment, often resulting in tooth loss. Although the potential contribution to the disease of *Aggregatibacter actinomycetemcomitans, Campylobacter rectus, Prevotella intermedia, Fusobacterium nucleatum,*

Table 1: Primers used to amplify a fragment of the 16S rRNA gene from *P. gingivalis* and *S. intermedius* in a multiplex PCR system.

Strain	Primer (5'—3')	key	Position within gene	Product Size (pb)	Accession No.
P. gingivalis	AAGGATTGTAAACTTCTTTTATAC	VJ-1	427	705	L16492
	ACTGTTAGCAACTACCGATGT	VC-2	1132		
S. intermedius	GTTAAGGAAGAACGAGTGTGAGAA	VJ-5	343	833	X58311
	TGCCGTCACCGGCTTGCGACTCGT	VC-6	1176		

Streptococcus intermedius, Porphyromonas gingivalis and *Tannerella forshytia,* also known as "putative periodontal pathogens", is recognized, few of these have been demonstrated to have a direct pathogenic effect on the host [1]. It is estimated that approximately 50% of the human oral flora has yet to be isolated and cultured; therefore, it is likely that currently unknown and uncharacterized bacterial species may play a role in the etiology of periodontitis[2]. Molecular techniques, including polymerase chain reaction (PCR), allow the identification of types and/or concentration of bacterial flora in healthy subjects, gingivitis and periondontitis patients, thus providing support for specific diagnosis[3]. The aim of the present study was to identify bacteria associated with chronic periodontitis, using PCR multiplex type amplification tests, and design specific 16S rRNA gene primers for *P. gingivalis* and *S. intermedius;* these genes are present in every bacterium and are highly conserved within a species[4]. This approach is valuable for the detection of microorganisms that cannot be cultured or easily isolated in culture[5].

MATERIAL AND METHODS

Oligonucleotide PCR primers design

Suitable primers and PCR products were designed by using all 16S rDNA sequences of *P. gingivalis* and *S. intermedius* available in the GenBank database. This survey was done using BLAST (National Center for Biotechnology Information) and the ClustalW program. Sequence of primers, their location within the respective gene sequences, and the expected sizes of their PCR products are shown in Table 1. Oligonucleotides were synthesized using a DNA synthesizer (Microsyn 1450A; Systec Inc., Minneapolis, Minn) following the manufacturer's instructions[6].

Specificity and sensitivity of primers and PCR amplifications

The specificity and sensitivity of the primers and PCR conditions were evaluated by testing *P. gingivalis* ATCC 33277^T and *S. intermedius* ATCC 27335^T, as well as the closely related *Tannerella forshytia and P. intermedia* ATCC 25611^T as positive controls; and the more distant *Escherichia coli*, *Proteus vulgaris, Bacillus thuringiensis* and *Bacillus subtilis* as negative bacteria. The oral strains *P. gingivalis* ATCC 33277^T, *S. intermedius* ATCC 27335^T, *P. intermedia* ATCC 25611^T and *Tannerella forshytia*, were kindly provided, as frozen cultures, by Dr. Stanley Holt (Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, Kentucky). The other strains were grown on nutrient agar (DIFCO, Ba Leewarden, Netherlands) for 24 h at 37°C and frozen in skim milk. All strains were maintained at -70°C until use. The specificity of the primers was evaluated by testing positive and negative bacterial strains. DNA samples were obtained as reported elsewhere[7]. In brief, 10^3 cells from each strain were transferred to 100 µl of sterile double distilled water and placed for 10 min in a boiling water bath. The resulting cell lysate was centrifuged for 30s at 12,000 rpm (Eppendorf model 5415C, Hamburg, Germany) and 35 µl of the supernatant were used as a DNA sample in the PCR assays. In order to evaluate the sensitivity of the PCR system, cultures of P. *gingivalis* ATCC33277^T and *S. intermedius* ATCC27335^T beginning at 8.2 x 10^3 cells were titrated. Serial dilutions of the original culture in 1.0 ml of sterile double distilled water were made and the number of cells was determined by using a Petroff-Hausser bacteria-counting chamber; DNA samples were then obtained as described above.

Clinical samples from individuals with and without periodontitis

A total of 180 samples were taken from 65 adults with untreated periodontitis and 17 healthy individuals (≤3 mm probing depths). The mean age of the healthy adults was 40.8±5.5 years (10 were females and 7 males). All patients were recruited from the Departament of Periodontics, Faculty of Dentistry, Autonomal University of Nuevo Leon, Monterrey, Mexico. Periodontitis was determined by clinical and radiographic examination. Patients were 35-45 years-old with at least four teeth with periodontal

probing depths of 4 to 7 mm. Patients were divided in two age groups as follows: group 1 consisted of 35-40 year-old individuals (13 males and 29 females) and group 2 consisted of 41-45 year-old subjects (10 males and 13 females). Patients who had used systemic antibiotics in the previous six months or had any systemic disease, were excluded. Samples were obtained from three or four periodontal pockets per patient with a sterile curette, pooled, and suspended in a 0.5 ml tube containing 100 µl of double distilled sterile water and stored at –70°C until use. The DNA samples were obtained by placing the tubes in a boiling water bath, and heating for 10 min at 94°C. The cell lysate was centrifuged as described above and a 35-µl aliquot was used as DNA source for PCR assays.

Table 2: Detection of *P. gingivalis* and *S. intermedius* in untreated patients patients with periodontitis.

Patients: age in years	Sex		Probing Pocket Depth (mm)				Presence of		
	M	F	4	5	6	7	*P. gingivalis*	*S. intermedius*	*P. gingivalis and S. intermedius*
35-40	13	29	11	17	10	4	14	7	10
41-45	10	13	6	10	5	2	10	4	6
Total	*23*	*42*	*17*	*27*	*15*	*6*	*24 (37%)*	*11 (17%)*	*16 (25%)*

Fisher exact test p= 0.34

PCR amplification

PCR amplification was performed in a 50 µl volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 2.5 U of *Taq* polymerase (Boehringer, Mannheim), 0.2 µM of each deoxynucleoside triphosphate (Boehringer, Mannheim), 0.2 µM of the primer and 35 µl of DNA sample. Amplification was carried out in a DNA thermal cycler (Perkin-Elmer model 2400 Applied Biosystems, and Foster City, California, USA). The process included 30 cycles of each of the following temperature programs: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min. After that, an extra elongation step of 10 min at 72°C was added. A 10-µl aliquot of amplified samples from each PCR tube was placed in an electrophoresis gel containing 2% agarose or 4% polyacrylamide in 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) for 1.5 h at 100 V, and stained with ethidium bromide.

Statistical analysis

It was performed with the aid of SPSS version 10.0. Software. Patients were grouped by age, gender and depth of pockets. The presence and ratio of *P. gingivalis* and *S. intermedius* was recorded. The associations between these variables were calculated by Fisher's exact test, p values < 0.05 were considered significant.

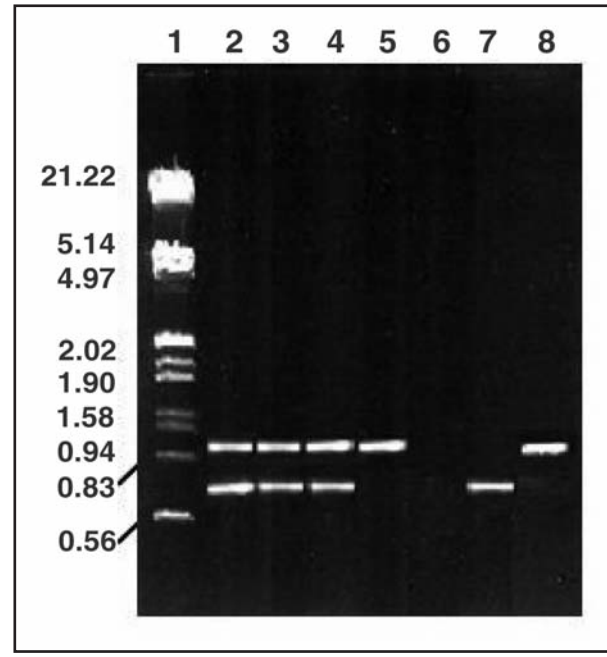

Fig. 1: Specific DNA amplification for P. gingivalis and S. intermedius from periodontitis clinical samples using a multiplex PCR system. Lanes: 1, Lambda DNA/EcoRI+HindIII as molecular marker size (kbp); 2-5, samples from patients; 6, negative control without DNA; 7, positive control for P.gingivalis; 8, positive control for S. intermedius.

RESULTS

Detection of *P. gingivalis* and *S. intermedius* in clinical samples

A representative multiplex PCR result for clinical samples is shown in Fig. 1, where several patients (lines 2-4) were positive for both microorganisms and one patient was positive for *S. intermedius* (line 5). The detection of *P. gingivalis*, *S. intermedius* or both was positive in 51 of 65 (78.5%) patients with untreated periodontitis. On the other hand, only 4 healthy volunteers (21.5%) were positive for *P. gingivalis*, and none for *S. intermedius* (data not shown). Tables 2 and 3 summarize the results obtained from the clinical samples. It can be observed that 37% of the patients were

Table 3: Relationships between periodontal pocket depth and the presence of *P. gingivalis* and *S. intermedius* in positive patients with periodontitis.

Probing Depth (mm)	n	Presence of			Total
		P. gingivalis	*S. intermedius*	*P. gingivalis and S. intermedius*	
4 - 5	44	14	7	10	31 (70.4%)
6 - 7	21	10	4	6	20 (95.2%)
Total	*65*	*24*	*11*	*16*	*51 (78.4%)*

Fisher exact test p= 0.02

positive for *P. gingivalis*, 17% for *S. intermedius*, and 24.5% for both. Thus 61.5 % of the patients (40/65) were positive for *P. gingivalis*, alone or in combination with *S. intermedius*, whereas *S. intermedius* alone or in combination with *P. gingivalis* was present in 41.5% of the patients (27/65) (Table 2). It is important to note that the total value is higher than 100% because 16 patients had both microorganisms. An analysis according to age revealed that in group 1 (35-40 years) 31/42 (73.8%) were positive, whereas in group 2 (41-45 years) 20/23 (87%) were positive for one or both bacteria tested (*p*: 0.34). Another important finding is that the depth of the periodontal pocket was shown to be significant (*p*: 0.026) since 70.45% (31/44) of patients with 4-5 mm pockets were positive for one or both bacteria, compared to 95.23 % (20/21) of the patients with 6-7 mm pockets (Table 3).

DISCUSSION

In order to specifically detect two bacteria of several involved in chronic periodontitis, 65 patients with untreated periodontitis and 17 healthy volunteers were evaluated for the presence of *P. gingivalis* and *S. intermedius* in a single and easy step using multiplex PCR. The main concern for DNA amplification by PCR techniques is related to the pretreatment of the sample prior to the PCR assay to obtain the DNA. It is well known that some biological samples may contain several PCR inhibitors and different methods have been suggested to eliminate or neutralize their activity[8,9]. However, many of these methods are laborious and expensive. In the present study, a cell lysate from a simple boiling step was used as the DNA source for multiplex PCR. Although Conrads et al.[10] suggested that clinical samples should be processed with Chelex 100 resin prior to boiling to decrease potential PCR inhibition and increase sensitivity, our boiling step yielded positive and specific DNA amplification in 78.5% of the patients. This result was comparable with that found for *A. actinomycetemcomitans*, *T. forshytia*, *P. intermedia* and *P. gingivalis*[10,11]. On the other hand, 23.5% of healthy volunteers were positive for *P.* gingivalis, but none for *S. intermedius*, in keeping with previous studies[12]. The specificity of the primers was demonstrated by using positive and negative cultures and clinical samples where no additional DNA band was visualized. Because these samples may contain around 50 to 300 different species, this finding supports the specificity of our method. Although we cannot determine the precise number of pathogen cells in plaque samples, our multiplex PCR assay detected as few as 8.2 *P. gingivalis* and *S. intermedius* cells (data not shown) in pure cultures. This detection level is comparable to those obtained for other periodontal pathogens such as *A. actinomycetemcomitans*[11,13]. In this sense, our approach is simple, highly sensitive, cost-effective and specific.

Another important feature of our work was to evaluate the presence of *S. intermedius* in oral samples from both healthy tissues and periodontitis lesions. Early reports recognized that this bacterium could be associated not only with periodontitis[14,15,16], but also with infections in deep sites such as liver and brain[17]. In spite of the clinical significance of *S. intermedius* as a pathogen, this is the first report where it has been included and detected by multiplex PCR in patients with untreated periodontitis. In conclusion, our findings reveal that multiplex PCRs might detect relevant numbers of putative periodontal pathogens in a rapid, easy to perform and inexpensive fashion.

ACKNOWLEDGEMENTS

We thank Dr. S. Holt for providing the oral strains. This work was supported in part by the National Research Council of Mexico (CONACYT) grant 25587-M. M. De la Garza Ramos was recipient of a fellowship from CONACYT.

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