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A single tube PCR assay for simultaneous amplification of HSV-1/-2, VZV, CMV, HHV-6A/-6B, and EBV DNAs in cerebrospinal fluid from patients with virus-related neurological diseases

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Cerebrospinal fluid (CSF) specimens from 27 patients with encephalitis, meningitis, and other neurological diseases were studied for the presence of herpes simplex virus types 1 and 2 (HSV-1/-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), human herpesviruses 6A and 6B (HHV-6A/-6B) and Epstein-Barr virus (EBV) DNA using the polymerase chain reaction (PCR) method. The DNAs were amplified using two sets of consensus primer pairs in a single tube, bringing simultaneous amplification of the herpesviruses. The PCR products were analyzed by agarose gel electrophoresis, and Southern blot hybridization with virus-type specific probes, thus allowing discrimination between the different types of herpesviruses to be made. Each virus-specific probe was highly specific for identifying the PCR product. Thirty CSF specimens from 13 patients with encephalitis and 10 specimens from 10 patients with meningitis, respectively, were examined using this method. Eight patients with encephalitis and six with meningitis were positive for different herpesviruses, including patients with coinfections (HSV-1/-2 and VZV, VZV and CMV). Among four CSF specimens from four patients with other neurological disorders, dual amplification of CMV and EBV was present. Since identification of the types of herpesviruses in this system requires a very small amount of CSF, and is completed with one PCR, it is useful for routine diagnosis of herpesvirus infections in diagnostic laboratories. The viruses responsible for central nervous system infection are easily detected with various coinfection and serial patterns of herpesviruses, by this consensus primer-based PCR method. This may give an insight into the relationship between virus-related neurological diseases (VRNDS) and herpesvirus infections. *Journal of NeuroVirology* (2000) **6,** 410–417.

Keywords: herpesviruses; consensus primer; PCR; cerebrospinal fluid; encephalitis; meningitis

Introduction

Herpesviruses, such as herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), human herpesvirus 6 (HHV-6) and Epstein-Barr virus (EBV) are neurotropic viruses. Epidemiological studies have indicated that the majority of aseptic encephalitis results from viral infections. In adults, viral encephalitis is very often caused by herpesviruses, and herpes simplex encephalitis (HSE) is the most common cause of sporadic viral encephalitis. VZV is implicated in the pathogenesis of meningoencephalitis, myelitis and neuritis. Furthermore, CMV encephalitis is one of the common complications in patients with AIDS. The neurological complications of EBV infections are various, including meningoencephalitis, Guillain-Barré syndrome, and Fisher's syndrome. Moreover, it was reported that HHV-6 DNA and protein were found in the brains of patients with necrotizing encephalitis (Novoa *et al*, 1997) and Griscelli's syndrome (Wagner *et al*, 1997). Thus, herpesviruses

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are associated with various types of central nervous system (CNS) infections (Weber *et al*, 1996). Conventional virus detection techniques using cerebrospinal fluid (CSF), virus isolation and serology are insufficient for detecting most of these viruses. As the detection reliability is poor, a specific pathogen is identified only in 50% of patients with encephalitis, indicating that viral DNA is frequently present at low copy numbers in CSF. The early diagnosis of viral infection in CNS is necessary for effective and specific anti-viral drug therapy, since it progresses so rapidly. Therefore, a highly sensitive PCR method has been used with virus-specific primers to identify the target virus (Read *et al*, 1997; Hosoya *et al*, 1998). However, different types of herpesvirus infections cause similar clinical symptoms. In addition, in cases where it is suspected that there are simultaneous herpesviral infections, PCR has to be performed changing the primer and the conditions to target each virus. Therefore, we developed a single PCR method in a single tube with consensus primers combined with Southern blot hybridization with virus-specific oligonucleotide probes. In this system, PCR can be economically applied to detect any seven herpesviruses routinely in a diagnostic laboratory. We also detail the results of CSF specimens from patients with virus-related neurological diseases (VRNDS) using this system.

Results

Amplification of herpesviral DNAs with consensus primers

A fragment of the DNA polymerase gene from different herpesviruses was successfully amplified from 1000 copies/μl of each plasmid DNA using a mixture of two sets of primer pairs (P1 and P2, P3 and P4). PCR products of the expected size (516, 514, 588, 510 and 522 bp corresponding to HSV-1/-2, VZV, CMV, HHV-6, and EBV, respectively) were detected on an ethidium bromide-stained gel. One hundred ng of DNA from uninfected HEL cells was not amplified (Figure 2, top). To confirm the specificity of the PCR assay, and discriminate between the virus types, 10^8 copies/μl of each plasmid DNA were amplified and Southern blot hybridization with virus-specific and digoxigenin (DIG)-labeled probes (Table 1) was performed. Each probe was specific for a target virus, and no cross hybridization was observed among the viruses. Extracts from uninfected HEL cells were not

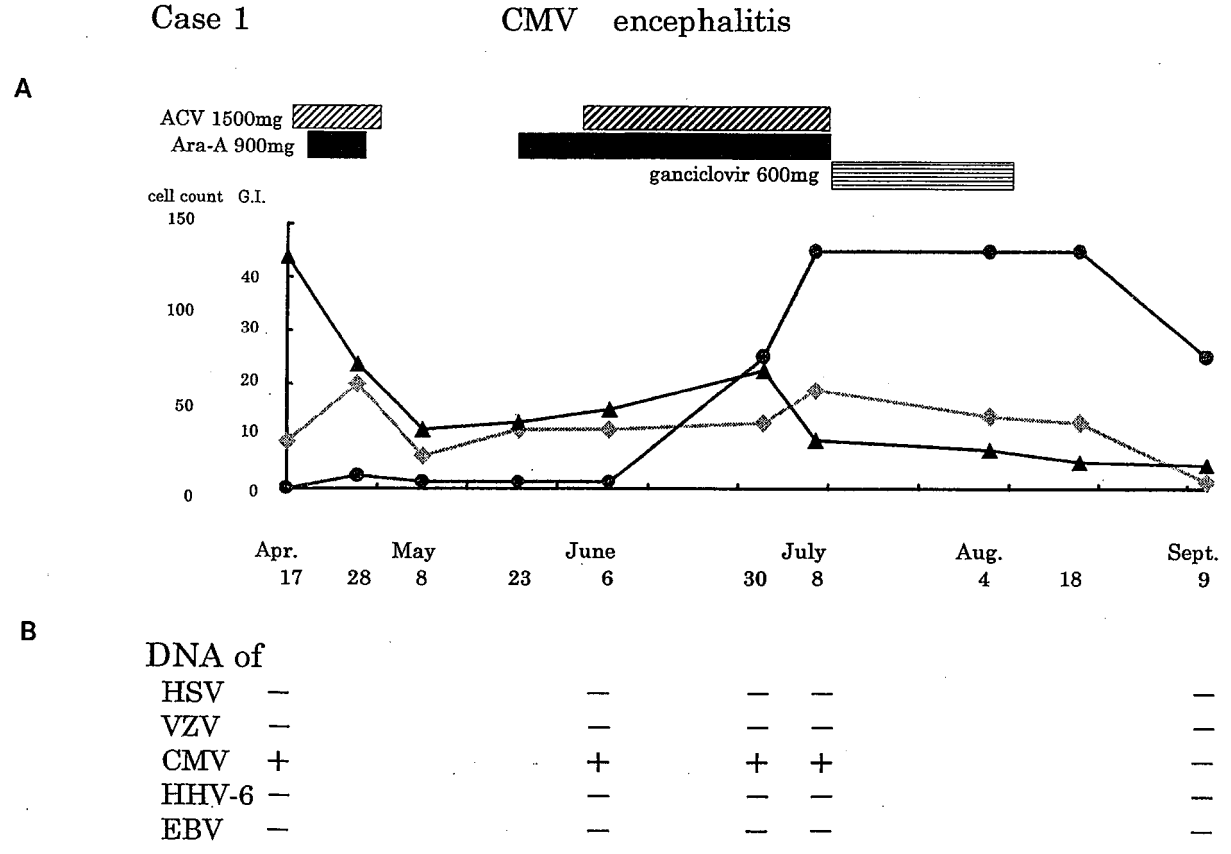

Figure 1 A 33-year-old patient with CMV encephalitis. Changes in CSF cell counts (▲), antibody titers and detection of herpesvirus DNA. Specific IgG antibodies to HSV (◆) and CMV (●) in CSF specimens were measured with a BEP-III (Boehringer ELISA Processor III, Hoechst, Tokyo, Japan). The presence of CMV DNA was verified by the methods used here. ACV: acyclovir.

hybridized with either probe (Figure 2, bottom). To determine the sensitivity of the PCR assay, 10^5 copies/μl of HSV-1, VZV, CMV, HHV6-A and EBV plasmid DNAs were serially diluted in 10-fold steps to one copy/μl, and PCR followed by Southern blot hybridization was performed. These DNAs were detected at a level of 100 copies by an ethidium bromide-stained agarose gel, and at a level of 10 copies by Southern blot hybridization (Figure 3). Similar results were obtained for HSV-2, and HHV-6B DNAs (data not shown).

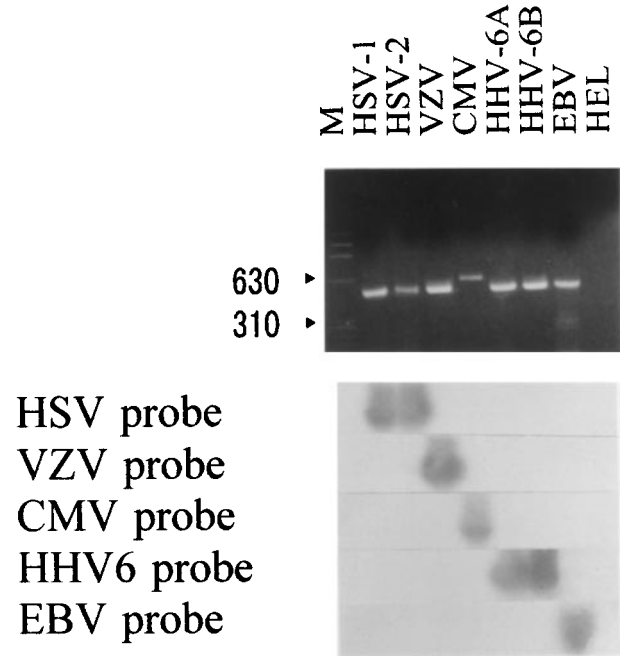

Figure 2 Specificity of PCR amplification. Top: Detection of HSV-1, HSV-2, VZV, CMV, HHV-6A, HHV-6B and EBV. One thousand copies/μl of the plasmid DNAs were amplified by a PCR assay using two sets of consensus primers, and the amplified products were analyzed with electrophoresis on a 1.8% agarose gel. Lane M: DNA molecular weight marker of $\phi \times 174$RF DNA/*Hae*III fragments. Bottom: Southern blot hybridization assay of the amplified products (10^8 copies/μl of the plasmid DNAs) by virus specific probes.

Analysis of clinical specimens

In order to prove the usefulness of the PCR assay for routine work, we tested for the presence of viral genomes in five CSF specimens obtained from a 33-year-old male patient (case 1). Only CMV DNA was specifically detected in four of the specimens (Figure 1B), and HSV encephalitis (HSE) was denied. The results using this system were in agreement with those of PCR using virus-specific primers (data not shown).

We also tested for the presence of viral genomes in four CSF specimens obtained from an 18-year-old female patient (case 2). Dual amplification of VZV and CMV was initially observed in an early CSF sample on the day of administration. Negative results were obtained at a follow-up CSF taken 11 days from onset. After 18 days from onset, aciclovir was reinitiated, since there was strong evidence for HSE because of MRI and SPECT findings. VZV DNA was amplified at a follow-up CSF taken 25 days from onset. No DNAs were amplified at a follow-up CSF taken 33 days from onset when the patient was discharged.

Next, we screened 31 CSF specimens from 11 encephalitis and 10 meningitis, and four CSF specimens from patients with other neurological disorders. In patients with encephalitis, HSV-1/-2

Table 1 Sequences of primers and probes for DNA polymerase genes

Primer	*Sequence* (5′→ 3′)	*Specificity*
P1	GACTTTGCCAGCCTSTACC	HSV-1/-2, CMV, EBV
	*	
HSV-1/2	G	
CMV	C	
EBV	C	
P2	GTCCGTGTCCCCGTAGATA	HSV-1/2, CMV, EBV
P3	GATTTTSMAAGTTTRTATCC	VZV, HHV-6A/-6B
	** *	
VZV	GC A	
HHV-6	CA G	
P4	CGTATCWCCATAWATWACCT	VZV, HHV-6A/-6B
	* * *	
VZV	T T A	
HHV-6	A A T	
Probe		
HSV	GTGCGCACTGCGTCGGCCCTCAGGGAGAG	HSV-1/-2
VZV	CCTCGTACGCTTTTTGGGAGAGAACGCTAC	VSV
CMV	ATTGTTGTGAGAAGCCGAGGGAAAAGCGGCG	CMV
HHV-6	CCGTGAAGTTGGGGGATGAGACTCATCGGT	HHV-6A/-6B
EBV	CGGGCGCAGGCCGGCTAGCCTGTGCTCTTC	EBV

S: G/C, M: C/A, R: A/G, W: A/T.

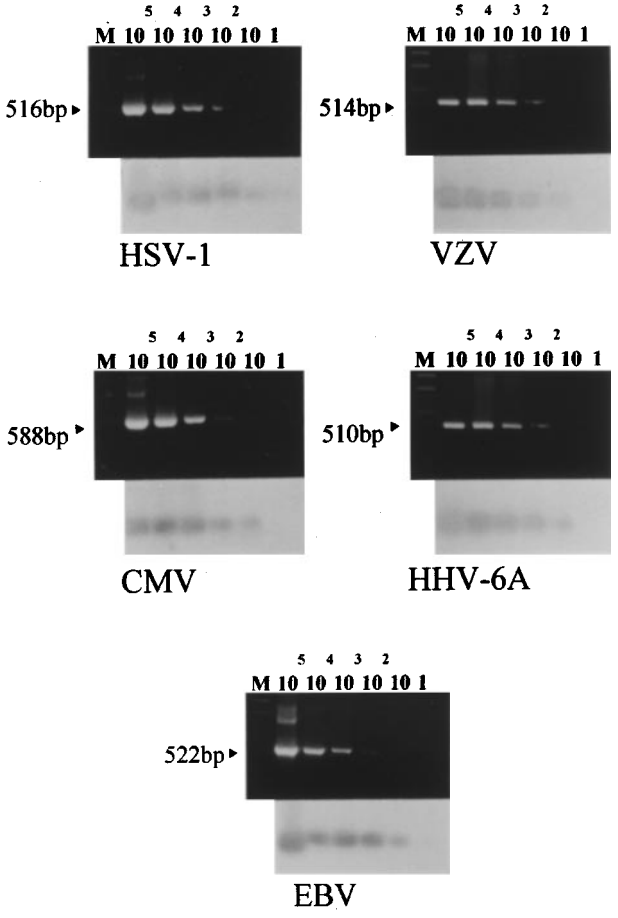

Figure 3 Sensitivity of PCR amplification. A 10-fold dilution series of HSV-1, VZV, CMV, HHV-6A and EBV DNAs was subjected to PCR assay followed by Southern blot hybridization. The endpoint dilution was 100 copies with ethidium bromide-stained agarose gel, and 10 copies with Southern blot hybridization. Lane M: DNA molecular weight marker of $\phi \times 174$RF DNA/*Hae*III fragments.

DNA was detected in five specimens obtained from four patients (cases 3, 4, 5 and 6). Cases 3 and 4 were positive for HSV-1/-2 in early CSF specimens. No DNAs were amplified in the CSF sample from a patient who had suspected HSE (case 7). Eight specimens (five patients) were positive for VZV DNA (cases 3, 4, 5, 6 and 8). Cases 3 and 4 were positive for VZV in both early and late CSF specimens. Case 8 was positive for VZV only in a late CSF sample. CMV DNA was found in one sample (case 11). Negative results were obtained in cases 7, 9, 10, 12 and 13. In patients with meningitis, single positive results were obtained in six cases. One had HSV-1/-2 (case 17), three had VZV (cases 14, 18 and 19), one had CMV (case 21), and one had EBV (case 16) (Tables 2 and 3). In patients with AML, dual amplification of CMV and EBV was observed in 1 CSF sample (data not shown).

Table 2 Detection of viral DNA in patients with encephalitis with PCR assay using consensus primers

Case no	*Days from onset*	*Age*	*Sex*	*Diagnosis*	*HSV*	*VZV*	*CMV*	*HHV-6*	*EBV*
1	1	33	M	CMVE	–	–	+	–	–
	51				–	–	+	–	–
	75				–	–	+	–	–
	83				–	–	+	–	–
	147				–	–	–	–	–
2	1	18	F	VE	–	+	+	–	–
	11				–	–	–	–	–
	25				–	+	–	–	–
	33				–	–	–	–	–
3	1	54	M	HSE	+	+	–	–	–
	27				+	+	–	–	–
	33				–	+	–	–	–
4	1	51	M	HSE	+	+	–	–	–
	18				–	+	–	–	–
5		48	F	HSE	+	+	–	–	–
6		49	M	HSE	+	+	–	–	–
7		46	M	HSE susp.	–	–	–	–	–
8	1	43	M	VE	–	–	–	–	–
	18				–	+	–	–	–
9		35	F	VE	–	–	–	–	–
10		27	M	VE	–	–	–	–	–
11		25	F	VE	–	–	+	–	–
12	1	37	F	VE	–	–	–	–	–
	2				–	–	–	–	–
	7				–	–	–	–	–
	11				–	–	–	–	–
	14				–	–	–	–	–
13	1	55	F	VE	–	–	–	–	–
	14				–	–	–	–	–
	20				–	–	–	–	–

HSV, herpes simplex virus; VZV, varicella-zoster virus; CMV, cytomegalovirus; HHV-6, human herpes virus 6; EBV, Epstein–Barr virus; CMVE, cytomegalovirus encephalitis; HSE, herpes simplex encephalitis; VE, viral encephalitis.

Table 3 Detection of viral DNA in patients with meningitis with PCR assay using consensus primers

Case no.	*Age*	*Sex*	*Diagnosis*	*HSV*	*VZV*	*CMV*	*HHV-6*	*EBV*
14	3m	M	ME	–	+	–	–	–
15	59	M	ME, ML	–	–	–	–	–
16	32	M	ME	–	–	–	–	+
17	50	F	ME	+	–	–	–	–
18	18	M	ME	–	+	–	–	–
19	25	M	ME	–	+	–	–	–
20	32	F	ME	–	–	–	–	–
21	37	M	ME	–	–	–	–	–
22	56	F	P ME	–	–	+	–	–
23	19	M	P ME, HD	–	–	–	–	–

HSV, herpes simplex virus; VZV, varicella-zoster virus; CMV, cytomegalovirus; HHV-6, human herpes virus 6; EBV, Epstein–Barr virus; ME, meningitis; ML, malignant lymphoma, P ME, purulent meningitis; HD, Harada's disease.

Discussion

The shared clinical features of herpesviruses in patients with CNS infections led us to develop a

rapid and simple diagnostic PCR system for simultaneous detection of seven herpesviruses in a single tube assay. It may also be possible to amplify HHV-7 DNA using these primers, when comparing the sequences between HHV-6 and HHV-7. Rozenberg and Lebon (1991) developed a simultaneous amplification system using a single pair of consensus primers. Recently, several studies have been reported a multiplex PCR method in the diagnosis of presumed viral infections of the nervous system (Pozo and Tenorio, 1999; Read and Kurtz, 1999; Tenorio *et al*, 1993). One of the problems with multiplex PCR methods combined with several different primers may be less sensitive, and these protocols require a nested amplification for the detection and typing of herpesviruses. The other problem with PCR using consensus primers is that it detects only a few herpesviruses (Aono *et al*, 1994; Kidd *et al*, 1998; Moschettini *et al*, 1998; Rozenberg and Lebon, 1991). In our PCR method combined with two consensus primers selected from the DNA polymerase genes, seven herpesviruses could be detected without affecting the sensitivity; superior to the previous reports. In this study, four of five patients with HSE were positive for both HSV and VZV. One patient with CMV encephalitis (CMVE) was positive for CMV. Three of seven patients who were diagnosed with viral encephalitis were positive for VZV, CMV, and both VZV and CMV, respectively. Six of 10 patients with meningitis were implicated to have HSV, VZV, CMV or EBV. In patients with other neurological disorders, no herpesviruses were detected in three of four patients. One patient with AML was positive for CMV and EBV DNAs, suggesting reactivation of these viruses under immunosuppression. We have previously reported that coinfection with CMV and EBV occurred in patients whose dialysis period was less than 3 months, and who were in an immunosuppressive state (Yamamoto *et al*, 1995b). Aalto *et al* (1998) reported that primary CMV infection could induce EBV immunoreactivation. In contrast, EBV primary infection did not induce immunoreactivation of CMV. Moreover, CMV immediate early gene products can trans-activate a variety of heterologous promoters, which have been shown to be mediated at the RNA level (Hermiston *et al*, 1987; Pizzorno *et al*, 1988; Davis *et al*, 1987). One patient with viral encephalitis was positive for VZV and CMV DNAs. It was reported that rising IgG for VZV was observed in a patient with primary CMV infection (Aalto *et al*, 1998). CMV appears to be an active inducer of some, but not all herpesviruses. Four patients with HSE were positive for both HSV-1/-2 and VZV. It has been reported that HSV and VZV share antigenic epitopes that may produce cross-reactivity in the antibody test (Fosgren *et al*, 1989). This combination of coinfection determined by PCR was also demonstrated in patients with acute encephalitis, and dual antibodies to both HSV and VZV were present in some of these patients (Casas *et al*, 1996). It seems likely from these reports that coinfection with HSV and VZV in the CNS may be present in patients with suspected HSE, thus implying that both agents are etiologically involved. Several studies have reported the presence of two or more herpesvirus infections, determined by PCR, in CSF specimens (Casas *et al*, 1996; Landgren *et al*, 1994; McElhinney *et al*, 1995; Minjolle *et al*, 1999; Moschettini *et al*, 1998; Read *et al*, 1997; Sudahl *et al*, 1994; Tang *et al*, 1997; Tenorio *et al*, 1993). Herpesviruses induce a concomitant release of IL-6 (Gosselin *et al*, 1992; Kanangat *et al*, 1996; Lagneaux *et al*, 1996) and this could allow the establishment or reactivation of other infectious agents within the host, enhancing the occurrence of coinfection in the CNS. However, it is still unclear whether herpesvirus coinfection with various patterns of combination in the CNS increases the general severity, or alters the clinical features. These points remain to be clarified.

One advantage of our PCR method is its ability to identify the presence of two or more herpesviruses within the same sample. This method was in agreement with the results of PCR using virus-specific primers as previously reported (Yamamoto *et al*, 1995a). Seven herpesviruses could be detected at levels of 10 copies of DNA. However, the detection level in this system is 10-fold less than that of virus-specific PCR. Patients with higher HSV-1 DNA levels (>100 copies/μl) tended to have a more severe diseases and poorer outcomes than patients with lower viral DNA levels (Domingues *et al*, 1998). Patients with active CMV disease of the CNS had over 1000 copies/μl (Shinkai *et al*, 1995) in the CSF or 1500 copies/10^5 CSF cells (Wildemann *et al*, 1998). The copy number of viral DNA in the CSF of patients with mild, atypical or recurrent infections may be lower than the level of viral DNA present in patients with more severe clinical courses (Monteyne *et al*, 1996; Rose *et al*, 1992). The negative results may have been under the detection levels of viral DNA in the CSF. Alternatively, other pathogens may have been present. Therefore, the detection level in this system is reliable to give a rapid diagnosis of herpesvirus-associated CNS diseases. This system can be conveniently and economically applied to detect and identify herpesviruses with routine work in a diagnostic laboratory. The procedure requires as little as 50 μl of CSF, and can be completed in a single tube for amplification of seven herpesviruses. A successful viral isolation from CSF is exceptional, and it takes more than 10 days to reach a significant antibody level (Echevarría *et al*, 1997; Linde *et al*, 1997). Since CSF specimens from

patients without overt CNS infection commonly do not have detectable viral DNA in the CSF (Aslanzadeh *et al*, 1992; Rose *et al*, 1992), this detection system is suitable in CSF specimens from patients with VRNDS. When a sample is positive for HSV or HHV-6 DNA, it is necessary to identify the virus by performing PCR using virus-type specific primers. We selected primers from a highly conserved region in the DNA polymerase genes of herpesviruses. The sequences of HSV-1 and HSV-2 are highly conserved in this region, and it is difficult to select virus-type specific probes. As only partial sequences of HHV-6B DNA are available by computer search, until the full sequences of HHV-6B are available, we cannot distinguish the HHV-6A from HHV-6B.

It is necessary for accurate diagnosis and monitoring of anti-viral drug therapy to quantify the copy number of DNA in specimens. This may give an insight into the relationship between VRNDS and herpesviruses infections. The presence or absence of viral DNA is very important to elucidate the pathogenetical role of viruses in VRNDS. In the future, it would be useful for diagnostic laboratories to develop a microplate hybridization technique to quantify DNA in PCR samples.

Materials and methods

Patients and CSF specimens

Case 1 Five CSF specimens were taken from a 33-year-old male patient who had signs and symptoms including conjunctival hyperemia, headache, and cognitive dysfunction. As he was positive for HSV-IgG, VZV-IgG, and CMV-IgG titers, and had an increasing CSF cell count, HSE was suspected. Acyclovir and Ara-A were administered, and his condition improved. Cognitive dysfunction recurred 1 month later, and elevated anti-CMV-IgG in the CSF was observed. A brain CT demonstrated brain edema without focal lesions. CMV encephalitis was suspected, and ganciclovir was administered. No underlying diseases were found, and he was serologically negative for HIV. The patient made a complete recovery, and was discharged 3 months after treatment. The patient's clinical course is shown in Figure 1A.

Case 2 Four CSF specimens were taken from an 18-year-old female patient who developed tonic-clonic seizure after common cold symptoms. She was healthy before this episode. The only neurological abnormality was a single convulsion at onset and there were no other abnormal physical or neurological signs except for a low-grade fever. Her electroencephalogram showed a spike and slow wave complex of 2 Hz focused on a right posteriotemporal point (T6) and an MR FLAIR (Fluid-attenuated inversion recovery) image revealed a high signal intensity area in her right temporal cortex. There was a decreased cerebral blood flow in the same area on a SPECT study. This lesion was obscure on T1 and T2 MR images. CSF findings showed pleocytosis with normal glucose levels and protein concentration. Bacterial and fungal cultures of CSF were negative and a test for tubercles using PCR method was also negative. Although the CSF findings suggested a CNS viral infection, virological study indicated no infection with HSV-1, HSV-2, VZV, CMV, measles virus, mumps virus, Japanese encephalitis virus, influenza virus type A or type B. Finally, HSV-1/-2 DNA was detected in the CSF. After infusion of acyclovir and antibiotics, the patient was discharged from our hospital without further encephalitis symptoms. EEG was normal at this point and a high intensity area of MR FLAIR image diminished 2 months later. SPECT findings normalized 6 months later.

In addition, 31 specimens from 11 encephalitis and 10 meningitis patients, and four specimens from four other patients with neurological disorders were screened for the presence of herpesviruses. Fourteen of the patients were males, and 11 were females. They ranged in age from 3 months to 61 years (mean $\pm$ S.D., 37.0 ± 17.1 years). Five patients had HSE, including suspected HSE, six had viral encephalitis, eight had meningitis, two had purulent meningitis, and four had other neurological disorders as complications; one had mucocutaneous lymphnode syndrome (MCLS) and sepsis, one had sepsis, one had associated malignancy (acute myeloblastic leukemia; AML), and one had a subarachnoid hemorrhage. The clinical diagnoses of 21 patients with encephalitis and meningitis are shown in Tables 2 and 3.

Cell culture and preparation of negative and positive controls

Vero cells infected with HSV-1 (HF strain) and HSV-2 (UW strain) and human embryo lung (HEL) cells infected with CMV (Town strain) were harvested when a cytopathic effect greater than 80% was observed. A cell suspension of the EBV-containing Raji cells line was used. Human cellular DNA was prepared from HEL cells. The cells were centrifuged and resuspended in a TE buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA) containing 1% sodium dodecyl sulfate (SDS) and 100 μ/ml proteinase K. After incubation at 56°C for 60 min, the lysate was extracted with phenol/$CHCl_3$, and the DNA was precipitated using 2 volumes of ethanol/300 mM sodium acetate (pH 5.2) prior to harvesting by centrifugation. The pellet was resuspended in TE buffer. VZV (a gift from Dr K Hirai), HHV-6A (HST strain) and -6B (U1102 strain) (a gift from Dr K Yamanishi) DNAs were also used.

To obtain well-characterized positive controls and to determine the sensitivity of the PCR assay using consensus primers, a clone containing the complete target sequence for each herpesvirus was

obtained. A region of the DNA polymerase gene from each herpesviral DNA was amplified and cloned into a pGEM-T Easy vector (pGEM-T Easy vector system: Promega, Madison, WI, USA).

PCR amplification by consensus primers

DNA was extracted from CSF by treatment with glass powder (Glassmilk. BIO101 Inc., CA, USA), as described previously (Yamamoto *et al*, 1995a). Briefly, 50 μl of 6 M Guanidine thiocyanate, 3.5 μl of 2 M NaCl, and 10 μl of Glassmilk were added to 50 μl of a CSF sample. After incubation and washing in a washing buffer (50% ethanol, 10 mM Tris-HCl, pH 7.4, and 50 mM NaCl), the DNA bound to glass powder was extracted using 50 μl of DW at 55°C for 15 min followed by centrifugation. To detect the DNAs of HSV-1/-2, VZV, CMV, HHV-6, and EBV simultaneously, consensus primers should be selected from highly conserved sequences among the target genes. Two sets of primer pairs were chosen within the DNA polymerase genes of herpesviruses (Teo *et al*, 1991). 5′-DIG-labeled oligonucleotide probes, which enable distinction between the different types of herpesviruses, were synthesized by Roche Diagnostic Inc. (Tokyo, Japan). The sequences of primers and probes are shown in Table 1.

One μl of DNA was added per 20 μl of reaction mixture. Two pairs of primers were combined in the reaction mixture, after ensuring the sensitivity was unaffected. The reaction mixture contained 1 μM of each primer, 250 μM of each deoxynucleoside triphosphate, 1 × PCR buffer, and 1.5 U of Taq polymerase (Roche Diagnostic Inc.). PCR was performed under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min (40 cycles), and final extension at 72°C for 5 min. Five μl of the amplified products were analyzed by electrophoresis on 1.8% agarose containing 0.5 μg/ml of ethidium bromide, and visualized under UV light.

Southern blot hybridization

Southern blot hybridization with DIG-labeled oligonucleotide probes specific for each virus was carried out according to the manufacturer's method (Roche Diagnostic) at 42°C. After hybridization, positive signals were visualized by development with nitroblue tetrazolium chloride (NBT)/bromochloroindolyl phosphate (BCIP).

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References

Aalto SM, Linnavuori K, Peltola H, Vuori E, Weissbrich B, Schubert J, Hedman L, Hedman K (1998). Immunoreactivation of Epstein-Barr virus due to cytomegalovirus primary infection. *J Med Virol* **56:** 186–191.

Aono T, Murakami S, Yanagihara N, Yamanishi K (1994). Detection of human alpha-herpesvirus DNA using consensus primers and specific probes. *Acta Otolaryngol (Stockh)* **514 (Suppl):** 132–134.

Aslanzadeh J, Osmon DR, Wilhelm MP, Espy MJ, Smith TF (1992). A prospective study of the polymerase chain reaction for detection of herpes simplex virus in cerebrospinal fluid submitted to the clinical virology laboratory. *Mol Cell Probes* **6:** 367–373.

Casas I, Tenorio A, de Ory F, Lozano A, Echevarría JM (1996). Detection of both herpes simplex and varicella-zoster viruses in cerebrospinal fluid from patients with encephalitis. *J Med Virol* **50:** 82–92.

Davis MG, Kenney SC, Kamine J, Pagano J, Huang ES (1987). Immediate-early gene region of human cytomegalovirus transactivates the promoter of human immunodeficiency virus. *Proc Natl Acad Sci USA* **84:** 8642–8646.

Domingues RB, Lakeman FD, Mayo MS, Whitley RJ (1998). Application of competitive PCR to cerebrospinal fluid samples from patients with herpes simplex encephalitis. *J Clin Microbiol* **36:** 2229–2234.

Echevarría JM, Casas I, Martinez-Martin P (1997). Infections of the nervous system caused by varicella-zoster virus/A review. *Intervirology* **40:** 72–84.

Fosgren M, Sköldenberg B, Jeansson S, Grandien M, Blomberg J, Juto P, Berström T, Olding-Stenkvist E (1989). Serodiagnosis of herpes encephalitis by indirect enzyme-linked immunosorbent assay: experience from Swedish antiviral trial. *Serodiagn Immunother Infect Dis* **3:** 259–271.

Gosselin J, Flamand L, D'Addario M, Hiscott J, Stefanescu I, Ablashi DV, Gallo RC, Menezes J (1992). Modulatory effects of Epstein-Barr, herpes simplex, and human herpes-6 viral infections and coinfections on cytokine synthesis. A comparative study. *J Immunol* **149:** 181–187.

Hermiston TW, Malone CL, Witte PR, Stinski MF (1987). Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. *J Virol* **61:** 3214–3221.

Hosoya M, Honzumi K, Sato M, Katayose M, Kato K, Suzuki H (1998). Application of PCR for various neurotropic viruses on the diagnosis of viral meningitis. *J Clin Virol* **11:** 117–124.

Kanangat SJ, Babu S, Knipe DM, Rouse BT (1996). HSV-1-mediated modulation of cytokine gene expression in a permissive cell line: selective upregulation of IL-6 gene expression. *Virology* **219:** 295–300.

Kidd IM, Clark DA, Bremner JAG, Pillay D, Griffiths PD, Emery VC (1998). A multiplex PCR assay for the simultaneous detection of human herpesvirus 6 and human herpesvirus 7, with typing of HHV-6 by enzyme cleavage of PCR products. *J Virol Methods* **70:** 29–36.

Lagneaux L, Delforge A, Snoeck R, Bosmans E, Moreau JF, Taupin JL, De Clercq E, Stryckmans P, Bron D (1996). Human cytomegalovirus increases constitutive production of interleukin-6 and leukemia inhibitory factor by bone marrow stromal cells. *Blood* **87:** 59–66.

Landgren M, Kyllerman M, Bergstrom T, Dotevall L, Ljunstrom L, Ricksten A (1994). Diagnosis of Epstein-Barr virus-induced central nervous system infections by DNA amplification from cerebrospinal fluid. *Ann Neurol* **35:** 631–635.

Linde A, Klapper PE, Monteyne P, Echevarrí JM, Cinque P, Rozenberg F, Vestergaard BF, Ciardi M, Lebon P, Cleator GN, the EU concerted action on virus meningitis and encephalitis (1997). Specific diagnostic methods for herpesviruses infections of the central nervous system: a consensus review by the European Union Concerted Action on Virus Meningitis and Encephalitis. *Clin Diagn Virol* **8:** 83–104.

McElhinney LM, Cooper RJ, Morris DJ (1995). Multiplex polymerase chain reaction for human herpesvirus-6, human cytomegalovirus, and human β-globin DNA. *J Virol Methods* **53:** 223–233.

Minjolle S, Michelet C, Jusselin I, Joannes M, Cartier F, Colomon R (1999). Amplification of the six major human herpesviruses from cerebrospinal fluid by a single PCR. *J Clin Microbiol* **37:** 950–953.

Monteyne P, Sindic CJM, Laterre EC (1996). Recurrent meningitis and encephalitis associated with herpes simplex type 2: demonstration by polymerase chain reaction. *Eur Neurol* **36:** 176–177.

Moschettini D, De Milito A, Catucci M, Marconi A, Rinina C, Bianchi-Bandinelli ML, Valesin PE (1998). Detection of human herpesviruses 6 and 7 in heart transplant recipients by a multiplex polymerase chain reaction method. *Eur J Clin Microbiol Infect Dis* **17:** 117–119.

Novoa LJ, Nagra RM, Nakawatase T, Edwards-Lee T, Tourtellotte WW, Cornford ME (1997). Fulminant demyelinating encephalitis associated with productive HHV-6 infection in an immunocompetent adult. *J Med Virol* **52:** 301–308.

Pizzorno MC, O'Hare P, Sha L, LaFemina RL, Hayward GS (1988). Transactivation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. *J Virol* **62:** 1167–1179.

Pozo F, Tenorio A (1999). Detection and typing of lymphotropic herpesviruses by multiplex polymerase chain reaction. *J Virol Methods* **79:** 9–19.

Read SJ, Jeffery KJM, Bangham CRM (1997). Aseptic meningitis and encephalitis: the role of PCR in the diagnostic laboratory. *J Clin Microbiol* **35:** 691–696.

Read SJ, Kurtz JB (1999). Laboratory diagnosis of common viral infections of the central nervous system by using a single multiplex PCR screening assay. *J Clin Microbiol* **37:** 1352–1355.

Rose JW, Stroop WG, Matsuo F, Henkel J (1992). Atypical herpes simplex encephalitis: clinical, virologic, and neuropathologic evaluation. *Neurology* **42:** 1809–1812.

Rozenberg F, Lebon P (1991). Amplification and characterization of herpesvirus DNA in cerebrospinal fluid from patients with acute encephalitis. *J Clin Microbiol* **29:** 2412–2417.

Shinkai M, Spector SA (1995). Quantitation of human CMV DNA in plasma and cerebrospinal fluid (CSF) by competitive PCR identifies AIDS patients at highest risk for CMV diseases. *The fifth international cytomegalovirus conference. Program and abstracts 67.*

Studahl M, Ricksten A, Sandberg T, Elowson S, Herner S, Sall C, Berstrom T (1994). Cytomegalovirus infection of the CNS in non-compromised patients. *Acta Neurol Scand* **89:** 451–457.

Tang Y-W, Espy MJ, Persing DH, Smith TF (1997). Molecular evidence and clinical significance of herpesvirus coinfection in the central nervous system. *J Clin Microbiol* **35:** 2869–2872.

Tenorio A, Echevarría JE, Casas I, Echevarría JM, Tabarés E (1993). Detection and typing of human herpesviruses by multiplex polymerase chain reaction. *J Virol Methods* **44:** 261–269.

Teo IA, Griffin BE, Jones MI (1991). Characterization of the DNA polymerase gene of human herpesviruses 6. *J Virol* **65:** 4670–4680.

Wagner M, Müller-Berghaus J, Schroeder R, Sollberg S, Luka J, Leyssens N, Schneider B, Krueger GRF (1997). Human herpesvirus-6 (HHV-6)-associated necrotizing encephalitis in Griscelli's syndrome. *J Med Virol* **53:** 306–312.

Weber T, Frye S, Bodemer M, Otto M, Luke W (1996). Clinical implications of nucleic acid amplification methods for diagnosis of viral infections of the nervous system. *J Neurovirol* **2:** 175–190.

Wildemann B, Hass J, Lynen N, Stingele K, Storch-Hagenlocher B (1998). Diagnosis of cytomegalovirus encephalitis in patients with AIDS by quantitation of cytomegalovirus genomes in cells of cerebrospinal fluid. *Neurology* **50:** 693–697.

Yamamoto T, Nakajima Y, Hironaka T, Hirai K, Nakamura Y (1995a). Rapid detection of cytomegalovirus DNA in sera using the polymerase chain reaction: relationship with clinical diagnosis of cytomegalovirus infection after renal transplantation. *Nephron* **70:** 100–103.

Yamamoto T, Nakajima Y, Yamamoto M, Hironaka T, Hirai K, Nakamura Y (1995b). Epstein-Barr virus activity in patients on chronic hemodialysis. *Nephron* **70:** 449–454.