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Short communication

Detection of herpetic viruses in gingival crevicular fluid of patients suffering from periodontal diseases: prevalence and effect of treatment

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Background/Aim: Although the role of bacteria in the etiology of periodontitis is well established, it has been suggested that herpetic viruses could contribute to the initiation and progression of this disease. The aim of this study was to determine the prevalence of human cytomegalovirus (HCMV), Epstein–Barr virus (EBV) and herpes simplex virus (HSV) in gingival crevicular fluid (GCF) samples obtained from periodontally healthy, gingivitis and periodontitis patients. In addition, the effect of periodontal treatment (scaling and root planing) on the persistence of herpetic viruses was evaluated in a sub-group of patients suffering from chronic periodontitis.
Methods: The presence of viruses in GCF samples was assessed by a nested PCR amplification technique. The persistence of viruses in periodontal sites was evaluated following a scaling and root planing therapy.
Results: A statistically significant higher prevalence of HCMV was observed in periodontitis patients as compared to healthy control subjects (35 vs. 8%, respectively; $P = 0.0377$). A trend for a higher prevalence of HSV was also noted in the periodontitis group, in comparison with healthy control subjects. In addition, a higher prevalence of HCMV was associated with deep periodontal pockets in subjects suffering from periodontitis. In the sub-group of periodontitis patients, periodontal therapy resulted in the elimination (HCMV and EBV) or reduction (HSV) of the herpetic viruses.
Conclusions: This study showed that the prevalence of HCMV and HSV viruses in GCF is higher in patients suffering from periodontitis compared to periodontally healthy subjects, and that the prevalence of HCMV is higher in deep periodontal pockets. It also brought evidences that periodontal therapy may be associated with virus elimination in diseased sites.

Key words: gingival crevicular fluid; herpetic viruses; periodontitis

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Periodontal diseases comprise a variety of conditions, lesions and pathological processes affecting the tooth supporting tissues. It is now well accepted that specific bacterial species, mostly Gram negative and strictly anaerobic, play a key role in the etiology of periodontitis (16). Among them, *Treponema denticola*, *Porphyromonas gingivalis* and *Tannerella forsythia* have been strongly associated with the severity of periodontitis (16). These bacteria stimulate the host immune response, which results in production of inflammatory mediators and matrix metalloproteinases, leading to connective tissue destruction and bone loss (5, 6, 10). Although the role of bacteria in the etiology of periodontitis is well established, it has been suggested that herpetic viruses, including the human cytomegalovirus (HCMV), the Epstein–Barr virus

(EBV) and the herpes simplex virus (HSV), could contribute to the initiation and progression of periodontitis (4, 9, 14). Furthermore, the gingival sulcus or periodontal pocket has been proposed to act as a reservoir between periods of recurrence of the herpetic infections (19).

Contreras and Slots (3) observed that the prevalence of virus in periodontitis patients correlated with periodontal pocket depth. In addition, viral co-infections were also reported to be more frequent in deep periodontal pockets (3, 13). Interestingly, Sunde et al. (17) recently reported that an antiviral treatment given to a patient with recurrent periodontitis and showing a high load of EBV in subgingival sites resulted in a decreased detection of EBV and an improved periodontal condition. Although additional studies are required to clarify their exact roles in the pathogenesis of periodontitis, viruses may cause direct damages to host tissue or alter the host defense system (14). The aim of this study was to determine the prevalence of HCMV, EBV, and HSV in gingival crevicular fluid (GCF) samples obtained from periodontally healthy, gingivitis and periodontitis Caucasian subjects. In addition, the effect of periodontal treatment (scaling and root planing) on the persistence of herpetic viruses was evaluated in a subgroup of patients suffering from chronic periodontitis.

A total of 48 subjects (22 men, 26 women), aged 21–63 years, were recruited from the dental clinics of the Faculté de Médecine Dentaire (Université Laval), after having signed a written informed consent. The protocol has been approved by the Université Laval ethics committee (# 2003-063). Patients having taken antibiotics in the 6 months preceding periodontal examination or having received periodontal treatments were excluded from the study. At the initial examination, periodontal pocket depth (PD), clinical attachment loss (CAL), and bleeding on probing (BOP) were recorded at six sites per tooth for all teeth in the oral cavity. A diagnosis of gingivitis was assigned to four subjects presenting generalized clinical signs of gingival inflammation with PD ≤ 4 mm and CAL ≤ 1 mm, whereas a diagnosis of periodontitis was given to 31 patients having more than five sites with PD ≥ 6 mm and BOP, with CAL ≥ 3 mm at these sites. Thirteen subjects who did not bleed on probing and did not have PD > 3 mm were considered periodontally healthy. Each participant was recalled 1 week after the initial periodontal examination and GCF samples were then collected from at least three healthy sites from each subject and from sites showing PD ≥ 4 mm with BOP (up to 10 sites per subject). Prior to sampling, supragingival plaque was removed with sterile cotton pellets and the sites were isolated with cotton rolls to avoid saliva contamination. The samples were taken with paper strips (2 × 10 mm; 3 MM; Whatman, Clifton, NJ) inserted to the depth of the periodontal pocket/gingival sulcus for 30 s. Each paper band was then placed in a microtube containing 250 μl of 10 mM Tris–HCl buffer – 1 mM EDTA (pH 8.0) and stored at −20°C until processed. Following the initial periodontal examination and GCF samples collection, patients suffering from periodontitis were given oral hygiene instructions and were treated by scaling and root planing with periodontal curettes and ultrasonic instruments. At least 6 weeks after scaling and root planing, a periodontal re-evaluation was performed (PD, CAL and BOP) on a sub-group of eight periodontitis patients. These subjects were recalled a week later to collect GCF samples from the initially collected sites.

DNA was isolated from the buffer solution containing the paper band with the DNeasy Tissue System (QIAGEN Inc, Mississauga, ON, Canada) according to the manufacturer's instructions. The nested PCR amplification technique (nested-PCR) described by Parra and Slots (11) was used to identify viral DNA from EBV, HSV and HCMV. Detection of EBV was performed using outer primers (5′-AGGGATGCCTGGACACAAGA-3′ and 5′-TGGTGCTGCTGGTGGCAA-3′) and inner primers (5′-TCTTGATAGGGATCCGCTAGGATA-3′ and 5′-ACCGTGGTTCTGGACTATTCGGATC-3′). Detection of HSV was carried out using outer primers (5′-AGGGATGCCTGGACACAAGA-3′ and 5′-TACATCGGCGTCATCTGCGGGG-3′) and inner primers (5′-CAGTTCGGCGGTGAGGACAAA-3′ and 5′-GCGTTTATCAACCGCACCTCC-3′). Lastly, the outer primers (5′-CAGACACAGTGTCCTCCCGCTCCTC-3′ and 5′-CCTAGTGTGGATGACCTACGGGCCA-3′) and inner primers (5′-CAGACACAGTGTCCTCCCGCTCCTC-3′ and 5′-CCAGAGTCCCCTGTACCCGC-3′) were used to monitor HCMV. For each viral amplification, the following number of amplification cycles and annealing temperatures were respectively used. For HSV, outer amplification was 25 cycles at 55°C and inner amplification was 30 cycles at 55°C. For EBV, outer amplification was 25 cycles at 60°C and inner amplification was 30 cycles at 55°C. For HCMV, outer amplification was 25 cycles at 60°C and inner amplification was 30 cycles at 55°C. For each round of PCR performed, five negative controls were included. Ten microlitres of PCR products were loaded on a 1% agarose gel stained with ethidium bromide and amplicons were detected under a 312 nm UV light. Sensibility thresholds of HCMV, EBV, and HSV detection were determined by using serial dilutions of known concentrations of viral amplicons as DNA template in the nested-PCR protocol. This analysis showed that detection levels for HCMV, EBV, and HSV with the nested-PCR technique were 11, 25 and 3 viral copies, respectively.

A total of 300 GCF samples were collected from the 48 volunteers. In order to eliminate the bias associated with the collection of a higher number of GCF samples from patients suffering from periodontitis, a correction by statistical resampling has been applied to the raw data, where the three deepest sampling sites have been retained for each patient. Chi square statistical tests were performed to verify the relationship between the presence of HCMV, EBV, and HSV and periodontal status. As reported in Table 1, 45% of the subjects suffering from periodontitis harboured at least one viral species, while the prevalence was slightly lower in the gingivitis and healthy groups. A higher prevalence of HCMV was observed in periodontitis patients as compared to healthy control subjects (35 vs. 8%, respectively; $P = 0.0377$), and a trend for a higher prevalence of HSV was noted in the periodontitis group, in comparison with healthy control subjects (13 vs. 0%; $P = 0.0747$). Interestingly, a difference in EBV detection was also observed between periodontitis patients and periodontally healthy subjects, the virus being isolated more frequently from GCF samples of healthy subjects (3 vs. 23%, respectively; $P = 0.0298$).

Logistic regression tests at 5% threshold were applied to the raw data (no correction by statistical sampling) to verify the effect of the sampling site depth and the presence of herpetic viruses. Table 2 reports the detection of herpetic viruses in periodontal sites subcategorized by pocket depth for healthy, gingivitis and periodontitis subjects. A trend was observed between HCMV prevalence and pocket depth, in subjects suffering from periodontitis ($P = 0.114$). No correlation between pocket depth and detection of HSV or EBV could be found.

Eight of the fourteen patients suffering from periodontitis and harbouring herpetic

Table 1. Detection of herpetic viruses in GCF samples according to the patients' periodontal status

Patients' periodontal status	Number of subjects	Age range (mean)	Male/Female	Subjects with at least one virus species	Subjects with at least two virus species	Subjects with viruses (% of subjects)		
						HCMV	EBV	HSV
Health	13	21–26 (23)	8/5	4 (31%)	0	1 (8)	3 (23)*	0
Gingivitis	4	22–34 (25)	1/3	1 (25%)	0	1 (25)	0	0
Periodontitis	31	28–63 (45)	14/19	14 (45%)	2 (6%)	11 (35)**	1 (3)	4 (13)***

A correction by statistical resampling has been applied to the raw data in order to eliminate the bias associated with the collection of a higher number of GCF samples from patients suffering from periodontitis.
*Different from periodontitis patients at $P = 0.0298$.
**Different from healthy subjects at $P = 0.0377$.
***Different from healthy subjects at $P = 0.0747$.

Table 2. Detection of herpetic viruses in GCF samples subcategorized by periodontal pocket depth in healthy, gingivitis and periodontitis patients

Periodontal status	Probing depth (mm)	Number of sites	Sites with at least one virus species	Sites with viruses (% of sites)		
				HCMV	EBV	HSV
Health	≤3	63	8 (13%)	2 (3)	4 (6)	2 (3)
Gingivitis	≤3	16	2 (13%)	1 (6)	1 (6)	0 (0)
	4	4	4 (100%)	4 (6)	0 (0)	0 (0)
Periodontitis	≤3	47	11 (23%)	6 (13)	2 (4)	3 (6)
	4–5	28	7 (25%)	6 (21)	2 (7)	0 (0)
	6–7	110	27 (25%)	22 (20)	4 (4)	2 (2)
	8–10	32	14 (44%)	14 (44)	4 (12)	1 (3)

Raw data with no correction by statistical resampling were used. This explains why some sites are positive for certain viruses while they may have been recorded as negative in Table 1.

viruses agreed to participate in the second phase of the study. They all received a periodontal treatment consisting of scaling and root planing, and at least 6 weeks following this therapeutic modality, GCF samples were collected from the previously collected sites. The presence of herpetic viruses before and after treatment for each individual is reported in Table 3. Although the small number of sites analyzed did not allow to perform statistical analysis, a clear trend for the elimination of herpetic viruses, more specifically HCMV, following periodontal treatment was noted.

The correlation between the presence of herpetic viruses in gingival tissues and in GCF samples was previously assessed by Contreras et al. (2). In GCF samples collected from periodontitis sites, EBV-1 was present in 43% of GCF samples and HCMV in 64% of GCF samples, whereas biopsies did show the presence of EBV-1 in 79% of sites and HCMV in 86% of sites. This suggests that although GCF samples slightly underestimate viral infection, it may be appropriate for analyzing the presence of herpetic viruses in periodontal sites. In the present study, the prevalence of herpetic viruses in GCF samples was found to be higher in patients suffering from periodontitis (45%) than in periodontally healthy subjects (31%). A similar trend was reported by Parra and Slots (11) who observed that 78% of patients suffering from periodontitis possessed at least one of the five studied viruses (HCMV, EBV, HSV, human immuno-deficiency virus [HIV] and human papovavirus [HPV]) in GCF samples. More particularly, these authors reported that, among the patients suffering from advanced periodontitis, HCMV was detected in 60% of them, EBV in 30% and HSV in 20%. A more recent study by Contreras et al. (2) showed that HCMV was present in 64% of subjects suffering from moderate/advanced periodontitis, EBV-1 in 43% and HSV in 21% of these patients. In addition, Saygun et al. (13) showed that HCMV is detected in 43% of their chronic periodontitis patients (30 subjects), EBV in 17% and HSV in 7%, whereas in the periodontally healthy group, HCMV and EBV were both found in 14% of the patients, while HSV could not be detected. The above results are in agreement with our study showing that HCMV is more prevalent that EBV and HSV in diseased sites from periodontitis patients. However, a recent report by Imbronito et al. (8) indicated that HSV-1 was the most prevalent virus detected in Brazilian patients with aggressive periodontitis, followed by HCMV and EBV-1. Our data also supports previous reports (1, 3, 18) showing a positive correlation between HCMV detection and periodontal pocket depth in patients suffering from periodontitis.

Table 3. Effect of scaling and root planing on viral detection at 6 weeks post-treatment of periodontal sites initially presenting viral species

Virus	Number of sites with virus	
	Before treatment	After treatment
HCMV	7	0
EBV	1	0
HSV	4	1

The second part of our study did compare the prevalence of HCMV, EBV and HSV viruses before, and at least 6 weeks following scaling and root planing in a sub-group of eight patients suffering from generalized periodontitis harbouring at least one viral species at initial presentation. In this study, scaling and root planing did allow the elimination or reduction of HCMV concentration to a non-detectable level in all sites ($n = 7$) initially presenting this viral species. While scaling and root planing led to complete elimination of HCMV at reevaluation, one should consider that these viruses may be periodically active, and that prevalence of viruses may be lower at a later time point regardless of the treatment. However, for ethics reasons we could not have an untreated control group. It could be hypothesized that the elimination of HCMV may result from the reduction of the bacterial load following periodontal therapy. This is supported by the fact that positive correlations were found between HCMV and important periodontopathogens such as *P. gingivalis*, *T. forsythia* and *Campylobacter rectus* (12). Slots et al. (15) suggested that the relationship between HCMV and *P. gingivalis* is bi-directional. Indeed, HCMV reactivation may suppress host defenses and allow growth of *P. gingivalis*, while this bacterium, through its inflammatory-inducing products, has the potential to activate periodontal HCMV (15). EBV,

which was present in only one site of patients involved in the second phase of the study, was also eliminated following the periodontal therapy, while HSV persisted only in 1 out of 4 sites where it was initially present. Unfortunately, statistical analysis could not be performed due to the small number of sites analyzed. Our data differs from those of Hanookai et al. (7) who examined the effects of scaling and root planing on the subgingival presence of HCMV, EBV and HSV. They showed that, 1 month following periodontal treatment, HCMV and EBV were still present in deep sites, even if there has been a notable decrease of viral presence at 7 days post-treatment. Consequently, these authors concluded that scaling and root planing therapy had a temporary effect of viral load reduction, but no long term effect on viral detection in crevicular fluid of patients presenting deep pockets (7). Additional studies with a larger number of sample sites are needed to clarify this difference.

In summary, our study showed that the prevalence of HCMV and HSV viruses in GCF is higher in patients suffering from periodontitis compared to healthy control subjects, and that the prevalence of HCMV increases with periodontal pocket depth. Although additional studies involving more subjects are required, we brought evidences that periodontal therapy may be associated with reduction of HCMV viral load to a non-detectable level at 6 weeks post scaling and root planing.

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