

Original Article

HHV-6 DNA and Antibody Detection in Plasma and PBMC of Multiple Sclerosis Patients in North of Iran

Ahmadi A¹, Naeimi MH², Javid N¹, Moradi A¹, Vakili MA³, Tabarraei A^{4*}

1. Department of Microbiology, Golestan University of Medical Sciences, Gorgan, Iran.
2. Department of Neurology, Sayyad Hospital, Golestan University of Medical Sciences, Gorgan, Iran.
3. Health Management and Social Development Research Center, Golestan University of Medical Sciences, Gorgan, Iran.
4. Infectious Diseases Research Center, Golestan University of Medical Sciences, Gorgan, Iran.

Abstract

Background and Aims: Multiple sclerosis (MS) is defined by the presence of inflammatory demyelinating plaques in the central nervous system (CNS). It has been indicated that human herpes virus 6 (HHV-6) may play role in pathogenesis of MS. The aim of this study was to determine the presence of HHV-6 DNA and anti HHV-6 IgG in MS patients and controls.

Materials and Methods: Blood samples from 59 patients with MS and 59 healthy controls were collected. DNA extraction and real-time PCR based on (Syber Green) were done in the peripheral blood mononuclear cells (PBMCs) and in plasma specimens. Anti-HHV-6 IgG was measured by ELISA technique. Demographic and clinical data were collected and entered in SPSS 16 and analyzed.

Results: HHV-6 DNA was found in the plasma sample of 1 (1.7%) of 59 MS patients. There was no HHV-6 DNA in PBMCs of the MS patients and controls and in plasma of control group. Anti- HHV-6 IgG antibody was present in 84.7% of MS patients and 82.1% of control group.

Conclusion: There was no significant relation based on presence of HHV-6 DNA and antibody between MS patients and controls in north of Iran.

Keywords: HHV-6, Multiple sclerosis, Real-Time PCR, ELISA

Introduction

Multiple sclerosis (MS) has originally been explained as presence of inflammatory demyelinating plaques in the central nervous system (CNS) and etiology of disease is not clear. It should be noted that genetic and environmental factors could play a pathogenic role in MS (1-3). Among environmental factors, Infection with

different viruses has been implicated as a possible triggering event for the invasion of MS (1). Viruses have not been definitively implicated as an agent causing MS, but certain members of human herpes viruses family (HHVs) have been linked with the pathogenesis of MS, because they show neurotropic manner and have ability to establish latent infection and are ubiquitous (4). HHV-6 belongs to the Roseolavirus genus of the Subfamily β - herpesvirus and causes Exantem Subitum disease or (Roseola) a benign febrile illness with skin rash (5). It is classified to variants A and B both can enter the CNS (6, 7). In recent years, HHV-6 has been suggested to be a possible causative agent for MS. Several studies have examined anti

* **Corresponding author:** Alijan Tabarraei, PhD; Infectious Diseases Research Center, Department of microbiology, Golestan University of Medical Sciences, Gorgan, Iran.
Tel: (+98) 17 32422652, Fax: (+98) 17 32440225
Email: alijant@yahoo.com

HHV-6-specific antibody responses, HHV-6 DNA, or HHV-6 presence in the CNS tissue in both MS patients and controls groups (8-12). Although the results reported don't indicate relation HHV-6 and MS disease clearly. The goal of this work was to clarify the incidence of HHV-6 infection in MS patients and healthy control to determine association between the presence of HHV-6 in MS patients as compared with the control group.

Methods

Fifty nine MS patients and 59 healthy individuals as control group from Golestan province in north of Iran were selected in 2014. Control group did not have any symptoms of MS and were matched for age, gender, and socioeconomic status. Subsequently demographic and clinical data were recorded. 5 mL of whole blood with EDTA were obtained from MS patients (RRMS samples were collected in remission phase) and control group and separated its plasma and PBMC for DNA extraction and antibody detection. All samples were frozen in -80°C until further examinations.

DNA Extraction. DNA extraction of plasma samples was performed according to Özaslan *et al.*(13) which is summarized below. HHV-6 DNA was extracted from 200 μl plasma, with addition of 500 μl K buffer (20m M Tris, 10 mM EDTA, 0.2%SDS, 150 mM NaCl, 20 $\mu\text{l}/\text{ml}$ Proteinase K 25mg/ml) and incubated at 37°C for 2h. The microtubes were centrifuged at 11,000 rpm at 4°C for 6 min, then DNA was extracted by phenol-chloroform-isoamyl alcohol (25:24:1) allowed by ethanol precipitation. Then, DNA was dissolved in 30 μl sterile distilled water stored at -20°C until use for PCR.

DNA extraction of PBMC samples was performed according to Shahbazi *et al.*(14) which is summarized below. The protocol applied for DNA extraction of the PBMC samples was based on the lyses of the erythrocytes with R buffer (ammonium carbonate, potassium hydrogen carbonate, EDTA 0.5 M). Then 700 μl salt EDTA, 25 μl proteinase K (25 mg/ml) and 30 μl SDS 10%

were added to the pellet, which was incubated for 1h at 65°C . After incubation, mixture of phenol-chloroform-isoamyl alcohol was added to samples, which were then centrifuged. To precipitate the DNA, with adding isopropanol and sodium acetate to the supernatant, DNA was extracted after centrifugation. All samples were dissolved in 100 μl sterile distill water and stored at -20°C for future analysis.

Genomic DNA PCR (Internal control). PCR test (Internal control) was done for controlling DNA extraction in both samples (plasma and PBMC) for a sequence 250 bp of the human GAPDH gene, (Genetbio, Korea per kit, Eppendorf thermocycler).

Reverse primer Human GAPDH: GAAGGTGAAGGTCGGAGTC, Forward primer Human GAPDH: GAAGATGGTGATGGGATTTC (15). Each 25 μl PCR mixture contained 2.5 μl of 10X PCR buffer, 0.5 μl dNTPs 10mM, 2 μl Mgcl2 25 mM, 0.1 μl of each primers with 10pmol/ μl concentration, 0.3 Unit of Taq DNA polymerase, 14.5 μl ddH2O and 5 μl (500 ng/ml) of extracted DNA. The amplification process was conducted under following conditions: 95°C for 5 min; then 35 cycles of 94°C for 30s, 60°C for 30s and 72°C for 30s and 72°C for 7 min.

Detection of HHV-6 DNA by Real-Time PCR (Syber Green). The detection of HHV-6 DNA was carried out according to Yavarian *et al* (16) The Plasma and PBMC specimens were tested for HHV-6 DNA. Real-time PCR was done based on SYBR Green/ROX q PCR Master Mix (2X) (Ampliqon, Denmark) kit using ABI system 7300. The primers were designed for the U22 ORF of both HHV-6 variants (A, B); forward primer: 5'-TCGAAATAAGCATTAAATAGGCACACT-3', reverse primer: 5'-CGGAGTTAAGGCATTGGTTGA-3' which amplified a 98-bp fragment. Thermocycling conditions were as follows, initial heat activation for three minutes at 95°C , followed by 35 cycles of denaturation at 95°C for 15seconds, annealing at 56°C for 30 seconds and final extension step of at 60°C for 30 seconds. Dissociation curve and amplification plot are shown in (fig 1 and 2) with positive control and clinical sample.

Detection of Anti-HHV-6 IgG antibody.

Anti-HHV-6 IgG Antibody in the plasma specimens were assayed by ELISA Technique according to manufacturer’s instruction (Abnova, Taiwan). Briefly, 100 µl of plasma diluted 1:100 were added to the wells coated with the HHV-6 antigen. Samples were incubated 60 minutes at 37°C and washed 4 times. 100 µL of Px-conjugate was added into each well and incubated 20 minutes at 37°C. After the wells were washed 4 times, 100µL of TMB was added, incubated 20 minutes at room temperature and reaction was stopped and read in an ELISA reader at 630 nm. The presence or absence of anti- HHV-6 IgG was measured in relation to the cut-off calibrator. By dividing the sample or control absorbance by the cut-off calibrator factor an index value <0.9 was calculated to be negative, between 0.9 and 1.1 was equivocal and >1.1 was computed to be positive.

Statistical analysis. Clinical and demographic data were entered in SPSS-16 and analyzed. Statistical analysis was assessed by the X² test and independent T-Test. P values were determined, and those less than 0.05 were considered to be significant.

Results

Of 59 MS patients, 17 (28%) males and 42 (72%) females, 55 patients suffered from relapsing–remitting MS (RRMS) and four of them had secondary-progressive MS (SPMS). Patients age were ranging from 15 to 55 years (mean±SD, 32±8 years) and in control group it was ranging from 18 to 55 years (mean±SD 33.2 ± 8.2) (Table 1). All of the MS patients were under treatment with different drugs. Most of MS patients (71.1%) were taking Cinovex and rest of them treated with Avonex, Recigen, Betafron, Actafron, Rebif, Zifron and Azaram. 89.83% of MS patients belonged to ethnicity of Fars, 3.38% to Turk, 5.08% to Turkman and 1.06% to Sistani.

Detection HHV-6 Genome in plasma and PBMC. Only one (1.7%) of MS patients had shown HHV-6 DNA in its plasma sample. PBMC of all MS patients and plasma and PBMC samples of control group were negative

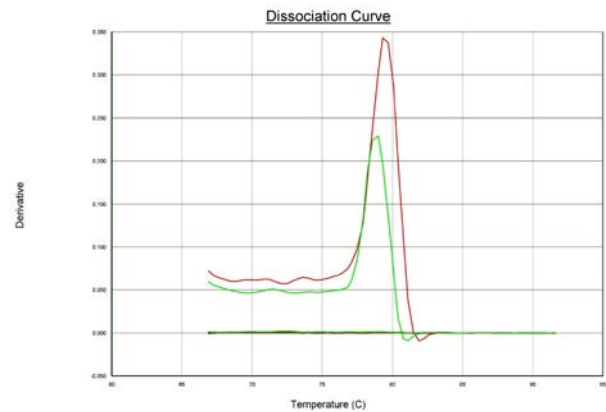


Fig. 1. Dissociation curve. Sample of Positive control (red line) and clinical sample (green line).

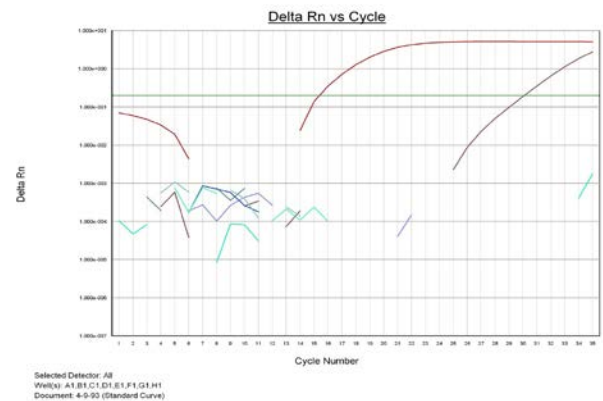


Fig. 1. Amplification plot indicated Ct number in positive control and clinical sample (red line: Control positive and brown line: clinical sample).

for HHV-6 DNA (Table 2). Positive sample belonged to RRMS patient. There was no significant difference between HHV-6 and MS. **Detection of IgG Antibody against HHV-6.** Anti HHV-6 antibody were demonstrated in 50/59 (84.70%) of MS patients in contrast with 23/28 (82.1%) of healthy controls. All of SPMS patients had anti- HHV-6 IgG antibody of their plasma samples compared to 83.63% of the RRMS patents. The average index antibody for MS patients were 2.94 (Std. Deviation= 2.12) and control group 2.87 (Std. Deviation = 1.83). Normality in these data were analyzed with Kolmogorov-Smirnov test and assumptions of normality didn’t reject. According to the independent t test, this

Table 1: Demographic characteristics of the Iran multiple sclerosis patients and the control Group.

Group	N	Gender	Mean age \pm SD
		Male / Female	
MS patients	59	17/ 42	32.9 \pm 8.6
RRMS	55	16/ 39	
SPMS	4	1/ 3	
Control group	59	17/ 42	33.2 \pm 8.2

MS – multiple sclerosis, SD – standard division

Table 2: Presence of HHV-6 DNA and anti-HHV-6 IgG in MS patients and control group.

Groups	HHV-6 DNA in plasma n (%)	HHV-6 DNA in PBMC	HHV-6 IgG ⁺ (%)	P value	Average index Ab/ Std. Deviation
MS (n=59)			50 (84.7)	NS	2.94 /2.12
RRMS (n=55)	1(1.7)	0	46(83.63)	-	
SPMS (n=4)	0	0	4(100)	-	
Control group (n=59)	0	0	23/28 (82.1)	NS	2.87/ 1.83

NS=Not significant , Std. Deviation=Standard deviation

Table 2: Presence of HHV-6 DNA and anti-HHV-6 IgG in MS patients and control group.

Sample	MS (%)	Control (%)	Significant Correlation	Reference
PBMC	3.2	0	No	(20)
PBMC	0	0	No	(21)
Total Blood	6.66	3.17	No	(19)
Serum	26.7		No	(18)
Serum	0	0	No	(9)

PBMC = peripheral blood mononuclear cell

difference was not significant and we didn't find significant difference in level of anti-HHV-6 IgG antibody between MS patients and the control group (Table 2).

Discussion

Family members of the herpesviruses are possible candidates for association with MS disease. Many studies have been conducted on the association between HHV-6 and MS, however, we are faced with controversial results. According to various reports HHV-6 DNA was found in high levels in the CNS, CSF and serum of MS patients instead of many others with no HHV-6 infection in both MS cases and control groups (17). In the level of active infection our results demonstrated presence of HHV-6 DNA in plasma sample of only one MS patients instead of no HHV-6

infection in peripheral blood mononuclear cells (PBMCs) of MS patients as well as plasma and PBMC of control group.

Different studies reported low and high level of HHV-6 DNA presence in MS patients in various areas of Iran and other countries. According to table 3 in recent studies (9, 18-21) there has been no any report showing results for significant relation between presence of HHV-6 DNA in MS patients and control group. However some of them showed low level of HHV-6 DNA in MS patients as well as detection of viral DNA in both of MS patients and control group. These are in agreement with our finding with low presence of HHV-6 DNA in Blood sample and no association with MS. Instead of these data several studies reported before 2010 (22, 23) showed high frequency of HHV-6 DNA in MS patients and significant relation between MS

patients and controls (Table 3). Recent literature in determination of role of HHV-6 in etiology of MS has failed regarding to disability of most of studies in support of play of HHV-6 in MS onset or progression (24). On the other hand our results were in contrast with two studies published recently from south of Iran(11, 25) demonstrated high percentage of HHV-6 DNA and IgM antibody detection and strong relation to support role of HHV-6 in MS diseases. It makes it difficult to discuss the results we have reported however we could say the finding in patients and control group may be the reflection of population ethnicity as well as type of sample and techniques.

We have demonstrated anti-HHV-6 IgG antibody in plasma samples of 84.70% MS patients and 82.10% of control group. A few studies concentrated on demonstration of titer of anti HHV-6 IgG antibody in MS patients and control group in relation to find out any role in MS diseases. We did not show any relation based on statistical analysis. This is in agreement with recent report from South of Iran by Behzad-Behbahani et al (11) and Ramroodi et al (25) Supported that level of anti HHV-6 antibody in two group was close and did not show difference. As we do not have any official report about prevalence of HHV-6 infection in Iran we do not able to discuss completely in this area.

According to results of other literature from South of Iran and the rest of the world many variables such as population, experimental techniques at the level of DNA extraction, type of polymerization protocols, primer selection as well as type of samples could influence the results. However pathobiology of MS is not definitely clear and is known as a multi factorial and complex disease still immune system seems to be under pressure of abnormality for diseases.

Conclusions

According to the results we concluded that there is no a definite relation in role of Human herpesvirus-6 in etiology of multiple sclerosis. The possible correlation of HHV-6 and MS is

still controversial and far from being established.

Acknowledgement

We thank Golestan University of Medical Sciences for the continuous encouragement during this study and Dr. Jila Yavarian for providing HHV-6 positive control. This article was extracted from a medical virology Msc thesis.

References

1. Lassmann H. Pathology and disease mechanisms in different stages of multiple sclerosis. *Neurological Sciences*. 2013;1-4.
2. Franciotta D, Bestetti A, Sala S, Perucca P, Jarius S, Price RW, et al. Broad screening for human herpesviridae DNA in multiple sclerosis cerebrospinal fluid and serum. *Acta Neurol Belg*. 2009;109:277-82.
3. Lassmann H. Neuropathology in multiple sclerosis: new concepts. *Multiple sclerosis*. 1998;4(3):93-8.
4. HS P. Influence of infection on exacerbations of multiple sclerosis. *Ann Neurol*. 1994;36(suppl):S25-S8.
5. Reynaud JM, Horvat B. Human herpesvirus 6 and neuroinflammation. *ISRN Virology*. 2013;2013.
6. Dockrell D. Human herpesvirus 6: molecular biology and clinical features. *Journal of medical microbiology*. 2003;52(1):5-18.
7. Swanborg RH, Whittum-Hudson JA, Hudson AP. Human herpesvirus 6 and Chlamydia pneumoniae as etiologic agents in multiple sclerosis—a critical review. *Microbes and infection*. 2002;4(13):1327-33.
8. Chapenko S, Millers A, Nora Z, Logina I, Kukaine R, Murovska M. Correlation between HHV-6 reactivation and multiple sclerosis disease activity. *Journal of medical virology*. 2003;69(1):111-7.
9. Al-Shammari S, Nelson R, Voevodin A. HHV-6 DNAemia in patients with multiple sclerosis in Kuwait. *Acta Neurologica Scandinavica*. 2003;107(2):122-4.
10. Tejada-Simon MV, Zang YC, Hong J, Rivera VM, Zhang JZ. Cross-reactivity with myelin basic protein and human herpesvirus-6 in multiple sclerosis. *Annals of neurology*. 2003;53(2):189-97.

11. Behzad-Behbahani A, Mikaeili MH, Entezam M, Mojiri A, Pour GY, Arasteh MM, et al. Human herpesvirus-6 viral load and antibody titer in serum samples of patients with multiple sclerosis. *Journal of Microbiology, Immunology and Infection*. 2011;44(4):247-51.
12. Xu Y, Linde A, Fredrikson S, Dahl H, Winberg G. HHV-6 A-or B-specific P41 antigens do not reveal virus variant-specific IgG or IgM responses in human serum. *Journal of medical virology*. 2002;66(3):394-9.
13. Özaslan M, Özaslan E, Barsgan A, Koruk M. Mutations in the S gene region of hepatitis B virus genotype D in Turkish patients. *Journal of genetics*. 2007;86(3):195-201.
14. Shahbazi M, Roshandel D, Rshaidbaghan A. Interaction of HLA-DRB1* 1501 allele and TNF-alpha- 308 G/A single nucleotide polymorphism in the susceptibility to multiple sclerosis. *Clinical Immunology*. 2011;139(3):277-81.
15. Cuccato G, Polynikis A, Siciliano V, Graziano M, di Bernardo M, di Bernardo D. Modeling RNA interference in mammalian cells. *BMC systems biology*. 2011;5(1):19.
16. Yavarian J, Gavvami N, Mamishi S. Detection of Human Herpesvirus 6 in Cerebrospinal Fluid of Children With Possible Encephalitis. *Jundishapur J Microbiol*. 2014;7(9):e11821.
17. Álvarez-Lafuente R, Martín-Estefanía C, De las Heras V, Castrillo C, Cour I, Picazo J, et al. Prevalence of herpesvirus DNA in MS patients and healthy blood donors. *Acta Neurologica Scandinavica*. 2002;105(2):95-9.
18. Ahram M, El-Omar A, Baho Y, Lubad M. Association between human herpesvirus 6 and occurrence of multiple sclerosis among Jordanian patients. *Acta Neurologica Scandinavica*. 2009;120(6):430-5.
19. Ben-Fredj N, Ben-Selma W, Rotola A, Nefzi F, Benedetti S, Frih-Ayed M, et al. Prevalence of human herpesvirus U94/REP antibodies and DNA in Tunisian multiple sclerosis patients. *Journal of neurovirology*. 2013;19(1):42-7.
20. Hon GM, Erasmus RT, Matsha T. Low prevalence of human herpesvirus-6 and varicella zoster virus in blood of multiple sclerosis patients, irrespective of inflammatory status or disease progression. *Journal of Clinical Neuroscience*. 2014;21(8):1437-40.
21. Sotelo J, Ordoñez G, Pineda B, Flores J. The participation of varicella zoster virus in relapses of multiple sclerosis. *Clinical neurology and neurosurgery*. 2014;119:44-8.
22. Álvarez-Lafuente R, Heras VD, Bartolomé M, García-Montojo M, Arroyo R. Human Herpesvirus 6 and Multiple Sclerosis: A One-Year Follow-up Study. *Brain pathology*. 2006;16(1):20-7.
23. Alvarez-Lafuente R, de Las Heras V, Garcia-Montojo M, Bartolome M, Arroyo R. Human herpesvirus-6 and multiple sclerosis: relapsing-remitting versus secondary progressive. *Multiple sclerosis*. 2007;13(5):578-83.
24. Hona GM. Human herpesvirus-6 and the etiology of multiple sclerosis: a literature review. *Asian Biomed*. 2014;8(3).
25. Ramroodi N, Sanadgol N, Ganjali Z, Niazi AA, Sarabandi V, Moghtaderi A. Monitoring of active human herpes virus 6 infection in Iranian patients with different subtypes of multiple sclerosis. *Journal of pathogens*. 2013;2013.