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Molecular Detection of Epstein–Barr Virus, Human Herpes Virus 6, with Multiple Sclerosis

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ABSTRACT

Background and Aims: Multiple sclerosis (MS) is an immune inflammatory disease, where the underlying etiological cause remains elusive. Multiple triggering factors have been suggested, including environmental, genetic and gender components. However, underlying infectious triggers to the disease are also suspected. There is an increasing abundance of evidence supporting a viral etiology to MS, including the efficacy of interferon therapy and over-detection of viral antibodies and nucleic acids when compared with healthy patients, Furthermore, this study aimed to evaluate the relationship between viral diseases, including Epstein–Barr virus (EBV), human herpes virus and MS in the present case-control study.

Materials and Methods: Patients with MS were recruited from our clinic population, and blood was drawn while they were in clinic for standard clinical care. The study was enrolled-with 60 MS patients (40 female/20 males, rang years of age 10-65), and 50 healthy-looking controls (25 female/25 males, Rang 17-70 years of age). All patients were admitted in Al-Sadder- Medical City in Al-Najaf Province from December up to March 2024.all subjects signed informed consent. five milliliters of blood were collected in EDTA tube, the samples were allowed to stand at room temperature for 15 min and maintained at -20°C in order to viral DNA Extraction using a specific viral DNA extraction kit (Patho Gene-spinTM DNA/RNA Extraction Kit, Intron/Korea); as a preliminary step to amplify the target HHV-6 DNA and EBV-DNA, according to the manufacturer's instructions in order to use in Real Time PCR.

Results: One hundred and ten cases included in this study (60 out of 110 was diagnosis with MS 21 male and 39 female) with Mean+-SDE age (31.66 ± 11.10) years, ranging from 9 to 63 years, and 50 out of 110 23 males and 27female apparently healthy control while the mean age of healthy controls was 32.42 ± 11.89 years, ranging from 17 to 70 years. The results of RT-PCR amplification revealed that the ratio of positive detection of *Human herpesvirus 6 (HHV-6)* 23 (38.3%) out of 60 patients with MS and 6 (12.0%) of healthy control has positive detection of *HHV-6*, and the difference was significant (P= 0.002) as shown in Table (4). Furthermore, the detection of Epstein-*Barr virus (EBV)* in patients with MS and healthy control show 38 (63.3%) out of 60 patients with MS and 12 (24.0 %) of healthy control have positive detection of *EBV*, and the difference was significant (P= 0.001).

Conclusion: We detected a significantly higher number of individuals with DNA of EBV and HHV6 in their blood among patient with MS compared with the control group, the results suggest association between the presence of DNA of HHV6, EBV and MS.

Keywords: Multiple sclerosis, HHV6, EBV, Real time-PCR.

Introduction

Multiple sclerosis (MS) is a severely debilitating progressive inflammatory disease of the central nervous system (CNS) [1]. The basic pathology is thought to be auto-immune mediated damage to the myelin sheaths of the central nerves [2]. This is supported by the finding of plaques, areas of the damage, particularly within the white matter around the lateral ventricles of the brain and optic nerves [3,4]. Demyelination of the white mater in MS is

routinely demonstrated by conventional MRI techniques [5]; however, lesions in the grey matter are also demonstrated [6]. It appears that the degree of cortical demyelization reflects the clinical progression of MS with demyelination of the grey matter associated with the progressive form of the disease along with neuronal loss, while myelin destruction is detected in relapsing–remitting MS [7,8]. Cortical lesions can also be detected at the early stages and they correlate with the disease severity [9].

There is an established epidemiological link between herpesvirus infection status and the risk of MS. Herpesviruses have a near ubiquitous prevalence in adult populations and are usually contracted in early childhood with little overt disease [10]. There are several herpes virus types known to be human pathogens: alpha, beta and gamma [11]. Members of each group, namely, alpha (varicella–zoster virus, VZV), beta (cytomegalovirus (CMV) and human herpesvirus 6 (HHV-6)) and gamma (Epstein–Barr virus EBV), are all suspected of having a potential role in MS. Herpesviruses can establish two replication cycles: latency and reactivation. Herpesviruses have multiple targets, including neuronal (alpha-herpesviruses), non-neuronal (beta and gamma herpesviruses), macrophages and B cells [12,13]. Herpesviruses targeting neurons directly or indirectly can contribute to tissue damage detected in MS.

EBV and HHV-6 belong to the Herpesviridae family, are DNA viruses surrounded by an icosahedral capsid and have an envelope consisting of a lipid bilayer of cell origin and viral glycoproteins. Both are latent viruses responsible for infections that can reactivate over the years and is among the most well-established environmental risk factors in MS (14).

The best evidence linking EBV and MS comes from cross-sectional and prospective serologic studies. Studies comparing adult MS patients with controls have consistently found a higher prevalence of antibodies against EBV in the patients with MS [15]. Although the association between EBV and MS is well-established, the relation of the virus to the disease is not clear. If EBV plays a pathogenic role, then the characteristics of EBV infection in MS might differ from those in controls. The viral load in the blood might be higher, the virus might be more active with more frequent lytic infections, and viral activity might correspond with disease activity [16]. human herpesvirus-6 (HHV-6) may play a role in the pathogenesis of RRMS, there is not enough information related to SPMS patients; although they evolve from RRMS, both types could differ in the role of HHV-6 in the triggering or in the maintenance of the disease [17]. this study aimed to evaluate the relationship between viral diseases, including Epstein–Barr virus (EBV), human herpes virus and MS in the present case-control study.

Materials and Methods

Study Population

This research was carried out between the 15th of November 2023 and the 15th of February 2024 on sixty Multiple Sclerosis Iraqi patients (21 males and 39 females) with ages ranged 9-63 years who were clinically diagnosed by a consultant medical staff in gastrointestinal center in AL-Saader Medical City /Najaf/Iraq, in addition to fifty healthy individuals (control group) who had been randomly selected to be matched with the patients regarding age and gender.

Blood Sample Collection & viral DNA Extraction

After obtaining informed consent, five milliliters venous blood sample was collected aseptically from all enrolled subjects by an expert phlebotomist. Three milliliters of Blood samples from the patient and control groups were separate to extraction of viral DNA from leukocytes by using a specific viral DNA extraction kit (Patho Gene-spinTM

DNA/RNA Extraction Kit, Intron/Korea) according to company instruction. DNA quality was assessed by using agarose gel electrophoresis (0.8% agarose gel, 80 volts, 30 minutes) with subsequent visualization under UV light. Good quality DNA samples were kept on -20 OC until further analysis. However, samples with low quality or quantity of DNA were subjected to another round of viral DNA extraction.

Detection of EBV and HHV-6 by Real-Time Polymerase Chain Reaction

Firstly, the viral genome (DNA) is isolated from samples, Secondly, amplified for each sample in real-time PCR (qPCR). PCR amplification was performed with SYBR Green PCR Mix, GoTaq 1-Step RT-qPCR System (Promega, USA). The primers sequence was mentioned in Tables (1) to detect the EBV and HHV-6 genome. Using specific primers according to the mentioned conditions as illustrated in **Table (1)**

Table 1: PCR primer information for Detection of EBV and HHV-6

	Primer name	Sequence(5′-3′)	Product size (bp)
EBV	Forward Reverse	TTGTGCGGGTCCGTTCCCATCATA TCGGGATAGAAAAACCTAATCCCT	214 bp
HHV-6	Forward Reverse	CCTTAGGAGGAACAAGTCCC GGCTGGTGTCACCTGTGTTA	145 bp

The conditions of amplification reactions were describing in Table (2)

Table 2: PCR amplification conditions for detecting HHV-6 and EBV

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of Cycle
EBV	95	95	60	72	72	35
HHV-6	95	95	60	72	72	35

Statistical Analysis

All the tests were performed on SPSS version 20 (IBM Inc. Armonk, NY, USA). Chi square test helped in assessing the association between EBV and HHV-6 polymorphism and Multiple Scleroses. It also calculated the genotypic frequencies, while allelic frequencies were calculated by gene counting method.

Ethical approval

Following the ethical guidelines provided in the Declaration of Helsinki, the inquiry was conducted. The operation was performed following the acquisition of both verbal and written consent from the patients prior to sample collection. The study protocol, the subject information, and the consent form were evaluated and granted permission by a local ethics committee, in line with document number 3342, which specifies the date in December 2023, for the purpose of obtaining this approval.

Result

Blood samples were collected from individuals in both case and control groups and analyzed for detection of EBV and HHV-6 DNA, one hundred and ten cases included in this study (60 out of 110 was diagnosis with MS 21 male and 39 female) with Mean+-SDE age (31.66 ± 11.10) years, ranging from 9 to 63 years, and 50 out of 110 23 males and 27female apparently healthy control while the mean age of healthy controls was 32.42 ± 11.89 years, ranging

from 17 to 70 years. The majority of participants in the study were under 30 years of age. Among the MS patients, 35.0% were male, with the remaining 65.0% being female. In contrast, the control group comprised 46.0% males and 54.0% females. This demographic distribution is visually represented in Figure 1.

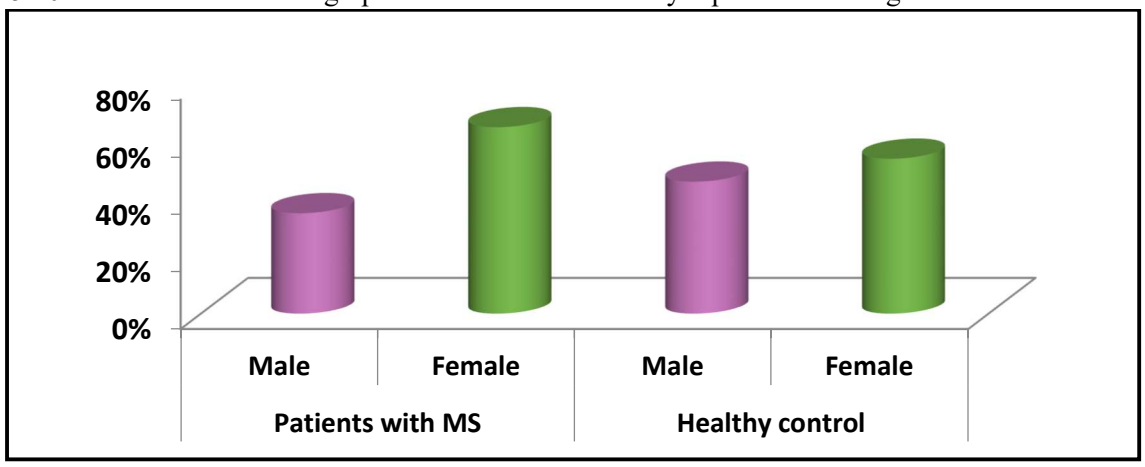

Figure (1): Distribution of patients and control subject according to gender.

Furthermore, the study revealed no statistically significant difference in the gender distribution between patients with MS and the control (P = 0.241), as illustrated in Table (3). It is noteworthy that a majority of the participants enrolled in this study were female.

Table (3): Demographic characteristics of healthy patients with MS and control

Characteristic	Patients with MS *n* = 60	Healthy control *n* = 50	*P*
Age (years)			
Mean ±SD	31.66 ± 11.10	32.42 ± 11.89	0.732 † NS
Range	9–63 years	17– 70 years	
< 30, *n* (%)	29 (48.3%)	22 (44.0%)	0.686 ¥ NS
30-39, *n* (%)	17 (28.3%)	18 (36.0%)	
≥ 40, *n* (%)	14 (23.4%)	10 (20.0%)	
Gender			
Male, *n* (%)	21 (35.0 %)	23 (46.0 %)	0.241 ¥ NS
Female, *n* (%)	39 (65.0%)	27 (54.0%)	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; ¥: Chi-square test; NS: not significant at P > 0.05; MS: multiple sclerosis

The results of RT-PCR amplification revealed that the ratio of positive detection of *Human herpesvirus 6 (HHV-6)* 23 (38.3%) out of 60 patients with MS and 6 (12.0%) of healthy control has positive detection of *HHV-6*, and the difference was significant (P= 0.002) as shown in Table (4). Furthermore, the detection of Epstein-*Barr virus (EBV)* in patients with MS and healthy control show 38 (63.3%) out of 60 patients with MS and 12 (24.0 %) of healthy control have positive detection of *EBV*, and the difference was significant (P= 0.001).

Table (4): Detection of *Human herpesvirus 6 (HHV-6) and Epstein-Barr virus (EBV)* in patients with MS and healthy control by Real-time PCR

Characteristic	Patients with MS *n* = 60	Healthy control *n* = 50	*P*
Human herpesvirus 6 (HHV-6)			
Positive, *n* (%)	**23 (38.3%)**	**6 (12.0%)**	**0.002 ¥ S**
Negative, *n* (%)	**37 (61.7%)**	**44 (88.0%)**	
Epstein-Barr virus (EBV)			
Positive, *n* (%)	**38 (63.3 %)**	**12 (24.0 %)**	**0.001 ¥ S**
Negative, *n* (%)	**22 (36.7%)**	**38 (76.0%)**	

n: number of cases; ¥: Chi-square test; S: significant at P < 0.05;

The frequency distribution of HHV-6 positive patients by age group included 14 cases in less than 30 years age group, 5 cases in the 30-39 years group and 4 cases in more than 40 years age group. While the frequency distribution of HHV-6 negative patients by age group was, less than 15 years age group, 12 cases in the 30-39 years group and 10 cases in more than 40 years age group, and the difference was non-significant (p=0.309). While it included frequency distribution of EBV positive patients according to age group, 17 cases in less than 30 years age group, 9 cases in the 30-39 years group and 12 cases in more than 40 years age group,distribution EBV negative according to the age group, 12 cases in less than 30 years age group, 8 cases in the 30-39 years group and 2 cases in more than 40 years age group and the difference was non-significant at (P= 0.130). While the frequency distribution of patients with HHV-6 positive by gender included 5 cases with male gender and 18 cases with female gender, while the frequency distribution of patients infected with HHV-6 negative by gender included 16 cases with male gender and 21 cases with female gender, while the frequency distribution of patients positive for EBV positive by gender included 15 cases with male gender and 23 cases with female gender, while the frequency distribution of patients with EBV negative by residency included 6 cases with male gender and 16 cases with female gender, and the difference was non-significant at (P = 0.340).

Table (5): Number and percentage of *HHV-6 and EBV* according to the age interval and gender by Real-time PCR

Age group	*HHV-6 +/-*	*EBV +/-*	X^2	*P value*
< 30, *n* (%)	14/15 (48.3%)	17/12 (58.6%)	0.624	0.430
30-39, *n* (%)	5/12 (29.4%)	9/8 (52.9%)	1.943	0.163
≥ 40, *n* (%)	4/10 (28.6%)	12/2 (85.7%)	9.333	0.002*
X^2	2.350	4.088		
P value	0.309	0.130		
Gender				
Male, *n* (%)	5/16 (23.8%)	15/6 (71.4%)	9.545	0.002*
Female, *n* (%)	18/21 (46.2%)	23/16 (59.0%)	1.285	0.257
X^2	2.883	0.912		

P value	0.090	0.340	

¥: Chi-square test; *: significant at P ≤ 0.05

Discussion

MS is a chronic inflammatory disease characterized by demyelination of CNS. Several viruses have been suggested to be involved in the pathology of MS, and the most often sited viruses are EBV and HHV-6 [18]. The present study investigated the prevalence of EBV and HHV-6 among patients with and without MS. Our results showed significantly differences positive detection of HHV6 compared to healthy individuals at (P=0.002), our finding agreement with many available reports showed that the presence of HHV-6 in MS is significantly differences [19] [20]. HHV-6 is neurotropic and have capacity to establish lifelong latency in cells of the central nervous system, with potential to reactivate and cause complications later in life [21]. A breakdown in the Blood Brain Barrier (BBB) during an acute infection with HHV-6, may result in the same antibody detection in sera or CSF samples. In both cases the antibodies will originate from the periphery which, of course, does not suggest a direct implication of HHV-6 in the pathogenesis of MS [22].

Our study show association between HHV6 and MS this similar result of Wilborn and colleagues suggested a potential role for HHV6 in the development of MS [23]. In a systematic review, Pormohammad and others reported a relationship between HHV6 infection and MS [24]. However, Hon and colleagues did not support a causative role for HHV6 in the development of MS [25]. Some other studies also did not confirm any association between HHV6 and the development of MS [26] [27].

In addition, there are many alternative scenarios that theoretically may lead to the presence of HHV-6 antibodies in the biological fluids of MS patients. Some of those are reactivation of a latent HHV-6 infection, a subclinical infectious course without any clinical symptoms to i.e. immunocompromised patients and immune system hyperactivity in the case of MS relapse. Moreover, a positive sample for HHV-6 antibodies can be the result of an infection in childhood or an active infection in adult life. It remains to be seen whether these two conditions can have the same influence in pathogenesis of MS, given the fact that their chronologic encounter is quite different [28].

MS is the most prevalent chronic inflammatory and neurodegenerative disease of the central nervous system and is thought to be triggered in genetically predisposed individuals by an infectious agent, with EBV as the lead candidate, is a ubiquitous human lymphotropic herpesvirus with a well- established causal role in several cancers [29]. The risk of MS increases approximately 32- fold with EBV infection, and more with symptomatic to severe infectious mononucleosis and HLA- DR2b (HLA- DRB1*1501b and HLA- DRA1*0101a) [30]. The result of detection of Epstein-*Barr virus (EBV)* in patients with MS and healthy control show 38 (63.3%) out of 60 patients with MS and 12 (24.0 %) of healthy control have positive detection of *EBV*, and the difference was significant (P= 0.001), our finding disagreement with study conducted by Asouri *et al* that find a significantly higher prevalence of EBV among the control group, while there was no significant association between MS and other viral pathogens [31]. Furthermore, Ramroodi and co-workers reported a significant association between the detrimental effects of EBV and MS attacks [32] that agreement with our result. However, Cocuzza and others did not find any association between EBV and MS disease [33] In addition to Cocuzza, Franciotta and colleagues did not report any significant relationship between viral infections and MS disease [34].

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