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# EBV DNA load in the saliva of healthy people in south-eastern Poland

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## Abstract

**Introduction and Objective.** Epstein-Barr virus (EBV) is widespread in the world's population. Transmitted mainly through saliva, the EBV virus infects the epithelial cells of the oropharynx, where it can replicate, establishing latency in B lymphocytes. Many authors analyse EBV viremia in saliva as a possible biomarker of various diseases. The aim of the study is to analyse the frequency and level of EBV viremia in the saliva of a healthy population of children and adults. The level of anti-EBV antibodies and a possible correlation between EBV viral load in saliva and antibody titres in serum were also examined.

**Materials and Method.** The study involved 66 healthy people, including 22 children aged 5–10 and 44 adults. EBV DNA was isolated from the saliva of all patients, and the presence and level of EBV DNA was detected using the qPCR method. Anti-EBV antibodies in serum were detected by ELISA.

**Results.** The higher percentage of positive results was detected in children aged 5–10 (81.8%) and a smaller percentage in adults aged 20–49 (75.0%), this difference was not statistically significant. EBV DNA was most often detected in adults aged 50–70 (85.7%). The mean level of EBVCA IgG antibodies was similar in both age groups. Similarly, no difference in EBNA-1 antibody titers was observed between younger and older groups of adult patients. In three healthy people, despite the presence of EBV DNA in saliva, no anti-EBVCA or EBNA-1 antibodies were detected.

**Conclusions.** The prevalence of EBV DNA in the healthy population ranged from 75% – 85.7%. Additionally, no correlation was observed between the EBV load in saliva and the titer of tested antibodies in the serum of healthy people.

## Key words

saliva, healthy people, anti-EBV antibodies, EBV DNA load

## INTRODUCTION

Epstein–Barr virus (EBV), classified as *Lymphocryptovirus humangamma4* according to the new ICTV 2025 taxonomy, is a ubiquitous virus infecting more than 90–95% of the global adult population [1, 2]. Primary infection typically occurs in early childhood in developing regions, whereas in industrialized countries seroconversion is often delayed until adolescence or early adulthood [3]. The timing of primary infection is influenced by socio-economic conditions, population density, and hygiene standards. Despite the usually asymptomatic course of early-life infection, EBV establishes lifelong persistence within the host.

Following primary infection, EBV establishes latency predominantly in memory B lymphocytes. A hallmark of EBV biology is its ability to alternate between latent and lytic replication programs [1, 3, 4]. During lytic reactivation, viral replication occurs in epithelial cells of the oropharynx, leading to the release of infectious virions into saliva. This phenomenon, referred to as salivary shedding, occurs intermittently and may be detected even in immunocompetent and clinically healthy individuals [4, 5]. The frequency and magnitude of EBV DNA detection in saliva show considerable interindividual variability, suggesting complex regulation of viral reactivation and immune control.

Saliva has emerged as a particularly valuable non-invasive biological specimen for EBV detection. Advances in quantitative polymerase chain reaction (qPCR) techniques have enabled highly sensitive and specific quantification of EBV DNA in oral samples [6]. The use of saliva is especially advantageous in paediatric populations, where venipuncture may be challenging due to procedural, ethical, or compliance-related considerations [6]. However, the biological interpretation of EBV DNA detection in saliva remains complex. The presence of viral DNA does not necessarily indicate systemic viraemia, nor does it invariably correlate with serological markers of immune response [7]. Serological diagnostics of EBV infection rely primarily on antibodies directed against viral capsid antigen (VCA), early antigen (EA), and Epstein–Barr nuclear antigen (EBNA). These antibody profiles allow differentiation between primary infection, past infection, and reactivation states [8]. Nevertheless, antibody titers may remain stable for years following primary infection and do not consistently reflect current lytic activity in epithelial tissues. Several studies have reported a lack of significant association between serum EBV antibody levels and the quantity of EBV DNA detected in saliva among immunocompetent individuals [7]. These findings suggest that humoral immune status and

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local viral shedding may represent partially independent biological processes.

From an epidemiological perspective, age-related differences in EBV infection dynamics are of particular interest. Seroprevalence studies demonstrate progressive increases in EBV seropositivity with age, with lower prevalence observed in early childhood compared to adulthood [2]. However, even among seropositive and clinically healthy individuals, intermittent episodes of high-level salivary viral shedding have been documented [4, 5]. The determinants of such variability remain incompletely understood and may involve host immune regulation, viral latency programs, and environmental cofactors.

Understanding the relationship between systemic humoral immunity and local viral replication has important clinical and translational implications. EBV has been implicated in the pathogenesis of multiple autoimmune diseases and malignancies, including multiple sclerosis, nasopharyngeal carcinoma, and certain lymphoproliferative disorders [1, 3]. Therefore, clarification of whether salivary EBV DNA levels correlate with serum antibody titers in healthy individuals may improve interpretation of molecular and serological findings in both research and clinical settings.

The main aim of the study was to assess the prevalence and level of EBV DNA load in the saliva of a healthy population of children and adults in south-eastern Poland. Additionally, the potential correlation between EBV load in saliva and the level of anti-EBV antibodies in serum was analyzed.

## MATERIALS AND METHOD

**Patients.** A total of 66 healthy people took part in the study, including 22 children aged 5–10 years and 44 adults divided into two age groups, i.e. 16 people aged 20–29 and 28 aged 50–70 (Table1).

**Table 1.** Structure of the study group according to gender and age

| Adults; *N=44* | EBV + | EBV - | Total |
|---|---|---|---|
| | N % | N % | N % |
| Age | | | |
| 20–49 | 12 75.0 | 4 25.0 | 16 36.4 |
| 50–70 | 24 85.7 | 4 14.3 | 28 63.6 |
| Gender | | | |
| Female | 20 55.5 | 4 50.0 | 24 54.5 |
| Male | 16 45.5 | 4 50.0 | 20 45.5 |
| Children; *N = 22* | | | |
| Age 5–10 | 18 81.8 | 4 18.2 | 22 100.0 |
| Gender | | | |
| Female | 14 77.8 | 2 50.0 | 16 72.7 |
| Male | 4 22.2 | 2 50.0 | 6 27.3 |

Saliva was collected from patients who agreed to participate in the study during a preventive dental examination at the Dental Centre in Lublin. Inclusion criteria for the study were: absence of head and neck cancer or pre-cancerous lesions, absence of severe systemic diseases or infections, and good general health. All patients were divided according to age and gender.

**Clinical materials – saliva collection.** To detect EBV DNA, saliva samples were collected from both children and adults. However, blood for testing the presence and level of antibodies was collected only from adults. About 5 mL of unstimulated whole saliva was collected. All subjects were advised not to eat or drink anything 30 minutes before sample collection. Each patient was then instructed to rinse their mouth with 10 mL of physiological saline (0.9% NaCl). After brief instructions from the nurse, all patients collected saliva themselves by spitting into a sterile tube. 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 DNA extraction. The saliva was thawed and centrifuged (3,000 × g, 10 min, 4 °C) to remove cellular debris and other impurities.

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

**EBV DNA extraction and detection.** DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After isolation, the purity and yield of the obtained eluate were assessed using an Epoch spectrophotometer (Biotek Instruments, Winooski, VT, USA). The measurement was performed on a Take 3 plate (Biotek Instruments, Winooski, VT, USA) enabling measurement in microvolumes (2 µL). A Microplate Reader Software Gen 5.2.0 (Biotek Instruments, Winooski, VT, USA) was used to analyze the results. The isolates were stored at –20 °C until the test was performed. In order to check the quality of the obtained DNA (presence of polymerase chain reaction inhibitors), a test for β-globin was performed.

EBV DNA was detected using a commercially available diagnostic kit [Gene Proof EBV virus (Brno, Czech Republic)] according to the manufacturer's instruction. All samples were analysed twice. The specific conserved DNA sequence for the EBV nuclear antigen 1 (EBNA-1) gene was amplified using Light Cycle 2.0 software, version 4.1. (Roche Applied Science System). Results were normalized for DNA isolation efficiency; the detection limit in saliva was 100 EBV DNA copies/mL.

**Antibodies detection.** The presence and titer of anti-EBNA-1 IgG and anti-EBVCA IgG antibodies were determined in serum using the Microblot-Array kit (Test Line Clinical Diagnostics Ltd. Brno, Czech Republic) in accordance with the manufacturer's protocol. 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 Medical University of Lublin Ethics Committee (No. KE-0254/295/2019, 26 September 2019).

**Statistical analysis.** The obtained results were subjected to statistical analysis using Tibco Statistica 13.3 (StatSoft, Kraków, Poland) and GraphPad Prism software version 10.4.0. (San Diego, CA, USA). The Shapiro–Wilk test was used to check the normal distribution of continuous variables. The Mann-Whitney and Kruskal Walis tests were used to compare the EBV DNA load as well as the antibody levels between groups. The correlation between salivary EBV DNA load and anti-EBV antibodies was assessed using the Spearman rank test.

## RESULTS

In the first stage, the frequency of EBV DNA detection in the saliva of the examined patients was assessed (Fig. 1). Although the higher percentage of positive results was detected in children aged 5–10 (81.8%) and a smaller percentage in adults aged 20–49 (75.0%), this difference was not statistically significant (p = 0.6047). EBV DNA was most often detected in adults aged 50–70 (85.7%).

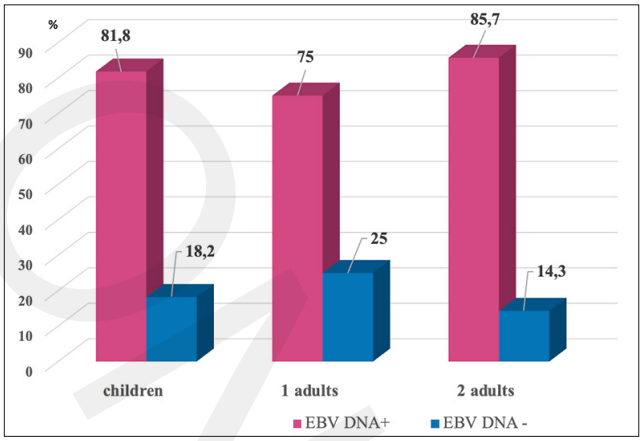


**Figure 1.** Prevalence of EBV DNA in saliva of healthy people by age (children: 5–10; 1 adults: 20–49; 2 adults: 50–70)

The EBV DNA load level was analyzed in all three study groups (Fig. 2). There was no statistically significant difference in viral load between the study groups (p = 0.9002). In the saliva of children, the average level of EBV DNA load was 777.2 copies/mL, in adults aged 20–49 years – 746.7 copies/mL, while the highest level was in older people aged 50–70 years – 863.1 copies/mL. Moreover, a much higher viral load was observed in three people, i.e. 1,100 – 2,100 copies/mL.

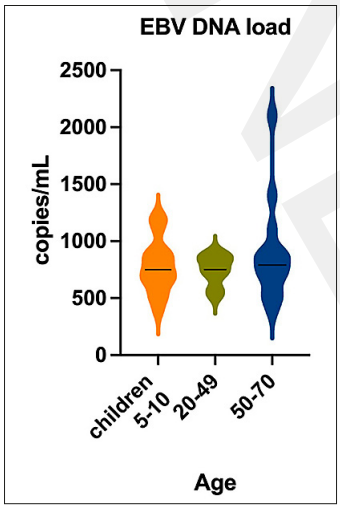


**Figure 2.** EBV DNA load in saliva in healthy individuals p=0.9002

In the next stage, an attempt was made to check whether there is a correlation between the level of viral load in saliva and the titer of antibodies in the serum. At this stage, only a group of adult patients was analyzed because there were difficulties in collecting blood from children. The titer of EBVCA and EBNA-1 IgG antibodies is graphically presented in Figure 3.

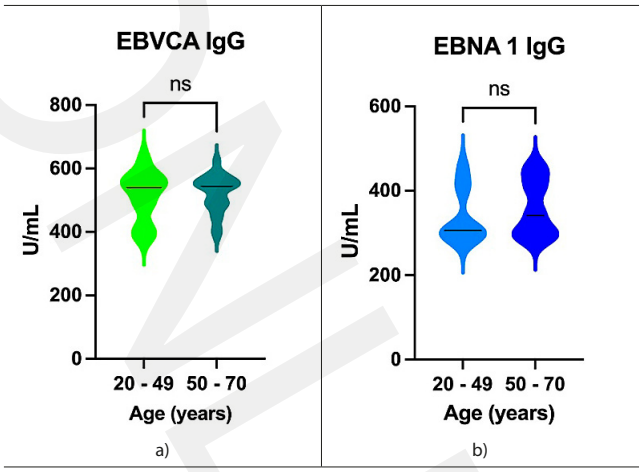


**Figure 3.** The level of anti-EBVCA IgG and EBNA 1 IgG antibodies in healthy adults

The average level of EBVCA IgG antibodies was similar in both age groups (p=0.8838) and amounted to 513.0 and 522.0 U/mL, respectively. Similarly, no difference was observed in the level of EBNA-1 IgG antibodies between the younger and older groups of adult patients (p= 0.4564), which was respectively 336.9 and 357.3U/mL. In three healthy individuals, despite the presence of EBV DNA in saliva, no anti-EBVCA or EBNA-1 IgG antibodies were detected.

Moreover, the analysis showed no correlation between EBV load in saliva and the titer of tested antibodies in the serum of healthy people (Tab. 2).

**Table 2.** Correlation between salivary EBV DNA load and serum level of anti-EBV antibodies

| Parameter | Spearman r | 95% CI | p value |
|---|---|---|---|
| EBVCA IgG | -0.086 | -0.5197 – 0.3825 | 0.718 |
| EBNA 1 IgG | 0.334 | -0.1892 – 0.7099 | 0.188 |

## DISCUSSION

Transmitted primarily through saliva, EBV infects oropharyngeal epithelial cells, where it can replicate and then infect B cells, establishing latency during which several viral proteins are expressed [9].

In the available scientific literature, there are few studies on the prevalence and the level of EBV viraemia in a healthy population. Most often, healthy people are selected as a control group when examining various EBV-related diseases.

Salivary EBV viral load has been studied as biomarkers of various diseases, including lymphoproliferative diseases [10], periodontitis [11], human immunodeficiency virus (HIV) [12], nasopharyngeal carcinoma (NPC) [13, 14] and other EBV-related cancers.

Ikuta et al. [15] analysed the prevalence of EBV in saliva and throat washings from healthy people in Japan. EBV DNA was detected in 90% of throat wash samples from healthy adults (aged 21–57 years) and in 38% of saliva samples from healthy children (aged 0–6 years). The percentage of EBV DNA in saliva was higher in older children. Additionally, EBV DNA was detected in throat washes from two healthy

adults who did not have antibodies to the EBV. In turn, Gieß et al. [7] detected EBVDNA in 78% of healthy individuals and the average viral load was 28370 copies/mL. However, quite significant numbers of EBV DNA copies can be detected in some people (maximum 1,506,000 copies/mL).

In the current study, EBV DNA was most often detected in adults aged 50–70 (85.7%) with the highest viral load 863.1 copies/mL. However, in children aged 5–10 years, 81.8% samples were positive. In the saliva of children, the average level of EBV DNA load was 777.2 copies/mL. Oral A. et al. [16], analysing the presence of EBV DNA and the level of viral load among patients with oral lichen planus (OLP), lichenoid contact lesions (OLCL) compared to the control group, did not observe any significant differences. However, the level of EBV viraemia varied depending on the type of clinical sample (biopsy – 46 copies/mL; saliva – 19 copies/mL). Zheng et al. [17] assessed the EBV content in the saliva of people at high risk of nasopharyngeal cancer compared to a control group of healthy people. EBV DNA was detected in 84.2% in controls, and the EBV viral load was 394,739 copies/mL. According to the authors, saliva testing for EBV viral load and DNA methylation is a useful triage tool after screening for anti-EBV antibodies in serum. The ability to self-collect samples at home further increases its usefulness in the diagnosis of people at high risk of NPC.

The prevalence of EBV antibodies varies and depends on the geographic region, ethnicity and socio-economic status of a given population [18, 19]. In a study conducted at the Charité-Universitätsmedizin in Berlin, Germany, anti-EBVCA antibodies were detected in 95% and anti-EBNA antibodies in 73% of healthy adults [7].

Studies conducted in the USA have shown that the incidence of IgG antibodies against EBV varies in different age groups. The highest percentage of EBV IgG antibodies was observed in people aged 18–19 years (89%), while the lowest in the age group 6–8 years (50%) [20]. According to Dowd [21], the overall EBV seroprevalence was 66.5%, increasing with age from 54.1% for children aged 6–8 years to 82.9% for those aged 18–19 years. In turn, in the study by Levine et al. [22] the overall seroprevalence rate for EBV was 87% among people aged 17–26.1 years. However, Condon's [23] results showed that the percentage of anti-EBV IgG antibodies in children aged 18 months to 19 years ranged from 26–74%.

In current study, due to procedural limitations related to blood collection from children, antibody analysis was performed only in the adult cohort. The mean level of EBVCA IgG antibodies was similar in both age groups. Similarly, no difference in EBNA-1 antibody titers was observed between younger and older groups of adult patients. In three healthy people, despite the presence of EBV DNA in saliva, no anti-EBVCA or EBNA-1 antibodies were detected. Moreover, no correlation was observed between the EBV virus load in saliva and the titer of tested antibodies in the serum of healthy people.

Low titers of antibodies against Epstein-Barr virus nuclear antigen (EBNA-1) are associated with a reduced risk of diseases associated with Epstein-Barr virus [24]. Researchers have generally not found a strong correlation between the frequency of salivary virus shedding or salivary viral load and the level of EBV VCA-IgG or EBNA-1 antibodies in healthy individuals. Mahler et al. [24] compared virus shedding and antibody diversity in healthy seropositive individuals. They hypothesized that individuals with extremely low levels of anti-EBNA-1 constitute a biologically distinct subgroup because no EBV DNA was detected in their saliva. Therefore, the authors suggest the existence of a distinct non-spreader phenotype that inhibits viral replication, despite the presence of latent virus. A similar situation was observed in the current study, where very low levels of anti-EBNA-1 antibodies were found in two adults with EBV DNA detected in saliva.

A recent study by the authors of the presented study showed high EBV DNA viraemia in advanced clinical stages of EBV-related OPSCC [25]. Therefore, it seems that EBV DNA testing combined with the assessment of the level of anti-EBV antibodies and other biomarkers may be a useful tool in the diagnosis and prognosis of EBV-related diseases.

Although more than 50 years have passed since the discovery of the EBV virus in 1964, knowledge about it has developed significantly, but many issues remain unresolved. EBV is a human oncogenic virus etiologically associated with many diseases, ranging from mild infectious diseases to EBV-related cancers. More than 250,000 cases of cancer are associated with EBV each year, and approximately 2% of all cancer deaths are due to malignancies attributed to EBV. Although EBV is widespread in the human population, only some people develop diseases or cancers etiologically related to this virus. Primary EBV infection is often subclinical, but persistent EBV infection may cause immune system dysregulation and a variety of complications. The factors that determine EBV persistence after primary infection are not fully understood, although human genetic variability may play a role. Nyeo et al. [26] demonstrated that whole-genome sequencing of human populations could be used to quantify persistent EBV DNA. Single-cell and pathway analyses indicate that variable antigen processing is a major determinant of EBV DNA persistence.

Therefore, further research is necessary on the role of EBV in the pathogenesis of cancer, as well as on the explanation of the interactions between the EBV virus and the infected cell, and between EBV and the immune system.

**Limitations of our study.** The authors are aware of certain limitations of their own research resulting from the too small study group, Moreover, we were unable and the inability to collect blood from the children due to lack of parental consent. Nevertheless, it is believed that the study can be considered as preliminary to further in-depth research on a larger population.

## CONCLUSIONS

The prevalence of EBV DNA in saliva ranged from 75%-85%. It was highest in adults aged 50–70, but this difference was not statistically significant. EBV DNA viral load levels were similar in children and adults. However, in adults aged 50–70 years, a high viral load was observed in several cases.

Both anti-EBVCA IgG and EBNA-1 IgG antibody levels were similar in younger and older adults. There was no correlation between the level of viral load in saliva and the titre of the tested antibodies in serum. Taking into account the lack of correlation between viral load and antibody titres in healthy individuals, it seems that EBV DNA testing combined with the assessment of the level of anti-EBV antibodies, as well as other biomarkers, may be a useful tool in the diagnosis and prognosis of EBV-related diseases.

In two adults in whom EBV DNA was detected in their saliva, very low levels of anti-EBNA-1 antibodies were found, which may suggest the existence of a distinct non-spreader phenotype that inhibits viral replication despite the presence of latent virus.

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