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Detection of the Predominant Strain of Epstein-Barr Virus in Systemic Autoimmune and Thalassemia Patients

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ABSTRACT

EBV, is a member of the herpesvirus family and one of the most common human viruses, Epidemiological data suggest that EBV is associated with polytransfused blood β-thalassemia and several autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis. We examined the presence of IgM antibodies against EBV in serum of 35 Thalassemic patients, 75 autoimmune patients among as 35 rheumatoid arthritis patients, 20 Systemic lupus erythrematosus and 20 autoimmune hypothyroid diseases, and 20 healthy controls by ELISA assay then detected the predominant strain in positive samples. The results show that the highest EBV infection percent was in SLE 15% whilst the lowest infection percent was in Thalassemia 5.7%., and according to gender, the results showed that the highest infection percentage recorded in females with rheumatoid arthritis 30 %, whilst the infection does not appear in males with rheumatoid arthritis and autoimmune thyroid disease and females of thalassemia patients. On the other hand, this study reveals that EBV-1 is the predominant strain in autoimmune diseases and thalassemia in Iraq.

Keywords: EBV, Thalassemia, Autoimmune diseases.

التحري عن السلالة السائدة لفايروس الابشتاين- بار في مرضى المناعة الذاتية الجهازية والثلاسيميا

الملخص

ابشتاين - بار فايروس هو احد افراد عائلة Herpesvirus، ويعد من الفيروسات الشائعة في الإنسان. تشير البيانات الوبائية أن الابشتاين - بار فايروس يرتبط مع مرضى الثلاسيميا الذين يتعرضون لنقل دم متكرر والعديد من أمراض المناعة الذاتية، مثل داء الذئب الاحمراري، التهاب المفاصل الرثواني والتصلب المتعدد، ومتلازمة سجوجرن، المناعة الذاتية للغدة الدرقية والتهاب الكبد ويمكن أن يسهم في زيادة امراضية هذه الأمراض. تم فحص وجود الأجسام المضادة المناعية (IgG) ضد EBV في 35 مصل دم من مرضى

الثلاسيميا، 75 من مرضى المناعة الذاتية موزعة بين 35 مريضا بالتهاب المفاصل الرثواني، داء الذئب الاحمراري 20 و 20 الأمراض المناعة الذاتية للغدة الدرقية، و 20 الاصحاء بواسطة ELISA ثم الكشف عن السلالة السائدة في العينات الإيجابية. أظهرت نتائج الدراسة أن أعلى نسبة اصابة كانت في مرضى داء الذئب الاحمراري 15 ٪ بينما أقل نسبة اصابة في مرضى الثلاسيميا 5.7٪. حسب نوع الجنس، أظهرت النتائج أن أعلى نسبة إصابة كانت في اناث مرضى التهاب المفاصل الرثواني 30٪، في حين لم تظهر الإصابة في ذكور المرضى المصابين بالتهاب المفاصل الرثواني والمناعة الذاتية للغدة الدرقية واناث مرضى الثلاسيميا. من جهة أخرى كشفت هذه الدراسة أن EBV-1 هي السلالة السائدة في أمراض المناعة الذاتية والثلاسيميا في العراق.

الكلمات الدالة: ابشتاين- بار فايروس، فقر دم البحر المتوسط، أمراض المناعة الذاتية.

INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous double stranded DNA virus from human herpes virus family, which has B-lymphotropism. More than 90% of adults in the world have serologic evidence of infection with this virus. It is acquired during early childhood and the age of infection is much lower in undeveloped countries with low socioeconomic condition (Rickinson and Kieff, 2001). A typical EBV virion consists of a linear double-strand DNA genome packaged into an icosahedral capsid, which is surrounded by a proteinaceous structure called the tegument, and an envelope composed of several viral glycoproteins embedded in a lipid bilayer (Morissette and Flamand, 2010). It is replicated during each cell division by the host DNA polymerase together with the host chromosomes. The EBV genome encodes over 85 open reading frames ORFs (Kieff and Liebowitz, 2001).

EBV has been associated with a number of diseases, such as Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma, conditions associated with human immunodeficiency virus HIV (Balandraud *et al*., 2004) and multiple transfused beta Thalassemia (Ergazaki *et al*., 1994) also associated with some non-B cell malignancies like extranodal T-cell and NK cell lymphomas, nodal T-cell lymphomas, nasopharyngeal carcinoma (NPC), gastric carcinoma and some others (Middeldorp *et al*., 2003) indicating the capacity of EBV to infect non-B cells as well.

EBV was first associated with autoimmune diseases in 1971, when a high prevalence of the virus was found in the sera of systemic lupus erythematosus (SLE) patients (Evans, 1971). Over the years, this relationship was strongly established, and today it is known that EBV infection preceding the development of autoimmune diseases may contribute to the pathogenesis of SLE via the mechanism of molecular mimicry of its nuclear antigen 1 (EBV NA-1) and lupus-associated autoantibodies (ANAs), and its subtypes such as anti-Sm and anti-Ro.(Poole *et al*., 2006). Aside from SLE, EBV infection is currently associated with multiple chronic autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome (SS), autoimmune thyroiditis, and autoimmune hepatitis. (Pender, 2003)

The aim of this study is to indicate the presence of EBV antibody in the serum of some systemic autoimmune diseases and thalassemia patients and to know the predominant strain

of EBV in a common autoimmune disorders and thalassemia by using ELISA and PCR techniques.

MATERIALS AND METHODS

Patients

A cohort of 130 serum samples has been collected of different autoimmune diseases as (RA, SLE, and ATD), Thalassemia and controls. Among those samples have been 35 RA, 20 SLE, 20 ATD, 35 thalassemia and 20 control samples, which were paid a visit Al-Salam Teaching, Ibn-Atheer Teaching and Nuclear medicine hospitals from date of 15\8\2012 to 15\2\2013 and diagnosed by the treating physicians and confirm the diagnosis by specific tests for each disease. Their ages are ranging from 3- 84 years.

Methods

A primary detection of EBV in all samples has been done by using Anti-EBV-CA ELISA (IgM) kit (Euroimmune, Germany), which provides a semi-quantitative *in vitro* assay for human anti-bodies of IgM class against Epstein Barr Virus capsid antigen (EBV-CA) in serum or plasma. In brief, the first reaction steps, diluted patient samples are incubated for 30 minutes in wells then wash three times used 300 µl of working wash buffer and empty the wells. In the case of positive samples, specific IgM antibodies will bind to antigens. To detect the bound antibodies, a second incubation has been carried out after adding conjugate enzyme (peroxidase-labelled anti-human IgM) into each well, after wash 100 µl of substrate was added into microplate wells and incubated in dark for 15 minutes at room temperature then finally stopped the reaction and read the absorbance at 450nm within 30 minutes by using Microelisa (washer and reader) applied by Biotek, USA in Al-Salam Teaching hospital. The result is evaluated semi-quantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator by using the following formula to calculate the ratio:

$$\text{Ratio} = \frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator}}$$

Interpreting result as following:
Ratio < 0.8: Negative
Ratio > 0.8 <1.1: Borderline
Ratio > 1.1: Positive

Viral DNA extraction

The Geneaid Viral DNA extraction kit (Geneaid, USA) has been used for quickly and conveniently extract of DNA from serum. The method has been comprised of concentrating viral particles from serum, lysis of viral particles, viral DNA binding to the surface of the glass fiber membrane, and releasing of DNA into the release buffer. DNA extraction was done for 10 positive serum samples of Anti-EBV-CA ELISA (IgM) and the DNA concentration has been measured using a (Genova- Nano Spectrophotometer, Jenway) with nuclease free water used to reset the machine. The Abs 260 of the dsDNA has been measured and the concentration has been calculated.

Conventional PCR

AccuPower PCR PreMix has been used to screen EBV for the presence of EBNA2 regions, as to the supplier's instructions (Bioneer, South Korea). This premix consists of a lyophilized pellet of 1 U Top DNA polymerase, 250 µM dNTP (dATP, dCTP, dGTP, and dTTP), 10 mM Tris–HCl (pH 9.0), 30 mM KCl, 1.5 mM MgCl2, a tracking dye, and a stabilizer and DNA in a final volume of 20µl Table(1).

Table 1: Primers of EBV (Synthesized by Bioneer)

Primer name	orientation	Sequences(5́ -3́)	Amplicon size (bp.)
EBNA2	Forward	AGG CTG CCC ACC CTG AGG AT	type-1EBV 168 type-2EBV 184
	Reverse	GCC ACC TGG CAG CCC TAA AG	

Accomplished by Jin *et al.,* 2010

PCR has been carried out on a Thermo cycler (^{3}Prime Thermal Cycler, Techne). The reaction consists of an initial denaturation step 95°c, 5 minutes, followed by 35 cycles of denaturation 95°c, 45 seconds, annealing 56°C, 45 seconds and elongation 72°C, 1 minute. A final elongation step 72°C, 10 minutes have been followed by cooling to 4°c before visualization on a 2% (w/v) agarose gel (Jin *et al.,* 2010).

Agarose Gel Electrophoresis

Agarose has been dissolved in the appropriate volume of TBE buffer by heating in a hotplate stirrer. Ethidium bromide to a final concentration of 100µg/l has been added to the molten gel to allow visualization of the DNA. Samples are loaded into the set gel, and run at 100V in TBE for 1 hour. The nucleic acid bands have been visualized on a UV transilluminator.

RESULTS

According to ELISA technique, the results revealed that 10 of the patients have seropositive of EBV (Table 2) and the highest EBV infection percent was in SLE 15% followed by ATD 10% and RA 8.6% while the lowest infection percent was in Thalassemia 5.7% as (Table 3).

Table 2: EBV seropositive in study group

Study groups	RA	SLE	ATD	Thala	Cont.
EBV seropositive (No.)	3	3	2	2	-
Total	10				

Thala= Thalassemia, R.A= Rheumatoid arthritis, ATD= Autoimmune thyroid disease, SLE= Systemic Lupus Erythromatosis, Cont.= control

Table 3: Percentage of EBV seropositive in study group

Study groups	RA	SLE	ATD	Thala	Cont.
EBV seropositive (%)	8.6	15	10	5.7	-
Total cases	35	20	20	35	20

Thala= Thalassemia, R.A= Rheumatoid arthritis, ATD= Autoimmune thyroid disease, SLE= Systemic Lupus Erythromatosis, Cont.= control

On the other hand, the results according to gender showed that the highest infection percentage recorded in females with rheumatoid arthritis 30 %, followed by females with SLE, autoimmune thyroid diseases and males of thalassemia patients 20% and the lowest infection percentage in male with SLE 10%, while no infection in males with rheumatoid arthritis and autoimmune thyroid disease and females of thalassemia patients (Table 4).

Table 4: Percentage seropositivly EBV infection in relation to gender

Study groups / Gender	Thala	RA	SLE	ATD	Total
	N0. (%)				
Males	2(20%)	0(0%)	1(10%)	0(0%)	3(30%)
Females	0(0%)	3(30%)	2(20%)	2(20%)	7(70%)
Total	2(20%)	3(30%)	3(30%)	2(20%)	10(100%)

The frequency of type 1 or type 2 EBV infection has been determined for all positive samples of the study groups. Collectively, type 1 EBV is present in 9 of 10 positively samples 90% while type 2 EBV is not found in samples 0% and one sample was missed (Fig. 1).

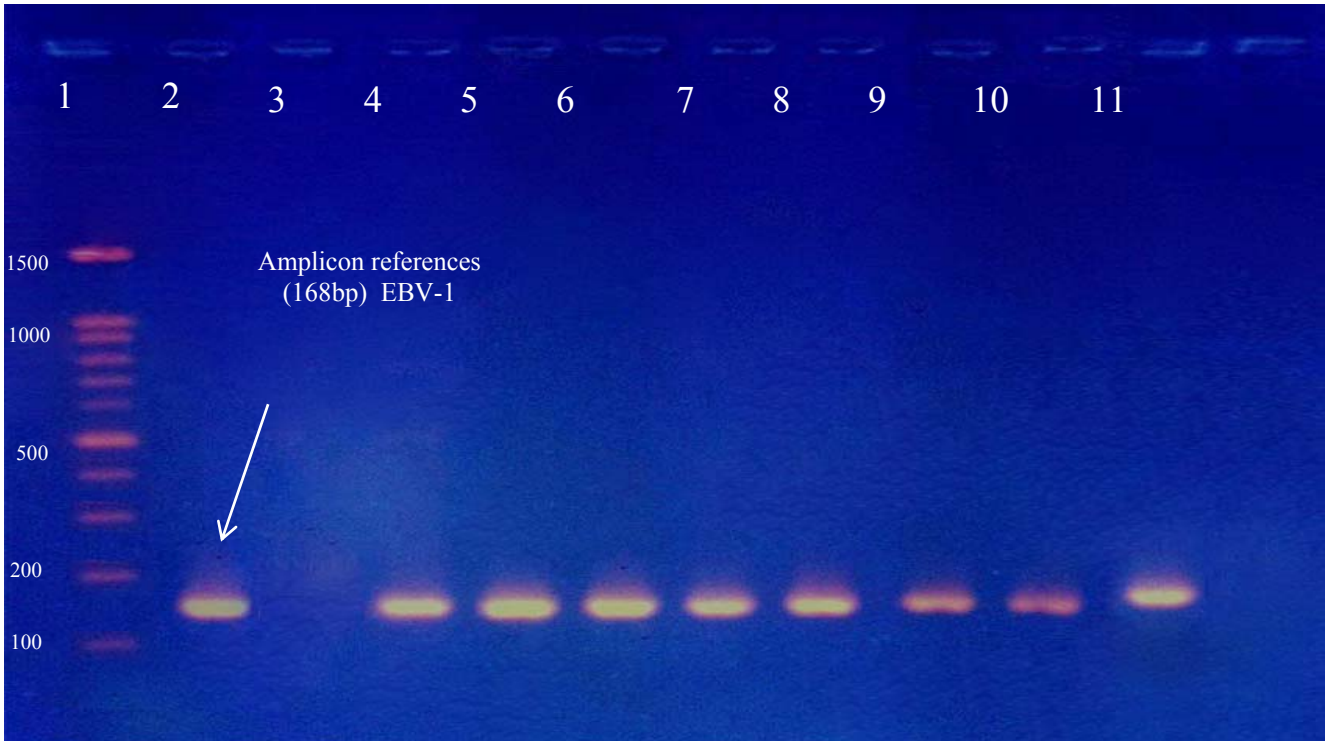

Fig. 1: Ethidium bromide-stained agarose gel of PCR amplified products from extracted EBV DNA amplified with primers *EBNA2* gene. Lane 1 DNA molecular sizes marker (100-bp. ladder). Lanes (2-11) EBV extracted show positive results for *EBNA2* gene (amplicon size 168 bp.).

DISCUSSION

In the 1970s, IARC demonstrated that more than 90% of adults worldwide are infected with EBV, based on the detection of antibodies to EBV especially antibodies to viral capsid (VCA) and complement-fixing soluble (CF/S) antigens (IARC, 1997). On one side the results of the present study agree with Kannangai *et al.*, (2010), who documents that autoimmune diseases have been positive for EBV and on the other side, our study disagrees with the recent study on EBV in rheumatoid arthritis that shows a higher positivity of anti-EBV compared to controls (Balandraud *et al.*, 2005; Balandraud *et al.*, 2003). In patients with rheumatoid arthritis (RA), the immune response to the EBV is slightly impaired, which usually have high titer anti-EBV antibodies in their sera, and also have impaired peripheral blood T-cell responses to EBV proteins. This can result in inefficient control of the outgrowth of EBV infected B cells and systemic EBV overload (Balandraud *et al.*, 2005). Subsequent studies have shown that 99% of SLE patients are seropositive for EBV (James *et al.*, 2001). SLE patients have higher titers of anti-EBV antibodies than control populations (Verdolini *et al.*, 2002). T cells from patients with SLE cannot control the numbers of EBV-infected B cells from SLE patients or normal subjects but T cells from normal EBV-seropositive subjects can control infected B cells from SLE patients (Tsokos *et al.*, 1983). Patients with autoimmune thyroiditis have increased titers of anti-EBV antibodies in their sera compared to healthy subjects (Vrbikova *et al.*, 1996). As well Jasim, (2010) who found that the highly titer of IgG antibodies to EBV-VCA in Hashimoto's and Graves' disease as compared with healthy people. On the other hand, a previous study shows about 15% of thalassemia patients have EBV DNA whom are on

frequent transfusion treatment (Ergazaki *et al*., 1994), suggested that EBV can be transmitted via transfussion of blood or blood products given and/or bone marrow transplantation (Liloglou *et al.*, 1994). In one side Arjmandi *et al*., (2008) conducted a study on EBV in beta thalassemia and he documents that thalassemic patients show positivity of Anti-EBV (0.75 %). On the other side, Papaevangelou *et al*., (1979) who document that there are no evidence of repeated infection or recent infection with EBV in the polytransfused thalassemia patients.

Moreever, The low income and crowded family conditions have also been found to increase titer of EBV seropositive in children from other geographical locales, such as Thailand (Mekmullica *et al*., 2003), Turkey (Ozkan *et al*., 2003).

On the other hand, antibody titers seem to be higher in females than in males (Wagner *et al*., 1994). This difference, which also has been observed for other viruses, is in accordance with the notion that women in general mount more vigorous antibody- and cell-mediated immune response following infection or vaccination than men (Beagley and Gockel, 2003).

Generally, two major types of EBV (EBV-1 and EBV-2) have been identified and differ in geographic distribution. The role of specific EBV types in the etiology of different cancers is unknown. Immunocompromised patients are more commonly harbour both subtypes of EBV (Thompson and Kurzrock, 2004). In the present study, EBV-1 was predominant. This is consistent with the previous studies, Abdirad *et al.*, (2007) report that type 1 EBV is predominant in Iran and with other previous studies that reveal that type 1 EBV is the most prevalent in Asia (Sidagis *et al*., 1997), An interesting finding is the high frequency of EBV type -2 with latent membrane protein-1 (LMP-1) deletion in the Mexican and found a similar prevalence of EBV diffuse large B-cell lymphoma of the elderly in a Mexican population compared with that has been reported from Asian countries, and in contrast to the low frequency in Western populations (Hofscheier *et al*., 2011). Type 2 EBV is predominant in equatorial Africa (Yao *et al*., 1996) and the high incidence of type 2 EBV infections among HIV positive patients especially during the first 10 years of the AIDS epidemic (Biggar and Rabkin, 1996). EBV-2 may be more common in Africa (Gratama and Ernberg, 1995), and in homosexual men (Higgins *et al.*, 2007). However, other studies show that HIV-infected haemophiliacs have lower rates of EBV-2 infection than HIV-infected homosexuals have challenged this hypothesis, and suggest that the acquisition of EBV-1 versus EBV-2 would rather be due to the opportunity for exposure (Thompson and Kurzrock, 2004). However, we need first to evaluate the genotype of EBV in healthy people and more advanced research in Iraq before any conclusion.

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