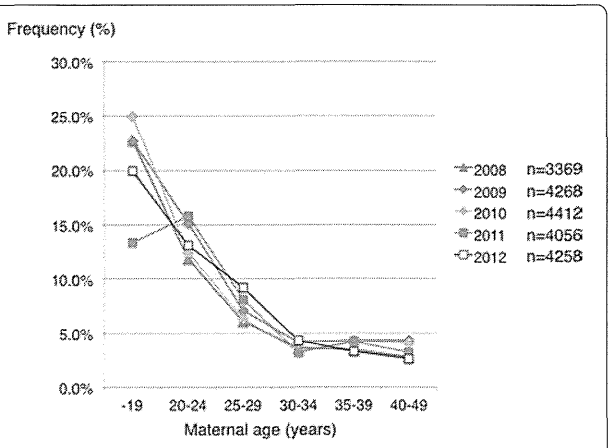

Figure 2 Prevalence rate of pregnant women susceptible to rubella (HI titer < 8×) according to maternal age and year.

aged ≤ 19, 12.6% [99/787] for those aged 20 – 24 years, 7.0% [233/3313] for those aged 25 – 29 years, 3.6% [244/6871] for those aged 30 – 34 years, 3.8% [265/6966] for those aged 35 – 39 years, and 3.5% [81/2300] for those aged ≥ 40 years. Overall seronegative rates according to year did not change greatly: 4.5%[150/3369], 5.2%[221/4268], 4.4%[195/4412], 4.6%[186/4056], and 4.6%[195/4258] for 2008, 2009, 2010, 2011, and 2012, respectively.

Effects of a history of prior birth on the prevalence rate of susceptible pregnant women

Overall, the prevalence rate of susceptibility to rubella was significantly lower in multiparous than in primiparous women (Table 2). However, statistically significant differences in seronegative rate were seen in only a limited number of areas. Furthermore, significant differences in seronegative rates were seen in a limited number of groups of women stratified by age: women aged 30 – 31 years (2.3% [22/967] for multiparous women vs. 4.5% [66/1454] for primiparous women) and women aged 36 – 37 years (3.4% [55/1601] for multiparous women vs. 5.7% [79/1389] for primiparous women) (Table 3).

Table 2 Effects of a history of prior birth on frequency of susceptible women to rubella according to area

	Primiparous		Multiparous	
Area	Age (years)	% (a/b)†	Age (years)	% (a/b)†
A	30.9 ± 5.3	7.0 (57/816)	32.4 ± 4.9	3.3 (21/634)*
B	28.2 ± 4.8	4.8 (20/416)	34.3 ± 4.3*	3.4 (13/387)
C	30.4 ± 7.7	3.7 (46/1252)	33.0 ± 6.6	3.3 (40/1215)
D	33.2 ± 5.9	4.3 (181/4241)	34.2 ± 5.7	2.5 (76/3088)*
E	29.5 ± 5.6	6.4 (132/2070)	33.4 ± 6.4*	6.2 (98/1572)
F	30.2 ± 7.5	7.1 (161/2253)	32.8 ± 6.7*	4.2 (102/2419)*
Overall	33.2 ± 5.9	5.4 (597/11048)	34.7 ± 5.2*	3.8 (350/9315)*

(a/b)†, number of women with HI titer < 8×/total number of women;
*, $P < 0.05$ vs. corresponding counterpart.

Discussion

This study demonstrated that a history of prior birth had some favorable effect on reduction in the number of women susceptible to rubella, although this effect was limited, and did not prevent the occurrence of CRS.

As Japanese guidelines for obstetric practice recommend determination of rubella immunity during the 1st trimester with HI test [2], a considerable number of multiparous women with seronegative results (HI titer < 8×) may have realized that they were susceptible to rubella in their previous pregnancies. In addition, as the guidelines recommend postnatal vaccination in women with seronegative results and low HI titer (≤ 16×) [2], a very low frequency of susceptible multiparous women was expected in this study. However, the susceptible fraction decreased only by 30% (from 5.4% to 3.8%) in this study (Table 2). These observations indicated that some women ignored the recommendation and or some obstetricians forgot to recommend the postpartum vaccination. Furthermore, there were no significant differences in seronegative rates between primiparous and multiparous women in some areas. This suggested that the strength of vaccination campaigns for postpartum women with seronegative results differed between areas. A low postpartum vaccination rate of 11% among eligible women has also been reported in other countries [6]. Thus, it was evident based on this study that some women ignore or underestimate the risk of rubella infection during subsequent pregnancies even after the recognition of susceptibility to rubella.

The higher seronegative rate among younger pregnant women is a cause for concern (Figure 2). Although some fluctuations in seronegative rate according to year were seen mainly due to the small size of the study population, seronegative rate was consistently high among teenage pregnant women ranging from 13.0% in 2011 to 23.5% in 2012 and among young women aged 20 – 24 years ranging from 10.7% in 2010 to 14.3% in 2009. Those women may become pregnant in future. According to the serosurvey conducted by the Japanese National Institute of Infectious Diseases (JNIID) in the 5-year period between 2008 and 2012, seronegative rate among females according to age are as follows: 6.7% for women aged 15 – 19 years, 5.5% for those aged 20 – 24, 4.0% for those aged 25 – 29, 3.6% for those aged 30 – 34, 3.2% (41/1299) for those aged 35 – 39, and 3.9% (64/1655) for those aged ≥ 40 [7]. Although there was a large discrepancy in seronegative rate between pregnant women in this study and female participants in the serosurvey, especially in younger women, trends such as the higher seronegative rate in younger women were similar between the results of the JNIID serosurvery and our observations. In addition, one in five male Japanese adults in their 30s and 40s was susceptible to rubella

Table 3 Effects of a history of prior birth on frequency of susceptible women to rubella according to maternal age

	No immunity against rubella (HI < 8×)			
Age (year)	**Overall**	**Primiparous**	**Multiparous**	***P*-value**
≤ 19	25/126 (19.8%)	23/111 (20.7%)	2/15 (13.3%)	0.7335
20 – 21	29/184 (15.8%)	23/141 (16.3%)	6/43 (14.0%)	0.7102
22 – 23	43/335 (12.8%)	30/236 (12.7%)	13/99 (13.1%)	0.9166
24 – 25	57/642 (8.9%)	37/451 (8.2%)	20/191 (10.5%)	0.3558
26 – 27	101/1171 (8.6%)	72/816 (8.8%)	29/355 (8.2%)	0.7138
28 – 29	100/1768 (5.7%)	69/1132 (6.1%)	31/636 (5.0%)	0.3549
30 – 31	88/2421 (3.6%)	66/1454 (4.5%)	22/967 (2.3%)	0.0036
32 – 33	103/2881 (3.6%)	60/1484 (4.0%)	43/1397 (3.1%)	0.1632
34 – 35	105/3184 (3.4%)	54/1556 (3.5%)	51/1628 (3.3%)	0.7366
36 – 37	134/2990 (4.5%)	79/1389 (5.7%)	55/1601 (3.4%)	0.0030
38 – 39	81/2363 (3.5%)	43/1105 (3.9%)	38/1258 (3.2%)	0.3484
40 – 49	81/2298 (3.5%)	41/1173 (3.4%)	40/1125 (3.6%)	0.8491
Overall	947/20363 (4.7%)	597/11048 (5.4%)	350/9315 (3.8%)	< 0.0001

The mean (SD) age was 33.2 ± 5.9 for the 11048 primiparous women and 34.7 ± 5.2 for the 9315 multiparous women ($P < 0.0001$).

[3,7]. The large discrepancy in fraction size susceptible to rubella between younger pregnant women and younger female participants in the serosurvey by the JNIID may be explained as follows: the participants in the serosurvey by the JNIID may have had a greater interest in healthcare than in the general population and teenage pregnant Japanese women may have rather constituted a group at risk for non-vaccination. These speculations were based on the findings that the risk of no antenatal care was high among women with teenage pregnancies in Japan [8], suggesting that women becoming pregnant as teenagers may have been less likely to receive social support from the community. The actual percentage of women susceptible to rubella may have fallen to a figure intermediate between those of pregnant women and female participants in the serosurvey. All of these observations suggested that the current Japanese vaccination strategy has been ineffective for elimination of CRS.

The current Japanese vaccination strategy is ineffective for elimination of CRS for several reasons as follows. With continuing circulation of rubella virus, there is a persistent risk of infection in susceptible pregnant women, even when only 2% – 3% of pregnant women are non-immune [9]. The circulation of rubella virus can occur in the presence of a low vaccination coverage rate in some populations in the community [10-12], as was confirmed in the current outbreak in Japan. The principal rationale for an accelerated vaccination strategy is to reduce the time needed to interrupt rubella virus circulation and to prevent CRS [13]. Eradication of only one manifestation (such as CRS) of a prevalent rubella infection is not a realistic goal. Samuel and John [14] stated that "To eliminate CRS, virus transmission should be interrupted." Our data combined with those obtained in the serosurvey and the current outbreak strongly suggest that more intensified universal vaccination programs targeting adolescents and children are required and that supplementary immunization activity should be focused on male adults to interrupt endemic rubella transmission. Programs to eliminate rubella have indeed been successful in the USA [15] and appear to have been successful in some European countries [16] and the Americas [12]. It is necessary to realize that "Treatment of CRS is costly and rubella vaccination programs are highly cost-effective" [17].

Japanese guidelines for obstetric practice recommend taking diagnostic measures in women with HI titer ≥ 256× during early pregnancy [2]. The total number of women with HI titer ≥ 256× was 14.1% (2876/20363) in this study, consistent with the results of a previous study conducted in the 3-year period between July 2003 and June 2006 [18] in which 469 (17.1%) of 2741 women had HI titers ≥ 256×. In that study, 411 of the 469 women underwent determination of rubella-specific IgM antibody, 6 women exhibit a positive IgM test result, and none gave birth to a CRS infant [18]. As there are approximately 1.05 to 1.1 million annual births in Japan, these results suggested that the number of women who should undergo determination of rubella-specific IgM would be approximately 150000 yearly in Japan. However, only one infant contracted CRS each year from 2000 to 2003 and 10 infants contracted CRS in the previous rubella outbreak in 2004 [1]. Although several infants may be diagnosed early as having CRS through the diagnostic measures using rubella-specific IgM for pregnant women with HI titers ≥ 256×, this strategy for the early detection of CRS may not be cost-effective.

Conclusion

The experience of prior birth may have favorably affected the reduction in number of pregnant women susceptible to rubella. Only 4.7% (947/20363) of all pregnant women were susceptible to rubella. However, 20 infants with CRS were born during the 12-month period between October 2012 and September 2013 in Japan [3]. Younger women less than 25 years old are more susceptible to rubella than other women of more advanced age. There is still a large fraction of male adults susceptible to rubella in Japan. We may have another rubella outbreak in the near future unless a new vaccination strategy is implemented for the elimination of rubella in Japan.

Competing interests

All authors declare that they have no financial relationships with biotechnology manufacturers, pharmaceutical companies, or other commercial entities with an interest in the subject matter or materials discussed in the manuscript.

Authors' contributions

TY and HM performed statistical analysis, data interpretation and wrote the paper. TY, JM, MH, EH, AO, MI, AN, SS, NU and SM collected and analyzed the data. All authors read and approved the final manuscript.

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Analysis of Ganciclovir-Resistant Human Herpesvirus 6B Clinical Isolates Using Quenching Probe PCR Methodology

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Quenching probe PCR (QP-PCR) analysis was used to determine the frequency of ganciclovir (GCV) resistance among clinical isolates of human herpesvirus 6B (HHV-6B) obtained from patients with primary viral infection and viral reactivation. Forty-two HHV-6B clinical isolates were repeatedly recovered from 15 hematopoietic stem cell transplant (HSCT) recipients, and 20 isolates were recovered from 20 exanthem subitum (ES) patients. Of the 15 HSCT recipients, 9 received GCV during the observation period; however, none of the ES patients were treated with GCV. Two established laboratory strains, Z29 and HST, were used as standards in this study. Regions 1 and 2 of the U69 gene of all of the clinical isolates demonstrated the same melting temperature as regions 1 and 2 of the Z29 strain. For region 3, the melting temperatures of all clinical isolates fell between the melting temperature of the plasmid containing the A462D single nucleotide polymorphism (SNP) and the melting temperature of the AQ: B **Z29 strain, and the melting temperatures profiles of all clinical isolates were similar to the melting temperature profile of the Japanese HST strain. As expected, none of the 20 clinical isolates recovered from the ES patients and the 14 isolates recovered from the HSCT recipients who did not receive GCV treatment carried the six known SNPs associated with GCV resistance. Interestingly, these six SNPs were not detected in the 28 clinical isolates recovered from the 9 HSCT recipients who received GCV. Additional sequence analysis of the U69 gene from the 15 representative isolates from the 15 HSCT recipients identified other SNPs. These SNPs were identical to those identified in the HST strain. Therefore, the rate of emergence of GCV-resistant HHV-6B strains appears to be relatively low, even in HSCT recipients treated with GCV.**

All clinical isolates were similar to HST strain.

Primary human herpesvirus 6B (HHV-6B) infection causes exanthem subitum (ES) (1). Although this disease generally manifests as a benign self-limiting febrile illness (2), in rare cases it can cause severe complications, including encephalitis (3, 4) and fulminant hepatitis (5). HHV-6B reactivation has clinical manifestations in transplant recipients, such as skin rash and fever (6, 7) and bone marrow suppression and posttransplant acute limbic encephalitis (8–13). Antiviral drugs, such as ganciclovir (GCV) and foscarnet, have been used to treat transplant recipients with HHV-6B-associated encephalitis (8–13). To date there are limited data on the emergence of drug-resistant HHV-6B in these patients.

GCV was initially developed as an antiviral drug against human cytomegalovirus, which belongs to the same herpesvirus subfamily (the *Betaherpesvirinae* subfamily) as HHV-6A and HHV-6B. Resistance to GCV in cytomegalovirus has been mapped to the UL97 protein kinase that is responsible for the monophosphorylation of GCV (14–16). The antiviral effect of GCV against HHV-6B has been demonstrated using *in vitro* susceptibility assays (17–19). The *in vivo* efficacy of GCV remains inconclusive, AQ: C despite its general use in patients with HHV-6 encephalitis posttransplantation (8–13). The U69 gene in HHV-6 is a homologue of the HCMV UL97 gene, and it phosphorylates GCV (20, 21). *In vitro* assays have revealed several hot spots in the HHV-6 U69 gene that were associated with GCV resistance (22, 23).

Although *in vitro* drug susceptibility assays are a reliable tool for determining the sensitivity of HHV-6B isolates to antiviral drugs, they are inappropriate for the rapid detection of drug-resistant viruses because of the long incubation period required for HHV-6B replication. Molecular methods for the detection of mutations in the U69 gene that are associated with GCV resistance would be useful for monitoring the emergence of GCV-resistant HHV-6B strains. The quenching probe PCR (QP-PCR) assay is a novel technique that can detect deviations as small as a single-base substitution in the sequence of a DNA fragment. Recently, a new and convenient molecular method for the screening of GCV-resistant HHV-6B strains using the QP-PCR technique was developed (24). This method could be appropriate for the rapid detection of GCV-resistant virus in transplant recipients being treated with GCV.

In the study described here, we utilized the QP-PCR method to elucidate the frequency of GCV-resistant HHV-6B strains in clinical isolates obtained from patients with primary viral infection and during viral reactivation. In contrast to a previous study of the emergence of GCV-resistant HHV-6B strains in transplant recipients based on viral DNA amplified by PCR (25), the present study

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TABLE 1 Characteristics of the 15 HSCT recipients with HHV-6B viremia[a]

Case patient no.	Gender	Age (yr)	Underlying disease	No. of HHV-6B clinical isolates analyzed	Length of GCV treatment (days)
1	F	62	MDS	3	22
2	M	48	AML	5	144
3	F	11	AML	4	7
4	M	13	AML	9	14
5	F	10	AA	1	24
6	M	9	ALD	1	11
7	F	48	AML	2	24
8	F	32	AML	1	14
9	M	52	AML	2	14
10	M	37	MDS	2	–
11	F	17	MDS	2	–
12	M	13	AML	2	–
13	M	10	NB	2	–
14	F	36	AML	4	–
15	M	14	AA	2	–

[a] GCV, ganciclovir; F, female; M, male; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; AA, aplastic anemia; ALD, adrenoleukodystrophy; NB, neuroblastoma. –; no GCV treatment.

analyzed isolated viruses, which are reliable for the evaluation of active viral infections but not latency in transplant recipients.

MATERIALS AND METHODS

Clinical and laboratory isolates. Forty-two HHV-6B clinical isolates recovered from 15 hematopoietic stem cell transplant (HSCT) recipients (Table 1) and 20 isolates recovered from 20 ES patients were used in this study. Nine of the 15 HSCT recipients received GCV during the observation period. None of the ES patients were treated with GCV during the observation period. This study was approved by the Ethical Review Board at the Fujita Health University School of Medicine (no. 08-183). The patients or their guardians consented to participation in this study.

HHV-6B isolation and identification were performed as previously described (26). In brief, peripheral blood mononuclear cells were cocultured with cord blood mononuclear cells in culture medium. Viruses were identified primarily by morphological changes in cultured cells (i.e., characteristics of pleomorphic, balloon-like large cells), and then viral isolation was confirmed by immunofluorescence staining with an HHV-6B monoclonal antibody (OHV-3; provided by T. Okuno, Department of Microbiology, Hyogo College of Medicine, Hyogo, Japan). Cocultured cord blood mononuclear cells that were infected with each isolate were stored after 2 to 3 passages at −80°C.

The previously characterized HHV-6B strains HST and Z29 are established laboratory strains and were used as standards in the current study. HST was originally isolated from an ES patient in Japan, while Z29 was originally isolated from an AIDS patient in Zaire (27). The HST and Z29 strains were also propagated by cocultivation with cord blood mononuclear cells.

Control plasmids. DNAs containing the six known mutations in the U69 gene that are associated with GCV resistance (A to G, C to A, T to G, T to C, C to A, and G to A at positions 952, 1340, 1342, 1349, 1385, and 1388, respectively) were constructed using a PrimeStar mutagenesis basal kit (23). These mutations correspond to the amino acid substitutions M318V, A447D, C448G, L450S, A462D, and C463Y, respectively, in functional subdomains VIb and XII (Fig. 1). The DNAs were amplified by PCR and subcloned into pGEM-T vectors (Promega, Madison, WI). Plasmid DNAs were isolated using a Wizard Plus SV miniprep DNA purification system (Promega, Madison, WI).

DNA extraction. Viral DNAs were extracted from the stored clinical isolates and cord blood mononuclear cells infected with the HHV-6B laboratory strain (strains Z29 and HST) using a QIAamp DNA blood minikit (Qiagen, Chatsworth, CA). Extracted DNAs were eluted in 100 μl buffer and stored at −20°C until QP-PCR analysis.

QP-PCR for detection of GCV resistance-associated mutations in U69. The PCR amplification protocol used in this study to detect GCV resistance-associated mutations in the HHV-6B U69 gene was modified

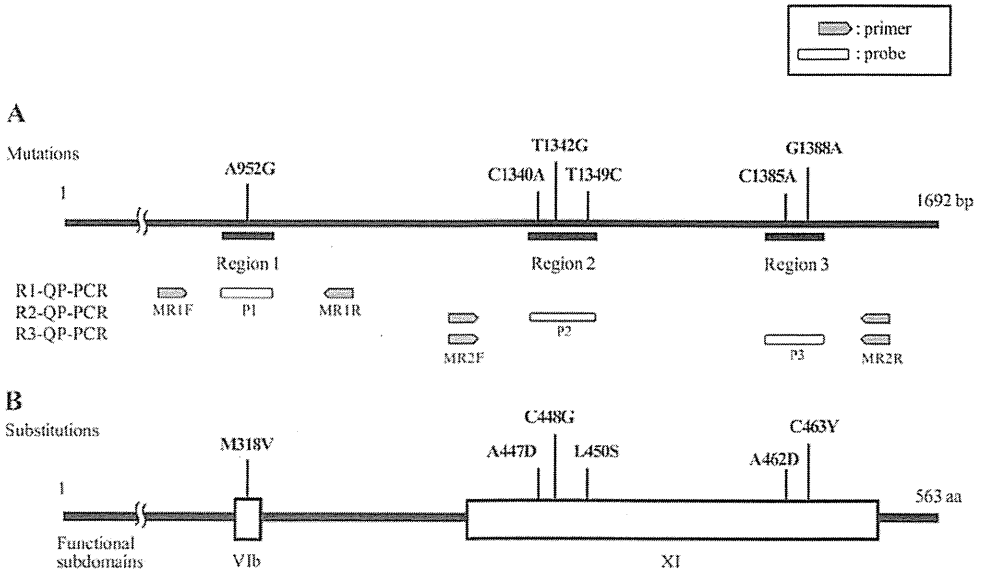

FIG 1 Map of the HHV-6B U69 (protein kinase) gene and protein. The U69 protein contains two functional domains. The six mutations (and the substitutions that they encode) that have been associated with GCV resistance are A952G (M318V), C1340A (A447D), T1342G (C448G), T1349C (L450S), C1385A (A462D), and G1388A (C463Y). (A) Positions of the synthesized primers and probes in the nucleotide sequence; (B) the corresponding positions of the six amino acid substitutions in the U69 protein.

TABLE 2 Primers and probes used for detection of GCV resistance mutations in U69 gene by QP-PCR analysis

Primer or probe name	Region(s)	Sequence (orientation)
Primers		
MR1F	1	5′-TCTATAAGTTCGAAGATTGGGATGTC-3′ (sense)
MR1R	1	5′-GGACAAGCTGTAATCCGCCAAC-3′ (antisense)
MR2F	2 and 3	5′-GATGCCGCGAAGCTCAGATATAC-3′ (sense)
MR2R	2 and 3	5′-TATATCAACCACGTCTCGGTAAAAC-3′ (antisense)
Probes		
P1	1	5′-CCTATGAATATCTTTATAAATC-3′
P2	2	5′-GATTCAATCGACAGGCCTC-3′
P3	3	5′-CAATACTTTACAGCAAGCGTC-3′

from the protocol described previously (24). As shown in Fig. 1, the six SNPs are located in three distinct regions (regions 1, 2, and 3 [R1, R2, and R3, respectively]). Therefore, three different QP-PCRs (a QP-PCR with a probe specific for R1 [R1-QP-PCR], an R2-QP-PCR, and an R3-QP-PCR) were developed to detect mutations in each of the three regions. The PCR and subsequent melting curve analyses were performed using an Applied Biosystems StepOne real-time PCR system. Two primer sets were designed for the amplification of regions 1, 2, and 3 containing the six single nucleotide polymorphism (SNPs). Table 2 shows the sequences of the primers for region 1 (MR1F and MR1R) and regions 2 and 3 (MR2F and MR2R) as well as the sequences of the probes for region 1 (P1), region 2 (P2), and region 3 (P3). Fluorophores were linked to cytosine residues located in the 3′ end of the probe sequences. The PCR mixture (20 μl) contained 5 μl of extracted DNA, 5 μl of primers, 0.2 μM probe, and 10 μl of a master mix (TaqMan universal PCR master mix [Applied Biosystems, Foster City, CA]). The primers were made at a ratio of 1:5 (0.1 μM MR1F to 0.5 μM MR1R or 0.1 μM MR2R to 0.5 μM MR2F). The PCR was performed under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s; 72°C for 10 min; and 4°C for storage. Melting curve thermal cycle conditions were as follows: 95°C (a 1-s holding time), followed by a 60-s annealing step at 30°C with a slow ramp (0.3°C/s) up to 70°C with continuous detection through the ramp.

Direct sequencing. To confirm the results of the QP-PCR analysis, sequencing analysis of the entire U69 gene was performed using PCR. Table 3 shows the four primer pairs that were designed to amplify the four distinct regions of the U69 gene, the 5′ terminal region (the U69-S1 primer pair), the first middle region (the U69-S2 primer pair), the second middle region (the U69-S3 primer pair), and the 3′ terminal region (the U69-S4 primer pair). The PCR products amplified from the selected isolates were sequenced using a BigDye Terminator (v3.1) cycle sequencing kit and a Prism 3100 Avant sequencer (Applied Biosystems, Foster City, CA). The sequences of the HHV-6B U69 region were compared using the Clustal W computer program (DNA Data Bank of Japan).

RESULTS

Screening of the six SNPs associated with GCV resistance in clinical isolates. As the probes dissociate from unmatched PCR products at lower temperatures than perfectly matched PCR products, it is possible to detect mutations by melting curve analyses of the PCR products. Typical melting curves from the R1-QP-PCR, R2-QP-PCR, and R3-QP-PCR analyses are shown in Fig. 2. For regions 1 and 2, all clinical isolates demonstrated the same melting temperature as the Z29 strain (Fig. 2A and B). However, the melting temperatures of all clinical isolates were located between the melting temperatures of the A462D SNP and the Z29 strain (Fig. 2C). Then, the melting curves from the R3-QP-PCR for clinical isolates and DNA extracted from the laboratory strain, HST, were compared. As shown in Fig. 2C, the melting temperatures of all clinical isolates were the same as the melting temperature of HST. Sequence analysis of region 3 identified the C1383T SNP in HST and all of the clinical isolates (data not shown). Finally, as we expected, none of the 20 clinical isolates recovered from the ES patients and the 14 isolates recovered from the HSCT recipients who did not receive GCV contained any of the six GCV resistance-associated SNPs. Moreover, the six SNPs were not detected in any of the 28 clinical isolates recovered from the 9 HSCT recipients treated with GCV (Table 1).

In order to confirm our findings, sequence analysis of the entire U69 gene from 15 representative isolates consisting of the latest isolate recovered from each of the 15 HSCT recipients was carried out. The SNPs T63C, C132T, T240C, C348G, C405A, A438G, T693C, T1287C, and C1383T were discovered in all 15 isolates and were identical to the SNPs detected in the HST strain. Some additional unique silent mutations (C154T, G372T, C486T, C637A, and C1582A) were identified in two isolates that were recovered from recipients who were not treated with GCV.

Clinical courses and kinetics of viral DNA loads in nine HSCT recipients treated with GCV. As preemptive GCV administration for the prevention of cytomegalovirus diseases is well established in Japan, GCV was administered to patients with elevated cytomegalovirus DNA loads (cases 1, 2, 5, 6, 7, 8, and 9). In cases 2, 3, and 4, GCV was administered to control HHV-6B infection. An extended period of GCV treatment (more than 14 days) was carried out in cases 1, 2, 4, 5, 7, 8, and 9, and these patients were considered to be at high risk for the emergence of GCV-resistant viruses. Elevation of the HHV-6B DNA load occurred between approximately 2 and 4 weeks after they received the transplant, as demonstrated in cases 1, 3, 5, 6, 7, and 9, and HHV-6B DNA loads peaked at later time periods in the most

TABLE 3 Primers used for sequence analysis of U69 gene

Primer name	Region	Primer sequence (orientation)
U69-S1	5′ terminal region	5′-GAGAAATTGGACGTTGATGAAA-3′ (sense)
		5′-GCCATAGTCTTTTCAGGTTGACA-3′ (antisense)
U69-S2	First middle region	5′-TGGAAACTGGAGCTAGAGAAAA-3′ (sense)
		5′-CACACGTGCCGTTATACTCG-3′ (antisense)
U69-S3	Second middle region	5′-AAATCGGGAGCCGACTTATT-3′ (sense)
		5′-TCAACCACGTCTCGGTAAAA-3′ (antisense)
U69-S4	3′ terminal region	5′-TTGTCGCCAATGCAATGATA-3′ (sense)
		5′-GCCCAAAATGCAATTATTGTT-3′ (antisense)

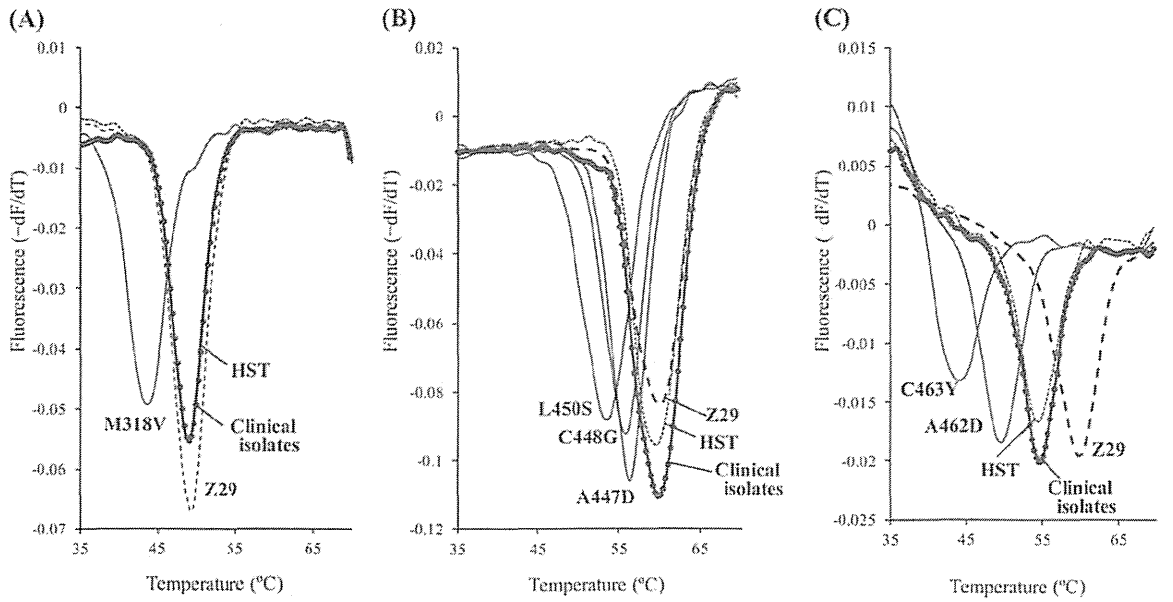

FIG 2 Melting curve analysis of the clinical isolates using QP-PCR with probes specific for region 1 (A), region 2 (B), and region 3 (C).

AQ: G critically ill ~~critical~~ patients (cases 4, 5, and 9). In most cases, the HHV-6B DNA load peaked once during the observation period, but either repeated elevation of the viral load or repeated isolation of the virus was demonstrated in several patients with severe immunosuppression (cases 1, 2, 3, 4, 5, 7, and 9). Persistent active viral infection (viremia) due to long periods of GCV administration was demonstrated in cases 2 and 4, which placed these patients at the highest risk for the emergence of GCV-resistant HHV-6B.

DISCUSSION

In order to elucidate the reliability of QP-PCR methods for the detection of GCV resistance-associated SNPs in HHV-6B clinical isolates, we initially examined clinical isolates recovered from ES patients not treated with GCV as representative samples without GCV resistance-associated SNPs. Regions 1 and 2 of all clinical isolates demonstrated the same melting temperatures as regions 1 and 2 of the Z29 strain; however, the melting temperatures of region 3 differed (Fig. 2C). Although both the Z29 and HST strains belong to HHV-6B, the Z29 strain was isolated from an AIDS patient in Zaire (27) and the HST strain was isolated from a Japanese ES patient (1). The melting temperature of the HST strain, which was same as that of the clinical isolates recovered from ES patients (Fig. 2C), differed from the melting temperature of the Z29 strain. Furthermore, sequence analysis of region 3 identified several sequence differences between the Z29 and HST strains (data not shown), which were consistent with the previously reported sequence data (GenBank accession numAQ: H bers AB021506 and AF157706). These results indicated that the R3-QP-PCR is useful for the detection of GCV resistance-associated SNPs not only in the Z29 strain but also in the HST strain. Therefore, our QP-PCR method performed with probes specific for regions 1, 2, and 3 was effective for the discrimination of GCV resistance-associated SNPs from the wild-type sequences.

Manichanh et al. demonstrated that one of the five peripheral blood mononuclear cell specimens obtained from AIDS patients who were treated with GCV contained the A952G mutation in region 1 (25). To the best of our knowledge, although the sample size was too small, our study is the first one to analyze the frequency of GCV-resistant HHV-6B isolates in patients with primary viral infections and HSCT recipients with viral reactivation. As we expected, no GCV resistance-associated SNPs were detected in the isolates recovered from ES patients who were not treated with GCV. Additionally, no GCV resistance-associated SNPs were detected in any of the clinical isolates obtained from HSCT recipients irrespective of GCV treatment. As demonstrated by *in vitro* experiments, persistent viral replication under GCV treatment is required for the emergence of GCV-resistant HHV-6B strains (22, 23). In contrast to cytomegalovirus reactivation, HHV-6B reactivation (viremia) regresses without antiviral treatment in most HSCT recipients. Although HHV-6B was repeatedly isolated from several HSCT recipients, these patients (cases 1, 3, 5, 7, and 9) did not receive concurrent prolonged GCV administration. Thus, the self-limiting clinical course of HHV-6B reactivation in HSCT recipients and the low frequency of active viral infection under GCV treatment may result in the low rate of emergence of GCV-resistant HHV-6B strains. As cases with prolonged HHV-6 viremia were rare in the recipients after HSCT, the number of study subjects was limited in this study. Therefore, a large number of patients with prolonged viremia during GCV treatment, which may be very rare, should be analyzed to elucidate the precise incidence of the emergence of GCV-resistant HHV-6 in HSCT recipients.

We failed to detect GCV resistance-associated SNPs in the isolates recovered from the two patients (cases 2 and 4) with persistent GCV administration (Fig. 3). Mutations in the U38 gene, F3
which is another candidate gene involved in GCV metabolism, occurred in only one GCV-resistant clinical isolate obtained from an HSCT recipient (28). Therefore, further studies are needed to determine whether the isolates recovered from the two patients in AQ: I
the current study may also have mutations in the U38 gene which

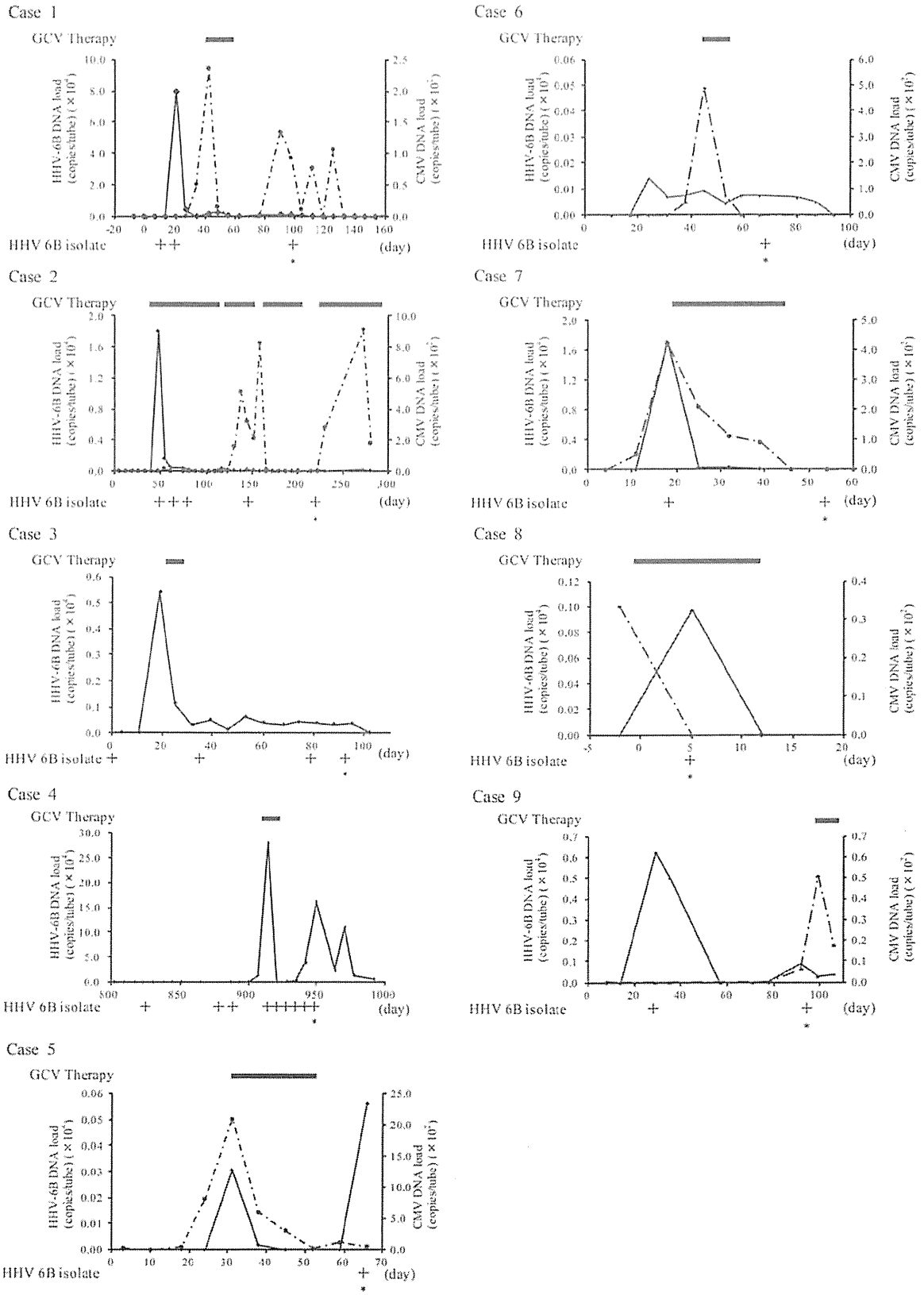

FIG 3 Clinical courses and kinetics of viral DNA loads in nine HSCT recipients treated with GCV. HHV-6 was isolated from peripheral blood mononuclear cells collected from the patients weekly. +, an HHV-6B isolate was obtained; *, sequencing analysis was performed; dotted lines, cytomegalovirus (CMV) DNA loads; solid lines, HHV-6B DNA loads.

may mediate their GCV resistance. We are currently developing a QP-PCR method for the detection of mutations in the HHV-6B U38 gene that mediate GCV resistance.

The use of clinical isolates in this study may have resulted in the selection of the virus during the virus isolation step; however, this is unlikely because no differences in the growth curves between the wild-type and GCV-resistant strains were demonstrated on the basis of *in vitro* assays (25). Previous studies suggested that mixed cytomegalovirus populations composed of wild-type and GCV-resistant strains occurred in immunosuppressed patients following GCV treatment (29, 30). Thus, further pyrosequencing analyses may be needed to determine the precise incidence of the emergence of GCV-resistant HHV-6B strains in immunocompromised patients following GCV administration.

Previous studies demonstrated HHV-6B reactivation between 2 and 4 weeks after transplantation (6, 7, 31), and similarly, in the current study we found repeated viremia (cases 2 and 4) or viremia at a later time period (case 6) in several HSCT recipients. Repeated HHV-6B viremia at later time points after transplantation, generally immediately before the death of the patients, was consistent with a previous report of a high rate of HHV-6B reactivation in critically ill patients (32). The role of HHV-6B reactivation at the later time points after transplantation remains unclear, and HHV-6B reactivation suggests that sufficient antiviral treatment was not carried out in these transplant recipients. In order to improve the patient's prognosis, it is important to elucidate the clinical significance of HHV-6B reactivation at later time periods after transplantation as well as to monitor the emergence of GCV-resistant HHV-6B strains in transplant recipients with persistent GCV administration.

The current study demonstrates that QP-PCR analysis can be used to monitor GCV-resistant HHV-6B strains in high-risk patients, such as transplant recipients. In contrast to the rate of emergence of GCV-resistant cytomegalovirus, our findings suggest that the rate of emergence of GCV-resistant HHV-6B strains appears to be relatively low even in HSCT recipients who have received GCV treatment.

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AQB—Au: Insertion of "melting temperatures profiles of all clinical isolates" correct for complete comparison? If not, please indicate the things that were similar. All clinical isolates were similar to HST.

AQC—Au: HHV-6B meant throughout the remainder of this paragraph? Please check use of HHV-6B versus HHV-6 throughout. If "HHV-6" was used in the original paper, we used that word,

AQD—Au: The dashes were deleted from the body of Table 1, as dashes are retained as placeholders only if they are defined in a footnote, ASM style. "–" indicated no GCV treatment.

AQE—Au: "pGEM-T Easy" meant? "pGEM-T" was correct.

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AQG—Au: "critically ill" meant? Yes.

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The sequences of the clinical isolates were same as those of the HST strain (not Z-9 strain). So, I don't think that we have to submit the sequences.

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AQI—Au: Would "isolates without GCV resistance-associated SNPs" be more correct to specify the isolates meant? I think that original sentence is better.

AQJ—Au: Would "GCV-resistant virus" be more correct to specify the virus meant?

AQK—Au: Please provide a doi if available for this reference.
We don't have the information.

This is also original is better.

Analysis of the Shedding of Three β-Herpesviruses in Urine and Saliva of Children With Renal Disease

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Cytomegalovirus (CMV), human herpesvirus 6 (HHV-6) and 7 (HHV-7) are important pathogens in immunocompromised patients. To elucidate the kinetics of the three β-herpesviruses in saliva and urine samples were collected serially from children with renal diseases. Twenty children with renal diseases were enrolled in this study. A total of 240 saliva and urine samples were collected monthly from the patients over a 1-year period. Viral DNAs loads were measured by real-time PCR. In 10 CMV seropositive patients CMV DNA was detected rarely in saliva and CMV DNA load was lower than the other two β-herpesviruses DNA loads. All patients were seropositive for HHV-6B and the virus was detected frequently in saliva. Two of 20 patients were HHV-7 seronegative. High copies of viral DNA were detected continuously in saliva of the HHV-7 seropositive patients. Although neither CMV nor HHV-6B DNA load was different among the three renal diseases, HHV-7 DNA load was different among the diseases ($P=0.039$). HHV-6B DNA loads were significantly higher in patients with immunosuppressive treatment compared to those without treatment ($P=0.013$). Although CMV DNA was detected in urine samples collected from 5 of 10 CMV seropositive patients, HHV-6B and HHV-7 DNA were detected at relatively low frequencies in urine. No remarkable temporal associations between viral DNA excretion and proteinuria or immunosuppressive treatment were demonstrated. The pattern of viral DNA excretion in saliva and urine were different among the three viruses. No temporal correlation was observed between viral infection and renal diseases. ***J. Med. Virol. 9999:1–7, 2013.***

KEY WORDS: CMV; HHV-6B; HHV-7; real-time PCR; renal diseases

INTRODUCTION

Cytomegalovirus (CMV), human herpesvirus (HHV)-6A, HHV-6B, and HHV-7 are members of the β herpesviridae subfamily. After primary viral infection, these viruses infect persistently several tissues, such as the kidney, salivary gland, and monocyte/macrophage lineage cells. These viruses reactivate intermittently in both immunocompromised and immunocompetent hosts; indeed clinical importance of viral reactivations is quite different between the immunocompromised and immunocompetent hosts. Previously, the kinetics of viral shedding in saliva was found to vary depending on the viral infection and to some extent the host immune status [Yoshikawa et al., 2005]. The long term shedding of CMV in the urine of infants and young children after primary viral infection is well established; however, there is limited data of primary HHV-6 and HHV-7 infection resulting in unusual urinary shedding [Suga et al., 1997, 1998].

CMV has been implicated in nephritis and rejection in renal transplant recipients [De Keyzer et al., 2011]. Additionally, it has been suggested that CMV may be involved in childhood nephritis [Platt et al., 1985; Matsukura et al., 2006; Georgaki-Angelaki et al., 2009] and congenital nephrotic syndrome [Evans and Lyon, 1991; Batisky et al., 1993; Giani

Abbreviations: CMV, cytomegalovirus; HHV-6, human herpesvirus 6; HHV-7, human herpesvirus 7; PCR, polymerase chain reaction

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

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et al., 1996; Eddleston et al., 1997; Barbi et al., 1998; Frishberg et al., 2003; Besbas et al., 2006]. Since children with renal disease are treated generally by immunosuppressive drugs, these patients are considered to be at high risk for reactivation of herpesviruses. However, the impact of β-herpesviruses on the pathogenesis of pediatric renal diseases treated by immunosuppressive drugs remains unclear. Therefore, in order to elucidate the pathogenic role of these viruses in childhood renal diseases, viral loads of CMV, HHV-6, and HHV-7 were examined in saliva and urine samples that were collected serially from children with renal diseases.

MATERIALS AND METHODS

Patients and Sample Collection

This study was approved by the Institutional Review Board of Fujita Health University. Informed consent was obtained from the parents or guardians of the patients. Urine and saliva samples were collected monthly for one year from 20 patients (11 females and 9 males) with renal diseases (8 IgA nephropathy, 6 Henoch–Schönlein purpura nephritis, and 6 nephrotic syndrome) to monitor the three β-herpesvirus shedding. A total of 240 saliva and urine samples were collected prospectively from the patients. One milliliter of saliva and urine samples were collected from the patients at the same time, then these samples were used to measure viral DNA load of the three β-herpesviruses by real-time PCR. The patient characteristics, such as immunosuppressive treatments and sero-status for the three viruses, are summarized in Table I.

In order to evaluate the effect of immunosuppressive drugs on salivary viral shedding, the patients were divided into two different groups: with immunosuppressive treatment (n = 13) and without immunosuppressive treatment (n = 7). If a patient was administered at least one immunosuppressive drug during the observation period, they were placed in the immunosuppressive treatment group.

The seropositive patients (CMV; n = 10, HHV-6; n = 20, HHV-7; n = 18) were subjected in the analysis for the effect of the host factors including with and without immunosuppressive treatments and types of the renal diseases on salivary viral shedding.

DNA Extraction and Real-Time PCR

DNA was extracted from 200 μl of whole saliva and whole urine specimens using QIAamp Blood Kit (QIAGEN Chatsworth, CA), eluted finally in 100 μl of TE buffer and stored at −20°C until assayed. Ten microliters of extracted DNA was used to measure CMV, HHV-6B, and HHV-7 DNA load by real-time PCR. The detail of the real-time PCR method for measurement of viral DNA load has been described previously [Tanaka et al., 2000]. The detection limit for viral DNA was 10 copies per reaction for all three viruses. In this current study HHV-6A DNA load was not examined because the frequency of HHV-6A infected individuals is considered to be low in Japan [Tanaka-Taya et al., 1996].

Serological Analysis

Serum samples were collected from the patients at the time of entry into this study. IgG antibody titers to HHV-6 and 7 were measured using an indirect-immunofluorescence assay as described previously [Yoshikawa et al., 1989; Yoshikawa et al., 1993]. IgG antibody titers to CMV were measured by a

TABLE I. Patient Characteristics

No. of cases	Age (years)	Gender	Disease	Steroid (months)[a]	Other immunosuppressive drugs
1	14	F	IgA nephropathy	No	No
2	7	F	IgA nephropathy	Yes (7)	No
3	8	M	IgA nephropathy	No	No
4	15	M	IgA nephropathy	No	No
5	12	F	IgA nephropathy	Yes (7)	No
6	5	F	IgA nephropathy	Yes (6)	No
7	9	F	IgA nephropathy	Yes (7)	No
8	15	M	IgA nephropathy	No	No
9	7	F	Nephrotic syndrome	Yes (12)	Cyclosporine
10	10	F	Nephrotic syndrome	Yes (6)	No
11	11	M	Nephrotic syndrome	Yes (12)	Cyclosporine
12	4	M	Nephrotic syndrome	Yes (7)	No
13	6	M	Nephrotic syndrome	Yes (12)	Cyclosporine
14	11	M	Nephrotic syndrome	Yes (4)	No
15	6	M	HSPN	No	No
16	8	F	HSPN	No	No
17	7	F	HSPN	No	No
18	11	M	HSPN	Yes (10)	No
19	14	F	HSPN	Yes (5)	No
20	9	F	HSPN	Yes (5)	No

M, male; F, female; HSPN, Henoch–Schönlein purpura nephritis.
[a]Duration of steroid treatments.

commercial laboratory (SRL, Hachioji, Tokyo, Japan) using an enzyme linked immunosorbent assay (ELISA).

Statistical Analysis

Viral DNA loads in saliva were compared among the three renal diseases using Kruskal–Wallis test, and were compared between the patients with and without immunosuppressive treatment using the Mann–Whitney *U*-test. The gender and age differences between the patients with and without immunosuppressive treatments were compared using Fisher's exact tests and Student's *t*-tests, respectively. $P < 0.05$ was considered to be statistically significant. The statistical analysis was performed with StatView software, version J-5.0 (SAS Institute, Cary, NC).

RESULTS

Viral DNA Load of CMV, HHV-6, and HHV-7 in Saliva Samples

Ten (50%) of 20 patients were CMV seropositive at the time of study entry. Four (40%) of 10 CMV seropositive patients did not excrete the virus in saliva during the observation period. As shown in Figure 1, two CMV seropositive patients excreted the virus in saliva at only one time point and the remaining four patients excreted the virus several times (median, 6.8 times; range, 2–12 times). CMV DNA load in saliva was similar to HHV-6B DNA load, however CMV DNA shedding was infrequent in comparison to HHV-6B DNA. Additionally, CMV DNA load in saliva was clearly lower than HHV-7 DNA load in saliva. All patients were seropositive for HHV-6 and excreted the virus in saliva several times (median, 10.0 times; range, 4–12 times). As shown in Figure 1, HHV-6B DNA load in saliva was lower than HHV-7 DNA load. Two (10%) of 20 patients were HHV-7 seronegative at the time of entry into the study. HHV-7 shedding started from the 10th saliva sample in one (case 9) of the two seronegative patients. Although no HHV-7 DNA was detected in 12 saliva samples collected from one HHV-7 seropositive patient (case 3), high viral DNA copy numbers were detected in the saliva samples collected from 17 other HHV-7 seropositive patients (Fig. 1).

An Association Between Viral DNA Loads in Saliva and Host Factors

In order to determine whether host factors affect salivary shedding of the three β-herpesviruses, the

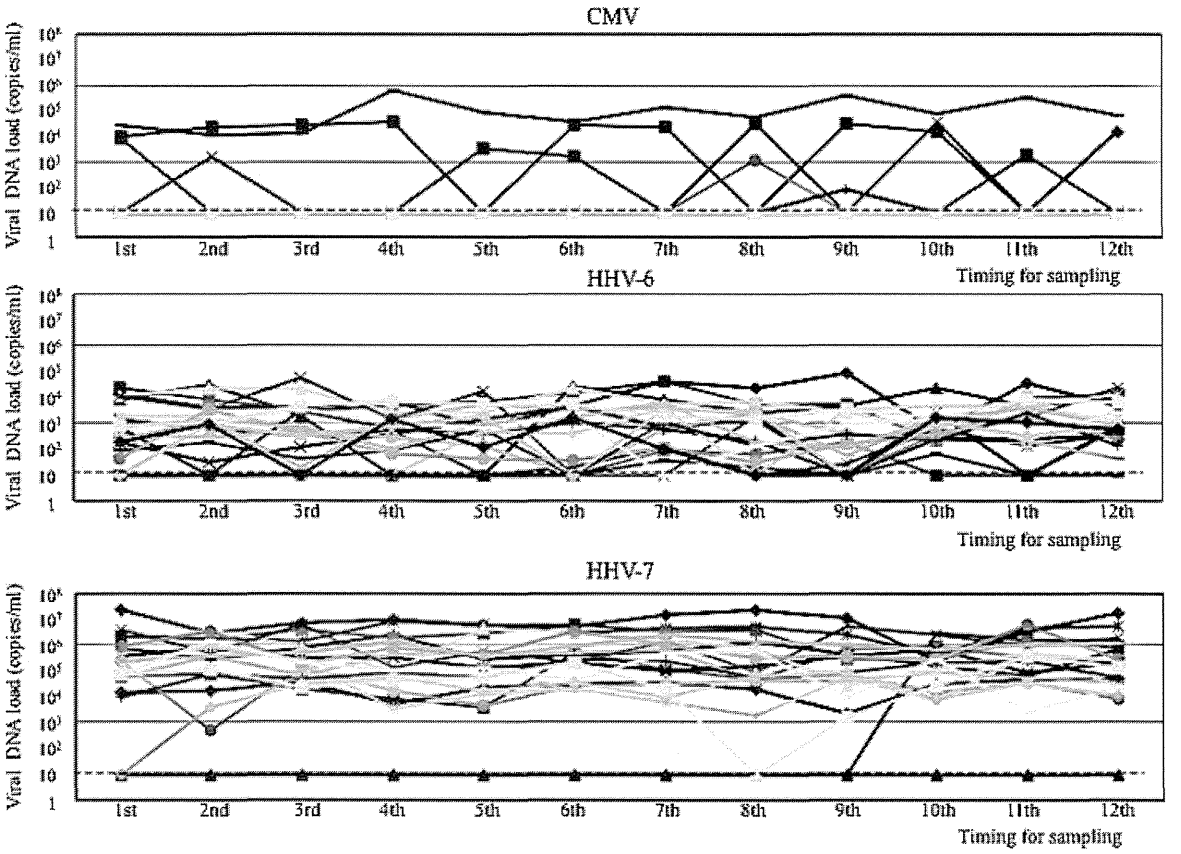

Fig. 1. Kinetics of CMV, HHV-6, and HHV-7 viral DNA loads in 12 series of saliva samples obtained from 20 children with renal diseases. Each line denotes the viral DNA loads of a single patient. The dotted lines indicate threshold levels of the real-time PCRs.

three viral DNA loads in saliva were compared among the seropositive patients with nephrotic syndrome (CMV, n = 4; HHV-6, n = 6; HHV-7, n = 4), IgA nephropathy (CMV, n = 3; HHV-6, n = 8; HHV-7, n = 8), and Henoch–Schönlein purpura nephritis (CMV, n = 3; HHV-6, n = 6; HHV-7, n = 6). Mean viral DNA copy numbers in the positive samples from each subject were calculated and then the mean viral DNA loads were compared among the three groups. Although neither CMV nor HHV-6B DNA load was different among the three renal diseases, HHV-7 DNA load was different among the diseases (median, interquartile range [IQR]; nephrotic syndrome; 2195143.5, 1,780,774–4410873.2 copies/ml, IgA nephropathy; 245,469, 45619.5–406,378 copies/ml; Henoch–Schönlein purpura nephritis; 150,713, 99346.2–780583.2 copies/ml), and was high in the patients with nephrotic syndrome ($P = 0.039$; Fig. 2).

Next, in order to compare the three viral DNA loads in saliva between the seropositive patients with (CMV, n = 8; HHV-6, n = 13; HHV-7, n = 11) and without (CMV, n = 2; HHV-6, n = 7; HHV-7, n = 7) immunosuppressive treatments, the mean viral DNA copy numbers in the positive samples from each subject were calculated, and then the mean viral DNA loads were compared between the two groups. As shown in Figure 3, no statistical differences were observed in CMV or HHV-7 DNA loads between the patients with and without immunosuppressive treatments; however, HHV-6B DNA load was significantly higher in the patients with immunosuppressive treatment than those without immunosuppressive treatment (median, IQR; 2,813, 717.0–9619.0 copies/ml vs. 377, 218.5–1041.5 copies/ml; $P = 0.013$). No statistical differences were detected in the patient characteristics between the patients with and without immunosuppressive treatments. For example, age (patients with immunosuppressive treatments, 8.9 ± 3.0 years old; patients without immunosuppressive treatments, 10.4 ± 4.0 years old; $P = 0.449$) and gender (patients with immunosuppressive treatments, five males and eight females; patients without immunosuppressive treatments, four males and three females; $P = 0.423$).

Viral DNA Load of CMV, HHV-6B, and HHV-7 in Urine Samples

Kinetics of DNA loads for three viruses in serially collected urine samples are shown in Table II. CMV DNA was detected in the urine samples collected from five (50%) of 10 CMV seropositive patients. Additionally, one urine sample collected from a CMV seronegative patient (case 5) was positive CMV DNA. Only three (15%) of 20 HHV-6 seropositive patients rarely excreted low copies of HHV-6B DNA in urine. Similar to HHV-6B, low copy numbers of HHV-7 DNA were detected rarely in urine samples collected from five (27.8%) of 18 HHV-7 seropositive patients. Additionally, two urine samples collected from one HHV-7 seronegative patient (case 9) contained relatively high copy numbers of HHV-7 DNA.

As an aim of this study was to determine whether the three β-herpesviruses were involved in the pathophysiology of childhood renal diseases, an association between the kinetics of the viral DNA loads in urine and proteinuria, which served as a surrogate marker for disease activity was examined. As shown in Table II, no remarkable temporal association between

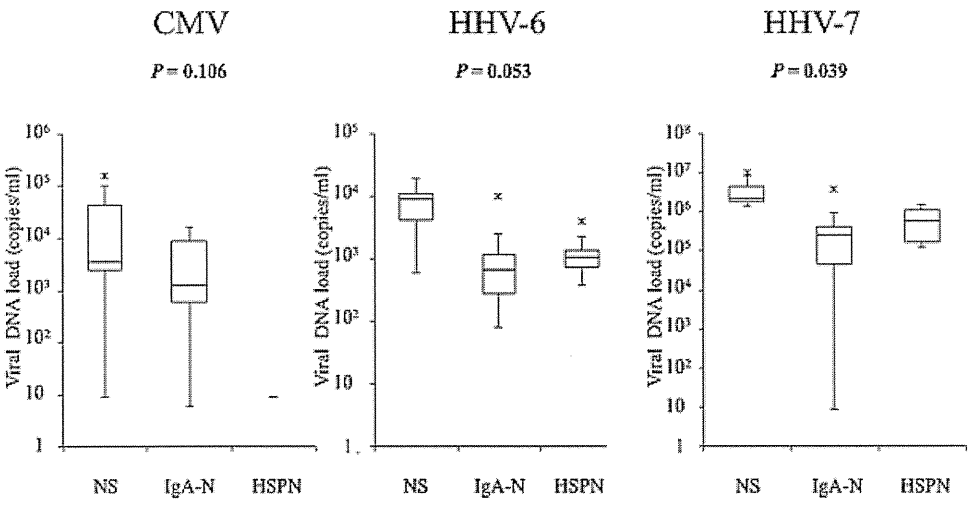

Fig. 2. Comparison of CMV, HHV-6, and HHV-7 viral DNA loads based on the type of renal disease nephrotic syndrome (CMV, n = 4; HHV-6, n = 6; HHV-7, n = 4), IgA nephropathy (CMV, n = 3; HHV-6, n = 8; HHV-7, n = 8), and Henoch–Schönlein purpura nephritis (CMV, n = 3; HHV-6, n = 6; HHV-7, n = 6). Data are shown as box plots, where the boxes represent the first through third quartile, the lines within the boxes represent the median, and the lines outside the boxes represent the minimum and maximum values (excluding outliers). NS, nephrotic syndrome; IgA-N, IgA nephropathy; and HSPN, Henoch–Schönlein purpura nephritis

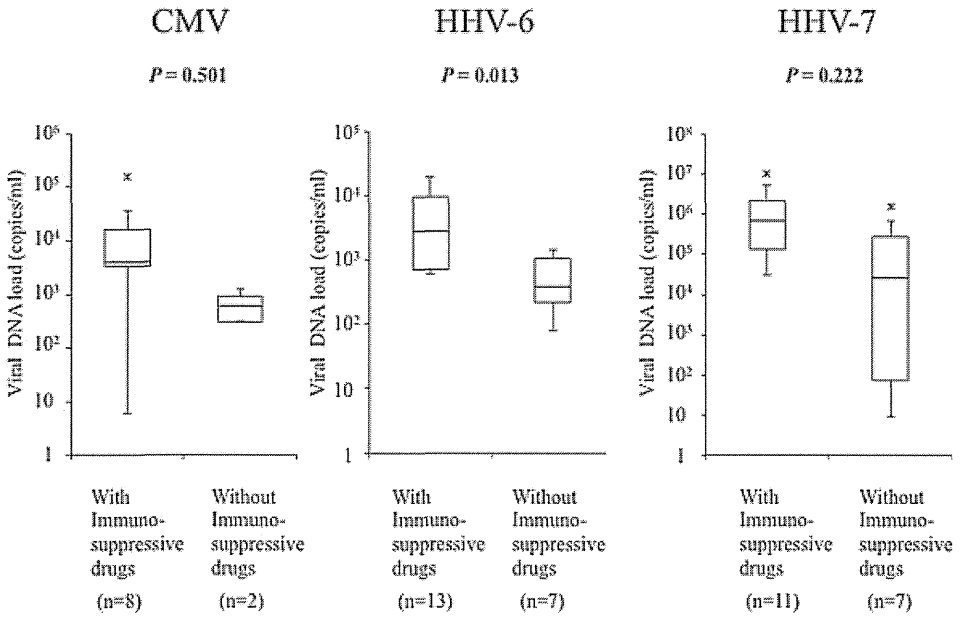

Fig. 3. Comparison of CMV, HHV-6, and HHV-7 viral DNA loads between the seropositive patients with (CMV, n = 8; HHV-6, n = 13; HHV-7, n = 11) and without (CMV, n = 2; HHV-6, n = 7; HHV-7, n = 7) immunosuppressive treatments. Data are shown as box plots, where the boxes represent the first through third quartile, the lines within the boxes represent the median, and the lines outside the boxes represent the minimum and maximum values (excluding outliers).

TABLE II. An Association Between Urine Protein Levels and Each Viral DNA Load (Log 10) (Copies/ml) in Urine

	No. of cases	Antibody	Timing for sampling											
			1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
CMV	2	+	0	0	4.0	3.8	0	4.9	4.9	3.8	0	0	5.0	4.1
			+	±	±	−	−	±	−	−	−	−	−	−
	5	−	0	0	0	0	0	0	0	0	0	0	2.8	0
			−	−	−	−	−	−	−	−	−	−	−	−
	9	+	5.1	3.6	4.5	4.4	4.1	4.2	4.7	4.6	4.5	4.3	5.1	4.4
			−	+++*	−	−	−	+++*	−	−	−	−	−	−
	11	+	0	3.7	0	0	0	0	0	0	0	0	0	0
			−	−	−	++*	+*	+*	++*	−	+*	±	−	−
	13	+	0	0	0	0	0	0	5.1	0	0	0	0	0
			+++*	−	−	−	+++*	−	−	−	−	−	−	−
	14	+	0	0	0	4.0	0	1.8	1.3	0	0	1.3	0	0
			−	−	−	−	−	−	±	−	−	−	−	−
HHV-6	2	+	0	0	0	0	0	0	2.1	0	0	0	0	0
			+	±	±	−	−	±	−	−	−	−	−	−
	15	+	0	0	0	0	0	0	0	0	0	2.4	2.4	0
			−	−	−	−	−	−	−	−	−	−	−	−
	20	+	0	0	0	1.6	0	0	0	0	0	0	0	0
			++	+++	+	+	+	±	−	−	−	−	−	−
HHV-7	4	+	3.5	0	0	0	0	0	0	0	0	0	0	0
			−	−	−	+++	±	−	−	−	+	±	±	±
	7	+	0	0	0	0	0	0	2.8	0	0	0	0	0
			+	±	±	±	±	−	−	−	+	−	−	−
	9	−	0	0	0	0	0	0	5.6	0	0	0	5.1	0
			−	+++*	−	−	−	+++*	−	−	−	−	−	−
	10	+	0	0	0	0	0	0	0	0	0	0	1.5	0
			+*	−	+*	++*	−	−	−	−	−	−	−	+
	12	+	0	0	0	0	2.5	0	0	0	0	0	0	0
			−	−	−	−	−	−	−	−	−	−	−	−
	19	+	0	0	0	0	0	0	0	3.7	0	1.4	0	0
			++	+++	+++	++	++	++	+	+	++	+	+	+

Shaded bocks indicate period for immunosuppressive treatments.
*At the time of relapse of nephrotic syndrome.

viral DNA loads and proteinuria was demonstrated. Moreover, immunosuppressive treatments appear to have no effect on the kinetics of viral DNA excretion in urine of these patients.

DISCUSSION

Previously, the kinetics of the three β-herpesviruses DNA loads in saliva collected from an adult population that were generally seropositive for all of the β-herpesviruses were reported [Yoshikawa et al., 2005]. Most of the children in the current study were seropositive for HHV-6 and 7; however, only half of the patients were seropositive for CMV. In fact, most infants and young children experience primary HHV-6 infection [Yoshikawa et al., 1989], and it precedes typically primary HHV-7 infection [Yoshikawa et al., 1993] in Japan. Meanwhile, recent seroepidemiological data has shown a decline in the seroprevalence of CMV in Japan [Nishimura et al., 1999] as similar to other industrialized countries. It is considered that the seroprevalence of these three β-herpesviruses in this present study reflects the current seroepidemiology of these viruses in the pediatric population in Japan.

Viral DNAs were detected in the samples obtained from the two seronegative patients (case 5, CMV DNA positive in 11th urine sample; case 9, HHV-7 DNA positive in 10th saliva sample and 7th and 11th urine sample) during the observation period. As serological tests were carried out at the time of entry into the study, it is likely that these two patients received the primary infection of each virus during the observation period. Although primary HHV-7 infection causes exanthem subitum [Asano et al., 1995; Suga et al., 1997; Caserta et al., 1998; Suga et al., 1998], no typical clinical symptoms of disease were identified in case 9. It has also been demonstrated that primary HHV-7 infection is associated with febrile illness without any specific clinical symptom [Caserta et al., 1998], which makes it difficult to diagnose based on clinical features.

As shown in Figure 1, frequency and quantity of viral shedding in saliva were different among the three β-herpesviruses. HHV-7 shedding was detected most frequently, and HHV-7 DNA loads were also high. CMV shedding was less frequent in comparison to the other two viruses, and CMV DNA loads were also low. These findings were consistent with previous data based on the analysis of an adult population [Yoshikawa et al., 2005]. Additionally, other studies demonstrated that the HHV-7 DNA load in saliva was relatively high in comparison to the other two β-herpesviruses [Gautheret-Dejean et al., 1997; Fujiwara et al., 2000]. In addition to the kinetic data, only HHV-7 DNA load in saliva was different among the three different renal diseases (Fig. 2). Viral DNA load was high in the patients with nephrotic syndrome in comparison to other two renal diseases. It has been suggested that abnormality of systemic immunity may be associated with the pathogenesis of nephrotic syndrome [Eddy and Symons, 2003]. Interestingly, variations were found in salivary HHV-7 shedding based on viral isolation analysis [Ihira et al., 2003] and higher HHV-7 DNA loads in the saliva of patients with adult onset Still disease compared to healthy controls [Yoshikawa et al., 2005]. These findings suggested an association between host immunity and salivary HHV-7 shedding.

In this current study, HHV-6B DNA load in saliva was significantly higher in patients with immunosuppressive treatments than those without immunosuppressive treatments (Fig. 3). It is important to note that there were no differences in the patient characteristics between the patients with and without immunosuppressive treatments. Stress hormones including cortisol have been associated with CMV reactivation in the salivary gland based on the analysis of astronauts [Mehta et al., 2000]. Moreover, it has been suggested that steroids may be a risk factor for HHV-6 reactivation in hematopoietic stem cell transplant recipients [Ogata et al., 2006]. Thus, the present finding supports the notion that corticosteroids may play an important role in the upregulation of HHV-6B reactivation in the salivary gland.

In previous studies, very low levels of urinary excretions of HHV-6 [Suga et al., 1998] and HHV-7 [Suga et al., 1997] were demonstrated at the time of primary viral infections. As expected, the frequency of HHV-6B and HHV-7 DNA shedding in urine were clearly lower than CMV in this current study. Additionally, although large amounts of HHV-6B and HHV-7 DNA were detected in saliva, the copy numbers of the HHV-6B and HHV-7 DNA in urine were low. Thus, the present study suggests that although the CMV, HHV-6B, and HHV-7 can infect latently both the salivary gland and kidney, the main site of persistent infection and viral reactivation are different between CMV and HHV-6B or HHV-7, as suggested by Gautheret-Dejean et al. [1997]. Furthermore, no temporal relationship between urinary shedding of these viruses and proteinuria was demonstrated, and viral DNA loads did not increase during immunosuppressive treatments. Therefore, immunosuppressive treatment did not impact the replication of the three viruses in renal tissues. The present data suggest that neither CMV nor HHV-6B and HHV-7 play an important role in the pathogenesis of pediatric renal diseases even though the patients received immunosuppressive treatments. However, as new immunosuppressive drugs, such as cyclosporine and rituximab, have been introduced for treatment of pediatric renal diseases, viral shedding in urine should be monitored in these patients in future studies.

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Dual roles for the telomeric repeats in chromosomally integrated human herpesvirus-6

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Approximately 1 percent of healthy individuals carry human herpesvirus-6 within a host chromosome. This is referred to as chromosomally integrated herpesvirus-6 (CIHHV-6). In this study, we investigated the chromosomal integration site in six individuals harboring CIHHV-6B. Using FISH, we found that HHV-6B signals are consistently located at the telomeric region. The proximal endpoints of the integrated virus were mapped at one of two telomere-repeat-like sequences (TRSs) within the DR-R in all cases. In two cases, we isolated junction fragments between the viral TRS and human telomere repeats. The distal endpoints were mapped at the distal TRS in all cases. The size of the distal TRS was found to be ~5 kb which is sufficient to fulfill cellular telomeric functions. We conclude that the viral TRS in the DR regions fulfill dual functions for CIHHV-6: homology-mediated integration into the telomeric region of the chromosome and neo-telomere formation that is then stably transmitted.

Human herpesvirus 6 (HHV-6) is one of the best characterized family members of the nine human herpesviruses. HHV-6 is classified as two distinct species, designated HHV-6A and HHV-6B, with an overall nucleotide sequence identity of 90%[1–3]. It has been demonstrated that primary HHV-6B infection occurs in infancy and causes a common febrile exanthematous illness, exanthem subitum[4,5]. However, neither the clinical features of primary HHV-6A infection nor the diseases directly associated with it have been identified to date. Following primary infection, HHV-6 remains latent in monocytes/macrophages and persists in the salivary glands[6,7]. In transplant recipients, HHV-6B reactivation can cause several clinical conditions such as encephalitis, bone marrow suppression, and pneumonitis[8,9].

Accumulating evidence now indicates that a subset of normal healthy individuals carry the HHV-6 genome within their chromosomes, which is known as chromosomally integrated herpesvirus-6 (CIHHV-6)[10]. The virus genome in these cases is transmitted by Mendelian inheritance. The integrated virus itself does not appear to be pathogenic, but CIHHV-6 carriers are often identified as high-titer virus carriers during screenings for HHV-6 reactivation in immunocompromised hosts. The presence of CIHHV-6 is not a rare condition with a reported incidence in healthy individuals of 1% in Caucasians and 0.21% in Japanese populations[11–13].

Based on consistently detectable FISH signals at chromosome ends in all previously analyzed independent CIHHV-6 cases, it had been speculated that the HHV-6 viral genome is integrated into human telomeres through an unknown mechanism that is specific to HHV-6[14]. The HHV-6 genome comprises a linear double stranded DNA of 159 kb flanked by identical 8 kb direct repeats at the left and right ends (DR-L and DR-R). Each DR contains two human telomere repeat (TTAGGG)-like sequences (TRS) proximal to both ends of the DRs[15,16]. The function of these motifs is uncertain, but it is not unreasonable to hypothesize that they plays a role in protection of viral genome ends from host defense systems such as nucleases in a similar manner to the telomere protection