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## ORIGINAL ARTICLE

# Human herpesvirus-6 viral load and antibody titer in serum samples of patients with multiple sclerosis

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**KEYWORDS**

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**Background:** Despite the number of cases with definite diagnosis of multiple sclerosis (MS) being on increase, the role of human herpesvirus-6 (HHV-6) infection as a trigger for MS disease still is deliberated. Based on antibody detection and quantitative HHV-6 polymerase chain reaction assay, this study was achieved to find out the possible association between infection with HHV-6 and clinical progression of MS disease.

**Methods:** A total of 108 serum samples were obtained from 30 MS patients followed prospectively for a 6-month period. These samples were analyzed for the presence of HHV-6 DNA by nested polymerase chain reaction enzyme-linked immunosorbent assay and for anti-HHV-6 IgG titer. Activation of the disease was determined by either magnetic resonance imaging or by clinical status of the patients. Control groups were also included.

**Results:** The average antibody index for the MS patients in the first sample collection was higher than both control groups ( $p = 0.001$ ). HHV-6 DNA was detected in the serum samples of 10 of 30 MS patients. The mean HHV-6 viral load in patients with relapsing-remitting multiple sclerosis (RRMS) with and without relapse was 973 and 714, respectively. Seven patients showed an exacerbation during the study period. Of those, four patients had HHV-6 DNA in their collected samples. The prevalence of HHV-6 DNA was significantly higher in patients with MS as compared with control groups ( $p = 0.001$ ).

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**Conclusions:** The results indicate that HHV-6 is implicated somehow in MS disease. Over time, rising HHV-6 IgG antibody titers together with an exacerbation and detection of HHV-6 DNA in serum samples of some MS patients suggests possible association between the reactivation of the virus and disease progression.

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## Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS).<sup>1–3</sup> Several types of studies over the past decade support the hypothesis that human herpesvirus-6 (HHV-6), which is associated with a common childhood illness, roseola, is a strong candidate to play a role in the course of MS, either as a causal agent or as a cofactor. In recent years, HHV-6 has been investigated as a possible causative agent for MS. These studies have examined anti-HHV-6-specific antibody responses, HHV-6 viral DNA, or HHV-6 presence in the CNS tissue in both MS patients and controls.<sup>4–11</sup> However, there are still discrepancies between the results obtained from studies concerning how the presence of HHV-6 is linked with clinical activity in MS patients. Active HHV-6 viral infection in some MS patients may fluctuate over time during the progression of the disease,<sup>12</sup> causing variation in viral detection.

The prevalence of MS, according to World Health Organization reports, should be about 4 in 100,000. However, a recent study has shown that Iran could be considered as an area with a medium to high risk of MS.<sup>13</sup>

Despite the number of cases with definite diagnosis of MS being on the increase in this region, the role of HHV-6 active infection in this group of patients has not yet been studied. Therefore, based on IgG antibody titer and quantitative HHV-6 polymerase chain reaction (PCR) assay, this study was performed to determine the possible association between infection with HHV-6 and clinical parameters of MS disease progression. The results were then compared with HHV-6 infection in normal subjects and in a group of patients with other neurological diseases (OND).

## Methods

### Patients and samples

Thirty MS patients (21 females and 9 males) with clinically definite MS were randomly selected from those attending the Neurology Clinic at the Namazi Hospital, Shiraz University of Medical Sciences, Shiraz, Iran during February 2008 and January 2009. The mean duration of the MS disease was 4 years (2–20 years). The mean age of the patients with MS was 33 years (18–45 years). Twenty-two patients had relapsing-remitting MS (RRMS), seven had secondary progressive MS (SPMS), and one patient had primary progressive MS (PPMS). These patients suffered from various symptoms and clinical manifestations. Activation of the disease (exacerbation) was determined by either gadolinium-enhancing magnetic

resonance imaging (MRI) or clinical status according to the criteria of Poser et al.<sup>14</sup>

The control patients included 20 individuals (11 females and 9 males) with OND including motor neuron disease, dizziness, cerebrovascular disease, migraine, meningitis, and febrile seizure, and 20 randomly selected healthy blood donors (10 females and 10 males). The mean age of the control groups were 32 years (18–48 years) and 28 years (18–35 years), respectively. All the participants gave informed consent before the examination.

A total of 108 serum samples were collected from 30 MS patients. Two to four serum samples were collected from each patient during the 6 months follow-up of the study. Surveillance serum samples were collected at first admission and then every 4–6 weeks for 6 months. MRI was performed for the entire patients on the day of admission and for 18 subjects at the end of the study. During the study period, 15 MS patients were under treatment of Interferon beta-1a (Avonex, Biogen Idec Brazil, Brazil). Serum samples were also collected from the patients with OND and for the normal subjects as well. All the serum samples were frozen at  $-70^{\circ}\text{C}$  to be used for DNA detection by PCR and enzyme-linked immunosorbent assay (ELISA) for detection of specific antibodies against HHV-6.

### Extraction and amplification of viral DNA

DNA extraction from serum samples was performed by the methods detailed in Kramvis et al.,<sup>15</sup> with some modifications. Serum (100  $\mu\text{L}$ ) was extracted with a proteinase K-sodium dodecyl sulfate (SDS) extraction mix (267  $\mu\text{g}$  of proteinase K per mL, 1% sodium dodecyl sulfate, 2.5 mM EDTA, 25 mM sodium acetate) by incubation at  $70^{\circ}\text{C}$  for 2 hours. DNA was then purified by phenol-chloroform extraction and ethanol precipitation. The resulting DNA pellet was resuspended in 20  $\mu\text{L}$  of sterile water and incubated at  $95^{\circ}\text{C}$  for 10 minutes to reduce or inactivate PCR inhibitory factors. DNA extracts were then allowed to cool and were amplified immediately.

DNA–enzyme-linked immunosorbent assay (DELISA) was designed for quantifying HHV-6 DNA molecules in patient serum samples. The nested primer set complementary to the gene coding the major capsid protein (MCP) was used with the aim of recognizing both HHV-6A and HHV-6B variants. The nucleotide sequence of the primers 5'-GCTA GAACGTATTTGCTG-3' and 5'-ACAACGTCTGACTGGCA-3' (outer pairs); 5'-TCACGCACATCGGTATAT-3' and 5'-CTCAA GATCAACAAGTTG-3' (inner pairs) were used for the first and second round of the PCR assay, respectively. The inner primers amplify a 124-bp fragment of HHV-6. Each PCR contained 5  $\mu\text{L}$  of extracted DNA in a final volume of 50  $\mu\text{L}$

with PCR buffer (Fermentas Life Sciences, UK), 0.2 mM of each deoxyribonucleoside triphosphate, 1.5 mM MgCl<sub>2</sub>, 2 U Taq DNA polymerase (Fermentas), and 0.5 μM of each specific primer. Plasmid DNA was used as the positive control. To exclude the possibility of contamination during the PCR, HHV-6 negative DNA, as well as water controls, was included in each experiment. The first round of PCR was carried out at 94°C for 3 minutes, followed by 30 cycles of 94°C for 40 seconds, 51°C for 1 minute, and 72°C for 40 seconds. Terminal extension of 72°C for 5 minutes was carried out after the completion of the 30 cycles. A sample of the first round product (3 μL) was used as template for the second round using, the conditions described for the first round except that 10 μM of digoxigenin-11 dUTP (Roche, Mannheim, Germany) was also added to the PCR mixture.

### Detection of amplified products

The PCR ELISA method was also used to determine the virus load. Digoxigenin-labeled PCR products were detected by using the commercially PCR ELISA digoxigenin detection kit (Roche). The method was optimized for the detection of either HHV-6A or HHV-6B DNA in the amplified product, using 5'-end-labeled biotin oligonucleotide probe (5'-CT CAAGATCAACAAGTTGCCATTTTCG-3'). The manufacturer's instructions were followed to perform PCR ELISA detection. The final concentration of 16 pmol/mL biotin end-labeled HHV-6 oligonucleotide probe was used as a capture probe during hybridization. The results were expressed as net absorbance (<sup>405</sup>/<sub>492</sub> nm) after the optical density (OD) of the substrate blank was automatically subtracted for each microwell. The cut-off point for test positives was defined as the mean of negative values plus three times the standard deviation; the values below this were defined as negative.

### Determination of the PCR sensitivity

The threshold of sensitivity of the PCR was determined using 10-fold serial dilution of the plasmid DNA containing MCP genes. The amplified products were analyzed by microplate hybridization method.

### Anti-HHV-6 IgG antibody

IgG antibodies to HHV-6 in the serum samples were determined by enzyme immunoassay according to manufacturer's instruction (Biotin, Dublin, Ireland). The presence or absence of HHV-6 antibodies was determined in relation to the cut-off calibrator. An index value was calculated by dividing the sample or control absorbance by the cut-off calibrator value. An index value <0.9 was calculated to be negative, an index value between 0.9 and 1.1 was equivocal, and an index value >1.1 was calculated to be positive.

### Statistical analysis

Statistical difference in the prevalence of HHV-6 infection between the tested groups was assessed by the Fisher's exact test. The  $\chi^2$  test was used to analyze the significance of differences in serology and DNA detection.

## Results

### Determination of PCR ELISA positive/negative cut-off values

The "cut-off" OD <sup>405</sup>/<sub>492</sub> nm point between positive and negative for detection of HHV-6 DNA by PCR ELISA in the serum samples was found to be OD = 0.5. To evaluate the concentration of HHV-6 DNA in the serum samples, the standard curve was plotted using  $8 \times 10^6$  to 8.0 copies of plasmid DNA versus OD (<sup>405</sup>/<sub>492</sub> nm). The sensitivity limit of the PCR ELISA was found to be 370 copies/mL of the HHV-6 genome in the serum samples.

### Prevalence of HHV-6 DNA in MS patients and in control groups

Using nested PCR and DELISA detection methods for the HHV-6 MCP gene, cell-free DNA was detected in the serum samples of 10 of 30 (33.3%) patients with MS. These results were in contrast to 5% (one of 20) either in OND or normal blood donors control groups. The difference between prevalence of HHV-6 DNA in MS patients and in control groups was statistically significant ( $p = 0.001$ ).

The viral DNA was detected at least in one serum sample along the 6 months follow-up in 41% of RRMS samples, 14% of SPMS samples, and none of the primary progressive MS (Table 1). We did not find any significant differences ( $p = 0.36$ ) between the presence of viral DNA in serum samples of RRMS and the SPMS group. However, a larger sample size is needed for better evaluation.

With respect to viral load in serum samples of MS patients, HHV-6 DNA copy number ranged from undetectable to  $1.5 \times 10^3$ /mL. The lowest HHV-6 DNA molecules (427 copies/mL) were found in patients with remission and the highest HHV-6 DNA copy numbers ( $1.5 \times 10^3$  copies/mL) were found in the serum samples of the patients with exacerbation. HHV-6 viral load during active infection was significantly greater ( $p < 0.001$ ) than remission: 765 versus 491 genome/mL of collected serum samples (64% of increase in viral load during acute attack). During study time, 7 of 24 (28.6%) MS patients whose results were completely available were exacerbated. HHV-6 DNA was detected in four of seven patients with exacerbation (Table 1).

MRI was performed for 18 subjects at the end of study. The last MRI results revealed some new demyelinating plaques in three HHV-6 positive individuals with exacerbation, when compared with the previous MRI findings. HHV-6 DNA was detected either in 4 of 15 (27%) of Avonex treated or in 6 of 15 (40%) untreated MS patients. HHV-6 DNA was also detected in one serum sample of the normal controls (1,200 DNA molecules/mL) and in one serum sample of the patients with OND (900 DNA molecules/mL). Both of them were negative for IgG antibody in their serum samples.

### Prevalence of anti-IgG in MS patients and in control groups

The prevalence of positive HHV-6 IgG antibody in MS patients, in patients with neurological diseases other than MS, and in normal subjects was found to be 100% (30 of 30),

**Table 1** Mean viral load for HHV-6 and clinical findings in patients with multiple sclerosis

	HHV-6 DNA (+)	Mean HHV-6 DNA copies/mL	HHV-6 IgG (+)
MS (n = 30)			
RRMS (n = 22)	9	766	22
SPMS (n = 7)	1	610	7
PPMS (n = 1)	None	None	1
Exacerbation (n = 7)	4	882	7
New plaque in MRI	3	759	3
Avonex treatment (n = 15)	4	828	4
Other neurologic disorders (n = 20)	1	900	14
Healthy controls (n = 20)	1	1,200	15

HHV-6 = human herpesvirus-6; MRI = magnetic resonance imaging; MS = multiple sclerosis; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SPMS = secondary progressive MS.

70% (14 of 20), and 75% (15 of 20), respectively. The average antibody index for the MS patients in the first sample collection was 2.68 versus 1.4 for both the control groups ( $p = 0.001$ ).

## Discussion

Many studies have implicated HHV-6 in the etiology of MS; however, this association is controversial, and, as is the case, experimental evidence linking this organism to MS is equivocal. Immunohistochemical analysis of MS plaques has revealed a concentration of an HHV-6 antigen in oligodendrocytes, the CNS cell responsible for the formation and maintenance of myelin.<sup>7,16</sup> An increased incidence of IgG and IgM antibodies against HHV-6 in both serum and CSF in MS patients has been reported.<sup>17,18</sup> It has been indicated that HHV-6 DNA is more frequent in MS patients than in healthy blood donors.<sup>19</sup> In several studies, on the other hand, HHV-6 DNA was not detected in different clinical samples. However, there is still controversy about this theory. In the present study, we have determined the seroprevalance and the quantitative frequency of HHV-6 DNA in serum samples of the patients with MS in different courses of illness. The results were then compared with two control groups including normal individuals and patients with neurological diseases other than MS. Since the presence of HHV-6 DNA in the serum would be an indicator of active infection, we tried to determine the viral load during active infection. The prevalence of HHV-6 DNA was significantly higher in patients with MS (33.3%) compared with those with other neurological disorders and the normal individuals (5%). We found a higher HHV-6 frequency of active infection in RRMS patients than in the other groups. A significant increase in viral load during active infection in comparison with remission ( $p < 0.001$ ) suggesting the possible exposure of HHV-6 antigen to the immune system of the host. To realize the outcome of active HHV-6 infection on clinical symptoms of patients with MS, a quantitative DELISA was established. In four patients with exacerbation, viral DNA load in the serum samples was found to be 830, 610, 1,400, and 690 molecules of DNA/mL respectively. The absence of HHV-6 in serum sample of patients with active MS may be associated with an early stage of viral replication.<sup>19</sup> The last MRI results revealed some new demyelinating plaques in three HHV-6 positive individuals, when compared with their previous MRI results. Of those, two patients had exacerbation during study time,

but the third one had no exacerbation. Our results confirm the relationship between the active lesions on MRI scans and patterns of disease activity in some MS patients. Although our DELISA was not able to detect less than 370 molecules of HHV-6 DNA/mL of the serum samples, using an advanced quantitative real time PCR technique, might improve detection of more active HHV-6 infections in MS patients.

MS patients have increased titer of serum antibody in comparison with control groups. The analysis by ELISA showed that the MS patients had increased prevalence and higher titer of HHV-6 antibody than the control groups. The comparison of the antibody titer in the first sample collection of the MS patients with control groups suggests that either the virus may possibly replicate in MS patients intermittently or the patients had been exposed to reinfection. The prevalence of HHV-6 IgG antibody in all serum samples collected from MS patients (100%) indicates that all MS patients were infected by HHV-6.

Among our results, the prevalence of HHV-6 DNA was not influenced by treatment with beta interferon. These results are compatible with those reported by Alvarez-Lafuente et al.<sup>20</sup>

However, it has not been indicated that the current dosage regimens (for MS patients) produce serum levels of the drug capable of suppressing the replication of HHV-6.

In conclusion, results indicate that HHV-6 is implicated somehow in MS disease. The rise in HHV-6 IgG antibody titer together with an exacerbation and detection of HHV-6 DNA in the serum samples of some MS patients suggests possible association between the reactivation of the virus and disease progression. Nevertheless, further studies concerning the role of HHV-6 in MS disease are clearly warranted.

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