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ORIGINAL ARTICLE

Salivary levels of Epstein-Barr virus DNA correlate with subgingival levels, not severity of periodontitis

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OBJECTIVE: The aim of this study was to determine the presence and quantity of human cytomegalovirus (CMV) and Epstein-Barr virus (EBV) DNA in the saliva of patients with periodontitis, and investigate the correlation between these factors.

METHODS: Presence and amounts of viral DNA in saliva and subgingival plaque samples, from healthy and disease sites, of 65 adults diagnosed with chronic periodontitis were determined using quantitative real-time polymerase chain reaction.

RESULTS: Epstein-Barr virus DNA was detected in saliva of 81.5% (53/65) of patients at a median concentration of 4325 copies ml^{-1}. CMV DNA was detected in saliva of one individual (1.5%) at low copy number. Patients who had EBV in saliva were 10 times more likely to have EBV in subgingival plaque than patients lacking EBV in saliva (odds ratio = 10.1, 95% confidence interval = 2.6–39.5; $P = 0.0009$). EBV DNA burden in saliva positively correlated with the amounts detected in plaque and with amounts detected in increasing number of affected sites ($P < 0.0001$). EBV DNA presence and quantity in saliva did not correlate with increasing severity of disease as measured by periodontal indices.

CONCLUSIONS: Epstein-Barr virus DNA presence and burden in saliva are associated with its presence and burden in subgingival plaque, but presence and burden in saliva does not correlate with periodontal disease severity.

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Keywords: Epstein-Barr virus; cytomegalovirus; saliva; real-time polymerase chain reaction; periodontal disease

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Introduction

Human herpesviruses (HHV) are large, ubiquitous DNA viruses that cause significant human disease, ranging from but not limited to mucosal ulcerations, infectious mononucleosis and a range of malignancies (Roizman and Pellet, 2001; Whitley and Roizman, 2001; Reeves and Sinclair, 2008; Thorley-Lawson *et al*, 2008). These viruses typically infect the oral cavity and induce a lytic or subclinical primary infection at a young age. Subsequently, latency is established and their pathogenesis is elicited following episodic reactivation and/or activation of oncogenic pathways (Roizman and Pellet, 2001; Young and Murray, 2003; Masucci, 2004). Recrudescence in and around the oral cavity is integrally linked to immune function, levels of stress, trauma and inflammation, as well as genotypic differences associated with viral strains (Johnson *et al*, 2007; Sheridan *et al*, 2007; Steiner *et al*, 2007; Reeves and Sinclair, 2008; Sinclair, 2008).

Of the eight HHV, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) have been detected in subgingival sites of patients with periodontitis (Contreras and Slots, 1998; Contreras *et al*, 1999a,b; Botero *et al*, 2007). CMV is harbored within myeloid lineage cells (i.e., monocytes/macrophages) and T-lymphocytes, and EBV resides in B-lymphocytes. These cell types target regions of inflammation. As periodontitis is a major contributor of oral inflammation, it has been hypothesized that the inflammatory cells that harbor these viruses may be directed to sites of periodontal inflammation where CMV and EBV could contribute to periods of disease exacerbation (Socransky *et al*, 1984; Slots, 2007). It is possible, however, that presence of viral DNA in plaque is merely a consequence of its presence in saliva. Few studies to date have investigated the prevalence of these two viruses in the saliva of patients who have periodontal disease and determined if viral DNA burden in salivary secretions relates with the genomic burden in subgingival plaque (Idesawa *et al*, 2004; Sugano *et al*, 2004; Imbronito *et al*, 2008).

The purpose of this study was to determine the presence and amount of CMV and EBV DNA in saliva and determine the relationship of these viruses in saliva with subgingival plaque and periodontal disease. The hypothesis tested was that salivary levels of EBV and CMV DNA correlate with periodontal disease severity.

Materials and methods

Subjects

Subjects at least 18 years of age who were in good systemic health, had at least 20 erupted teeth and had existing moderate to severe periodontitis were eligible to participate (Armitage, 1999, 2004). Subjects were included if they had at least five qualifying sites in two quadrants with two affected teeth in each quadrant. Each qualifying disease site had probing depths (PD) ≥5 mm, clinical attachment loss (CAL) ≥3 mm and bleeding on probing (BOP). Patients were excluded if they had a history of aggressive periodontitis, alcoholism, liver, kidney or salivary gland dysfunction, inflammatory bowel disease, granulomatous disease, immunosuppression or were undergoing or had undergone organ transplant or cancer therapy. Pregnancy, lactation, previous periodontal treatment within 6 months, use of antibiotics or immunosuppressant medication within the last 3 months, need of antibiotics for infective endocarditis prophylaxis during dental procedures, symptoms of acute illness (i.e., fever, sore throat, body aches, and diarrhea), unable or unwilling to provide informed consent, or detection of an oral mucosal inflammatory condition (e.g., aphthous, lichen planus, leukoplakia, and oral cancer) were additional exclusion criteria. The study was performed at the University of Kentucky between August 2005 and January 2008 and was approved by the University Institutional Review Board. All subjects provided written informed consent and received incentives (i.e., monetary compensation, a clinical exam and had their teeth cleaned at the conclusion) as part of the study protocol.

Study procedures

Saliva was collected from each subject prior to periodontal examinations. Each subject rinsed their mouth with tap water then provided whole saliva in an upright position by expectoration every 30 s into a sterile plastic tube until 5 ml was collected. Clinical periodontal examinations for each subject were performed by one investigator (DRD) and included measures of PD, BOP, and CAL. PD were measured at six sites per tooth (mesial-buccal, mid-buccal, distal-buccal, mesial-lingual, mid-lingual, and distal-lingual) using a UNC 15 probe (HU-Friedy, Chicago, IL, USA). BOP was recorded at the same six sites using a bleeding index (0, no bleeding; 1, single bleeding point or fine line of blood; 2, interdental triangle or direct margin fills with blood; and 3, profuse bleeding immediately after probing) (Polson *et al*, 1997). In each subject, dental plaque from three diseased sites (≥5 mm PD, ≥3 mm CAL and BOP) and three healthy sites (≤3 mm and no BOP) were obtained. Prior to sampling, the site was gently cleaned of supragingival plaque and saliva with sterile cotton pellets, isolated with cotton rolls and air-dried. A sterile periodontal curette was gently inserted to the depth of the pocket and the subgingival material was removed by a single stroke. A separate sterile curette was used for each site. Plaque samples were not pooled, rather each was placed in separate vial, containing 0.4 ml of sterile phosphate-buffered saline, and homogenized by vigorous mixing. All plaque specimens and 1 ml aliquots of the saliva were stored at −80°C and assayed within 6 months of collection. Serostatus for antibodies against CMV and EBV was determined as described previously (Dawson *et al*, 2009).

DNA extraction and real-time PCR

DNA was extracted from sample pellets following centrifugation using the MasterPure DNA Purification Kit (Epicentre, Madison, WI, USA) and resuspended into 0.1 ml of Tris and ethylenediaminetetraacetic acid Buffer. Extracted DNA was tested for the presence and quantity of CMV, EBV and *Fusobacterium nucleatum* DNA by Taqman polymerase chain reaction (PCR) assays using an ABI Prism 7700 Sequence Detection system (PE Applied Biosystems, Foster City, CA, USA) as we previously described (Miller *et al*, 2005). The primers and probes used were derived from published sources as we have previously reported (Miller *et al*, 2005). Briefly, two primer-probe sets were used to detect CMV DNA. The glycoprotein B (gB) gene was first targeted using primer-probe set (254-bp amplicon spanning nucleotides 83 906–84 159) described elsewhere (Bai *et al*, 1997; Li *et al*, 2003). The CMV polymerase (POL) gene was targeted second using primer-probe set (66-bp amplicon spanning nucleotides 79 128–79 193) described previously (Yun *et al*, 2003; Nye *et al*, 2005). Primers and probes for EBV were directed to the BALF5 gene encoding viral DNA polymerase (Kimura *et al*, 1999). All probes were labeled as previously described (Miller *et al*, 2005). DNA (10 μl) of each sample was assessed in duplicate PCR reactions and recorded as positive if either reaction showed positive exponential amplification above the threshold value. These primers and probes detect single copies of CMV and EBV DNA in 10 μl purified template (Miller *et al*, 2005) and are specific when tested with known HHV (i.e., cross-reactivity is not observed between the viral assays) (Kimura *et al*, 1999; Collot *et al*, 2002; Li *et al*, 2003). Each PCR run included controls without template and serially 10-fold diluted DNA standards (1–10^5 genome equivalents per μl) of either genomic CMV DNA for POL, plasmid pCR2.1 that contained a cloned 254-bp fragment of CMV gB (kindly provided by Y.-W. Tang, Vanderbilt University, Nashville, TN, USA) for gB, or plasmid pGEM-BALF5 containing the EBV BALF5 gene (kindly provided by Dr H. Kimura, Nagoya University, Nagoya, Japan). Copy numbers of genomic viral DNA are reported as the average of duplicate reactions. As a positive control for the amplification reaction, all plaque samples were analyzed for *Fusobacterium nucleatum* using the primers and probe previously described (Combs *et al*, 2008).

Statistical analysis

A generalized linear mixed model (GLMM) with a logit link was used to correlate the presence of viral DNA in saliva with plaque sites taking into account the status of the site (diseased *vs* healthy) and the nesting of sites within health status. Similar analyzes were conducted to correlate the patient's age, gender, and race with the presence of viral DNA. Odds ratios (OR) and 95% confidence intervals (CI) to measure the association between viral DNA in saliva and/or plaque were based on these GLMM models. Cross tabulations were analyzed using chi-square statistics or Fisher's exact test. Statistical significance was determined at the 0.05 level. All data were analyzed with the use of the SAS statistical analysis software (SAS Institute, Cary, NC, USA).

Results

Characteristics of the study population

Sixty-five adults ranging in age from 25 to 66 years (mean 41.2 years) were evaluated. The demographics and serostatus of the subject population were 70.8% male, 40% Caucasian, 35.4% Hispanic, 16.9% African-American and 7.7% Asian. All had moderate to severe periodontal disease, and the majority had antibodies against CMV and EBV. The mean percentage and standard deviations of sites affected were: BOP (53.2 ± 21.0%), PD ≥ 4 mm (29.3 ± 20.9%), PD ≥ 5 mm (14.7 ± 10.2%), and CAL ≥ 2 mm (23.9%). Mean values of the clinical indices of periodontal disease for the entire mouth for the study group were: PD 4.12 ± 0.5 mm, CAL 2.41 ± 1.27 mm, and BOP 1.99 ± 0.96.

Prevalence of EBV and CMV DNA in saliva and relationships with subgingival plaque and periodontitis

DNA from a single saliva specimen and six subgingival plaque samples from each patient were examined for EBV and CMV using quantitative PCR. EBV DNA was detected in 81.5% of salivas (53/65) and in 72 subgingival plaque sites (55 disease, 17 healthy) of 44.6% (29/65) of patients. By contrast, CMV DNA was detected in one saliva (1.5%) and one subgingival plaque sample (0.3%). As shown in Table 1, the mouth means for PD, CAL and BOP did not significantly differ between the EBV positive individuals and the EBV negative individuals. Consistent with this finding, the presence of EBV DNA in saliva did not correlate with increasing severity of periodontal disease (i.e., PD, CAL or BOP) as determined using univariate logistic regression (data not shown).

Epstein-Barr virus DNA was detected simultaneously in the saliva and plaque of 41.5% (27/65) of patients, in the saliva but not plaque in 40% (26/65), and in the plaque but not saliva in 3.1% (2/65) of patients. The presence of EBV DNA in saliva was significantly associated with the presence of EBV DNA in subgingival plaque ($P < 0.0001$). Logit link function analysis indicated that persons with EBV DNA in their saliva were 10 times more likely to have EBV DNA in their plaque than those lacking EBV in saliva (OR = 10.1, 95% CI = 2.6–39.5; $P = 0.0009$). Interestingly, when EBV DNA was found at two or more plaque sites, 100% of patients had EBV DNA in saliva (data not shown). The presence of EBV in saliva was also influenced by the status of the disease site, in that patients who had EBV DNA in saliva were significantly more likely to have EBV DNA at periodontal disease sites 34.6% (1.28 OR, 95% CI = 1.13–1.44) than periodontal healthy sites (10.1%). Age, race and gender were not observed influences of EBV DNA prevalence in saliva.

Quantity of EBV and CMV DNA in saliva and relationship with subgingival plaque and periodontitis

In quantitative PCR reactions that can detect single copies of viral DNA per reaction, the median EBV DNA load was 4325 copies per ml of saliva (range 4–75 million copies). By contrast, only 44 copies of CMV DNA per ml of saliva were detected in one individual. Significant positive correlations were identified between EBV burden in saliva and EBV burden in plaque. EBV burden in saliva correlated with EBV burden in plaque from healthy sites (coefficient 0.30; $P = 0.02$), with EBV burden in plaque from disease sites (coefficient 0.56; $P < 0.0001$) and with EBV burden in the total number of subgingival sites affected (correlation coefficient 0.53, $P < 0.0001$; Figure 1). In addition, EBV was more likely to be present at disease sites when saliva contained a threshold of 4718 copies per ml; however, the threshold had to exceed 1.86×10^7

Table 1 Comparison of mouth means for PD, CAL, and BOP

	EBV DNA positive in saliva, n *[mean (s.d.)]*	*EBV DNA negative in saliva,* n *[mean (s.d.)]*	P-*value**
PD	53 [4.06 (0.98)]	12 [4.5 (0.92)]	0.22
CAL	53 [2.37 (1.15)]	12 [2.63 (1.99)]	0.57
BOP	53 [1.98 (0.53)]	12 [2.04 (0.38)]	0.73

PB, probing depths; CAL, clinical attachment loss; BOP, bleeding on probing.
*Two-sample *t*-tests.

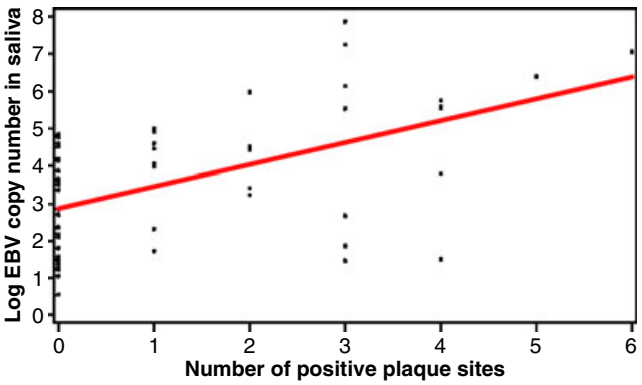

Figure 1 Relationship of Epstein-Barr virus (EBV) DNA copy number in saliva with total number of subgingival sites positive for EBV per person

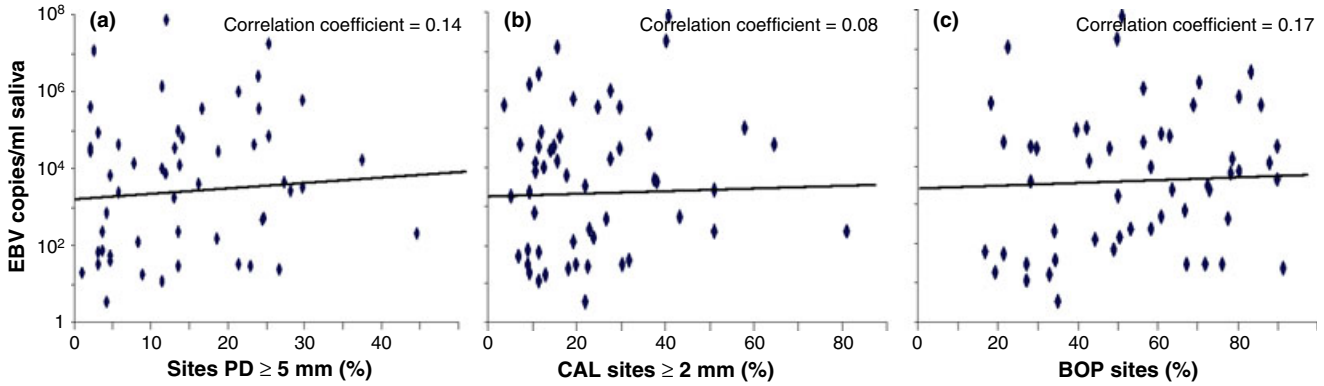

Figure 2 Relationship of Epstein-Barr virus (EBV) copy number in saliva with measures of periodontal disease severity (**a**) probing depths, (**b**) clinical attachment loss, and (**c**) bleeding on probing

copies per ml in saliva for EBV to be likely at healthy sites. By contrast, none of the clinical parameters of periodontitis as measured by PD, CAL, and BOP significantly correlated with EBV copy number in saliva (Figure 2).

The accuracy of the assays was demonstrated in that positive controls were amplified and detected in all cases in each assay performed (data not shown), whereas no amplification or detection was observed in all negative controls. Moreover, *F. nucleatum* was detected in all plaque samples analyzed.

Discussion

This study was undertaken to determine the presence and amount of CMV and EBV DNA in saliva and to determine if their presence was associated with periodontal disease. At present, only a limited number of studies, consisting of relatively small numbers of subjects, exist on this topic (Idesawa *et al*, 2004; Sugano *et al*, 2004; Saygun *et al*, 2005; Imbronito *et al*, 2008). Our analyzes of saliva and subgingival plaque specimens from 65 persons who had moderate to severe adult periodontitis make this one of the larger studies to date on this topic. We found that EBV DNA is frequently present in the saliva of patients suffering from periodontal disease, whereas CMV DNA is rare in this population. Interestingly, the presence of EBV in saliva did not correlate the severity of periodontal disease as determined by several measures. By contrast, EBV DNA in saliva was significantly associated with the presence of EBV DNA in subgingival plaque and significantly more likely to appear at periodontal disease sites than periodontal healthy sites when saliva contained EBV. In addition, an increasing EBV DNA burden in saliva was predictive of an increasing viral DNA burden in plaque and the number of subgingival sites affected. These findings reject our hypothesis that salivary levels of EBV and CMV DNA correlate with periodontal disease severity, and instead suggest that EBV DNA in saliva is a predictor of EBV DNA in plaque, or vice versa that EBV DNA in plaque is a predictor of EBV DNA in saliva.

The prevalence of CMV in saliva is generally accepted to be low. Studies indicate that salivary CMV occurs in $<10\%$ of immunocompetent persons on any given day (Lucht *et al*, 1993; Druce *et al*, 2002; Miller *et al*, 2005). By contrast, CMV is detected more frequently in immunosuppressed individuals possibly because of changes in the host defense system as well as the presence of oral inflammatory lesions (Fons *et al*, 1994; Greenberg *et al*, 1995; Lucht *et al*, 1998; Nowzari *et al*, 2003). In this study of immunocompetent patients with untreated inflammatory periodontal disease, CMV was detected in the saliva of only one of 65 persons. This low rate of detection is comparable with the rate we and others have previously reported in the saliva of adults as a result of the use of similar assay techniques (i.e., quantitative real-time PCR) (Lucht *et al*, 1998; Druce *et al*, 2002; Miller *et al*, 2005; Combs *et al*, 2008). However, the prevalence we observed in this study is lower than that reported in studies that have employed nested PCR techniques which are known to encounter spurious amplification (Don *et al*, 1991; Saygun *et al*, 2004; Imbronito *et al*, 2008). This, as well as different patient populations studied, could explain the different observations obtained from these studies.

Epstein-Barr virus is more prevalent in saliva than CMV. EBV is detected in the oral fluids of 22–88% of the adult population on any given day (Sixbey *et al*, 1989; Oosterveer *et al*, 1993; Apolloni and Sculley, 1994; Ikuta *et al*, 2000; Idesawa *et al*, 2004; Miller *et al*, 2005, 2006). Rates have been reported to be higher in the saliva of persons with periodontitis than the healthy controls (Idesawa *et al*, 2004; Sugano *et al*, 2004), and our high rate of detection (81.5%) could suggest that periodontal disease sites contribute to the presence of EBV in saliva. However, the presence of EBV DNA in saliva did not correlate with the severity of any of the parameters of periodontal disease measured in our study population. This is in contrast to the observation that EBV-positive patients have had higher BOP scores than EBV-negative patients in one study (Idesawa *et al*, 2004). However, our findings were similar to the reports that the presence of salivary EBV DNA did not correlate with PD or CAL (Idesawa *et al*, 2004; Saygun

et al, 2005). Thus, these findings suggest that EBV DNA presence in saliva may correlate more with inflammation than other aspects of periodontal disease.

The relationship between EBV salivary burden and periodontal status has been described previously but remains ill defined. Saygun *et al* (2005) reported the lack of correlation between EBV DNA counts in saliva and plaque from periodontal pockets, and also found a positive correlation between PD and salivary EBV DNA burden. Our observations contrast with these findings. We found that the salivary EBV DNA burden was closely related to the viral DNA burden in plaque as well as the amounts found at increasing number of plaque sites. This suggests that persons harboring EBV DNA in saliva are more likely to have more EBV DNA in plaque and at an increasing number of subgingival sites. This could mean that there is communication between the two compartments before or during plaque collection, although the latter is unlikely as all tissue surfaces were dried and isolated during the collection process. Active interaction between the two sites could help explain the findings of Idesawa *et al* (2004) and Saygun *et al* (2005) who reported that EBV DNA levels in saliva decreased after initial periodontal treatment. It remains to be seen, if we will observe similar findings when our treatment component arm of this study concludes in the near future.

While our findings shed light on the relationships between EBV, CMV, saliva and periodontitis, the results presented should be viewed with respect to the limitations of this clinical study. First, an orally healthy control group was absent which makes comparisons difficult in this regard. However, instead of recruiting healthy individuals, we used the patient as their own control. That is, plaque samples were procured from three healthy periodontal sites and three periodontal disease sites. Although this is not the same as recruiting healthy patients, it does provide a comparison between disease and healthy sites, albeit the healthy sites generally yielded less volume of plaque and the disease sites had the risk of contamination with blood. Thus, the detection of EBV in the plaque from disease sites could reflect EBV's presence in the blood or the inflamed gingival sulcus.

In conclusion, the present findings are consistent with the premise that high levels of EBV DNA in saliva reflect increased likelihood of several subgingival periodontal sites having high EBV DNA burden. By contrast, the low prevalence of CMV DNA in plaque and saliva suggest that this virus is an infrequent participant of periodontal disease in immunocompetent individuals. Interestingly, we did not observe a relationship between the presence or amount of these viruses in saliva and severity of periodontal disease. The contrasting findings between ours and published studies and the limited number of studies that have investigated these relationships to date should prompt larger studies that include periodontal disease and healthy controls to further define the relationships of EBV and CMV in periodontitis and determine whether periodontal therapy and/or antiviral therapy can reduce oral herpesvirus loads and improve periodontal health.

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Author contributions

DR Dawson, RJ Jacob, RJ Kryscio and CS Miller designed the study. C Wang and RJ Danaher performed all laboratory analyses. Y Lin and RJ Kryscio performed all data analyses. CS Miller drafted the manuscript and all authors participated in the editing and final preparation of the manuscript.

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