Periodontopathogen and Epstein–Barr Virus-Associated Periapical Periodontitis May Be the Source of Retrograde Infectious Peri-Implantitis

Fernando Verdugo, DDS, PhD;* Ana Castillo, MD, PhD;† Krikor Simonian, DDS;‡ Francisca Castillo, MS;§ Esther Farez-Vidal, MD, PhD;¶ Antonio D'Addona, DDS**

ABSTRACT

Background: Herpesviral-bacterial synergism may play a role in periodontitis and peri-implantitis etiopathogenesis. Periapical periodontitis (PP) lesions can predict future apical peri-implantitis complications.

Purpose: This pilot study aimed to substantiate herpesviral-bacterial coinfection in symptomatic (SP) and asymptomatic (AP) PP and assess associations with periodontopathogen salivary contamination in patients receiving implants.

Materials and Methods: Polymerase chain reaction (PCR)-based identification was performed on PP granulation tissue (GT) from 33 SP and AP patients and compared with unstimulated whole saliva. Quantitative PCR evaluated Epstein–Barr virus (EBV) and cytomegalovirus copy counts.

Results: SP GT had higher proportions of periodontopathogens. Symptomatic patients were 3.7 times more likely to be infected with EBV than AP ($p = .07$; 95% CI: 0.8–16.2). SP were 2.9, 2.1, 3.6, and 1.6 times more likely to be infected with *Treponema denticola*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*, respectively. The odds ratio of EBV infecting PP lesions was two times higher in those positive for the virus in saliva. Saliva *Tannerella forsythia*-positive patients were 15 times more likely to present this pathogen in PP lesions ($p = .038$). Saliva EBV-positive individuals were 7 and 3.5 times more likely to yield GT contamination with *T. forsythia* and *T. denticola*, respectively. EBV copy counts were significantly higher in SP ($p < .01$).

Conclusions: A causal association between EBV, specific bacterial anaerobic infection, and symptomatic PP is likely. EBV high prevalence underscores the viral etiological importance. Salivary EBV contamination is likely to be associated with viral and bacterial GT infection. Saliva PCR analysis can be a good predictor of GT specific infection and help establish antimicrobial therapy. If confirmed by prospective longitudinal clinical trials, antiviral therapy could possibly benefit SP and nonresponsive to treatment individuals and help prevent potential peri-implant infectious complications.

KEY WORDS: *Actinobacillus actinomycetemcomitans*, human herpesvirus 4, microbiology, periapical periodontitis, polymerase chain reaction, *Porphyromonas gingivalis*, saliva

INTRODUCTION

Periodontopathic bacterial proliferation has been associated with periodontal herpesvirus infections.[1–3] Clinical studies suggest that both Epstein–Barr virus (EBV) and cytomegalovirus (CMV) may play an important role in the pathogenesis of human periodontitis.[4,5] Periodontal EBV infection may influence pathogenic bacterial overgrowth, and impact their adhesion potential to infected host cells by altering the inflammatory cells involved in the immune response.[3,6]

Specific microbial contamination can impair osteogenesis. Increased bone volume loss is associated with

*Consultant, Periodontics Department, VA Hospital Greater Los Angeles Healthcare System, Los Angeles, CA, USA, and private practice, Altadena, CA, USA; †professor, Department of Microbiology, School of Medicine, University of Granada, Granada, Spain; ‡clinical professor, Department of Periodontics/Advanced Periodontology, University of Southern California School of Dentistry, Los Angeles, CA, USA and private practice, Pasadena, CA, USA; §researcher, Department of Microbiology, School of Dentistry, University of Granada, Granada, Spain; ¶professor, Department of Biochemistry & Molecular Biology, University of Granada, School of Medicine, Granada, Spain; **professor and director, Odontology Clinic, Catholic University Sacro Cuore, Rome, Italy

Reprint requests: Dr. Fernando Verdugo, 2028 North Lake Avenue, Altadena, CA 91001, USA; e-mail: fverdugo@perio.org

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the occurrence of key anaerobic species and salivary EBV-1.[7] The likelihood of pronounced bone volume loss was over 16 times higher in individuals contaminated with a combination of salivary EBV-1 and at least three of the following species: *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, or *Prevotella intermedia*.[7] EBV distinctive pathogenic assets could readily boost the progression of human periodontitis and predispose to bone loss. An active herpesvirus infection may impair neutrophil periodontal first line of defense and evade T lymphocytic response, vital in eliminating and containing periodontitis-causing bacteria.[8,9] Moreover, herpesviruses may induce a direct cytopathic impact on fibroblasts, keratinocytes, endothelial, inflammatory, and bone cells.[6]

EBV infects roughly 80% to 95% of the adult population in developed countries.[10] The alternate EBV cellular tropism capability between B and epithelial cells could further amplify virion output by fostering viral replication.[11] Evaluating specific periodontopathogen salivary copy count could help establish and predict future periodontal breakdown.[12]

The etiopathogenesis of symptomatic (SP) and asymptomatic (AP) periapical periodontitis (PP) lesions seems to be orchestrated and fueled by a combination of herpesviruses types 4 and 5, and Gram-negative anaerobic rods.[5,13–17] Researchers have hypothesized that active herpesviral infections would induce local immunosuppression and allow destructive bacterial overgrowth favoring the initiation and progression of tissue breakdown.[4,6] However, the exact mechanisms of action, etiopathogenesis, and pathogen proportions for disease progression are still unclear.[18]

Furthermore, past PP lesions around teeth could predict potential future periapical peri-implantitis complications.[19] Implant sites that had previous teeth with periapical lesions were 7.2 times more likely to develop similar peri-implant lesions with *P. gingivalis* being the most common infecting pathogen. An endodontic pathosis on extracted or adjacent teeth is likely to be a source of infection for future neighboring implants.[19]

The present study aimed to substantiate herpesviral-bacterial coinfection in established SP and AP periapical pathosis and assess potential associations with specific periodontopathogen salivary contamination in patients receiving dental implants.

MATERIALS AND METHODS

Study Population

This study research was conducted in accordance to the requirements of the Helsinki Declaration of 1975 as revised in Edinburgh 2000. Patients were verbally informed about the tissue samples to be taken at the time of tooth extraction and gave their written consent approved by the institutional review board. Ethical approval was previously obtained from the University of Basque Country Ethics Committee as part of a larger thesis research protocol.

Thirty-three consecutive patients, 15 females and 18 males, with an age range of 35 to 91 years and presenting PP entered this study. All study patients were systemically healthy nonsmokers. Patients with uncontrolled diabetes, long-term corticosteroid therapy, history of antibiotic use for the last 3 months, or uncontrolled hypertension were excluded from the study. Also, the exclusion criteria extended to those who had an oral-periradicular lesion communication or fractured teeth. Extracted teeth were deemed as having a questionable/poor mid and long-term prognosis based on extensive restorative work and individual treatment planning. Individuals were evaluated for periodontal disease status based on full mouth radiographs and patient records. They were either healthy with no history of periodontitis or healthy with history of the disease and under supportive periodontal therapy on a 3 to 6-month recall program.

Periapical radiographs using the long cone paralleling technique were used to identify apical pathosis. None of the study teeth showed signs of moderate or severe types of marginal periodontitis. Patients were divided into SP and AP groups. Symptomatic teeth presented pain, swelling, suppuration, or sensitive percussion. The procedures were performed between the years 2010 to 2012 in two private offices (Altadena and Pasadena, CA, USA).

Saliva samples were taken first using sterile empty containers or microcentrifuge tubes. Unstimulated whole saliva was obtained before anesthetic delivery. Thereafter, patients rinsed with 0.12% chlorhexidine mouthwash for 1 minute.[20] Following extraction, teeth were washed with a sterile saline solution.[15,21] Granulation tissue (GT) from periapical lesions or attached root apexes was sampled using sterile curettes or detached from the apex using a sterile #15 blade and placed in a second container.

Sample Processing

Nucleic Acid Extraction. Saliva and GT sample DNA was extracted following conventional protocols and manufacturer's instructions. The "QIAamp DNA Mini Kit" (Qiagen, GmbH, Hilden, Germany) was used for saliva samples and the "High Pure PCR Template Preparation Kit" (Roche Diagnostics, GmbH, Penzberg, Germany) was used for tissue samples. DNA extracts were cryopreserved at –20°C until their use.

PCR Analysis. Table 1 lists all polymerase chain reaction (PCR) primers used in the study that have been previously described: *P. gingivalis*, *T. forsythia*, *P. intermedia*, *P. nigrescens*, *A. actinomycetemcomitans*, and *Treponema denticola*,[1] and EBV and CMV.[22] All primers were synthesized on Tib Molbiol (Berlin, Germany). Reference strains used as controls were: *P. gingivalis* ATCC 33277, *T. forsythia* JCM 10827, *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, *A. actinomycetemcomitans* CCUG 1210, and *T. denticola* ATCC 35405.

PCR mixtures for amplification were prepared using Master Mix 2× (PCR Master Mix M7505, Promega Corporation, Madison, WI, USA) adjusting the final concentrations of each of the components. PCR temperature profiles for *P. intermedia*, *P. nigrescens*, and *A. actinomycetemcomitans* included an initial denaturation step at 95°C for 2 minutes, 36 cycles of a denaturation at 94°C 30 seconds, a 55°C primer-annealing step for 1 minute, and an extension step at 72°C for 2 minutes, with a final step of 72°C for 10 min. Temperature profiles for *P. gingivalis*, *T. forsythia*, and *T. denticola* included an initial 2-minute step at 95°C, followed by 36 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, and a final step of 2 minutes of 72°C. Experimental viral PCR assay conditions have been described before.[1] PCR products were analyzed by 1.5% agarose gel electrophoresis in TBE buffer 1×, with the addition of an ethidium bromide solution (Fluka, BioChemika, Steinheim, Switzerland), until a final concentration of 0.5 μg/mL was obtained. A molecular weight marker was included in all cases. After 60-minute 80 V electrophoresis, agarose gel was photographed (Nikon Coolpix 4500, Tokyo, Japan) over an ultraviolet light source (Figure 1).

Real-Time Assay. Amplification, data acquisition, and analyses were carried out using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) following standard procedures. Quantitative detection of EBV and CMV DNA was performed using the ready-to-use artus® EBV/CMV TM PCR Kit (Artus, Qiagen, Hilden, Germany). Detection of the amplified fragments was carried out by measuring fluorescence in the FAM channel in the ABI PRISM® SDS system. The artus® EBV/CMV TM PCR Kit contains a second system for heterologous amplification that allows to check for PCR reaction inhibition. This reaction is detected as an internal control measuring VIC fluorescence. Positive external controls (EBV RG/TM QS 1–4 & CMV LC/RG/TM QS 1–4) allowed determining viral load. The lower limit of detection for the assay was

TABLE 1 Primers Used for PCR Detection of Putative Periodontopathogens: Bacteria and Viruses

Periodontopathogen	Forward	Reverse
Porphyromonas gingivalis	F729 5′-AGG CAG CTT GCC ATA CTG CG-3′	R1132 5′-ACT GTT AGC AAC TAC CGA TGT-3′
Aggregatibacter actinomycetemcomitans	F478 5′-AAA CCC ATC TCT GAG TTC TTC TTC-3′	R1034 5′-ATG CCA ACT TGA CGT TAA AT-3′
Prevotella intermedia	F458 5′-TTT GTT GGG GAG TAA AGC GGG-3′	R1032 5′-TCA ACA TCT CTG TAT CCT GCG T-3′
Tannerella forsythia	F120 5′-GCG TAT GTA ACC TGC CCG CA-3′	R760 5′-TGC TTC AGT GTC AGT TAT ACC T-3′
Treponema denticola	F193 5′-TAA TAC CGA ATG TGC TCA TTT ACA T-3′	R508 5′-TCA AAG AAG CAT TCC CTC TTCTTC TTA-3′
Prevotella nigrescens	5′- ATG AAA CAA AGG TTT TCC GGT AAG –3′	5′- CCC ACG TCT CTG TGG GCT GCG A –3′
Cytomegalovirus	5′-GAG CGC GTC CAC AAA GTC TA- 3′	5′-GTG ATC CGA CTG GGC GAA AA-3′
Epstein–Barr virus type 1	5′-GCC AGA GGT AAG TGG ACT TTA ATT T-3′	5′-TGG AGA GGT CAG GTT ACT TAC C-3′

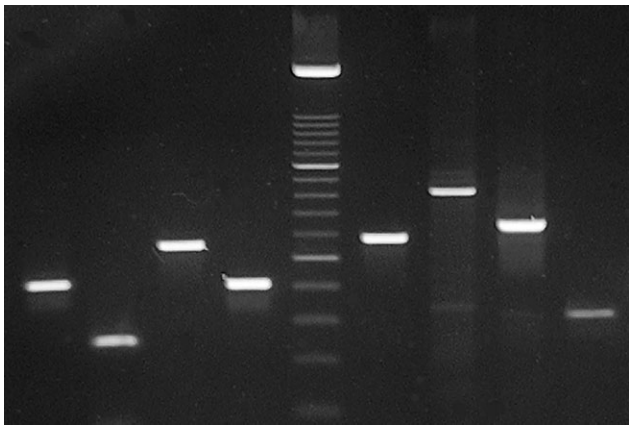

Figure 1 Electrophoresis of viral and bacterial PCR-amplified DNA products in 1.5% agarose gel under UV illumination. Lanes and sizes of amplified fragments from left to right: (1) Cytomegalovirus (406 bp), (2) Epstein–Barr virus type 1 (240 bp), (3) *Aggregatibacter actinomycetemcomitans* (557 bp), (4) *Porphyromona gingivalis* (404 bp), (5) DNA 100 bp ladder, (6) *Prevotella intermedia* (575 bp), (7) *Prevotella nigrescens* (804 bp), (8) *Tannerella forsythia* (641 bp), and (9) *Treponema denticola* (300 bp).

5.3 copies/μL of sample for EBV and 0.2 copies/μL for CMV.

EBV and CMV copy numbers were calculated using the formula below:

$$\text{Result (copies/mL)} = \frac{\text{Result (copies/μL)} \times \text{Elution volume (μL)}}{\text{Sample volume (mL)}}$$

Data Analysis

Descriptive analysis of data was expressed as mean ± standard deviation. Significance of group comparisons was determined by chi-square test (χ^2) using a commercially available software program (SPSS®, version 12.0, SPSS Inc., Chicago, IL, USA). Pearson's chi-square was applied to all contingency tables and the test for independence run to evaluate differences between SP and AP. The likelihood of GT microbial periapical infection in the presence of specific salivary pathogen contamination was expressed as the odds ratio (OR) and relative risk. Independent sample *t*-test was run to compare mean differences between groups for viral copy counts. Statistical significance was set at $p < .05$.

RESULTS

Thirty-three consecutive patients, 15 females and 18 males, with an average age of 58.7 ± 13 years (range: 35–91 years) and presenting PP, 20 SP and 13 AP, entered this study.

Thirty-two (96.9%) out of the 33 patients examined yielded PCR positive results, for the targeted periodontopathogens, in GT samples.

GT samples (Table 2) showed that 19 patients (57.6%) were positive for EBV and three patients were positive (9.1%) for CMV. Specific bacterial DNA detection was positive in 27 (81.8%) out of 33 GT samples for *T. forsythia*, 19 (57.6%) for *P. nigrescens*, 15 (45.5%) for *P. gingivalis*, 9 (27.3%) for *T. denticola*, 4 (12.1%) for *P. intermedia*, and 2 (6.1%) for *A. actinomycetemcomitans*.

Saliva samples were taken in a subgroup of 15 patients (Table 3). Fourteen (93.3%) out of the 15 saliva samples examined yielded PCR positive results for the targeted periodontopathogens. Saliva PCR analysis revealed to be a relatively accurate predictor of GT microbial contamination in the present study population for the targeted periodontopathogens. The positive predictive value (PPV) was defined as the probability that a patient being saliva PCR positive for a specific pathogen would also yield GT-positive results for the same microorganism. The PPV of saliva PCR positive patients were high in detecting GT positive contamination with the same pathogen. PPV were 96.2% for *T. forsythia*, 92.6% for *T. denticola*, 90% for *P. gingivalis* and *P. nigrescens*, 75% for *P. intermedia* and *A. actinomycetemcomitans*, and 50% for EBV.

Salivary EBV-positive patients were 2.3 times more likely to have at least one pathogenic bacterial species isolated in PP GT than those who were salivary EBV negative. The OR of EBV infecting PP lesions was two times higher in those individuals positive for the virus in saliva. Individuals that were salivary *T. forsythia* positive were 15 times more likely to present this pathogen in a PP lesion than those who were salivary *T. forsythia* negative ($p = .038$). Patients saliva EBV positive were 7 and 3.5 times more likely to yield positive GT contamination with *T. forsythia* and *T. denticola*, respectively.

Overall, SP were more likely to present higher proportions of GT-specific periodontopathogen contamination (Table 4). GT samples from SP were 3.7 times more likely to be infected with EBV ($p = .07$; 95% CI: 0.8–16.2) than AP.

Whenever EBV DNA was detected, copy counts ranged from as little as 6 copies to as much as 5,893,890.

TABLE 2 Qualitative and Quantitative PCR Detection of Periodontopathogens in Granulation Tissue from Symptomatic versus Asymptomatic Patients

GT Aa	GT Pn	GT Pi	GT Pg	GT Tf	GT Td	GT CMV	GT EBV	GT CMV	GT EBV	Clinical
0	0	0	1	1	0	1	1	430	554	Sympto
0	0	0	0	0	0	0	1	0	66,110	Sympto
0	0	0	0	1	0	0	1	0	1,120	Sympto
0	0	0	0	0	0	0	1	0	3,815	Sympto
0	0	0	1	1	0	0	1	0	360	Sympto
0	1	1	1	1	0	0	0	0	0	Sympto
0	1	0	1	1	0	0	1	0	498	Sympto
0	0	0	0	0	0	1	1	371,700	5,893,890	Sympto
0	1	1	1	1	1	0	1	0	1,749,225	Sympto
0	1	0	1	1	1	0	0	0	0	Sympto
1	1	0	1	1	1	1	1	100	61,840	Sympto
0	0	0	0	0	0	0	0	0	0	Sympto
0	0	1	0	1	1	0	1	0	25,130	Sympto
0	1	0	0	1	0	0	1	0	1,410	Sympto
0	1	0	1	1	1	0	0	0	0	Sympto
0	1	0	0	1	0	0	1	0	21,550	Sympto
0	1	0	0	1	1	0	0	0	0	Sympto
0	0	0	0	1	0	0	1	0	11,920	Sympto
1	1	0	1	1	1	0	0	0	0	Sympto
0	0	0	1	1	0	0	1	0	640	Sympto
0	0	0	1	1	0	0	1	0	195	Asympto
0	0	0	0	1	0	0	1	0	125	Asympto
0	1	1	1	1	0	0	0	0	0	Asympto
0	1	0	0	1	0	0	0	0	0	Asympto
0	1	0	1	1	0	0	0	0	0	Asympto
0	1	0	1	1	1	0	1	0	3,650	Asympto
0	0	0	0	1	0	0	1	0	4,125	Asympto
0	1	0	0	1	1	0	1	0	6	Asympto
0	1	0	0	0	0	0	0	0	0	Asympto
0	1	0	0	0	0	0	0	0	0	Asympto
0	1	0	0	1	0	0	0	0	0	Asympto
0	0	0	0	1	0	0	0	0	0	Asympto
0	1	0	1	1	0	0	0	0	0	Asympto

0 = absence, 1 = presence; Aa, *Aggregatibacter actinomycetemcomitans*; Asympto, asymptomatic patient; CMV, cytomegalovirus; EBV, Epstein–Barr virus type 1; GT, granulation tissue; Pi, *Prevotella intermedia*; Pg, *Porphyromonas gingivalis*; Pn, *Prevotella nigrescens*; Sympto, symptomatic patient; Td, *Treponema denticola*; Tf, *Tannerella forsythia*.

EBV copies were significantly higher in SP, 391,903 (range: 360–5,893,890) versus 623 (range: 6–4,125) for AP ($p < .01$). Only three patients were CMV GT positive (100–371,700 copies) and were all SP and EBV positive.

DISCUSSION

The present study was designed to validate herpesviral-bacterial coinfection in established SP and AP PP patients receiving dental implants and evaluate potential associations with specific periodontopathogen salivary contamination. The outcome assessment shows that EBV plays a significant role in the etiopathogenesis of PP, particularly in SP. EBV mean copy counts are significantly elevated in SP as compared with AP, 391,903 versus 623, respectively ($p < .01$). The single SP individual harboring the largest viral copy count had 5,893,890 and 371,700 copies of EBV and CMV, respectively, and no target bacteria. This does not rule out

TABLE 3 PCR Periodontopathogen Detection in Saliva and GT

Pathogen/Saliva versus GT	Saliva $n = 15$ (%)	Granulation Tissue $n = 15$ (%)	Saliva and GT*	p Value	Odds Ratio	Relative Risk
Porphyromonas gingivalis†	5 (33.3)	4 (26.7)	4	**<0.05**	60	16.7
Aggregatibacter actinomycetemcomitans†	2 (13.3)	1 (6.7)	1	**<0.05**	27	14.2
Prevotella intermedia†	4 (26.7)	1 (6.7)	1	>0.05	9.8	7.5
Prevotella nigrescens†	13 (86.7)	10 (66.7)	9	>0.05	2.3	1.4
Tannerella forsythia†	14 (93.3)	12 (80)	12	**<0.05**	15	3.3
Treponema denticola†	6 (40)	5 (33.3)	5	**<0.05**	70	15.7
Epstein–Barr virus†	6 (40)	6 (40)	3	>0.05	2	1.5
Cytomegalovirus†	1 (6.7)	0 (0)	0	>0.05	9.7	7.6

*PCR detection positive for both saliva and GT (granulation tissue).
†χ^2 test.
Odds ratio and relative risk of salivary positive subjects being GT positive for the same pathogen. Bold denotes statistical significance.

other specific bacteria infecting the GT lesion. The microbiota infecting PP lesions is complex and could harbor an average of 40 different genera per sample.[18] Pyrosequencing technology has allowed to identify large arrays of bacteria infecting PP lesions.[18,23,24]

SP patients are more likely to harbor higher proportions of aggressive periodontopathogens in GT (see Table 2). The OR of GT EBV infection was 3.7 times higher for SP than for AP samples, with a proportion of up to 70% positive samples versus 38.5%, respectively ($p = .07$; 95% CI: 0.8–16.2). One AP, considered GT EBV positive, yielded only 6 copies of EBV. This suggests that the sample could have been possibly contaminated with saliva or the virus was on a latent phase. Patients rinsed with 0.12% chlorhexidine and teeth were washed to reduce contamination. Had this subject been considered EBV negative, chi-square would have been significant ($p = .027$; 95% CI: 1.2–23.9; OR = 5.3). The kit used has a low limit of detection for EBV (5.3 copies/μL), so even if the virus is in a cryptic phase, DNA viral detection would still be feasible. Two more AP patients had low GT EBV counts (125 and 195 copies). Chi-square would have been further significant ($p = .002$; 95% CI: 2.2–76.4; OR 12.8) if they had been considered EBV negative. The statistical power would have been overall greater if the study sample size had been larger. Sample size was not predetermined in the present study. Historical data are usually used to estimate variances and other parameters in the power function. There is insufficient historical data in this regard to establish the right sample size and a literature search showed no meta-analysis performed for PP etiopathogenesis.

SP were 2.9, 2.1, 3.6, and 1.6 times more likely than AP to be infected with *T. denticola*, *P. intermedia*,

TABLE 4 Frequency of Granulation Tissue Periodontopathogen PCR Detection: Symptomatic vs. Asymptomatic

Pathogen/Symptomatic Asymptomatic Patients	Symptomatic $n = 20$ (%)	Asymptomatic $n = 13$ (%)	p Value	Odds Ratio
*Porphyromonas gingivalis**	10 (50)	5 (38.5)	=0.3	1.6
*Aggregatibacter actinomycetemcomitans**	2 (10)	0 (0)	=0.2	3.6
*Prevotella intermedia**	3 (15)	1 (7.7)	=0.5	2.1
*Prevotella nigrescens**	10 (50)	9 (69.2)	>0.05	0.4
*Tannerella forsythia**	16 (80)	11 (84.6)	>0.05	0.7
*Treponema denticola**	7 (35)	2 (15.4)	=0.2	2.9
Epstein–Barr virus*	14 (70)	5 (38.5)	**=0.07**	**3.7**
Cytomegalovirus*	3 (15)	0 (0)	**=0.07**	**5.4**

*χ^2 test: Symptomatic versus Asymptomatic.
Bold denotes statistical significance or close to being statistically significant.

A. actinomycetemcomitans, and *P. gingivalis*, respectively. However, the proportions of *T. forsythia* and *P. nigrescens* did not differ significantly between SP and AP ($p > .05$). These differences could be due to bacterial biofilm adherence specificity and herpesvirus tissue and host cell tropism.[3,6,11,25–27] Moreover, the Waldeyer tonsilar ring acts as a reservoir of EBV and could potentially shed virions into saliva and blood stream at a continuous pace in healthy carriers. Saliva viral levels could be replaced in as little as 2 minutes.[11,28] The alternate EBV cytotropism capability between B and epithelial cells could further amplify virion output by nurturing viral replication.[11] Latent EBV status can be switched into a dynamic lytic phase by different inducers.[10] The pathogenic interaction between EBV and Gram-negative anaerobic bacteria, such as *P. gingivalis*, seems to be bi-directional.[29] Herpesviruses reactivation can suppress local host defenses and allow bacterial overgrowth while specific bacterial structural components have shown to have the potential to stimulate EBV reactivation and production.[29] EBV can also develop local vascular damage and contaminate a variety of cellular lineages such as epithelial, endothelial, and B cells favoring periodontal tissue breakdown.[27,30]

Saliva EBV-positive patients were 2.3 times more likely to harbor, at least, one aggressive bacterial specie in GT. The OR of EBV infecting PP lesions was 2 times higher in those individuals positive for the virus in saliva. Patients saliva EBV positive were 7 and 3.5 times more likely to yield positive GT contamination with *T. forsythia* and *T. denticola*, respectively.

Saliva PCR analysis seems to be an accurate predictor of GT microbial contamination in the present study population. These data should be interpreted with caution considering the relatively small sample size of our study population. Salivary copy counts of common periodontopathogens could help predict forthcoming periodontal breakdown.[12] Salivary copy counts of *P. gingivalis*, *T. forsythia*, and *P. intermedia* seem to have the potential to detect periodontitis with diagnostic sensitivities of 86% to 89%.[12]

The low prevalence of CMV in the present study, one positive patient for saliva and two for GT, could be partly explained by the fact that study patients had no active periodontal disease and were following maintenance protocols. EBV high prevalence (GT and saliva samples) underscores the importance of this pathogen in the etiopathgogenesis of human periodontitis. The present results are in agreement with previous studies for EBV prevalence[4,5,13,14] and differ for CMV and EBV prevalence with others.[21,31] Different susceptible populations might be at greater risk of EBV-bacterial active infection or reactivation and therefore prevalence rates could significantly fluctuate.[3]

Different clones of *P. intermedia* and *P. gingivalis* can colonize the same anatomic sites in endodontal–periodontal infections, suggesting a genetic intraindividual diversity.[17] It is plausible that certain bacterial strains display greater synergism for herpesviral coinfection favoring immune response evasion in SP and, therefore, influencing treatment outcomes. This could explain why EBV-positive individuals were 7 and 3.5 times more likely to yield positive contamination with *T. forsythia* and *T. denticola* in the present study.

This research supports the hypothesis of a causal relationship between EBV infection and symptomatic PP. Symptomatic PP may progress when a latent or cryptic EBV infection reactivates. Herpesvirus reactivation will occur with a weakened or impaired immune system and a large virion load shedding.

The present study outcomes on EBV may have therapeutic implications in SP and help prevent future peri-implant infectious complications.

Periapical or retrograde peri-implantitis has recently emerged as a potential clinically challenging complication. PP lesions around teeth seem to be the source of infectious complications around implants placed at the same area or adjacent locations after tooth extraction.[19,32–34] Residual granulomatous or scar tissue at the recipient socket and marrow spaces could act as a periodontopathogen and herpesvirus latent reservoir.[32] EBV is a likely candidate in the etiopathogenesis of periapical lesions around teeth and implants.

Successful antiviral therapy to treat human EBV-associated severe periodontitis with valacyclovir 500 mg/10 days has been reported.[35] Antiviral therapy significantly decreased viral loads, resulting in a considerable clinical and periodontal improvement. Antiviral therapy in mice with established vascular disease resulted in clearance of viral antigens from the affected vessel and drastic improvement of arteritic lesions.[36]

Further studies are needed to elucidate the etiopathogenesis of periapical lesions around teeth and implants to help establish preventive therapeutic measures.

CONCLUSION

The present observational study supports the notion of a causal association between EBV infection and symptomatic PP. If confirmed by controlled prospective longitudinal clinical trials, antiviral therapy could possibly benefit SP and nonresponsive to treatment individuals and help prevent potential peri-implant infectious complications. The etiopathogenesis of SP and AP PP seems to be orchestrated and fueled by a combination of EBV and Gram-negative anaerobic rods. SP are more likely to display higher proportions of GT-specific pathogen contamination than AP. Saliva EBV-positive patients are more likely to yield positive GT contamination with specific anaerobic species such as *T. forsythia* or *T. denticola*. Saliva PCR analysis could be a potentially good predictor of GT microbial contamination for specific pathogenic bacteria and herpesviruses.

CONFLICT OF INTEREST

The authors deny any conflicts of interest related to this study.

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REFERENCES

1. Saygun I, Kubar A, Ozdemir A, Yapar M, Slots J. Herpesviral-bacterial interrelationships in aggressive periodontitis. J Periodontal Res 2004; 39:207–212.
2. Kantarci A, Hasturk H, Van Dyke T. Host-mediated resolution of inflammation in periodontal diseases. Periodontol 2000 2006; 40:144–163.
3. Slots J. Herpesviral-bacterial interactions in periodontal diseases. Periodontol 2000 2010; 52:117–140.
4. Rotola A, Cassai E, Farina R, et al. Human herpesvirus 7, Epstein–Barr virus and human cytomegalovirus in periodontal tissues of periodontally diseased and healthy subjects. J Clin Periodontol 2008; 35:831–837.
5. Hernádi K, Szalmás A, Mogyorósi R, et al. Prevalence and activity of Epstein-Barr virus and human cytomegalovirus in symptomatic and asymptomatic apical periodontitis lesions. J Endod 2010; 36:1485–1489.
6. Slots J, Saygun I, Sabeti M, Kubar A. Epstein–Barr virus in oral diseases. J Periodontal Res 2006; 41:235–244.
7. Verdugo F, Castillo A, Simonian K, et al. Periodontopathogen and Epstein-Barr virus contamination affects transplanted bone volume in sinus augmentation. J Periodontol 2012; 83:162–173.
8. Savard M, Gosselin J. Epstein-Barr virus immunossuppression of innate immunity mediated by phagocytes. Virus Res 2006; 119:134–145.
9. Ressing ME, Horst D, Griffin BD, et al. Epstein-Barr virus evasion of CD8(+) and CD4(+) T cell immunity via concerted actions of multiple gene products. Semin Cancer Biol 2008; 18:397–408.
10. Kieff E, Rickinson AB. Epstein-Barr virus and its replication. In: Fields BN, Knipe DM, Howley PM, eds. Fields virology. 5th ed. Philadelphia, PA: Lippincott-Williams & Wilkins, 2007:2603–2654.
11. Borza CM, Hutt-Fletcher LM. Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. Nat Med 2002; 8:594–599.
12. Saygun I, Nizam N, Keskiner I, et al. Salivary infectious agents and periodontal disease status. J Periodontal Res 2011; 46:235–239.
13. Sabeti M, Simon JH, Slots J. Cytomegalovirus and Epstein-Barr virus are associated with symptomatic periapical pathosis. Oral Microbiol Immunol 2003; 18:327–328.
14. Sabeti M, Slots J. Herpesviral-bacterial coinfection in periapical pathosis. J Endod 2004; 30:69–72.
15. Saboia-Dantas CJ, Coutrin de Toledo LF, Sampaio-Filho HR, Siqueira JF Jr. Herpesviruses in asymptomatic apical periodontitis lesions: an immunohistochemical approach. Oral Microbiol Immunol 2007; 22:320–325.
16. Buonavoglia A, Latronico F, Pirani C, Greco MF, Corrente M, Prati C. Symptomatic and asymptomatic apical periodontitis associated with red complex bacteria: clinical and microbiological evaluation. Odontology 2013; 101: 84–88.
17. Pereira CV, Stipp RN, Fonseca DC, Pereira LJ, Höfling JF. Detection and clonal analysis of anaerobic bacteria associated to endodontic-periodontal lesions. J Periodontol 2011; 82:1767–1775.
18. Saber MH, Schwarzberg K, Alonaizan FA, et al. Bacterial flora of dental periradicular lesions analyzed by the 454-pyrosequencing technology. J Endod 2012; 38:1484–1488.
19. Lefever D, Van Assche N, Temmerman A, Teughels W, Quirynen M. Aetiology, microbiology and therapy of periapical lesions around oral implants: a retrospective analysis. J Clin Periodontol 2013; 40:296–302.
20. Young MPJ, Korachi M, Carter DH, Worthington H, McCord JF, Drucker DB. The effects of an immediately pre-surgical chlorhexidine oral rinse on the bacterial contaminants of bone debris collected during dental implant surgery. Clin Oral Implants Res 2002; 13:20–29.
21. Sabeti M, Daneshmand A, Simon JH, Slots J. Cytomegalovirus-infected inflammatory cells in dental

apical lesions. Oral Microbiol Immunol 2009; 24:434–436.

22. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996; 11:266–273.
23. Li L, Hsiao WW, Nandakumar R, et al. Analyzing endodontic infections by deep coverage pyrosequencing. J Dent Res 2010; 89:980–984.
24. Siqueira JF Jr, Alves FR, Rôças IN. Pyrosequencing analysis of the apical root canal microbiota. J Endod 2011; 37:1499–1503.
25. Scrivano L, Sinzger C, Nitschko H, Koszinowski UH, Adler B. HCMV spread and cell tropism are determined by distinct virus populations. Plos Pathog 2011; 7:e1001256.
26. Adler B, Sinzger C. Endothelial cells in human cytomegalovirus infection: one host cell out of many or a crucial target for virus spread? Thromb Haemost 2009; 102:1057–1063.
27. Fishman JA, Rubin RH. Infection in organ-transplant recipients. N Engl J Med 1998; 338:1741–1751.
28. Hadinoto V, Shapiro M, Sun CC, Thorley-Lawson DA. The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. Plos Pathog 2009; 5:1–15.
29. Sugano N, Ikeda K, Oshikawa M, et al. Relationship between Porphyromonas gingivalis, Epstein-Barr virus infection and reactivation in periodontitis. J Oral Sci 2004; 46:203–206.
30. Kanai K, Kuwabara S, Mori M, Arai K, Yamamoto T, Hattori T. Leukocytoclastic-vasculitic neuropathy associated with chronic Epstein-Barr virus infection. Muscle Nerve 2003; 27:113–116.
31. Yazdi KA, Sabeti M, Jabalameli F, Eman M, Kolahdouzan SA, Slots J. Relationship between human cytomegalovirus transcription and symptomatic apical periodontitis in Iran. Oral Microbiol Immunol 2008; 23:510–514.
32. Quirynen M, Vogels R, Alsaadi G, Naert I, Jacobs R, van Steenberghe D. Predisposing conditions for retrograde peri-implantitis, and treatment suggestions. Clin Oral Implants Res 2005; 16:599–608.
33. Zhou W, Han C, Li D, Li Y, Song Y, Zhao Y. Endodontic treatment of teeth induces retrograde peri-implantitis. Clin Oral Implants Res 2009; 20:1326–1332.
34. Ayangco L, Sheridan PJ. Development and treatment of retrograde peri-implantitis involving a site with a history of failed endodontic and apicoectomy procedures: a series of reports. Int J Oral Maxillofac Implants 2001; 16:412–417.
35. Sunde PT, Olsen I, Enersen M, Grinde B. Patient with severe periodontitis and subgingival Epstein–Barr virus treated with antiviral therapy. J Clin Virol 2008; 42:176–178.
36. Dal Canto AJ, Virgin HW 4th, Speck SH. Ongoing viral replication is required for gammaherpesvirus 68-induced vascular damage. J Virol 2000; 74:11304–11310.