Oral Microbiology Immunology 2004: 19: 230–232
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Oral Microbiology and Immunology

Detection of Epstein–Barr virus in saliva by real-time PCR

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Idesawa M, Sugano N, Ikeda K, Oshikawa M, Takane M, Seki K, Ito K. Detection of Epstein–Barr virus in saliva by real-time PCR.
Oral Microbiol Immunol 2004: 19: 230–232. © Blackwell Munksgaard, 2004.

The purpose of this investigation was to assess the salivary levels of Epstein–Barr virus (EBV) in patients with periodontitis using real-time PCR. EBV was detected in 16 out of 33 (48.5%) periodontitis patients and in 3 out of 20(15%) healthy subjects. The baseline mean values for bleeding on probing in EBV-positive patients were significantly higher than those in EBV-negative patients. A significant decrease in EBV levels was observed after initial periodontal treatment. Our findings indicate that levels of EBV in saliva may reflect the status of periodontal inflammation.

Key words: Epstein–Barr virus; periodontal treatment; real-time PCR; saliva

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Accepted for publication January 30, 2004

Epstein–Barr virus (EBV), a ubiquitous human herpes virus, is associated with infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, and chronic fatigue syndromes (17). Recent studies have also shown an association between oral EBV and periodontal diseases such as chronic periodontitis, juvenile periodontitis, Down's syndrome periodontitis, HIV-associated periodontitis and acute necrotizing ulcerative gingivitis (4–6, 18, 20, 22, 26, 29). EBV in subgingival plaque is significantly associated with clinical parameters and the presence of periodontopathic bacteria (7, 13, 25, 27). EBV infects periodontal B lymphocytes, which during reactivation shed the virus into saliva, allowing it to spread to new hosts (3, 8). Several studies have shown that EBV DNA in saliva is elevated in lymphoproliferative disorders, transplant recipients, and people with AIDS (10–12, 23, 24). Despite many recent clinical studies of plaque samples of patients with periodontitis, there has been little investigation into the prevalence of EBV in the saliva of periodontitis patients or the effect of periodontal treatment on salivary levels of EBV.

There are several methods for detecting EBV. The quantity of EBV in clinical samples has previously been studied by *in situ* hybridization, EBV clonality assay, immunohistochemistry, ELISA and polymerase chain reaction (PCR). Real-time PCR with specific labeled probes can provide a precise and sensitive method for more accurate quantification of EBV (15).

The purpose of this investigation was to assess the salivary levels of EBV in periodontitis patients using real-time PCR.

The study group comprised 33 systemically healthy subjects with periodontitis, mean age 49.9 years (range 25–68 years). The clinical criteria of periodontitis were judged from standard measurements of clinical pocket depths. Periodontitis patients had at least two sites showing probing depths greater than 4 mm. Twenty systemically healthy subjects with clinically healthy periodontium served as controls (mean age 35.2 years, range 25–65 years). Bleeding on probing and periodontal pocket depth were recorded for six sites of all teeth present. Clinical records were taken at the first examination and after initial periodontal treatment in 11 periodontitis patients (four women and seven men, age range 28–64 years). The 2–4-month-long initial periodontal treatment consisted mainly of oral hygiene instruction, and scaling and root planing. At clinical examination, paraffin wax-stimulated whole saliva was collected, and the samples were stored at −80°C until analyzed.

Saliva samples were boiled for 10 min and then centrifuged at $10{,}000 \times g$ for 5 min, and 5 μl of the supernatant was used as a template for PCR. Real-time PCR was performed using the ABI PRISM 7700 Sequence Detection System (ABI, Foster City, CA). Each reaction tube contained 50 μl of reaction mixture, including 5 μl of sample, 1 × Universal PCR Master Mix (ABI), 900 nM of each primer and 250 nM probe.

The primer and probe set for EBV are listed in Table 1 (15). To use the quantity of total bacteria as a positive control, conserved sequences were selected from the *16S* gene as reported previously (16). Total bacteria were determined using total bacteria primers and probes that hybridized to all bacterial 16S rDNA. Amplification of total bacterial 16S rDNA was

Table 1. Sequences of oligonucleotide primers and probes

	Sequence (5′ → 3′)
EBV	
Forward	AGT CCT TCT TGG CTA CTC TGT TGA C
Reverse	CTT TGG CGC GGA TCC TC
Probe	CAT CAA GAA GCT GCT GGC GGC C
Total bacteria	
Forward	GGATTAGATACCCTGGTAGTC
Reverse	TACCTTGTTACGACTT
Probe	TGACGGGCGGTGTGTACAAGGC

Table 2. Detection of salivary EBV in periodontitis patients and clinically healthy subjects. all values are expressed as mean ± SE

Variable	Periodontitis patients	Healthy subjects
No. of subjects	33	20
Positive subjects	16	3
No. of EBV/ml	$4.48 \pm 2.19 \times 10^5$	238 ± 133
No. of total bacteria/ml	$9.17 \pm 0.24 \times 10^8$	$2.16 \pm 0.63 \times 10^8$

carried out in a separate reaction at the same time and under the same conditions as those used for the EBV-specific amplification. The PCR probes were labeled at the 5′ end with the reporter dye 6-carboxyfluorescein (6-FAM) and at the 3′ end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). Data were analyzed using the Sequence Detection System software from ABI. The amount of EBV was determined using synthesized nucleotides of the amplified region (98mer). EBV levels were expressed as a percentage of total bacterial number. The number of bacterial cells was determined using DNA from known amounts of bacteria (14, 16).

Differences in probing depth and bleeding on probing between EBV-positive patients and -negative patients were analyzed using the Mann–Whitney *U*-test. Differences in the salivary levels of EBV, probing depth and bleeding on probing before and after the initial periodontal treatment were analyzed using Student's *t*-test. Statistical analyses were performed using SPSS® software (SPSS Inc., Chicago, IL).

Only those C_T-values smaller than 36 for total bacteria and 38 for EBV were considered positive to avoid errors resulting from inaccurate and unreliable quantification values. The detection limit of the real-time PCR assay was about 2000 bacterial cells ($C_T = 36$, 100 copies/reaction) and of EBV, 500 copies ($C_T = 38$, 25 copies/reaction) per ml of saliva (data not shown). By PCR analysis, EBV was detected in 16 of 33 (48.5%) periodontitis patients and in 3 of 20 (15%) healthy subjects (Table 2). The mean bleeding on probing values in positive patients were significantly higher than those in negative patients, but there was no significant difference in probing depth (Table 3).

Table 3. Detection of salivary EBV and clinical measurements in periodontitis patients (n = 33). All values are expressed as mean ± SE

Variable	Positive (n = 16)	Negative (n = 17)
EBV levels (%)	$7.8 \pm 3.7 \times 10^{-2}$	0*
No. of EBV/ml	$7.11 \pm 3.80 \times 10^5$	0*
No. of total bacteria/ml	$9.02 \pm 0.28 \times 10^8$	$9.26 \pm 0.47 \times 10^8$
Age in years	46.4 ± 3.7	53.2 ± 3.1
Probing depth (mm)	3.59 ± 0.20	3.40 ± 0.28
Bleeding on probing (%)	58.0 ± 4.8	31.0 ± 3.3*

*$P < 0.01$. Mann–Whitney *U*-test, statistically significant difference between EBV-positive and EBV-negative periodontitis patients.

Table 4. Changes in clinical measurements and salivary EBV levels in periodontitis patients (n = 11) before and after treatment. All values are expressed as mean ± SE

Variable	Before	After
Positive subjects	11	5
EBV levels (%)	$5.1 \pm 2.6 \times 10^{-2}$	$0.2 \pm 0.1 \times 10^{-2}$
No. of EBV/ml	$9.46 \pm 5.40 \times 10^5$	$9.01 \pm 4.80 \times 10^3$
No. of total bacteria/ml	$2.05 \pm 0.78 \times 10^9$	$2.36 \pm 0.73 \times 10^9$
Probing depth (mm)	3.60 ± 0.27	2.72 ± 0.21*
Bleeding on probing (%)	57.7 ± 6.2	23.5 ± 3.2*

*$P < 0.01$. Student's *t*-test, statistically significant difference before, and after periodontal treatment.

EBV levels and numbers in whole saliva were compared before and after periodontal treatment in 11 periodontitis patients. Periodontal status improved following the initial periodontal treatment (Table 4). A significant decrease in EBV was observed after the treatment. The mean bleeding on probing and pocket depth values in positive patients post-treatment (bleeding on probing 25.9 ± 12.3%, pocket depth 3.01 ± 0.73 mm) were slightly higher than those in negative patients (bleeding on probing 20.6 ± 5.8%, pocket depth 2.38 ± 0.38 mm).

Overall, more than 90% of the world's population carries EBV as a life-long persistent infection, with latent infection of B lymphocytes and virus being shed into saliva (1, 21, 28, 30).

Saliva represents an easy means of obtaining samples containing infectious agents from all oral sites such as mucosa and supragingival and subgingival plaque (2, 9, 19). In our study, EBV was detected in 48.5% of periodontitis patients and in 15.6% of healthy subjects. The mean bleeding on probing values in positive patients were significantly higher than those in negative patients. Previous research has demonstrated that EBV can be found in B lymphocytes from adult patients with periodontitis (8), and that the number of B lymphocytes in sites with active periodontitis is significantly higher when compared to stable sites (31). Although other factors may also be involved, inflammation of periodontal tissue may play an important role in the prevalence of salivary EBV.

Salivary EBV levels decreased in response to initial periodontal treatment. Eliminating inflammation may contribute to a decrease in salivary EBV. These results suggest that initial periodontal treatment is useful for reducing EBV levels in saliva. Further investigation is needed to reach a conclusive result.

Acknowledgments

This work was supported by a Grant-in-Aid for Technology to Promote Multidisciplinary Research Projects from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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