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Prevalence of *Porphyromonas gingivalis* and *Bacteroides forsythus* in Chronic Periodontitis by Multiplex PCR

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Abstract: The present research decided to study prevalence of *Porphyromonas gingivalis* and *Bacteroides forsythus* in chronic periodontitis patient by use of Multiplex PCR. The subgingival plaque samples from 61 patients suffering from chronic periodontitis with probing depth PD$\geq$6 and 40 healthy controls were collected by sterile curette. In this study we used two species-specific Forward primers in combination with a single Reverse primer. These primers target variable and conserved region of 16S rRNA gene, respectively. The study included 61 patients (34 women, 27 men; 24-69 years of age; mean 43) and 40 periodontally healthy controls (22 Women, 18 men, 21-69 years in age; mean 41.35%). *Porphyromonas gingivalis* was detected in 51 samples (83.61%) and 16 samples (40%) of chronic periodontitis patients and healthy subjects, respectively and *Bacteroides forsythus* was detected in 32 samples (52.50%) of chronic periodontitis patients and was not detected in any sample from healthy persons. We set up Multiplex PCR in order to detect *P. gingivalis* and *B. forsythus* simultaneously. The present data suggest that *P. gingivalis* is a more important cofactor in etiology of chronic periodontitis. Further studies are needed to determine spectrum of pathogenicity of the disease and effective management of diagnosis and treatment in order to decrease the risk of periodontic complicates such as systemic infection.

Key words: *Porphyromonas gingivalis*, *Bacteroides forsythus*, periodontitis, multiplex PCR

INTRODUCTION

Periodontitis describes an inflammation of the supporting tissue of the teeth (Loesche and Grossman, 2001). Periodontitis with its various clinical forms represent the most widely distributed type of oral disease (Smola *et al*., 2003). A growing number of scientific reports point toward a causative link between periodontal disease and various systemic illnesses for example endocarditic and other cardiovascular disease, respiratory disease, preterm birth and diabetes mellitus (Li *et al*., 2000; Beck *et al*., 1998, 2005; Fiehn *et al*., 2005; Khader and Ta'ani, 2005). Dental plaque is the major causative factor of periodontitis. The major putative pathogens known to be involved in severe periodontitis notably *P. gingivalis* and *B. forsythus* are widely regarded as major periodontal pathogens (Kumar *et al*., 2003). Anaerobic culture is most commonly used to detect of the subgingival plaque, however it is time consuming and has a low level of sensitivity. This is due to the extremely slow growth of some oral pathogens (Smola *et al*., 2003; Boutaga *et al*., 2003). The use of PCR assay has resulted in large saving in time, costs and experimental effort when compared with the other bacterial identification methods (Kook *et al*., 2005). In recent years, there has been great interest in PCR-based tests which use the bacterial small-subunit 16S rRNA gene (16SrDNA) to detect bacterial pathogens. Nucleotide sequences of some portions of 16SrDNA have been highly conserved. However, other regions of this gene are hyper variable. Most tests have emphasized the detection of only a single species. However, sets of 16SrDNA-based primers can be combined to detect more than one species in a single patient sample. The general approach of combining multiple primers in a single reaction mixture is called multiplex PCR (Tran and Rudney, 1996).

MATERIALS AND METHODS

Study population: Sub gingival plaque samples were obtained from 61 patients (34 women, 27 men; 24-69 years of age; mean age 43) and 40 periodontally healthy controls (22 women, 18 men, 21-69 years in age; mean age 41.35%) undergoing periodontal surgery at the

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Department of Periodontology, School of Dentistry, University of Isfahan, Iran. All volunteers gave their informed consent prior to participating in this study. Present study was performed in 2006.

Subjects: The selection criteria were as follows: (a) the presence of a site of a Probing Depth (PD) 6 mm or more in at least two quadrants, (b) without known systemic disease, (c) not pregnant, (d) no periodontal treatment within the last 6 months, (e) high gingival index (GI 2 or 3) (f) presence of inflammation and (g) tendency to bleed after probing at a probing depth≥3 mm.

Sample collection: After removing supragingival plaque with sterile cotton pellets, a sterile periodontal curette was gently inserted to bottom of the test periodontal pocket and subgingival material was removed by a single stroke (Slots, 2005). The subgingival specimens were suspended in 500 μL of sterile (TE) buffer (10 mM Tris-hydrochloride, 1 mM EDTA, pH 8) and homogenized by vigorous vortex mixing and then samples were stored at -70°C prior to analysis.

Isolation of DNA from plaque samples: The samples were thawed and genomic DNA was extracted by phenol-chloroform. Briefly, the samples were recovered by centrifugation at 8000xg for 3 min. Supernatant was combined with the 0.1 volume of each sample by SDS 10%. The mixture was incubated at 37°C for 30 min each of samples was extracted three times with phenol/chloroform (1:1). After precipitating DNA with 0.1 volume of 3M sodium acetate and 0.5 mL of cold absolute ethanol, DNA was washed in 70% ethanol, dried and redissolved in 1×TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA]. DNA was stored at -70°C.

Primer selection: The Multiplex PCR analysis for detection of *B. forsythus* and *P. gingivalis* using primers based on the 16S rRNA genes, was performed as previously described by Tran and Rudney (1999). The expected product lengths were 745 bp for *B. forsythus* and 197 bp for *P. gingivalis* (Fig. 1). The sequences of selected primers were as follow.

B. forsythus-specific forward primer (BfF), 5'-TAC AGGGGA ATA AAA TGA GAT ACG-3'; *P. gingivalis* specific forward primer (PgF), 5'-TGT AGA TGA CTG ATG GTG AAAACC-3' conserved reverse primer (C11R),5'-ACG TCA TCC CCA CCT TCC TC-3'

Multiplex PCR with conserved and species-specific primers: In brief, to each Multiplex PCR reaction test tube 5 μL of the template DNA was added to a master mix

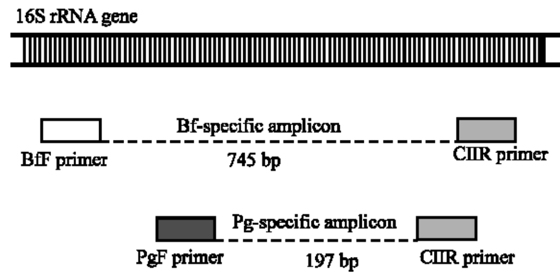

Fig. 1: Multiplex PCR with conserved and species-specific 16SrDNA primers for simultaneous detection *B. forsythus* (Bf) and *P. gingivalis* (Pg). The drawing is a schematic of the location where the primers anneal to the bacterial 16S rDNA. The approximate sizes of the species specific amplicons generated are also depicted. The 16S rDNA forward primer specific for *B. forsythus* labeled. BfF PgF is the 16S rDNA forward primer specific for *P. gingivalis*. C11R is the 16S rDNA conserved (universal) reverse primer

solution containing 5 units of Taq DNA polymerase, 1× PCR buffer with 2 mM $MgCl_2$, 0.5 mM mixture of dNTP, 12.5 pM of primer PgF, 12.5 pM primer of BfF, 25 pM primer C11R and water to the final volume of 50 μL. PCR amplification denotation was performed in a thermal cycler Master (Eppendrof, Germany). Samples were initially denaturized at 95°C for 5 min, followed by 34 cycles, which included denaturation for 1 min at 94°C, annealing for 45 sec at 59°C, extension for 1 min at 72°C, with final extension at 72°C for 2 min. Each set of experiments included negative controls with sterile distilled water instead of template DNA and purified genomic DNA from *B. forsythus* and *P. gingivalis* was used as a positive controls. An adequate DNA template for PCR and absence of PCR inhibitors were confirmed in clinical materials with amplification of beta-globin gene.

Post-multiplex PCR gel electrophoresis: Amplicons for all Multiplex PCR reactions were detected by electrophoresis at 4 V/Cm in Tris-Boric acid buffer of a 10 μL sample in a 1.2% agarose gel (Fermentas) containing 0.5 μg mL ethidium bromide. A 100 bp DNA ladder (Fast Ruller, Fermentas) served as molecular weight markers in electrophoresis analysis. Electrophoresis DNA bands were visualized under 300 nm ultraviolet light.

Statistical analysis: Chi-squire test was used to compare the presence of *Porphyromonas gingivalis* and Bacteroides forsythus in periodontits patients and health subject, p-value equal to or below 0.05 were considered statistically significant.

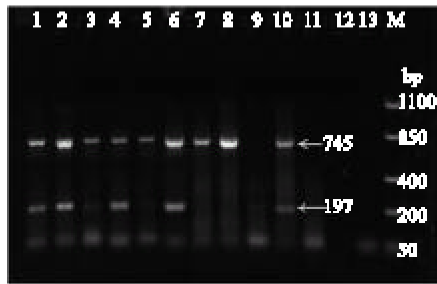

Fig. 2: *Porphyromonas gingivalis* and Bacteroides *forsythus* DNA, amplified by multiplex PCR with specific universal reverse primer (C11R) and forward primers PgF and BfF. Lanes: 1, positive control (745 and 197 bp); 13, negative control; 2, 3, 4, 5, 6, 7, 8, 10 positive samples and the other lanes are negative samples

RESULTS

The prevalence of each species was computed for each subject. The detection of periodontopathogen distribution and frequency differed for healthy and patients site.

The prevalence of *B. forsythus* in periodontitis patients was 52.5%, whereas the bacteria were not detected in healthy controls. *P. gingivalis* was found 83.61 and 40% in periodontitis patients and normal subject, respectively.

Statistically significant differences were observed between chronic periodontitis and healthy control groups. In this study statistically significant differences were found between men and women. Prevalence of disease was detected more frequently in men than in women patients. Multiplex PCR detection of Bacteroides forsythus and *Porphyromonas gingivalis* DNA in agarose gel electrophoresis is shown in Fig. 2.

DISCUSSION

Microbiological studies have demonstrated that the composition of subgingival plaque is highly complex and variable. So far, about 500 bacterial species have been identified in healthy or disease periodontal tissue (Boutaga *et al*., 2003). Studies comparing the efficacy of different periodontal therapeutic regimens report that the suppression or elimination of these bacterial species improves the clinical treatment response. Therefore, microbial monitoring for periodontal pathogenic bacteria might be useful for predicting the treatment outcome (Kook *et al*., 2005).

Early studies attempting to relate bacteria to specific periodontal disease, indicated their presence in periodontally healthy subject to be a rare occurrence, suggesting such bacteria may be considered exogenous pathogens (Genco *et al*., 1986) however, within the last decade, cross-sectional studies in subjects from developing countries, where the natural history of infection is less likely to be influenced by external factors (such as antibiotics and regular exposure to oral hygiene measures) have frequently detected these pathogens in subject with no clinical evidence of periodontal disease (Dahlen*et al*., 1992; Madianos*et al*., 1997; Ali *et al*., 1997; Papapanou *et al*., 1993). Eick and Pfister detected *P. gingivalis* in 76% and *B. forsythus* in 80% of subgingival plaque in chronic periodontitis (2002). In the study of Zhan *et al*. (2005) the prevalence of 91.5% *P. gingivalis* in periodontal plaque was reported (2005).Sosransky *et al*. (1998) also compared the microflora of periodontal pockets with different depths and found a higher prevalence of *P. gingivalis*, *B. forsythus* in deep pockets than in shallow pockets. Conrads *et al*. (1998) showed the prevalence of *B. forsythus* in 28.9% of patients. Zambon (1996). suggested that *P. gingivalis* and *B. forsythus* are closely associated with pathogenesis of periodontitis, as well as that loss of connective tissue and severe resorption of alveolar bone (1996). Similarity, in some studies an association between deep pockets and high numbers of *P. gingivalis* and *B. forsythus* were reported; suggested that these bacteria might have an etiologic role in periodontal disease progression (Loesche *et al*., 1992; Slots, 1986; Takeuchi *et al*., 2001). However some other scientist has not found this correlation (Loesche *et al*., 1985; Dzink *et al*., 1985).

In our study, higher prevalence of *P. gingivalis* (83.61%) and *B. forsythus* (52.5%) was detected in periodontal site. The present data suggests that *Porphyromonas gingivalis* is more important cofactor in etiology of chronic periodontitis Than Bacteroides forsythus. Further studies are needed to determine spectrum of pathogenecity of the disease and effective management of diagnosis and treatment in order to decrease the risk of periodontic complication such as systemic infections. The use of clinical parameters in sample site selection, particularly probing depth measurements, is likely to considerably enhance the chance of detecting *P. gingivalis* and *B. forsythus*. Similarly, sample site selection based on loss of clinical attachment level and bleeding upon probing is likely to increase the chances of detecting these two bacterial species.

This study forms the baseline of a continuing longitudinal study that may help elucidate factors that may be involved in periodontal disease initiation and or progression.

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