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Prevalence of Epstein-Barr Virus Type 1 in Patients with Chronic Periodontitis by Nested-PCR

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Abstract: The present study evaluated the subgingival presence of Epstein-Barr virus type 1 (EBV-1) in patients with chronic periodontitis with nested-PCR. Subgingival plaque samples from 61 patients with chronic periodontitis with Probing Depth (PD) ≥ 6 and 40 healthy controls were collected by sterile curette. DNA was extracted. A nested Polymerase Chain Reaction (PCR) method determined presence of EBV-1. The study included 61 patients (34 women, 27 men; 24-69 years of age; mean 43) and 40 periodontally health controls (22 Women, 18 men, 21-69 years in age; mean 41.35%). EBV type 1 was detected in 37 samples (60.7%) and 1 samples (2.5%) of chronic periodontitis patients and healthy subjects, respectively. This study demonstrated that EBV-1 infection is associated with the activity of chronic periodontitis.

Key words: Epstein-Barr virus type 1, periodontitis, subgingival, nested-PCR method, infection, method, Iran

INTRODUCTION

Periodontitis is a disease attributable to multiple infectious agents and interconnected cellular and humeral host immune responses (Ezzo and Cutler, 2003). It is important to realize that the composition of the subgingival microflora viruses remarkably in periodontal health and in various form of periodontal diseases (Umeda *et al*., 1998; Page and Beck, 1997). Previous research on periodontal disease has focused on putative periodontopathic bacteria and their products (Moore and Moore, 1994; Haffajee and Socransky, 1994). Recent finding have showed herpesviruses especially EBV can infect or alter structural cells and host defense cells of the periodontium (Contreras *et al*., 2000). EBV-1 assumes particularly close relationship with human periodontitis (Contreras and Slots, 1996). EBV-2 seems to exhibit little or no associated with most of destructive periodontal disease (Contreras *et al*., 1997, 1999).

These herpesviruses are capable of infecting and impairing polymorphonuclear leukocytes (PMNs) and lymphocytes. Herpesvirus infected inflammatory cells can reduce the host defense and give rise to overgrowth of pathogenic bacteria and invade cells more efficiently (Contreras and Slots, 2000; Contreras *et al*., 1999).

EBV infections have the potential to increase the virulence of resident bacteria pathogens. By expressing virally-induced proteins on eukaryotic cell membranes (Mackowiak *et al*., 1984). Herpesviruses may also infected epithelia cells (Imai *et al*., 2004), thereby facilitating the penetration of pathogenic bacteria into connective tissue (Saygun *et al*., 2004). Genome of EBV-1 occur at high frequency in progressive periodontits in adults, localized and generalized aggressive (juvenile) periodontitis, acute necrotizing ulcerative gingivitis, periodontal abbesses and some rate type of advanced periodontitis associated with medical disorders (Slots, 2005).

The aim of present study was to examine the presence EBV-1 in subgingival plaque samples from patients with chronic periodontitis.

MATERIALS AND METHODS

Dental plaque samples were obtained from 61 patients (34 women, 27 men; 24-69 years of age; mean 43). and 40 periodontally health controls (22 Women, 18 men, 21-69 years in age; mean 41.35%) undergoing periodontal surgery at the Department of Periodontology, School of Dentistry, University of Isfahan, Iran. All participants were systematically healthy and had not received periodontal treatment or antibiotics for at least 6 months prior to sampling. The periodontitis patients had at least 9 posterior teeth, 6 and 3 mm Probing Depths (PD) and attachment loss ≥ 6 mm.

After removing supragingival plaque with sterile cotton pellets, a sterile periodontal curette was gently inserted to bottom of the test periodontal pocket and subgivgival material was removed by a single stroke (Saygun *et al*., 2005). The subgingival specimens were suspended in 500 μL of sterile TE buffer (10 mM Tris-

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Table 1: Sequences of primers used to amplify EBV-1 DNA with product size

Name	Nucleotide sequences (5'-3')	Product size (bp)
EBNA-2.1.A	AGG GAT GCC TGG ACA CAA	600
EBNA-2.1.B	GTG CTG GTG CTG CTG GTG G	
EBNA-2.2.A	TCT TGA TAG GGA TCC GCT AGG ATA	497
EBNA-2.2.B	ACC GTG GTT CTG GAC TAT CTG GAT C	
PC03	ACA CAA CTG TGT TCA CTA GC	123
PC04	CAA CTT CAT CCA CGT TCA CC	

hydrochloride, 1 mM EDTA, pH 8) and homogenized by vigorous vortex mixing and then samples were stored at -70°C prior to analysis.

The samples were thawed and genomic DNA was extracted by phenol-chloroform. Briefly, the sample was extracted three times with mixture of phenol-chloroform (1:1). After precipitating DNA with 0.1 volume of 3 M 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, 1mM EDTA, pH 8). DNA was stored at -70°C.

The nested-PCR method was used to detect species-specific DNA of EBV-1. We used EBNA-2.1.A and EBNA-2.1.B as outer primers; inner primers for EBV type 1 were EBNA-2.2.A and EBNA-2.2.B and primers for β-globin (GMW Co., Germany). All the primers used are listed in Table 1 (Borisch *et al*., 1993).

In brief, to each PCR reaction test tube 5 μL of the template DNA was added to a master mix solution containing 1.5 units of Taq DNA polymerase, 1×PCR buffer with 1 mM $MgCl_2$, 0.1 mM mixture of dNTP, 10 pM of each primer and water to the final volume of 50 μL.

PCR amplification was performed in a thermal cycler Master (Eppendrof, Germany). Samples were initially denaturized at 94°C for 4 min, followed by 30 cycles, which included denaturation for 1 min at 94°C, annealing for 1 min at 63.5°C, extension for 1 min and 30 sec at 72°C, with final extension at 72°C for 5 min. For nested-PCR reactions, the second round of amplification was performed in a new tube using (50 μL), 2 μL of the first PCR products, 30 pM of the inner primers, 5 μL of 10×PCR buffer, 1.5 U of enzyme, 1 mM $MgCl_2$, 0.1 mM dNTP. The second PCR program was 30 cycles of a denaturizing step at 94°C for 1 min, an annealing step at 60°C for 1 min and a final extension step at 72°C for 1 min. The PCR detection limit was calculated using serially diluted viral DNA positive control. Positive control included EBV positive cell line (B95-8, Pasteur Institute of Iran). Specificity was confirmed by determining the size of the amplicons and retesting of positive samples. Negative control was the reaction mixture without DNA template. Amplicons for all 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 (Fastfuller, Fermentas) served as molecular weight markers in electrophoresis analysis. Electrophoretic DNA bands were visualized under 300 nm ultraviolet light.

Statistical analysis: Chi-squire test was used to compare difference EBV-positive and EBV-negative periodontits patients, p-value equal to or below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

An adequate DNA template for PCR and absence of PCR inhibitors were confirmed in clinical materials with amplification of β-globin gene. All 61 clinical samples taken from the patients with periodontitis were analyzed according to PD for the presence of DNA from EBV. DNA of EBV-1 was confirmed in 37 of 61 (60.7%) periodontitis patients and in 1 of 40 (2.5%) healthy subjects. 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 were detected more frequently in men than in women patients. PCR detection of EBV-1 DNA in agarose gel electrophoresis is shown in Fig. 1.

This study evaluated the presence of EBV-1 in periodontitis patients and healthy subjects. It confirmed the previous data from other authors associating herpesvirus with periodontal diseases (Contreras and Slots, 2000; Parra and Slots, 1996; Klemenc *et al*., 2005; Slots *et al*., 2006). In previous studies, investigators identified a significant relationship between EBV and periodontitis. Contreras *et al*. (2000) EBV-1 in 79 and 27% of chronic periodontitis and healthy periodontal, respectively by nested-PCR method. In Greece patients, detected EBV-1 in 56% of chronic periodontitis (Konstantinidis *et al*., 2005). Kubar *et al*. (2005) found EBV-1 in 46% of periodontal patients. Klemenc *et al*. (2005) showed 44% of patients had EBV-1. In Japan and China 49 and 37% of chronic periodontitis, respectively (Wu *et al*., 2005; Idesawa *et al*., 2004). In this study higher prevalence of EBV-1 (60.7%) in patients and low prevalence (2.5%) in healthy persons were detected in periodontal site. These results similar to the previous data from other authors. According to report of Ling *et al*. (2004) in Taiwan chronic periodontitis is very low (4%). The reasons for variation in EBV-occurrence among studies may include differing EBV detection technique, dissimilar periodontal diseases states studies and true geographic variation in EBV prevalence.

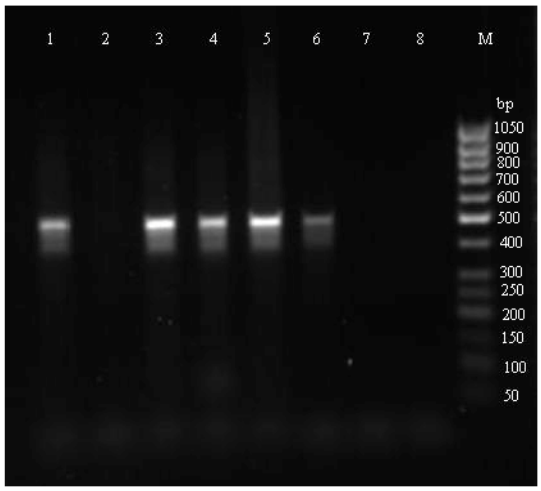

Fig. 1: Electrophoresis results of nested-PCR amplification. M: size marker; Lane 1: Negative control; Lane 2: Positive control; Lanes 4, 5, 6, 7, 9, 10: Positive samples; Lanes 3, 8, 11, 12: Negative samples

All herpesviruses after primary infection persist in different parts of human body for life time, but reactivation may occur spontaneously or as result of every factor reducing the host immune defense (Salvi *et al*., 1997). The severe forms of periodontal diseases that occur with increased frequency in population of developing countries and among individual of low socioeconomic status (Baelum and Scheutz, 2002). The other factors cause reducing host immune system and lead to periodontitis diseases are smoking, genetics, increasing age. For example with increasing age capacity of immune system reduces and reactivation of herpesviruses, especially EBV occur (Locker *et al*., 1998; Stanford and Rees, 2003; Amit *et al*., 1992). 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. It may be for this reason that smoking is more usual between men groups in our country.

In the future, controlled clinical and virological studies, which should include quantitative PCR and other methods, might better assess the importance all herpesviruses and specific type in periodontal diseases.

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