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ORIGINAL ARTICLE

Salivary EBV DNA in periodontitis – analysis of clinicopathological and serological parameters

Karol Paradowski[1,A-B,D], Małgorzata Goździewska[2,D-F], Mirosław J. Jarosz[3,A-F], Małgorzata Polz-Dacewicz[1,A-F]

[1] *Department of Virology with Viral Diagnostics Laboratory, Medical University, Lublin, Poland*
[2] *Departament of Medical Anthropology, Institute of Rural Health, Lublin, Poland*
[3] *Faculty of Human Sciences, University of Economics and Innovation, Lublin, Poland*
A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

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Abstract
Introduction and Objective. Oral diseases affect about 3.5 billion people worldwide, with periodontitis being a major concern due to its potential to cause tooth loss. The Epstein–Barr virus (EBV), which infects most of the global population, is linked to several cancers and may contribute to periodontal disease. Growing evidence suggests a significant role of the EBV in the etiopathogenesis of periodontitis. The aim of the study was to assess the EBV load in saliva and crevice-gingival fluid (CGF) in patients with periodontitis. The study also took into account the clinical stage of periodontitis and the OHI.
Materials and Method. The study included a total of 60 patients (36 women and 24 men) treated for periodontitis at the Dental Centre in Lublin. The research used crevice-gingival fluid (CGF), saliva and sera. EBV DNA was detected by PCR in the CGF and saliva, and anti-EBV antibodies were detected in the serum (ELISA test).
Results. EBV DNA was detected in 39.5% of patients vs. 16.7% in healthy people. Both anti-EBNA and anti-EBVCA antibodies level was statistically significantly higher among patients with periodontitis compared to the control group. The obtained results encourage further in-depth research in this area.
Conclusions. In more advanced stages of periodontitis, higher levels of viral load and higher levels of anti-EBV antibodies were observed. Moreover, the higher the level of antibodies in the serum, the higher the level of EBV viremia was detected. Saliva is a good clinical diagnostic material for EBV in oral diseases.

Key words
periodontitis, saliva, EBV DNA, anti-EBV antibodies

INTRODUCTION

According to data from the World Health Organization (WHO), approximately 3.5 billion people – nearly half of the global population – suffer from oral diseases, including untreated dental caries, periodontal diseases, and oral cancers [1]. Among these, periodontitis is a significant public health concern. It is a chronic, multifactorial inflammatory disease that leads to the destruction of the tooth-supporting structures, formation of periodontal pockets, and gingival bleeding, ultimately resulting in tooth loss if left untreated.

The Epstein–Barr virus (EBV) is a widespread member of *Herpesviridae* family infecting more than 90% of the world human population. EBV infects both epithelial cells and B lymphocytes, establishing latency with periodically reactivation. It is also the first known human oncogenic virus. EBV has been associated with the development and progression of various cancers derived from B cells, e.g., Burkitt's lymphoma and Hodgkin's lymphoma, as well as with epithelial malignancies, such as gastric cancer and nasopharyngeal carcinoma (NPC) [2–5].

Growing evidence suggests a significant role of EBV in the etiopathogenesis of periodontitis [6, 7]. Therefore, the aim of our study was to assess the level of EBV viremia in saliva and crevice-gingival fluid (CGF) in patients with periodontitis. Comparison was also made between the serological status of the examined patients (prevalence and level of anti-EBV antibodies) in a group of healthy individuals. A possible relationship between salivary EBV load, anti-EBV antibody levels and the clinical stage and OHI index was also analysed.

Corresponding author: Małgorzata Goździewska, Departament of Medical Anthropology, Institute of Rural Health, Lublin, Jaczewskiego 2, 20-090 Lublin, Poland
E-mail: malgorzata.gozdziewska@gmail.com

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MATERIALS AND METHOD

Patients. First, a screening test was performed in a group of 152 patients diagnosed with periodontitis; tests were performed for the presence of EBV DNA in the gingival crevice fluid. The further study included 60 EBV-positive patients (36 women and 24 men) treated for periodontitis at the Dental Centre in Lublin. Patients in whom EBV DNA was detected in gingival fluid were qualified for the study. The control group consisted of 40 people, appropriately selected according to gender and age, treated in the same centre for other reasons (Tab. 1).

Clinical materials. During the dental examination, samples of crevice-gingival fluid (CGF) were collected from all patients with periodontitis using sterile paper points and placed in sterile sample tubes containing 1 mL of sterile phosphate buffered saline. Saliva and blood were also collected. Information on socio-demographic characteristics

Table 1. Baseline characteristics of patients and control group

Characteristics of study groups		Patients N = 60		Control group N = 40		p
		N	%	N	%	
Gender	Male	24	40.0	16	40.0	>0.9999
	Female	36	60.0	24	60.0	
Age	55–64	25	41.7	16	40.0	>0.9999
	65+	35	58.3	24	60.0	
Stage	I	9	15.0			
	II	13	21.7			
	III	17	28.3			
	IV	21	35.0			
OHI index	0	4	6.7			
	1	8	13.3			
	2	14	23.3			
	3	18	30.0			
	4	16	26.7			

and data on periodontal examinations were obtained from a medical history.

In response to the need for a more precise diagnostic framework, the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions, organized by the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP), introduced a new classification system [8, 9]. This classification replaced the previous division into chronic and aggressive periodontitis and introduced a two-dimensional system of staging (I–IV) and grading (A–C). The current study included two periodontal indicators – staging and OHI.

Staging – Extent and severity.

- *Stage I (Initial).* Interdental clinical attachment loss (CAL) of 1–2 mm, probing depth ≤4 mm, mostly horizontal bone loss.
- *Stage II (Moderate).* CAL of 3–4 mm, probing depth ≤5 mm, radiographic bone loss up to 15–33% of root length.
- *Stage III (Severe with potential for tooth loss).* CAL ≥5 mm, probing depths ≥6 mm, vertical bone loss ≥3 mm, furcation involvement, and potential tooth loss (up to 4 teeth).
- *Stage IV (Advanced with significant damage).* All features of Stage III, plus occlusal trauma, bite collapse, drifting, masticatory dysfunction, and potential for loss of ≥5 teeth.

In the assessment of periodontal status and oral hygiene, clinicians often rely on standardized indices to quantify the presence of plaque and calculus. Among these, the Oral Hygiene Index (OHI) is considered one of the most comprehensive tools used in dental practice, as it evaluates both dental plaque and calculus accumulation [10]. The OHI is composed of two parts: Debris Index (DI) – assessing the amount of soft plaque, and the Calculus Index (CI) – evaluating mineralized deposits (tartar). Each component is scored on a scale from 0–3, based on the extent of surface coverage:

- 0 – no plaque or calculus presen;
- 1 – supragingival plaque or calculus covers less than one-third of the tooth surface;
- 2 – supragingival plaque or calculus covers one-third to two-thirds of the surface, or isolated subgingival deposits are present;
- 3 – more than two-thirds of the surface is covered, or a heavy subgingival band of calculus is observed along the cervical margin.

To perform the assessment, the dentist may examine all teeth or limit the evaluation to six representative teeth: typically, two molars, two premolars, and two incisors from various quadrants. Each tooth is scored separately for debris and calculus. The final OHI score is obtained by adding both indices (DI + CI) for each tooth and dividing the total by the number of teeth examined, resulting in an average value between 0–6. This index is particularly useful in research and clinical monitoring of patients with periodontitis, as it provides a quantifiable measure of oral hygiene effectiveness and correlates well with disease severity.

CGF preparation. All tubes were vortexed for about 1 min and then centrifuged at 3,000 × *g* rpm for 20 min at room temperature. All supernatants were then collected and stored at -80 °C until further analysis.

Saliva Collection. About 5 mL of unstimulated whole saliva was collected. The saliva samples were centrifuged at 1,500 × *g* rpm at room temperature for 10 min and diluted (1:1) in PBS and frozen at –80 °C until analysis.

Serum collection. Both patients and controls venous blood samples were centrifuged at 1,500× *g* rpm for 15 min at room temperature; the serum was collected and frozen at –80 °C until analysis.

EBV DNA extraction and detection. The presence of EBV DNA was determined in GCF and saliva samples. DNA was extracted using the QIAampDNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A commercially available diagnostic kit was used to detect the EBV DNA (Gene Proof EBV virus, Brno, Czech Republic), in accordance with the manufacturer's protocol. All samples were analyzed in duplicate. A specific conserved DNA sequence for the EBV nuclear antigen 1 gene (EBNA-1) was amplified using Light Cycle 2.0 Software Version 4.1. (Roche Applied Science System).

Antibodies detection. Serum anti-EBNA and anti-EBVCA antibodies in IgG class were determined by the commercially available Microblot-Array test (TestLine Clinical Diagnostics Ltd., Brno, Czech Republic), according to the manufacturer's recommendations. Test results are presented in U/mL.

Ethical considerations. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee at the Medical University of Lublin (No. KE-0254/295/2019, 26 September 2019).

Statistical analysis. GraphPad Prism software version 10.4.0. (San Diego, CA, USA) was used to conduct data analysis. The normal distribution of continuous variables was checked using the Shapiro–Wilk test. The relationship between clinical and demographic parameters was calculated using the Pearson chi-square test. Pearson's chi-square test and

Fisher's exact test were used to compare the frequency of the examined clinicopathological features in both groups. The Mann–Whitney U test was used to compare differences in antibody levels between the study groups. Results were considered significant at $p \leq 0.05$.

RESULTS

EBV DNA was detected in 60/152 patients (39.5%). However, in the control group, EBV DNA was detected in 20/120 patients (16.7%). This difference is statistically significant ($p < 0.0001$). EBV positive patients were qualified for further analyses (60 individuals).

1) EBV load in saliva and CGF. EBV DNA was detected in both saliva and CGF of patients with periodontitis. Therefore, the level of viral load was compared in both types of clinical samples (Fig. 1). The viral load in CGF was higher than in saliva. However, the viral load in saliva was so high that this aspect was taken into account in further analysis.

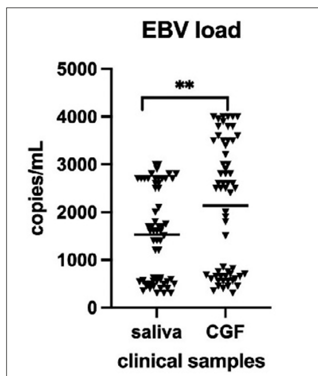

Figure 1. Comparison of EBV load in saliva and CGF samples collected from patients with periodontitis

EBV load was then analysed in relation to clinical stage and OHI (Fig. 2). A higher level of viral load was observed in patients with a more advanced clinical stage of periodontitis as well as a higher OHI.

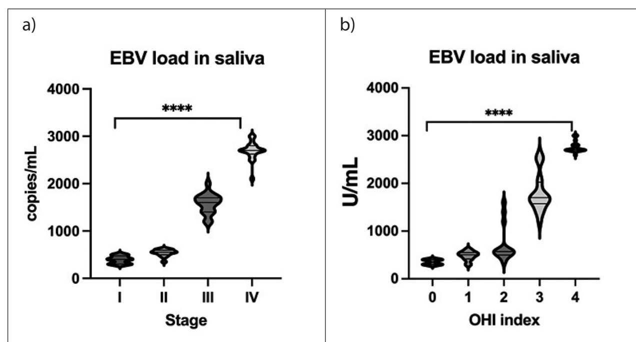

Figure 2. Assessment of the viral load in relation to the clinical condition (a) and OHI (b)

2) Evaluation of anti-EBV antibody levels in serum patients with periodontitis. The serological status of patients with periodontitis was also analyzed compared to the control group. It was found that anti-EBVCA IgG antibodies were detected statistically more often in the group of patients with periodontitis (Tab. 2).

Table 2. Prevalence of anti-EBNA IgG and anti-EBVCA IgG antibodies in periodontal patients compared to control group

Anti-EBV antibodies	Patients EBV+ N=60		Controls N=40		p
	N	*%*	*N*	*%*	
EBNA IgG	47	78.3	30	75.0	0.8093
EBVCA IgG	52	86.7	22	55.0	0.0009*

Chi squared test; * statistically significant

However, the level of IgG antibodies, both anti-EBNA and anti-EBVCA was statistically significantly higher among patients with periodontitis (Fig. 3).

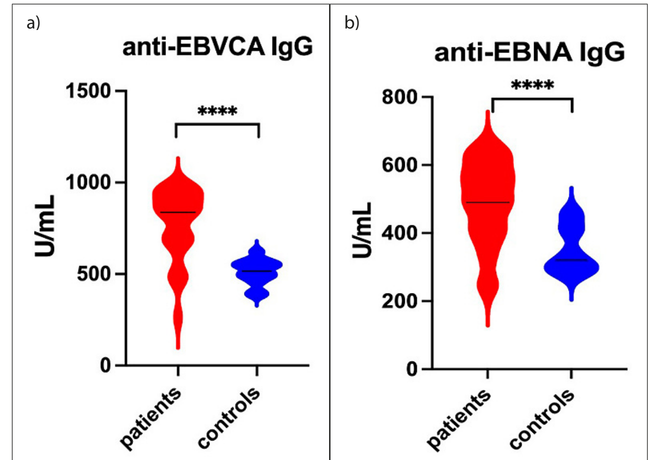

Figure 3. Comparison of anti-EBVCA IgG (a), anti-EBNA IgG (b) level in periodontitis patients and control group

In the next stage, the level of the above-mentioned antibodies was assessed in the context of stage of periodontitis and OHI index (Fig. 4). It was observed that only the level of anti-EBNA IgG antibodies was higher in more advanced stages of periodontitis and in people with a higher OHI.

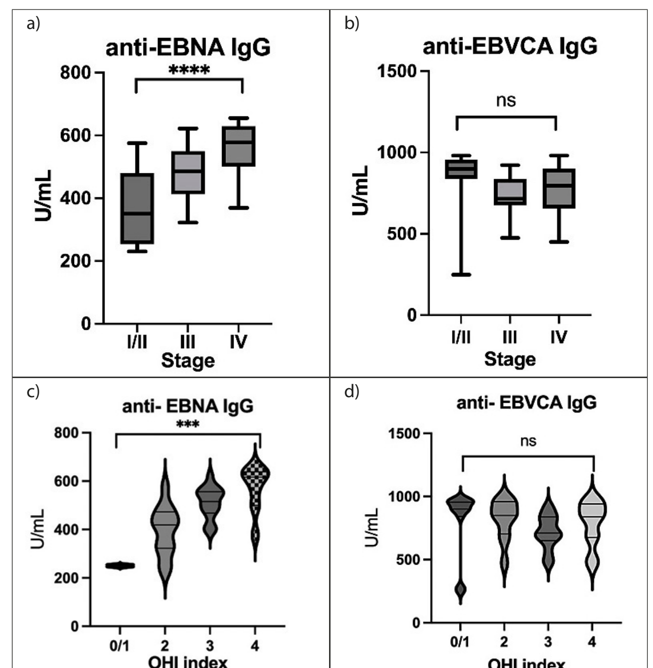

Figure 4. Anti-EBV antibodies level in relation to stage (a, b) and OHI (c, d)

In the last stage of this research, a possible correlation between EBV viremia in serum and the level of anti-EBNA IgG antibodies was checked (Fig. 5). It was observed that the higher the level of antibodies in the serum, the higher the level of EBV viremia was detected.

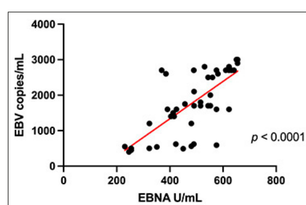

Figure 5. Correlation between salivary EBV load and serum level of EBNA IgG

DISCUSSION

The etiopathogenesis of periodontitis is a complex process in which both pathogens and host cell factors play a role. Initially, only bacteria such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were taken into account as the etiological factor of periodontitis. In the late 1990s, attention was paid to the role of viruses, especially herpes viruses, in the development of periodontal diseases [11]. This relationship was observed by researchers from different parts of the world, i.e. Asia, Europe and America, except Africa [12]. Some researchers have found the presence of EBV DNA in both saliva and gingival crevices fluid, subgingival plaque and periodontal pockets [13, 14, 15].

Observations in the current study are consistent with the results of other researchers. The percentage of patients with positive EBV was 39.5%, compared to 16,7% in control group. Different results, however, were presented by Tonoyan et al. [16], where the incidence of EBV was slightly higher in periodontitis tissues than in healthy control subjects (81.5% vs. 70.4%), and this difference was not statistically significant. These authors suggest frequent EBV infection even before clinical symptoms appear. The discrepancies in the frequency of EBV DNA detection by different researchers result from, among others, the size of the research group and/or the detection methods used.

EBV establishes a latent infection in affected host cells and reactivates in the head and neck epithelium, influencing the pathogenesis of EBV-related diseases. Very interesting research was carried out by Tonoyan et al., detecting the lytic form of the EBV virus in healthy tissues, and in periodontitis tissues – a mixture of latent and lytic genomes [16]. The main site of latent EBV infection is the periodontal epithelium, which may be important in persistent infection of healthy carriers and the subsequent development of periodontitis [17].

Moreover, EBV DNA was detected more often among patients with poor periodontal condition [18].

The detection of various antibodies has been used in the diagnosis of many diseases, including cancers associated with EBV. Therefore, the current study aimed to check the serological status of patients with periodontitis compared to the control group. Particular attention should be paid to EBNA1 which plays a dual role. On the one hand, it maintains latency, on the other hand, it plays a role in virus reactivation and lytic infection, and additionally responsible for virus replication. When reactivated in periodontal tissues, EBV can induce a strong pro-inflammatory response by activating the TLR9/MyD88/NF-κB pathway, leading to the increased production of pro-inflammatory cytokines, such as IL-1β and TNF-α, and chemokines, such as IL-8 and MCP-1 [19]. It is known that EBV has the ability to suppress host immunity, and it is possible that EBV is a causative agent for periodontitis, although the role of EBV in the development and progression of periodontitis has not yet been fully understood. Therefore, further research is necessary to elucidate the mechanisms in which EBV is involved.

Limitation of the study. Due to the fact that this is a pilot study, the main aim of which was to investigate whether EBV DNA is detected in periodontitis, comorbidities were not taken into account; therefore, the grading index was not analysed in this study. This will be the subject of further research.

No data was collected on bacterial infection because the focus of the study was only on EBV. However, it seems that understanding the interaction of EBV with bacteria would provide a more complete picture of the mechanism of periodontitis.

CONCLUSIONS

Both higher levels of viral load and higher levels of anti-EBV antibodies occurred in more advanced stages of periodontitis. Furthermore, the higher the serum antibody level, the higher the EBV viral load. Saliva may be a good material for clinical diagnosis of EBV in oral diseases.

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