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Table 1
Cytokine levels in serially collected sera samples in patients with and without primary HHV-6 infection.

Sampling days	Cytokines (pg/ml)	Normal control	With primary HHV-6 inf.		W/O primary HHV-6 inf.		*P*-Value[a]
		Mean ± SD	Median (25–75%)		Median (25–75%)		
Days 0–5 (acute)	IL-1β	0.0 ± 0.0	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.3921
	IL-2	0.0 ± 0.0	**24.1**	**(15.7–26.8)**	**8.4**	**(0.0–17.6)**	**0.0366**
	IL-4	0.0 ± 0.0	**13.5**	**(0.0–23.0)**	**0.0**	**(0.0–0.0)**	**0.0255**
	IL-5	0.0 ± 0.0	11.5	(8.9–12.2)	9.5	(2.3–10.1)	0.2227
	IL-6	1.1 ± 4.2	14.2	(0.0–21.2)	7.8	(0.0–22.5)	0.8001
	IL-10	3.9 ± 4.8	40.3	(18.9–64.0)	9.0	(0.0–25.5)	0.0513
	IL12p70	0.0 ± 0.0	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.3237
	IFN-γ	0.0 ± 0.0	**168.2**	**(119.1–291.7)**	**66.7**	**(38.0–85.5)**	**0.0046**
	TNF-α	0.0 ± 0.0	0.0	(0.0–9.3)	0.0	(0.0–6.6)	0.0874
Days 6–15 (convalescent)	IL-1β	0.0 ± 0.0	0.0	(0.0–12.4)	0.0	(0.0–0.0)	0.1264
	IL-2	0.0 ± 0.0	23.6	(6.9–33.5)	7.8	(0.0–16.4)	0.0701
	IL-4	0.0 ± 0.0	18.5	(0.0–21.7)	0.0	(0.0–0.0)	0.0535
	IL-5	0.0 ± 0.0	**10.9**	**(8.4–13.4)**	**0.0**	**(0.0–6.2)**	**0.0205**
	IL-6	1.1 ± 4.2	0.0	(0.0–17.5)	4.2	(0.0–9.0)	0.4503
	IL-10	3.9 ± 4.8	0.0	(0.0–15.4)	0.0	(0.0–0.0)	0.0653
	IL12p70	0.0 ± 0.0	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.4173
	IFN-γ	0.0 ± 0.0	45.8	(35.2–52.0)	13.4	(0.0–45.2)	0.3033
	TNF-α	0.0 ± 0.0	15.9	(6.6–19.7)	8.8	(0.0–17.7)	0.6276
1 month after onset of illness	IL-1β	0.0 ± 0.0	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.8195
	IL-2	0.0 ± 0.0	22.2	(9.6–29.5)	17.2	(4.2–17.8)	0.6367
	IL-4	0.0 ± 0.0	0.0	(0.0–13.9)	0.0	(0.0–7.8)	0.7676
	IL-5	0.0 ± 0.0	10.8	(0.0–14.1)	9.6	(1.7–13.5)	0.9729
	IL-6	1.1 ± 4.2	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.6859
	IL-10	3.9 ± 4.8	0.0	(0.0–1.8)	0.0	(0.0–4.7)	0.8627
	IL12p70	0.0 ± 0.0	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.4945
	IFN-γ	0.0 ± 0.0	31.1	(9.8–43.7)	16.1	(3.3–21.2)	0.3625
	TNF-α	0.0 ± 0.0	5.6	(0.0–16.9)	0.0	(0.0–11.4)	0.8853

Inf.: infection, W/O: without.
Bold value indicates the biomarkers, in which there were significant difference between the 2 groups.
[a] Comparison between patients with and without primary HHV-6 infection.

was approved by the review boards of Fujita Health University. EDTA-treated peripheral blood and serum were serially collected from the patients at the initial visit to the clinic (days 0–5, acute), days 6–15 (convalescent), and 1 month after onset of the illness. Meanwhile, 1 ml of serum was collated from 14 healthy controls in Konan Kosei Hospital.

3.2. Virus isolation and serological analysis

The procedures for the isolation and identification of HHV-6 have been described elsewhere.[2] IgG antibody titers to HHV-6 were measured using an indirect-immunofluorescence assay as described previously.[2] We defined the patients with either seroconversion or HHV-6 viremia as the patients with primary HHV-6 infection.

3.3. Measurements of cytokines and chemokines

Serially collected serum samples were processed immediately after collection and stored at −70 °C for the subsequent measurement of cytokines and chemokines. The quantification of nine cytokines (interleukin 1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α)) and five chemokines (IL-8, regulated on activation normal T-cell expressed and secreted (RANTES), monokine induced by interferon-γ (MIG), monocyte chemotactic protein-1 (MCP-1), interferon inducible protein-10 (IP-10)) in serum were determined by the cytometric bead array kit (BD Biosciences, CA, USA). Assays were carried out according to the manufacturer's instructions.

3.4. Statistical analysis

Demographic factors and clinical data were compared between patients with and without primary HHV-6 infection by Fisher's exact test or Student's *t*-test. Mean peak cytokine and chemokine levels were compared between the two groups using the Mann–Whitney *U*-test. The statistical analysis was performed with StatView software, version J-5.0.

4. Results

4.1. Virological analysis and demographic factors

Seroconversion of HHV-6 IgG antibody titers was observed in 20 of the 26 (76.9%) patients. Additionally, HHV-6 was isolated from 13 of the 20 patients that had seroconverted. All of the patients with primary HHV-6 infection showed the typical clinical course of exanthem subitum. None of the patients had complications such as febrile seizure and hepatitis. No skin rash was observed in the patients without primary HHV-6 infection. In these patients, initial hematological examinations revealed normal range of leukocyte counts and C-reactive protein. The mean age of the patients with and without primary HHV-6 infection was 9.1 months (ranged between 6 and 16 months) and 12 months (ranged between 7 and 16 months), respectively ($P = 0.1289$). The proportion of males to females was not statistically different between the two categories (with primary HHV-6 infection, 6/14; without primary HHV-6 infection; 2/4, $P = 0.9999$).

4.2. Cytokine and chemokine levels

A total of 74 serially collected sera samples (days 0–5, 25 samples; days 6–15, 24 samples; 1 month after the onset of the illness, 25 samples) were analyzed in this study. The cytokine levels for the patients with and without primary HHV-6 infection are reported in Table 1. The expression of three cytokines (IFN-γ, IL-2, and IL-4) at days 0–5 were significantly higher in patients with primary HHV-6 infection than those without primary HHV-6 infection. The level of

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Table 2
Chemokine levels in serially collected sera samples in patients with and without primary HHV-6 infection.

Sampling days	Chemokines (pg/ml)	Normal control	With primary HHV-6 inf.		W/O primary HHV-6 inf.		*P*-Value[a]
		Mean ± SD	Median (25–75%)		Median (25–75%)		
Days 0–5 (acute)	IL-8	12.3 ± 4.2	30.7	(23.0–44.2)	31.1	(24.6–36.0)	0.8313
	RANTES	19,238.3 ± 6592.1	2745.4	(724.3–19,822.6)	3513.3	(2302.2–18,470.3)	0.5839
	MIG	648.9 ± 499.4	859.9	(534.9–1204.8)	850.1	(539.0–1569.6)	0.6701
	MCP-1	148.1 ± 72.7	**974.7**	**(732.1–1385.0)**	**296.6**	**(250.6–519.7)**	**0.0019**
	IP-10	660.1 ± 333.5	3130.8	(2560.3–3985.5)	1977.6	(1448.5–3678.1)	0.2733
Days 6–15 (convalescent)	IL-8	12.3 ± 4.3	28.6	(17.7–43.0)	28.0	(18.1–30.3)	0.4836
	RANTES	19,238.3 ± 6592.1	3506.0	(2059.8–22,030.1)	12,459.2	(3229.5–22,535.5)	0.6057
	MIG	648.9 ± 499.4	339.0	(189.9–842.5)	615.8	(450.6–1195.8)	0.2267
	MCP-1	148.1 ± 72.7	402.1	(310.4–455.5)	315.6	(274.8–353.0)	0.1046
	IP-10	660.1 ± 333.5	792.6	(489.7–1066.4)	517.7	(268.6–832.1)	0.1815
1 month after onset of illness	IL-8	12.3 ± 4.3	25.9	(9.7–40.8)	18.0	(15.9–21.3)	0.4611
	RANTES	19,238.3 ± 6592.1	3893.5	(858.0–22,256.7)	12,463.3	(1524.4–24,455.6)	0.3412
	MIG	648.9 ± 499.4	325.7	(202.7–482.7)	503.0	(440.0–561.6)	0.2849
	MCP-1	148.1 ± 72.7	469.1	(333.1–611.8)	394.8	(288.5–440.8)	0.2040
	IP-10	660.1 ± 333.5	548.7	(425.2–687.8)	444.8	(384.6–505.1)	0.5475

Inf.: infection, W/O: without.
Bold value indicates the biomarkers, in which there were significant difference between the 2 groups.
[a] Comparison between patients with and without primary HHV-6 infection.

IL-5 during the convalescent period (days 6–15) was significantly higher in patients with primary HHV-6 infection than those without primary HHV-6 infection (10.0 ± 5.2 pg/ml vs. 3.2 ± 5.0 pg/ml, $P = 0.0205$).

The chemokine levels are shown in Table 2. MCP-1 expression was significantly higher in patients with primary HHV-6 infection than those without primary HHV-6 infection (1028.2 ± 459.3 pg/ml vs. 385.2 ± 224.0 pg/ml, $P = 0.0019$). No other chemokine levels were statistically different between the patients with primary HHV-6 infection and those without primary HHV-6 infection.

5. Discussion

At the time of the acute phase of disease (days 0–5), the expression of 3/9 cytokines (IFN-γ, IL-2, and IL-4) and 1/5 chemokines (MCP-1) were significantly higher in patients with primary HHV-6 infection than those without HHV-6 infection, which might contain various type of viral infections. Additionally, IL-5 levels during the convalescent period (days 6–15) was significantly higher in patients with primary HHV-6 infection. Moreover, as shown in Table 1, all of the cytokines and chemokines analyzed in this study were returned to normal levels by 1 month after onset of the illness, such that there was no statistical difference between the patients with and without primary HHV-6 infection. Therefore, the upregulation of cytokines and chemokines observed during the acute and convalescent periods of the disease are considered to be specific finding. Many in vitro analyses have been carried out to determine whether HHV-6 can modulate with cytokine and chemokine production,[8–12] however, to our knowledge, this is the first study, which analyzed cytokine and chemokine expression in vivo, specifically in patients with primary HHV-6 infection.

Interpretation of increased levels of cytokines or chemokines in patients with HHV-6 infection might be difficult because of the complex cytokine and chemokine networks in vivo. It has been demonstrated that IL-2, which is strong T-cell activator, is necessary for efficient HHV-6 replication in cultured lymphocytes.[13] Therefore, upregulation of IL-2 in patients with primary HHV-6 infection may enhance viral replication in vivo. However, a previous in vitro study reported reduced IL-2 production by HHV-6 infected T-cells, which may contribute to immune suppression.[14] These conflicting data suggest that the in vitro findings may not fully recapitulate the cytokine and chemokine responses to primary HHV-6 infection in vivo. Meanwhile, an increase in production of IL-4 and IL-5, which are associated with allergic reaction, has been demonstrated in patients with drug induced hypersensitivity syndrome with HHV-6 reactivation.[15] However, no remarkable observation with regard to these cytokines has been reported on the basis of in vitro experiments. Thus, further in vitro and in vivo analysis is needed to the functional biological relevance of the expression of these cytokines and chemokines during HHV-6 infection.

Upregulation of MCP-1 has been demonstrated in HHV-6 infected human peripheral blood monocytes in vitro.[11] Thus, our observation of increased MCP-1 levels in patients with primary HHV-6 infection is consistent with the previous in vitro study. MCP-1 is strong mediator of monocyte chemoattraction. As it has been suggested that HHV-6 can latently infect monocytes/macrophage cells after primary infection,[16] upregulation of this chemokine might be beneficial for efficient establishment of HHV-6 latency. Furthermore, it has been demonstrated that HHV-6 encodes viral chemokines,[17] which can mediate monocyte chemoattraction. Collectively, these data suggest that HHV-6 might use various mechanisms to establish latency in monocytes/macrophage.

Conflict of interest

The authors do not have any commercial or other associations that might pose a conflict of interest.

Ethical approval

This study was approved by the review boards of Fujita Health University.

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Different characteristics of human herpesvirus 6 encephalitis between primary infection and viral reactivation

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ABSTRACT

Background: Pathogenesis of human herpesvirus 6 (HHV-6) encephalitis, in particular difference between HHV-6 encephalitis at the time of primary infection and reactivation remains unclear.
Objectives: To elucidate the mechanism of HHV-6 encephalitis at the time of primary infection and reactivation.
Study design: Twenty-two HHV-6 encephalitis patients at the time of primary infection, 6 febrile convulsion (FC) patients caused by HHV-6 infection, and 14 FC patients without HHV-6 infection (non HHV-6 FC) were enrolled. Additionally, 7 stem cell transplant recipients with HHV-6 encephalitis and eight adult controls were also enrolled in this study. Cerebrospinal fluid (CSF) HHV-6 DNA copy numbers and biomarkers levels were compared.
Results: Low copy number of CSF HHV-6 DNA was detected in 7 of the 22 patients with HHV-6 encephalitis in primary infection, whereas all seven CSF samples collected from post-transplant HHV-6 encephalitis patients contained high viral DNA copy numbers ($P<0.001$). CSF concentrations of IL-6 ($P=0.032$), IL-8 ($P=0.014$), MMP-9 ($P=0.004$), and TIMP-1 ($P=0.002$) were significantly higher in patients with HHV-6 encephalitis in primary infection than non-HHV-6 FC. CSF IL-6 ($P=0.008$), IL-8 ($P=0.015$), and IL-10 ($P=0.019$) concentrations were significantly higher in patients with post-transplant HHV-6 encephalitis than adult controls.
Conclusion: The present study suggests that the characteristics of HHV-6 encephalitis are different between HHV-6 encephalitis at the time of primary infection and reactivation in transplant recipients.
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1. Background

Primary human herpesvirus 6 (HHV-6) B infection (exanthem subitum)[1,2] is considered a benign febrile illness that rarely causes neurological complications such as febrile convulsion and encephalitis. According to a recent survey,[3] an annual incidence of exanthem subitum-associated encephalitis/encephalopathy was estimated at 60 cases per year in Japan, and the prognosis of the disease was unexpectedly poor. In addition to primary infection, it has been suggested that HHV-6 reactivation can cause encephalitis in transplant recipients[4–8] and patients with drug-induced hypersensitivity syndrome.[9–11]

Highly sensitive nested polymerase chain reaction (PCR) detected HHV-6 DNA in cerebrospinal fluid (CSF) collected from HHV-6 encephalitis patients[12–14] and exanthem subitum patients with febrile convulsion,[15] suggesting direct invasion of the virus into central nervous system (CNS). Furthermore, both viral DNA and viral antigens were detected in the postmortem brain tissues[16] and brain tissues collected from mesial temporal lobe epilepsy patients.[17] In addition, HHV-6 also infects neuroglial cell lines and modulates cytokine synthesis in HHV-6-infected an astrocytoma cell line.[18] Collectively, these findings highlight the neurovirulence of HHV-6. However, the pathogenesis of HHV-6 encephalitis, in particular the difference between HHV-6 encephalitis at the time of primary infection and viral reactivation remains unclear.

2. Objective

In the present study, we assessed viral copy number and expression of several biomarkers in CSF samples collected from HHV-6 encephalitis patients to elucidate the mechanism of

Abbreviations: HHV-6, human herpesvirus 6; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; CNS, central nervous system; IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

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Table 1
Characteristics of the patients.

	HHV-6 encephalitis at primary inf. ($n=22$)	FC with HHV-6 inf. ($n=6$)	FC w/o HHV-6 inf. ($n=14$)	Post-transplant HHV-6 encephalitis ($n=7$)	Adult controls ($n=8$)
Mean age ± SD (years old)	0.7 ± 0.8	0.8 ± 0.8	1.1 ± 0.6	43.9 ± 15.2	66.9 ± 11.0
Gender (M/F)	11/11	2/4	4/10	5/2	4/4

FC; febrile convulsion, inf.; infection.
Adults controls: 2 patients with involuntary movement, 2 patients with spinocerebellar degeneration, 1 patient with frontotemporal dementia, 1 patient with normal pressure hydrocephalus, 1 patient with progressive supranuclear palsy, 1 patient with Parkinson disease.

HHV-6 encephalitis at the time of primary infection and reactivation.

3. Study design

3.1. Patients

Twenty-two patients with HHV-6 encephalitis at the time of primary infection (HHV-6 encephalitis at primary infection) were enrolled. Thirteen of the 22 patients had convulsions and became comatose within a few days after the onset of fever, and 9 of the 22 patients had cluster convulsions at the eruptive phase. As control groups, six patients with febrile convulsion due to primary HHV-6 infection (HHV-6 FC) and 14 patients with febrile convulsion without HHV-6 infection (non HHV-6 FC) were also examined. The viral reactivation group included seven allogeneic hematopoietic stem cell transplant recipients with HHV-6 encephalitis. Eight adult control patients were enrolled. Patient demographics are summarized in Table 1. This study was approved by the review boards of Fujita Health University. Guardians or the patients consented to their participation in this study.

Primary HHV-6 infection was defined on the basis of viral isolation or detection of viral DNA in serum and serological analysis. HHV-6 encephalitis in primary infection was defined as the patients with stupor and/or convulsion at the time of primary HHV-6 infection. To exclude patients with a severe form of febrile convulsion, abnormal radiological examinations were required for diagnosis of HHV-6 encephalitis in this study. Reactivation of HHV-6 was confirmed by a presence of HHV-6 IgG antibody and either detection of viral DNA in serum or viral isolation from peripheral blood at the time of disease onset in transplant recipients. All seven post-transplant HHV-6 encephalitis patients had typical clinical symptoms and radiological findings for limbic encephalitis.

3.2. Measurement of CSF cytokines, MMP-9 and TIMP-1

CSF samples were collected from patients within 48 h of the onset of neurological symptoms and stored at −70 °C. The concentrations of interleukin (IL)-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 in CSF were determined using the cytometric bead array kit according to the manufacturer's instructions (BD Biosciences, CA, USA). Matrix metalloproteinase (MMP)-9 (pro- and active forms) concentrations in the CSF (1:5 dilution) were determined by enzyme-linked immunosorbent assay (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. Tissue inhibitor of metalloproteinase (TIMP)-1 concentration in CSF (1:8 dilution) was also determined by enzyme-linked immunosorbent assay (Invitrogen, CA, USA).

3.3. DNA extraction and real-time PCR

DNA was extracted from 200 μl of CSF or serum using QIAamp Blood Kit (QIAGEN, Chatsworth, CA) and eluted in 50 μl of elution buffer, then stored at −20 °C. The details of the real-time PCR methods for measuring viral DNA copy numbers have been described elsewhere.[19] Detection limit of the real-time PCR was 125 copies/ml.

3.4. Statistical analysis

CSF viral DNA copy number or concentration of the biomarkers between the two different groups of the patients were compared: (1) HHV-6 encephalitis in primary infection vs HHV-6 FC; (2) HHV-6 encephalitis in primary infection vs non-HHV-6 FC; (3) HHV-6 FC vs non-HHV-6 FC; (4) HHV-6 encephalitis in primary infection without neurological sequelae vs HHV-6 encephalitis in primary infection with neurological sequelae; (5) HHV-6 encepalitis in primary infection vs post-transplant HHV-6 encephalitis; and (6) post-transplant HHV-6 encephalitis vs adult control. The statistical comparison of age and gender was evaluated by Student's *t*-test and Fisher's exact test, respectively. The statistical comparison of CSF biomarkers levels was evaluated by Mann–Whitney *U*-test. $P<.05$ was considered to be statistically significant. The statistical analysis was performed with StatView software, version J-5.0.

4. Results

4.1. Patients

As shown in Table 1, there was no significant age and gender difference among 22 patients with HHV-6 encephalitis in primary infection, 6 patients of HHV-6 FC, and 14 patients of non-HHV-6 FC. Although only one encephalitis patient had mild pleocytosis (13 cell/μl), CSF cell counts were normal in the remaining 19 patients. Seven of the 22 HHV-6 encephalitis patients with primary infection had severe neurological sequelae such as hemiplegia (2 cases), quadriplegia (2 cases), and mental retardation (3 cases). The 13 patients revealed no severe neurological sequelae. No detail information about sequelae was available from remaining the two cases. All seven post-transplant HHV-6 encephalitis patients were adult. Although no statistical gender difference was demonstrated between post-transplant encephalitis patients and adult control ($P=0.608$), mean age of the controls (66.9 ± 11.0 years) was significantly higher than post-transplant HHV-6 encephalitis patients (43.9 ± 15.2 years) ($P=0.008$). In order to exclude possibility for chromosomal integration of the HHV-6 genome in post-transplant HHV-6 encephalitis patients, copy numbers of HHV-6 DNA was measured in serum or CSF samples collected after antiviral treatment. CSF HHV-6 DNA decreased to undetectable level in 3 of the 7 patients. No viral DNA in serum was demonstrated in the remaining 4 recipients (data not shown). These results suggested that the 7 post-transplant HHV-6 encephalitis patients were not chromosomal integrated HHV-6 patients.

4.2. HHV-6 DNA copy numbers in CSF

Detection rate and copy numbers of HHV-6 DNA in CSF samples are shown in Table 2. Small amounts of HHV-6 DNA was detected in CSF from 7 of the 22 patients (31.8%) with HHV-6 encephalitis in primary infection (median, interquartile range

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Table 2
Copy numbers and detection rate of HHV-6 DNA in cerebrospinal fluid.

	Number of positive cases/ number of tested (%)	Copy numbers of HHV-6 DNA; mean ± SD (copies/ml)	*P*-value
HHV-6 encephalitis at primary infection	7/22 (31.8)	13.22 ± 39.16	a, c, d
HHV-6 FC	1/6 (16.7)	48.75 ± 119.41	b, c, d
Non HHV-6 FC	0/14 (0)	0 ± 0	d
Post-transplant HHV-6 encephalitis	7/7 (100)	464090.4 ± 1185622.0	e
Adult controls	0/8 (0)	0 ± 0	e

(a) Comparison between patients with HHV-6 encephalitis at primary infection and those with HHV-6 FC ($P=0.648$). (b) Comparison between patients with HHV-6 FC and those with non-HHV-6 FC ($P=0.127$). (c) Comparison between patients with HHV-6 encephalitis at primary infection and those with non-HHV-6 FC ($P=0.021$). (d) Comparison between patients with HHV-6 encephalitis at primary infection and those with post-transplant HHV-6 encephalitis ($P<0.001$). (e) Comparison between patients with post-transplant HHV-6 encephalitis and adult controls ($P<0.001$).

(IQR); 0, 0–3.8 copies/ml), and in 1 of the 6 patients (16.7%) with HHV-6 FC (292.5 copies/ml). However, no HHV-6 DNA was detected from CSF in any patients with non HHV-6 FC (control group). In contrast to HHV-6 encephalitis in primary infection, all seven CSF samples collected from post-transplant HHV-6 encephalitis contained high copy of HHV-6 DNA (median, IQR; 1820.0, 848.3–46120.8 copies/ml), which was significantly higher than the patients with HHV-6 encephalitis at primary infection ($P<0.001$).

4.3. CSF concentrations of cytokines, MMP-9, and TIMP-1

Comparison of the biomarkers among HHV-6 encephalitis at primary infection, HHV-6 FC, and non-HHV-6 FC is presented in Fig. 1. CSF IL-8 concentrations were significantly higher in patients with HHV-6 encephalitis at primary infection (median, IQR; 291.0, 115.1–801.6 pg/ml) than those with HHV-6 FC (median, IQR; 63.5, 56.2–173.2 pg/ml, $P=0.025$) or those with non-HHV-6 FC (median, IQR; 105.5, 77.6–196.8 pg/ml, $P=0.014$). CSF IL-6 concentrations were also significantly higher in patients with HHV-6 encephalitis at primary infection (median, IQR; 22.6, 9.2–64.0 pg/ml) than those with non-HHV-6 FC (median, IQR; 10.7, 7.9–16.5 pg/ml, $P=0.032$). Moreover, the concentration of MMP-9 (median, IQR; 7.9, 7.1–8.3 pg/ml) and TIMP-1 (median, IQR; 110.2, 87.0–153.3 pg/ml) was significantly higher in patients with HHV-6 encephalitis at primary infection than those with non-HHV-6 FC (MMP-9, median, IQR; 6.3, 4.7–7.3 pg/ml, $P=0.004$; TIMP-1, median, IQR; 55.2, 47.9–77.5 pg/ml, $P=0.002$). Additionally, HHV-6 encephalitis in primary infection patients had a significantly higher concentration of TIMP-1 (median, IQR; 110.2, 87.0–153.3 pg/ml) than patients with HHV-6 FC (median, IQR; 71.6, 65.9–82.1 pg/ml, $P=0.025$).

Interestingly, CSF IL-6 concentrations were significantly higher in patients with HHV-6 encephalitis at primary infection with neurological sequelae (median, IQR; 29.7, 22.6–127.4 pg/ml) than those without neurological sequelae (median, IQR; 13.3, 5.5–25.6 pg/ml, $P=0.017$) (Fig. 2). No other biomarkers were significantly different between the two groups.

Next, concentrations of CSF biomarkers were compared between post-transplant HHV-6 encephalitis patients and adult controls or patients with HHV-6 encephalitis at primary infection (Fig. 3). CSF IL-8 (median, IQR; 200.0, 107.1–316.5 pg/ml vs 60.3, 41.7–74.6 pg/ml; $P=0.015$), IL-6 (median, IQR; 23.8, 7.8–143.3 pg/ml vs 2.7, 2.3–4.1 pg/ml; $P=0.008$), and IL-10 (median, IQR; 2.5, 0.9–5.1 pg/ml vs 0.0, 0.0–0.0 pg/ml; $P=0.019$) concentrations were significantly higher in post-transplant HHV-6 encephalitis than adult controls. No statistical difference was observed in all of the biomarkers between post-transplant HHV-6 encephalitis patients and HHV-6 encephalitis at primary infection patients. Although CSF MMP-9 concentrations were not significantly different between post-transplant HHV-6 encephalitis patients and adult controls, MMP-9 concentrations were significantly lower in post-transplant HHV-6 encephalitis (median, IQR; 4.2, 3.6–6.0 pg/ml) than HHV-6 encephalitis at primary infection (median, IQR; 7.9, 7.1–8.3 pg/ml; $P=0.01$). In contrast to MMP-9, its inhibitor, CSF TIMP-1 was significantly higher in post-transplant HHV-6 encephalitis patients (median, IQR; 207.4, 180.1–221.8 pg/ml) than either adult controls (median, IQR; 77.7, 66.8–95.0 pg/ml; $P=0.001$) or HHV-6 encephalitis at primary infection (median, IQR; 110.2, 87.0–153.3 pg/ml; $P=0.001$).

5. Discussion

Quantification of viral DNA copy number in CSF using real-time PCR provided valuable information regarding the pathogenesis of HHV-6 encephalitis. Although high copy numbers of HHV-6 DNA have been detected in CSF collected from post-transplant HHV-6 encephalitis,[8,17,20,21] to our knowledge, this is the first report demonstrating a difference in CSF HHV-6 DNA copy numbers between HHV-6 encephalitis in primary infection and post-transplant HHV-6 encephalitis patients using same real-time PCR method. These data suggest that replication of HHV-6 in the CNS plays an important role in the pathogenesis of post-transplant HHV-6 encephalitis. However, it seems that direct viral replication in the brain tissue is not main pathological mechanism for HHV-6 encephalitis in primary infection. Thus, the pathogenesis of HHV-6 encephalitis would be different between primary infection and reactivation in transplant recipients.

Elevation of two (IL-6 and IL-8) of the six cytokines in CSF was striking in patients with HHV-6 encephalitis in primary infec-

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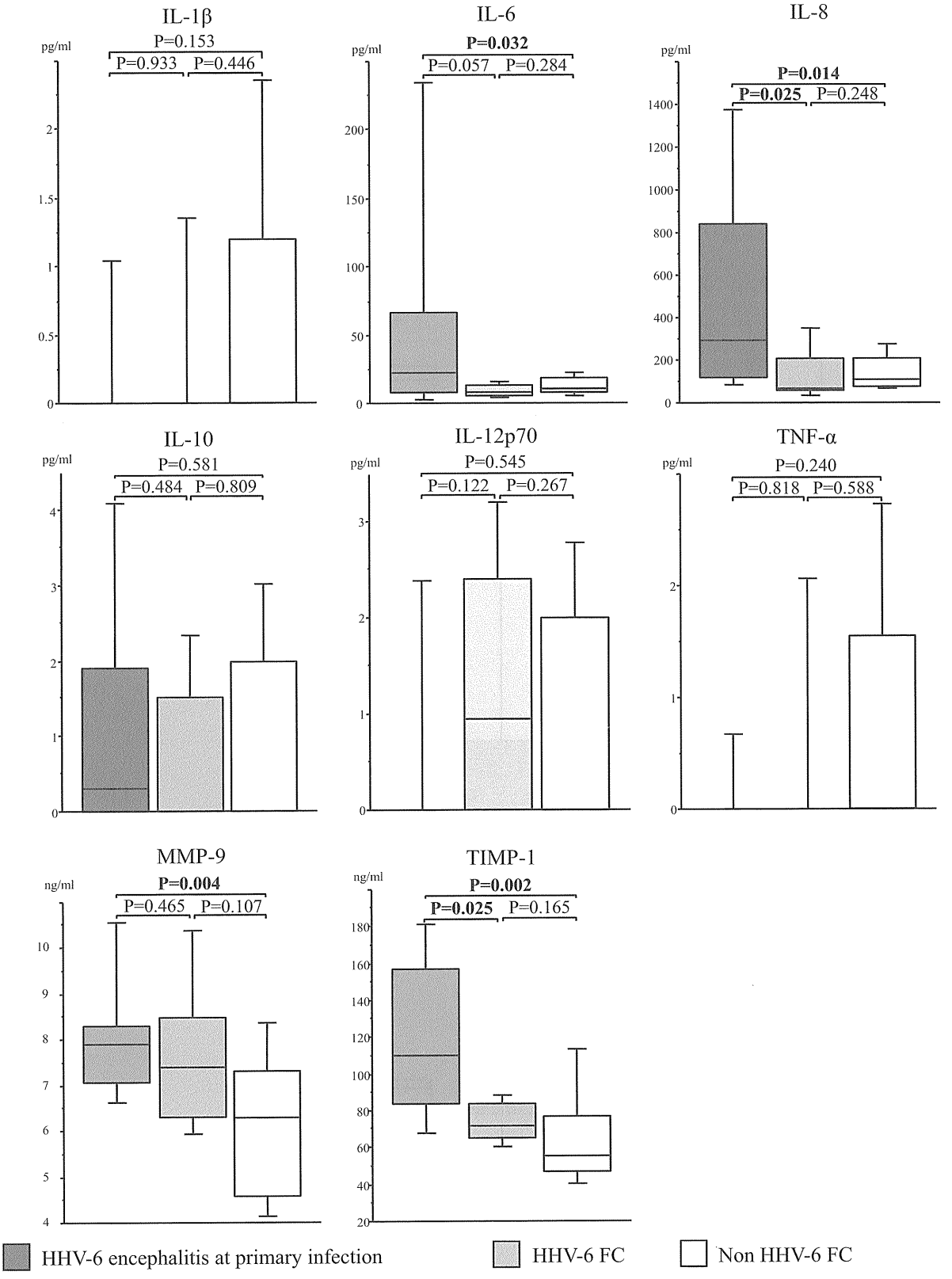

Fig. 1. Comparison of the eight cerebrospinal fluid (CSF) biomarkers (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, MMP-9, and TIMP-1) levels among patients with HHV-6 encephalitis in primary infection ($n = 22$), febrile convulsion due to primary HHV-6 infection (HHV-6 FC) ($n = 6$), and febrile convulsion without HHV-6 infection (non HHV-6 FC) ($n = 19$). Data are shown as box plots, where the boxes represent the first through third quartiles, the lines within the boxes represent the median, and the lines outside the boxes represent the minimum and maximum values.

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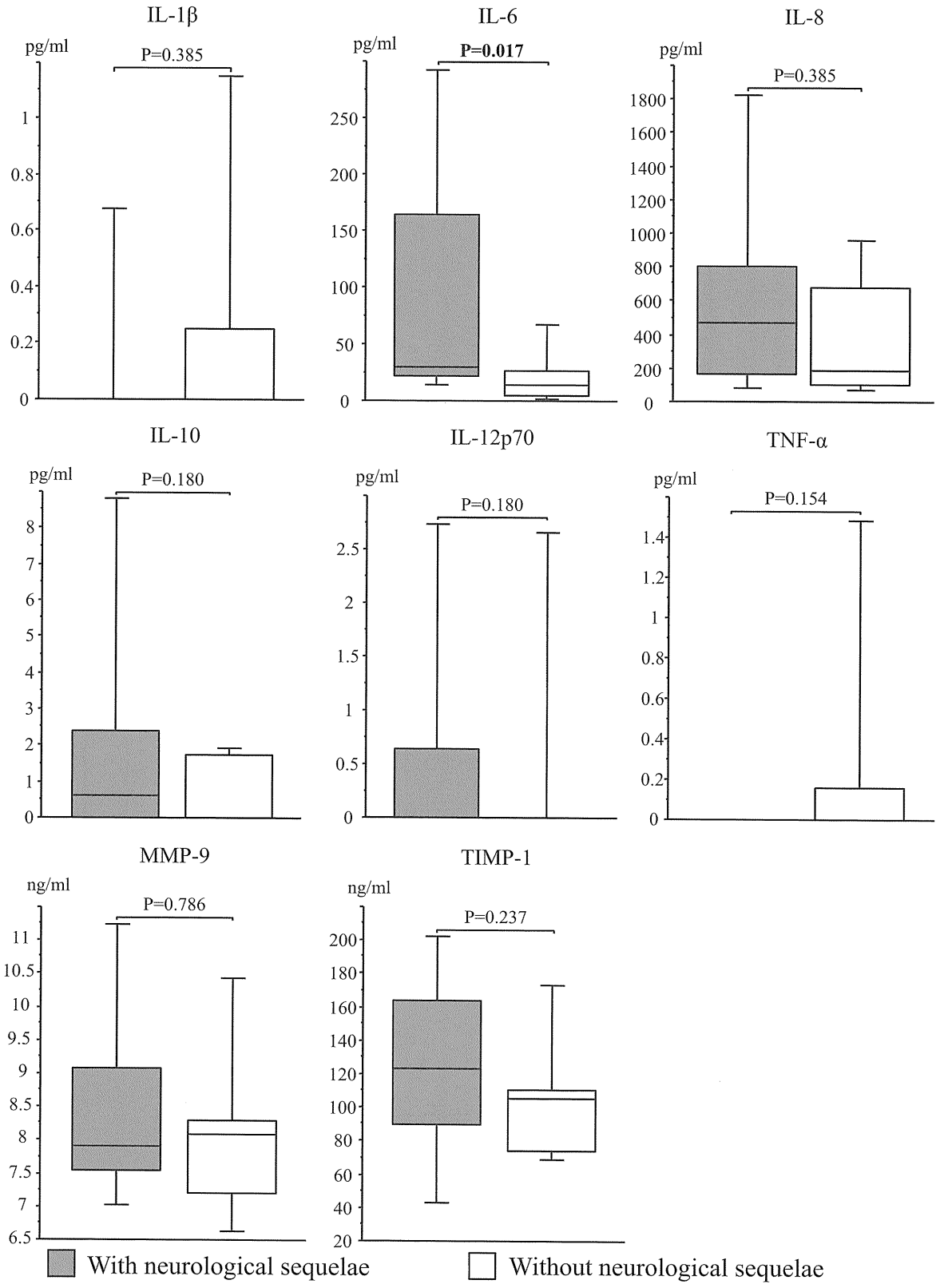

Fig. 2. Comparison of the eight cerebrospinal fluid (CSF) biomarkers (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, MMP-9, and TIMP-1) levels between HHV-6 encephalitis patients (at primary infection) with neurological sequelae ($n=7$) and those without neurological sequelae ($n=13$). Data are shown as box plots, where the boxes represent the first through third quartiles, the lines within the boxes represent the median, and the lines outside the boxes represent the minimum and maximum values.

tion. Recently, Ichiyama et al.[22] reported similar findings in HHV-6 encephalitis in primary infection. These findings suggest that IL-6 may play an important role in the pathogenesis of HHV-6 encephalitis in primary infection. Furthermore, significantly higher concentration of CSF IL-6 was observed in HHV-6 encephalitis in primary infection with severe neurological sequelae. Similar observations have been demonstrated in herpes simplex virus encephalitis patients[23] and Japanese encephalitis patients.[24]

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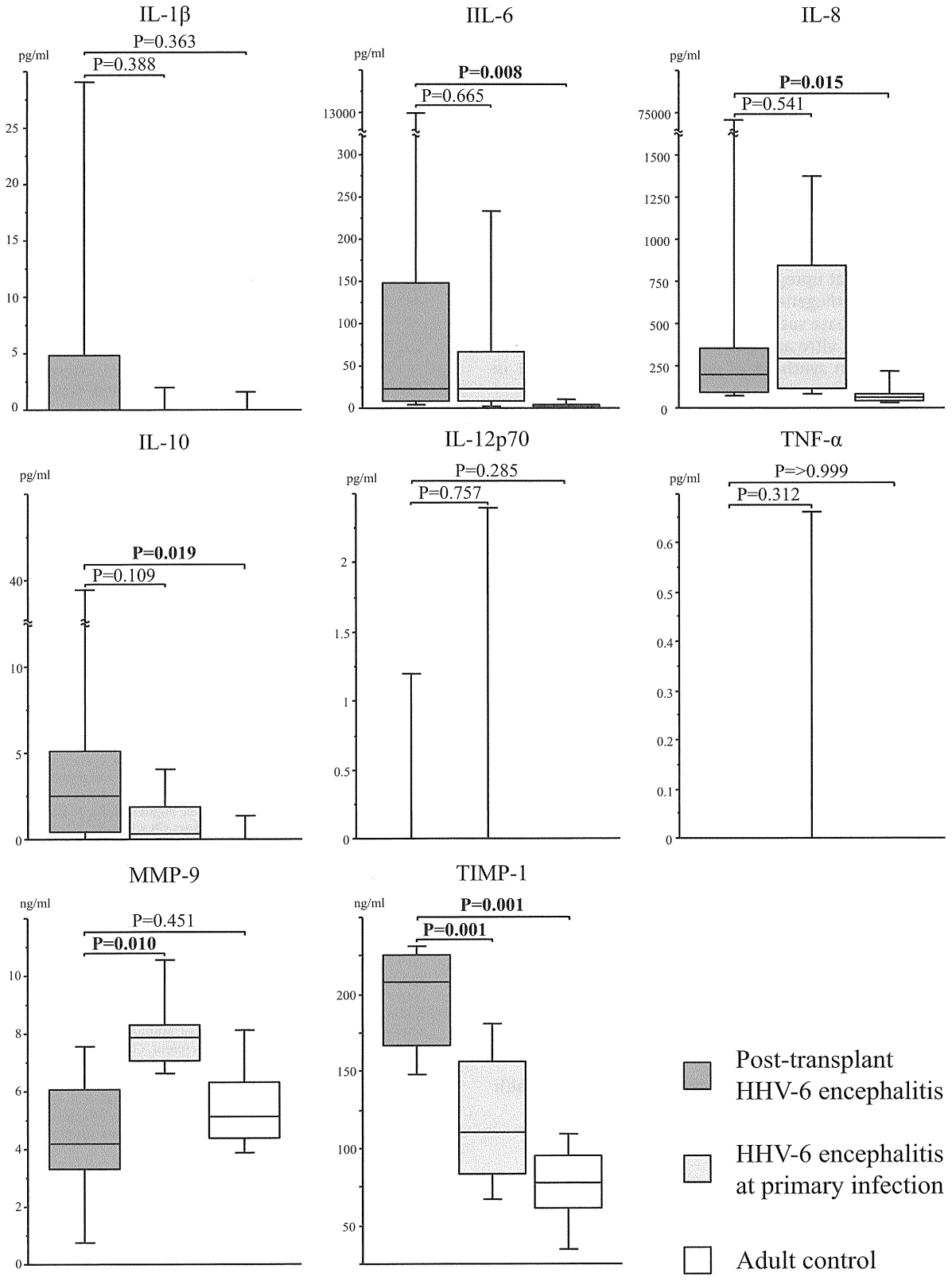

Fig. 3. Comparison of the eight cerebrospinal fluid (CSF) biomarkers (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, MMP-9, and TIMP-1) levels among patients with post-transplant HHV-6 encephalitis ($n = 7$), HHV-6 encephalitis in primary infection ($n = 22$), and adult controls ($n = 8$). Data are shown as box plots, where the boxes represent the first through third quartiles, the lines within the boxes represent the median, and the lines outside the boxes represent the minimum and maximum values (excluding outliers).

Although transient CSF IL-8 elevation has been reported in patients with aseptic meningitis or bacterial meningitis,[25,26] the role of elevated CSF IL-8 in the HHV-6 encephalitis in primary infection patients remains undefined, because most of the CSF collected from the patients did not show pleocytosis. The primary role of IL-8 is the recruitment and activation of neutrophils, but it also serves as a chemotactic for T cells[27] and monocytes.[28] Therefore, it is possible that elevated CSF IL-8 may accelerate invasion of HHV-6 resident

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cells into CNS, which establishes a suitable condition for HHV-6 latency in brain tissue.

In CNS infections, MMPs are thought to play an important role in promoting destructive inflammatory processes including disruption of the blood brain barrier, edema, and disintegration of the neurovascular unit.[29,30] As in the case with other viral encephalitis,[31] MMP-9 and TIMP-1 were significantly higher in patients with HHV-6 encephalitis in primary infection than those patients with non-HHV-6 FC. The expression and activity of MMP-9 can be upregulated by cytokines and chemokines.[32] Therefore, we speculated that elevated IL-8 or IL-6 would induce MMP-9 elevation in HHV-6 encephalitis in primary infection patients. It has been suggested that upregulation of MMP-9 and TIMP-1 was associated with the pathogenesis of multiple sclerosis, which is a major neurological disease suspected to be associated with HHV-6 infection.[33–35] Thus, in vitro analysis to determine whether HHV-6 might be involved in synthesis of either MMP-9 or TIMP-1 in neurological cell lines is needed.

Although adult control subjects were not perfect in this study because of differences in host conditions and age from post-transplant HHV-6 encephalitis patients, similar cytokines elevation was observed in post-transplant HHV-6 encephalitis patients as HHV-6 encephalitis at primary infection. Furthermore, CSF IL-10 concentrations were also significantly higher in post-transplant HHV-6 encephalitis patients than controls ($P = 0.019$). IL-10 is the prototypical anti-inflammatory cytokine, and elevation of CSF IL-10 concentration might reflect strong protective reaction against CNS inflammation caused by direct HHV-6 replication. Additionally, as the ratio of MMP-9 and TIMP-1 was different between HHV-6 encephalitis in primary infection and post-transplant HHV-6 encephalitis, this discrepancy may be associated with the distinct pathological mechanisms between HHV-6 encephalitis in primary infection and viral reactivation in transplant recipient. Thus, in addition to different concentrations of direct HHV-6 replication in brain tissue, a complex network of cytokines and inflammatory mediators is likely to be associated with the pathological mechanisms that distinguish HHV-6 encephalitis in primary infection from viral reactivation in transplant recipient.

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Ethical approval

This study was approved by the review boards of Fujita Health University (No. 08-183).

Conflict of interest

The authors do not have any commercial or other associations that might pose a conflict of interest.

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Evaluation of Reverse Transcription Loop-Mediated Isothermal Amplification Assays for Rapid Diagnosis of Pandemic Influenza A/H1N1 2009 Virus

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Two genetic diagnosis systems using reverse transcription-loop-mediated isothermal amplification (RT-LAMP) technology were evaluated: one for detecting the HA gene of the pandemic influenza A/H1N1 2009 virus (H1pdm RT-LAMP) and the other for detecting the matrix gene of the influenza A virus (TypeA RT-LAMP). The competence of these two RT-LAMP assay kits for the diagnosis of the pandemic influenza A/H1N1 2009 virus was compared using real-time RT-PCR assays developed recently on viruses isolated and clinical specimens collected from patients with suspected infection. TypeA RT-LAMP and H1pdm RT-LAMP showed almost the same sensitivity as real-time RT-PCR for viruses isolated. The sensitivity and specificity of TypeA RT-LAMP and H1pdm RT-LAMP were 96.3% and 88.9%, respectively, for clinical specimens. Considering that the ability of the two RT-LAMP assay kits for detection of the pandemic influenza A/H1N1 2009 virus was comparable to that of the real-time RT-PCR assays, and that the assays were completed within 1 hr and did not require any expensive equipment, these two RT-LAMP assays are promising rapid diagnostic tests for the pandemic influenza A/H1N1 2009 virus at the hospital bedside. ***J. Med. Virol. 83:10–15, 2011.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: rapid diagnosis; RT-LAMP; pandemic influenza A/H1N1 2009 virus

INTRODUCTION

The novel H1N1 subtype influenza A virus (pandemic influenza A/H1N1 2009 virus) has spread worldwide since it was identified in Mexico and the United States in March and April 2009 [WHO, 2009a,b]. As of November 6, 2009, the mortality rates of pandemic influenza A/H1N1 2009 infection (deaths per million population) ranged from 1.8 to 14.6 in temperate zone countries, while it was only 0.2 in Japan [WHO, 2009c]. In Japan, only 197 deaths from pandemic influenza A/H1N1 2009 infection were confirmed as of March 9, 2010, although the estimated number of cases was about 20.6 million according to the Japanese Ministry of Health, Labor, and Welfare. The low case to fatality rate in Japan may have resulted from the aggressive early treatment strategy adopted by hospitals; thus, it is important to develop highly specific and sensitive early diagnostic tests for the pandemic influenza A/H1N1 2009 virus that can be performed easily in the clinic.

A novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), was described by Notomi et al. [2000]. Amplification is conducted under isothermal conditions ranging from 60 to 65°C with DNA polymerase and usually with 4 primers recognizing 6 distinct target regions (4-primer-based LAMP), making this assay highly specific. If two additional "loop primers" are included in the LAMP assay (6-primer-based LAMP), the reaction time can be reduced [Nagamine et al., 2002]. Nucleic acid amplification-based diagnostic assays have become the gold standard for the rapid diagnosis of viral infections. Several PCR assays, such as conventional RT-PCR and real-time RT-PCR (rRT-PCR), have been reported for

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the pandemic influenza A/H1N1 2009 virus [Bose et al., 2009; Carr et al., 2009; Hall et al., 2009; He et al., 2009; Lam et al., 2009; Lau et al., 2009; LeBlanc et al., 2009; Pabbaraju et al., 2009; Wang et al., 2009; Yang et al., 2009; Nakauchi et al., 2010; Chidlow et al., 2010]; however, these methods require high-precision instruments, such as the LightCycler Real-Time PCR System (Roche Diagnostics Ltd., Mannheim, Germany). On the other hand, the LAMP assay can be carried out without using such instruments; furthermore, viral genomes can be detected within a shorter time and in a real-time manner. Recently, LAMP-based assays for several virus infections [Hong et al., 2004; Mori et al., 2006; Shirato et al., 2007; Yoneyama et al., 2007; Iizuka et al., 2009] and influenza virus infections [Poon et al., 2005; Imai et al., 2006, 2007; Ito et al., 2006; Kubo et al., 2010] have been reported.

In this study, two genetic diagnosis kits using RT-LAMP technology were evaluated: one for detecting the pandemic influenza A/H1N1 2009 virus and the other for detecting the influenza A virus. These two RT-LAMP assay kits (Eiken Chemical, Tokyo, Japan) contain the Loopamp Extraction Reagent that does not require an RNA purification step, RNA Amplification Reagent (Dried Form) that eliminates the need to dispense the enzyme and reaction buffer, and Primer Set for FluA (for Dried Form) or Primer Set for H1 pdm 2009 (for Dried Form); as a result, RT-LAMP is simpler and easier to perform using these kits than conventional RT-LAMP assays. The competence of the two RT-LAMP assay kits for the diagnosis of the pandemic influenza A/H1N1 2009 virus was compared with rRT-PCR assays using isolated viruses and clinical specimens collected from patients with suspected infection.

MATERIALS AND METHODS

Viruses and Cells

All influenza virus isolates included in this study were grown in Madin–Darby Canine Kidney (MDCK) cells. Pandemic influenza A/H1N1 2009 virus isolates are listed in Table III.

The following influenza viruses were used to evaluate the specificity of the RT-LAMP assays: A/New Caledonia/20/1999 (H1N1); A/Moscow/13/1998 (H1N1); A/Panama/2007/1999 (H3N2); A/Wyoming/03/2003 (H3N2); A/New York/55/2004 (H3N2); A/Sydney/05/1997 (H3N2); A/Hong Kong/156/1997 (H5N1); A/Hong Kong/213/2003 (H5N1); A/Vietnam/HN30259/2004 (H5N1); A/Vietnam/HN30262/2004 (H5N1); A/Netherlands/219/2003 (H7N7); A/Netherlands/33/2003 (H7N7); A/duck/Hokkaido/55/96 (H1N1); A/duck/Hong Kong/278/78 (H2N9); A/duck/Ukraine/1/63 (H3N8); A/duck/Hong Kong/365/78 (H4N6); A/chicken/Yamaguchi/7/04 (H5N1); A/duck/Hong Kong/716/79 (H6N1); A/duck/Hong Kong/293/78 (H7N2); A/turkey/Ontario/6118/68 (H8N4); A/duck/Hong Kong/702/79 (H9N5); A/duck/Hong Kong/560/79 (H10N8); A/duck/England/56 (H11N6); A/duck/Alberta/60/76 (H12N6); A/gull/Maryland/704/77 (H13N6); A/mallard/Gurjev/263/82 (H14N5); A/duck/Australia/341/83 (H15N8); B/Shangdong/07/2002; B/Shanghai/361/2002; and B/Brisbane/32/2002.

Clinical Specimens

Nasal swabs were taken in duplicate from patients with suspected infection at the same time. One of the two swabs, designated as swab 1, was suspended in 4 ml of Loopamp Extraction Reagent for Influenza virus (Eiken Chemical). The other swab, designated as swab 2, was suspended in 4 ml of viral transport medium (VTM) (Becton-Dickinson and Company, Franklin Lakes, NJ), and was used for RNA extraction and virus isolation. The study protocol was approved by the Ethics Committee at NIID and Fujita Health University School of Medicine, and the study was performed in compliance with the Declaration of Helsinki. Informed consent was obtained from all patients.

RNA Extraction

Supernatants of the cultured MDCK cells were cleared by centrifugation at 10,000*g* for 10 min. Viral RNA was prepared using the MagMAX 96 Viral Isolation Kit (Ambion, Austin, TX) from 50 μl of the supernatant with a KingFisher Flex (Thermo Fisher Scientific, Waltham, MA) according to the manufacturers' instructions. Total RNA from the clinical specimens was also prepared using the MagMAX 96 Viral Isolation Kit (Ambion) from 50 μl of sample. Total RNA was eluted in 30 μl of elution buffer (Ambion) and stored at −70°C until used.

RT-LAMP

RT-LAMP was carried out using the RNA Amplification Reagent (Dried Form) (Eiken Chemical) that consists of 200 μl tubes with dried enzyme and reaction buffer for RT-LAMP fixed on the inside of the cap. Ten microliters of purified RNA or a nasal swab suspended in Loopamp Extraction Reagent for Influenza virus (Eiken Chemical) was added to the bottom of a tube containing 15 μl of Primer Set for FluA (for Dried Form) or Primer Set for H1 pdm 2009 (for Dried Form) (Eiken Chemical), and the tube was then inverted to resuspend the enzyme and buffer. The reaction mixture was collected at the bottom of the tube by a quick spin down. The mixture was incubated using a Loopamp real-time turbidimeter (LA-320C; Eiken Chemical) for 35 min at 62.5°C and then for 5 min at 80°C to terminate the reaction. The locations, names, and sequences of the RT-LAMP primers specific for the pandemic influenza A/H1N1 2009 virus HA gene (Primer Set for H1 pdm 2009 (for Dried Form)) and the influenza A virus matrix gene (Primer Set for FluA (for Dried Form)) are given in Tables I and II, respectively (information provided by Eiken Chemical).

Real-Time RT-PCR

Real-time RT-PCR assays for detecting the Type A influenza virus and specifically the pandemic influenza

TABLE I. Primer Set for H1 pdm 2009 (for Dried Form) (Eiken Chemical)

Primer name	Sequence (5'–3')	Genome position	Length (bp)
H1F3-1	AGCTAAGAGAGCAATT	350–365	16
H1B3-1	TTTCCCTTTATCATTAATGTAGGATTTG	537–564	28
H1FIP-1[a]	ACCTTTGTTCGAGTCATGATTGG-	422–444 (F1c-1)	
(F1c-1 + (T) + F2-1)	(T)CTCAGTGTCATCATTTGAAAGGTTT	369–393 (F2-1)	49
H1BIP-1[b]	TAACGGCAGCATGTCCTCA-	446–464 (B1c-1)	
(B1c-1 + B2-1)	GTATGAATTTCCTTTTTTAACTAGCCA	499–525 (B2-1)	46
H1FL-1	CCATGAACTTGTCTTGGGGAATA	398–420	23
H1BL-1	TGCTGGAGCAAAAAGCTTCTAC	465–486	22
H1F3-2	ACCTTCTAGAAGACAAGCATAA	143–164	22
H1B3-2	TCCTCATAATCGAT	337–350	14
H1FIP-2[c]	TGGATTTCCCAGGATCCAGC-	227–246 (F1c-2)	
(F1c-2 + F2-2)	GGAAACTATGCAAACTAAGAGG	167–188 (F2-2)	42
H1BIP-2[d]	TCCACAGCAAGCTCATGGTC-	262–281 (B1c-2)	
(B1c-2 + B2-2)	TCCTGGGTAACACGTTCC	313–330 (B2-2)	38
H1FL-2	CCAAATGCAATGGGGCTAC	190–208	19
H1BL-2	CTACATTGTGGAAACATCTAGTTCAG	282–307	26

[a]H1FIP-1 primer consisted of F1c-1, a T linker, and F2-1.
[b]H1BIP-1 primer consisted of B1c-1 and B2-1.
[c]H1FIP-2 primer consisted of F1c-2 and F2-2.
[d]H1BIP-2 primer consisted of B1c-2 and B2-2.

A/H1N1 2009 virus were performed as described previously [Nakauchi et al., 2010].

Virus Isolation

One hundred microliters of VTM containing a nasal swab was diluted with an equal volume of Opti-MEM (Invitrogen, Carlsbad, CA). This was then added to MDCK cells in a 12.5 cm^2 flask and incubated at 34°C for 1 hr. The cells were washed twice with Opti-MEM, and then cultured in 2 ml of Opti-MEM containing 5 μg/ml acetylated trypsin (Sigma-Aldrich Corp., St. Louis, MO), 200 μg/ml penicillin/streptomycin (Invitrogen), 100 μg/ml gentamicin (Invitrogen), and 0.5 μg/ml fungizone (Invitrogen) until a cytopathic effect was observed.

RESULTS

Sensitivity of RT-LAMP Assays

The sensitivity of the RT-LAMP assay using primer set Influenza A (TypeA RT-LAMP) and primer set AH1pdm (H1pdm RT-LAMP) was evaluated and compared with that of the TypeA rRT-PCR or H1pdm rRT-PCR assays [Nakauchi et al., 2010] using RNA samples diluted serially that were prepared from the pandemic influenza A/H1N1 2009 virus, as indicated in Table III. The assays were carried out twice independently. As shown in Table III, the 100% detectable concentration of each strain of the pandemic influenza A/H1N1 2009 virus by TypeA RT-LAMP or H1pdm RT-LAMP was almost identical to the 100% detectable concentration of each strain by TypeA rRT-PCR or H1pdm rRT-PCR, respectively.

The specificity of the LAMP assays was evaluated using 27 strains of human and avian influenza A viruses (subtypes H1–H15) and 3 strains of influenza B viruses. TypeA RT-LAMP detected all influenza A viruses with no cross-reactivity against influenza B viruses. H1pdm RT-LAMP reacted specifically to pandemic influenza A/H1N1 2009 viruses with no cross-reactivity against other subtype influenza A viruses, except for pandemic influenza A/H1N1 2009 viruses and influenza B viruses.

Evaluation of RT-LAMP Using Clinical Specimens

The TypeA RT-LAMP and H1pdm RT-LAMP assays were evaluated using 45 nasal swabs from patients with

TABLE II. Primer Set for FluA (for Dried Form) (Eiken Chemical)

Primer name	Sequence (5'–3')	Genome position	Length (bp)
FluAF3-1	GACTTGAAGATGTCTTTGC	80–98	19
FluAF3-2	GACTGGAAAGTGTCTTTGC	80–98	19
FluAB3-1	TGTTATTTGGATCCCCATT	259–277	19
FluAB3-2	TGTTGTTCGGGTCCCCATT	259–277	19
FluAFIP[a]	TTAGTCAGAGGTGACAGGATTG-	149–170(F1c)	
(F1c + F2)	CAGATCTTGAGGCTCTC	110–126(F2)	39
FluABIP[b]	TTGTGTTCACGCTCACCGTG-	185–204(B1c)	
(B1c + B2)	TTTGGACAAAGCGTCTACG	226–244(B2)	39
FluAFL	GTCTTGTCTTTAGCCA	133–148	16
FluABL	CAGTGAGCGAGGACTG	207–222	16

[a]FluAFIP primer consisted of F1c and F2.
[b]FluABIP primer consisted of B1c and B2.

TABLE III. The Sensitivity of RT-LAMP Was Directly Compared With Real-Time RT-PCR Assay Using Series Dilutions of Viral RNA

	Dilution rate of virus			
	10^7		10^8	
Virus (TCID50/ml)	TypeA	H1pdm	TypeA	H1pdm
A/Aichi/198/2009 ($10^{6.5}$)				
RT-LAMP	2/2	2/2	1/2	1/2
rRT-PCR	2/2	2/2	2/2	1/2
A/Saitama/85/2009 ($10^{7.5}$)				
RT-LAMP	2/2	2/2	2/2	0/2
rRT-PCR	2/2	2/2	2/2	1/2
A/Shiga/44/2009 ($10^{8.0}$)				
RT-LAMP	2/2	2/2	2/2	2/2
rRT-PCR	2/2	2/2	1/2	1/2
A/Kagoshima/56/2009 ($10^{6.3}$)				
RT-LAMP	2/2	2/2	1/2	0/2
rRT-PCR	2/2	2/2	2/2	0/2
A/Kobe/1/2009 ($10^{8.0}$)				
RT-LAMP	2/2	2/2	2/2	1/2
rRT-PCR	2/2	2/2	2/2	2/2
A/Shiga/2/2009 ($10^{6.7}$)				
RT-LAMP	2/2	2/2	0/2	1/2
rRT-PCR	2/2	2/2	2/2	1/2
A/Kanagawa/140/2009 ($10^{7.3}$)				
RT-LAMP	2/2	2/2	0/2	2/2
rRT-PCR	2/2	2/2	0/2	1/2
A/Hiroshima/310/2009 ($10^{7.5}$)				
RT-LAMP	2/2	2/2	2/2	2/2
rRT-PCR	2/2	2/2	2/2	1/2

suspected pandemic influenza A/H1N1 2009 infection, and the results were compared with those obtained using rRT-PCR (Table IV). Compared with TypeA rRT-PCR, the sensitivity and specificity of TypeA RT-LAMP were 96.3% and 88.9%, respectively. Similarly, compared with H1pdm rRT-PCR, the sensitivity and specificity of H1pdm RT-LAMP were 96.3% and 88.9%, respectively. Among the 45 samples, 2 samples (Samples 27 and 46) were positive for TypeA and H1pdm when tested by RT-LAMP; however, they were negative for TypeA and H1pdm when tested by rRT-PCR (Table V). Two samples (samples 23 and 40) were positive for TypeA and H1pdm when tested by rRT-PCR; however, they were negative for TypeA or H1pdm when tested by RT-LAMP (Table V).

In addition to comparing them with rRT-PCR, the TypeA RT-LAMP and H1pdm RT-LAMP assays were also compared with viral isolation from 45 nasal swabs. As shown in Table IV, 53.3% (24/45) were positive by viral isolation, 60% (27/45) by TypeA rRT-PCR and H1pdm rRT-PCR, and 62.2% (27/45) by TypeA RT-LAMP and H1pdm RT-LAMP.

DISCUSSION

Two RT-LAMP assays were evaluated, namely, TypeA RT-LAMP and H1pdm RT-LAMP and demonstrated that these assays have approximately the same sensitivity as the TypeA and H1pdm rRT-PCR assays, respectively (Table III). The results of assays using

TABLE IV. Comparison of the Results of RT-LAMP and Those of rRT-PCR and Virus Isolation

	RT-LAMP: H1pdm		
	+		−
TypeA rRT-PCR (+) (n = 27)	26 (23)		1 (1)
TypeA rRT-PCR (−) (n = 18)	2 (0)		16 (0)
Sensitivity		96.3%	
Specificity		88.9%	
	RT-LAMP: H1pdm		
	+		−
H1pdm rRT-PCR (+) (n = 27)	26 (23)		1 (1)
H1pdm rRT-PCR (−) (n = 18)	2 (0)		16 (0)
Sensitivity		96.3%	
Specificity		88.9%	

The number of the samples which were virus isolation positive were shown in parentheses.

TABLE V. Comparison of the Results of Assays Performed on Four Clinical Specimens With RT-LAMP, rRT-PCR, and Virus Isolation

	RT-LAMP		rRT-PCR		
Sample ID	TypeA	H1pdm	TypeA	H1pdm	Virus isolation
23	+	−	+	+	+
27	+	+	−	−	−
40	−	+	+	+	+
46	+	+	−	−	−

clinical specimens (Table IV) also suggested that the RT-LAMP assays have the same sensitivity as the rRT-PCR assays. However, four samples showed different results when tested with RT-LAMP and rRT-PCR (Table V). It was presumed that these discrepancies were due to the fact that swab 1 (suspended directly in Loopamp Extraction Reagent for Influenza virus), which was used for the LAMP assay, and swab 2 (suspended in VTM), which was used for the real-time RT-PCR assay and virus isolation, did not necessarily contain the same quantity of virus even though they were collected from the same patient. For samples 23 and 40, swab 2 was positive for TypeA and H1pdm by rRT-PCR and also positive for virus isolation, whereas swab 1 was negative for Type A or H1pdm by RT-LAMP (Table V). The remaining RNA extracted from swab 2 of samples 23 and 40 was further tested using the RT-LAMP assays. As a result, it was demonstrated that swab 2 from samples 23 and 40 was positive for TypeA and H1pdm using the RT-LAMP assays (data not shown). Swab 1 from samples 27 and 46 was positive for TypeA and H1pdm by the RT-LAMP assays, whereas both swab 2 samples were negative for TypeA and H1pdm by rRT-PCR and also negative for virus isolation. The rRT-PCR assays were carried out after RNA extraction from swab 1 of samples 27 and 46 using the Loopamp Extraction Reagent for Influenza virus. As a result, it was demonstrated that both swab 1 samples were positive for TypeA and H1pdm by rRT-PCR (data not shown). Unfortunately, virus isolation could not be performed using swab 1 because the viral particles were disrupted by some component of the Loopamp Extraction Reagent for Influenza virus. These facts suggests that swab 1 from samples 23 and 40 contained insufficient amounts or quality of virus for the RT-LAMP assays, and swab 2 from samples 27 and 46 contained insufficient amounts or quality of virus for the rRT-PCR assays and virus isolation. Considering these results, the ability of the TypeA and H1pdm RT-LAMP assays to detect the pandemic influenza A/H1N1 2009 virus was comparable to the ability of the TypeA and H1pdm rRT-PCR assays, respectively.

A diagnostic method was developed recently to detect specifically the pandemic influenza A/H1N1 2009 virus using RT-LAMP [Kubo et al., 2010]; however, as this assay targeted only one region of the pandemic influenza A/H1N1 2009 virus HA gene, the RT-LAMP assay may fail to detect viruses with mutations in the target region that are difficult to amplify. Although the H1pdm RT-LAMP assay is more tolerant to mutations of the HA gene than the assay of Kubo et al. because it includes two primer sets targeted to different regions of the HA gene (Table I), it is important to check the nucleotide sequence of recently circulating viruses and to modify the primers when viruses emerge with mutations in the target regions. When only the TypeA RT-LAMP assay is positive, a mutant of the pandemic influenza A/H1N1 2009 virus or a different subtype influenza virus circulating in the community is considered because of the failure of the H1pdm assay and if necessary, further analysis, such as sequencing, should be performed. Furthermore, to reduce the risk of detection failure of the influenza A virus, it is important to perform simultaneously the subtype-specific H1pdm RT-LAMP assay and the TypeA RT-LAMP assay on the same samples.

The positive rate of pandemic influenza A/H1N1 2009 virus detection in the clinical specimens using TypeA or H1pdm RT-LAMP was higher than that obtained with virus isolation and it was almost identical to the rates of the TypeA and H1pdm rRT-PCR assays. The virus isolation method is only able to detect an infectious virus, and the quantity and amount of infectious viral particles depend on the preparation and storage conditions of the sample. Thus, the detection rate of the pandemic influenza A/H1N1 2009 virus using the virus isolation method is lower than that using genetic diagnosis methods such as RT-LAMP and rRT-PCR. Three out of 27 samples were positive for TypeA and H1pdm by rRT-PCR, but were negative for virus isolation (Table IV); however, the crossing point (C_p) values for the TypeA and H1pdm rRT-PCR assays for these three samples were high (data not shown), suggesting that there was only a low amount of virus in these three samples.

The two RT-LAMP assay kits did not require the enzyme or reaction buffer to be aliquoted. In addition, by using the Loopamp Extraction Reagent for Influenza virus (Eiken Chemical), the assays did not require RNA purification. Thus, the RT-LAMP assay kits are easy to use compared with the RT-PCR assays that are employed routinely for the diagnosis of pandemic influenza A/H1N1 2009 infection. The RT-LAMP assays can be completed within 1 hr without the need for any expensive equipment, such as a thermal cycler. The two RT-LAMP assay kits are considered valuable for the diagnosis of the pandemic influenza A/H1N1 2009 virus in clinics and show promise for bedside diagnosis.

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Analysis of rotavirus antigenemia in hematopoietic stem cell transplant recipients

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Abstract : Systemic rotavirus infection, such as rotavirus antigenemia, has been found in immunocompetent rotavirus gastroenteritis patients. However, the pathogenesis of rotavirus infection in immunocompromised transplant recipients remains unclear. Enzyme-linked immunosorbent assay was used to measure rotavirus antigen levels in serially collected serum samples obtained from 62 pediatric patients receiving allogeneic hematopoietic stem cell transplants (HSCT). Rotavirus antigen was detected in 43 (6.8%) of 633 serum samples (8 of 62 patients). The duration of rotavirus antigenemia ranged between 1 and 10 weeks, and diarrhea was concurrent with rotavirus antigenemia in Cases 3, 6, 7, and 8. The level of viral antigen in the transplant recipients (0.19 ± 0.20) was significantly lower than that observed in serum samples collected from immunocompetent patients on either day 1 (0.49 ± 0.18, $P = 0.0011$) or day 3 (0.63 ± 0.09, $P = 0.0005$). A patient who received a graft from a human leukocyte antigen (HLA)-mismatched donor was at significant risk for rotavirus antigenemia ($P = 0.024$; odds ratio = 9.44) in comparison to patients who received grafts from HLA-matched donors. Although the duration of antigenemia was clearly longer in HSCT patients than in immunocompetent rotavirus gastroenteritis patients, the levels of viral antigen were not as high. Therefore, mismatched HLA may be a risk factor for rotavirus antigenemia after HSCT.

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Rotavirus is the major cause of gastroenteritis in young children worldwide. Severe dehydration caused by rotavirus-induced diarrhea and vomiting can be fatal in developing countries. In addition, the gastroenteritis induced by rotavirus infection causes a large economic burden in developed countries. Rotaviral infection is generally benign and self-limited in immunocompetent children. In contrast, it has been reported that rotavirus can cause severe diarrhea resulting in fatal outcomes for immunocompromised transplant recipients (1–5). Thus, the pathogenesis of rotaviral infection may differ between immunocompetent and immunocompromised individuals.

Initially, rotavirus replication was thought to be limited to the gastrointestinal tract in patients with rotavirus gastroenteritis. However, recently, rotavirus antigen and RNA were detected in the sera of rotavirus-infected children (6–9). In addition, rotavirus antigen was detected not only in the serum but also in several organs, including the stomach, intestine, liver, lung, spleen, kidney, pancreas, thymus, and bladder in rotavirus-infected animals (10). These findings suggest that rotavirus spreads beyond the intestine in children with rotavirus gastroenteritis, resulting in systemic viral infection. Recently, we found that rotavirus antigenemia was frequently observed during the acute phase of rotavirus

gastroenteritis (11). Rotavirus antigen peaked on day 2 of the illness, with the amount of viral antigen gradually decreasing to nearly undetectable levels by day 6. We also found that cytokines were involved in controlling antigenemia levels. The results of this study, together with those from previous studies (7, 8, 12, 13), suggested that host immune responses have important roles in regulating viral replication. Therefore, the kinetics of rotavirus antigenemia in transplant recipients may be different from those in immunocompetent rotavirus gastroenteritis patients. In this study, we sought to elucidate the kinetics of rotavirus antigenemia in hematopoietic stem cell transplant (HSCT) recipients. We measured rotavirus antigen levels in serum samples serially collected from pediatric HSCT recipients and analyzed the associations between antigenemia and clinical features.

Patients and methods

Patient characteristics

Between September 2004 and February 2007, 62 patients received allogeneic HSCT (17 with allogeneic bone marrow transplant [BMT] from human leukocyte antigen [HLA]-matched siblings, 9 with allogeneic BMT from HLA-mismatched siblings, 22 with allogeneic BMT from unrelated donors, 2 with peripheral blood stem cell transplants, and 12 with umbilical cord blood transplants) at the Division of Hematology-Oncology at the Children's Medical Center, the Japanese Red Cross Nagoya First Hospital, or the Department of Pediatrics at the Nagoya University Graduate School of Medicine. The patients' guardians provided written consent for their participation in this study. This study was approved by the review boards of the 3 institutes. Patient characteristics are summarized in Table 1 and include age, gender, underlying diseases, type of graft, HLA matching, having received total body irradiation (TBI) or anti-thymocyte globulin (ATG) in conditioning regimen, and occurrence of acute graft-versus-host disease (GVHD).

Patient management

Details of the conditioning regimen and GVHD prophylaxis have been previously described (14, 15). In brief, patients with hematologic malignancies were conditioned with high-dose chemotherapy consisting of melphalan (180 mg/m^2) plus busulfan (16 mg/kg) or TBI (12 Gy). Patients with severe aplastic anemia were conditioned with 200 mg/kg cyclophosphamide and 10 mg/kg rabbit ATG for transplantation from a matched sibling donor. For patients transplanted with an unrelated bone marrow donor, TBI (5–10 Gy) was added (16). GVHD prophylaxis consisted of cyclosporine or tacrolimus with short-term methotrexate. All patients received trimethoprim–sulfamethoxazole orally or inhaled pentamidine as prophylaxis against *Pneumocystis jirovecii*. The standard doses of oral amphotericin B and acyclovir were administered as prophylaxis for fungal and herpes simplex virus infections. Intravenous γ-globulin preparations were administered weekly during the first 3 months as prophylaxis for cytomegalovirus (CMV) infection. In addition, ganciclovir was given as preemptive therapy against CMV infection following a positive result from a CMV antigenemia assay. Acute and chronic GVHD was diagnosed and graded according to established criteria.

Experimental design

Serum samples were collected from 62 recipients at the time of HSCT, weekly for 3 or 4 months post transplant. Ultimately, 633 serum samples were analyzed in this study. In addition to these samples, 15 serum samples were collected from rotavirus gastroenteritis patients on days 1, 3, and 5 of illness and used as controls. Clinical data were collected retrospectively and assessed to determine associations with rotavirus antigenemia.

Rotavirus antigen detection

Rotavirus antigen was measured using a previously described enzyme-linked immunosorbent assay (ELISA) for the detection of VP6 antigen of the virus (11). Diluted serum (1:16, 50 μL) was used to detect rotavirus antigen. Ninety-six–well well plates (Nalgen Nunc, Rochester, New York, USA) coated with a monoclonal antibody against the VP6 antigen of rotavirus (YO-156) (17) were used for the ELISA (18). The plates were incubated with 50 μL of diluted serum at 4°C overnight. Then, 50 μL of anti-human rotavirus hyperimmune rabbit serum (diluted 1:5000 with phosphate buffered saline [PBS] containing 0.05% Tween-20 [PBST] and 2.5% skim milk) was added to each well. Peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) (diluted

Patient characteristics and risk factors for rotavirus antigenemia after hematopoietic stem cell transplantation

	Rotavirus antigenemia			
Categories	Yes (n = 8)	No (n = 54)	Odds ratio (95% CI)	P
Age (years)	7.4 ± 5.6	8.3 ± 5.1	...	0.652
Sex				
Male	4	37	0.46 (0.10–2.06)	0.312
Female	4	17		
Underlying disease*				
Malignancy	4	36	2.0 (0.44–8.93)	0.368
Non-malignancy	4	18		
Total body irradiation				
Yes	6	41	1.05 (0.18–5.85)	0.955
No	2	13		
Anti-thymocyte globulin				
Yes	4	11	0.25 (0.05–1.19)	0.080
No	4	43		
Acute graft-versus-host disease (grade 2–4)				
Yes	3	17	0.77 (0.16–3.58)	0.705
No	5	37		
Source of the graft				
Related donor	3	23	1.0	
Unrelated donor	3	19	1.21	0.827
CBT	1	11	0.70	0.760
PBSCT	1	1	7.67	0.206
HLA matching				
Match	1	31	9.44 (1.09–82.11)	0.024
Mismatch	7	23		

*Acute lymphoblastic leukemia, 23; aplastic anemia, 12; acute myeloid leukemia 11; myelodysplastic syndrome, 4; rhabdomyosarcoma, 1; malignant lymphoma, 2; neuroblastoma, 1; others, 8.
CI, confidence interval; CBT, cord blood transplant; PBSCT, peripheral blood stem cell transplant; HLA, human leukocyte antigen.

Table 1

1:5000, Jackson ImmunoResearch Laboratory Inc., West Grove, Pennsylvania, USA) was used as a secondary antibody. Finally, the amount of rotavirus VP6 antigen bound to specific monoclonal antibody was assessed by adding substrate. The optical density (OD) was read using spectrophotometry at 492 nm, and an appropriate cut-off value was established based on data from 20 serum samples collected from control subjects. As the mean OD of the control samples was 0.084 ± 0.014, we defined 0.13 (mean + 3 standard deviations [SD]) as the baseline value in this study.

Measurement of rotavirus-specific IgG

Serum anti-rotavirus IgG antibody titer was determined using sandwich ELISA. Briefly, 96-well plates were coated with rabbit anti-rotavirus serum diluted 1:10,000 in 10 mM PBS overnight at 4°C. After the plates were washed twice with 10 mM PBS containing 0.05% PBST, then 1% bovine serum albumin in PBST was added, and the plates were incubated for 4 h at 4°C. The plates were washed twice with PBST. SA-11-infected culture fluid was then added to the plates and incubated for 1 h at 37°C. Serum samples diluted with 2.5% skim milk in