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ORIGINAL ARTICLE

The prevalence of human herpes viruses in the saliva of chronic periodontitis patients compared to oral health providers and healthy controls

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Abstract The causative agents in periodontal disease are periopathogenic bacteria; however, viruses have been implicated. The aim of this study was to examine the prevalence of different HHVs in the saliva of chronic periodontitis patients and to compare it to two groups of healthy controls. Three groups were included: chronic periodontitis patients (CP), periodontally healthy patients (NP) and oral health providers with a healthy periodontium (NPOHP). For each subject, 1 ml of unstimulated whole saliva was collected and mixed with 2 ml lysis buffer. HHVs assays were performed using real-time PCR. Fifteen percent of the subjects in the CP group tested positive for CMV compared to none in the NP and NPOHP groups ($p = 0.04$). Recurrent herpes was more frequent in females (51.7 %) than in males (33.3 %), and this was statistically significant ($p = 0.038$). The higher prevalence of CMV in the unstimulated saliva of CP patients suggests that CMV may play a role in the pathogenesis of chronic periodontitis.

Introduction

The major causative agents in periodontal disease are periopathogenic bacteria [1]. Yet, while these bacteria have been identified (to a greater or lesser extent), some other microorganisms, mainly viruses, have been implicated in the pathogenic flora of the periodontium [2, 3]. In particular, human herpesviruses (HHVs) have been detected in subjects with both chronic and aggressive forms of periodontal disease [2]. HHVs (order *Herpesvirales*, family *Herpesviridae*) are ubiquitous pathogens that intermittently reactivate from latency. Eight distinct members of the family *Herpesviridae* that infect humans have been identified. They include herpes simplex virus type1 (HSV-1) and HSV-2 (subfamily *Alphaherpesvirinae*, genus *Simplexvirus*), varicella-zoster virus (VZV; subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, species *Human herpesvirus 3*), Epstein-Barr virus (EBV; subfamily *Gammaherpesvirinae*, genus *Lymphocryptovirus*), cytomegalovirus (CMV; subfamily *Betaherpesvirinae*, genus *Cytomegalovirus*), human herpesvirus 6 (HHV-6; subfamily *Betaherpesvirinae*, genus *Roseolovirus*], HHV-7, and HHV-8 (also known as Kaposi's sarcoma-associated herpesvirus, KSHV; subfamily *Gammaherpesvirinae*, genus *Rhadinovirus*) [4]. Following the initial lytic infection, HHVs establish a latent state in a diverse group of cells that ensures survival of the viral genome throughout the life of the host. Periodic reactivation and viral recrudescence crop up after stress and alterations in immune surveillance. Frank immunosuppression is associated with more-severe disease and complications (e.g., encephalitis, pneumonia, hepatitis, and various forms of cancer) [5–7]. Over 95 % of the adult population is infected with HHVs [8–11]. Herpesviruses, especially human cytomegalovirus (HCMV), Epstein-Barr virus type 1 (EBV-1) and HCMV/EBV-1 dual infection, have recently been identified in most of the advanced periodontitis lesions of children, adolescents, and adults [12]. HSV-1, HHV-6, HHV-7 and HHV-8 could also be detected in some periodontitis lesions [12].

Imbronito et al. studied the presence of HHVs and periodontal pathogens in sub-gingival plaque samples of

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patients with chronic periodontitis (CP), generalized aggressive periodontitis (GAgP), and gingivitis [13]. They detected HSV-1, CMV, and EBV-1 in both the GAgP and CP groups. However, they reported that HSV-1 and EBV-1, rather than CMV were more frequently associated with CP and GAgP. Conversely, Nibali et al. [14], in a similar study, could not detect CMV DNA in any of the samples. EBV DNA was detected in LAgP (25 %), GAgP (3 %) subjects and healthy individuals (10 %).

Tantivanich et al. used nested PCR to evaluate CMV, HHV-6 and EBV-1 in CP patients and compared them to healthy controls [15]. In the CP patient group, CMV was found in 34 %, HHV-6 was found in 4 %, and EBV was not detected. In the healthy controls, CMV was found in one (3.3 %) subject while HHV-6 and EBV-1 were not detected at all. In contrast to this study, other authors found an important association between CMV and EBV-1 among patients with chronic periodontitis [16].

CMV and HSV have been detected in gingival crevicular fluid (GCF) samples obtained from chronic periodontitis patients [17]. Contreras and Slots [2] and Contreras et al. [18] concluded that subgingival co-infections with EBV-1 and CMV are associated with the subgingival presence of some periodontal pathogens and consequent periodontitis. These authors have suggested that HHV infection might potentiate the virulence effect of oral bacteria by decreasing host resistance against subgingival colonization and multiplication of periodontal pathogens. Thus, alteration between latent and active HHV infection in the periodontium might lead to a transient local immunosuppression and explain in part the episodic progressive nature of human periodontitis. The tissue tropism of herpesvirus infections might also explain the localized pattern of tissue destruction in periodontitis. The absence of herpesvirus infection or viral reactivation might also explain why some individuals carry periodontopathic bacteria while still maintaining periodontal health. These conflicting reports may be attributed to methodological differences (medium that was sampled, i.e., GCF versus saliva or plaque and tissue biopsies) [15]. In addition, variations in demographic characteristics such as age, gender and occupation between studies may affect the prevalence and detection rate in these assays [19, 20].

Oral health care providers might be at greater risk of contracting airborne and fluid-borne microorganisms because, in most treatment scenarios, their mouth is in close proximity to that of the patient. Browning and McCarthy [21] have reported several cases in which HSV-1 was contracted by dental team members following treatment of infected patients. Herbert et al. [22] showed higher EBV serum antibody titers in dentists and dental students compared to pre-clinical dental students, while other assays showed similar titers for HSV-1, CMV and HHV6, but the occupational risk of HHV infection for dental workers has not yet been established.

Over the years, technological advances have expanded the usefulness of oral fluids in the diagnosis of diseases, prediction of disease progression, monitoring of therapeutic drug levels, and detection of illicit drugs. The simple noninvasive nature of sample collection and the relationship between oral fluid and other diagnostic fluid make them valuable clinical tools [23–27].

The aim of this study was to examine the prevalence of HHVs in the saliva of chronic periodontitis patients and to compare them to those found in periodontally healthy oral care providers and non-periodontitis controls.

Materials and methods

This study was initially approved by the ethics committee of the Rambam Health Care Campus. Patients that were screened at the department of Periodontology and Oral Medicine were invited to join the study providing that they had chronic periodontitis with no recent periodontal treatment. To be eligible for the study, subjects were required to have a minimum of 20 teeth. Chronic periodontitis patients had to exhibit two or more teeth with clinical attachment loss (CAL) $\geq$6 mm and at least one site with a pocket depth (PD) $\geq$5 mm [28]. All subjects with PD values below 4 mm were considered periodontally healthy. Data on recurrent herpes was collected via a patient questionnaire. The nature of the study was conveyed, and written informed consent was obtained from all of these subjects prior to commencement. Age-matched controls were identified among periodontally healthy patients seeking consultation for possible implant placement and were invited to join the study (NP group). Finally, the staff of the department (dentists, dental hygienists and dental assistants) were screened, and those individuals with a healthy periodontium formed the third group of oral health providers (NPOHP). Twenty patients were included in each of these groups.

Patients under 18 years of age and pregnant and lactating woman were excluded from the study. Subjects were also excluded if they received medications such as antibiotics within the previous six months or had had periodontal treatment in the past twelve months.

Saliva collection and sampling

All subjects were requested to eject $\geq$2 ml of unstimulated saliva into tubes containing sterile viral transport medium (M-199 containing 2 % FCS; Biological Industries, Kibbutz Beit Haemak, Israel). Samples were immediately placed on ice or cold packs for transport to the laboratory.

One milliliter of the saliva specimen was mixed with 2 ml of lysis buffer (nucliSENS EasyMAG lysis buffer; BioMerieux, Marcy I'Etoile, France) and kept at 4 °C for no longer than 4 days until nucleic acid extraction. Sample collection and analysis were carried out using the same protocol that was used in a previous investigation by our group [27]. In short, nucleic acids were extracted from the saliva samples using an Easymag NucliSENSE instrument (BioMerieux, Marcy I'Etoile, France). The off-board lysis-specific B 2.0 protocol was used according to the manufacturer's instructions (elution volume 100 µl). Ten microliters (or 5 µl in case of HSV-1) of the nucleic acid samples was used as template for the 25-µl real-time PCR reaction containing Absolute Blue QPCR mix (Thermo scientific UK), 300 nM each primer and 200 nM TaqMan probe. A positive and a negative control were used for each test. The primers and probes for the detection of CMV (gB gene), EBV (BNRF1 gene), HHV6 (U6 gene) and HSV-1 (gpG gene) were described previously by Boeckh et al. [29], Niesters et al. [30], Tavakoli et al. [31], Filén et al. [32], respectively. Real-time PCR assays were performed using a Rotor-Gene 3000 or 6000 instrument (Corbett Research/QIAGEN, Hilden, Germany).

Statistical analysis

Descriptive statistics were initially used to characterize the entire population and the three groups. Next, analysis of variance (ANOVA) with Scheffe's LSD was used to compare continuous data between the three groups. A chi-square test was used to compare categorical data between the three groups. A 5 % significance level was employed.

Results

Sixty subjects were recruited into this study. One sample (in the NP group) was deemed unreadable; thus, a total of 59 subjects are included in this study (Table 1). Age ranged from 20 to 81 (mean, 46.4 ± 13.8 years). The subjects in the NPOHP group were slightly younger (45.0 ± 11.5 years) compared to the CP subjects (47.25 ± 16.7 years), and NP healthy subjects (47.05 ± 13.5 years); however, these differences were not statistically significant ($p = 0.8497$). Fifty-five percent (11/20) of the subjects in the CP group were males, while their proportions in the other NP and NPOHP groups were much lower (21.5 % and 30 %, respectively). These differences were marginally significant ($p = 0.073$).

Fifteen percent of the subjects in the CP group tested positive for CMV in their unstimulated whole saliva, compared to none in both the NP and NPOHP groups (Table 2). These differences were statistically significant ($p = 0.0459$). Likewise, HSV1 was found in a small proportion of the study population, with a frequency ranging from 5 % (CP group) to 21 % (NP group), though not statistically significant.

In contrast, HHV6 was frequently found in all groups, with the highest proportions in the NPOHP group (75 %), followed by the NP (68.4 %) and CP (55 %) groups; however, these differences were not statistically significant ($p = 0.3961$). Similarly, EBV was present in about one-third of these samples (40 %, 36.8 % and 35 %) for the CP, NP and NPOHP groups, respectively.

Recurrent herpes was reported in approximately half of these subjects (51.2 %) with similar proportions across all groups. A larger number of females reported having recurrent herpes (15/29, 51.7 %) compared to males (5/15, 33.3 %) and these differences were statistically significant ($p = 0.038$). Nonetheless, all of the viruses that were tested appeared to be similarly distributed in the saliva of males and females (Table 3).

Finally, age did not seem to have an effect on either the self-reported prevalence of recurrent herpes or the distribution of any of the tested viruses in the saliva of healthy and CP patients.

Discussion

In the current study, 15 % of the chronic periodontitis patients exhibited CMV in their saliva, while none was detected in either of the periodontally healthy groups. Tantivanich et al. [15] reported a somewhat higher proportion (34 %) in saliva samples from CP patients and a low proportion (3.3 %) in healthy controls. Other studies have reported even higher proportions of CMV detection in salivary samples from CP patients, ranging from 63 % to 75 % [13, 33] Chalabi et al. [3] studied subgingival and supragingival plaque samples from patients with chronic periodontitis and observed that 59 % of these patients were positive for CMV, while no CMV was detected in healthy control samples. Similar results were reported by Sahin et al. [34]. In this study, EBV was found to be present in approximately 1/3 of all subjects regardless of their periodontal condition and occupation. Similar results were observed previously [14]. In contrast, much higher proportions were reported by Ling et al. [33] (63 %) and Dawson et al. [35] (81.5 %) in salivary samples of CP patients. Another study found salivary EBV in 79 % of CP patients but only in 33 % of gingivitis patients; however, 54 % of completely edentulous patients were also found to be positive for EBV [34].

This phenomenon might be associated with patients wearing dentures and mixed infections associated with it. Saygun et al. [36] studied EBV in plaque and salivary

Table 1 Demographics and patient data

Variable	Group				
	Chronic periodontitis Mean ± SD (%)	Non-periodontitis Mean ± SD (%)	Non-periodontitis oral health professionals Mean ± SD (%)	p-value	All subjects Mean ± SD (%)
Age (years)	47.25 ± 16.7	47.05 ± 13.5	45.0 ± 11.5	0.8497‡	46.40 ± 13.8
Gender	9:11 (55)	15:4 (21.5)	14:6 (30)	0.0730^	38:21 (35.6)
F:M ratio (% males)					
Recurrent Herpes Y:N (%)	4:2 (66.6)	9:10 (47.4)	8:8 (50)	0.6233	21:20 (51.2)

‡ Analysis of variance with Scheffe LDS

^ Chi-square test

Table 2 Frequency distribution of different viruses in patients' unstimulated salivary samples

Group Virus/condition/	Chronic periodontitis Y:N (% positive)	No Periodontitis Y:N (% positive)	No Periodontitis oral health professionals Y:N (% positive)	p-value*
HHV6	11:9 (55)	13:6 (68.4)	15:5 (75)	0.3961
EBV	8:12 (40)	7:12 (36.8)	7:13 (35)	0.9468
CMV	3:17 (15)	0:19 (0)	0:20 (0)	0.0459
HSV1	1:19 (5)	4:15 (21)	2:18 (10)	0.2863
Recurrent Herpes	4:2 (66.6)	9:10 (47.4)	8:8 (50)	0.7063

*Chi-square analysis

Table 3 HHVs and recurrent herpes—comparison between genders

Group Virus/condition	Females Y:N (% positive)	Males Y:N (% positive)	p-value*
HHV6	27:11 (71.1)	12:9 (57.1)	0.2798
EBV	12:26 (31.6)	11:10 (51.4)	0.2225
HSV1	5:33 (13.2)	2:19 (9.5)	0.6794
CMV	3:35 (7.9)	0:21 (0)	0.1863
Recurrent Herpes	15:14 (51.7)	5:10 (33.3)	0.038

*Chi-square analysis

samples of patients with different periodontal status. Higher readings were reported in the saliva compared to the plaque samples. These differences were statistically significant for the gingivitis group. Therefore, comparison of studies utilizing different sampling media might have an intrinsic methodological error.

The prevalence of HHV6 in the CP group (55 %) was lower than but not statistically different from that of the other groups (68-75 %). Similarly, Pereira et al. [37] found HHV6 in 67.8 % of saliva samples from healthy individuals. Several other studies reported higher prevalence of HHVs in saliva of periodontitis patients compared with healthy controls [13, 15, 38–40]. In our study HSV-1 (gpG gene) was present in 5 % to 21 % of the salivary samples, with no statistical difference between the groups. Beydoun et al. [41] reported from the 1999-2004 National Health and Nutrition Examination Survey a 60 % prevalence in adults between 18 and 49 years of age. These rates are of the same order of magnitude as those reported in other industrialized countries. In a recent study, describing the seroepidemiology of HSV-1 in eight European countries, age-standardized HSV-1 seroprevalence ranged from 52 % in Finland to 84 % in Bulgaria [42].

In the United States, the seroprevalence of HSV-1 decreased from 62 % between 1988 and 1994 to 57.7 % between 1999 and 2004 [43]. Approximately one-half of the subjects in all groups had reported having recurrent herpes at least once in the past. These findings are in accordance with Beydoun et al. [41] and Xu et al. [43]. In our investigation, a higher frequency of past episodes of recurrent herpes labialis was observed in females than in males ($p = 0.038$). Similarly, in a study of 2000 individuals, about 45 % of them reported having at least one episode of herpes labialis infection; females were found to be affected more commonly than men [OR 1.42 (1.18-1.70)] [44].

Human herpes viruses are thought to be a potential threat to healthcare workers. However, in view of their ability to establish latent infections with intermittent shedding, particularly in the oral cavity and peri-oral regions, in the present study, we could not find differences in the prevalence of HHVs amongst periodontally healthy oral health care providers and other periodontally healthy controls. The literature pertaining to HHV infection as an

occupational hazard is very limited. While generally accepted as a potential risk, the evidence level is very low. Browning and McCarthy [21] have recently published a small case series that is of anecdotal nature. Thus, the present findings further highlight the urgent need for more research into the potential of HHV as a true occupational hazard for dental professionals

A possible limitation of our study is the small number of patients as well as the fact that sampling of saliva was performed during different seasons (winter, spring and summer). This may have affected our results, as the prevalence of viruses is known to fluctuate with seasonal changes. In spite of these limitations, it can still be concluded that higher concentrations of CMV were found in CP patients. These results and those of other reports substantiate the need for further investigations on the correlation of viruses in the aetiopathogenesis of periodontitis.

Conclusion

Higher prevalence of CMV was detected in unstimulated saliva of CP patients. This study shows an association between CMV and chronic periodontitis. However, further studies are necessary in order to ascertain a causal relationship.

Conflict of interest The authors report no conflicts of interest related to this study, which was wholly supported by the authors.

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