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Increased detection of serum HHV-6 DNA sequences during multiple sclerosis (MS) exacerbations and correlation with parameters of MS disease progression

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In recent years, human herpesvirus 6 (HHV-6) has been investigated as a possible causative agent for MS. To determine if the detection of HHV-6 DNA in the serum of MS patients correlates with clinical parameters of MS disease progression, a total of 215 serum samples was obtained from 59 MS patients followed prospectively for a 5-month period. These samples were analyzed for the presence of HHV-6 DNA by nested PCR and compared in parallel to MS disease activity. HHV-6 DNA was amplified in 22% (4/18) of samples obtained during a period of clinical exacerbation. Significantly fewer ($P = 0.008$) sera, 5.6% (11/197), obtained from MS patients during clinical remission tested positive for the presence of HHV-6 DNA. This work demonstrates that the detection of serum HHV-6 DNA is significantly correlated with clinical exacerbations in MS. Moreover, the findings presented in this study have confirmed previous reports supporting an association between MS and HHV-6 and suggest a role for this human herpesvirus in the pathogenesis of MS. *Journal of NeuroVirology* (2002) **8,** 250–256.

Keywords: HHV-6; multiple sclerosis; exacerbation

Introduction

Multiple sclerosis (MS) is the most prevalent demyelinating disease of the central nervous system (CNS). The hallmarks of MS pathology include demyelinating white matter lesions, perivascular inflammation, and relative sparing of the axons. Although the cause of MS is unknown, it is generally believed that an autoimmune component is involved in the pathogenesis of this disease (Hunter and Rodriguez, 1995). This hypothesis is supported by similarities between MS and experimental immune-mediated demyelinating diseases (Martin and McFarland, 1997), the association of MS with genes involved in the immune response, and the clinical response of MS symptoms to immunomodulatory treatments. Genetic factors have also been shown to be important in the pathogenesis of MS (Ebers, 1994). The lifetime risk of developing this disease is higher in biological relatives of MS patients (Sadovnick, 1993). However, the concordance rate among monozygotic twins is only 28%, which suggests that a susceptible genetic background alone is not sufficient to cause the disease (Cook, 1997).

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It has been suggested that environmental stimuli may play an important role in triggering MS symptoms and pathology. Familial and regional clustering studies indicate that an environmental factor, possibly an infectious agent, may induce disease exacerbations in susceptible individuals (Panitch, 1994). In the search for an infectious etiology in the pathogenesis of MS, the historic focus has been on the identification of a unique virus predominately associated with MS and absent in unaffected individuals. This avenue of research has failed to convincingly identify any such 'MS virus.' More recently, the attention has shifted to the possible association of MS with a common, ubiquitous virus which persists as a commensal infection in the general population, but may induce disease in a subset of susceptible individuals (Berti and Jacobson, 1999; Blumberg *et al*, 2000). In recent years, human herpesvirus 6 (HHV-6), a β-herpesvirus closely related to cytomegalovirus and HHV-7 (Salahuddin *et al*, 1986), has been investigated extensively as a possible causative agent for MS (Challoner *et al*, 1995) (Soldan *et al*, 1997). HHV-6 is the etiologic agent of a childhood febrile illness, *exantem subitum* (*roseola infantum*) (Yamanishi *et al*, 1988) and it has been suggested that HHV-6 may be the most neuroinvasive member of the human herpesvirus family (Caserta *et al*, 1994; Knox and Carrigan, 1995; Mackenzie *et al*, 1995; Wilborn *et al*, 1994). Several immunological and molecular studies have supported a relationship between HHV-6 and MS (Sola *et al*, 1993; Challoner *et al*, 1995; Soldan *et al*, 1997; Ablashi *et al*, 1998; Friedman *et al*, 1999; Akhyani *et al*, 2000; Soldan *et al*, 2000). Most recently, Tomsone *et al*, 2001, have confirmed the higher prevalence of HHV-6 in plasma of MS patients compared to controls. However, others have failed to support these observations (Martin *et al*, 1997; Enbom *et al*, 1999; Goldberg *et al*, 1999) and the association between HHV-6 and MS remains open (Soldan and Jacobson, 2001).

HHV-6 DNA has been reported in the serum of patients with primary HHV-6 infection (*roseola infantum*) (Secchiero *et al*, 1995) and is believed to be a marker for active virus replication. Of interest, cell-free serum HHV-6 DNA has also been reported in approximately 23% of MS patients but not in either healthy controls or other inflammatory disease and other neurologic disease controls (Akhyani *et al*, 2000; Berti *et al*, 2000; Soldan *et al*, 1997). To further investigate the role of HHV-6 in MS, it is important to determine if active virus replication, as measured by the detection of serum HHV-6 DNA, correlates with parameters of MS disease progression. To address this question, a total of 215 serum samples was obtained from 59 MS patients who were enrolled in clinical protocols at the Neuroimmunology Branch of the National Institutes of Health and followed prospectively for a 5-month period. These samples were analyzed for the presence of HHV-6 DNA by nested PCR and compared in parallel to MS disease activity.

Results

In previous studies examining the presence of serum HHV-6 DNA in MS patients from the NIH cohort, HHV-6 DNA was amplified from the serum of approximately 23% (25 of 108) MS patients (Akhyani *et al*, 2000). In contrast, no HHV-6 DNA was detected from control sera (Akhyani *et al*, 2000; Berti *et al*, 2000; Soldan *et al*, 1997). The present work extends these observations to include a total of 167 MS patients (Table 1). To date, the presence of HHV-6 DNA has been detected in 39 out of 167 (23%) of MS patients compared to 0 out of 70 (0%) of controls (Table 1), including sera from normal control subjects ($n = 36$), patients with other inflammatory ($n = 15$), and other neurologic diseases ($n = 19$) ($P = 0.0001$, χ^2 test). To verify that sequences amplified from sera of MS patients were HHV-6, PCR products were sequenced and analyzed from representative patients using the modules ContigExpress and AlignX from the VectorNTI Suite 5.5 (InforMax, Inc, North Bethesda, MD). All sequences obtained from MS patient samples aligned with known HHV-6 sequences (Figure 1).

The possible relationship between MS exacerbations and the detection of serum HHV-6 DNA was examined over time in a large cohort of MS patients. Fifty-nine MS patients (17 men, 42 women) were evaluated from two to five times over a 5-month period. A total of 215 serum samples was collected from these 59 patients. Among those, 18 sera were obtained during an exacerbation. HHV-6 DNA was amplified from 4 of 18 exacerbation samples (Table 2). Serum HHV-6 DNA was detected from significantly fewer ($P = 0.008$, χ^2 test) sera obtained during periods of remission. From 197 sera obtained from MS patients during remission, only 11 tested positive for the presence of HHV-6 DNA (Table 2). Because 29 of the 59 patients in this study were under an interferon β regimen (28 Betaseron, Berlex, 1 Avonex), it was of interest to determine if the ability to detect serum HHV-6 DNA in MS patients was influenced by

Table 1 Detection of serum HHV-6 DNA in MS patients and controls

Patient populations	*PCR reactivity*		*Percent HHV-6 reactive*
	HHV-6+	*HHV-6−*	
MS $n = 167$	39	128	23**
*RRMS $n = 149$	36	113	24**
*PPMS $n = 2$	0	2	0
*RPMS $n = 9$	2	7	22**
*SPMS $n = 7$	1	6	14**
Non-MS $n = 70$	0	70	0
*ND $n = 36$	0	36	0
*OID $n = 15$	0	15	0
*OND $n = 19$	0	19	0

*RRMS = relapsing remitting MS; PPMS = primary progressive MS; RPMS = relapsing progressive MS; SPMS = secondary progressive MS; ND = normal donor; OID = other inflammatory disease; OND = other neurologic disease.
** $P < 0.0001$.

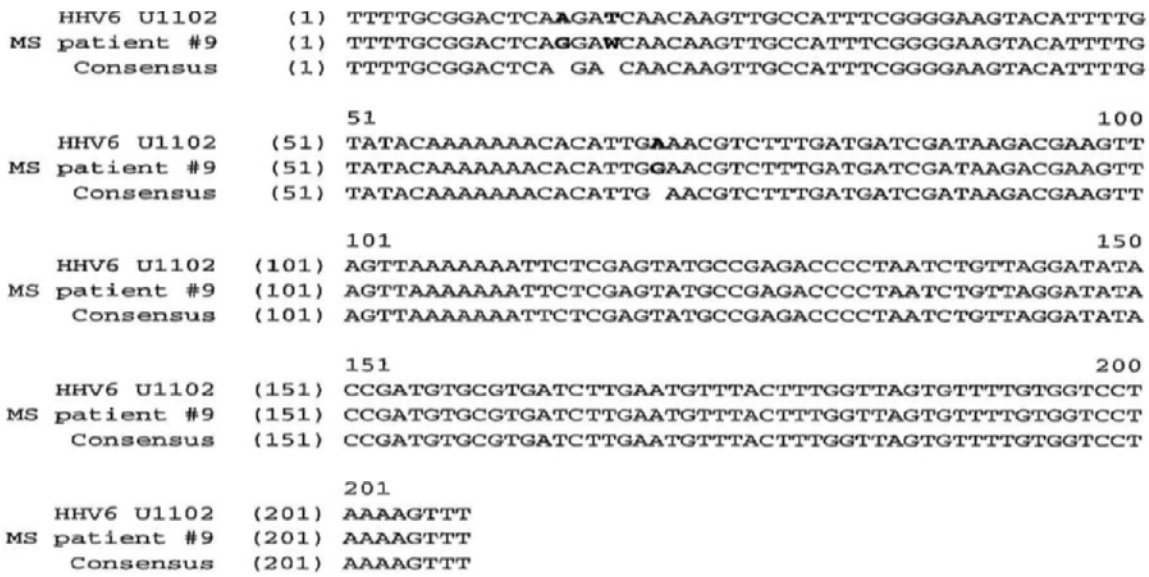

Figure 1 Verification of MS serum HHV-6-nested PCR products by sequence analysis. The DNA amplified from MS patient sera by primers specific for the major capsid protein region of HHV-6 demonstrated 98.6% sequence homology to the HHV-6A variant isolate U1102.

β-interferon therapy. Of the 103 serum samples collected from patients using β-interferon, 6/103 (5.8%) were positive for HHV-6 DNA. When samples collected from patients not on β-interferon therapy were analyzed separately, HHV-6 DNA was found in 9/112 (8.0%) samples. The difference between these two groups did not reach statistical significance ($P = 0.12$, χ^2 test).

To further examine the relationship between the detection of HHV-6 DNA in the serum of MS patients with clinical parameters of MS disease progression over time, two MS patients with early secondary progressive MS and frequent periods of clinical worsening were tested extensively for the presence of serum HHV-6 DNA. Numerous samples from a period of over 19 months were available from these two patients for a retrospective analysis of HHV-6 serum DNA (Table 3). MS patient 1 was treated with β-interferon for the 19-month period under study and experienced four episodes of clinical relapse. Serum HHV-6 DNA was amplified from three of four of these serum samples obtained during defined periods of clinical worsening (Table 3). In contrast, HHV-6 DNA was detected only once during the 14 time points sampled in which clinical worsening was not evident (Table 3). Similarly, MS patient 2 who started β-interferon treatment on the 13th month of this 23-month study period, experienced four clinical exacerbations. HHV-6 DNA was amplified from two of four serum samples

Table 2 Detection of serum HHV-6 DNA in MS patients during clinical exacerbations and remissions

Serum samples	*PCR reactivity*		*Percent HHV-6 reactive*
	HHV-6+	*HHV-6−*	
Exacerbations $n = 18$	4	14	22*
Remissions $n = 197$	11	186	5.6

*$P = 0.008$.

Table 3 Correlation of serum HHV-6 DNA and clinical MS disease activity

Serum samples	*MS patient 1*			*MS patient 2*		
	HHV-6 PCR	*MRI # Gd-lesions*	**Exac.*	*HHV-6 PCR*	*MRI # Gd-lesions*	*Exac.*
Month 1	+	0	+	−	0	−
Month 2	−	0	−	−	0	−
Month 3	−	0	−	−	3	−
Month 4	−	0	−	−	0	−
Month 5	−	0	−	−	2	−
Month 6	−	0	−	−	0	−
Month 7	−	0	−	−	0	−
Month 8	−	0	−	+	1	+
Month 9	−	0	+	−	0	−
Month 10	−	0	−	−	6	−
Month 11	+	0	+	−	11	−
Month 12	−	0	−	−	25	−
Month 13	−	0	−	−	8	+
Month 14	+	0	−	−	10	−
Month 15	−	0	−	−	4	−
Month 16	−	0	−	−	0	−
Month 17	+	0	+	−	0	−
Month 18	−	0	−	−	0	+
Month 19	−	0	−	−	0	−
Month 20	**NT	NT	NT	−	0	−
Month 21	NT	NT	NT	+	5	−
Month 22	NT	NT	NT	+	0	+
Month 23	NT	NT	NT	−	0	−

*Exac. = exacerbation.
**NT = not tested.

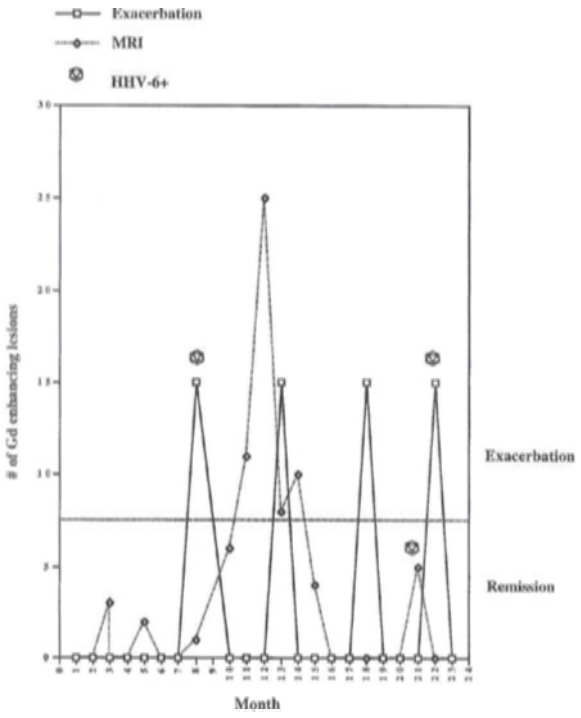

Figure 2 The number of new gadolinium (Gd)-enhancing lesions observed by MRI (triangles and dotted line) was compared to the presence of serum HHV-6 DNA (indicated by icosohedrals) for patient 2. Although both the number of Gd-enhancing lesions and the detection of HHV-6 DNA fluctuated over time in this individual, a significant correlation between lesion load and the presence of serum HHV-6 DNA could not be demonstrated.

obtained during exacerbation (Table 3). HHV-6 DNA was detected only once during 19 time points corresponding with periods of remission in this patient (Table 3).

It has been demonstrated that the number of gadolinium-enhancing lesions as measured by magnetic resonance imaging (MRI) vary in MS patients over time (McFarland *et al*, 1992). Therefore, it was of interest to compare MRI lesion load with the detection of HHV-6 DNA in the serum of these two MS patients followed over a period of at least 19 months. Although MS patient 1 had no Gd-enhancing lesions throughout the 19-month study period, MS patient 2 experienced periodic fluctuations of lesion load over 23 months (Figure 2). There was no apparent correlation between the number of Gd-enhancing lesions and the detection of HHV-6 serum DNA in this patient.

Discussion

Throughout the years, a number of viruses have been associated with the pathogenesis of MS (Berti and Jacobson, 1999). Recently, HHV-6 has been suggested as a possible candidate based on a number of characteristics of the virus. First, primary infection with HHV-6 usually occurs during the first 2 years of life (Caserta and Hall, 1993), which is consistent with epidemiological evidence suggesting that childhood exposure to an etiologic agent may contribute to the pathogenesis of MS. Second, herpesviruses, in general, are highly neurotropic. HHV-6 has recently been described as neuroinvasive (Wilborn *et al*, 1994; Kimberlin and Whitley, 1998) and oligodendrocytes within MS lesions have been shown to express HHV-6 proteins (Challoner *et al*, 1995). Third, one of the main characteristics of herpesviruses is their ability to reactivate. The same factors that can lead to herpesvirus reactivation, such as stress and infection with another pathogen, have been associated with MS exacerbations. Finally, although HHV-6 predominantly infects T-lymphocytes, it has also been found to infect other cells of both lymphoid and nonlymphoid origin (He *et al*, 1996). A ubiquitous virus, such HHV-6, which has the ability to infect cells of both the immune and nervous systems, could cause abnormalities in both.

It has been previously reported (Secchiero *et al*, 1995) that cell-free viral HHV-6 DNA in serum is a marker of active infection/replication for this ubiquitous virus. Previous reports from this laboratory have demonstrated that HHV-6 DNA sequences can be detected in approximately 23% of MS patients but not in serum of patients with other neurological and other immunological disorders or normal controls (Soldan *et al*, 1997; Akhyani *et al*, 2000; Berti *et al*, 2000). This report extends these findings by addressing the detection of serum HHV-6 DNA in the context of the natural history of this disorder and relevant clinical parameters including disease exacerbations and remissions, MRI lesion load, and treatment modalities. In this study, a cohort of 59 MS patients were followed for a period of 5 months from which 215 serum samples were obtained. As demonstrated in Table 2, there was a significant correlation between the detection of serum HHV-6 DNA and the number of patients who exhibited clinical exacerbations during the study period ($P = 0.008$). The detection of HHV-6 DNA in the serum of this new cohort of MS patients extends and supports previous findings (Soldan *et al*, 1997; Berti *et al*, 2000). Whereas data from previous studies represent single time points, the present study afforded the opportunity to analyze a large group of MS patients over time. Our results suggest that there is a statistically greater likelihood of detection of HHV-6 DNA in the serum of an MS patient during an exacerbation than in a remission ($P = 0.008$).

This relatively short term prospective study (5 months) was supplemented by a more comprehensive retrospective study of longitudinal samples obtained from two patients followed for over 19 months to better correlate MS disease activity with the presence of HHV-6 DNA. In one MS patient, four episodes of clinical worsening were observed over an 18-month period. Serum HHV-6 DNA was detected

from three of the four serum samples obtained during clinical relapse compared to 1 of 14 serum samples obtained during disease remission. Similarly, in a second MS patient, HHV-6 DNA was detected in two of four serum samples obtained during exacerbations but from only 1 of 19 serum samples during remissions. In these MS patients, there was a significant correlation with disease activity and detection of HHV-6 in serum samples. The MS patients described in this report were evaluated as part of a larger natural history and MRI study. Although not the primary focus of these experiments, this study allowed for preliminary observations on the correlation of serum HHV-6 DNA with MRI lesion frequency. Of the 59 MS patients studied, 2 were identified from which larger numbers of archived serum samples (greater than the 5 months of this study) were available. Of these 2 MS patients, one demonstrated a cyclicity in Gd-enhancing lesions typical of MS. From the data presented in Figure 2, an increase in the amount Gd-enhancing lesions did not appear to have a direct correlate with the detection of serum HHV-6 in this patient.

Of the 59 MS patients in this report, 29 were enrolled in a β-interferon clinical protocol (protocol 89-N-0045). While not the primary focus of this study, it was of interest to determine if interferon β had an effect on the serum HHV-6 levels. Of interest, 5 of 29 (17.2%) patients on β-interferon therapy compared to 9 of 30 (30%) MS patients not under β-interferon therapy were positive for HHV-6 DNA at least once during the course of this study. Although fewer MS patients under interferon β treatment had detectable HHV-6 DNA sequences in serum than untreated MS patients this observed trend did not reach statistical significance ($P = 0.12$). Therefore, this cohort of MS patients may be too small to make firm conclusions on the effect of β-interferon on the presence of serum HHV-6 DNA and warrants further investigation.

The association of HHV-6 and MS has been supported by several studies demonstrating the detection of HHV-6 protein in active plaques of MS patients (Challoner *et al*, 1995), the detection of HHV-6 DNA in the serum of MS patients (8, 11, 12, 18, 22), and an increased humoral and cellular immune responses to HHV-6 (Sola *et al*, 1993; Soldan *et al*, 1997; Ablashi *et al*, 1998; Soldan *et al*, 2000). This study has confirmed previous reports from our group (Soldan *et al*, 1997; Berti and Jacobson, 1999; Akhyani *et al*, 2000) and others (Sola *et al*, 1993; Challoner *et al*, 1995), supporting an association between MS and HHV-6 by demonstrating the presence of HHV-6 DNA sequences in the serum of a subset (approximately 23%) of MS patients. The present study extends these observations by examining the presence of serum HHV-6 DNA in the context of disease activity and by demonstrating a higher percentage of serum HHV-6 during the relapsing phase of the disease. This report further supports a role for this agent in the pathogenesis of MS by suggesting that the presence of serum HHV-6 DNA, similar to the presence of gadolinium-enhancing lesions, coincides with clinical worsening in a subset of patients. It is currently unknown whether active replication of HHV-6 causes worsening of the disease or if events associated with clinical relapse (e.g., stress) reactivate HHV-6. Although the pathophysiology of MS has not been fully defined, a complex series of events incorporating genetic, immunologic, and environmental factors are involved. Accumulating evidence suggests that HHV-6 is associated with MS and may be one of the environmental "triggers" involved in disease progression.

Methods

Patients

Serum samples were obtained from a total of 59 patients with clinically and laboratory-supported definite MS according to Poser *et al* (Poser *et al*, 1983). This group of MS patients represented three MS phenotypes and included 48 relapsing-remitting (RRMS), 6 relapsing progressive (RPMS), and 5 secondary progressive patients (SPMS). Patient blood samples were collected as part of clinical natural history and treatment protocols approved by the National Institute of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH) Institutional Review Board and informed consent was obtained from all patients. Of this MS cohort, 29 patients were on interferon-β-1b therapy and 30 were not on immunomodulatory or antiviral medications. During clinical exacerbations, intravenous methylprednisolone (1000 mg/d for 5 days) followed by an oral prednisolone taper were administered. All specimens were obtained prior to any steroid therapy. Exacerbations were diagnosed on the basis of the patient complaints, neurological examination and changes in either timed ambulation, nine-hole-peg test, or Scripps Neurologic Rating scale.

Additionally, 70 control sera were obtained from 36 normal donors, 15 other inflammatory disease controls (including 4 rheumatoid arthritis, 4 systemic lupus erythematosus, 2 polymyositis, 2 psoriatic arthritis, and 3 mitochondrial myositis), and 19 other neurologic disease controls (including 6 epilepsy, 9 Parkinson's disease, and 4 amyotrophic lateral sclerosis).

Blood samples

Serum was isolated by centrifugation after clotting, divided into aliquots, and stored at -70°C.

DNA extraction

Serum DNA was extracted as described by Boom *et al* (1990). Briefly 300 to 400 μl of human serum were added to a solution containing 40 μl size-fractionated

silica (Sigma St. Louis, MO) and 1.2 ml of a lysis buffer containing guanidinium thiocyanate (Fluka, Ronkokoma, NY), were thoroughly mixed and incubated at room temperature for 15 min. The solution was centrifuged for 1 min at 15000 RPM. The resulting supernatant was discarded by aspiration and the pellet was washed twice with a guanidinium thiocyanate buffer, twice with 70% ethanol, and once with acetone. Acetone was aspirated after the final wash and the pellet was dried for 10 min at 56°C. The pellet was resuspended in 50 μl of DEPC-treated water and incubated for 10 min at 56°C to elute DNA. After centrifugation for 10 min at 15000 RPM, 10 μl of the supernatant were used for each PCR reaction. DNA extraction and PCR analysis of sera were performed independent of clinical status.

Nested polymerase chain reaction (PCR)
The two sets of nested primers used were derived from different regions of the HHV-6 genome. The first set was chosen from a highly conserved sequence corresponding to the major capsid protein gene (MCP) (Secchiero *et al*, 1995). The outer pair of primers amplified a 520-bp sequence; the inner pair a region 258-bp long. The second set of primers was derived from a region shown to encode a putative large tegument protein (LTP). This set consisted of an outer primer pair defining a target sequence of 834 bp and an inner primer pair amplifying a 658-bp fragment. All oligonucleotides were synthesized by Genset, Inc (La Jolla, CA). PCR was performed as described (Secchiero *et al*, 1995). Breifly, 10 μl of the extracted DNA were added to a 90-μl mixture containing 1× PCR buffer (50 mM KCl, 10 mM TRIS-HCl, pH 8.3, 15 mM $MgCl_2$, 0.01% gelatin), 0.2 mM each dNTP and 2.5 units of Ampli Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The primers' concentration was 0.5 μM and the reaction was repeated for 35 cycles (denaturation at 92°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min). A 10-min additional extension step was done after the last cycle. Then, 10 μl of the first round PCR product was amplified further by using the inner set of primers. The same conditions were used except the annealing temperature was increased to 55°C. Then, 10 μl of the final PCR product was electrophoresed on 2% agarose gel and visualized by ethidium bromide staining. Each positive result was confirmed by an ECL assay. When the LTP set of primers was used, the reaction was performed with 0.5 μM of each primer for 40 cycles (denaturation at 92°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min in the first 15 cycles, and an extension of 2 s in the subsequent 25 cycles). An additional 10-min extension step was included after the last cycle. Following the first amplification step 10 μl of the PCR product were amplified with the inner primer pair following the same conditions described for the primary PCR. HHV-6 DNA extracted from infected cells was added to normal human serum and used as positive control both for the extraction and the PCR. One negative control every three patients, consisting of normal human serum (Sigma, St, Louis, MO), was also used. The extraction, PCR reaction, and gel electrophoresis were done in separate rooms to avoid contamination.

Enzyme chemioluminescence assay (ECL)
Each positive result was confirmed for specificity with a non-radioactive probe for the MCP gene (Secchiero *et al*, 1995; ECL detection system; Amersham Life Science).

Sequences
The sequences were analyzed using the modules ContigExpress and AlignX from the VectorNTI Suite 5.5 (InforMax, Inc, Bethesda, MD). Each fragment received from the ABI sequencer was inspected for quality using ContigExpress and, when appropriate, secondary peaks above 95% of the threshold were called and bases substituted. Ambiguous end-bases were also trimmed. The final fragments were then aligned to the GenBank references for HHV-6 using the AlignX module (ClustalW algorithm) and the percentage of similarity between the sequences was determined.

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