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Qualitative and Quantitative Molecular Analysis of Epstein-Barr Virus in Iraqi Patients with Relapsing-Remitting Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a neuro-inflammatory disorder in which the Epstein-Barr virus (EBV) is proposed to have a pathogenic role. Therefore, a case-control study was performed (93 patients with relapsing-remitting MS and 113 healthy controls (HC) to analyze the prevalence and viral load of EBV infection using real time-polymerase chain reaction. Prevalence of EBV infection was lower in patients compared to HC but the difference was not significant (12.9 vs. 21.2%; probability [p] = 0.187). EBV-positive MS cases were more common in females than in males (83.3 vs. 16.7%), while an opposite distribution was observed in HC (37.5 vs. 62.5%), and the difference was significant (p = 0.041). Blood group O frequency was higher in EBV-positive patients compared to the corresponding HC but the difference was not significant (33.3 vs. 20.8%; p = 0.443). EBV-positive MS cases showed similar frequency in the two groups of the expanded disability status scale (EDSS: < 3.0 and ≥ 3.0; 50% each). EBV load was significantly elevated in EBV-positive MS cases compared to EBV-positive HC (94.6 ± 84.2 vs. 17.0 ± 16.3 DNA copy/100 cells; p = 0.009). When EBV-positive MS cases were classified by gender, EDSS groups or ABO blood groups, there were no significant differences between EBV loads in each stratum. However, a significant correlation between EBV load and EDSS was found (correlation coefficient = 0.620; p = 0.031). In conclusion, the prevalence of EBV infection showed no significant differences between MS patients and HC, while EBV load was significantly higher in patients.

Keywords: Relapsing-remitting multiple sclerosis; Epstein-Barr virus; Viral load; Expanded disability status scale.

تحليل جزيئي نوعي وكمي لفيروس ابشتاين بار بين المرضى العراقيين المصابين بالتصلب المتعدد الانتكاسي المتحول

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الخلاصة

أن التصلب المتعدد (MS) اضطراب التهابي عصبي يُقترح فيه أن يكون لفيروس إبشتاين بار (EBV) دور مُمْرِض. لذلك، تم إجراء دراسة الحالات والشواهد (93 مريضًا يعانون من التصلب المتعدد الانتكاسي

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المتحول و 113 عنصر سيطرة) لتحليل الانتشار والحمل الفيروسي لعدوى EBV باستخدام تفاعل البلمرة المتسلسل. كان انتشار عدوى EBV أقل في المرضى مقارنة بالسيطرة لكن الفرق لم يكن معنويا (12.9 مقابل 21.2٪ ؛ الاحتمالية = 0.187). كانت حالات التصلب المتعدد إيجابية EBV أكثر شيوعًا في الإناث منها عند الذكور (83.3 مقابل 16.7٪) ، بينما لوحظ توزيع معاكس في السيطرة 37.5 مقابل 62.5٪) ، وكان الاختلاف معنويًا (الاحتمالية = 0.041). كان تكرار مجموعة الدم O أعلى في المرضى الموجبين لـ EBV مقارنةً بالسيطرة المقابل لكن الفرق لم يكن معنويًا (33.3 مقابل 20.8٪ ؛ الاحتمالية = 0.443). أظهرت حالات MS إيجابية EBV تكرارًا مشابهًا في مجموعتي مقياس حالة الإعاقة الموسع (EDSS: <3.0 و 3.0 ؛ 50٪ لكل منهما). ارتفع حمل EBV بشكل ملحوظ في حالات MS إيجابية EBV مقارنة بالسيطرة إيجابي EBV (84.2 ± 94.6 مقابل 17.0 ± 16.3 نسخة DNA / 100 خلية ؛ الاحتمالية = 0.009). وعندا تصنيف حالات MS إيجابية EBV حسب الجنس أو مجموعات EDSS أو مجموعات الدم ABO ، لم تكن هناك فروق ذات دلالة إحصائية بين أحمال EBV في كل طبقة. ومع ذلك ، تم العثور على ارتباط كبير بين حمل EBV و EDSS (معامل الارتباط = 0.620 ؛ ع = 0.031). في الاستنتاج ، لم يظهر انتشار عدوى الـ EBV فروق ذات دلالة إحصائية بين مرضى التصلب المتعدد و HC ، بينما كان حمل الـ EBV أعلى بشكل ملحوظ في المرضى.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disorder of the central nervous system (CNS), in which the myelin sheath surrounding neurons is damaged by cell-mediated immunity [1]. It is characterized by multifocal zones of inflammation, demyelination, gliosis, and various degrees of axonal pathology that lead to progressive physical and cognitive disabilities [2]. MS is the most common type of neurological disability and the estimated number of people with MS worldwide has increased from 29.26 cases/100,000 population in 2013 to 43.95 cases/100,000 population in 2020 [3]. In Iraq, MS is also showing an increased incidence; from 4.4 cases/100,000 population in 2007 to 11.73 cases/100,000 population in 2020 [4], [5]. Two main subtypes of MS are clinically recognized; relapsing-remitting MS (RRMS) and progressive MS. The most common subtype is RRMS, which accounts for 85% of MS cases, and is characterized by acute exacerbations followed by periods of remission [6].

Although MS etiology has not been fully identified, a markedly dysregulated chronic immune homeostasis has been described [7]. This dysregulation results from a complex interaction between genetic predisposition and infectious agents [8]. Other factors that promote inflammatory reactions also have a role in this context, including tobacco smoking, adolescent obesity, and lack of sunlight exposure/vitamin D deficiency [9]. Regarding infectious agents, various viruses, particularly those of the human herpesvirus (HHV) group, have been described as playing a pathogenic role in MS; including HHV-6 and -7, herpes simplex virus (HSV) 1 and 2, cytomegalovirus (CMV), Varicella Zoster virus (VZV), Kaposi sarcoma virus (KSV) and Epstein-Barr virus (EBV) [10], [11]. Most studies have focused on the latter virus (EBV) and its primary role in the pathogenesis of MS has been proposed [12]–[14].

EBV, also known as HHV-4, is an enveloped virus with a linear, double-stranded DNA genome of a molecular size of approximately 172 kb and contains more than 85 open reading frames that encode proteins involved in regulating DNA replication and gene expression, and maintaining genome integrity [15]. It is an ubiquitous oncovirus that has been identified to infect humans exclusively and causes latent asymptomatic infection in approximately 90% of the adult population worldwide, but EBV can also be etiologically associated with the clinical syndrome of infectious mononucleosis [16]. Further, several systemic autoimmune diseases, including MS, are associated with chronic relapsing EBV infection and inefficiency of the immune system to control the virus [17]. In the case of MS, EBV has been closely associated

with disease risk as EBV seropositivity has been associated with increased susceptibility to MS, and the virus has been suggested to be involved in the pathogenesis of MS and may be considered a prerequisite for disease progression [11], [18]. However, most studies have targeted EBV in MS in terms of antiviral antibody detection, while molecular evaluation of EBV has shown inconsistent results in different ethnic groups [19]. Therefore, the current study was conducted to evaluate the relationship between MS and the incidence of EBV infection in Iraqi patients with RRMS using real time-polymerase chain reaction (RT-PCR) assay as a sensitive molecular method for detection of EBV DNA. Viral load was assessed in this context and correlated with gender, ABO blood groups, expanded disability status scale (EDSS) and medication type.

2. Materials and methods

2.1. Populations studied

A case-control study was conducted on 93 patients with RRMS and 113 healthy controls (HC) to evaluate EBV infection in terms of prevalence and viral load. Patients were referred to the General Hospital for Neurosciences in Baghdad for diagnosis and treatment during the period from January 2020 to June 2021. Patients with RRMS were diagnosed following the 2010 revised Macdonald criteria, which were based on clinical features, magnetic resonance imaging (MRI), and visual evoked potential (VEP) testing [20]. The physical disability of MS patients was assessed using the EDSS, which ranges from 0 (normal neurological status) to 10 (death due to MS). For simplicity, MS patients were divided into two groups regarding EDSS, which were < 3.0 and ≥ 3.0 [21]. All patients were on medication but under two lines of therapy. First-line therapy included interferon beta 1-alpha, while second-line therapy included fingolimod or natalizumab.

The control sample included age- and gender-matched healthy subjects who were blood donors and health service personnel. They did not suffer from neurological disorders or autoimmune diseases. The Ethics Committee of the Iraqi Ministry of Health and Environment and Department of Biology (College of Science, University of Baghdad) approved the study protocol and written consent was obtained from all participants.

2.2. Laboratory tests

Five milliliters of blood were obtained from each participant and dispensed in ethylene-diamine-tetra-acetic acid (EDTA) tube. The blood was initially tested for ABO blood groups using specific anti-A and anti-B sera (Atlas Diagnostics, Germany). Then, genomic DNA was isolated from EDTA blood using the gSYNC DNA extraction kit following the manufacturer's instructions (Geneaid Biotech Ltd, Taiwan). Isolated DNA was subjected to RT-PCR analysis to detect EBV qualitatively (positive or negative) and quantitatively (viral load). The Real-TM Quant kit was used to perform this analysis following manufacturer's instructions (Sacace Biotechnologies Srl, Italy). Briefly, after DNA isolation, amplification and detection were performed using fluorescent reporter dye probes specific for EBV DNA, internal control (IC), and endogenous IC gene (β-globin gene). The RT-PCR mix consisted of 10 µL DNA and 15 µL reaction mix (total volume = 25 µL). The Applied Biosystem 7300 Fast Real-Time PCR System (USA) was programmed to create the following temperature profile: one cycle hold for 15 minutes at 95 °C, five cycles of 5 seconds at 95 °C, 20 seconds at 60 °C, 15 seconds at 72 °C and 5 seconds at 95 °C, and 40 cycles of 30 seconds at 60 °C and 15 seconds at 72 °C. The target amplification region was the latent membrane protein (LMP) gene of EBV. Viral load was expressed as EBV DNA copy/100 cells.

2.3. Statistical analysis

Categorical variables were given as numbers and percentages, and significant differences were assessed using Fisher exact test or Pearson Chi-square test. Continuous were given as mean and standard deviation, and significant differences were assessed using a Welch-corrected t-test. Receiver operating characteristic (ROC) curve analysis was conducted to estimate the area under the curve (AUC), 95% confidence interval (CI), cut-off value, sensitivity and specificity. Logistic regression analysis was performed to estimate the odds ratio (OR) and 95% confidence interval (CI) for EBV load under three models; unadjusted, age-adjusted, and age- and gender-adjusted. Pearson correlation analysis was applied to detect the correlation coefficient (r). A probability (p) ≤ 0.05 was considered statistically significant. The statistical package IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.) and GraphPad Prism version 8.0.0 (San Diego, California USA) were used to perform these analyses.

3. Results and Discussion

3.1. Baseline characteristics

MS patients and HC showed an approximate mean age and no significant difference was observed (35.2 ± 8.6 vs. 37.0 ± 9.4 years; p = 0.171). In addition, males and females showed approximate frequencies in patients and HC (Males: 46.2 *vs*. 41.6%; Females: 53.8 *vs*. 58.4%; p = 0.503). With regard to ABO blood groups, the frequency of type O was higher in patients than in HC (43.0 *vs*. 30.1%), but the distribution of the four blood types in patients and HC did not show significant differences (p = 0.138). According to the EDSS, MS patients were divided into two groups; < 3.0 and ≥ 3.0, and their frequencies were 59.1 and 40.9%, respectively. All patients were on medication, but most received first-line therapy (78.5%), while 21.5% received second-line therapy (Table 1).

Table 1: Baseline characteristics of multiple sclerosis patients and healthy subjects

Characteristic		MS; N = 93	HC; N =113	*p*-value
Age; mean ± SD (year)		35.2 ± 8.6	37.0 ± 9.4	0.171
Gender; N (%)	**Male**	43 (46.2)	47 (41.6)	0.503
	Female	50 (53.8)	66 (58.4)	
ABO blood group; N (%)	**A**	27 (29.0)	32 (28.3)	0.138
	B	15 (16.1)	31 (27.4)	
	AB	11 (11.8)	16 (14.2)	
	O	40 (43.0)	34 (30.1)	
EDSS		2.63 ± 1.66	NA	
EDSS group; N (%)	**< 3.0**	55 (59.1)	NA	
	≥ 3.0	38 (40.9)	NA	
Medication; N (%)	**First line**	73 (78.5)	NA	
	Second line	20 (21.5)	NA	

EDSS: Expanded disability status scale; Multiple sclerosis; HC: Healthy controls; p: the probability of Welch-corrected t-test (to compare continues continuous variables), two-tailed Fisher exact test or Pearson Chi-square test (to compare categorical variables).

3.2. Prevalence of EBV infection

Prevalence of EBV infection was lower in MS patients compared to HC but the difference was not significant (12.9 *vs*. 21.2%; $p = 0.187$) (Figure 1). When gender and ABO blood groups were considered, a different profile of EBV prevalence was observed in patients and HC. Among MS patients, EBV-positive cases were more frequent in females than in males (83.3 *vs*. 16.7%), while an opposite distribution was observed in HC (37.5 *vs*. 62.5%), and the difference was significant ($p = 0.041$). With regard to ABO blood groups, type O patients showed a higher EBV-positive frequency than the corresponding HC frequency but the difference was not significant (33.3 *vs*. 20.8%; p = 0.443) (Figure 2). The distribution of EBV-positive MS cases in EDSS groups (< 3.0 and ≥ 3.0) was similar (50% each). In the case of medication, most EBV-positive MS cases were under first-line therapy (83.3%) (data not shown).

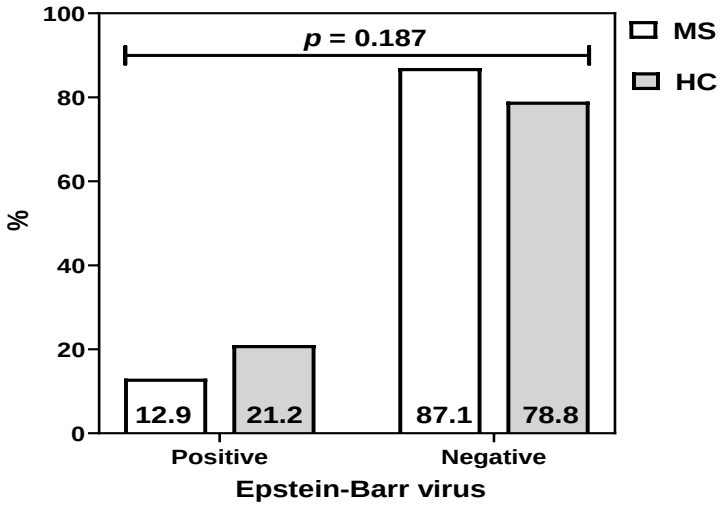

Figure 1: Prevalence of Epstein-Barr virus infection in multiple sclerosis (MS) patients and healthy controls (HC). *p*: Two-tailed Fisher exact probability.

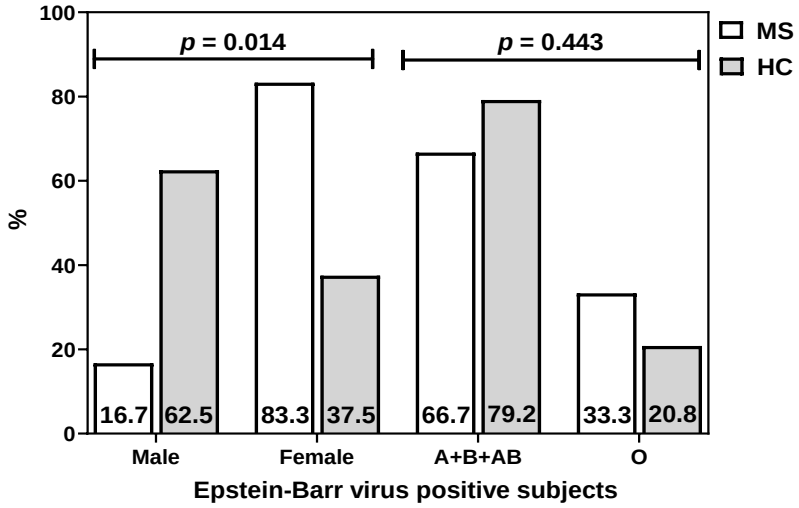

Figure 2: Epstein-Barr virus positive multiple sclerosis (MS) patients and healthy controls (HC) distributed according to gender and ABO blood group. *p*: Two-tailed Fisher exact probability.

3.3. EBV load

The mean EBV load was significantly elevated in EBV-positive MS cases compared to EBV-positive HC (94.6 ± 84.2 *vs.* 17.0 ± 16.3 DNA copy/100 cells; $p = 0.009$) (Figure 3).

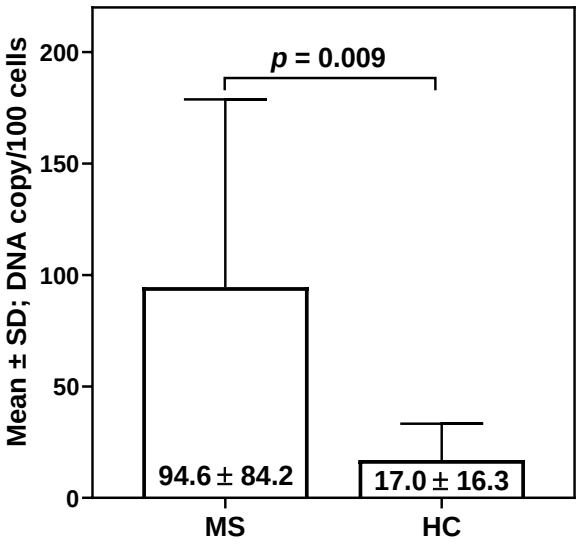

Figure 3: Viral load means of Epstein-Barr virus among multiple sclerosis (MS) patients and healthy controls (HC). p: Welch-corrected t-test probability.

ROC curve analysis revealed that EBV load was a very good predictor in discriminating between EBV-positive cases and HC (AUC = 0.816; 95% CI = 0.653-0.979; $p = 0.002$; cut-off value = 25.70; sensitivity = 66.7%; specificity = 66.7%) (Figure 4).

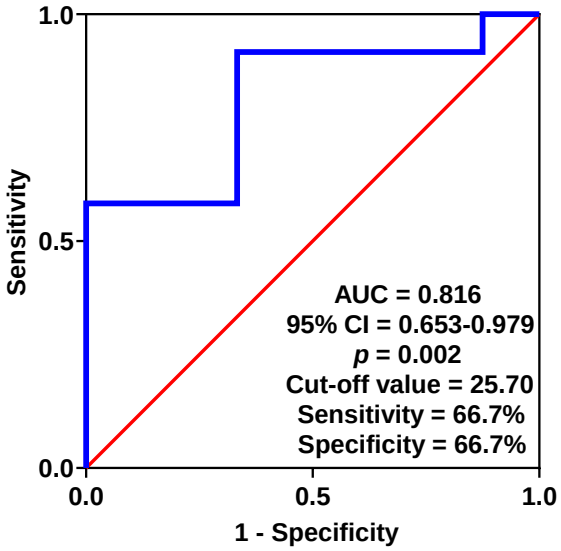

Figure 4: ROC curve analysis of Epstein-Barr virus load in multiple sclerosis patients *versus* healthy controls. AUC: Area under the curve; CI: Confidence interval; *p*: Two-tailed probability.

Further, logistic regression analysis demonstrated that EBV load was associated with an increased risk of developing MS whether the analysis was unadjusted (OR = 1.04; 95% CI = 1.01 - 1.08; $p = 0.021$) or adjusted for age and gender (OR = 1.05; 95% CI = 1.00 - 1.09; $p = 0.041$) (Table 2).

Table 2: Logistic regression analysis of Epstein-Barr virus load in multiple sclerosis patients *versus* healthy controls

Logistic regression analysis	OR	95% CI	*p*-value
Unadjusted	1.04	1.01 - 1.08	**0.021**
Age-adjusted	1.04	1.00 - 1.08	**0.036**
Age- and gender-adjusted	1.05	1.00 - 1.09	**0.041**

OR: Odds ratio; CI: Confidence interval; *p*: Probability (significant *p*-value is indicated in bold).

When EBV-positive MS cases were classified by gender, EDSS groups, or ABO blood groups, there were no significant differences between the means of EBV load in each stratum. Although there was no significant difference between the EDSS groups (< 3.0 and ≥ 3.0), EBV load showed a higher mean in ≥ 3.0 cases compared to < 3.0 cases (120.80 ± 95.99 vs. 68.45 ± 68.83 DNA copy/100 cells; p = 0.306) (Figure 5).

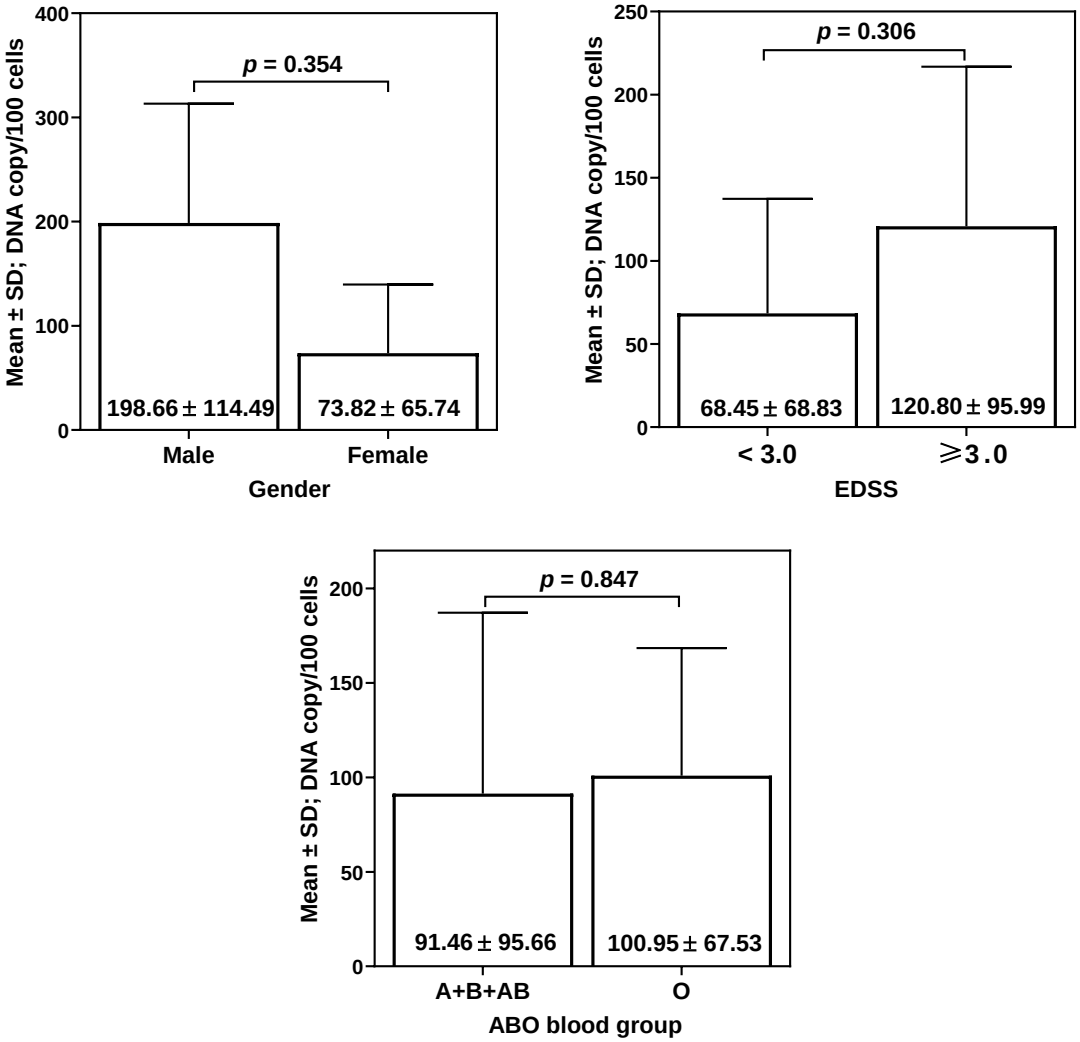

Figure 5: Epstein-Barr virus load in multiple sclerosis patients classified by gender, expanded disability status scale (EDSS), and ABO blood groups. p: Welch-corrected t-test probability.

To understand the relation between EBV load and EDSS in MS patients, Pearson correlation analysis between the two variables was performed. A positive correlation was

found and the estimated correlation coefficient (r) was 0.620 with a significant *p*-value (p = 0.031) (Figure 6).

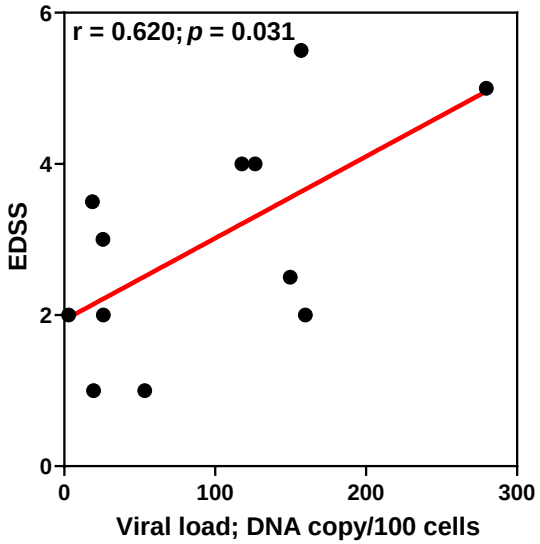

Figure 6: Scatter dot plot showing Pearson correlation coefficient (r) between viral load of Epstein-Barr virus and expanded disability status scale (EDSS) among multiple sclerosis patients.

Observational studies have indicated that viral infection is among the most important risk factors involved in mediating MS pathogenesis and/or exacerbation of disease [10]. In this context, different viruses have been described but EBV has been a cornerstone of research aimed to understand the causal relationship between the virus and MS [18]. Most studies have recognized the link between axonal pathology and demyelination mediated by autoimmune response and epitopes of EBV. Accordingly, EBV has been proposed as a potential trigger for MS episodes and an association between this viral infection and susceptibility to MS has been indicated, although the evidence is debatable [7], [17]. Molecular qualitative analysis of EBV in the current cohort of MS patients and HC may not favor such a potential role for EBV in MS pathogenesis. Indeed, the study revealed that EBV prevalence showed a higher frequency in HC than in MS patients, and that only a small percentage of MS patients had EBV DNA in peripheral blood (12.9%). Consistent with our observation, a recent Iranian study showed that EBV infection was more prevalent in non-MS subjects than in MS patients and no significant association was found between the occurrence of EBV infection and the development of MS [19]. Another Italian study used RT-PCR to examine the DNA of a group of HHVs (HSV-1 and -2, CMV, VZV and EBV) in the cerebrospinal fluid (CSF) and serum of MS patients and all samples were negative for HHV DNA except for one CSF sample among 56 MS patients, which was positive for EBV DNA [22]. Two additional studies also showed no significant differences in EBV positivity rates between MS patients and HC [23], [24].

Quantitative analysis of EBV DNA may be more predictable than qualitative analysis in revealing the role of EBV in the pathogenesis of MS. The EBV-positive subjects in the current study showed a significantly higher EBV load in MS patients compared to the corresponding HC. ROC curve analysis demonstrated the predictive significance of EBV load in distinguishing MS patients from HC. Further, logistic regression analysis confirmed the potential of EBV load in susceptibility to MS. These results may indicate that the development of MS is related to latent EBV reactivation, but the underlying mechanism is still not precisely defined. It is well known that B lymphocytes are the primary cellular target

of EBV, and in most cases, this viral infection remains asymptomatic due to the highly effective immune response of the host [25]. However, under certain physiological and immunological conditions, EBV reactivation can occur in some individuals and is associated with the development of a variety of diseases such as autoimmune diseases. Therefore, it has been proposed that chronic reactivation of EBV is an important mechanism that may underlie the pathogenesis of these diseases [26]. In MS, it was evident that a significantly higher rate of EBV reactivation was found. Besides, EBV infection is associated with a decreased ability of EBV-specific cytotoxic T cells to reduce EBV reactivation and this may contribute to progressive aspects of MS [12]. Further, the association of severe gray matter and lesion pathology with an exaggerated humoral response against EBV was reported in patients with RRMS [27]. In the current study, EBV load was found to be elevated in EBV-positive MS patients with EDSS $\geq$ 3.0 compared to EBV-positive patients with EDSS < 3.0, and although the difference was not significant, a significant correlation was found between EBV load and EDSS. There is no direct evidence to support an association between EBV load and EDSS, but it has been suggested that EBV reactivation may be associated with MS activity [23]. Further, anti-EBV latent nuclear antigen (EBNA1) IgG antibody titer has been suggested to be an indicator of alteration in EDSS, and an association between these antibodies and MS progression was implicated [28]. On the contrary, neither EBV antibody levels nor EBV DNA load in the saliva of MS patients were associated with EDSS alteration in a follow-up study [29]. Therefore, the link between EBV load and EDSS may require further investigations due to these conflicting results.

The qualitative analysis also showed that most of the EBV-positive MS cases were females (83.3%), while the opposite observation was in HC and males were more prevalent in EBV-positive HC. There are no well-documented data regarding this point, but it is well known that females are more likely to develop RRMS than males and environmental factors (for instance vitamin D) may have a role in this gender disparity [30]. When EBV load was considered, it was higher in male patients compared to female patients but the difference was not significant (Figure 5). However, it should be noted that the mean in males was based on only two cases and this might have contributed to the higher mean of EBV load in males.

The study also revealed that MS patients tended to have a higher frequency of blood type O compared to HC, but the general distribution of ABO blood groups in patients and HC did not show significant differences. Similarly, the distribution of EBV-positive cases and EBV viral load also showed no significant differences between MS patients with A+B+AB blood groups compared to those with O blood group. In contrast to the current study, blood type O was considered as a protective factor against the development of MS in Cuban, Croatian and Spanish populations [31]. This discrepancy may be related to sample size; otherwise, racial differences may have an account because ABO blood groups show significant racial differences [32].

The study encountered some limitations. First, the sample size of MS patients and controls was relatively small, particularly those who tested positive for EBV. Second, the anti-EBV antibody profile was not determined. Third, vitamin D status was not evaluated.

Conclusions

Prevalence of EBV infection showed no significant differences between MS patients and HC, while the EBV load was significantly higher in patients. These findings might have been influenced by gender and EDSS.

ETHICAL CLEARANCE

The Ethics Committee of the Iraqi Ministry of Health and Environment and Department of Biology (College of Science, University of Baghdad) approved the study protocol and written consent was obtained from all participants.

CONFLICT OF INTEREST

The authors declare that there were no conflicts of interest.

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