Detection of Epstein-Barr virus and human cytomegalovirus in blood and oral samples: comparison of three sampling methods

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Abstract: The purpose of this study was to identify and compare the presence of HCMV and EBV-1 in subgingival plaque, unstimulated saliva and peripheral blood of patients with chronic periodontitis. Forty patients diagnosed with chronic periodontitis (mean age, 41.7 years) were recruited. Unstimulated saliva, subgingival plaque and peripheral blood were collected from each patient and the DNA of each sample was isolated. The viruses were detected using the nested PCR technique. The detection frequency of EBV-1 in subgingival plaque, saliva and peripheral blood was 45%, 37.5% and 25%, respectively. HCMV was detected in 82.5% of subgingival plaque samples and peripheral blood and in 75% of salivary samples. The sensitivity for detecting EBV-1 in saliva and peripheral blood when EBV-1 was detected in subgingival plaque samples was low (22% and 27.7%, respectively) and the sensitivity for detecting HCMV in saliva and peripheral blood when compared to subgingival plaque was high (81.8% and 87.8%, respectively). There is a high agreement among the three sampling methods in detection of HCMV, but the detection of EBV-1 would require a combination of saliva and subgingival plaque sampling to avoid false negative results. (J. Oral Sci. 50, 25-31, 2008)

Keywords: periodontitis; EBV-1; HCMV; saliva; chronic periodontitis; subgingival plaque.

Introduction

Periodontitis is an infectious disease caused by bacterial pathogens and modulated by characteristic humoral and cellular host responses (1-3). The composition of subgingival microbiota varies in periodontal health and various forms of periodontal diseases (4). More recently, herpes viruses, especially human cytomegalovirus (HCMV) and Epstein-Barr virus-1 (EBV-1) have been identified in the subgingival plaque of patients with advanced periodontitis, acute necrotizing ulcerative gingivitis and periodontal abscesses (5). As HCMV infects periodontal monocytes/macrophages and T-lymphocytes, and EBV infects B-lymphocytes (6), the presence of these viruses may increase the virulence of resident bacterial invasiveness into epithelial cells (7), facilitating the penetration of pathogenic bacteria into connective tissues (8).

Herpes viruses are transmitted from person to person during the period of primary infection or reactivation. The balance between latency and activation involves the regulation of gene expression and reactivation may be triggered by stress, hormonal changes, infections and immunosuppressive medication (9,10).

Salivary glands are reservoirs for salivary herpes viruses (11). Acquisition of EBV often occurs through exchange of saliva infected by herpes virus (12). EBV DNA in saliva is elevated in lymphoproliferative disorders, transplanted patients, and HIV-positive patients (13,14). In periodontally-compromised patients, Saygun et al. reported that there is a correlation between gingival tissue and salivary counts of HCMV DNA and EBV DNA, suggesting that the periodontal pocket may be a source of salivary herpes viruses (15). In addition, periodontal therapy decreases the
subgingival herpes virus counts, a reduction which is also reflected in salivary counts (16).

The detection of periodontopathogens is commonly performed by subgingival plaque sampling (17). Curettes and paper points have been used for collecting samples, and the technique used may influence the results of the microbiological analysis (18). Saliva is easier to obtain and contains infectious agents from all oral sites, including mucosa, supragingival and subgingival plaque (19-21). Many recent clinical studies have identified herpes viruses in subgingival plaque, but few investigations have evaluated the utility of whole saliva as a diagnostic method (15,16). The aim of the present study was to compare the presence of Human Cytomegalovirus (HCMV) and Epstein-Barr virus type 1 (EBV-1) in subgingival plaque, saliva and peripheral blood of patients with chronic periodontitis.

Materials and Methods

Study Population

Forty systemically healthy adult patients (mean age = 41.7 years, 17 females and 23 males), clinically and radiographically diagnosed with untreated chronic periodontitis, were enrolled in the study. Patients were recruited from the Department of Periodontology at University of São Paulo, and they had signed an informed consent form approved by the Ethics Committee of the Dental School at University of São Paulo (FOUSP). Patients were enrolled in the study from January to October 2006.

Clinical Examination

Periodontal examination consisted of measurements of probing depth and clinical attachment level (6 sites per tooth), using a periodontal probe calibrated in millimeters (PCPUNC 15, HuFriedy, Chicago, IL, USA), bleeding sites, mobility and furcation involvement. Patients had periodontal pockets greater than 5 mm with clinical attachment loss that showed bleeding on probing in four quadrants. Patients had not received previous periodontal treatment and had not used antibiotics in the past 3 months. Patients were excluded if pregnant or with a history of systemic disorders. Full-mouth intra-oral radiographs were taken.

Sampling Procedures

Samples were obtained at least one week after periodontal examination. Patients were asked not to brush their teeth or eat at least 2 hours before the appointment. Samples were taken in the following order: unstimulated saliva, peripheral blood and subgingival plaque. Approximately, 3 ml of expectorated unstimulated whole saliva was collected from the patient in a sterile plastic tube and stored at -70°C. DNA was extracted from the clinical sample material using a method described by Hochmesiter et al. (22) and adapted by Rivero et al (23). Briefly, 500 µl of saliva was placed in a 2 ml tube. Saliva was centrifuged at 100 rpm for 5 min, and the pellet was washed twice in 1000 µl of PBS 1X. It was then added to 200 to 400 µl of collection buffer [NaCl 1M; Tris 1M, pH = 8; EDTA (Sigma Chemical, St Louis, MO, USA) 0.5M, pH 8.0; SDS 10% and Proteinase K (Invitrogen, Carlsbad, CA, USA)] to a final concentration of 500 µg/l. Tubes were incubated at 55°C for 7 days to complete the dissolution of pellet. Proteinase K (200-400 µg/l) was added to the collection buffer every 24 h. Enzyme was inactivated at 95°C for 10 min, then 200 µl of ammonium acetate (Synth, Brazil) were added to the lyase. Tubes were homogenized for 20 s, incubated for 5 min and centrifuged at 13000 rpm for 3 min. The supernatant was transferred to another tube and 600 µl of 100% isopropanol was added to the DNA precipitate and the material was centrifuged at 16000 rpm for 5 min. The precipitate was washed in 600 µl of 70% ethanol and centrifuged at 16000 rpm for 2 min. The precipitate was dissolved in 30 or 50 µl of TE buffer. The samples were stored at 4°C.

Peripheral blood was collected by finger puncture and stored in filter paper without anticoagulant. The samples from the filter papers were punched out with a stainless steel punch, yielding discs of 3 mm in diameter. Prior to subgingival sampling, supragingival plaque was removed using curettes or cotton balls. In order to avoid saliva contamination, the test sites were air dried and kept dry using cotton rolls. Subgingival plaque was collected from the deepest site in each quadrant by using sterile paper points. Two paper points were inserted to the base of the pocket in each site and kept in place for 30 s. A pooled sample was obtained from each patient and the paper points were stored in an Eppendorf tube in a freezer at -70°C until DNA extraction.

The extraction of nucleic acid from peripheral blood and subgingival plaque samples was based on preferential binding to silica particles in the presence of a high concentration of guanidium thiocyanate (GUSCN). This method was described by Parra and Slots (24). Briefly, 0.4 ml of sample was mixed with 5 µl of silica particles (Sigma Chemical Co., USA) in 80 µl of lysis buffer (120 g of GuSCN; 100ml of 0.1mM Tris HCl, pH = 6.4; 22 ml of 0.2 M EDTA, pH = 8; 2.6 g of Triton X 100), vortexed for 10 s and kept at room temperature for 10 min. Nucleic acid/silica combinations were recovered by centrifugation at 12000 rpm for 1 min, washed twice in buffer (GUSCN-Tris HCl), twice in 70% ethanol and one time in acetone. The sample was then dried in a heating block at 56°C for...
10 min. The nucleic acid pellet was resuspended in 100 µl of TE buffer. The sample was incubated at 56°C for 10 min. After centrifugation at 12000 rpm for 2 min, the supernatant was stored at -70°C.

**PCR procedures**

The nested PCR method was used to detect viral DNA of HCMV and EBV-1 using specific primers for each gene with sequences shown in Table 1. Amplicons for all PCR reactions were visualized after electrophoresis in a 1% agarose gel with ethidium bromide.

**Statistical analysis**

The comparison among the three sampling methods was performed using the Q Cochran test. A *P*-value < 0.05 indicated statistically significant comparisons. For data analysis, sensitivity and specificity were calculated when comparisons were made between the presence of HCMV or EBV-1 in subgingival plaque and saliva, subgingival plaque and peripheral blood and saliva and peripheral blood.

**Results**

Forty patients with moderate to severe chronic periodontitis showed a mean clinical attachment loss of 4.1 mm (± 0.3), mean bleeding on probing percentage of 82% (± 7.2) and mean supragingival plaque detection of 74% (± 4.1). The frequencies of detection of EBV-1 and HCMV in subgingival plaque, saliva and peripheral blood are presented in Fig. 1. Only 1 subject (2.5%) was positive for EBV-1 in all the three studied samples, while 25 (62.5%) were positive for HCMV in the three samples. Seven (17.5%) subjects were negative for EBV-1 in all the samples and 2 (5%) were negative for HCMV in the samples. None of the subjects were positive or negative for both EBV-1 and HCMV as detected by the three studied methods.

EBV-1 was detected in 45% of subgingival plaque samples, 37.5% of saliva samples and 25% of peripheral blood samples (Q Cochran test, *p*=0.18). HCMV was detected in 82.5% of subgingival plaque samples, 75% of saliva samples and 82.5% of peripheral blood samples (Q Cochran test, *P* = 0.91) (Fig. 1).

The calculated sensitivity and specificity values are presented in Table 2. Subgingival plaque samples were used as reference criteria. The sensitivity for detection of EBV-1 in saliva and peripheral blood when EBV-1 was detected in subgingival plaque samples was low (22% and 27.7%, respectively), although the calculated specificity was higher for saliva (50%) and peripheral blood (77.3%). The sensitivity for detection of HCMV in saliva and peripheral blood when compared to subgingival plaque was high (81.8% and 87.8%, respectively). The specificity was also calculated and it was 57.1% for saliva and 42.8% for peripheral blood.

**Discussion**

The accuracy in detecting pathogens relies on the sampling methods. In periodontal research, bacteria are detected by collecting samples of subgingival plaque from

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**Table 1 Primers used in nested PCR**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Target gene size (pb)</th>
<th>Nucleotide sequence 5’-3’</th>
<th>Annealing temperature °C (mM MgCl₂)</th>
<th>Reference</th>
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<tr>
<td>HCMV 1st round</td>
<td>150</td>
<td>ACCACCGCACGTAGGAATGTGAG TCAATCATGCGTTTGAAGAGGA</td>
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<td>25</td>
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<tr>
<td>HCMV 2nd round</td>
<td>100</td>
<td>ACCACCGCACGTAGGAATGTGAG TCAATCATGCGTTTGAAGAGGA</td>
<td>50 (1.0)</td>
<td>26</td>
</tr>
<tr>
<td>EBV-1 1st round</td>
<td>602</td>
<td>AGGGATGCCTGGACACAAAGA TGTGCTGTGCTGCTGCTG</td>
<td>56 (1.5)</td>
<td>26</td>
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<tr>
<td>EBV-1 2nd round</td>
<td>116</td>
<td>AACTTCAACCCACACATCA TTCTGGACTATCTGGATCAT</td>
<td>46 (1.5)</td>
<td>26</td>
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</table>

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![Fig. 1 Prevalence of EBV-1 and HCMV in subgingival plaque, saliva and peripheral blood of 40 patients with chronic periodontitis.](image)
single or pooled sites, unstimulated or stimulated saliva, supragingival and subgingival plaque or even mucosal surfaces (27-30). To assess the presence of periodontopathogens with sufficient probability, it would be necessary to evaluate whole saliva and a pooled subgingival plaque sample (27). A combination of supragingival and subgingival plaque samples was also proposed (28). However, Cortelli et al. considered that sampling only the whole saliva may be an easier alternative to individual plaque samples (31). Although different from saliva, oral lavage may also be superior to the subgingival sampling method (29). Since herpes viruses have been implicated in the pathogenesis of periodontal diseases, an important issue is how to detect these viruses.

Most clinical studies evaluating the prevalence of herpes viruses and their association with periodontopathogenic bacteria evaluated subgingival plaque samples (24,32-39).

Saygun et al. observed a positive correlation between periodontal pocket and salivary counts of EBV DNA and between gingival tissue and salivary counts of HCMV DNA, and suggested that periodontitis lesions are a source for salivary HCMV and EBV (40). In the present study, the prevalence of HCMV and EBV-1 was compared according to the sampling method, i.e. saliva, subgingival plaque and peripheral blood of patients with chronic periodontitis. The results showed that the frequency of EBV-1 and HCMV in subgingival plaque was comparable to that in saliva and peripheral blood, but this does not mean that there was an agreement among the samples within each patient. There was a lack of agreement among the methods in detecting EBV-1, meaning that the identification of EBV-1 in subgingival plaque was not accompanied by the detection in saliva or peripheral blood. In fact, the saliva samples were positive in only 4 of 18 patients who had EBV-1 positive subgingival plaque samples. Although Idesawa et al. (16) observed that the levels of EBV-1 in saliva may reflect the periodontal status of the patients, the results of the present study showed that the absence of EBV-1 in saliva may not indicate that EBV-1 is absent in subgingival plaque. There was a significant agreement between subgingival plaque and saliva and subgingival samples and peripheral blood in the detection of HCMV, probably due to the high detection frequency of this virus, with high sensitivity and specificity. It is important to point out that the high detection of HCMV in saliva and subgingival samples leads to a high agreement between the two methods, since only two patients were negative to HCMV.

The detection frequency of EBV-1 and HCMV in subgingival plaque in the present study was consistent with previous reports. The reported prevalence of EBV-1 infections in periodontitis patients ranges between 17.7% (37) and 70.6% (38) and the prevalence of HCMV varies between 26% (35) and 89% (33). EBV-1 infections in healthy individuals range broadly between 0 and 100% (40).

Walling et al. (41) detected EBV in saliva specimens in 25 of 28 healthy subjects and Braz-Silva et al. (42)...

<table>
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<tr>
<th>EBV subgingival plaque</th>
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<tr>
<td>-</td>
<td>14</td>
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<th>peripheral blood</th>
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<tr>
<td>specificity</td>
<td>50%</td>
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Table 2 Comparison of detection of EBV-1 and HCMV in subgingival plaque and saliva, subgingival plaque and peripheral blood in 40 chronic periodontitis patients
recovered EBV DNA from the oral mucosa of 86.6% of renal transplant recipients and in 46.6% of healthy subjects. HCMV is an ubiquitous agent and seropositivity rates in the adult population over 40 years of age worldwide vary between 60 and 100%, possibly due to transmission through breastfeeding, sexual contact and spread from children (43,44). The wide range of detection of HCMV and EBV may be attributed to differences in the studied populations and methodological approaches. In this study, nested PCR was used and although time consuming, nested PCR showed comparable sensitivity and specificity to qRT-PCR (45). Of the chronic periodontitis patients, 82.5% were positive to HCMV both in subgingival plaque and peripheral blood. A quantitative analysis of virus DNA might show differences between the local and systemic HCMV DNA load. The subgingival and salivary detection of HCMV may therefore reflect the systemic presence of the virus.

Additionally, it is important to evaluate the frequency of HCMV and EBV-1 in subgingival plaque and saliva of gingivitis patients and healthy subjects.

In conclusion, there is a high correlation among the three sampling methods in detecting HCMV, suggesting that the detection of HCMV in subgingival plaque is comparable to that in saliva. But the detection of EBV-1 in oral sites of periodontal patients would require a combination of saliva and subgingival plaque analysis to avoid false negative results when relying on only one sampling method, since there is a discrepancy between subgingival and salivary detection of EBV-1.

Acknowledgments
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References


