J Periodont Res 2005; 40; 294–298

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2005.00796.x

Real-time polymerase chain reaction quantification of Epstein–Barr virus in chronic periodontitis patients

Antonis Konstantinidis[1], Dimitra Sakellari[1], Anna Papa[2], Antonis Antoniadis[2]

[1]Department of Periodontology, School of Dentistry, Aristotle University of Thessaloniki, Greece, [2]'A' Department of Microbiology, School of Medicine, Aristotle University of Thessaloniki, Greece

Konstantinidis A, Sakellari D, Papa A, Antoniadis A. Real-time polymerase chain reaction quantification of Epstein–Barr virus in chronic periodontitis patients. J Periodont Res 2005; 40: 294–298. © Blackwell Munksgaard 2005

Background: Although herpes viruses have been implicated in the pathogenesis of chronic and aggressive periodontitis, few data in the literature refer to quantification of these viruses in periodontal sites, especially in relation to serological findings.

Objective: The aim of the present study was to compare Epstein–Barr virus (EBV) DNA load in subgingival specimens from chronic periodontitis patients and in periodontally healthy subjects, in relation to serologic testing of IgM and IgG antibodies to EBV.

Methods: A total of 22 chronic periodontitis patients and 13 controls participated in the present study. Seventy-nine subgingival specimens (one pooled, one from a deep and one from a shallow site), sampled with paper points, were analysed with real-time polymerase chain reaction for EBV. Subjects were also examined for anti-EBV IgG and IgM levels in serum, using an enzyme-linked immunosorbent assay.

Results: One subject was seronegative for EBV. Three subjects (one patient and two controls) displayed anti-EBV IgM. Their data were excluded from further analysis. All three displayed EBV in their subgingival samples. Nine out of the remaining 20 chronic periodontitis patients and 10 out of 11 controls did not display EBV subgingivally. A statistically significant difference in viral load was observed between pooled and shallow-pocket samples from periodontitis patients but not between samples from deep and shallow pockets (Kruskall–Wallis ANOVA, Dunn's multiple comparisons test).

Conclusions: Data from the present study do not strongly support the pathogenetic significance of EBV in chronic periodontitis lesions. The data do, however, suggest that parallel serological assessments provide a useful insight into the association of viruses with periodontal disease.

Antonis Konstantinidis, 4 Agias Sofias str., Thessaloniki, 54622, Greece
Tel: +30 2310 238351
Fax: +30 2310 999613
e-mail: antkon@otenet.gr,konstant@dent.auth.gr

Keywords: chronic periodontitis; Epstein–Barr virus; real-time polymerase chain reaction

Accepted for publication October 27, 2004

A new concept has emerged during the last decade concerning the contribution of herpesviruses in aetiopathogenesis of periodontal diseases.

Taken collectively up to now, data in the literature suggest an increased frequency of detection of specific members of the Herpesviridae family, such as Epstein–Barr virus (EBV), human cytomegalovirus (HCMV) and herpes simplex-1 (HSV-1), in various forms of periodontal disease (1–3). As extensively reviewed in recent literature, these viruses possess the biological mechanisms to potentially modify the periodontal microenvironment and therefore might both favour the establishment of a more pathogenic flora, and affect the pathogenetic processes (1, 2).

Specifically, an increased frequency of detection of EBV, HCMV and HSV-1 has been reported for chronic periodontitis patients compared to gingivitis patients (3, 4), for periodontitis sites compared to gingivitis sites in the same patient (5), for deep sites from localized juvenile periodontitis (localized aggressive periodontitis) compared to shallow sites from the same patients (6), and for active sites compared to stable sites of early onset (aggressive) periodontitis in maintenance phase (7). In the majority of these studies, the viruses implicated have been detected in subgingival samples using nested polymerase chain reaction (PCR).

EBV, a double-stranded DNA virus, is one of the eight human herpes viruses (HHV4). It is transmitted by salivary contact and establishes a lifelong latent infection, usually asymptomatic. Approximately 50% of human infections of EBV at a young age involve the form of infectious mononucleosis, a disease associated with fever and swollen lymph glands.

Real-time PCR, a reliable and sensitive technique allowing quantification of the viral load, is being extensively applied for monitoring viral infections. Recently, a study using this technique has shown increased numbers of copies of CMV in pooled subgingival samples from aggressive periodontitis patients, whereas the virus was not detected in any of the samples from periodontally healthy individuals (8).

The aim of the present study was to estimate EBV DNA load in chronic periodontitis patients, using Lightcycler® real-time quantitative PCR (LC-PCR), in conjunction with serological data.

Material and methods

Subject sample

Patients participating in the present study ($n = 22$, mean age 48 ± 8.6) were recruited from the Clinic of the Department of Periodontology and Implant Biology, Dental School, Aristotle University of Thessaloniki, and provided informed consent. Personnel from the Department ($n = 13$, mean age 45.9 ± 6.7) with no attachment loss, and bleeding on probing < 10%, also volunteered to participate in the present study. Criteria for inclusion were the following:

1. absence of systemic conditions;
2. absence of herpetic infection (self-reported) during the last six months;
3. no history of antibiotics within the previous six-months;
4. no history of periodontal treatment during the last 12 months;
5. diagnosis of generalized chronic periodontitis based on clinical and radiographic findings (9);
6. presence of at least 20 teeth.

Clinical sample collection

Before subgingival sampling, supragingival plaque was carefully removed using sterilized cotton pellets. After isolation and drying with cotton rolls, sterilized paper points were inserted to the depth of pre-selected sites and left in place for 30 s. Three samples were collected from each patient. One pooled sample was obtained from the four mesial sites of molar teeth. In addition, one sample was obtained from a deep pocket (> 7 mm) and one sample from a shallow site (< 4 mm). Paper points were placed in sterile Dnase and Rnase-free microcentrifuge tubes, and stored at −80°C until processed.

Blood collection and analysis

Samples of peripheral blood were collected by venipuncture in tubes without anticoagulant. Blood samples were taken on the same day as subgingival sampling. Serum was separated after centrifugation at 1600 *g* for 20 min and stored at −20°C until assayed. Specific anti-VCA (Viral Capsid Antigen) IgG and IgM antibodies to EBV in patients' serum samples were detected by enzyme-linked immunosorbent assay using commercial kits (DIA.-PRO. Diagnostic Bioprobes Srl, Milano, Italy).

Real-time polymerase chain reaction for Epstein–Barr virus

All samples were analysed at the 'A' Department of Microbiology, School of Medicine, Aristotle University of Thessaloniki. Two hundred microlitres of binding buffer supplemented with poly(A) and 50 µl of Proteinase K (included in the commercial kit described below) were added to the eppendorfs with the paper points and viral DNA was extracted from the clinical samples with the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Penzberg, Germany). The final elution volume was 50 µl. Viral DNA was quantified using the Lightcycler EBV Quantification Kit and the Lightcycler instrument (Roche Applied Science, Penzberg, Germany), following the manufacturer's instructions. LightCycler PCR is very well suited for monitoring EBV DNA load dynamics and its diagnostic value is comparable to that of Q-PCR (10, 11). Briefly, 5 µl of the extracted DNA was added to a glass capillary containing 15 µl of a ready-to-use buffered mixture of primers, hybridization probes, *Taq* polymerase and deoxynucleotide Triphosphates (dNTPs). A specific internal control at a particular amount close to the lower detection limit was used to prevent misinterpretation of false negative results due to inefficient extraction or interference from PCR inhibitors. The internal control was detected in channel F3, whereas EBV DNA was detected in channel F2. The kit allows quantification within a range

of 10^2–10^6 copies per reaction, and the lower detection limit of the kit is < 10 copies per reaction. Melting curve analysis was performed to increase the specificity of the assay. The T_m peak of the amplicon was 62 ± 2°C. All experiments included appropriate positive and negative controls. DNA extraction and PCR amplification took place in different laboratory areas in order to avoid contamination.

Statistical analysis

The median age of patients and controls were compared with the Mann–Whitney test. Differences of subgingival EBV counts (viral DNA copies per sample) among samples from chronic periodontitis patients (pooled, deep and shallow) and controls were sought with the Kruskall-Wallis ANOVA. Further differences of mean EBV counts between groups were sought using Dunn's multiple comparisons test. 2 × 2 contingency tables were used to determine any overall correlation between the presence of EBV subgingivally, and periodontal status, using Fisher's exact test. Differences between mean probing depths of the sampled sites were sought with the Mann–Whitney test between chronic periodontitis patients positive and negative for subgingival EBV. For all the above, the significance level was set at the 0.05. Statistical analysis was performed using the Instat® statistical package (Graphpad™, San Diego, CA, USA).

Results

No differences were observed between the median age of chronic periodontitis patients and controls (Mann–Whitney test, $p = 0.79$). The correlation between serological findings and the presence of EBV subgingivally, are presented in Table 1. Data are displayed separately for chronic periodontitis patients and controls. A high percentage of participants (95.5% of chronic periodontitis patients and 100% of controls) were seropositive for anti-EBV IgG. Of these subjects more than 50% of chronic periodontitis patients and 23% of controls displayed EBV subgingivally. When participants were stratified by combining IgG and IgM seropositivity, 4.5% of chronic periodontitis patients and 15.4% of controls were positive. Of these subjects, 100% displayed EBV subgingivally, regardless of periodontal status. When stratified combining IgG seropositivity and IgM seronegativity, 90.9% of chronic periodontitis patients and 84.6% of controls were positive. Of this subgroup, 55% of chronic periodontitis patients and 9% of controls displayed EBV subgingivally. Only data from this subset of participants was analysed further for EBV counts.

The 2 × 2 contingency table constructed to examine the relationship of presence of subgingival EBV with periodontal status is presented in Table 2. Only subjects seropositive for IgG but seronegative for IgM were include in the present analysis. A statistically significant correlation was found between presence of EBV subgingivally and chronic periodontitis (Fisher's exact test, $p = 0.021$).

Data referring to EBV counts subgingivally are presented in Table 3 (mean counts per sample and range). Although statistical analysis (Kruskall–Wallis ANOVA) displayed significant differences between the groups ($P = 0.003$), when statistical differences were sought between specific groups, the only significant difference observed was between the EBV load in pooled vs. shallow pocket samples in chronic periodontitis patients (Dunn's multiple comparisons test, $P < 0.01$). No significant differences were found between deep and shallow pockets or between the pooled sample from chronic periodontitis and controls (Dunn's multiple comparisons test, $p > 0.05$).

No correlation between mean probing depth and EBV detection was found in any of the tested subcategories (mean pooled, deep and shallow) of chronic periodontitis patients (data not shown).

Table 1. Serological data and subgingival Epstein–Barr virus in the subject sample

	CP $n = 22$ (%)	Controls $n = 13$ (%)
IgG positive	21 (95.5)	13 (100)
Subgingival EBV positive	12 (54.5)	3 (23)
IgG positive, IgM positive	1 (4.5)	2 (15.4)
Subgingival EBV positive	1 (100)	2 (100)
IgG positive, IgM negative	20 (90.9)	11 (84.6)
Subgingival EBV positive	11 (55)	1 (9)

CP, chronic periodontitis; EBV, Epstein–Barr virus.

Table 2. Correlation of subgingival Epstein–Barr virus with periodontal conditions

	CP	Controls	Total
EBV positive	11	1	12
EBV negative	9	10	19
Total	20	11	31

Fisher's exact test $p = 0.02$.
CP, chronic periodontitis; EBV, Epstein–Barr virus.

Table 3. Subgingival Epstein–Barr virus (viral copies/sample) in IgG positive, IgM negative chronic periodontitis patients and controls

	Mean EBV counts	Range
CP pooled	19830*	0–390000
CP deep	10476	0–208200
CP shallow	0*	0
Controls pooled	39.1	0–430

*Kruskall–Wallis ANOVA, $p = 0.009$. Dunn's multiple comparisons test, $p < 0.01$.
CP, chronic periodontitis; EBV, Epstein–Barr virus.

Discussion

According to the findings of the present study, assessment of the serological status of patients participating in virus-related protocols appears to be a useful procedure in order: (i) to determine an individual's seropositivity

and (ii) to avoid the possibility of a subclinical viral infection that might affect findings from the periodontal environment. Only data from subjects seropositive for anti-EBV IgG were analysed further in the present study for reasons of homogeneity. One out of the 35 participants did not display anti-EBV IgG in serum, therefore our subject sample was 97% seropositive (Table 1). In the present study, one chronic periodontitis patient displayed high IgM titres for EBV in serum. Most interestingly, the same patient displayed an extremely high number of viral copies in the pooled sample (274×10^4 copies/sample), as well as in both individual samples from the deep and the shallow pockets. Two periodontally healthy individuals also displayed IgM in serum and EBV in their pooled subgingival samples (240 and 6860 viral copies/sample, respectively). Elevated IgM titres are indicative of recent even subclinical infection with EBV, although none of the participants reported so. In the light of the above-mentioned findings from IgM positive cases, at this point we cannot exclude the possibility of a viral 'spill-over' to the periodontal environment and we excluded data from these subjects from further analysis.

It appears that individual samples are preferable to pooled samples for determining the pathogenetic significance of subgingival viruses. Although pooled samples have so far provided an initial clue about the increased frequency of detection of herpes viruses in periodontally diseased subjects and sites, more meaningful conclusions can be drawn from individual samples. In the present study, pooled samples from chronic periodontitis patients did indeed display higher mean viral load compared to shallow pockets (Table 3, Dunn's multiple comparisons test, $p = 0.003$). The application of highly sensitive techniques such as real-time PCR partly overcomes the issue of detection problems due to insufficient samples, especially from the shallow pockets, as described in several previous investigations (8). Quantification of the viral load, compared to evaluating the presence or absence of a virus subgingivally, strongly enhances the potential for correctly assessing of the pathogenetic significance of viruses under investigation.

Although the subgingival presence of EBV was statistically significantly correlated with the presence of chronic periodontitis (Table 2, Fisher's exact test $p = 0.02$), compared to controls in the present study, the virus was not detected in any of the three samples from nine out of 20 IgG-positive, IgM-negative chronic periodontitis patients. All remaining 11 patients displayed EBV (range $39–390 \times 10^3$ viral copies/sample) in their pooled samples and none in the shallow pocket sample. No differences were observed in probing pocket depth (mean pooled, deep and shallow pockets), between EBV positive and EBV negative chronic periodontitis patients. Four patients displayed higher EBV load (range $520–208 \times 10^3$ viral copies/sample) in deep pockets than in their pooled sample (range $39–10.37 \times 10$ viral copies/sample) and these four cases are suggestive of a specific pathogenetic role for EBV in deep pockets, although statistical analysis failed to demonstrate a difference of the mean EBV load between deep and shallow pockets (Dunn's multiple comparisons test $p > 0.05$).The above-mentioned findings should be interpreted as indicative since they derive from a relatively small subject sample (22 chronic periodontitis patients). Previous investigations have also shown higher prevalence of EBV in pooled subgingival samples of chronic periodontitis patients compared to gingivitis patients, and periodontitis-affected sites compared to gingivitis sites in the same patient (3–5). The higher prevalence of subgingival EBV (55%) in chronic periodontitis patients reported in the present study compared to previous ones (3–5) could be attributed to the highly sensitive real-time PCR for EBV.

The issue of correct sampling for microbiological and virological studies in periodontology remains unresolved. In the present study, as in most relevant studies, sampling for viruses was performed with paper points. This sampling procedure was chosen in the present study for reasons of comparability with previous studies, as well as being less 'invasive' than using a curette, and therefore more suitable for avoiding, as far as possible, inclusion of blood in the samples. Blood cells in the sample constitute a possible source of EBV and thus might have a confounding effect on the correct estimation of viral load in the subgingival area. Although a recent study using real-time PCR for CMV chose subgingival sampling with a curette over paper points in order to yield more specimen from the pocket, it appears that gingival biopsies, as used in a previous study by Contreras *et al.* (12), constitute the most reliable approach for determining viral cells in periodontal tissues rather than the ones shedding in the pocket environment or deriving from blood.

In conclusion, although data from the present study do not strongly support the pathogenetic significance of EBV in chronic periodontitis lesions, it is suggested that serological assesments provide a useful insight into the association of viruses with periodontal disease.

Acknowledgements

The valuable assistance of A. Baka, J. Ioannidis and P. Xatzisavvas, postgraduate students of the Department of Periodontology and the technical assistance of E. Papadimitriou, biologist of the 'A' Department of Microbiology are greatly appreciated by the authors.

References

1. Contreras A, Slots J. Herpesviruses in human periodontal disease. *J Periodont Res* 2000;**35:**3–16.
2. Slots J, Contreras A. Herpesviruses: a unifying causative factor in periodontitis? *Oral Microbiol Immunol* 2000;**15:**277–280.
3. Contreras A, Umeda M, Chen C *et al.* Relationship between herpesviruses and adult periodontitis and periodontopathic bacteria. *J Periodontol* 1999;**70:**478–484.
4. Parra B, Slots J. Detection of human viruses in periodontal pockets using polymerase chain reaction. *Oral Microbiol Immunol* 1996;**11:**289–293.
5. Contreras A, Slots J. Mammalian viruses in human periodontitis. *Oral Microbiol Immunol* 1996;**11:**381–386.

6. Ting M, Contreras A, Slots J. Herpesvirus in localized juvenile periodontitis. *J Periodont Res* 2000;**35:**17–25.
7. Kamma JJ, Contreras A, Slots J. Herpes viruses and periodontopathic bacteria in early-onset periodontitis. *J Clin Periodontol* 2001;**28:**879–885.
8. Kubar A, Saygun I, Yapar M *et al*. Real-time PCR quantification of cytomegalovirus in aggressive periodontitis lesions using TaqMan technology. *J Periodont Res* 2004;**39:**81–86.
9. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;**4:**1–6.
10. Servi S, Verkuijlen S, van der Brule A *et al*. Comparison of Quantitive Competitive PCR with LightCycler -Based PCR for measuring Epstein–Barr virus DNA load in clinical specimens. *J Clin Microbiol* 2002;**40:**3986–3992.
11. Stocher M, Holzl G, Stekel H *et al*. Automated detection of five human herpes virus DNAs by a set of LightCycler PCRs complemented with a single multiple internal control. *J Clin Virol* 2004;**29:**171–178.
12. Contreras A, Nowzari H, Slots J. Herpesviruses in periodontal pocket and gingival tissue specimens. *Oral Microbiol Immunol* 2000;**15:**15–18.