ORIGINAL RESEARCH

Differences of Clinical Manifestations According to the Patterns of Brain Lesions in Acute Encephalopathy with Reduced Diffusion in the Bilateral Hemispheres

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BACKGROUND AND PURPOSE: The precise clinical characteristics of acute encephalopathy with bilateral reduced diffusion are not fully understood. We compared clinical, laboratory, and neuroimaging findings according to the patterns of brain lesions among children with reduced diffusion in the bilateral hemispheres.

MATERIALS AND METHODS: Nine patients were analyzed. The patterns of brain lesions were divided into diffuse lesions and central-sparing lesions. Diffuse lesions were defined as reduced diffusion in the whole cortex and/or subcortical white matter. Central-sparing lesions were defined as the lack of reduced diffusion in the areas around the bilateral Sylvian fissures. Clinical, laboratory, and neuroimaging findings were compared between groups.

RESULTS: Five patients showed diffuse lesions and 4 showed central-sparing lesions. Coma was significantly more common in patients with diffuse lesions, whereas a biphasic clinical course was more common in those with central-sparing lesions. Outcome was worse in patients with diffuse lesions. Maximal aspartate aminotransferase, alanine aminotransferase, and kinase levels were also significantly higher in patients with diffuse lesions. In 2 patients with diffuse lesions, diffusion-weighted images during the acute phase revealed reduced diffusion in the bilateral frontal and occipital areas, followed by diffuse lesions. No patient with central-sparing lesions showed MR imaging abnormalities during the acute phase.

CONCLUSIONS: Clinical manifestations in patients with diffuse lesions were severe, whereas those in patients with central-sparing lesions were relatively mild.

Acute encephalopathy in association with infectious disease has attracted the attention of pediatricians and pediatric neurologists in Japan since the outbreak of influenza-associated encephalopathy during the 1997/1998 winter season. Every year, it is estimated that hundreds of Japanese children die or experience neurologic sequelae due to acute encephalopathy of infectious causes, which has prompted studies on acute encephalopathy in Japan. Recent studies have revealed several patterns of neuroimaging abnormalities in children with acute encephalopathy. Acute necrotizing encephalopathy is characterized by the presence of multiple symmetric brain lesions in the bilateral thalami and other specific brain regions, such as the periventricular white matter and internal capsule.[1] Clinically mild encephalitis/encephalopathy with a reversible splenial lesion is characterized by transient reduced diffusion in the splenium of the corpus callosum, with complete recovery.[2]

Recently, Takanashi et al[3] described a form of acute encephalopathy characterized by biphasic seizures and late reduced diffusion (AESD). In patients with AESD, a seizure, especially a prolonged one, is commonly observed at onset. The following day, patients appear relatively well and seem to have recovered consciousness almost fully, though slightly reduced responsiveness, an absent-minded appearance, or subtle disorientation may be apparent. Deterioration of consciousness, clustered seizures, and involuntary movements appear 3–7 days after the first seizure. Furthermore, this subtype of acute encephalopathy is characterized by widespread reduced diffusion on MR imaging from 3 to 9 days after onset. Although the outcome of patients with AESD is reportedly very poor,[3] a milder form of AESD without neurologic sequelae has also been described.[4] It is interesting that all reported cases of children with AESD have been of in those of East Asian descent.[5]

At present, the precise clinical characteristics of AESD are not fully understood. For example, it is uncertain whether a biphasic clinical course is always observed in children with AESD. It is also unclear whether reduced diffusion is always absent on MR imaging within 2–3 days after onset. We compared the clinical manifestations, laboratory data, and MR imaging features of children with reduced diffusion in the bilateral hemispheres, according to the patterns of brain lesions, to better understand the spectrum of this subtype of acute encephalopathy associated with infection.

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The data in this manuscript were collected from many hospitals. The first 9 authors were attending pediatric neurologists and contributed to the collection of clinical data. Dr Toshiaki Shimizu helped to integrate the clinical data. Dr Tsuneo Morishima supervised this study. These 2 coauthors also contributed to the writing of this manuscript.

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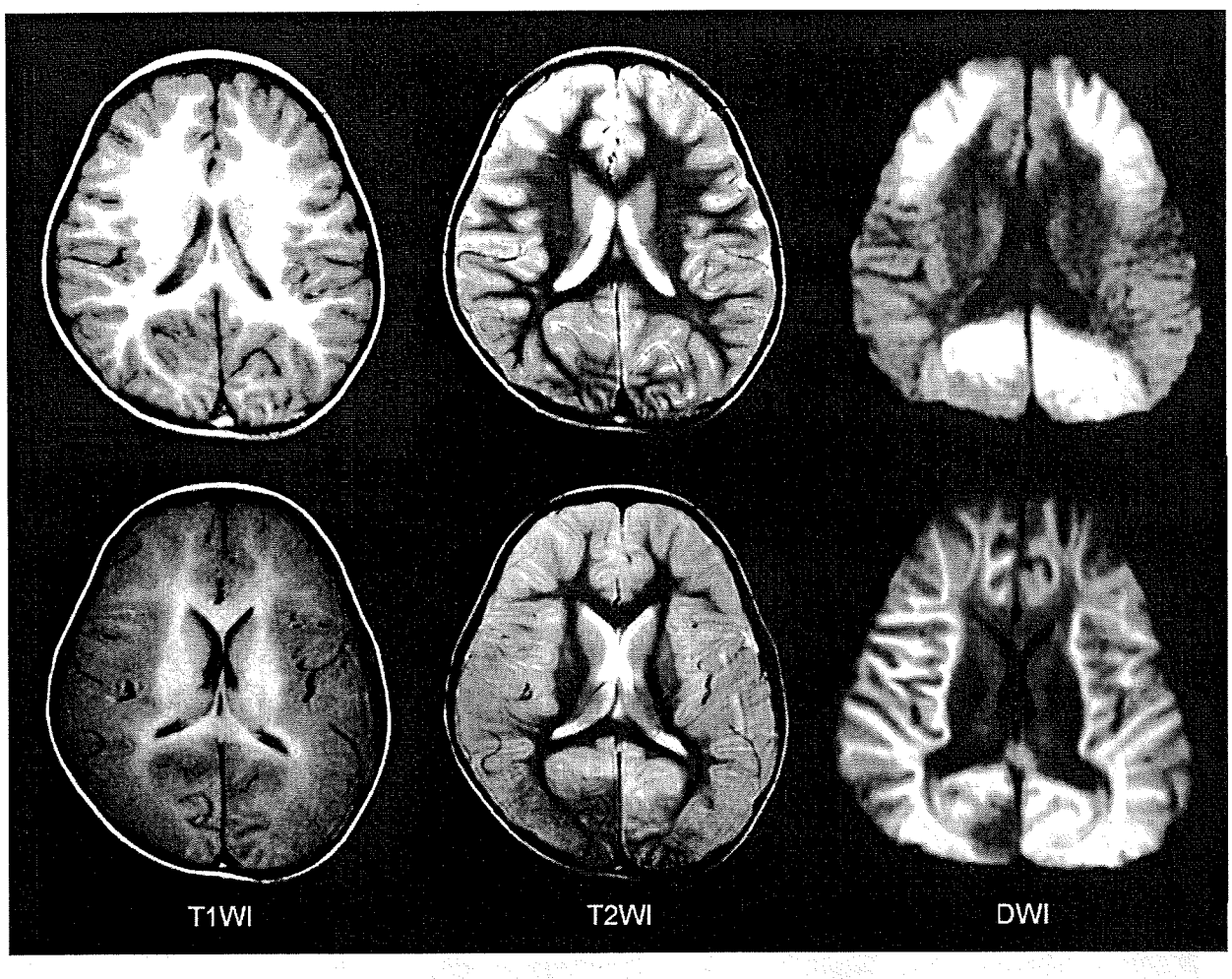

Fig 1. MR imaging findings of a patient with diffuse lesions. Top: The day after the onset, T1-weighted (T1WI) images show mild thickening of the cortex and T2-weighted (T2WI) images reveal mildly increased intensities in the cortex of the bilateral frontal lobes. Reduced diffusivity is observed in the bilateral frontal and occipital regions on DWIs (frontal occipital lesions). Bottom: Five days after the onset, T1WI and T2WI images demonstrate marked edematous changes in the entire cortex. Reduced diffusivity is observed in the entire subcortical white matter on DWIs.

Materials and Methods

We identified 79 children (age, ≤15 years) with acute encephalopathy who were admitted to the Department of Pediatrics at Nagoya University Hospital, Juntendo University Hospital, and 13 affiliated hospitals between January 2000 and August 2007. Acute encephalopathy was defined as a condition characterized by decreased consciousness with or without other neurologic symptoms, lasting for >24 hours in children with infectious symptoms, such as fever, cough, and diarrhea. We carefully excluded patients with sustained decreased consciousness after a febrile seizure, and those with delirious behavior without obviously reduced consciousness. We also excluded patients who were clinically diagnosed with status epilepticus by the attending physician. In this study, a prolonged seizure was defined as one lasting for >20 minutes. Coma was defined as a condition in which the patient was not arousable with maximal painful stimulation, which is consistent with a score of 3–5 on the Glasgow Coma Scale, modified for children, or a score of 100–300 on the Japan Coma Scale.

MR imaging was performed in 65 of 79 patients, and diffusion-weighted images (DWIs) were obtained in 37 patients. Among these, 9 patients showed widespread reduced diffusion in the cortex and/or subcortical white matter of the bilateral hemispheres. These 9 patients became the subjects of this study. In these patients, DWIs were generated by using a 1.5T unit, with a spin-echo echo-planar imaging sequence with variable settings (TE, 86–109 ms; TR, 3066–4100 ms; 952- to 1445-Hz/pixel bandwidth; echo-planar factor, 53–128; section thickness, 5.0–6.0 mm). MR spectroscopy was not performed in any patient.

Two distinct patterns of brain lesions were recognized in DWI: diffuse lesions and central-sparing lesions (Figs 1 and 2). Diffuse lesions were defined as reduced diffusion in the whole cortex and/or subcortical white matter in the bilateral hemisphere during the clinical course. In some patients, reduced diffusion in the frontal and occipital areas preceded diffuse lesions. Central-sparing lesions were defined as the lack of reduced diffusion in the areas around the bilateral Sylvian fissures, though diffuse abnormalities were typically present in other areas on MR imaging. All MR imaging data were reviewed by the chief author (A.O.), with the attending physicians.

Laboratory data were also assessed from medical records. We investigated the following values: platelet counts; aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase, creatinine kinase, blood urea nitrogen, creatinine, glucose, and ammonia levels; and cell counts and protein in the CSF. Some of these values were evaluated in previous studies as prognostic factors of acute encephalopathy.[6,7] Nagao et al[6] revealed that elevated AST levels, hyperglycemia, the presence of hematuria or proteinuria, and the use of diclofenac sodium were associated with poor outcomes in children with influenza-associated encephalopathy.

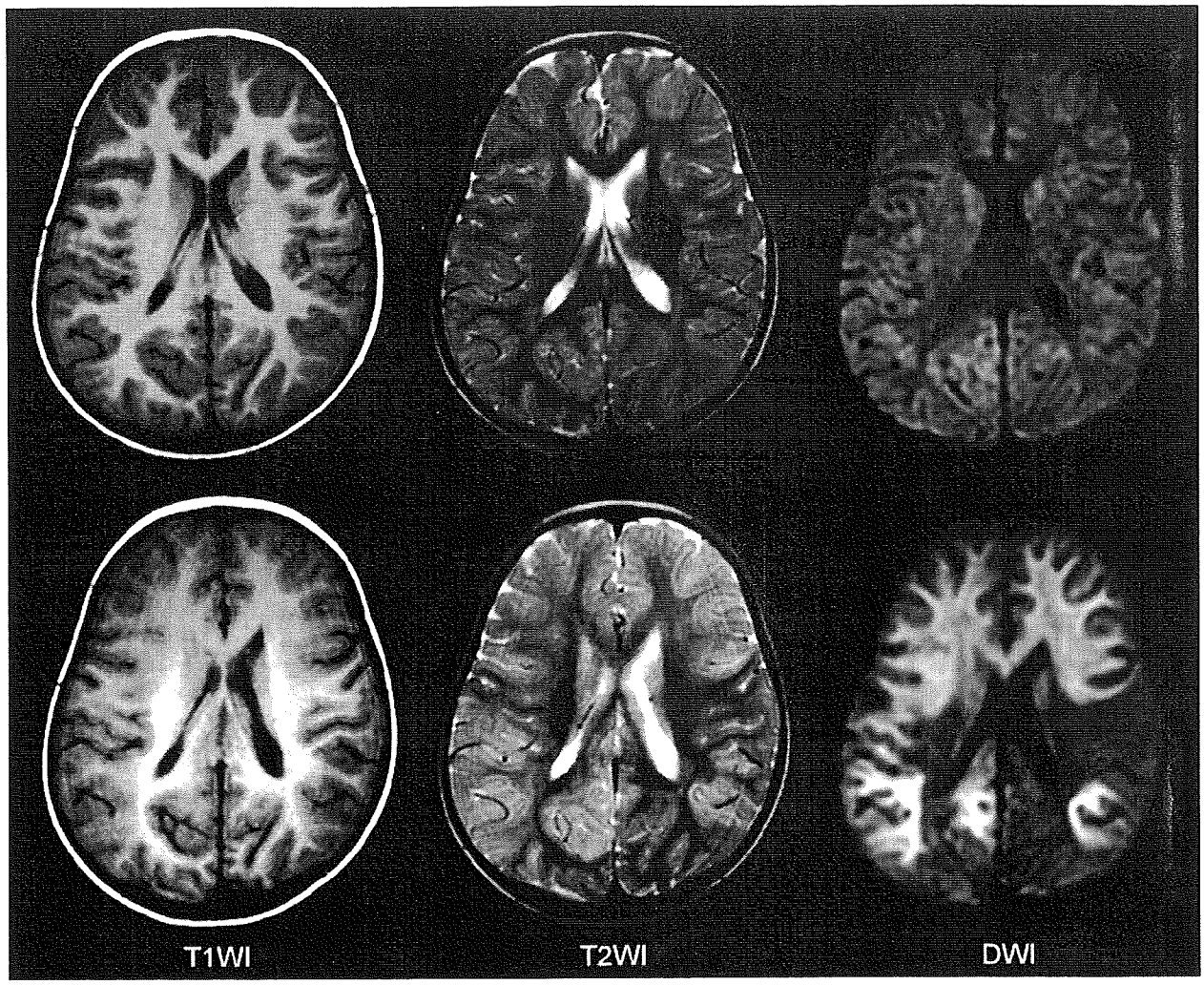

Fig 2. MR imaging findings of a patient with central sparing lesions. Top: Two days after the onset, no abnormalities are observed. Bottom: Four days after the onset, T1-weighted (T1WI) images show unclear gray-white matter differentiation in the bilateral frontal regions. T2-weighted (T2WI) images reveal diffuse thickening of the cortex and increased signal intensities in the bilateral caudate nuclei. Reduced diffusivity is observed in the bilateral frontal and parietooccipital regions on DWIs.

Cognitive impairment was assessed in all surviving patients 1–1.5 years after discharge. Cognitive impairment was usually measured by using the Kaufman Assessment Battery for Children, though it could not be applied in some patients with severe cognitive impairment. The severity of cognitive impairment was defined as mild when the patient's intelligence quotient (IQ) or development quotient (DQ) was between 50 and 70, moderate when IQ/DQ was between 30 and 50, and severe when IQ/DQ was below 30.

Statistical analyses between the 2 groups were performed by using the Mann-Whitney *U* test for numeric variables and the Fisher exact probability test for categoric variables. The outcome of patients was also analyzed by using the Mann-Whitney *U* test. *P* values $< .05$ were statistically significant.

Results

Patient Characteristics

Five boys and 4 girls were analyzed. The median age was 17 months (range, 3–66 months), and 6 of the children were 18 months of age or younger. One patient showed multiple congenital anomalies and developmental delay of unknown cause. Another had a history of febrile seizures. Five patients showed diffuse lesions, and 4 showed central-sparing lesions.

Patient characteristics are summarized in Table 1. Age and sex did not differ between groups. Prodromal illnesses were variable: Virologically proved influenza was observed in 3 patients; exanthem subitum, in 1; gastroenteritis, in 2; and nonspecific febrile illness, in 3.

Neurologic symptoms are also shown in Table 1. Coma was observed within 24 hours after onset in all patients with diffuse lesions. One patient with central-sparing lesions became comatose on the fourth day of illness. Coma was significantly more common in patients with diffuse lesions than in those with central-sparing lesions ($P = .048$). In contrast, a biphasic clinical course was more common in patients with central-sparing lesions than in those with diffuse lesions ($P = .048$). All patients with central-sparing lesions showed a biphasic clinical course. The reduction of consciousness was mild within the first few days after onset, followed by seizures appearing 3–6 days after onset in association with deteriorated consciousness. One patient with diffuse lesions also showed a

Table 1: Patients' characteristics, neurologic symptoms, and outcome

	Diffuse Lesions (n = 5)	Central-Sparing Lesions (n = 4)	P Value
Age (months)*	18 (3–52)	15 (10–66)	NS
Sex (M-F)	3:2	2:2	NS
Prodromal illness			Not done
Influenza	2	1	
Subitum	0	1	
Gastroenteritis	2	0	
NSFI	1	2	
Coma	5	1	.048
Biphasic clinical course	1	4	.048
Seizure at onset	3	4	NS
Prolonged seizure at onset	1	1	NS
Seizure after the first 24 hours	2	4	NS
Outcome			.056
Death	3	0	
Severe cognitive impairment	1	1	
Mild cognitive impairment	1	2	
Healthy	0	1	

Note:—NSFI indicates nonspecific febrile illness; NS, not significant.
* Data are shown as median (range).

biphasic clinical course. In this patient, coma without seizures was the initial presentation, but the reduction of consciousness became milder thereafter. Clustered seizures and worsening of consciousness were observed 2 days after onset. Seizures at onset or after the first 24 hours were observed in all patients with central-sparing lesions, whereas seizures were observed in 3 patients at onset and in 2 after the first 24 hours among those with diffuse lesions. A prolonged seizure was observed in 1 patient with diffuse lesions and in 1 with central-sparing lesions. The duration of seizures was 60 minutes in these patients.

Statistical analyses showed marginal differences in outcome between patients with diffuse-versus-central-sparing lesions (Table 1). All except 1 patient with diffuse lesions died or had severe cognitive impairment, whereas all patients with central-sparing lesions survived and only 1 showed severe cognitive impairment (P = .056). Postmortem examination was not performed in those who died.

Laboratory Data

Maximal AST, ALT, and creatinine kinase levels were significantly higher in patients with diffuse lesions than in those with central-sparing lesions (Table 2). Although abnormalities in platelet counts and lactate dehydrogenase, blood urea nitrogen, and creatinine levels tended to be more severe in patients with diffuse lesions, these differences were not statistically significant. Elevation in ammonia levels, if present, was mild. Hyperglycemia (serum glucose >200 mg/dL) tended to be more common in patients with diffuse lesions, though this difference did not reach statistical significance. CSF analyses did not reveal pleocytosis or increased protein levels in any patient. Disseminated intravascular coagulation was observed in only 1 patient with diffuse lesions. Metabolic acidosis was observed in all except 1 patient with diffuse lesions and in none of those with central-sparing lesions (P = .048).

Neuroimaging Findings

MR imaging during the acute phase (within the first 72 hours after onset) was obtained in 2 patients with diffuse lesions and in 2 with central-sparing lesions. In 2 of 3 patients with diffuse lesions, reduced diffusion was observed in the cortical and subcortical areas in the bilateral frontal and occipital areas (Fig 1). In addition, T1-weighted images demonstrated mild thickening of the cerebral cortex in the corresponding areas, and T2-weighted images revealed mildly increased intensities in the same areas. However, no abnormalities were observed in the remaining 3 patients.

During the subacute phase (from the fourth to the 12th day of illness), MR imaging demonstrated abnormal findings in all patients. As used in the categorization of the patients, markedly reduced diffusion and edematous changes in the entire cortical and subcortical areas were observed in 5 patients (Fig 1). Thickening of the cortex and T1 and T2 prolongation in the subcortical white matter were more remarkable compared with these findings during the acute period in all patients. Blurring of the gray-white matter junction was also prominent. In this group of patients, MR imaging was performed on the fourth day of illness in 1 patient, on the sixth in 2, on the eighth in 1, and on the 12th in 1. In 4 patients with central-sparing lesions, pre- and postcentral areas were clearly spared (Fig 2). Thickening of the cortex and T1 and T2 prolongation in the subcortical white matter were relatively mild, and blurring of the gray-white matter junction was not observed. In this group of patients, MR imaging was performed on the fifth day of illness in 1 patient, on the sixth in 1, on the eighth in 1, and on the 12th in 1.

During the late phase (>2 weeks after onset), MR imaging was conducted in all 7 surviving patients. Three of 5 patients with diffuse lesions survived. Marked cerebral atrophy was observed in 2 patients, and mild cerebral atrophy, in 1 patient on MR imaging during the late phase. Laminar necrosis and increased signal intensities in the subcortical white matter on T2-weighted images were observed in all of these patients. All 4 patients with central-sparing lesions survived. Late MR imaging revealed mild cerebral atrophy in 3 patients and no abnormality in 1. Laminar necrosis was not observed in any patient with central-sparing lesions, whereas mildly increased signal intensities in the subcortical white matter on T2-weighted images were recognized in 3 patients.

No patient showed markedly reduced diffusion in the basal ganglia, thalami, or corpus callosum throughout the clinical course. However, T2-weighted images showed increased signal intensities in the bilateral caudate nuclei in 2 patients with central-sparing lesions during the subacute period.

Discussion

This study demonstrated that acute encephalopathy with reduced diffusion in the bilateral hemispheres can be divided into 2 distinct groups according to the distribution of brain lesions: diffuse and central-sparing lesions. Clinical manifestations, laboratory data, and outcomes were markedly different between patients with diffuse-versus-central-sparing lesions. These results indicate that these 2 groups should be distinguished, though they share common MR imaging abnormalities (ie, widespread reduced diffusion in the cortex and/or subcortical white matter of the bilateral hemispheres).

Patients with diffuse lesions appear to represent a severe phenotype of acute encephalopathy. Clinical symptoms were characterized by rapid and severe deterioration of conscious-

Table 2: Laboratory data

	Diffuse Lesions (n = 5)	Central Sparing Lesions (n = 4)	P Value
Minimal Plt ($\times 10^3/\mu L$)*	14.3 (6.2–36.6)	17.8 (11.3–46.9)	NS
Maximal AST (IU/L)*	917 (189–4407)	107 (53–239)	.028
Maximal ALT (IU/L)*	403 (48–3200)	33 (19–77)	.028
Maximal LDH (IU/L)*	1325 (681–7758)	815 (351–1193)	NS
Maximal CK (IU/L)*	6500 (2057–128472)	320 (62–915)	.014
Maximal BUN (mg/dL)*	19 (7.0–79.1)	12.5 (8.3–15.2)	NS
Maximal Cr (mg/dL)*	0.59 (0.20–2.2)	0.29 (0.22–0.40)	NS
Maximal ammonia ($\mu g/dL$)*	147 (35–176)	83 (55–111)	NS
Blood glucose >200 mg/dL	4	1	NS
CSF cell >10/μL	0	0	NS
CSF protein >40 mg/dL	0	0	NS
DIC	1	0	NS
Metabolic acidosis	4	0	.048

Note:—Plt indicates platelet counts; LDH, lactate dehydrogenase; CK, creatinine kinase; BUN, blood urea nitrogen; Cr, creatinine; DIC, disseminated intravascular coagulation; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
* Data are shown as median (range).

ness, though seizures were not always observed. A biphasic clinical course was rare. Laboratory abnormalities were prominent, including elevated AST, ALT, and creatinine kinase levels; hyperglycemia; and metabolic acidosis. The outcome for patients with diffuse lesions was very poor. Death or severe neurologic sequelae were observed in 4 of 5 patients. These findings may be explained by a systemic inflammatory response, in which multiple organ failure, shock, and disseminated intravascular coagulation are often observed. During the acute stage of acute encephalopathy, the serum and CSF levels of inflammatory cytokines, such as interleukin-6 and tumor necrosis factor-α, were markedly elevated.[7-9] Several pathologic studies have suggested that vascular injury as a result of endothelial damage by inflammatory cytokines is the pathologic substrate of severe types of acute encephalopathy, such as acute necrotizing encephalopathy.[1,10] Although serum and CSF levels of inflammatory cytokines were not measured, it is possible that hypercytokinemia may contribute to the pathogenesis of diffuse lesions.

In contrast, patients with central-sparing lesions appear to represent a relatively mild phenotype of acute encephalopathy. Coma was uncommon and laboratory abnormalities were mild, if present. No patient died, though various degrees of cognitive impairment were observed as neurologic sequelae. A biphasic clinical course is characteristic of this group of patients, as described previously.[3] Several studies on acute encephalopathy have also reported a biphasic clinical course.[11-14] Onset is often marked by a prolonged seizure followed by improved consciousness. However, clustered seizures, signs of frontal lobe dysfunction, and worsening of consciousness become apparent at 3–4 days after onset. These features were observed in our patients with central-sparing lesions. The pathogenesis of acute encephalopathy with central-sparing lesions may be different from that of acute encephalopathy with diffuse lesions. Some authors have suggested that this subtype of acute encephalopathy is caused by excitotoxicity,[3,15] because prolonged seizures are often observed at the onset of AESD. MR spectroscopy has shown increased glutamate concentrations and decreased *N*-acetylaspartate levels in patients with AESD.[16] However, a prolonged seizure at onset was rare in our patients. Further studies are necessary to clarify the pathogenesis of acute encephalopathy with central-sparing lesions.

We consider it possible to distinguish central-sparing lesions from frontal occipital lesions, which may precede diffuse lesions. First, the appearance of reduced diffusion is earlier in frontal occipital lesions than in central-sparing lesions. In this study, central-sparing lesions were not observed in any patient within the first 3 days after onset, consistent with several previous reports.[3,12-14,17] In contrast, frontal occipital lesions were recognized within the first 3 days after onset and were followed by diffuse lesions. Second, the distribution of brain lesions is different in the 2 situations. In patients with central-sparing lesions, the areas without reduced diffusion were strictly limited, around the Sylvian fissures. In the occipital lobes, lesions in the lateral areas were more prominent than those in the mesial areas. In contrast, lesions were located in the anterior half of the frontal lobes and mesial areas of the occipital lobes. Diffusion abnormalities were absent in the posterior half of the frontal lobes and the parietotemporal lobes and were less prominent in the lateral areas of the occipital lobes. However, these observations were based on a small number of patients.

The results of our study are not conclusive. Neuroimaging evaluations of many patients will be necessary to clarify differences between central-sparing lesions and frontal occipital lesions. The recognition of frontal-occipital lesions is useful in the early diagnosis of acute encephalopathy with diffuse lesions and may contribute to early intensive treatment. Our previous study indicated that early steroid use was related to better outcomes in children with acute necrotizing encephalopathy without brain stem lesions.[18]

The DWI patterns of our patients were characteristic, though reduced diffusion in the bilateral hemispheres may be observed with other causes of brain injury, such as hypoxic-ischemic encephalopathy and shaken infant syndrome.[19,20] It is possible that encephalopathy due to substance abuse or intoxication may exhibit similar DWI abnormalities. Thus, the distinction between acute encephalopathy and brain injuries due to other causes may be problematic solely on the basis of imaging findings. For this reason, a diagnosis should be made only after considering clinical manifestations, physical and

neurologic examinations, and laboratory data, in combination with MR imaging abnormalities. In our patients, there was no evidence of hypoxia-ischemia, nonaccidental head injury, or substance intoxication.

To our knowledge, the subtypes of acute encephalopathy have not been sufficiently established at present. The clinical presentation and imaging features of our patients overlap partly with other acute encephalopathy syndromes, including AESD,[3] acute infantile encephalopathy predominantly affecting the frontal lobes,[11] human herpes virus-6 encephalopathy with clusters of convulsions during the eruptive stage,[12] and subacute encephalopathy.[14] These acute encephalopathy syndromes likely represent a spectrum of disorders that share a common process in terms of brain injury. Multidisciplinary studies and further clinical experience are required to clarify the relationships between these syndromes.

In conclusion, acute encephalopathy with reduced diffusion in the bilateral hemispheres can be divided according to the pattern of brain lesions. Patients with diffuse lesions were characterized by coma, severe abnormalities in laboratory test results, and poor neurologic outcome, whereas those with central-sparing lesions were characterized by a biphasic clinical course, less severe abnormalities on laboratory test results, and relatively mild neurologic sequelae. Further neuroimaging studies with larger numbers of patients are necessary to establish the subtypes of MR imaging for acute encephalopathy with reduced diffusion in the bilateral hemispheres and will contribute to clarifying its pathogenesis and effective treatments.

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ORIGINAL ARTICLE

Differences in clinical manifestations of influenza-associated encephalopathy by age

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ABSTRACT

Data from patients in Japan was analyzed to examine the age distribution and differences by age in the clinical manifestations of influenza-associated encephalopathy. Between 1998 and 2002, 472 cases of influenza-associated encephalopathy in patients aged 15 years or younger were reported to the Collaborative Study Group on Influenza-Associated Encephalopathy. These cases were divided into two groups by age: 0–5 and 6–15 years. The differences between the groups were estimated based on the data for those aged 0–5 years, and the odds ratios and 95% confidence intervals calculated. Distribution was inversely correlated with age, with a peak at 1–2 years old. In comparison with patients aged 0–5, those aged 6–15 years had a significantly greater incidence of type B infection, lower frequency of convulsions, higher frequency of loss of consciousness and altered consciousness as the initial neurological symptom, lower serum transaminase levels, lower frequency of low-density area for brain CT upon admission, and lower incidence of sequelae. Our analysis indicates that the clinical course, laboratory data, and brain imaging findings of influenza-associated encephalopathy exhibits patterns that vary with age.

Key words age distribution, age groups, influenza-associated encephalopathy.

INTRODUCTION

Influenza-associated encephalopathy occurs worldwide, but has been reported more often in Japan than in other countries (1–4). Although this disease was previously believed to occur only in Japan, case reports from other countries have increased (5–16). Influenza-associated encephalopathy is an abrupt disorder of the nervous system that is triggered by an influenza virus infection, often leading to death or severe sequelae. There have been no nationwide data on this disease, and frontline clinicians have difficulty identifying it and determining a course of management. Therefore, in the winter of 1998, we initiated a national survey to investigate various parameters of this disease in Japan. Our first comprehensive report, which included 148 cases of influenza-associated encephalopathy in Japan, was released in 2002 (1). Here, we shed further light on the characteristics of this disease in a wider age range of victims, based on data collected over four years.

One of the noteworthy characteristics of this disease is the age distribution of its victims. Influenza viruses have a broad geographic range and affect people of all ages

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List of Abbreviations: CT, computed tomography; HI, hemagglutination inhibition.

as a consequence of antigen drift each year. The scale of the epidemic varies with the virus type. Although school-aged children play an important role in the spread of influenza, most influenza-associated encephalopathy patients are children younger than five; adults are rarely affected (1, 3, 4). We believed that the clinical manifestations of the disease in older children differed from those in patients aged 0–5 years. Therefore, we analyzed the differences in the clinical manifestations of influenza-associated encephalopathy by age.

MATERIALS AND METHODS

Data collection

Questionnaires were developed by the Collaborative Study Group on Influenza-Associated Encephalopathy, which was organized by the Japanese Ministry of Health, Labor, and Welfare. This was a cross-sectional survey of influenza cases treated at all medical facilities. Between 1998 and 2002, hospitals, clinics, and local pediatric facilities reported 585 cases of influenza-associated encephalopathy. In addition to isolation of virus, the sudden onset of high fever with respiratory signs, myalgia, and headache were used as diagnostic markers.

Case definition

The diagnosis of encephalopathy was based on clinical signs. All patients had altered consciousness (*i.e.* delirium, confusion, and senselessness) or loss of consciousness (*i.e.* deep coma, coma, semicoma, stupor, and somnolence). Patients with meningitis, myelitis, and febrile seizures without prolonged unconsciousness were excluded. Postictal unconsciousness with prompt recovery was classified as febrile convulsion.

The diagnosis of influenza infection was based on viral isolation, the viral antigen test, or RT-PCR, or a fourfold or greater increase in paired serum antibody titers (hemagglutination inhibition or complement fixation test). Patients with none of those findings were excluded from further study. In all, 472 influenza-associated encephalopathy cases in patients aged $\leq$ 15 years were analyzed. The outcomes of influenza-associated encephalopathy were defined as normal resolution, mild sequelae, severe sequelae that necessitated personal help for the activities of daily living, and death. Mild sequelae include learning disorders, mental retardation, secondary epilepsy, and mild motor and sensory paralysis.

Statistical analysis

The 472 cases were divided into two groups based on patient age: 0–5 years (n = 382, 80.9%) and 6–15 years (n = 90, 19.1%). The group of patients aged 6–15 was compared to that aged 0–5 years. Odds ratios for the data of the 0–5 age group and their 95% confidence intervals were estimated. *P*-values of dichotomous variables were calculated using the χ^2 test or Fisher's exact test as appropriate. Epi Info version 3.3.2 was used to estimate odds ratios and their confidence intervals and to calculate *P*-values.

RESULTS

Patient background

The number of patients peaked at between one and two years of age. There were only three patients under six months of age; the youngest was two months old. No remarkable differences were observed in the age distribution of patients over each of the four years of the study compared to the combined distribution for all cases (Fig. 1). However, the total number of patients in each year varied (Table 1). Over the course of the study, mortality decreased each year, but the incidence of sequelae did not.

Two antiviral drugs were used for treatment: amantadine and a neuraminidase inhibitor. The former was used frequently in the 1999–2000 season, whereas the latter was used more frequently after it became commercially available in Japan in the 2000–2001 season. The percentage of patients who took amantadine and the neuraminidase inhibitor were similar in the two age groups across the four seasons studied. Some patients received both drugs.

A history of febrile seizures was present in 54 patients; 14 of these had epilepsy. Another three patients had epilepsy with no history of febrile seizures. One patient with propionic acidemia had convulsions and loss of consciousness and died on the day of fever onset.

Differences by age

The male-to-female ratios did not differ between the age groups (data not shown). The influenza virus type was identified in 436 of the 472 cases. The other 36 cases were diagnosed using a viral antigen test that cannot distinguish between type A and type B influenza. On comparing the age groups in the 436 cases, the ratio of type B to type A was significantly higher in patients aged 6–15 than in those aged 0–5 years (odds ratio, 2.35; 95% confidence interval, 1.11–4.91). There was no significant difference in peak body temperature (Table 2).

As an initial neurological symptom, convulsions occurred less frequently in patients aged 6–15 than in those aged 0–5 years (Table 2). However, loss of consciousness and altered consciousness as the initial neurological symptom occurred more frequently in patients aged 6–15 than

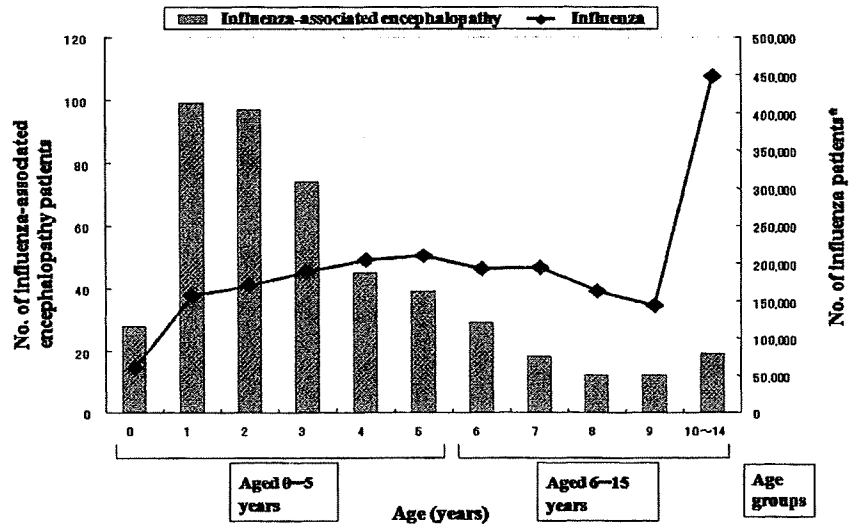

Fig. 1. Age distributions of patients with influenza-associated encephalopathy (vertical bars) and with influenza (line) in Japanese children between 1998 and 2002. *Influenza cases were reported as part of the National Epidemiological Surveillance of Infectious Diseases.

in those aged 0–5 years. The time of neurological onset (days after the onset of high-grade fever) did not differ significantly between the groups.

Concerning the relationship between age and laboratory findings on admission, patients aged 6–15 had lower serum transaminase levels than those aged 0–5 years (Table 3). Urinalysis and cerebrospinal fluid abnormalities did not differ significantly between the groups.

Brain CT was obtained on admission in 368 cases; approximately half of these were normal (Table 4). Brain edema occurred in approximately half of the cases in each age group. Low-density areas occurred less frequently in patients aged 6–15 than in those aged 0–5 years. The time from onset of neurological findings to the day of CT was no more than two days in 89% of the patients for whom both dates were reported.

Table 1 Baseline characteristics of influenza-associated encephalopathy in Japanese children between 1998 and 2002

Characteristic	1998–1999	1999–2000	2000–2001	2001–2002	Total
No. of patients	137	71	54	210	472
Age: no. (%)					
0–5 years	119 (86.9)	51 (71.8)	40 (74.1)	172 (81.9)	382 (80.9)
6–15 years	18 (13.1)	20 (28.2)	14 (25.9)	38 (18.1)	90 (19.1)
Sex: no. (%)					
Male	75 (54.7)	36 (50.7)	34 (63.0)	101 (48.1)	246 (52.1)
Female	62 (45.2)	35 (49.3)	20 (37.0)	109 (51.9)	226 (47.9)
Virus type: no./total no. (%)					
Type A	118/134 (88.1)	71/71 (100)	41/49 (83.7)	163/182 (89.6)	393/436 (90.1)
Type B	16/134 (11.9)	0/71 (0)	8/49 (16.3)	19/182 (10.4)	43/436 (9.9)
Unclassified	3	0	5	28	36
Outcome: no./total no.† (%)					
Normal resolution	54/137 (39.4)	37/71 (52.1)	28/51 (54.9)	106/183 (57.9)	225/442 (50.9)
Mild sequelae	27/137 (19.7)	11/71 (15.5)	11/51 (21.6)	27/183 (14.8)	76/442 (17.2)
Severe sequelae	12/137 (8.8)	7/71 (9.9)	5/51 (9.8)	20/183 (10.9)	44/442 (10.0)
Death	44/137 (32.1)	16/71 (22.5)	7/51 (13.7)	30/183 (16.4)	97/442 (22.0)
Medication: no./total no.‡ (%)					
Amantadine	55/133 (41.4)	52/70 (74.3)	31/39 (79.5)	91/130 (70.0)	229/372 (61.6)
Neuraminidase inhibitor	–	–	10/27 (37.0)	62/121 (51.2)	72/148 (48.6)

†Total no. consists of patients whose outcomes were ascertained.
‡Total no. consists of patients with a definite history of the medication and who took the medicine both before and after the onset of encephalopathy.

Table 2 Comparison of patient progress in the acute phase of influenza-associated encephalopathy

Parameter	No. of patients (%)		Odds ratio with respect to 0–5 years (95% CI)
	0–5 years	6–15 years	
Peak body temperature: no. of patients/total no. (%)			
≥40 °C	160/309 (51.8)	40/71 (56.3)	1.20 (0.69–2.09)
≥41 °C	25/309 (8.1)	11/71 (9.1)	2.08 (0.91–4.72)
Initial neurological symptom: no. of patients/total no. (%)			
Convulsion	270/343 (78.7)	42/76 (55.3)	0.33 (0.19–0.58)*
Loss of consciousness†	62/343 (18.1)	26/76 (34.2)	2.36 (1.31–4.22)**
Altered consciousness‡	11/343 (3.2)	8/76 (10.5)	3.55 (1.25–9.97)***
Time of neurological onset			
≥ day 3/≤ day 2 (% of ≥ day 3/total no.)	24/357 (6.3)	11/78 (12.4)	2.10 (0.92–4.71)

Total no. indicates the number of patients who responded to the question.
†Includes deep coma, coma, semicoma, stupor, and somnolence.
‡Includes delirium, confusion, and senselessness.
§Days after the onset of high-grade fever.
*: $P < 0.001$, **: $P = 0.002$, ***: $P = 0.011$ (Fisher exact).

There was no difference in mortality by age (Table 5). However, patients aged 6–15 had a significantly lower incidence of sequelae than did those aged 0–5 years. No significant differences in the severity of sequelae were observed.

DISCUSSION

Although, according to the report from the National Institute of Infectious Diseases, the age distribution of influenza-affected patients in Japan between 1998 and 2002 was generally flat from one to nine years of age, the distribution of influenza-associated encephalopathy was inversely correlated with age, with a peak at 1–2 years of age (17). The age distribution of influenza virus AH3N2 was similar to that of influenza-associated encephalopathy (data from the National Institute of Infectious Diseases, Japan [http://idsc.nih.go.jp/index.html] (17). Influenza virus AH3N2 is more likely than other types of the virus to trigger encephalopathy (1). We found that the ratio of virus type B to A among patients with encephalopathy was significantly higher in the 6–15 than in the 0–5 years group, possibly because of the prevalence rates of antibodies to each virus type. The National Institute of Infectious Diseases has reported that the antibody prevalence rate to type B virus is very low at all ages, because the scale of type B epidemics is smaller than those of other virus types (17). For example, among healthy

Table 3 Comparison of laboratory findings (blood, urine, and CSF)

	No. of patients (%)		Odds ratio with respect to 0–5 years (95% CI)
	0–5 years	6–15 years	
Blood			
AST ≥ 100 IU/L	119/313 (38.0)	13/68 (19.1)	0.39 (0.19–0.76)*
AST ≥ 500 IU/L	45/313 (14.4)	6/68 (8.8)	0.58 (0.21–1.49)
CPK ≥ 1000 IU/L	31/285 (10.9)	5/60 (8.3)	0.74 (0.24–2.13)
PT ≤ 70%	48/88 (54.5)	12/21 (57.1)	1.11 (0.39–3.23)
Platelet count ≤ $10 \times 10^4/\mu L$	55/293 (18.8)	9/65 (13.8)	0.70 (0.30–1.56)
Platelet count ≤ $5 \times 10^4/\mu L$	26/293 (8.9)	2/65 (3.1)	0.33 (0.05–1.47)
Urine			
Hematuria or proteinuria	79/240 (32.9)	13/55 (23.6)	0.63 (0.30–1.30)
CSF			
WBC count ≥ $8/\mu L$	25/237 (10.5)	10/53 (18.9)	1.97 (0.82–4.69)
Protein level ≥ 50 mg/dl	27/230 (11.7)	10/50 (20.0)	1.88 (0.78–4.45)

AST, aspartate transaminase; CPK, creatinine phosphokinase; PT, prothrombin time; CSF, cerebrospinal fluid; WBC, white blood cells.
*: $P = 0.005$

Table 4 Comparison of brain CT findings (on admission)

Finding	No. of patients/total no. (%)		Odds ratio with respect to 0–5 years (95% CI)
	0–5 years	6–15 years	
Normal	120/306 (39.2)	32/62 (51.6)	1.65 (0.92–2.96)
Edema	154/306 (50.3)	25/62 (40.3)	0.67 (0.37–1.20)
Low-density area	40/306 (13.1)	2/62 (3.2)	0.22 (0.04–0.97)*
Hemorrhage	4/306 (1.3)	1/62 (1.6)	1.24 (0.02–12.78)

*: $P = 0.045$

individuals stratified by age groups, the 2002 rates of HI titers $\geq$ 40 to virus type B/Shandong/7/97 (Victoria lineage) were < 10% in all age groups other than the 20–29 years group. However, the rates of HI titers of $\geq$ 40 to A/New Caledonia/20/99(H1N1) were 40–50% in the 5–19 years age group and approximately 20% in the 0–4 years age group. Finally, the rates of HI titers of $\geq$ 40 to A/Panama/2007/99(H3N2) were slightly less than 70% in patients aged 5–9 years, 55–65% in teens, and 25% in patients aged 0–4 years. Therefore, older children appear to be more susceptible to the type B virus.

Most of the influenza-associated encephalopathy patients had convulsions as an initial neurological sign (74.5% of all cases). However, older children were more likely to experience loss of consciousness or altered consciousness. This may represent information bias, as non-differential misclassification could occur as a result of underestimation of minor neurological signs like altered consciousness, especially in younger children. The age distribution of influenza-associated encephalopathy is similar to that of febrile seizures. Difficulty in distinguishing these two diseases could cause non-differential misclassification. However, differential misclassification might be low because there was almost no information about influenza-associated encephalopathy when we conducted our study, and our selection biases might be very low because almost every case in Japan was reported. There have been some reports that the incidence of febrile seizures is higher in Asia than in Western Europe and the USA (18). In turn, more cases of influenza-associated encephalopathy have been reported in Japan than in Western Europe and the USA (1, 2, 4, 6, 19). Cases of acute necrotizing encephalopathy, a type of acute encephalopathy, have also accumulated in East Asia (20), although reports from other areas have increased recently (9, 11, 12, 21, 22). Based on this data, a genetic background might be involved in the pathogenesis of these diseases.

Patients with influenza-associated encephalopathy often die from multiple organ failure, which is thought to be caused by mitochondria-mediated apoptosis (23). Hosoya *et al.* and Nunoi *et al.* have reported that cytochrome c, a mitochondrial protein found in the intermembrane space, is a good marker for evaluating the clinical severity of influenza-associated encephalopathy (24, 25). Our comparison showed that, upon admission, patients aged 6–15 had a lower incidence of liver dysfunction, and of low-density areas with brain CT, than did those in the group aged 0–5 years. Other studies have also shown that the prevalence of neuroimaging abnormalities is higher in younger than in older children (16, 26). A smaller extent of apoptosis may have caused these findings, which correlate with the low frequency of sequelae in the group aged 6–15 years. Clarke *et al.* have reported that young age is associated with a poor outcome, such as death or severe sequelae, in childhood encephalopathy (27). We suspect that age is a prognostic factor for the sequelae of influenza-associated encephalopathy, although it is not a prognostic factor for death (28). In summary, we

Table 5 Comparison of outcomes

Outcome	No. of patients (%)		Odds ratio with respect to 0–5 years (95% CI)
	0–5 years	6–15 years	
Mortality: no./total no.† (%)	80/359 (22.3)	17/83 (20.5)	0.90 (0.48–1.67)
Existence of sequelae: no./no. alive (%)	105/279 (37.6)	15/66 (22.7)	0.49 (0.25–0.95)*
Severity of sequelae			
Severe sequelae‡/mild sequelae (% of total no. of sequelae)	38/67 (36.2)	6/9 (40.0)	1.18 (0.34–3.99)

†Number of patients whose outcomes were reported.
‡Require personal help for activities of daily living.
*: $P = 0.032$

found that influenza-associated encephalopathy exhibits patterns that vary with age.

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ORIGINAL ARTICLE

Multiplex real-time PCR for the simultaneous detection of herpes simplex virus, human herpesvirus 6, and human herpesvirus 7

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ABSTRACT

A simultaneous detection system to quantify HSV, HHV-6, and HHV-7 DNA via multiplex real-time PCR using different fluorochromes was developed. The minimum quantitative level established via this multiplex assay was four copies per reaction for HSV type 1, four copies for HHV-6, and three copies for HHV-7, respectively. The dynamic range encompassed at least six orders of magnitude. The system was specific and reproducible even in the presence of large amounts of other viral DNA. We then applied this multiplex real-time PCR assay to 105 CSF specimens obtained from subjects less than 15 years old in whom a diagnosis of viral encephalitis/encephalopathy was suspected on clinical grounds. The detection rate for each viral DNA was 6.7% for HSV, 9.5% for HHV-6, and 1.9% for HHV-7. These results indicate that our system is reliable and may be useful for the rapid diagnosis of viral encephalitis/encephalopathy.

Key words cerebrospinal fluid, encephalitis, encephalopathy, viral load.

The human herpesviruses are ubiquitous within human populations; they remain latent within the body after primary infection and often become reactivated in immunocompromised individuals (1, 2). Both primary and recurrent herpesvirus infections may lead to CNS infection and disease. HSV-1 and HSV-2 belong to the genus *Simplexvirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae* (3). HSV-1 is the commonest cause of acute sporadic focal encephalitis (4), and accounts for 2 to 19% of all cases of viral encephalitis and 20 to 75% of all necrotising encephalitis (5, 6). HSV-2 causes encephalitis in neonates and recurrent meningitis in adults (3, 7). HHV-6 and HHV-7 are species within the genus *Roseolovirus*, subfamily *Betaherpesvirinae*, family *Herpesviridae* (8). HHV-6 and HHV-7 infections occur primarily during childhood; >95% of adults have been infected with these viruses (9–11). Primary HHV-6 and HHV-7 infections cause febrile illness, occasionally complicated by convulsions or, much more rarely, encephalopathy (12–16). Recently, it has been shown that reactivation of HHV-6 causes encephalitis, mainly in immunocompromised individuals (17–22).

Early diagnosis of viral encephalitis/encephalopathy is important to ensure adequate treatment and exclude other diseases with a similar clinical presentation. The diagnosis of viral CNS infections has been advanced by new molecular technologies, such as the amplification of viral DNA from CSF via PCR (23–25). PCR has been used for the early diagnosis of CNS HSV infections (26–29). We recently established a multiplex real-time PCR system to

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List of Abbreviations: BHQ, black-hole-quencher; CNS, central nervous system; CSF, cerebrospinal fluid; Ct, threshold cycle; Cy5, carbocyanine 5; FAM, 6-carboxyfluorescein; HHV-6, human herpesvirus 6; HHV-7, human herpesvirus 7; HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; JOE, 6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxyfluorescein; VZV, varicella-zoster virus.

Table 1 Sequences of primers and probes used in multiplex real-time PCR

Target gene (GenBank no.)			Sequence (5′→3′) Position
HSV-1 UL30	Forward primer	ACATCATCAACTTCGACTGG	64203–64222
(X14112)	Reverse primer	CTCAGGTCCTTCTTCTTGTCC	64453–64473
	Probe	FAM-ATGGTGAACATCGACATGTACGG-BHQ1a	64364–64386
HHV-6 U31	Forward primer	TTTGCAGTCATCACGATCGG	46661–46680
(AF157706)	Reverse primer	AGAGCGACAAATTGGAGGTTTC	46862–46883
	Probe	Cy5-AGCCACAGCAGCCATCTACATCTGTCAA-BHQ3a	46753–46780
HHV-7 U57	Forward primer	CGGAAGTCACTGGAGTAATGACAA	88307–88330
(AF037218)	Reverse primer	ATGCTTTAAACATCCTTTCTTTCGG	88390–88412
	Probe	JOE-CTCGCAGATTGCTTGTTGGCCATG-BHQ1a	88332–88355

simultaneously detect Epstein-Barr virus, cytomegalovirus, and HHV-6 DNA (30). This method is able to quantify more than one target per reaction by using different fluorochromes (31, 32), thus decreasing the expense, time, and labour required to diagnose viral CNS infections. Here, we present a multiplex real-time PCR assay for quantifying HSV, HHV-6, and HHV-7 DNA which will aid in the rapid diagnosis of viral encephalitis/encephalopathy.

MATERIALS AND METHODS

Viruses

HSV-1 KOS strain, HSV-2 186 strain, HHV-7 Sato strain, and a clinical HHV-6 strain isolated from a patient with exanthem subitum were used as positive controls. Genetically related viruses, including a VZV Kawaguchi strain, human cytomegalovirus AD169 strain, and Epstein-Barr virus B95–8 strain, were used for the cross-reactivity study.

Clinical specimens

CSF samples were collected from 105 patients and serum samples simultaneously obtained from 46 of them. In total, 151 specimens (CSF, 105; serum, 46) were included in this study. The patients were clinically examined for viral encephalitis/encephalopathy. The 105 patients ranged in age from 0 to 15 years (median, 0.9 years); however, more detailed patient profiles were not available. Informed consent was obtained from parents or guardians, and the ethical provisions of the Declaration of Helsinki were observed.

DNA extracts from either CSF or sera known to be negative for HSV-1, HHV-6, and HHV-7 were used for reconstruction studies. DNA extracted from sera obtained from 30 healthy volunteers was used as a negative control.

DNA extraction

DNA was extracted from 200 μl of each viral culture, CSF, or serum using QIAamp DNA blood kits (Qiagen, Hilden, Germany) and eluted into 50 μl of sterile, double-distilled water. The DNA specimens were stored at −20°C until use.

Primers and probes

The sequences of the primers and probes used in the multiplex real-time PCR assay are listed in Table 1. The target gene for HSV is UL30, encoding DNA polymerase. The target gene for HHV-6 is U31, which is a homologue of human cytomegalovirus UL48 and codes a large tegument protein. The target gene for HHV-7 is U57 which is a homologue of human cytomegalovirus UL86 and codes a major capsid protein. We searched reported sequences of target regions in 13 strains of HSV-1, 3 strains of HHV-6 (including HHV-6A and HHV-6B), and 2 strains of HHV-7. The primers and probes covered all the strains searched. With the exception of the HHV-7 reverse primer, the primer and probe sets used here have been described in previous studies (33–36). The HHV-7 reverse primer was modified because addition of the original primer suppressed amplification of HHV-6 DNA. A difference in only one nucleotide exists between the reverse primer sequences for HSV-1 and HSV-2 (34). A previous study has shown that the primer set for HSV-1 can detect HSV-2 with almost equal efficiency (34), so this primer set was used in the present study (Table 1). Each probe was labelled with different fluorochromes: the HSV-1 probe was labelled with FAM and quenched with BHQ 1a, the HHV-6 probe was labelled with Cy 5 and quenched with BHQ3a, and the HHV-7 probe was labelled with JOE and quenched with BHQ1a. All primers (Fasmac, Kanagawa, Japan) and probes (Operon Biotechnologies, Huntsville, AL, USA) were synthesised commercially.

Quantification of viral DNA by multiplex real-time PCR

Multiplex and single real-time PCR were performed using a QuantiTect multiplex PCR kit (Qiagen). The multiplex real-time PCR assay was performed in a total reaction mixture (25 μl) containing 5 μl of DNA extracts, 12.5 μl of 2× QuantiTect multiplex PCR master mix, 200 nM of each primer, and 100 nM of each probe. The passive reference dye Rox was included in the reaction mixture. Amplification and real-time fluorescence detection were performed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) and the following protocol: an initial denaturation and polymerase activation step for 15 min at 95°C, followed by 50 cycles of denaturation at 94°C for 60 sec and 62°C for 90 sec. Real-time fluorescent measurements were recorded and a Ct value for each sample was calculated by determining the point at which the fluorescence exceeded the threshold. Each real-time PCR assay contained a standard dilution series for DNA quantification, and all samples were analysed in duplicate. Negative controls were added to each run. The standards were plasmid controls that contained the PCR products amplified by each primer set as described previously (33–36). For multiplex real-time PCR, each plasmid control was mixed and diluted to produce standard curves. The number of viral DNA copies was calculated from these standard curves and expressed as copies/ml CSF or serum. A single real-time PCR assay was performed in the same manner as the multiplex assay, except that only one set of primers/probe was included.

Statistical analysis

SPSS for Windows 14.0 (SPSS, Chicago, IL, USA) was used to perform the data analysis. Probit analysis was used to determine the minimum quantitative level in multiplex real-time PCR (37).

RESULTS

Standard curve and dynamic range of multiplex real-time PCR

Serial dilutions of mixed plasmid standards were tested with the multiplex assay, and standard curves were constructed from the Ct values. The multiplex assay was able to detect each plasmid standard over a linear span of 5 to 5×10^6 copies per reaction (Fig. 1a). The standard curves generated from the multiplex assay were similar to those generated from the single assay (Fig. 1b). The dynamic range of the assay encompassed at least six orders of magnitude, with a strong linear relationship between the Ct values and the $\log_{10}$ of the input number of copies (HSV-1, $r^2 = 0.996$; HHV-6, $r^2 = 0.996$; HHV-7, $r^2 = 0.997$). The slope of each standard curve was −3.147 for HSV-1, −3.135 for HHV-6, and −3.255 for HHV-7. The minimum quantitative level, which was determined by probit analysis, was four copies per reaction for HSV-1 (95% confidence interval: 2.53–9.45), four copies for HHV-6 (2.81–9.25), and three copies for HHV-7 (1.99–7.28).

Reproducibility of multiplex real-time PCR

To investigate whether the background DNA of the clinical specimen influences amplification efficiency, we performed reconstructing studies using plasmid standard diluted in water (Fig. 1a) or in water containing HSV-1-, HHV-6-, and HHV-7-negative DNA from either CSF (Fig. 1c) or serum (Fig. 1d). Significant changes were not observed in the presence of DNA from CSF or serum, indicating that background DNA does not affect amplification efficiency.

To evaluate intra- and inter-assay variability, a tenfold serial dilution of the mixed standards was used in the multiplex assay. Ct values were measured in multiple replicates, and the mean of each Ct value and coefficients of variation were calculated. Intra-assay variability was determined using ten replicates per batch, and inter-assay variability was examined by running the same standards with five replicates on four consecutive days (Table 2). Except for the use of five copies of standard plasmids, the coefficients of variation were less than 5% in the intra- and inter-assays, indicating that the assay was highly reproducible.

Specificity of multiplex real-time PCR

To confirm the specificity of the primers and probes, viral genomic DNA extracts from standard strains were tested. No cross-reactivity was observed for VZV, human cytomegalovirus, or Epstein-Barr virus. On the other hand, up to five copies of HSV-2 186 strain were detected by the multiplex assay. To confirm cross reactivity between HSV-1 and HSV-2, the HSV-1 DNA plasmid was replaced with the HSV-2 DNA plasmid, and standard curves were constructed. The standard curves generated from the multiple assays with the HSV-2 plasmid (Fig. 1e) were similar to those generated with the HSV-1 plasmid (Fig. 1a). Probit analysis showed that the minimum quantitative level was 13 copies per reaction for HSV-2 (95% confidence interval: 10.41–23.37). These results indicate that the multiplex real-time PCR assay with the HSV-1 specific primer set can quantify HSV-2 with similar efficiency to HSV-1, although its sensitivity is somewhat lower.

To determine whether viral genomic DNA influences amplification efficiency, we tested our multiplex assay in the presence of large amounts of other viral DNA. In the

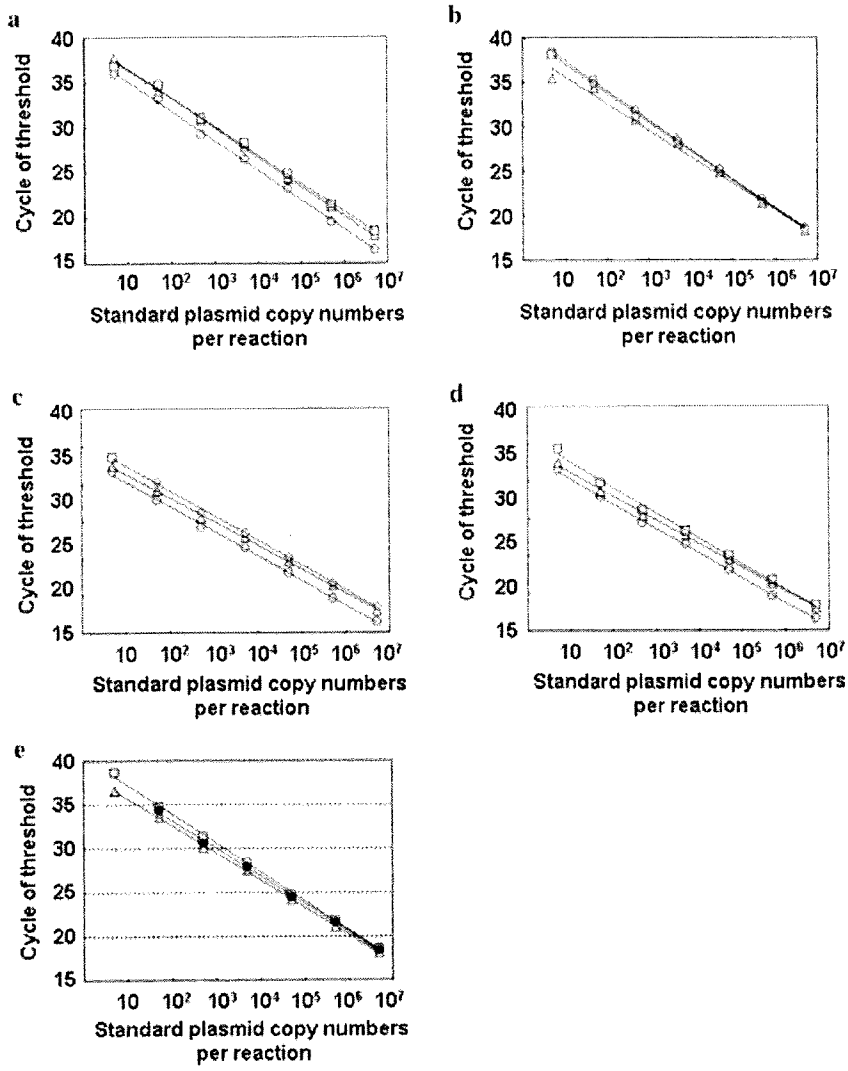

Fig. 1. Standard curves for multiplex and single real-time PCR. Serial dilutions of each viral standard ranging from 5 to 5 × 10^6 copies per reaction were used to generate the standard curves. The cycle of threshold values that corresponded to each PCR cycle number was plotted against the copy number of each viral standard. **(a)** Multiplex real-time PCR. **(b)** Single real-time PCR. **(c)** Multiplex real-time PCR with HSV-1-, HHV-6-, and HHV-7-negative DNA extracts from cerebrospinal fluid. **(d)** Multiplex real-time PCR with HSV-1-, HHV-6-, and HHV-7-negative DNA extracts from sera. **(e)** Multiplex real-time PCR. As a standard, HSV-1 DNA plasmid was replaced with HSV-2 DNA plasmid. (○), HSV-1 DNA plasmid standard; (△), HHV-6 DNA plasmid standard; (□), HHV-7 plasmid standard; (●), HSV-2 DNA plasmid standard.

presence of 10^5 copies of HSV-1 genomic DNA, the HHV-6 and HHV-7 standard curves were similar to those generated from the standard dilutions alone (Fig. 2a). Similarly, in the presence of 10^5 copies of HHV-7 genomic DNA, the HSV-1 and HHV-6 standard curves were similar (Fig. 2b). In contrast, the Ct values for HSV-1 increased and the standard curve for HSV-1 was altered in the presence of 10^5 copies of HHV-6 genomic DNA (Fig. 2c). However, in the presence of 10^4 copies of HHV-6 genomic DNA, the HSV-1 standard curve was similar to that generated from the standard dilutions alone (Fig. 2d). These results indicate that HHV-6 genomic DNA influences the quantification of HSV-1, but the assay remains reliably quantitative in the presence of less than 10^4 copies of each viral genomic DNA per reaction.

Detection of HSV, HHV-6, and HHV-7 DNA in clinical specimens

The multiplex real-time PCR assay was applied to a total of 151 clinical specimens (105 CSF and 46 sera). These specimens were obtained from neonates or children with

Table 2 Intra- and inter-assay variability

Copy number of plasmid standard	HSV-1		HHV-6		HHV-7	
	Mean of Ct	CV‡%	Mean of Ct	CV%	Mean of Ct	CV%
Intra-assay ($n = 10$)						
5×10^6	17.01	1.78	17.73	2.93	18.18	1.62
5×10^5	20.01	2.28	20.82	2.95	21.68	1.05
5×10^4	23.63	1.59	24.02	2.56	24.76	1.38
5×10^3	26.74	1.00	27.20	1.66	28.05	1.35
5×10^2	29.19	0.94	29.62	1.72	30.92	1.45
5×10	32.54	3.63	32.86	3.70	34.10	1.00
5	35.93	31.77	37.06	48.50	37.82	2.96
Inter-assay ($n = 20$, five replicates × four times)						
5×10^6	17.05	1.90	17.86	2.48	18.34	1.74
5×10^5	20.09	1.74	20.88	2.19	21.55	1.04
5×10^4	23.52	1.26	24.14	2.02	24.77	1.03
5×10^3	26.82	0.85	27.42	1.56	28.23	1.19
5×10^2	29.51	1.55	30.12	2.18	31.18	1.61
5×10	32.67	2.60	33.28	2.90	34.32	1.37
5	36.06	41.15	37.02	49.04	38.09	2.62

‡CV, coefficient of variation.

suspected encephalitis/encephalopathy. Since the system can quantify HSV-2 as well as HSV-1 and cannot discriminate between HSV-1 and HSV-2, the types of HSV are not stated in further experiments. The detection rates and amounts of each viral DNA are presented in Table 3. In CSF, all three viral DNA were detected. The detection rate of each viral DNA was 6.7% for HSV, 9.5% for HHV-6, and 1.9% for HHV-7. In one CSF specimen, HHV-6 and

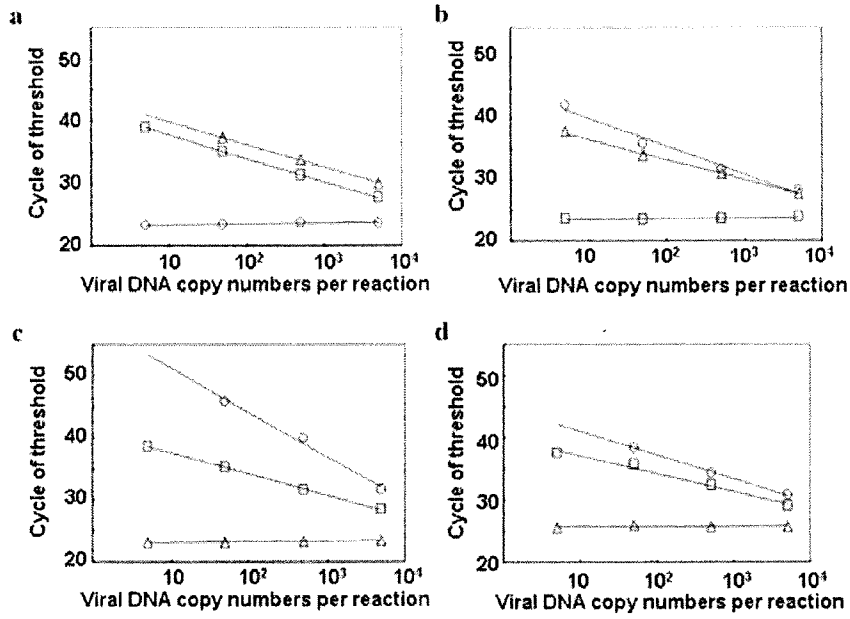

Fig. 2. Inhibition of PCR amplification by large amounts of viral genomic DNA during multiplex real-time PCR. (a) Tenfold dilution series of HHV-6 and HHV-7 plasmid standard mixed with 10^5 copies of HSV-1 genomic DNA were analysed via multiplex real-time PCR. **(b)** Tenfold dilution series of HSV-1 and HHV-6 plasmid standard mixed with 10^5 copies of HHV-7 genomic DNA. **(c)** Tenfold dilution series of HSV-1 and HHV-7 plasmid standard mixed with 10^5 copies HHV-6 genomic DNA. **(d)** Tenfold dilution series of both HSV-1 and HHV-7 plasmid standard mixed with 10^4 copies of HHV-6 genomic DNA. (○), HSV-1 DNA plasmid standard; (△), HHV-6 DNA plasmid standard; (□), HHV-7 plasmid standard.

Table 3 Detection and quantification of HSV, HHV-6, and HHV-7 DNA in cerebrospinal fluid and sera using multiplex real-time PCR

	Cerebrospinal fluid ($n = 105$)		Serum ($n = 46$)	
Detected viral DNA	No. of positive (%)	Mean copies/ml (range)	No. of positive (%)	Mean copies/ml (range)
HSV	7 (6.7)	15 813 (40–51 750)	2 (4.3)	18 860 (16 250–21 470)
HHV-6	10 (9.5)	435 (50–1.960)	12 (26.0)	18 041 (40–104 900)
HHV-7	2 (1.9)	27.0 (12–42)	0	

HHV-7 were detected at levels of 1960 copies/ml and 42 copies/ml, respectively. In contrast, only HSV and HHV-6 were detected in sera. HHV-6 was detected most frequently; the highest number of HHV-6 copies was 104 900 copies/ml, corresponding to 2090 copies/reaction.

DNA extracted from sera from 30 healthy volunteers was analysed as a negative control. HSV, HHV-6, and HHV-7 DNA were not detected in any samples.

DISCUSSION

We established a simultaneous detection system to quantify HSV, HHV-6, and HHV-7 DNA using multiplex real-time PCR. The multiplex assay was sensitive enough to detect and quantify a very low copy number without mutual interference. The system was also specific and reproducible even in the presence of background DNA or large amounts of other viral DNA. Although very large amounts of HHV-6 DNA decreased quantitative power, this assay was reliable in the presence of less than 10 000 copies of viral DNA per reaction. Because the highest copy number for HHV-6 in clinical samples was 2090 copies per reaction, this multiple real-time PCR assay may be useful for clinical samples. A recent study showed that serum viral loads in patients with primary HHV-6 infections were less than 1000 copies/ml (38). These results indicate that the multiplex system described here would be a reliable tool in clinical settings.

Only three genes can be analysed simultaneously when using the Mx3000P real-time PCR system. We selected HSV, HHV-6, and HHV-7 for several reasons. First, these three viruses are most representative of the DNA viruses that cause encephalitis/encephalopathy in children: HSV-1 is the commonest cause of acute sporadic focal encephalitis (4) and primary HHV-6 and HHV-7 infections cause significant neurological morbidity in children (15, 39, 40). HSV-2, which causes encephalitis in neonates and recurrent meningitis in adults, can be quantified by the multiplex real-time PCR system. Other DNA viruses exist that affect the CNS: VZV, which belongs to the *Alphaherpesvirinae* subfamily, is also neurotropic, and primary or reactivated VZV infections have been linked to CNS disease, including acute cerebellar ataxia, encephalitis, meningitis, myelitis, strokes, and Reye's syndrome (7). However, typical skin eruptions can be used to diagnose VZV-associated CNS diseases. Second, encephalitis caused by HSV-1 and HSV-2 has a poor prognosis, but can be treated with specific antiviral agents (3). Similarly, encephalitis caused by HHV-6 and HHV-7 occasionally leads to neurologic sequelae (7). Successful treatment with antiviral agents has been reported in HHV-6 encephalitis (22, 41–43). Therefore, early diagnosis is critical for encephalitis caused by HSV, HHV-6, and HHV-7. Furthermore, this system can be used to examine the effectiveness of anti-viral therapies by quantifying viral DNA loads in patients undergoing treatment. Finally, several RNA viruses can cause encephalitis/encephalopathy, including arboviruses, enteroviruses, and influenza virus (44), most of which are endemic or seasonal (4). Although real-time PCR is applicable to RNA viruses, the simultaneous detection of DNA and RNA viruses is difficult. Therefore, we examined only DNA viruses in this study.

The reported prevalence of herpesvirus DNA in CSF is variable because of different systems and populations. A report using a DNA microarray showed that the prevalence of viral DNA was 6.7% for HSV, 5.0% for HHV-6A and -6B, and 1.6% for HHV-7 in patients with clinically suspected meningitis or encephalitis (45). On the other hand, in another study the prevalence of HHV-6 DNA in CSF samples was 2.5% in young children (<2 years) with neurological illness (38). In this study, the detection rates for each viral DNA in CSF were 6.7% for HSV, 9.5% for HHV-6, and 1.9% for HHV-7. This study was based on samples from children and neonates with suspected viral encephalitis/encephalopathy. Because this group may be biased (i.e. cases without encephalitis/encephalopathy may have been included), we do not think that these rates represent the prevalence of each virus-associated encephalitis/encephalopathy in Japanese children. However,

our results indicate that the number of cases of these virus-associated CNS diseases in the population is substantial, which supports our decision to study these three viruses in particular.

Notably, both HHV-6 and HHV-7 were detected in CSF from one out of 105 patients (0.95%). Because of the study design, we could not obtain additional clinical information about this patient, so the significance of the double-positive is unclear. However, it does demonstrate that our multiplex assay is useful for detecting unexpected viruses. Further studies are necessary to determine the significance of co-existing HHV-6 and HHV-7 infections in the CNS.

Because these viruses are latent in lymphocytes, false-positive PCR detection can occur in CSF samples (16). In contrast, if viral replication does not occur in the CNS, viral DNA may not necessarily be present in the CSF. Interestingly, HHV-6 DNA was detected in 26% of the sera from patients with suspected encephalitis/encephalopathy, while it was positive in only 9.5% of the CSF. Meanwhile, we should be careful about the interpretation placed on the existence of HHV-6 DNA in the serum, since chromosomal viral integration is occasionally seen in immunocompetent individuals and may result in a mistaken association with encephalitis (38). The multiplex real-time PCR system described here may prove useful in elucidating the pathogenetic mechanism of these virus-associated CNS diseases.

In conclusion, we developed a multiplex real-time PCR system for the simultaneous detection of HSV, HHV-6, and HHV-7. This system was sensitive, specific, and reproducible without mutual viral interference. As such, this system offers potential benefits in clarifying the pathogenesis of viral encephalitis/encephalopathy and facilitating the early diagnosis of viral encephalitis.

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