



# Molecular Detection of Epstein–Barr Virus, Human Herpes Virus 6, Cytomegalovirus, and Hepatitis B Virus in Patients with Multiple Sclerosis

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## ABSTRACT

### BACKGROUND

Multiple sclerosis (MS) is a chronic disease with significant morbidity. A wide spectrum of risk factors has been suggested that triggers the development of MS. Among them, several viral infections have been implicated to play a role in MS pathogenesis.

We aimed to evaluate the relationship between viral diseases, including Epstein–Barr virus (EBV), human herpes virus 6 (HHV-6), cytomegalovirus (CMV), and hepatitis B virus (HBV) and MS in the present case-control study.

### METHODS

About 100 patients with confirmed MS and age- and sex-matched individuals were selected as case and control groups, respectively. The patients were randomly selected from individuals diagnosed by neurologists based on the clinical signs and symptoms and imaging procedures.

### RESULTS

More than 100 patients with MS and patients who were referred for other causes were analyzed for the presence of DNA of EBV, HHV6, CMV, and HBV separately. 9.37% of the control group had a positive test for the DNA of EBV in a real-time polymerase chain reaction (PCR), while the frequency of positive test result was zero in the case group ( $p = 0.0012$ ). HBV DNA was not detected in both the case and control groups. The prevalence of CMV was 0.88 and zero in the control and case groups, respectively ( $p = 0.3410$ ). For HHV6, 9.73% of the control group had a positive result, while this test was positive in 5.88% of the patients with MS ( $p = 0.2959$ ).

### CONCLUSION

We detected a significantly higher number of individuals with DNA of EBV in their blood among the control group compared with the case group. In conclusion, the results suggest a surprisingly adverse association between MS and EBV, and no association was found between the presence of DNA of HBV, CMV, and HHV6 and MS.

### KEYWORDS:

Multiple Sclerosis, EBV, HHV6, CMV, HBV.

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## INTRODUCTION

Multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system, is characterized by the inflammation and demyelination of white matter and neurodegeneration of the central nervous system (CNS) that leads to significant morbidity and disability. Multifocal demyelinating lesions eventually



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lead to a wide range of clinical symptoms such as cognitive decline, weakened motor skills, behavioral deficits, and vision loss.<sup>1,2</sup> This chronic immune-mediated disease is considered as the leading cause of non-traumatic related neurologic disability among young people.<sup>3-5</sup> People with MS may experience a lower life expectancy of up to 7 years based on some previous reports.<sup>6</sup> It is estimated that more than two million people are suffering from MS.<sup>7</sup> Based on one report, approximately 2.3 million people were suffering from MS in 2013.<sup>8</sup> Previous studies in Iran showed that the prevalence and incidence of MS are rapidly increasing, particularly in women.<sup>9</sup> Overall, it was shown that the prevalence and incidence of MS have critically increased in the last two decades.<sup>10</sup>

Both the environmental and genetic factors are involved in the etiology of MS. Many genes were reported to increase the disease susceptibility, including those who reside in HLA-DRB1\*15 loci and alleles in strong linkage with this allele.<sup>11</sup> There are multiple environmental risk factors for the development of this disease, including sex, age, smoking, vitamin D deficiency, sunshine (UVB), and some pathogens.<sup>3</sup> Analyzing the role of infectious agents in neurological diseases such as MS has been the goal of many studies.<sup>12-21</sup> It has been proposed that the activation of autoreactive lymphocytes in the CNS against the infectious agents expressing antigenic molecules, mimicking the glycoproteins and glycolipids, on the surface of the neural cells is conceivably the pathology behind the progression of MS.<sup>12,13</sup> Several viruses have been associated with the pathogenesis of MS. Among them, Epstein-Barr virus (EBV), human herpes virus 6 (HHV-6), cytomegalovirus (CMV), and hepatitis B virus (HBV) have been evaluated for a possible causal association with MS.<sup>14-21</sup> but inconsistent findings have been provided in different societies.

The aim of this study was to evaluate the relationship between MS and the incidence of infection with EBV, HHV6, CMV, and HBV in a case-control study using real-time PCR as a sensitive molecular method for DNA detection.

## MATERIALS AND METHODS

### Studied subjects

The study participants included 102 patients with MS

as the case group and 113 patients as the control group, that were randomly selected from individuals referring to Sina and Shomal Hospitals in Tehran and Amol, Iran. The patients with MS were selected from the individuals whose diseases were positively diagnosed by a neurologist based on McDonald criteria (2010), and confirmed by brain magnetic resonance imaging (MRI). These patients suffered from relapsing-remitting MS (RRMS) or secondary-progressive MS (SPMS). The control group was selected from the patients who were referred to the mentioned hospitals because of other health problems. Both groups were matched according to sex and age. The demographic data, including age, sex, educational level, and the job of the participants were collected. All procedures performed in this study, which involved human participants, were in accordance with the ethical standards of the Pasteur Institute of Iran's Research Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Research Ethics Committee at the Pasteur Institute of Iran (ir.pii.rec.1398.033). Written informed consent was obtained from the participants.

### DNA extraction

DNA was extracted from the blood samples using the QIAamp DNA Mini Kit (Cat. 51104; Qiagen Inc., USA) according to the manufacturer's instructions. Briefly, 200  $\mu$ L samples were lysed in the presence of lysis buffer and proteinase K and incubated for 10 min at 56 °C. Then, ethanol was added and mixed by pulse-vortexing. The mixture was loaded into the QIAamp Mini spin column and centrifuged at 8000 rpm for 60s. The DNA bound to the column was eluted using 60  $\mu$ L elution buffer.

### Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was used to screen the presence of DNA of EBV, CMV, HBV, and HHV6 in the samples on a StepOnePlus™ real-time PCR system (Applied Biosystems, Life Technologies) in the presence of specific primers and probe (table 1) and HotStarTaq® Plus DNA Polymerase (Qiagen, Hilden, Germany). The cycling conditions consisted of an initial denaturation at 95°C for 5 min followed by 50 cycles, 95°C for 15 s, and annealing at 60°C for 1 min. Data were collected during each the annealing phase.

**Table 1: Primers used for Viral DNA detection**

Virus	Viral target	Sequence 5–3	Ref
CMV	Forward	CAGTCCCGAGACMGTGAGAC	10
	Reverse	TGAACATCCCCAGCATCAACG	
	Probe	FAM-TGCCACATCTGCTTGCCCCGACGC-BHQ1	
HBV	Forward	GGCCATCAGCGCATGC	11
	Reverse	GCTGCGAGCAAAACA	
	Probe	FAM-CTCTGCCGATCCATACTGCGGAACTC-BHQ1	
EBV	Forward	CGGAAGCCCTCTGGACTTC	12
	Reverse	CCCTGTTTATCCGATGGAATG	
	Probe	FAM-TGTACACGCACGAGAAATGCGCC-BHQ1	
HHV6	Forward	TCGAAATAAGCATTAAATAGGCACACT	13
	Reverse	CGGAGTTAAGGCATTGGTTGA	
	Probe	FAM-CCAAGCAGTTCGTTTCTCTGAGCCA	

### Statistical analysis

To compare the continuity of data in the two studied groups, we performed independent t test for data with a normal distribution. When the data did not have the normal distribution, the non-parametric Mann-Whitney test was used to compare the related data in the two groups. To evaluate the association between nominal data, the Chi-square test was used. To remove the confounding effects of other potential mediators, logistic regression analysis with MS as the outcome and viral infections as independent variables were done on the data of patients with MS. The significance level was considered 0.05 for all analyses. All analyses were performed using SPSS software version 21.

### RESULTS

Blood samples were collected from individuals in both case and control groups and analyzed for detection of HBV, EBV, CMV, and HHV-6 DNA. Most of the samples (99 samples) in the case group were obtained from patients with RRMS, whereas three samples were taken from patients with SPMS. The general characteristics of the study populations in the case and control groups are presented in table 2. Individuals with MS had a significantly higher weight than the individuals in the control group ( $p < 0.001$ ). Furthermore, a significantly higher proportion of patients with MS had a positive family history of autoimmune diseases (table 2,  $p < 0.0001$ ). Most of the patients with MS were female (84 out of 102), showing the higher prevalence of MS among women (Table 3).

HBV DNA was absent in both control and case subjects, and EBV and CMV DNAs were detected only in the control group. 10 individuals (out of 11) in the control group and all individuals in the case group that were positive for HHV-6 were female. Overall, the majority of infected patients were female in both case and control groups (control: 85 women out of 112 [75.8%]; Case: 84 women out of 102, [82%]).

Table 4 shows the history of infection with HBV, EBV, CMV, and HHV6 in patients with and without MS based on the presence of viral DNA in their blood. The data show that patients without MS had a significantly higher frequency of EBV infection than patients with MS ( $p = 0.0012$ ). There was no significant difference between the case and control groups for the presence of other viral DNAs.

### 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. The present study investigated the prevalence of HBV, EBV, CMV, and HHV-6 among patients with and without MS. The results of this study showed a significantly higher prevalence of EBV among the control group, while there was no significant association between MS and other viral pathogens. Based on our results, a significantly higher proportion of people without MS had EBV infection compared with people with MS. This finding is not con-

**Table 2: General characteristics of individuals in case and control groups**

Characteristics	Mean ± SD		p- value
	Case	Control	
Age (year)	35.29 ± 14.66	31.96 ± 8.25	0.2851
Weight (Kg)	76.68 ± 14.57	64.56 ± 13.28	< 0.001
Height (cm)	164.29 ± 14.16	164.43 ± 8.65	0.7064
SBP (mmHg)	112.25 ± 14.36	109.50 ± 12.21	0.2613
DBP (mmHg)	69.00 ± 8.87	73.22 ± 9.10	0.0021
Characteristics	Proportion (95% CI)		p- value
	Case	Control	
A positive history of smoking	8.00 (2.68 – 13.32)	8.11 (3.03 – 13.19)	0.9770
A positive history of alcohol use	4.01 (0.16 – 7.85)	4.46 (0.64 – 8.29)	0.8671
A positive family history of Autoimmune disease	52.75 (42.49 – 63.00)	19.64 (12.28 – 27.00)	< 0.0001

SBP = Systolic blood pressure, DBP = Diastolic blood pressure

**Table 3: Detection of EBV, HBV, CMV, and HHV-6 in the blood samples of control and case groups**

Characteristics	Gender	Number	EBV	HBV	CMV	HHV-6
Control	Male	27	4	0	0	1
	Female	85	6	0	1	10
Case	Male	18	0	0	0	0
	Female	84	0	0	0	7

**Table 4: The prevalence of viral infection for HBV, EBV, CMV, and HHV6 in case and control groups**

Viral infection	Proportion (95% CI)		p- value
	Case	Control	
HBV	0.00 (-)	0.00 (-)	1.000
EBV	9.73 (4.26 – 15.20)	0.00 (-)	0.0012
CMV	0.88 (0 – 1.97)	0.00 (-)	0.3410
HHV6	9.73 (4.27 – 15.20)	5.88 (0.32 – 10.45)	0.2959

sistent with the association between EBV and MS development or exacerbation of MS attacks, as indicated by some previous studies.<sup>22</sup> Agostini and colleagues reported that DNA viral loads and EBV nuclear antigen-1 (EBNA-1) antibody titers were significantly higher in people with MS than those without it.<sup>23</sup> Furthermore, Ramroodi and co-workers reported a significant association between the detrimental effects of EBV and MS attacks.<sup>24</sup> However, Cocuzza and others did not find any association between EBV and MS disease.<sup>25</sup> In addition to Cocuzza, Franciotta and colleagues did not report any significant relationship between viral infections and MS disease.<sup>26</sup> They examined the serum and cerebrospinal fluid (CSF) of patients with MS for HSV, varicella-zoster virus (VZV), CMV, EBV, and HHV-6 by PCR amplification method. All serum and CSF samples but one were negative for the existence of

viral DNA. On the other hand, in a prospective cohort study, Munger and co-workers showed that anti-EBNA antibodies might be considered as strong markers for the risk of MS development.<sup>27</sup>

We did not find any HBsAg positive individual in our case and control groups. The wide spectrum application of hepatitis B vaccination in Iran could have a critical role in the reduction of hepatitis B prevalence, particularly in young adults. As a result, no detection of HBsAg in our subjects, both MS and control groups, could be expected considering the age of the patients with MS and their age-matched control group. Although some studies have suggested an association between hepatitis B vaccination and the development of MS, a large body of research studies rejects the possibility of any association between them.<sup>28-30</sup>

We also evaluated CMV in our case and control groups. While only one positive CMV infection was detected in the control group, no case was detected in those with MS. In a case-control study in northern Iran, Najafi and colleagues found the DNA of CMV in 28% of patients with MS compared with only 2% in the control group.<sup>31</sup> Zivadinov and others reported a relationship between positive titer of CMV antibody and better clinical outcomes of MS, including a later onset of disease development in the lifetime, lower relapse of the disease, and less brain atrophy through MRI evaluation.<sup>32</sup> In contrast to Zivadinov and others, Salim and co-workers reported that CMV infection could intensify the clinical symptoms in patients with MS<sup>32,33</sup>, but our study suggests no association between a positive CMV infection and MS.

Also, our study did not show any association between HHV6 and MS (table 3,  $p = 0.2959$ ). Wilborn and colleagues suggested a potential role for HHV6 in the development of MS.<sup>34</sup> In a systematic review, Pormohammad and others reported a relationship between HHV6 infection and MS.<sup>35</sup> However, Hon and colleagues did not support a causative role for HHV6 in the development of MS.<sup>36</sup> Some other studies also did not confirm any association between HHV6 and the development of MS.<sup>37,38</sup> Our results are in agreement with the latter reports.

Interestingly, the results regarding the relationship between viral infections, including HBV, CMV, and HHSV6 and the progression to MS are not consistent, and the higher incidence of EBV among the control group suggests an adverse relationship between MS and infection with EBV. While some studies reported a relationship between these viruses and MS, others did not confirm such a relationship. The presence of DNA of HHV6 was also higher in people without MS in comparison with patients with MS, though this was not statistically significant (table 4). This result can be explained by a higher self-care of people with MS for viral infections. Consequently, it may be possible to use more care to prevent viral diseases, as directed by physicians or some self-care measures. On the other hand, due to some disabilities, individuals with MS may play a passive role in the society and thus have lower active contact with other members of the society who are chronic or active carriers of related viruses; and this may result in the lower risk of

viral infections. Furthermore, differences in the genotypes of viral infections in different populations may explain the inconsistent results regarding the relationship between these viral infections and the development of MS. Differences in genetic and ethnicity among various populations evaluated by different studies can explain another aspect of this incontinuity.<sup>6,39,40</sup>

Like any other research, this study had some limitations. We conducted a case-control study to compare the prevalence of viral infections. In fact, we could not determine whether the infection among MS patients occurred before MS development or during the disease course. Regarding the results from EBV evaluation, we cannot exclude the possibility that EBV could exist in a latent form in any body-compartments, especially in peripheral blood mononuclear cells (PBMCs). As a result, a cause and effect relationship could not be established based on our findings. Although we did a group matching based on some demographic data, including age and sex, many other potential confounding mediators could muddle our findings. On the other hand, the strong associations can be affected by the rare cases of exposure to viruses in our case and control groups. Thus, large cohort population-based studies or high sample sizes can be more helpful in this context.

## CONCLUSIONS

A significantly higher incidence of viral DNA was detected in the control group in comparison with the case group suggesting a possible adverse effect of EBV on MS. No association was found between the presence of HBV, CMV, and HHV6 DNA and MS disease.

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**ETHICAL APPROVAL**

There is nothing to be declared.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest related to this work.

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