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Identification of Herpes virus in patients with chronic periodontitis from Northeastern Mexico

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Periodontitis is an inflammatory process that occurs in the tissues surrounding the teeth. It is caused by microorganisms and groups of microorganisms and results in loss of periodontal ligament and alveolar bone. Herpes virus has emerged as a putative pathogen in periodontal disease. Periodontal disease was demonstrated in 50 patients by careful probing of pockets and completion of a medical record. Three saliva samples were collected in individuals with deep pockets. Samples were obtained with a sterile swab and placed in 10% glucose solution for storage at -80°C. DNA purification and PCR, in addition to electrophoresis, were performed. Of the 50 samples processed, two were positive for HSV-1 and two for HSV-2. Three were from women with mild, moderate, and severe chronic periodontitis, respectively, and one (HSV-1) from a man with mild chronic periodontitis. Due to the low prevalence of herpes simplex virus in periodontal pockets, it was not possible to determine its role in the progression of periodontitis.

Key words: Herpes simplex virus, plaque index, gingival index, electrophoresis.

INTRODUCTION

Periodontitis is an inflammatory process that occurs in the tissues surrounding the teeth. It is caused by microorganisms and groups of microorganisms and results in progressive destruction of the periodontal ligament and alveolar bone (Loesche and Grossman, 2001). It is an asymptomatic, inflammatory infection characterized by colonization with microbial pathogens, microbial synergy, the host immune response, and different environmental risk factors, which are major determinants for the probability of acquiring destructive disease (Slots, 2002). Chronic periodontitis is the most common form and it is highly prevalent in adults over 35. Chronic periodontitis is linked to the accumulation of plaque and calculi and its progression can be mild or moderate; however, more rapid destruction of tissue and bone can be observed (Loesche and Grossman, 2001). It is important to consider the composition of the subgingival microflora since this varies significantly when the periodontium is pathologically affected. Previous research on this disorder has focused on putative periodontal bacteria and their products (Saygun et al., 2004).

Most human viruses known to cause oral disease are human herpesvirus (HHV) and human papillomavirus (HPV), DNA viruses that are acquired during childhood or young adulthood through contact with blood, saliva or genital secretions. Herpesviruses have emerged as putative pathogens in periodontal disease since the 1990s (Pawar et al., 2012). Herpesviruses can infect or alter structural cells and the host defense cells of

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periodontal tissue (Contreras et al., 2000). Human cytomegalovirus (HCMV), Epstein-Barr virus type 1 (EBV-1) and Herpes simplex virus (HSV) have been reported to play a role in the pathogenesis of severe types of periodontitis (Das et al., 2012; Pawar et al., 2012). Among the herpesviruses, herpes simplex 1 and 2 (HSV-1, HSV-2) are common causes of infection (Chayavichitsilp et al., 2009). Some studies have found a relationship between these subspecies and periodontal disease (Das et al., 2012). Other studies have found these subspecies both in patients with periodontal disease and in periodontally healthy patients, although HSV was more frequent in periodontitis lesions (Contreras et al., 2000). The action of herpesviruses is to infect and impair polymorphonuclear leukocytes, macrophages, and lymphocytes (Das et al., 2012). Endothelial cells infected with herpes virus can reduce host defenses and favor overgrowth of pathogenic bacteria.

Thanks to the development of molecular biology, laboratory tools can determine the genetic material in these particles. Viruses can be defined and analyzed in clinical specimens or biopsies. The viral genome can be measured directly by hybridization or after detection with nucleic acid amplification. Previous studies found that periodontitis lesions that are positive for herpesviruses harbor higher concentrations of pathogenic periodontal bacteria.

The objective of this study was to determine the presence of herpes simplex virus in patients with chronic periodontitis from a periodontics clinic in northeastern Mexico.

MATERIALS AND METHODS

Clinical samples (swabs) were obtained from patients presenting for the first time at the Undergraduate Periodontology Clinic of the School of Dentistry of the Universidad Autónoma de Nuevo León in Monterrey, Mexico. A general medical history and complete periodontal examination including diagnostic periapical radiographs, study models, photographs, and intraoral examination in order to diagnose the condition were performed.

Clinical parameters

Pocket depth was defined as the distance between the gingival margin and the base of the groove (Armitage, 2004). This measurement is performed with a calibrated periodontal #15 North Carolina probe and a #5 dental mirror, and the average physiological measures range from 1 to 3 mm.

The clinical attachment level was the distance between the cement-enamel junction and the base of the groove (Armitage, 2004). This measurement was performed with a calibrated periodontal #15 North Carolina probe and a #5 dental mirror.

The plaque index used was the Quegley and Hein index modified by Turesky (1970). This index measures the amount of plaque visible through a developing agent (fuchsine) on the buccal and lingual surfaces of all teeth except the third molars. The following criteria were used according to the clinical assessment: 0, no visible plaque on the tooth, the tooth surface is clean; 1, irregular plaque patches appear on the gingival third of the tooth, without forming a continuous band; 2, there is a continuous band of plaque on the gingival third, but without completely covering one third of the area examined; 3, the bacterial plaque covers the entire gingival third of the examined surface; 4, the plaque covers two thirds (gingival and middle) of the surface examined; 5, the plaque covers more than two thirds (gingival, middle, and incisal or occlusal) or completely covers the examined surface. After recording the values of individual teeth, these are added and divided by the number of teeth measured to obtain the mean values of the examined areas, which represents the bacterial plaque index.

The gingival index was proposed by Löe (1967) to objectively record the qualitative changes that occur in gum tissue, such as color changes, edema or swelling, bleeding and ulceration. The periodontal probe is gently slid along the soft wall of the groove and the value is determined for each tooth according to the following code: 0, absence of clinical inflammation, no bleeding on probing; 1, mild inflammation is clinically observed, characterized by a slight change in color, slight edema, a small change in texture and no gingival bleeding upon probing; 2, moderate inflammation can be observed clinically, characterized by frank gum redness, mild edema, tissue with a smooth texture and a shiny surface, an increase in volume with bleeding on probing; 3, severe inflammation with marked redness and edema, a smooth shiny surface, ulceration, an increase in bleeding with probing and a tendency to spontaneous bleeding. Values are recorded for mesial, middle, and distal points of the buccal surface and the midpoint of the lingual surface. Each of these values represents the Gingival Index. The values are added for the 4 areas of each tooth and divided by four to obtain the value of the tooth examined.

Sample collection

A careful examination was carried out in all patients to determine the presence of ulcers. The reason for the study was explained, and informed consent was obtained. Three samples were taken from each patient as follows: a swab (calcium alginate 0.9 mm) was inserted into the deepest pocket and then independently placed into 4 mL microtubes containing sterile PBS with 10% glucose at 4°C. The active part of the swab was cut with sterile forceps leaving the active portion in each tube, which were then deposited in a cooler for transport to a freezer where they were stored at -80°C until analysis.

Protocol used for DNA purification

DNA was purified from these samples using the Gentra Puregene Cell Kit® (Quiagen, Valencia, CA). The sample was thawed and then centrifuged at 14,500 rpm for 10 min. The glucose solution was carefully removed and 300 µL of cell lysis fluid and 1.5 µL of Puregene proteinase (Quiagen) were added. Afterwards, the Puregene procedure using a modified salting-out precipitation method was followed according to the manufacturer's instructions for obtaining DNA. A 10 µL Eppendorf pipette was used to place 16 µL of the kit mixture (PCR buffer [dNTP's, reagents, enhancer]) with the HSV oligos (Fwd AGCCTACTACTCGGAAAGCGAAGA, Rev TACATCGAGGCGTCATTTAGCGGA for Human herpesvirus 2 strain HG52, and Fwd AGTCCTACTGCGACCAGGA, Rev AGACATCCCATGGTACCAAAGACC for Human herpesvirus 1) (Table 1). Afterwards, 4 µL of the sample was added together with 0.2 µL of Taq polymerase. The components were mixed for placement in the thermocycler (MJ Mini Thermocycler BioRad Laboratories, Inc., Hercules, CA). PCR was carried out under the following conditions: DNA was initially heated for 1 min to 96°C, then denaturation was carried out for 30 s at 94°C for 30 cycles, annealing for 30 s at 58°C for 30 cycles followed by an extension period of 30 s at 72°C for 30 cycles and a final extension period of

Table 1. Primers used to identify herpesviruses.

Strand	Primer	ΔG selfdimer	ΔG harping	ΔG heterodimer	Tm	CG%	Size	Product size
Human herpesvirus 2 strain HG52, complete genome, accession no. JN561323.1								
Forward	AGCCTACTACTCGGAAAGCGAAGA	-5.19	-1.51	-7.81	59.3	50	24	200
Reverse	TACATCGAGGCGTCATTTAGCGGA	-6.76	-0.21		60	50	24	
Human herpesvirus 1 complete genome; accession no. X14112.1								
Forward	AGTCCTACTGCGACCAGGA	-6.24	-1.32	-6.37	57.6	57.9	19	634
Reverse	AGACATCCCATGGTACCAAAGACC	-13.69	-0.58		58.6	50	24	

Table 2. Clinical parameters of periodontitis found in the study population.

Parameter	Mild	Moderate	Advanced
Plaque index*	0.8	1.64	2.92
Gingival index**	0.9	1.8	2.3
Pocket depth (mm)	4.7	6.1	8.4
Insertion level (mm)	1.7	4.4	6.2

*Turesky, 1970; **Armitage, 2004.

10 min at 72°C for 1 cycle. Samples were then maintained at a temperature of 4 to 25°C. DNA was analyzed by electrophoresis in agarose gel; 30 µL of ethyl bromide was added and then electrophoresis was performed at 80 V for 30 min.

RESULTS

We studied 50 patients with chronic periodontitis; 32 women and 18 men with a mean age of 41 years and a range of 24 to 70 years. According to the attachment levels and pocket depth, 11 (22%) patients had mild chronic periodontitis, 29 (58%) moderate chronic periodontitis, and 10 (20%) advanced chronic periodontitis. According to age, mild (4) and moderate (3) periodontitis were predominant in women 20-35 years; mild (2) and moderate (12) disease with a few advanced cases (2) were predominant in those 36-50 years old; and in those >51 years, mild (3) and moderate (2) periodontitis were predominant with several advanced cases (3). In men mild (2) and moderate (7), periodontitis were predominant in the age group of 20-35 years. In those 36-50 years old, there was a predominance of moderate (2) and advanced (2) cases of periodontitis, which was also the case in those >51 years (3 and 3, respectively).

The clinical parameters found in the 50 patients studied are shown in Table 2. With regards to PCR, of the 50 samples processed, two were positive for HSV-1 and two for HSV-2. Three were women, one each with mild, moderate, and advanced chronic periodontitis, and one man (HSV-1) with mild chronic periodontitis (Figure 1).

DISCUSSION

Our results cannot define if herpes simplex virus is a catalyst for the advancement and progression of periodontal disease since only HSV-1 was found in only a small number of patients in our study population.

Dental plaque is directly related to periodontal disease, especially the subgingival plaque (Listgarten, 1976). Socransky and Haffajee in 2005 described the different bacterial groups associated with the pathogenesis of periodontal disease. They reported various groups associated with colonization, coaggregation, and pathogenic bacteria. It is important to point out that Socransky and Haffajee called the latter group the red complex, formed by *P. gingivalis*, *T. forsythensis* and *T. denticola*. The red complex is related to the magnitude of clinical parameters such as bleeding on probing, increased pocket depth, and loss of attachment levels.

Previous research has documented the presence of herpes viruses in peridonitis suggesting that there is a link between chronic periodontal disease and herpes viruses. Parra and Slots (1996) found five herpes viruses present in cases of advanced periodontitis and gingivitis. They detected cytomegalovirus in 60% of periodontitis patients, Epstein-Barr virus in 30%, herpes simplex virus in 20%, human papillomavirus in 17% and HIV in 7%. Contreras et al. (2000) and Saygun et al. (2008) stated that herpes virus is associated with periodontal disease and where clinical parameters are most affected, the presence of the virus was found. In their studies of healthy periodontal sites, they detected HCMV in 1 (9%) and EBV-1 in 2 (18%) pocket samples, and HCMV in 2 (18%) and EBV-1 in 3 (27%) gingival tissue samples. In periodontitis lesions, HCMV was found in 9 (64%) pocket samples and in 12 (86%) gingival tissue samples. EBV-1 was detected in 6 (43%) pocket samples and in 11 (79%) gingival tissue samples.

Other research has linked herpes virus to periodontal

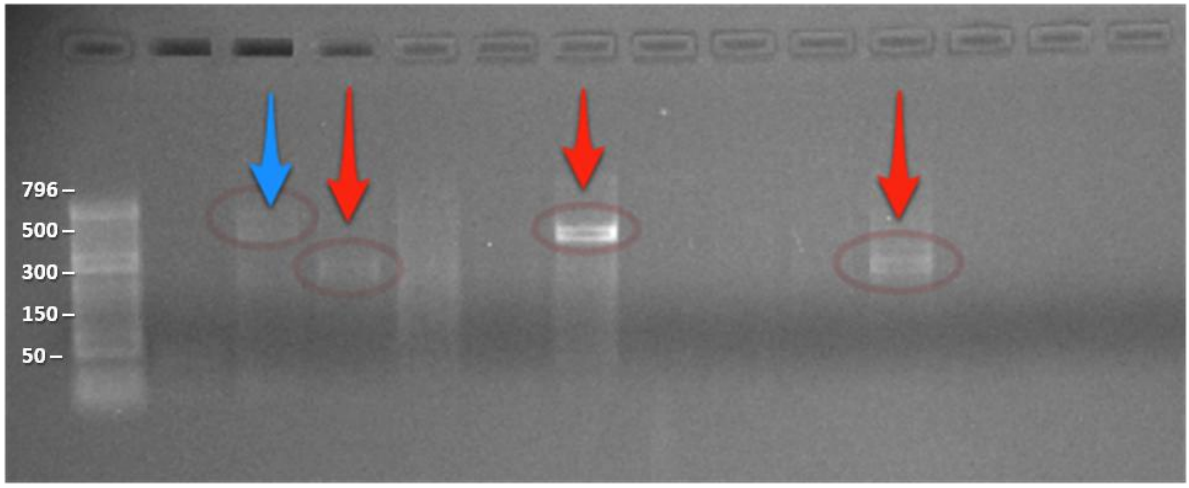

Figure 1. Positive electrophoresis samples for HSV type 1; three women (red arrows) and one man (blue arrow).

disease in marginal and apical periodontitis (Saygun et al. 2008; Slots 2005). In other research, it has been associated with aggressive and chronic periodontitis (Bilichodmath et al., 2009; Botero et al., 2007; Contreras et al., 2000; Ling et al., 2004, Yapar et al., 2003; Saygun et al., 2004).

We found that 8% of the individuals in our study from northeastern Mexico suffering from chronic periodontitis had HSV-1 present in the periodontal pockets. This is in contrast to the study by Kaufman et al. (2005) in which an incidence of 37.5% was found in saliva. The discrepancy in these figures could be due to the fact that study samples were taken throughout the oral cavity, unlike the present study in which only samples from periodontal pockets were obtained. Bilichodmath and colleagues in 2009 found the presence of HSV-1 in 100% of patients with chronic periodontitis and in 57% of patients with aggressive periodontitis. The possible reason why these same results were not found in our study are the age range, ethnic differences, the limited sample size, the methodology used, and the probable stage of herpes virus latency. Das et al. (2012) carried out a study to detect the presence of human cytomegalovirus (HCMV),

Epstein barr virus (EBV), and herpes simplex virus (HSV-1 and HSV-2) in chronic periodontitis, aggressive periodontitis, and healthy individuals divided into three groups: periodontally healthy individuals aged ≥ 21 years - 45 years with a sulcus depth ≤ 3 mm; chronic periodontitis in patients aged ≥ 35 years, with 30% of sites involved, a periodontal pocket depth ≥ 6 mm, and an attachment loss ≥ 3 mm, with severe bone loss (≥50% of root length) and patients with aggressive periodontitis aged ≥ 18 years, with 30% of sites involved, a periodontal pocket depth ≥ 6 mm, and an attachment loss ≥ 3 mm, with severe bone loss (≥50% of root length). In these patients HSV-1 was detected in sites with chronic periodontitis in 76% in sites with aggressive periodontitis in 80%; EBV was detected in 32% of sites of chronic periodontitis and aggressive periodontitis.

Our results were not able to define if HSV-1 is a catalyst for the advancement and progression of periodontitis; however, HSV-1 was present in our population. This opens the possibility of studying the presence of herpes viruses in a larger sample of patients, improving the sample collection and processing protocol.

Conclusion

The presence of HSV-1 in periodontal pockets in our study population opens the possibility of research in a greater number of patients and the possibility of searching for the presence of other herpesviruses. The low incidence of HSV in this study could be attributed to the low quantity of DNA collected by the swab, since its introduction into the periodontal pocket is limited.

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