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Original Article

Analysis of Epstein Barr Virus Genome in Serum and Ocular Samples of Patients with Inflammatory Eye Disease Using PCR Method

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Abstract

Background and Aims: Epstein-Barr virus (EBV) infection is very common in the population. Virus belongs to the family Herpesviridae, whose representatives are characterized by the ability to cause the human body latent persistent infections. The goal of this study was to assess EBV infection frequency using PCR method in samples from inflammatory eye disease, in comparison with EBV presence in Eye Infection and control group.

Materials and Methods: Primers were designed for conserved regions of the EBV genome. We have used PCR for rapid, accurate detection of EBV DNA in blood and from eye swabs. We have chosen to study patients with eye inflammation or infection symptom.

Results: EBV DNA was detectable in 5 of 130 control samples of serum (3.84%). EBV infection was seen in 1 out of 20 patients' serum samples. Compared with the controls, the presence of EBV DNA was in samples of the patient group. Three out of 20 patients in patient group and 2 out of 130 of control group had detectable EBV DNA in their ocular swab.

Conclusion: We have presented evidence for the presence of EBV sequences in normal and eye inflammation samples with PCR. The prevalence of EBV in ocular disease samples varied dramatically that this wide range may be due to variations and inconsistency in the techniques used for detection of the virus and its components and genetic susceptibility.

Keywords: Epstein-Barr virus, EBV, PCR analysis, serum and ocular swab, inflammatory eye disease

Introduction

Epstein-Barr (EBV) virus infection is very common in the population (1-5). Virus belongs to the family Herpesviridae, whose representatives are characterized by the ability to cause latent and persistent infections (1-3). The source of infection is a sick human being and vector. EBV viruses are lymphotropic. Infection can

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be transmitted through air-mist, contact, sexual contact and blood transfusions (3, 4).

EBV infects people of all ages and it ithrough congenital or acquired. Congenital CMV infection can cause fetal or infant problem usually violates the lungs, kidneys, liver and other organs or systems (5). EBV also causes Burkett's lymphoma carcinoma and other malignant diseases.

Normally, people with healthy immune response may have EBV infections without complications. EBV infections have the ability to go into a latent state (1-5). EBV belongs to the Herpesviridae family. Herpesvirus is one of the widespread human viral infectious agents that cause latent infections with recurrent viral activation. Their replication takes place in the cell nucleus. The viruses leave the cell nucleus into the cytoplasm through nuclear membrane (6, 7). EBV infection is spread throughout the world. In developed countries, 50% -70% of the general population has antibodies against EBV. The only source of infection is human (8, 9, 10).

In women illness of a primary infection during pregnancy, the risk of transmission to the fetus is about 50%. Postnatal CMV infection can occur due to the viruses in cervical secretions, but most often the source of infection is the mother's milk. Epstein-Barr virus infection is often transmitted by kissing, and was formerly a synonym for "kissing disease" (8, 11, 12).

EBV Infects B lymphocytes and epithelial cells. EBV causes infectious mononucleosis, pneumonia, hepatitis, hematological changes, Burkett's lymphoma, carcinoma, lymphomas and oral leukoplakia (1, 3, 7, 11). Often EBV infection may be asymptomatic or subclinical, people with the infection can distribute it. EBV is often found in healthy people, and even more so in patients with chronic periodontitis. Individuals and people living in asocial conditions, where there are close contacts may be endemic foci of infection (1, 14). EBV is associated with etiopathology of several cancers (1, 15, 16). In some region, especially in developing countries, EBV infection reaches up to 80% in some cancers (1, 16). The clinical diagnosis of some eye disease is difficult because of the lack of symptomatic systemic disease. The role of EBV in the etiology of eye disease is still not clear (17, 18). It has been associated with a variety of symptoms, such as conjunctivitis, keratitis, ocular disorders, choroiditis, and retinitis. In these reports, the diagnosis was based on virus culture from conjunctival or tears samples, or substantiated by serological findings in serum (19, 20). Only one group has provided evidence of intraocular EBV antibody synthesis in three immuno-competent patients with bilateral anterior ocular disorders, and no evidence of infectious mononucleosis. So far, the evidence for a possible role of EBV in the pathogenesis of ocular disorders is mainly based on serological evidence (17-20). With the availability of more specific laboratory techniques such as poly-merase chain reaction (PCR) for the identi-fication of infectious agents during intraocular inflammation we decided to investigate the role of EBV in ocular disorders and more specifically whether routine testing for this virus is useful in the laboratory examination of a patient with eye disorder. Previous reports have ascribed numerous ocular manifestations to EBV infection, including oculoglandular syndrome, conjunctivitis, dry eye, keratitis, ocular disorders, choroiditis, retinitis, papilli-tis, and ophthalmoplegia (17).

These correlations have been made mainly based on acute seroconversion to EBV or simultaneous signs of infectious mononuc-leosis. Research on EBV infections has been banned by the absence of routine cell culture techniques. Now, fast and accurate detection of primary or reactivated EBV infection is based on serologic testing (16). However, serology is an oblique marker of virus presence and is difficult to explain in the presence of immuno-suppression (21, 22).

Establishing the presence of EBV would have a deterministic role on perception of the different EBV disease, pathogenesis (21, 23). Moreover, it could reveal relation of EBV to febrile illnesses and other disorder (22, 24). The PCR allows fast and accurate diagnosis of viral DNA sequences in blood and tissue samples. Detection of heterophilic IgM anti-bodies with fast and easy slide agglutination test is rather specific for primary EBV

infection, with a low false-positive rate (<3%) (21-24). EBV nuclear antigen (EBNA) antibodies are not increased until weeks past to the invasion of clinical symptoms. The elevated levels of VCA IgM or IgG antibodies by increased of EBNA IgM antibodies are diagnostic for acute EBV infection (25, 26).

Antibody against EA elevates in primary infection of EBV reactivation (27). Specific sensitive methods for detecting EBV infection are based on in situ hybridization (ISH), Southern blotting and PCR (15, 28, 29). RNA-ISH (RISH) for EBERs (EBV transcripts highly expressed in latently infected cells) detection is the standard method for EBV diagnosis allowing identification of infected cell types (27–29). PCR-based methods are used for strain discrimination and PCR may have an important role in EBV diagnosis in high-grade non-Hodgkin lymphoma (NHL).

There is a rapid test for EBV diagnosis, but the test results may not be obtained before 24 to 48 hours. Serological tests are accurate, but antibodies can be ready at least one week after infection.

The goal of study is to assess EBV infection frequency and PCR method in the choice of EBV in inflammatory eye disease, Comparing with EBV presence in Eye Infection and control group.

Methods

The patients studied groups and the number of inquiries. All 150 samples were divided into two groups. The first group included individuals who have experienced eye inflammation, and the second group- individuals, inexperienced the infection. Group I consisted of 20 eye infection experienced in individuals. Group II for individuals with clinical symptoms, targeted, usually- once on suspicion of any infection.

Epstein-Barr virus DNA test was carried out by PCR method using Research thermal cycler. Isolation of nucleic acids in the laboratory was done manually, but in case of a large number of samples, extraction wass carried out with the Qiagen kit. Venous blood was taken into the test tube with EDTA. The study sampled 5-10 ml of blood. Immediately after sample collection tubes were mixed. Blood samples delivered to the laboratory on the same day after collection (if not possible, then the sample was stored at -20°C).

Laboratory checks whether the sample was taken correctly or it intact during transportation to the laboratory. Blood samples recorded and given a unique identification number. All swab samples were collected within maximum six hours transferred to our laboratory and manipulated within maximum 1 hour at room temperature or Swabs were kept at 4°C for 1-3 hours until processed or aliquoted and stocked at -70°C until later examination. Swabs were immersed in distilled water as eluent and resulted suspension was used for total DNA extraction. DNA was isolated using the QIAamp Tissue Kit (Qiagen Company). Extracted DNA samples were dissolved in 200 µl of sterile TRIS-EDTA, pH 8 and visualized by electrophoresis on 0.7% agarose gel.

Sample Preparation. Genomic DNA was prepared from PBMC of patients with of age- and sex-matched normal controls. In addition to PBMC samples, DNA was extracted from swab samples from patients, and from "normal" people. DNA was prepared from PBMC by lyophilization for 16h, digested with RNAase (10mg/ml) for 2h, and proteinase K (1 mg/ml) for 16 h at 37°C, followed by phenol extraction and ethanol precipitation.

DNA purification. Viral nucleic acid extraction procedure and PCR were performed in a separate room to prevent contamination of clean area of PCR reaction products.

We Used QIAamp DNA Blood Mini Kit. The kit contains: lyophilized proteinase (QIAGEN Protease); Lizzie ready solution (Buffer AL); washing buffer (Buffer AW1); washing buffer (Buffer AW2); DNA elution buffer (Buffer AE); internal control (IC). DNA extraction was performed at room temperature (+20°C to 24°C).

PCR reagents. PCR takes place by the following reaction components: isolated DNA, primers (oligonucleotide), Taq DNA polymerase, available nucleotides (dNTPs), and reaction buffer. All the reaction regents were then added to a PCR tube for holding the

PCR reaction. All components (except for isolated DNA) were prepared and submitted by commercial kits.

Polymerase Chain Reaction. The PCR amplification of sample DNA was performed for 30 cycles (1 cycle = 95°C 1 minute, 45°C 2 minutes, 72°C 2 minutes) on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) with Taq polymerase.

Primer sequences were derived from the internally repeated BamHI W region of the EBV genome. The immortalized human foreskin epithelial cell line RHEK-l and sterile deionized water served as negative controls; the approved EBV-positive, served as a positive control. The PCR products electrophoresed in 2% agarose gels.

Statistical analysis. Mann–Whitney U-test was used for the statistical analysis to assess the association between antigenemia and PCR results. The results were expressed as medians and P-values <0.05 were considered significant.

Pearson correlation and linear regression were used to compare EBV PCR results. Data were analyzed by mean values and standard deviations for frequency and percentages for qualitative categorical variables.

SPSS version 13.0 was used to analyze the data. A P value of 0.05 and 95% confidence intervals was accepted as statistically significant.

Result

EBV DNA was detectable in 5 out of 130 samples of serum (3.84%). No DNA of other infectious agents such as Toxoplasma gondii, HSV-1, and VZV could be detected in these controls. None of the controls was known to use any immunosuppressive therapy.

EBV infection was seen in 1 out of 20 patients' serum samples. Compared with the controls, the presence of EBV DNA was only significantly increased in samples of the patient group. 3 out of 20 in patients and 2 out of 130 of control group had detectable EBV DNA in their ocular swab. In four out of these six patients another infectious agent could be detected either by measurement of local antibody production or by PCR. The details of patients' demography were as follows.

The mean age of the patients was 42 (18–58) for men and 37 (19–65) years in Female. The male to female ratio was 1/1. 7 patients had used the antiviral agent within 12 month of the tear sampling. Next, we analyzed the total positive rate of tear PCR assay and the conditional positive rates according to the methods used to collect swab and the methods used to detect EBV. The total incidence rate of positive PCR was 5% (1 out of 20) in the swab. Finally, we investigated whether clinical factors affect the positive rates of swab PCR. The patients who presented with a typical eye lesion displayed higher positive rates of PCR than the patients with no lesion (p=0.006).

There was no significant statistical difference in the positive PCR rates between the patients by age and sex. Finally, the positive PCR rates were no greater in the patients without previous administration of anti-herpecidal medication than in the patients with previous administration of the antiviral agent within 12 month before the sampling (p<0.001).

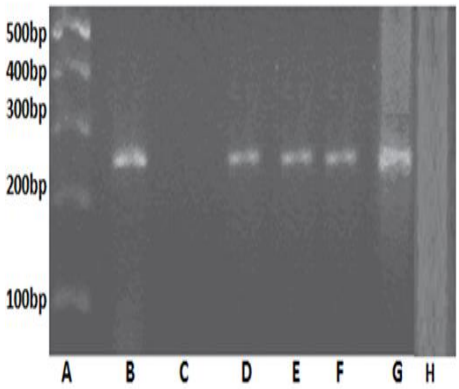

Fig. 1. Results of PCR for 240 bp of the EBV. A: marker; lane b: positive control; lane c: negative control; lanes D-E-F-G: positive samples; lanes H: Negative Sample.

Discussion

We have presented evidence for the presence of EBV sequences in normal and eye inflammation samples with PCR. The studies reported herein describe the detection of PCR-

amplified EBV sequences in 6 (42.3%) and 5 of 150 swabs and serum sample of both test and control groups, respectively. According to the literature, 50-70% of people have an antibodies against EBV infection and the EBV infects 95% of young people fewer than 25 years of age and 50% of individuals under 5 years of age. EBV infections of people in the population are more frequent than those of CMV infection, especially among young people (31-33).

Such literature confirms EBV infection is more frequent individuals with underlying disease. Seroepidemiologic studies have previously implicated EBV in anterior segment in eye diseases, including conjunc-tivitis, dacryoa-denitis, episcleritis, keratitis, and iritis (33, 34), as well as posterior segment processes such as retinitis and ocular diseases (17, 18, 35).

These observational study associations suggest that EBV can causes ocular disease, a hypothe-sis strengthened by detection of EBV genome by PCR in all ocular tissues except the optic nerve in normal cadaveric globes (17, 18, 36).

Even more decisive, definitive direct evidence for a pathogenic role was obtained by in situ hybridization studies of B cell lymphopro-liferations in lacrimal gland biopsies from patients with Sjogren's syndrome (31, 40).

Immunohistochemistry was used to suggest EBV as the pathoetiology of a conjunctival mass in an adult with acute, symptomatic EBV infection (1-40). In total, 150 inflammatory disordered and control individuals tested for EBV infection. In the Group I 20 individuals has been studied (13.3%), while in the second group (control group) there were 130 indivi-duals (86.6%). EBV infections agents in combination have been identified in 6serum samples of total individuals (Gr. I-5%, Gr. II–3.84%).

It was truly hypothesized that the sensitivity of the PCR technique could detect EBV sequ-ences in circulating lymphocyte DNA from EBV-seropositive patients, since circulating B cells are a known site of latency. EBV serology was available for large number of individuals. From a recent survey of the literature, we noted the prevalence of EBV in ocular disease samples varied dramatically (17, 18, 35-40). This wide range (0%–X %) may be due to variations and inconsistency in the techniques used to detect the virus and its Components, as well as geography and genetic susceptibility (41, 42).

Table 1: used EBV primer and PCR methods

location	Primer pair	[Mg2+] (M)	Annealing temperature (°C)	MELT TEMP	GC CONTENT	Product size (base pairs)
781	CTC TGG TAG TGA TTT GGA CC	0.002	60	52.5 ºC	50 %	240
1001	GTG AAG TCA CAA ACA AGC CC	0.003	62	54.4 ºC	50 %	240

Table 2: Demographics data of sample patients

Gender		
Male	50	
Female	50	
Race		
Iranian	100	
Age (mean)		
Male	42 (18–58)	
Female	37 (19–65)	
History of approved disease (n = 20)		
Ocular	20	
Cutaneous(cold sores)	5	
Genital	0	
Education	ND[1]	
EBV antibody	Positive	120(80)
	Negative	30(20)

ND: not determined

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