Brief Report

Epstein-Barr Virus Salivary Shedding in Patients With Acute Infectious Diseases – A Pilot Study

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Epstein-Barr Virus Salivary Shedding in Patients with Acute Infectious Diseases—A Pilot Study

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Abstract: Epstein-Barr virus (EBV) is a widely disseminated herpesvirus for which antibodies have been demonstrated in over 90% of adults worldwide. After subclinical primary EBV infections, as well as after infectious mononucleosis, the virus can be shed in saliva for a prolonged period of time. Diseases and disorders that can induce EBV salivary shedding include mental disorders and sex, connective tissue disease, multiple sclerosis, systemic lupus erythematosus, malaria and HIV infection. As the occurrence of EBV in saliva during acute infectious diseases has not been systematically researched so far, this pilot cross-sectional study aimed to investigate the possible relationship between acute infectious diseases and salivary shedding of EBV. A total of 40 patients with acute infectious diseases was enrolled, along with 41 adults free of acute infections. Peripheral venous blood samples for serodiagnosis and saliva samples for EBV PCR testing were collected from both groups. The most common acute infectious disease was COVID-19 pneumonia, followed by haemorrhagic fever with renal syndrome. Crude proportions of people with positive serological test results and those with saliva viral shedding were similar in the two groups. The presented preliminary data does not indicate acute infectious conditions as a marked "contributor" in increasing salivary EBV shedding.

Keywords: Epstein-Barr virus; acute infectious diseases; salivary shedding

1. Introduction

Epstein-Barr virus (EBV) is a widely disseminated herpesvirus that is spread by intimate contact between asymptomatic EBV shedders and susceptible persons. Antibodies to EBV have been demonstrated in over 90 percent of adults worldwide [1,2]. Most primary EBV infections that occur during childhood are subclinical, but infections in adolescents and adults frequently result in infectious mononucleosis (IM) which is the most common clinical presentation of EBV infection [1,3]. A typical clinical presentation of IM includes fever, pharyngitis, adenopathy, fatigue, and atypical lymphocytosis [1,3,4].

EBV has primary tropism for the type B lymphocytes. The hallmark of B lymphocyte EBV infections is the establishment of latency that results in a lifelong infection that the immune system cannot clear [1,3].

Following IM, the virus can be shed in saliva at high levels for a prolonged period of time, [5,6]. It is very important to emphasize the fact that the virus may be intermittently shed in the oropharynx for decades [5,7] and can be present in saliva and throat washings from healthy people [8].

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EBV reactivates under conditions of cellular immune response impairment, which is important in the long-term control and suppression of the replication of persistent and asymptomatic EBV in a healthy person [9]. EBV reactivations documented by serological tests can be caused by psychological stress [10] such as student examination stress [11,12], marital stress [13], attachment anxiety [14], loneliness [15], autoimmune diseases [16], chronic fatigue syndrome [17] and COVID-19 [18,19].

EBV reactivation identified by PCR testing in plasma has also been documented in patients in intensive care units [20,21], in patients with COVID-19 [22–24], long COVID-19 [19], malaria [25] and HIV [26,27].

Diseases and disorders that can induce shedding of EBV in saliva are also being investigated, but not as extensively. EBV salivary shedding is connected to psychological stress [10], mental disorders and sex [28], connective tissue diseases [29], multiple sclerosis [30], systemic lupus erythematosus [31], malaria [25] and HIV infection [26,27,32–35].

As the occurrence of EBV in saliva during acute infectious diseases (with the exception of HIV and malaria) has not been systematically researched so far, this pilot cross-sectional study aimed to investigate the possible relationship between acute infectious diseases and salivary shedding of EBV.

2. Patients and Methods

2.1. General Design and Ethics

This pilot cross-sectional study was conducted at the University Hospital for Infectious Diseases "Dr. Fran Mihaljević" Zagreb, Croatia, between September 2020 and December 2021, and was approved by the institutional Ethics Committee. Consenting adults hospitalized for acute infectious conditions and their peers free of acute infectious diseases provided blood and saliva samples for serological and virological tests related to EBV.

2.2. Subjects

Patients hospitalized for acute infectious diseases were eligible for inclusion during the first three days of hospitalization while febrile. All participants were 18 years of age or older at the time of the study and had provided a written informed consent. Patients with infectious mononucleosis, HIV infection, suspected but not confirmed infectious diseases and patients with fever of unknown origin or of non-infectious genesis were not included. Their peers free of acute infectious conditions were recruited on a voluntary basis among Hospital staff and students and were eligible if they were adults, free of any acute infectious condition within 3 months prior to being included in the study and had provided a written informed consent.

Hospitalized patients were managed in line with the standard procedures regarding their respective diagnoses, except for the provision of additional blood and saliva samples. The same was obtained from "healthy" control subjects, together with detailed medical histories.

Peripheral venous blood samples (5-10 mL) for serodiagnosis were collected into Vacutainer tubes. Sera were stored at -20°C until testing.

Unstimulated whole saliva samples (3 ml) for PCR testing were collected into sterile plastic containers before, or two hours after a meal. Samples were frozen at -72°C until assaying.

2.3. EBV Serological Diagnosis

EBV serostatus was defined by the presence of IgM and IgG antibodies against EBV viral capsid antigen (VCA), IgG against early antigen-diffuse (EA-(D)) and IgG against EBV nuclear antigen (EBNA) which were simultaneously tested using chemiluminescent immunoassay ((CLIA), DiaSorin, Saluggia, Italy). The tests were performed according to the manufacturer's instructions.

Acute infection was characterized by IgM anti-VCA and anti-EA(D) IgG without antibodies against EBNA. The presence of anti-EBNA IgG and anti-VCA IgG antibodies was interpreted as past infection. EBV reactivation was assumed when the level of anti-EA(D) IgG was high, and anti-VCA and anti-EBNA IgG were positive, and viremia or further increase of specific antibodies was performed for confirmation.

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2.4. EBV DNA Quantification

DNA was extracted from 200 µl of saliva using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Quantification of EBV DNA was performed by using RealStar® EBV PCR Kit 2.0 (CE-IVD, Altona Diagnostics, Hamburg, Germany) following the standard manufacturer's instructions (reaction volume 30 µl) by using LightCycler®480 System PCR Instrument (Roche Diagnostics, Mannheim, Germany). The kit contains reagents required for the PCR set up, internal control and four quantification standards (ranging from 10^1 to 10^4 IU/µl) that were calibrated against the 1st WHO International Standard for Epstein-Barr Virus for Nucleic Acid Amplification Techniques (NAT) (NIBSC code: 09/260). The assay utilizes a highly conserved (non-EBNA-2) primer binding site. The probes specific for EBV DNA are labelled with a fluorophore FAM whereas the probe specific for the internal control is labelled with the fluorophore JOE. The temperature time profile of the real-time PCR reaction includes a denaturation (stage hold, 1 cycle repeat, 90°C, 10 min) and amplification [(stage cycling, a total of 45 cycles, no acquisition (95°C, 15s) and acquisition (58°C, 1 min.)]. Analytical sensitivity of RealStar®EBV PCR Kit 2.0 is 1.59 IU/µl eluate [95% confidence interval (CI): 1.04 IU/µl to 3.37 IU/µl] with a linear range of 1.00E+08 IU/µl to 1.00E+01 IU/µl.

2.5. Data Analysis

Data are summarized for patients with acute infectious diseases and "healthy" controls. Serological test results are summarized as proportions of subjects with positive findings. Virological test results are summarized as proportions of subjects with viral presence in saliva and as numbers of viral copies in those with positive findings. We fitted logit and general linear models to proportions and to ln (viral copy counts) to generate adjusted proportions and geometric mean values in the two groups of subjects. We used SAS for Windows 9.4 (SAS Inc., Cary, NJ).

3. Results

A total of 40 patients with acute infectious diseases and 41 adults free of acute infections were enrolled in the study (Table 1). The former were somewhat older than the latter, while the prevalence of men, body mass index, prevalence of mild or heavy smokers and alcohol consumption habits (only sporadic) were similar in the two groups (Table 1). Hospitalized patients were somewhat more commonly diabetic and more commonly suffered from hypertension and other cardiovascular morbidity than the control subjects, while other background comorbidities were comparably rare (Table 1). The most common acute infectious disease was COVID-19 pneumonia (10/40 patients), followed by haemorrhagic fever with renal syndrome (6/40 patients) (Table 1). Crude proportions of people with positive serological test results and those with saliva viral shedding were similar in the two groups (Table 1). Age, sex and body mass index-adjusted proportions were also closely comparable in patients with acute infectious diseases and their peers free of acute infections (Table 2).

Table 1. Summary of subject characteristics: people with acute infectious conditions requiring hospitalization and people free of acute infections over the past 3 months. Data are mean±SD (range), count (%) and geometric mean (range) for the number of viral copies.

	Acute infection	**No infection**
N	40	41
Age (years)	56±16 (20-86)	42±16 (19-75)
Men	16 (40.0)	16 (39.0)
BMI (kg/m^2)	27.4±4.7 (19-41)	24.9±4.4 (18-37.1)
Non-smokers[1]	29 (72.5)	27 (65.9)
Mild smokers[1]	7 (17.5)	9 (22.0)
Heavy smokers[1]	4 (10.0)	5 (12.1)
Alcohol consumption[2]	7 (17.5)	11 (26.8)
Diabetic	9 (22.5)	1 (2.4)
Hypertension	18 (45.0)	6 (14.6)

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Other cardiovascular morbidity[3]	7 (17.5)	0
COPD/asthma	3 (7.5)	2 (4.9)
Gastrointestinal[4]	3 (7.5)	3 (7.3)
Immunocompromised[5]	4 (10.0)	3 (7.3)
Acute infectious diseases		
COVID-19 pneumonia (bilateral)	10 (25.0)	---
Haemorrhagic fever with renal syndrome	6 (15.0)	---
Legionella pneumonia	5 (12.5)	---
Pyelonephritis (*E. coli*)	4 (10.0)	---
Herpes zoster	3 (7.5)	---
Gram-negative sepsis (unknown infection site)	2 (5.0)	---
Meningitis (*Listeria*)	2 (5.0)	---
Other pneumonia	2 (5.0)	---
Salmonellosis	2 (5.0)	---
Various other (one case each)	4 (10.0)	---
Septic	6 (15.0)	---
VCA IgM positive	3 (7.5)	0
VCA IgG positive	38 (95.0)	36 (87.8)
EA IgG positive	4 (10.0)	2 (4.9)
EBNA IgG positive	36 (90.0)	34 (82.9)
Meet criteria of virus reactivation	4 (10.0)	2 (4.9)
Virus in saliva	12 (30.0)	10 (24.4)
Number of viral copies (if positive) x10^3	85.3 (2.3-4610)	16.3 (1-217)

[1] "Mild smokers"—<20 cigarettes a day; "heavy smokers"—≥20 cigarettes a day. [2] All declared only "sporadic/occasional" consumption of alcoholic beverages. [3] Includes cardiac arrhythmia history of occlusive cardio- or cerebrovascular incidents. [4] Practically exclusively peptic disease. [5] Suffered malignant or chronic autoimmune diseases or were substance addicted. COPD – chronic obstructive pulmonary disease; EA – early antigen; EBNA – Epstein Barr nuclear antigen, VCA – viral capsid antigen.

Table 2. Adjusted (for age, sex and body mass index) proportions of subjects with positive serological tests/viral presence in saliva and geometric means (95%CI) for the number of viral copies in patients with a positive viral detection in saliva, for patients with acute infection requiring hospitalization and people without acute infectious conditions.

	Acute Infection	**No infection**
N	40	41
VCA IgM positive	0	0
VCA IgG positive	98.2%	99.1%
EA IgG positive	1.7%	0.8%
EBNA IgG positive	91.0%	93.8%
Meet criteria of virus reactivation	1.7%	0.8%
Virus in saliva	29.1%	26.3%
Copies (if positive) x10^3	62.5 (12.4-315)	24.5 (4.0-152)

EA – early antigen; EBNA – Epstein Barr nuclear antigen, VCA – viral capsid antigen.

4. Discussion

EBV infection is very common in humans which is why the virus has been extensively researched [1,2]. It is known that it can occasionally appear in the blood during some diseases and conditions such as psychological stress [10], in patients in intensive care units [20,21], in patients with COVID-19 [22–24] and in patients with long COVID-19 [19]. In contrast, the occurrence of EBV in saliva has been less investigated. For this reason, it seems of interest to try to identify the diseases and conditions that lead to EBV shedding in saliva. Such findings would only help to further our understanding of the virus and its interaction with the human body.

Certain infections such as malaria [25] and HIV infection [26,27,32–35] have been associated with EBV occurrence in saliva.

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The study of Miller et al. [35] reported higher prevalence of EBV in saliva of HIV positive (90%) than in HIV negative group (48%) with significantly higher EBV viral loads in HIV- seropositive patients than in HIV-seronegative persons.

Scaggiante et al. [26] reported that the incidence of EBV in saliva in HIV-positive MSM with successful HIV viremia control was comparable to that in patients with unsuccessful HIV viremia control. But, patients with active plasma HIV replication had a significantly higher frequency of high viral load of EBV in saliva. When comparing EBV salivary shedding in HIV-positive MSM with controlled and those with uncontrolled plasma HIV viremia in the study of Basso et al. [32], EBV was proven to be present in both groups with a higher viral EBV load in HIV-viremic patients.

A similar observation was published by Byrne et al. [23]. In this study, HIV-infected patients had an increased risk of EBV presence in saliva and higher viral loads when compared with people not infected with HIV.

Agudelo-Hernandez et al. [27] investigated herpesvirus shedding in HIV-infected men in blood, semen, throat washings, urine and stool in comparison with HIV seronegative MSM. HIV-positive patients had significantly higher EBV shedding rates, and EBV was detected in the throat washes of all HIV-positive patients. Among all tested body compartments, the highest number of EBV shedding episodes was detected in throat wash.

An interesting observation was published by Donati et al. [25] concerning EBV DNA loads in the plasma and saliva of Ugandan children with acute malaria before and after antimalaria treatment. Plasma levels were higher in children with malaria than in those without malaria, but there were no significant differences in EBV DNA in saliva between these two groups. In the majority of cases, antimalaria treatment led to the clearance of plasma EBV DNA, but it did not affect the levels in saliva.

Considering these facts, it could be expected that other acute infectious diseases might also be associated with the occurrence of EBV in saliva. However, since infectious diseases are diverse in their aetiology (viruses, bacteria, parasites, fungi), they stimulate different types of immune responses in the human body, resulting in clinical presentations of varying severity.

Therefore, we did not initially select one infectious disease or group of diseases to investigate, rather we wanted to include patients with varying acute infectious conditions in order to "screen" the possibility that acute infections with moderate-severe clinical presentation might be associated with increased salivary EBV shedding. The present preliminary data are limited by their cross-sectional nature (and, hence, no insight into the possible dynamics of viral shedding during and after resolution of acute infections) and a rather limited sample size. Also, we did not screen the subjects for their level of stress at the time of sample collection although it may be a factor that could affect EBV shedding in saliva. But it is reasonable to assume that some level of stress is unavoidable in patients suffering from acute conditions, especially in hospitalized ones, regardless of how benign it might be. It seems almost impossible to separate the element of stress caused by the entire condition from the stress caused by an acute infectious disease.

Taking all limitations into account, it seems fair to state that data do not indicate acute infectious conditions (in general) as a marked "contributor" to increased salivary EBV shedding.

5. Conclusions

The aim of this study was to correlate the occurrence of Epstein-Barr virus copies in the saliva of patients with acute infectious diseases. Our preliminary results suggest that acute infectious diseases do not appear to increase the frequency of EBV copies in saliva. But, it should be taken into account that infectious diseases vary based on their aetiology and can therefore stimulate the immune system in many different ways. Therefore, we believe further studies on individual diseases and their causative agents will be needed to either confirm or deny their effect on the occurrence of EBV in saliva.

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