# The Assessment of the Association Between Herpesviruses and Subgingival Bacterial Plaque by Real-time PCR Analysis

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*This study used a real-time PCR analysis to determine possible correlations between periodontal presence of human cytomegalovirus, Epstein-Barr virus and various putative parodontopathogenic bacteria. The study included 18 patients (aged 18-38 years) with aggressive periodontitis, 12 patients (ages 37 to 62) with chronic periodontitis and 30 periodontally healthy subjects (aged 21-54 years). Clinical periodontal evaluation included plaque index, gingival index, percentage of bleeding on probing sites, and probing depth. In each patient, a subgingival bacterial plaque sample was obtained from the deepest periodontal pocket. The real-time fluorogenic PCR detection system was used to determine the number of infectious agents. Human cytomegalovirus was detected in 17 sites with periodontal lesions and in two healthy periodontal sites, and the Epstein-Barr virus was detected in 19 sites with periodontal lesions and in 3 normal periodontal sites. Positive correlations were found between human cytomegalovirus and P. gingivalis, T. forsythia and C. rectus. The Epstein-Barr virus positively correlated with P. gingivalis and T. forsythia. Unfavorable changes in environmental exposure or alteration of the immune system genes can periodically suppress the host's defence against periodontal aggression, which can then result in reactivation of resident herpesviruses and increased pro-inflammatory mediators followed by an increase in pathogenic bacteria.*

*Keywords: real-time PCR, periodontal pathogens, herpesviruses*

The pathogenic process of periodontitis includes dynamic interactions between different infectious agents and host, innate and specific interconnected responses [1, 2]. However, despite a long history of research in the pathobiology of periodontitis, a definitive claim about its probable molecular causes remains elusive. Bacterial pathogens are a causal antecedent for the development of periodontitis, but the simple bacterial plaque does not seem to provide a sufficient basis to explain important clinical-pathological features of the disease [3, 4].

Bacterial infection alone cannot explain the rapid tissues destruction around teeth showing small amounts of plaque, the tendency of periodontitis to continue with exacerbation and remission periods and the tendency to break down periodontal tissue to advance in a localized and bilateral symmetric model. In an attempt to accelerate progress in periodontal infections, we began to study the importance of human viruses in destructive periodontal disease. Identifying a viral agent in the development of periodontitis can help to clarify the clinical and pathophysiological features that are unexplainable to date with the disease.

There is a continuing increase in evidence for the etiopathogenic role of human cytomegalovirus (HCMV) in progressive periodontitis [5]. HCMV infected periodontal sites show a higher rate of disease activity than non-HCMV-detectable sites, even when compared to HCMV-positive and HCMV-negative sites with similar probing depths and clinical inflammation rates [6].

The Epstein-Barr virus (EBV) was also associated with periodontitis. In the case of non-oral diseases, it is known that HCMV infection can increase the incidence of bacterial and fungal infections [7,8], aggravate the severity of microbial infections and accelerate the rate of progression of infectious disease [9,10]. Although less studied, EBV and bacterial pathogens can also act synergistically in non-oral infectious diseases[11]. A similar theory for periodontitis focuses on the potential of periodontal HCMV and EBV to undermine host defence, thus enhancing the aggressiveness of subgingival bacteria.

Molecular detection methods, in particular polymerase chain reaction (PCR), greatly facilitated investigations of herpesviruses in oral and non-oral diseases, but the limitations of previous studies have generated discordant conclusions about the paradontopathogenic significance of herpesviruses.

Absence of longitudinal data prevents an assessment of the extent to which herpetic viruses are important risk factors for the subsequent development of periodontitis or only secondary to a lymphocytic infiltration of inflamed gingiva. Also, herpesviruses are a unique diagnostic challenge because of their ability to remain dormant in lymphocytes without active replication and disease, and early PCR studies did not differentiate between herpesvirus latency and higher replication levels.

Viral activation can be assessed by molecular techniques to determine gene transcription associated with viral reactivation, immunological methods for viral protein detection, and electron microscopy detection of intact virions to confirm viral assembly and delivery. Furthermore, false-positive errors and results of herpesvirus could occur in the shared regions of nucleotide sequences between unknown herpesvirus species and infectious agents. However, since periodontal HCMV was identified using a variety of primers in conventional PCR, non-PCR, reverse transcription PCR and real-time PCR [12], the risk of false HCMV identification is low. EBV was also identified in subgingival sites by a variety of PCR methods [13]. Finally, by providing only a dichotomy result, rapid qualitative PCR methods failed to differentiate between herpesvirus levels in disease-resistant periodontal sites. Real-time PCR

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methods allow for quantification of startup templates. Finding high levels of herpesvirus in active patients compared to inactive sites would reinforce the notion of parodontopathic role of viruses.

Real-time PCR of 5-nuclease (TaqMan) was used to quantify the genomes of HCMV and EBV [10] in periodontal pockets. TaqMan analysis is based on the ability of 5α-3α exonuclease 5a-3a Taq DNA polymerase to cleave an unexplained, double-stranded fluorogenic hybridization probe that directly refers to the fluorescence emission at the initial amount. The TaqMan fluorogenic probe hybridizes to the amplicon between the reverse and reverse primers and is cleaved by Taq DNA polymerase during the extension step.

The detachment of the probe separates the quencher reporter paint, generating a fluorescent signal proportional to the number of produced amplicons.

This study used a real-time polymerase chain reaction (PCR) analysis to determine possible correlations between periodontal presence of human cytomegalovirus, Epstein-Barr virus and various putative parodontopathogenic bacteria. Previous studies of herpesvirus-bacterial interactions in periodontal disease have used qualitative PCR methodologies that have underestimated the power of association between infectious periodontal agents.

## Experimental part

The study included 18 patients (18 to 38 years of age) with aggressive periodontitis, 12 patients (ages 37 to 62) with chronic periodontitis and 30 periodontal healthy subjects (aged 21 to 54 years). All patients were systemically healthy and did not receive periodontal treatment or antibiotics for at least 6 months before the start of the study.

Written informed consent was obtained from each subject after all procedures had been fully explained and understood.

Clinical periodontal evaluation included plaque index, gingival index, percentage of bleeding probing sites, and probing depth. In each patient, a subgingival bacterial plaque sample was obtained from the deepest periodontal pocket. Prior to sampling, the site was gently cleaned from the supragingival and saliva plaque using sterile cotton compresses, cotton rolled and air dried. A sterile periodontal curette was gently introduced until it reached the attachment epithelium and the bacterial plaque sample was extracted with one movement. The subgingival samples were suspended in 500 mL of 10 mM Tris-HCl containing 1 mM EDTA (*p*H 8) (TE buffer) and homogenized by vigorous vortex mixing.

The DNA was extracted from the sample material using a phenol-chloroform-isoamyl alcohol alcohol-based procedure. Briefly, 100 ìl of the sample was placed in 10 µl of protease solution (65 mg/mL) (Sigma-Aldrich Corp., St. Louis, MO, USA) and 250 µL potassium buffer for 60 min at 42°C. After centrifugation at 10,000 g for 10 min at 12°C, the DNA was extracted from the supernatant using a mixture of 250 µl of alkaline phenol and 250 µL of chloroform-isoamyl alcohol and then precipitated using 500 mL of isopropyl alcohol. The DNA was washed in 75% ethyl alcohol at 10,000 g for 5 min at 4°C, air-dried at 37°C and dissolved in 100 mL of distilled water.

The real-time fluorogenic PCR detection system was used to determine the number of infectious agents. The infectious agents studied included human cytomegalovirus, Epstein-Barr virus, *Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia, A. actinomycetem-comitans, Fusobacterium nucleatum* and *Campylobacter rectus*. PCR amplification was performed as individual analyzes for each infectious agent studied.

For each infectious agent tested, the TaqMan PCR assay was performed with a final volume of 25 µL of reaction mixture containing 5 µL of the extracted clinical sample, 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 5 pmol of primers and 3 or 4 pmol of the TaqMan probe. The cycling PCR program included 2 min at 50°C to eliminate the contamination transmitted for 10 min at 95°C to activate Taq DNA polymerase at warm start and then 40 cycles, each cycle consisting of two steps at 60°C for 1 min and one step at 95°C for 15 s.

No cross reactivity between human cytomegalovirus, Epstein-Barr virus, herpes simplex virus type 1 and 2, human herpes virus 6 and human herpes virus 8 was observed. The tested bacteria showed no cross-reactivity with nearby species such as *Prevotella nigrescens*.

The statistical evaluation was performed using the SPSS 10.0 statistical package. Descriptive data were reported in the frequency and percentage of the categorical variables and in the mean and standard deviation for the scale variables. Counting viruses and bacteria has been normalized by conversion to logarithmic values.

The t Student test was used to compare groups of topics.

The analysis of the Pearson correlation coefficient was used in the correlation calculations. A variable logistic regression analysis was used to calculate the risk based on the probability rate. Probability values (p) equal to or less than 0.05 were considered statistically significant.

## Results and discussions

The entire examination of the mouth in periodontal patients and healthy periodontal subjects revealed a mean of 4.2 and 1.5 mm respectively in the periodontal probing depth, 4.3 and 0.4 mm, respectively, in the loss of clinical attachment, 1.9 and 0.6 respectively in the plaque index, 1.8 and 0.5 respectively in the gingival index, and 67.9 and 5.1%, respectively, of the bleeding sites. The microbiological sample sites of periodontal patients and periodontal healthy subjects showed average of 4.9 and 1.6 mm, respectively,

| | Periodontal disease sites (n=30) | | Healthy sites (n=30) | |
|---|---|---|---|---|
| | % of PCR positive sites | Mean±SD (log) | % of PCR positive sites | Mean±SD (log) |
| Cytomegalovirus | 17 (56.66%) | 4.5 ±4.5 | 2 (6.66%) | 1.5 ±2.9 |
| Epstein–Barr virus | 19 (63.33%) | 6.6 ±4.4 | 3 (10%) | 1.9 ± 2.4 |
| *Porphyromonas gingivalis* | 25 (83.33%) | 9.5 ±5.1 | 3 (10%) | 1.6 ± 4.2 |
| *Prevotella intermedia* | 15 (50%) | 7.2 ± 6.2 | 4 (13.33%) | 1.5 ± 2.4 |
| *Tannerella forsythia* | 30 (100%) | 13.3 ±3.9 | 5 (16.66%) | 1.6 ±2.7 |
| *Fusobacterium nucleatum* | 20 (66.66%) | 9.6 ± 4.6 | 19 (63.33%) | 11.2 ± 2.9 |
| *Aggregatibacter actinomycetemcomitans* | 10 (33.33%) | 4.9 ± 5.3 | 1 (3.33%) | 0.8 ± 1.3 |
| *Campylobacter rectus* | 19 (63.33%) | 9.4 ± 2.7 | 3 (10%) | 1.7 ± 1.3 |
| PCR: Polymerase chain reaction; SD: Standard Deviation | | | | |

**Table1**
THE PRESENCE OF THE SUBGINGIVAL HERPESVIRUSES AND BACTERIAL SPECIES IN PERIODONTITIS AND HEALTHY PERIODONTAL SITES

in the periodontal probing depth, 4.5 and 0.7 mm, respectively, in clinical attachment loss, 1.9 and 0.5 respectively in the plaque index, 1.7 and 0.5 respectively in the gingival index, and 83.4 and 10.7%, respectively, of the bleeding sites. All clinical differences between periodontal patients and healthy periodontal subjects were statistically significant ($p < 0.001$).

Human cytomegalovirus was detected in 17 sites with periodontal lesions and in two healthy periodontal sites, and the Epstein-Barr virus was detected in 19 sites with periodontal lesions and in 3 normal periodontal sites.

Several statistical correlations have been detected between the number of viral strains and the number of bacteria. Positive correlations were found between human cytomegalovirus and *P. gingivalis, T. forsythia* and *C. rectus*. The Epstein-Barr virus positively correlated with *P. gingi*valis and *T. forsythia* (table 2).

The group of herpesviruses consists of large DNA molecules that replicate intranuclear and produce intranuclear inclusions. All viruses in this group are characterized by latency and reactivation. Herpesvirus hominis (herpes simplex virus (HSV) types 1 and 2) are members of this group. HSV-1 is usually acquired during childhood and is transmitted through direct muco-cutaneous contact or infection with drops of infected secretions. The severity and duration of the initial infection correlate approximately with the frequency and severity of subsequent recurrences. The virus (HSV-1) can be released by saliva and genital secretions from asymptomatic individuals, especially in the months following the first episode of the disease. The asymptomatic progression of HSV-1 in the oral cavity is one of the major sources of infection [14].

Various methods have been used to diagnose HSV-1, including HSV antibodies, direct immunofluorescence, DNA hybridization, cytological study and biopsies, viral culture. PCR can detect small amounts of viral DNA in suspect tissues, such as skin and mucous membranes [15]. The release of HSV-1 DNA into the oral cavity was monitored by PCR, and the virus was also found in the oral cavity of patients with recurrent subclinical labial herpes. Additionally, HSV DNA was detected by PCR in peripheral blood mononuclear cells concomitantly with recurrent labial herpes. This viremia may be responsible for the development of disseminated disease and may play a role in the pathogenesis of herpes multiforme erythema.

HSV viremia is more common than previously thought, and detection of HSV DNA in peripheral blood and plasma blood mononuclear cells is a useful diagnostic tool, especially in infants.

Using quantitative detection methods, this study revealed a parallel increase in the subgingival number of *P. gingivalis* and *T. forsythia* with gingival inflammation and loss of periodontal attachment. These findings are consistent with the parodontopathic role of these bacteria. However, data showing only an association between changes in bacterial load and the severity of the disease do not prove causality. It is possible that a bacterial change is, in essence, a secondary phenomenon to other key pathophysiological events. In fact, as demonstrated in other studies [16], the periodontal presence of human cytomegalovirus or Epstein-Barr virus was statistically associated with an increased occurrence of periodontal pathogens, especially *P. gingivalis* and *T. forsythia*, the two study species with the highest suspected pathogenic potential [17]. Interestingly, correlations with human cytomegalovirus and Epstein-Barr virus were found only for *P. gingivalis* and *T. forsythia*. The data strengthen the hypothesis of a causal relationship between herpesvirus-bacterial combined infection and destructive periodontal disease and indicate the potential of herpesviruses to disturb the delicate balance between host immunity and specific bacteria.

An active herpesvirus infection, as well as the bacterial infection, can initiate the activation of receptor activator of nuclear-kappa B (RANK) and its translocation into the nucleus, promoting the expression of cytokines, chemokines and proinflammatory adhesion molecules in infected cells [18-20], with periodontal therapy exerting significant changes, even in systemic context [21-28]. Pro-inflammatory cytokines recruit macrophages and natural killer cells at the site of infection and activate cellular expression of various effector functions. Nonspecific immune cells destroy infected cells and are a rich source of type 1 antiviral cytokines, including interleukin-1, interleukin-6, interleukin-12, interleukin-18, tumour necrosis factor $\alpha$ and interferons.

Periodontitis may develop due to impaired immune responses to herpesvirus-bacterial combined infection. Since the anti-herpesvirus and antibacterial immunity are partly antagonistic to each other, the diseased periodontal tissues can present a dominant change in herpetic or bacterial immune responses. For example, T helper T1

| Pathogen | Significant positive correlation with: |
|---|---|
| Cytomegalovirus | Epstein–Barr virus, Porphyromonas gingivalis, Tannerella forsythia, Campylobacter rectus |
| Epstein–Barr virus | Cytomegalovirus, Porphyromonas gingivalis, Tannerella forsythia |
| Porphyromonas gingivalis | Cytomegalovirus, Epstein–Barr virus, Tannerella forsythia, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Campylobacter rectus |
| Prevotella intermedia | Porphyromonas gingivalis, Tannerella forsythia, Campylobacter rectus,Fusobacterium nucleatum |
| Tannerella forsythia | Cytomegalovirus, Epstein–Barr virus, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Campylobacter rectus, Prevotella intermedia |
| Fusobacterium nucleatum | Prevotella intermedia, Tannerella forsythia |
| Aggregatibacter actinomycetemcomitans | Porphyromonas gingivalis, Tannerella forsythia, Campylobacter rectus |
| Campylobacter rectus | Cytomegalovirus, Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia, Aggregatibacter actinomycetemcomitans |

**Table 2**
CORRELATIONS AMONG SUBGINGIVAL INFECTIOUS AGENTS

proinflammatory cytokines can undermine T helper-type 2 mediated immunity and vice versa [26]. Immunosuppression may affect the innate immune defence mechanisms, which increases the risk of herpesvirus reactivation and an increase in the number of cytotoxic T cells and proinflammatory cytokines. Proinflammatory cytokines appear at elevated levels in severe periodontitis lesions, where they are related to collagen degradation and bone resorption [27]. Furthermore, the growth of cytotoxic / suppressor T cells in severe periodontal lesions, possibly due to infection with herpes viruses, may adversely affect the cells involved in antibacterial protection [28,29].

Recent studies proved that preventive measures in the dental office can decrease the severity of oral virus manifestations [30,31]. It is possible that an active herpesvirus infection has the potential to diminish antibacterial defences, triggering a microbial turn to a more virulent subgingival flora, leading to the destruction of periodontal tissue. If so, the (re)establishment of an effective antiviral immunity may be an important aspect of achieving a long-lasting remission of progressive periodontitis [32,33]. Other studies reported weak correlations between nitinol substrate on viral activity, but further research is necessary [34,35].

A better understanding of the role of herpesviruses in periodontitis can be crucial for elucidating the pathophysiology of the disease and for identifying new and more effective targets for disease prevention and long-term healing. Periodontal disease management can benefit in the future from antiviral immunotherapies: either prophylactic vaccines that use the immune system of healthy subjects to prevent infection with viruses that cause disease or therapeutic vaccines that stimulate the immune system to combat existing viruses and diseases.

## Conclusions

The present study suggests that adverse changes in environmental exposure or alterations in immune system genes may periodically suppress the host's defence against periodontal aggression, which may then result in the reactivation of resident herpesviruses and the proliferation of pro-inflammatory mediators, followed by an increase in pathogenic bacteria.

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