from DNA end repair systems in eukaryotes[17]. Recently, sequence analysis of the junction fragments of three individuals with CIHHV-6A revealed that the HHV-6 genome was directly joined with the human telomeric region via TTAGGG repeats in each case[18]. A homology-directed mechanism operated by host DNA damage repair response pathways such as homologous recombination is likely to mediate these rearrangements[19].

To further elucidate the mechanism of the viral integration into the human telomeres, we investigated the integration sites in six Japanese individuals harboring CIHHV-6B.

Results

Six cases that were suspected to CIHHV-6B by having genome-equivalent copy number of viral DNA in peripheral blood samples estimated by qPCR were analyzed[20]. Standard cytogenetic evaluations revealed no abnormalities in any of these subjects. FISH analysis with a HHV-6 genomic DNA probe detected virus-specific signals on the long arm of chromosome 22 in two cases (cases 18 and 19), on the long arm of chromosome 6 in one case (case 31), and on the short arm of chromosome X in the two cases (cases 28 and 63) (Fig. 1b–d). The mother of case 19 (case 20) was also analyzed and showed HHV-6 signals on the long arm of chromosome 22. Thus, a CIHHV-6B diagnosis was confirmed in all six study subjects. CIHHV-6B FISH signals were detectable on only one of the homologues in each case, suggesting that all six individuals were heterozygotes in terms of viral integration. HHV-6 signals were consistently detected at the end of the chromosomes, presumably at the telomeric regions, in all six cases.

To next determine the structure of the integrated viral genome in our subjects, we performed MLPA experiments which allowed us to determine copy number of the target sites of the viral genome relative to the chromosomal DNA in each case. The copy numbers for the UL regions were constant and similar to the chromosomal regions used as references (Fig. 2), suggesting that a single copy of the viral genome was integrated within the chromosomal DNA in our CIHHV-6 cases. Copy numbers for the DRs varied among the subjects; two-fold higher than those for the UL regions in cases 18, 19, and 20, three-fold higher in case 28, and at a similar level in case 31.

To map the breakpoints of the HHV-6 integration in more detail in our subjects, Southern analyses using several HHV-6 probes were performed. Since the two TRS regions in the DR-R are good candidates for viral integration breakpoints, DR probes located near to the TRS-2 site were used (Supplementary Fig. S1). These DR probes yielded two distinct bands of a similar intensity (Fig. 3a). One of the bands was detected at a similar position in all cases with a size that was expected for the DR-L, suggesting that the DR-L was intact. The sizes of second band were different in each case, although two cases that were found to carry the HHV-6B at the long arm of chromosome 22 showed a second band of similar size. This suggests that these fragments included the junction between the viral and human genome. According to the restriction map, the breakpoints were predicted to be located within the TRS-2. A similar band pattern in two cases with a 22q integration indicated a common founder for this integration event. The fact that two bands were detectable in these analyses suggests that the entire viral genome was inserted together with both the DR-L and DR-R. The fact that the intensities of the two detected bands were similar further supports the idea that only a single copy of entire HHV-6B genome had been inserted in each individual.

We next attempted to isolate the junction fragments and could fortunately rely on sequence information for the subtelomere-telomere junction in the Xp region. First, we performed PCR using a primer designed to amplify the subtelomeric region flanking the telomere repeats and a primer that recognized the UL region just outside of the DR-R. The amplification reactions appeared to yield no product, but subsequent Southern hybridization analysis detected

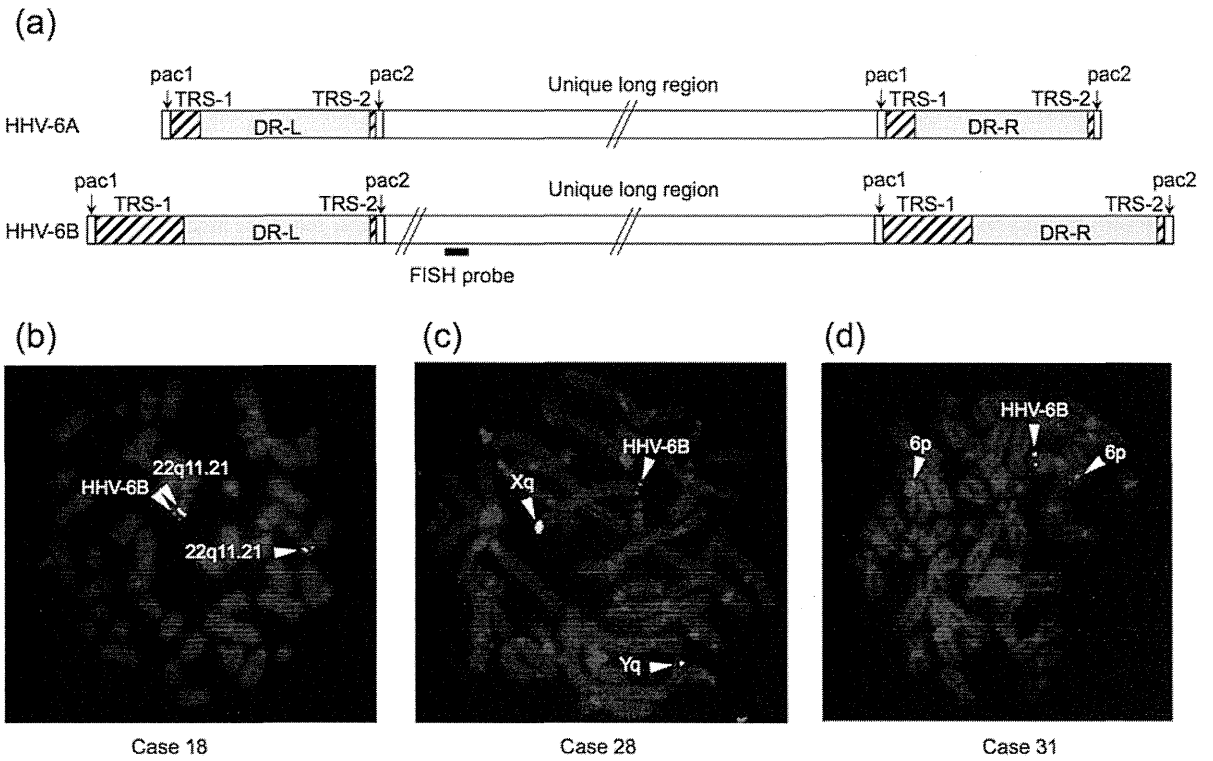

Figure 1 | Characterization of CIHHV-6B by FISH. (a) Schematic representation of the HHV-6 genomic structure. The 10 kb Pst I fragment used as s FISH probe is indicated. Gray boxes indicate DR sites and hatched boxes indicate TRS regions. (b) FISH analyses of metaphase chromosomes derived from the CIHHV-6B study subjects. A signal from the HHV-6B probe is detectable at the end of chromosome 22q (case 18), chromosome 6q (case 31) or chromosome Xp (case 28) (yellow arrowheads). The reference signals for chromosome 22q11.21, 6p and Xq are indicated by white arrowheads.

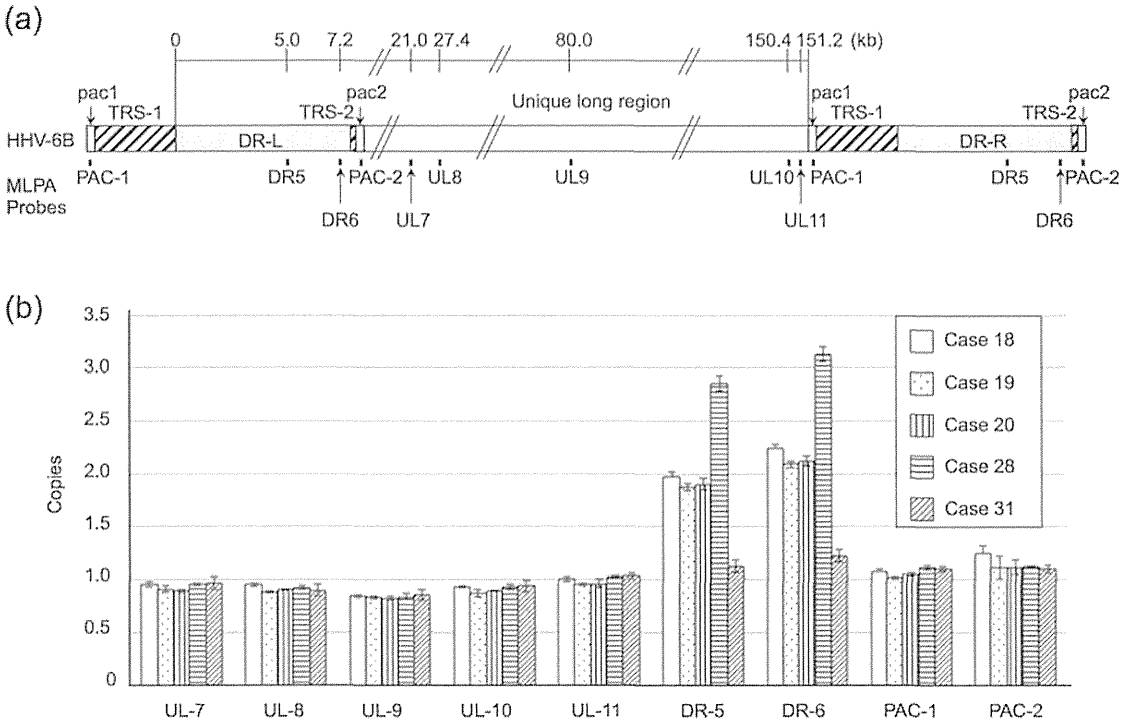

Figure 2 | Analyses of the CIHHV-6B genome structure by MLPA. (a) Schematic representation of the HHV-6B genome. The positions of the MLPA probes are indicated below the diagram. The scale bar indicates the distance from the starting point of DR-L. (b) Results of MLPA analyses. The vertical axis indicates the viral genome copy numbers relative to the single copy human genomic region. Data for cases 18, 19, 20, 28 and 31 are indicated from left to right.

a fragment exceeding 10 kb in length in case 28 with a HHV-6 integration at chromosome Xp (Fig. 3b). This indicated that the breakpoints of the virus were located within the DR-R. Unfortunately, less sequence information was available for the subtelomeric regions of chromosomes 6q and 22q. We attempted to perform junction PCR using a primer that bound to the most distal end of the reported subtelomeric sequence and a primer that recognized a region just outside of the DR-R, but no amplicon was obtained. We also tried inverse PCR but failed, because a short PCR product derived from short TTAGGG repeat present in the DR-L inhibited the amplification of real junction.

To further narrow down the positions of the HHV-6 breakpoints, multiple PCR primers were designed within the DR. Combined with a subtelomeric primer designed on the basis of sequence information for the Xp subtelomere-telomere junction (hg19, chrX: 60,427–60,445), all of the primers that recognized sites within the DR yielded PCR products of the expected size, which confirmed that the viral breakpoint is located within the TRS-2 (Fig. 3c). Sequencing of these amplicons revealed that the subtelomeric and viral DR-R regions were connected via 166 copies of the TTAGGG repeat, which is much shorter than the typical telomere repeat region in humans (9-15 kb; Fig. 3d) (GenBank accession number AB822541). These PCR experiments also yielded junction products in case 63 who had a HHV-6 integration at chromosome Xp. The sequence of this amplified fragment indicated that the integration sites are identical and the differences in the PCR product sizes was due to varying numbers of telomere repeats.

In one of our study subjects (case 31), the results of MLPA revealed only one copy of the DR, which was a similar level to the UL. Southern hybridization results for case 31 also produced a distinct pattern. A DR probe detected no bands corresponding to the DR-R, but constant bands only corresponding to the DR-L, suggesting that the TRS-2 had been deleted and that the HHV-6 breakpoint is located at a more distal region in this subject (Fig. 3a). Since TRS-1 is another candidate for the viral breakpoint, PCR for this region was performed using a DR primer flanking the TRS-1 site and a primer that was located just outside of the DR-R. No PCR product was obtained for case 31 although a TRS-1 amplicon was obtained in all other subjects, suggesting that the breakpoint in this one subject was located within the TRS-1 site in the DR-R (Supplementary Fig. S2a). Unexpectedly, the sizes of the TRS-1 PCR products from other cases were much larger (~5 kb) than that reported in the database (~300 bp), and also than TRS-2 (~500 bp). Although TRS-1 is referred to as heterogeneous $(TTAGGG)_n$ due to the reported presence of imperfect repeats, sequence analyses of our study subjects revealed a much longer stretch of perfect TTAGGG repeats than has been previously reported for the TRS-1 site, and greater also than those of TRS-2.

In case 28, MLPA results revealed a three-fold higher copy number for the DR compared with the UL region. Southern analyses further demonstrated the presence of additional DR copies in this subject, evidenced by three distinct bands (Fig. 3a). Sizes of the restriction fragments detected by DR probes suggested a proximal-DR-DR-UL-DR-distal structure within the genome of this individual (Supplementary Fig. S1, S3, and S4). To reveal the junction of the two distal DRs in this case, we performed PCR encompassing the TRS-2 – pac2 – pac1 – TRS-1 region. Only case 28 yielded a junction-specific PCR product that was also yielded from the subject including the replicating HHV-6B obtained from patients with exanthema subitum (Supplementary Fig. S5a). However, sequence analyses revealed that the junction between the two DRs in case 28 did not include pac1 or pac2, although the junction from the replicating HHV-6B carried the pac1 and pac2 regions.

To determine the structure of the other end of the HHV-6B genome, we mapped the endpoint of the viral genome within the DR-L. PCR amplification of the TRS-2 site in the DR-L was performed, and amplicons were obtained for all of our CIHHV-6B subjects. This suggested that the TRS-2 region in the DR-L had remained intact

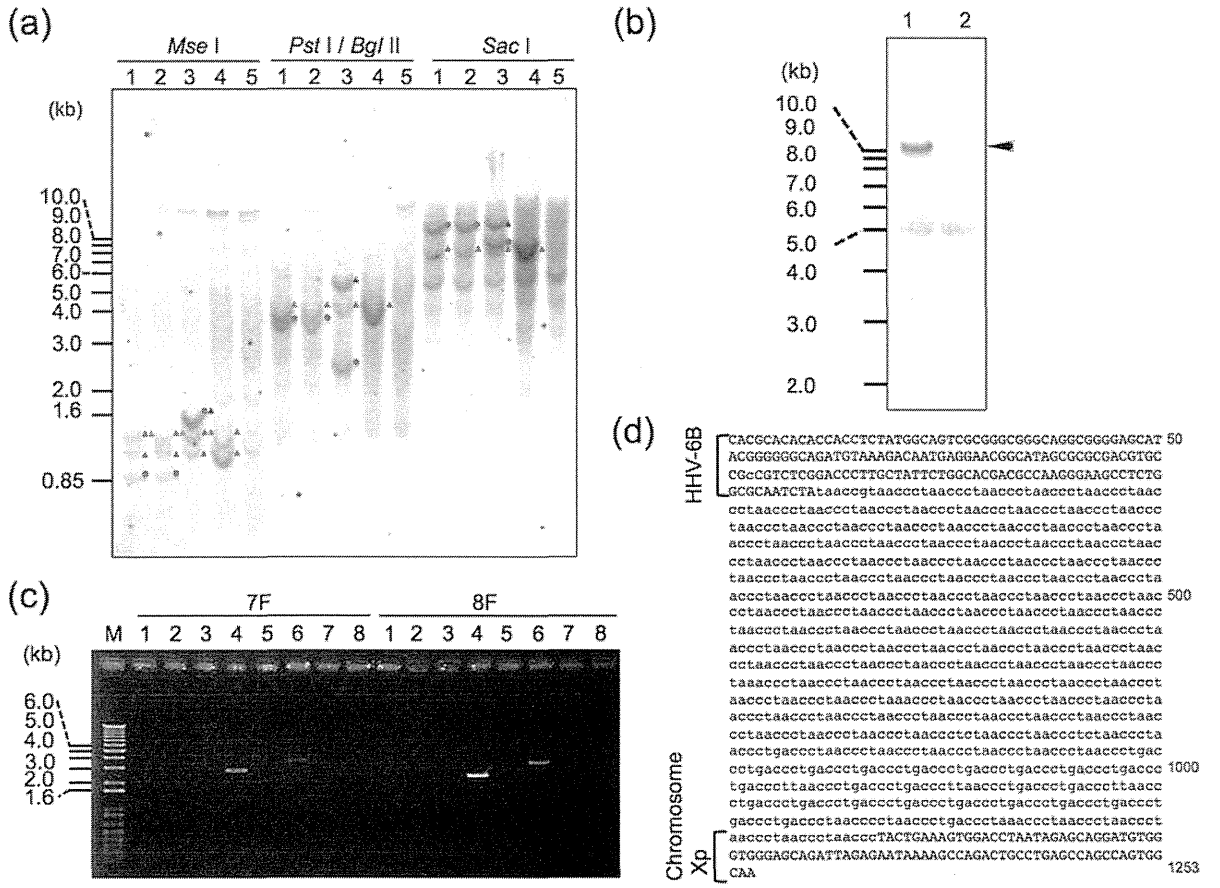

Figure 3 | Analyses of the junction between the human chromosome and viral genome. (a) Southern blot analyses of genomic DNA from the CIHHV-6B study subjects. The restriction enzymes used for these analyses are indicated above the panels; size markers are indicated on the left. Red dots indicate the rearranged bands derived from junction between human telomere and the HHV-6. Triangles indicate bands derived from the DR-L (green) and DR-R (orange), while blue triangles indicate the junction of the two DRs. Lane 1, case 18; lane 2, case 19; lane 3, case 28; lane 4, case 31; lane 5, non-CIHHV-6 control. (b) Southern blot analyses of PCR products. The arrowhead indicates a junction-specific PCR product. Lane 1, case 28; lane 2, non-CIHHV-6. Complete raw autoradiogram image can be seen in Supplementary Fig. S6. (c) PCR products that incorporate junction fragments. The primer sets were designed on the DR region (7F or 8F) and subtelomeric region on chromosomes X. Two CIHHV-6 cases yielded amplicons of different sizes. Lane M, size markers; lane 1, case 18; lane 2, case 19; lane 3, case 20; lane 4, case 28; lane 5, case 31; lane 6, case 63; lane 7, non-CIHHV-6 case; lane 8, water control. (d) Sequence of the junctions in case 28. The sequence shown covers the region from the DR-R of the viral genome to the human subtelomeric region. Lowercase letters denote telomere repeats.

in all cases (Supplementary Fig. S2b). We next performed PCR using one fixed primer for the site just outside of the DR-L and other primers for different sites within the DR. All of the primers within the DR successfully amplified specific products (Fig. 4). A telomere-repeat primer also yielded products appearing as a smear, the specificity of which was confirmed by Southern hybridization (Supplementary Fig. S5b). However, the use of a primer for the pacI site did not yield any specific product. We also designed MLPA probes within the pacI or pac2 region which produced a single copy signal which was similar to the UL region (Fig. 2), suggesting the absence of the pacI site. These results demonstrated that the CIHHV-6B chromosome ends within the TRS-1.

The presence of the TRS-2 in the DR-L not only indicated an intact TRS-2, but also gave us the opportunity to analyze individual variations in the TTAGGG repeat numbers. As expected, the sizes of the PCR products varied among individuals (Supplementary Fig. S2b)[21]. Among three of our study subjects with an integration at 22q, two cases from the same family (cases 19 and 20) showed an amplified product of the same size. The other case with a viral integration at 22q (case 18) showed a PCR product of a similar size but subsequent sequence analysis revealed different numbers of telomere repeats (32 for cases 19 and 20 versus 29 for case 18). Two cases with a viral integration at Xp showed different numbers of repeats (27 for case 28 and 23 for case 63).

Discussion

In our present study, we analyzed the structure of the integrated HHV-6B genome in six carriers of this virus using the MLPA technique. Achieving accuracy in copy number measurements poses particular challenges when attempting to characterize a tandem repeat region. The MLPA method shows utility in reproducibly distinguishing two copies of repeats from a single copy, which is not easily achievable using other standard methods. MPLA also overcomes the instability of the qPCR technique due to its high sensitivity to the amounts of template DNA, and thereby yields reproducible results[22]. Hence, MLPA is often used for the identification of deletion/duplication mutations in disease-causing genes or for determining the status of copy number variations at certain chromosomal loci in humans. In our current study, the use of MLPA allowed us to clearly determine the copy number of the integrated HHV-6B genome in the carriers and to characterize structural variations in the integrated viral genome among these CIHHV-6B cases. Our data indicate that human telomere repeats and the viral genomes had fused via one of the two TRSs within the DR-R in all six cases and

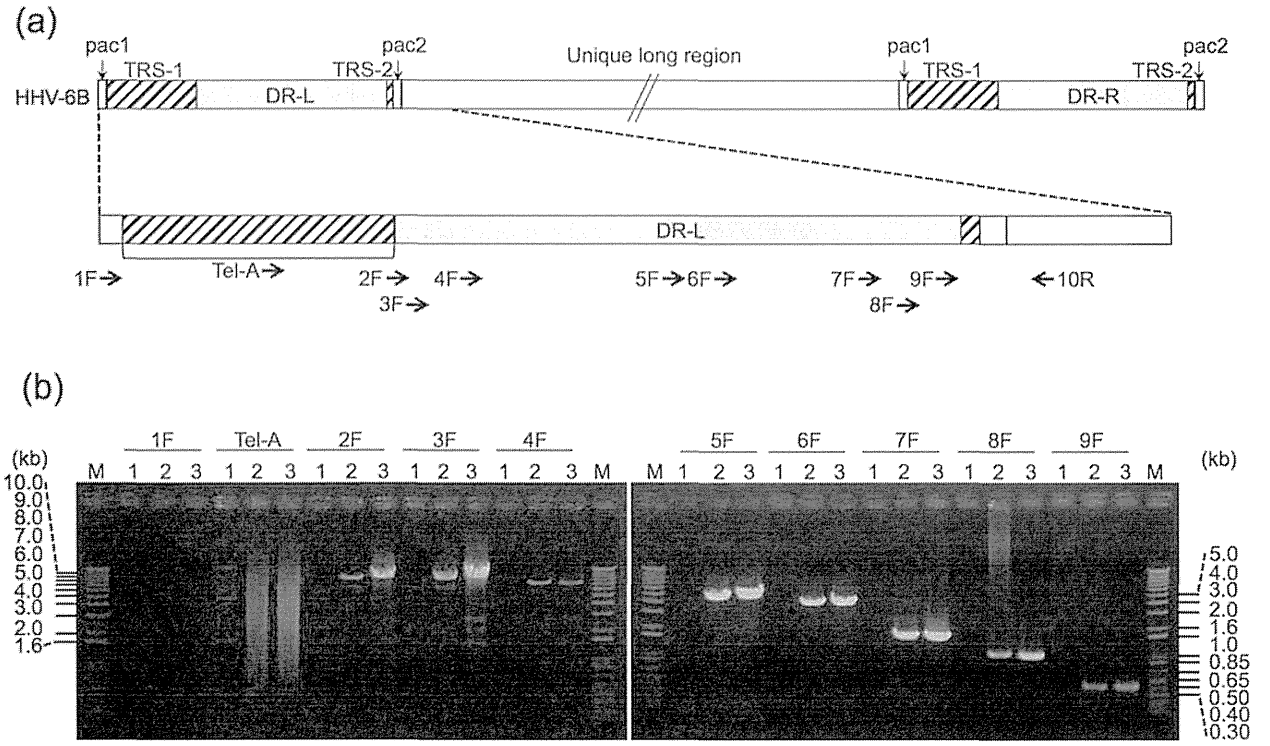

Figure 4 | Long range PCR analyses to determine the endpoints of the viral genomes. (a) Schematic representation of the HHV-6B genomic structure. The positions of the PCR primers are indicated below the diagram. (b) Results of long range PCR analyses. The 10R primer was used together with one of the DR primers for the long range PCR. The DR primers are indicated above the panels. Lane M, size markers; lane 1, non-CIHHV-6 case; lane 2, case 28; lane 3, case 18.

that the other end of the integrated HHV-6 virus is likely to be the distal TRS within the DR-L. Our findings are almost similar to those published previously[18,25]. These data implicate dual roles for the viral TRS sites in the manifestation of CIHHV-6.

The first important role of the viral TRS in the onset of CIHHV-6 is to trigger the homology-directed DNA repair mechanism for subsequent integration of the viral genome into the human genome. The ends of chromosomal DNA comprising the telomeres are generally structurally organized not to activate a DNA damage response by forming a stable T-loop DNA secondary structure and through the assembly of a shelterin complex[17]. Indeed, in normal cells, homologous recombination is repressed at the telomere[23]. However, the shortening of telomeres as a result of cell division might lead to a loss of chromosomal end protection and may cause improper DNA repair such as end-to-end fusions of the chromosomes by non-homologous end joining[24]. In our current study, the telomere repeats at the junction between the human subtelomere and the HHV-6 genome were found to be low in number, which has also been reported previously[18]. We thus speculate that telomere shortening, when it occurs in the cells infected with HHV-6, may activate a homology-directed DNA damage response that leads to viral integration.

A previous report has indicated that the viral breakpoints are located at TRS-2, both for CIHHV-6A and 6B[18,25]. The TRS-2 site has been shown to be longer and contain fewer degenerate TTAGGG repeats than TRS-1. However, our current analyses has revealed that the sizes of the TRS-1 region in our study subjects were much larger than TRS-2 and contained more perfect telomere repeats. If the free DNA end at the human telomere had activated the homology-directed DNA repair response pathway and searched for the appropriate template for DNA repair via homology, the integration event would have preferentially utilized TRS-1. However, we found that this was not the case. Thus, we speculate that the viral DNA end may be recognized as a bona fide DNA end by the host DNA repair system and thus subjected to end resection via homology-directed machinery. A small resection would be sufficient to reach the TRS-2 in the DR-R and enable the DNA end to find its homologous template, i.e. the human telomere. If the protection of the human telomere is incidentally removed at the time of this homology search due to age-related telomere shortening, the two DNA ends might be connected in a homology-dependent manner.

Once HHV-6 infection is established, the circularization of the linear double stranded viral DNA give rises to the formation of a stable episomal form in the nucleus whereby latency is achieved[26]. During HHV-6 replication, the episome produces a head-to-tail concatemer via a rolling circle mechanism, which is cleaved into a single unit of linear viral genome and is subsequently recircularized. A previous study has reported that some CIHHV individuals carry more than two copies of HHV[27]. However, in our current study we found only one copy of the viral genome in all of the CIHHV-6 cases in our cohort. In case 28, we identified tandem DRs repeat with no intervening pac1-pac2 region, which should be identified in a replicating concatemer or episomal virus. This suggests that a single linear form of replicated HHV-6 in latently infected cells gives rise to the integration event[10]. Normally, linear viral DNA ends are protected by episome formation, but it is possible that replication errors at the junction might result in an uncircularized virus genome, which may induce a DNA damage response. Although homologous recombination is suppressed at the telomere, a recent report has provided evidence of single strand annealing as a mechanism of telomere fusion[28].

The second pivotal role of the TRS regions in CIHHV-6 is the stabilization of the chromosomal end required for transmission of the virus. In our current analyses, we demonstrate that the end of the HHV-6B integrated chromosome was TRS-1, which others have also demonstrated using single telomere length analyses[25]. Once HHV-6 is integrated into the human telomere via homology with the TRS in the DR-R, the other end of the viral genome, DR-L, becomes a chromosomal end. Without the protection that normally operates

Table 1 | Characteristics of the Japanese study subjects with CIHHV-6B

Family	Case	Trigger for the diagnosis of CIHHV-6B	Chromosome
1	18	Monitoring after bone-marrow transplantation Aplastic anemia	22q
2	19	Differential diagnosis of mycoplasma Encephalitis	22q
	20	Mother of case19	22q
3	28	Monitoring after bone-marrow transplantation Congenital pure red cell aplasia	Xp
4	31	Differential diagnosis of febrile skin rash	6q
5	63	Uveitis	Xp

for chromosomal ends at the telomeres, the DR-L is subject to DNA end resection until the TRS-1 appears. Since the TRS-1 length is sufficient to recruit components of the shelterin complex, it can form a neo-telomere to maintain its size during some rounds of DNA replication and cell division until it forms gametes to transmit the viral genome to the offspring. We note that there is no reported case of chromosomally integrated HHV-7[29] and this might be due to a short TRS-1 (90 bp) in the genome of this virus which cannot maintain chromosomal end stabilization (U43400).

One of our current CIHHV-6B cases (case 31) was found to carry a different junction between the virus and human telomere, with a breakpoint in the TRS-1 region. One possibility for this occurrence is that a long DNA end resection took place that extended to the TRS1, which may be rare. Another possibility is a post-insertional genomic rearrangement[30]. Alternatively, non-allelic homologous recombination (NAHR) between the two TRS sites might have produced the deletion of DR-R in case 31, thereby mimicking the breakpoint at a different location for the initial integration. Another CIHHV-6B subject in our present analyses (case 28) was found to carry three DR copies. This is also possibly a product of an NAHR event between the two different TRS sites on the HHV-6 virus. In contrast to the normal repression of homologous recombination at the telomere, the subtelomeric region is known to be highly unstable and undergo frequent recombination[31]. The integration of HHV-6B in cases 28 and 63 in our present study are likely to have originated from a single ancestor since an identical genomic structure, three DR sites without a pac1-pac2 junction, was found in these two individuals. However, the repeat number at the TRS2-human telomere junction, as well as in the TRS2 site at the DR-L was found to be diverse between these two cases, suggesting that replication slippage or recombination may occur frequently at this new subtelomeric region.

A remaining question from our current observations is the timing of HHV-6 integration during the life cycle of a human host. Since CIHHV-6 appears to be transmitted from parent to child in a Mendelian fashion, the integration event must occur during germ cell development. HHV-6 uses CD46 as its receptor for entry into the cell[32]. Since CD46, a regulator of the complement activation receptor, is expressed ubiquitously, HHV-6 can theoretically infect all human cell types including those of a germ cell lineage. Since spermatogenic cells undergo a considerably greater number of DNA replication and cell division events than any other cells, there may be a higher chance of an integration event due to telomere dysfunction, although germ cells do possess telomerase activity[33]. Our current findings that some identified CIHHV-6 cases have similar molecular characteristics suggest that integration is a rare event. We propose from this that a small number of ancestral chromosomes that had undergone a HHV-6 integration have likely expanded throughout the general human population as a neutral polymorphism.

Methods

Human subjects. We analyzed six Japanese cases that were suspected to CIHHV-6B by having genome-equivalent copy number of viral DNA in peripheral blood samples estimated by qPCR[20]. The clinical features of the cases are listed in Table 1. After informed consent was obtained, peripheral blood samples were obtained again from each patient for our genomic analyses. Our study was approved by the Ethical Review Board for Human Genome Studies at Fujita Health University (Accession number 90, approved on 24 March 2010). All cases provided their written informed consent to participate in this study.

Fluorescent in situ hybridization (FISH). FISH was performed using a standard method. Briefly, PHA-stimulated lymphocytes or EB-transformed lymphoblasts were arrested by treatment with colcemid. Metaphase preparations were obtained by hypotonic treatment using 0.075 M KCl followed by methanol/acetate fixation. A 10 kb PstI fragment of HHV-6 was used as the probe (Fig. 1a)[3,12]. Probes were labeled by nick-translation with biotin-16-dUTP or digoxigenin-11-dUTP. After hybridization, the probes were detected using either Alexa Fluor® 488-conjugated streptavidin or rhodamine-conjugated anti-digoxigenin, respectively. Chromosomes were visualized by counter-staining with 4′,6-diamino-2-phenylindole (DAPI). As reference standards, we used RP11-186O8 (22q11.21), TelVysion 6p SpectrumGreen and TelVysion Xq/Yq SpectrumOrange (Abbott Molecular, IllinoisI, USA).

Multiplex ligation-dependent probe amplification (MLPA). MLPA probe pairs were designed using a standard methodology[22] so that they are strategically distributed throughout the viral genome, the central unique long (UL) region and two DRs, as well as pac1 and pac2. In this strategy, MLPA probes consist of two oligonucleotides, each containing a PCR primer sequence and a variable length sequence complementary to the target. Genomic DNA was denatured (1 min at 98°C) and subsequently hybridized to the MLPA probe pairs in accordance with the manufacturer's protocol (MRC-Holland, Amsterdam, Netherlands). After ligation, probe pairs were amplified using universal primers. The multiplex PCR products were then separated on a capillary sequencer. For normalization, we created a plasmid harboring a tandem array of probe sequences separated by 10-nucleotide spacer and used this construct as a standard for the copy number. We only had an EB-transformed cell line for case 63 and did not use this sample for MLPA because the presence of the EB virus genome may affect the results.

Analysis of junction fragments. Southern hybridization was performed using a standard methodology. Briefly, genomic DNA was cleaved with appropriate restriction enzymes, followed by size-separation via 0.8% agarose gel electrophoresis. After denaturation, the DNA was blotted onto a nylon membrane. Probes were labeled by crosslinking with alkaline-phosphatase and detected using CDP-Star detection reagent (GE Healthcare, Buckinghamshire, UK). To isolate a junction fragment, standard or long-range PCR was performed using LA Taq (TaKaRa, Siga, Japan). The amplification conditions were 35 cycles of 10 sec at 98°C, 30 sec at 63°C and 10 min at 72°C. A PCR primer was designed using the sequence data for the human chromosome X genomic contig (accession number NT_167191.1) and that of the HST strain of HHV-6B (AB021506). X-2R primer: 5′-TTGTCTCAGGGTCCTAGTG-3′. The PCR products were sequenced using the Sanger method.

Mapping of the distal breakpoints. To map distal breakpoints on the viral genomes, long-range PCR was performed using LA Taq. PCR primers were designed using the sequence data for the HST strain of HHV-6B (AB021506). The amplification conditions were 35 cycles of 10 sec at 98°C, 30 sec at 63°C and 10 min at 72°C. For amplification of larger fragments, PCR cycles were increased up to 38 cycles. To amplify telomere repeats, we performed the PCR using the Tel-A primer, 5′-CCCTAACCCTAACCCTAACCCTAACC-3′. To show the smear products really contain telomeric repeats Southern hybridization was performed with a telomere-specific probe[34].

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Author contributions

T.O., T.Y. and H.K. conceived and designed the experiments. T.O. performed all the experiments. I.H., M.I., Y.H., K.K., J.O., H.Y., T.N., Y.T., S.K. and T.Y., contributed regents and materials. T.O. and H.K. wrote the manuscript. All authors reviewed the manuscript.

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Copy Numbers of Telomeric Repeat Sequences of Human Herpesvirus 6B in Clinical Isolates: Possibility of Mixed Infections

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In order to determine whether mixed infections of human herpesvirus 6B (HHV-6B) occur in immunocompetent and immunocompromised individuals, we examined the copy numbers of telomeric repeat sequences (TRS) of clinical isolates. In clinical isolates obtained from patients with exanthem subitum caused by primary HHV-6B infection, PCR products with HHV-6B TRS ranging between 400 and 800 bp were amplified. PCR products of various sizes were amplified in four clinical isolates from drug-induced hypersensitivity syndrome (DIHS) patients and 15 isolates from hematopoietic stem cell transplant (HSCT) recipients with HHV-6B reactivation. Based on the sequence analysis of the PCR products, the copy numbers of TRS in DIHS and HSCT patients were between 42 and 82 and 22 and >90, respectively. For two of the HSCT recipients, HHV-6B TRS PCR products of different sizes were detected in several isolates from each patient, which suggests mixed HHV-6B infections. In two of the post-transplant HHV-6B encephalitis patients, the sizes of the TRS nested PCR products amplified from the reactivated virus detected in the central nervous system differed from those of the virus detected in initial isolates from peripheral blood mononuclear cells. Taken together, these results suggest that PCR analysis of TRS copy number is a reliable tool for the discrimination of HHV-6B clinical isolates. Additionally, mixed HHV-6B infections occurred in HSCT recipients, and in some cases, compartmentalization of the HHV-6B strains to the central nervous system versus the blood compartment occurred in posttransplant HHV-6B encephalitis patients.

Primary human herpesvirus 6B (HHV-6B) infection presenting as exanthem subitum (ES) (1, 2) is considered a benign febrile illness and rarely causes neurological complications, such as febrile convulsion and encephalitis (3, 4). In addition to the primary infection, HHV-6 reactivation may be associated with acute graft-versus-host disease (5–8), graft rejection (9), and encephalitis (10–13) in transplant recipients. Moreover, the virus can be reactivated in patients with drug-induced hypersensitivity syndrome (DIHS), which is a severe form of drug allergy that is characterized by fever, skin rash, lymphadenopathy, hepatitis, and leukocytosis (14–16). Active HHV-6B infection generally occurs only once throughout life (at the time of the primary infection) in immunocompetent individuals, but it may occur several times in transplant recipients (8) or DIHS patients (17). Frequent HHV-6B reactivation, as evidenced by the repeated isolation of the virus during an active viral infection, was observed in hematopoietic stem cell transplant (HSCT) recipients (8, 18).

Previous reports detected mixed infections with multiple cytomegalovirus (CMV) strains in the same human herpesvirus subfamily (*Betaherpesvirinae* subfamily) as HHV-6B in a variety of patient populations, including immunocompetent and immunocompromised patients (19–22). Furthermore, other studies have suggested that the genotype of the virus may be associated with the severity of the congenital cytomegalovirus infection (23). Although molecular epidemiological analysis of HHV-6 glycoprotein genes has been carried out, no specific genotype has been correlated with the pathogenicity of the virus (24). It remains unclear whether mixed HHV-6B infections occur in either immunocompetent or immunocompromised hosts.

The numbers of copies of telomeric repeat sequences (TRS) that are located in direct repeats of the HHV-6 genome are highly variable among laboratory strains and clinical specimens (25). In order to determine whether mixed infections of HHV-6B occur in immunocompetent and immunocompromised individuals, we examined the TRS copy numbers of clinical isolates obtained from ES and DIHS patients and HSCT recipients by using PCR and direct sequencing of the PCR products. Furthermore, in order to determine whether the virus was compartmentalized within the host, the TRS copy numbers of viral DNA that were detected in the peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid samples from two patients with posttransplant HHV-6B encephalitis were compared.

MATERIALS AND METHODS

Patients and samples. Twenty-nine patients were included in this study: 10 ES patients (6 to 23 months; median age, 11.9 months), 4 DIHS patients (11 to 75 years; median age, 53.7 years), and 15 HSCT recipients (2 to 62 years; median age, 30.6 years). One clinical isolate was obtained from each of the ES and DIHS patients. For the HSCT recipients, the numbers of clinical isolates obtained varied; there were 3 recipients with one clinical isolate, 7 recipients with two clinical isolates, 3 recipients with three isolates, 1 recipient with four clinical isolates, and 1 recipient with five isolates. A total of 49 isolates were analyzed retrospectively in this study. Furthermore, PBMCs and cerebrospinal fluid samples were obtained from the two posttransplant HHV-6B encephalitis patients (cases 14 and

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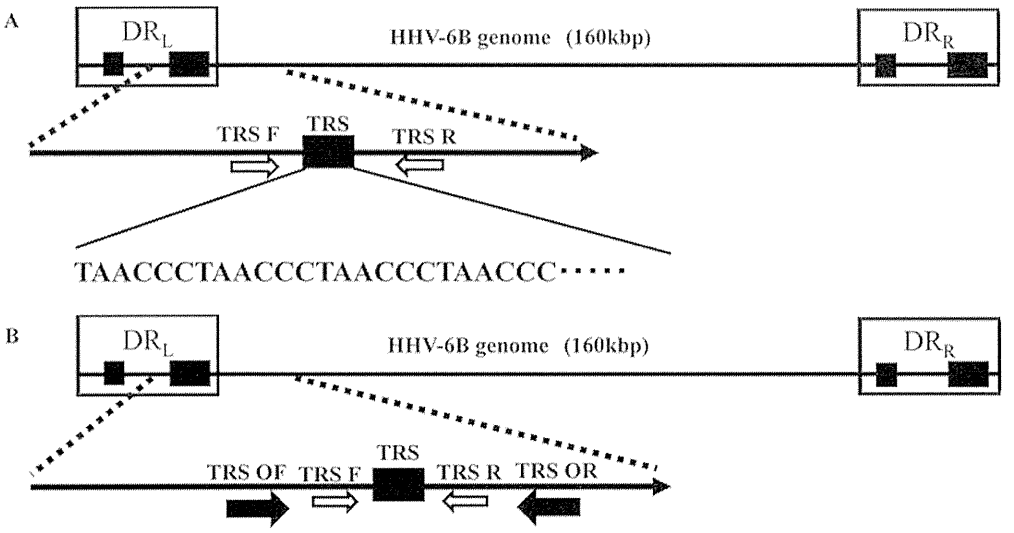

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Primer name	sequence	Location*
H6 TRS F	5'-CTCGGACCCATGCTATCCT-3'	8198- 8216
H6 TRS R	5'-CATATACCCTCGCCCGTTC-3'	8875- 8893
H6 TRS OF	5'-GGGGCAGATGTGAAGACAAT-3'	8146- 8165
H6 TRS OR	5'-AGTTTAAAGGCGAAGGTGCA-3'	8908- 8927

*Accession numbers: AF157706

FIG 1 HHV-6B genomic structure and the DNA sequence of the terminal repeat sequences (TRS) in the direct repeat region of the HHV-6B genome. The primer binding sites for PCR (TRS F and TRS R) (A) and nested PCR (TRS OF and TRS OR) (B) are shown. (C) Primer sequences for the PCR and nested PCR. DR$_L$, direct repeat left; DR$_R$, direct repeat right.

A). Patients or parents of the patients provided consent for participation in this study. This study was approved by the review boards of Fujita Health University.

HHV-6B isolation and identification were performed as previously described (1). In brief, PBMCs were cocultured with cord blood mononuclear cells that were infected using the clinical isolates. Infected cultures were identified on the basis of morphological changes in the cultured cells (i.e., characteristics of pleomorphic, balloon-like large cells). The presence of virus was confirmed by immunofluorescence staining of the cocultures with a specific HHV-6B monoclonal antibody (OHV-3; provided by T. Okuno, Department of Microbiology, Hyogo College of Medicine, Hyogo, Japan). Cocultivated cord blood mononuclear cells infected with the clinical isolates were stored after several passages at −80°C until assayed.

DNA extraction. Viral DNAs were extracted from the stored cord blood mononuclear cells and HHV-6B (strain Z29)-infected cord blood mononuclear cells using a QIAamp DNA blood minikit (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. Viral DNAs were also extracted from the stored PBMCs and cell-free cerebrospinal fluid samples that were obtained from the two posttransplant HHV-6B encephalitis patients using the same procedure. Extracted DNAs were eluted in 100 μl buffer and stored at −20°C until PCR analysis.

PCR assay. The TRS of HHV-6B were amplified using primers specific for the DNA sequences in the Z29 direct repeat region (Fig. 1A and B). The sequences of the forward primer (H6 TRS F) (5′-CTCGGACCCATGCT ATCCT-3′) and the reverse primer (H6 TRS R_) (5′-CATATACCCTCG CCCGTTC-3′) are shown in Fig. 1C. The binding sites for H6 TRS F and H6 TRS R were at bp 8,198 to 8,216 and bp 8,875 to 8,893, respectively (GenBank accession number AF157706) (Fig. 1A and B). TRS from the small amounts of viral DNA in PBMCs and cerebrospinal fluid samples were amplified using a nested PCR and forward (H6 TRS OF) (5′-GGGG CAGATGTGAAGACAAT-3′) and reverse (H6 TRS OR) (5′-AGTTTAA AGGCGAAGGTGCA-3′) primers (Fig. 1C). The binding sites for H6 TRS OF and H6 TRS OR were at bp 8,146 to 8,165 and bp 8,908 to 8,927, respectively (GenBank accession number AF157706) (Fig. 1B). LA Taq (TaKaRa Bio Inc., Otsu, Japan) and the following conditions were used for the PCRs: denaturation at 94°C for 1 min, followed by 30 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min. The sizes of the amplified products were analyzed by electrophoresis on a 1% agarose gel with ethidium bromide staining.

Sequence analysis. Direct sequencing of the purified PCR products amplified from the isolates and clinical specimens, including PBMCs and cerebrospinal fluid samples, was carried out using a BigDye Terminator cycle sequencing kit and a Prism 3100 Avant analyzer (Applied Biosystems, Foster City, CA). The PCR products were purified using a PCR purification kit (Qiagen) and then sequenced. The purified PCR products were sequenced using H6 TRS F (5′-CTCGGACCCATGCTATCCT-3′) according to the manufacturer's instructions. The viral sequences were compared using the ClustalW computer program (DNA Data Bank of Japan).

Statistical analysis. TRS copy numbers were compared among the three different patient populations (ES, DIHS, and HSCT) using the Kruskal-Wallis test. The statistical analysis was performed with JMP 7 (SAS Institute Inc., Cary, NC).

RESULTS

Stability of TRS copy numbers after numerous passages. In the initial validation analysis, we examined the stability of TRS copy numbers after 17 to 22 passages of cord blood mononuclear cells infected with HHV-6B strain Z29 and the three clinical isolates (HHV-6B) obtained from the ES and HSCT patients (see Fig. S1 in

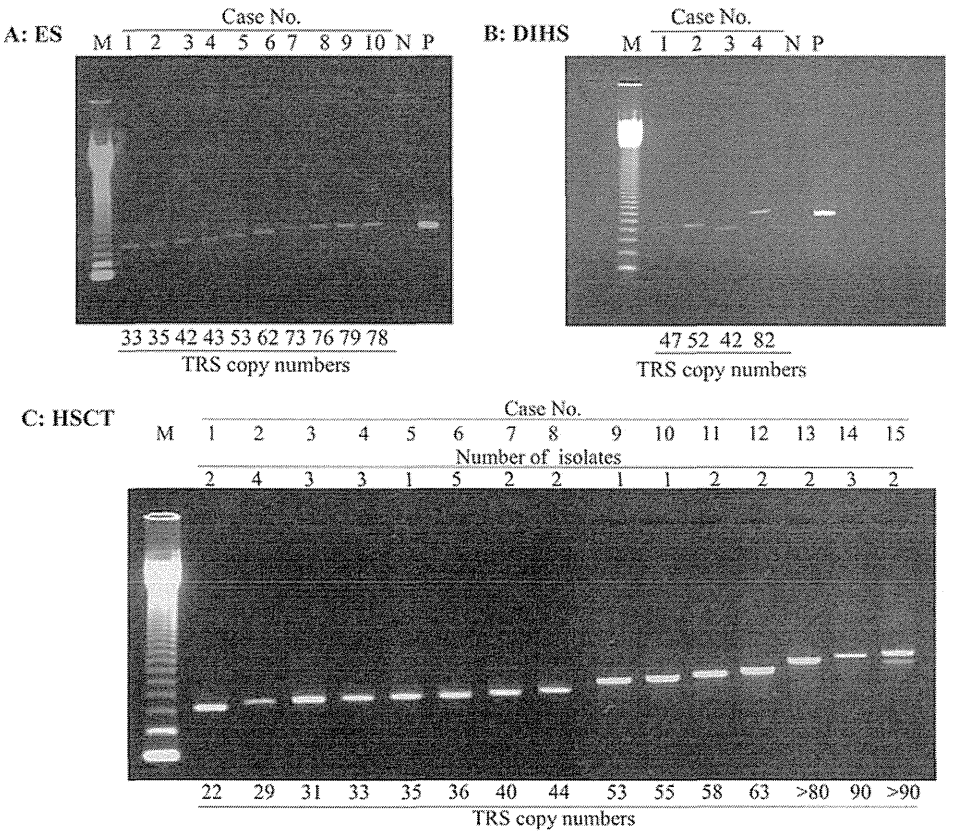

FIG 2 Determination of sizes and TRS copy numbers of PCR products amplified from clinical isolates. HHV-6B isolates were obtained from exanthem subitum (ES) patients ($n = 10$) (A), drug-induced hypersensitivity syndrome (DIHS) patients ($n = 4$) (B), and hematopoietic stem cell transplant (HSCT) recipients ($n = 15$) (C). TRS copy numbers of the PCR products were determined by DNA sequence analysis. Number of isolates (above the gel) indicates the number of HHV-6B isolates from each patient. M, marker; N, negative control; P, positive control.

the supplemental material). PCR products of different sizes were detected in the clinical isolates. It is important to note that the number of passages did not alter the sizes of the PCR products.

TRS copy numbers in isolates from three different types of patients. In clinical isolates obtained from the ES patients, HHV-6B TRS PCR products ranged in size between approximately 400 and 800 bp (Fig. 2A). Sequence analysis of the PCR products determined TRS copy numbers ranging between 33 and 79 copies. Additionally, the PCR products detected in the four clinical isolates from the DIHS patients (Fig. 2B) and the 15 HSCT recipients (Fig. 2C) were approximately 500 to 860 bp and 360 to 980 bp, respectively. Based on the sequence analyses of these PCR products, the TRS copy numbers of the clinical isolates obtained from the DIHS and HSCT patients ranged between 42 and 82 copies and 22 and >90 copies, respectively. Although TRS copy numbers were compared among the three different patient populations, no statistical difference was demonstrated in the copy numbers (ES: median, 57.5, and interquartile range [IQR], 42.3 to 75.3; DIHS: median, 49.5, and IQR, 45.8 to 59.5; HSCT: median, 44, and IQR, 34 to 60.5; $P = 0.669$) (see Fig. S2 in the supplemental material).

Isolates from HSCT recipients have different TRS copy numbers. HHV-6 TRS PCR products of different sizes were observed among several isolates from each HSCT recipient (cases 6 and 12) after transplant (Fig. 3A and B). In case 6, the TRS copy number in the two initial isolates was 36 and that in the 3rd isolate was 60. Further, the copy number of one of the two TRS PCR products obtained from the 4th isolate was 36, and that of another large-sized TRS PCR product was undetermined. Additionally, a larger faint band was also demonstrated in the PCR product obtained from the 1st isolate. The copy number of the final 5th isolate was 36. A minor change in TRS copy numbers (62 and 63) that was not

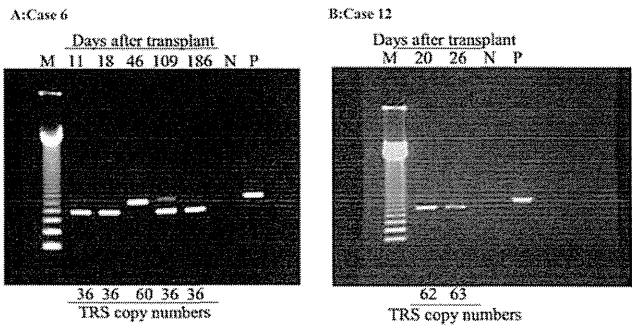

FIG 3 PCR products detected in the clinical isolates obtained from the two hematopoietic stem cell transplant recipients. TRS copy numbers were determined by sequence analysis. M, marker; N, negative control; P, positive control.

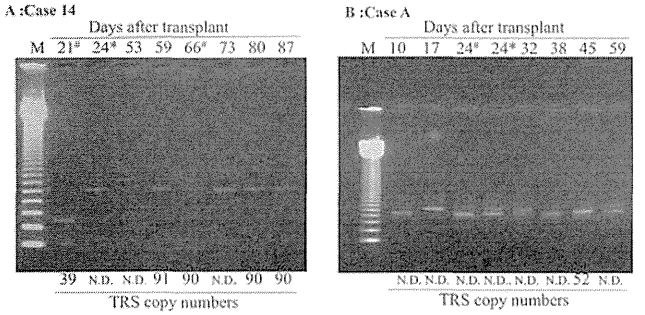

FIG 4 Comparison of TRS copy numbers in the CNS and peripheral blood compartment of the two posttransplant HHV-6B encephalitis patients (A, case 14; B, case A). #, HHV-6B isolates recovered from peripheral blood mononuclear cells; *, cerebrospinal fluid (CSF) samples containing HHV-6B DNA. Other samples were stored with peripheral blood mononuclear cells latently infected with HHV-6B. PCR products were amplified by a nested PCR. N.D., not determined.

differentiated on the basis of agarose gel electrophoresis analysis was discovered in case 12 by the sequence analysis.

TRS copy numbers in isolates from the peripheral blood and cerebrospinal fluid. The two posttransplant HHV-6B encephalitis patients (cases 14 and A) were analyzed to determine whether reactivated HHV-6B in the central nervous system was different from the virus detected in PBMCs. Figure 4A shows that in case 14, the sizes of the TRS nested PCR products that were amplified from cerebrospinal fluid samples (TRS copy numbers were not determined) were larger (approximately 600 bp) than that of the initial PCR product amplified from stored PBMC samples at the time of viremia (approximately 300 bp). The TRS copy number of the PCR product amplified from the PBMCs was 39 copies in case 14. In the remaining specimens from this patient, the resulting TRS nested PCR products were larger in size and the TRS copy number was 90 or 91. In case A, the sizes of the nested PCR products (approximately 500 bp) detected were similar in the PBMCs and cerebrospinal fluid samples collected at the same time (24 days after transplant); however, precise copy numbers of the PCR products were not determined. Additionally, the sizes of the nested PCR products amplified from the PBMCs collected on posttransplant days 17 and 32 were clearly larger.

DISCUSSION

Several passages of cultured cells are required to isolate HHV-6B from patient PBMC samples. Moreover, an additional 2 to 3 passages are necessary to produce sufficient amounts of infected cells for generating stocks of each clinical isolate. In order to use this PCR method for the differentiation between HHV-6B strains in clinical isolates, the stability of TRS copy numbers after several passages had to be confirmed. The initial validation analysis demonstrated that TRS copy numbers were stable after at least 17 passages of cultured cells (see Fig. S1 in the supplemental material), indicating that the passaged virus isolates were appropriate for analysis using the PCR method.

Measurement of viral DNA loads in peripheral blood samples using real-time PCR remains a popular method for monitoring active viral infection in transplant recipients (26). In addition to real-time PCR monitoring, viral isolation has been routinely carried out at our institute to demonstrate active HHV-6B infection in ES patients (27), transplant recipients (8), and DIHS patients (17). In contrast to the previous study (25), which used DNA extracted from PBMCs to identify latent HHV-6B infection, DNA extracted from clinical isolates was used in this study. Based on our findings, DNA extracted from clinical isolates was appropriate for determining whether mixed active HHV-6B infections (not latent infections) occurred in immunocompromised patients. Moreover, the clinical isolates obtained from ES patients contained PCR products that differed in length and in TRS copy numbers. Therefore, these data suggest that measurement of TRS copy number is a reliable tool for differentiation of HHV-6B strains in clinical isolates. Moreover, as no TRS copy number differences were observed among the three different patient populations (see Fig. S2 in the supplemental material), we considered TRS copy numbers to not be associated with primary HHV-6B infection and viral reactivation.

Although equivalent-sized PCR products were detected in most of the clinical isolates obtained from the HSCT recipients, which was suggestive of a common HHV-6B strain being repeatedly reactivated, in two HSCT recipients (cases 6 and 12), PCR products of different sizes were detected. Furthermore, TRS copy numbers were also different among the samples. Therefore, mixed HHV-6B infections were detected in two HSCT recipients, which supports the earlier molecular epidemiological study that was based on restriction fragment polymorphism analysis of the HHV-6B genome (28). To the best of our knowledge, this is the first study to demonstrate mixed HHV-6B infections in transplant recipients based on TRS analysis. Additionally, as two PCR products of differing sizes were demonstrated in clinical isolates 1 and 4, these isolates likely contain at least two distinct populations of HHV-6B. Further analysis, such as deep sequencing (29) or subcloning of the PCR product amplified from PBMCs obtained from ES patients, is required to confirm mixed populations of the virus in these isolates. Previous studies determined that mixed infections of CMV occurred in 15 to 50% of transplant recipients based on molecular epidemiological analysis of glycoprotein genes (30–35). Moreover, mixed viral infections have been linked to high viral loads (31, 32), delayed viral clearance (31), and higher rates of viral recurrence (31). Additional studies are needed to elucidate the significance of mixed HHV-6B infections in transplant recipients.

In a previous study, small amounts of HHV-6B DNA were detected in the cerebrospinal fluid samples of posttransplant HHV-6B encephalitis patients; however, HHV-6B was not isolated from these samples (36). It was unclear whether the same HHV-6B isolates were reactivated in both the central nervous system and systemically in patients with posttransplant HHV-6B encephalitis. We developed a highly sensitive nested PCR to amplify the TRS region in order to evaluate the TRS copy numbers in cerebrospinal fluid and PBMC samples. In case 14, the size of the PCR product amplified from the cerebrospinal fluid sample was distinct from that of the HHV-6B isolate that was recovered prior to the onset of neurological symptoms. On the other hand, PCR products similar in size to that amplified from the cerebrospinal fluid sample were detected in the PBMC samples obtained after onset of the illness in this patient (Fig. 4A). Meanwhile, in case A, we detected similar-sized PCR products in the cerebrospinal fluid samples and PBMCs that were collected simultaneously (24 days posttransplant). Subsequently, PCR products of differing sizes were also detected during the observation period for this patient (Fig. 4B); however, TRS copy numbers were not determined in

some of the samples due to the small amounts of amplified DNA. Taken together, these results suggest that not only the initially isolated virus but also additional strains may have been reactivated simultaneously, both systemically and in the central nervous system, in the posttransplant HHV-6B encephalitis patients. Similar findings have been reported in CMV infections in lung transplant recipients (37) and AIDS patients (38, 39). While this study analyzed only two posttransplant HHV-6B encephalitis patients, it clearly highlights the need to analyze a large number of cases in order to fully elucidate the spectrum of compartmentalization of HHV-6B strains in the central nervous system and peripheral blood compartment.

Herein, the utility of PCR for the analysis of TRS copy numbers to distinguish between HHV-6B strains in clinical isolates was demonstrated. Similar to cases of CMV infection, mixed infections of HHV-6B were found in HSCT recipients. Furthermore, compartmentalization of HHV-6B strains in the central nervous system and blood compartment was detected in two posttransplant HHV-6B encephalitis patients. We are currently conducting studies to elucidate the pathogenic role of mixed HHV-6B infections in transplant recipients.

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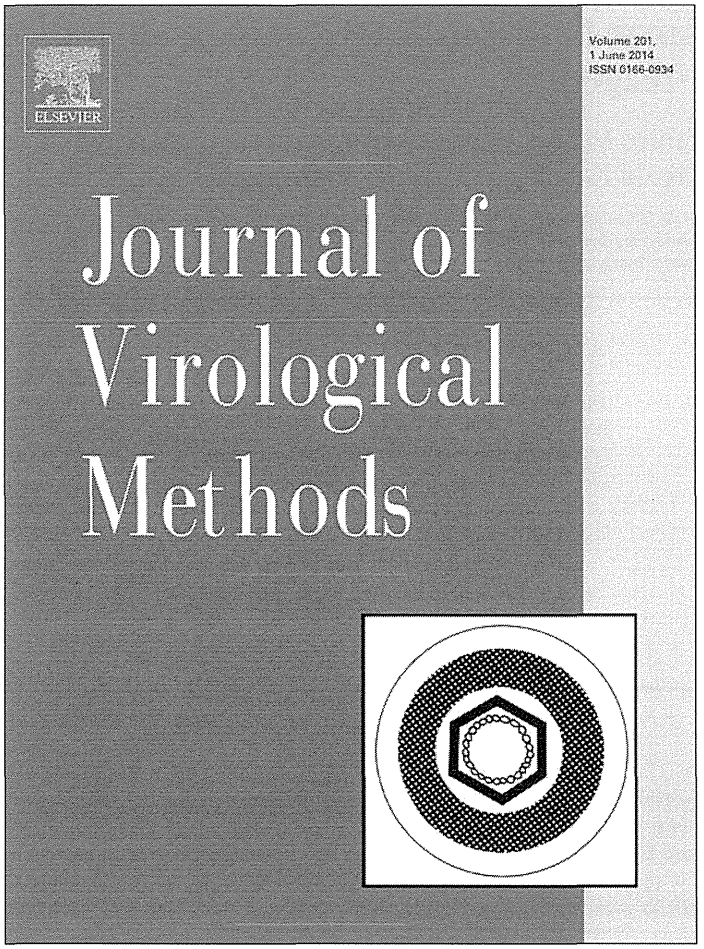

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Short communication

Direct detection of human herpesvirus 6B by the LAMP method using newly developed dry-reagents☆

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ABSTRACT

The reliability of the HHV-6B LAMP using the dry-reagent method was evaluated using serum samples obtained from febrile children. The sensitivity of the original and dry-reagent methods was 10 copies/reaction and 100 copies/reaction, respectively. The dry-reagent LAMP method was highly sensitive (94.0%) and specific (96.0%) for the detection of HHV-6B.

Human herpesvirus-6 (HHV-6) is classified into HHV-6A and HHV-6B species. HHV-6B is an ubiquitous virus and an etiological agent for exanthem subitum (Yamanishi et al., 1988). Primary HHV-6B infection is generally characterized by benign febrile illness in young children, but in some cases it may cause central nervous system complications, including febrile seizures and encephalitis (Suga et al., 1993; Nagasawa et al., 2007; Kawamura et al., 2011). Previous studies demonstrated that almost half of HHV-6B encephalitis patients had severe neurological sequelae (Yoshikawa et al., 2009). A new option for improving patient prognosis may be treatment with ganciclovir and foscarnet, which have an antiviral effect against HHV-6B (Agut et al., 1989; Burns and Sandford, 1990). Ganciclovir and foscarnet have side effects and should only be administered to patients with confirmed HHV-6B infection. Thus, an accurate method for the diagnosis of HHV-6B infection is required to restrict the use of these drugs. Additionally, because large numbers of patients with febrile seizure caused by primary HHV-6B infection visit emergency rooms (Hall et al., 1994), a rapid diagnostic test would be a valuable tool.

Direct detection of viral DNA in serum samples, without DNA extraction, using the loop-mediated isothermal amplification (LAMP) method is a valuable tool for the rapid diagnosis of primary HHV-6 infection (Ihira et al., 2004, 2007). However, the liquid reagent used in the original LAMP assay has to be stored at −20 °C, which requires a thawing step prior to beginning the assay. Recently, a dry-reagent that could be stored at 4 °C was developed to eliminate the thawing step. The aim of this study was to evaluate the reliability of the LAMP method using the dry-reagent for the rapid diagnosis of primary HHV-6B infection.

Previously designed primers against the HHV-6 B U31 gene (Ihira et al., 2004, 2007) (H6U31BIP, H6U31FIP, H6U31B3, H6U31F3, H6U31LPB, and H6U31LPF) were used to evaluate both the original and dry-reagent HHV-6B LAMP methods. The primers were diluted and stored at 4 °C in buffer (200 mM Tris pH 8.8, 1 mM DTT, 2 ng/uL ssDNA, 0.05% (w/v) NaN3). The primer solution was stable for one year under the condition (stored at 4 °C) (data not shown). In the original LAMP method, the LAMP reaction was conducted using the Loopamp DNA amplification kit (Eiken Chemical, Tokyo, Japan) as previously described (Ihira et al., 2004). In brief, patient serum

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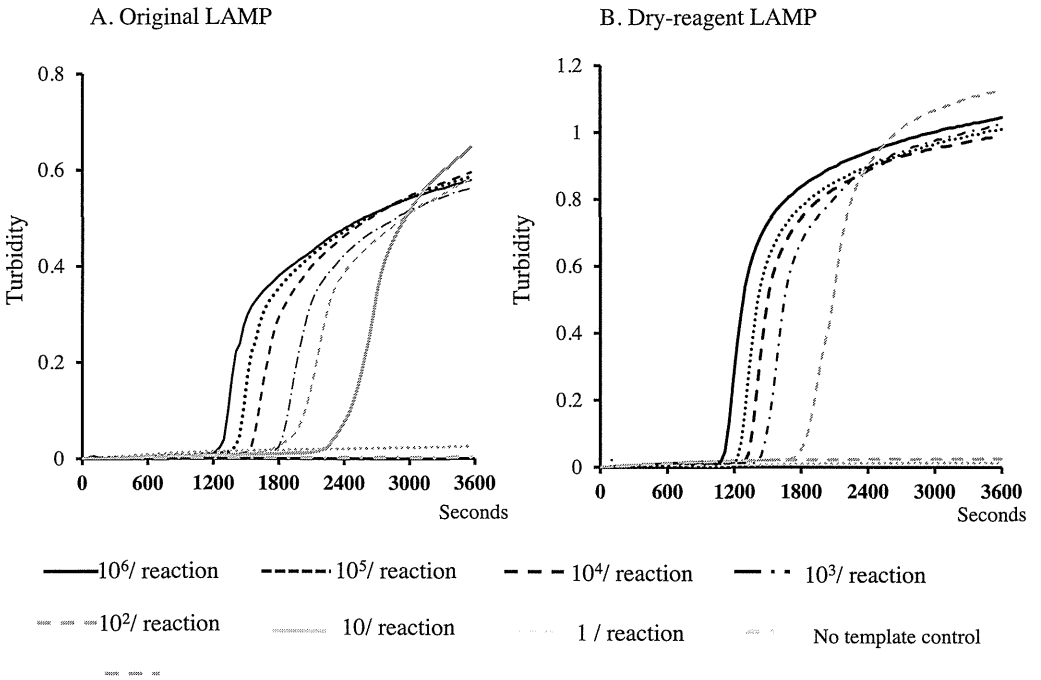

Fig. 1. The sensitivity of the original LAMP method (A) and dry-reagent LAMP method (B) were determined based on serial dilutions of the target plasmid (pGEMH6 S12) into HHV-6B negative serum samples. The lower detection limit of the original LAMP method and the dry-reagent LAMP method was 10 copies/reaction and 100 copies/reaction, respectively.

(5 μl) was added to 20 μl of the primer solution and the mixture was heat denatured at 96 °C for 3 min. *Bst* DNA polymerase (1 μl) was added to the mixture and incubated at 63 °C for 60 min. Meanwhile, for the dry-reagent LAMP method, patient serum (2 μl) was mixed with 13 μl of sterile water and 15 μl of primer solution, and then the mixture was incubated at 96 °C for 3 min. Although 5 μl serum was directly used in the initial experiment, dilution of the serum was necessary to increase the assay sensitivity (data not shown) probably due to some inhibitory effect. The mixture was transferred to a tube that was coated with the dry-reagent on inside of the lid (Loopamp "Fungi *Candida albicans*" Detection Kit, Eiken Chemical, Tokyo, Japan), and the tube was inverted five times. The LAMP reaction process and the detection of the LAMP products were the same as the original method.

The lower detection limit was determined using serial dilutions of the pGEMH6 S12 plasmid. After the initial validation analysis to determine the assay sensitivity, a total of 100 serum samples obtained from febrile children (58 male and 42 female; age, 10 ± 6.7 months) were used to evaluate reliability of the dry-reagent method in the rapid diagnosis of primary HHV-6B infection. These samples were stored at −70 °C until assayed. Direct detection of HHV-6B DNA in serum was tested using the original and the dry-reagent methods to evaluate the reliability of the dry-reagent LAMP method. Isolation of HHV-6B from patient's peripheral blood mononuclear cells was defined as the gold standard for active viral infection. Detail of the viral isolation was described in elsewhere (Yamanishi et al., 1988). HHV-6B was isolated from 50/100 (50%) patients samples.

In the initial validation analysis, the lower detection limit of the original LAMP method and the dry-reagent method were 10 copies/reaction and 100 copies/reaction, respectively (Fig. 1). The results of the retrospective clinical sample analysis are summarized in Table 1. Using the original LAMP method, HHV-6B DNA was detected in 48/50 (96%) serum samples with HHV-6B viremia and in 2/50 (4%) serum samples without viremia. Compared to the gold standard for the detection of active viral infection, the sensitivity, specificity, positive predictive value, and negative predictive value of the original LAMP method were 96.0%, 98.0%, 98.0%, and 96.1%, respectively. Using the dry-reagent method, HHV-6B DNA was detected in 47/50 (94%) serum samples with HHV-6B viremia and in 2/50 (4%) serum samples without viremia. Thus, sensitivity, specificity, positive predictive value, and negative predictive value of the dry-reagent LAMP method were 94.0%, 96.0%, 95.9%, and 94.1%, respectively. Although the sensitivity of the dry-reagent LAMP method was marginally lower than that of the original LAMP method, the sensitivity and specificity of the dry-reagent method was sufficient for the diagnosis of primary HHV-6B infection (Ihira et al., 2004, 2007).

No HHV-6B DNA was detected in one of the two false negative samples obtained using the original LAMP method and in one of the three false negative samples obtained using the dry-reagent method based on the real-time PCR analysis, which suggest that these samples might contain the small amounts of viral DNA below the detection limits of the methods. Meanwhile, remaining

Table 1
Results of original and dry-reagent HHV-6B LAMP methods (A) and sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the methods for detection of active HHV-6B infection (B).

HHV-6B viremia	Original LAMP		Dry-reagent LAMP	
	Positive	Negative	Positive	Negative
(*A*)				
Positive	48	2	47	3
Negative	1	49	2	48

	Original LAMP	Dry-reagent LAMP
(*B*)		
Sensitivity	96.0%	94.0%
Specificity	98.0%	96.0%
PPV	98.0%	95.9%
NPV	96.1%	94.1%

HHV-6B viremia was defined as the standard for active HHV-6B infection.

false negative samples contained certain copies of viral DNA (344, 461, and 530 copies/reaction). Additionally, in one of the three false negative samples that was obtained using the dry-reagent LAMP method, HHV-6B DNA was detected in the sample after DNA extraction from the serum. This sample may have contained an inhibitor of the LAMP reaction. Small amounts of HHV-6B DNA (277 and 33 copies/reaction) were detected in one false positive sample obtained using the original LAMP method and one of the two false positive samples obtained using the dry-reagent LAMP method by using the real-time PCR method. Furthermore, a significant increase in HHV-6B IgG antibody titers was demonstrated in one false positive sample suggesting an active viral infection. Although the serological analysis was not used for determining active viral infection in this study, it is considered that the serological assay in combination with viral isolation is required for precise evaluation of the reliability of the new diagnostic method.

Although this method is widely utilized in a variety of molecular diagnostic methods (Enomoto et al., 2005; Okafuji et al., 2005; Mori et al., 2006; Higashimoto et al., 2008; Nakauchi et al., 2011), to the best of our knowledge this is the first study that evaluated the reliability of the dry-reagent for the LAMP reaction to detect viral infection. The hands-on time per specimen was reduced by approximately 10 min by eliminating the thawing step. Additionally, the reduction of the handling steps for the preparation of the reaction mixture is also major advantage for rapid diagnostic methods. Furthermore, the LAMP method is a useful molecular diagnostic method for infectious diseases in developing countries (George et al., 2011; Nagdev et al., 2011; Ablordey et al., 2012). Because the dry-reagent method does not require the reagents to be stored at −20 °C, this method would be an advantageous tool in such situation. The development of the LAMP assay using the dry-reagent for the diagnosis of tropical infectious diseases is a major issue for the future studies.

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Low total IgM values and high cytomegalovirus loads in the blood of newborns with symptomatic congenital cytomegalovirus infection

Abstract

Aims: Neurological outcomes differ considerably between symptomatic and asymptomatic infants with congenital cytomegalovirus (CMV) infection. Our objective was to characterize laboratory markers in symptomatic newborns in comparison with asymptomatic newborns with congenital CMV infection.

Methods: Ten newborns with symptomatic and 13 newborns with asymptomatic congenital CMV infection were included in this 3-year prospective cohort study. Total immunoglobulin M (IgM), CMV-IgM, CMV antigenemia, and CMV-DNA in blood and urine were measured and their positive rates and quantitative values compared between the symptomatic and asymptomatic groups.

Results: Fifty percent of newborns in the symptomatic group were positive based on total IgM; this was significantly lower than in the asymptomatic group (100%). Quantitative total IgM values were significantly lower, and there were significantly more copies of CMV-DNA in the blood of symptomatic newborns than in asymptomatic newborns (median values for total IgM: 14 vs. 43 mg/dL and blood CMV-DNA: 3.2×10^2 vs. 3.5×10^1 copies/10^6 white blood cells). CMV-IgM, CMV antigenemia, and urine CMV-DNA did not differ significantly between groups.

Conclusion: Low total IgM values and high blood CMV loads were associated with the presence of symptoms in newborns with congenital CMV infection.

Keywords: Antigenemia; immunoglobulin; laboratory markers; serology; viral load.

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Introduction

Cytomegalovirus (CMV) is the main cause of congenital infection in developed countries [12]. In Japan, 0.31% of live newborns are infected with CMV [10]. Approximately 10%–15% of newborns with congenital CMV infection have clinical symptoms at birth, whereas the remaining 85%–90% of infected newborns are asymptomatic [12]. Approximately 85%–90% and 10%–15% of the symptomatic and asymptomatic newborns, respectively, develop neurological sequelae including sensorineural hearing loss and developmental disabilities [12]. Thus, neurological outcomes differ substantially between symptomatic and asymptomatic newborns with congenital CMV infection.

Newborn total immunoglobulin M (IgM) is the gold-standard diagnostic marker of mother-to-child infection. Additional laboratory markers of CMV infection in blood and urine include CMV-IgM, CMV antigenemia, and CMV-DNA copy number [4, 5, 7, 26]. However, few studies

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have assessed these markers concurrently in newborns with congenital CMV infection.

Our objectives were to assess the positive rates and quantitative values for these markers in symptomatic and asymptomatic newborns with congenital CMV infection and to find laboratory markers that characterize symptomatic newborns.

Methods

Study design

This study was conducted under the approval of the Ethical Committee of Kobe University Graduate School of Medicine with informed consent of the parents of the patients. In this 3-year prospective cohort study, 4364 newborns received routine urine screening for CMV-DNA from November 2009 to March 2013 at Kobe University Hospital and its affiliated hospitals. Twenty-three Japanese newborns with congenital CMV infection whose urine samples were positive for CMV-DNA within 1 week after birth [10, 23] were enrolled in the study. All had undergone extensive medical examination during their neonatal period at Kobe University Hospital and were classified as having symptomatic (n=10) or asymptomatic (n=13) infections. Total blood IgM, CMV-IgM, CMV antigenemia, and CMV-DNA in blood and urine were measured and then, 1) positive rates for these markers were calculated in all patients, 2) positive rates and quantitative values were compared between the symptomatic and asymptomatic groups, 3) values associated with the symptoms were scrutinized using receiver operating characteristic curve (ROC) analyses, and 4) the correlations between the values of each laboratory marker and the gestational ages of the newborns were assessed.

Definition of symptomatic congenital CMV infection

Newborns were categorized as having symptomatic infection if at least one of the following clinical manifestations was evident at their initial medical examination: small for gestational age (SGA), hepatosplenomegaly/hepatitis, thrombocytopenia, brain abnormality, chorioretinitis, or an abnormal auditory brainstem response (ABR). SGA was defined as a birth weight (BW) ≤1.5 standard deviations from the mean BW of Japanese newborns of the same gestational age. Hepatosplenomegaly was confirmed by ultrasound examination and/or abdominal X-ray. Hepatitis was defined as a serum alanine aminotransferase level >100 U/L, and thrombocytopenia as a platelet count $<1\times10^5/\mu L$ [9, 14, 19]. Brain abnormality was defined as intracranial calcifications, ventricular dilation, cortical dysplasia, or subependymal cyst detected by ultrasound, computed tomography, or magnetic resonance imaging. A pediatric ophthalmologist diagnosed chorioretinitis. ABR abnormalities were diagnosed using a Neuropack S1 (Nihon Kohden Co., Tokyo, Japan) according to the manufacturer's recommended protocol. A lack of response either unilaterally or bilaterally to a noise >40 dB for infants with a postconceptional age of ≥37 weeks and 50 dB for infants with a postconceptional age of 34–36 weeks was defined as abnormal [1, 14, 28].

Measurement methods and cut-off values

Total IgM and CMV-IgM were measured with commercially available kits (Siemens Healthcare Diagnostics Corp., Tokyo, Japan) using the nephelometry method [8] and enzyme immunoassay [24, 30], respectively. CMV antigenemia was measured at a commercial laboratory (Special References Laboratories, Inc., Tokyo, Japan) by a direct immunoperoxidase technique using the monoclonal antibody C7 (Teijin, Tokyo, Japan) as previously described [13, 16, 25]. The number of positive cells per 5×10^4 white blood cells (WBCs) was counted. For measurement of CMV-DNA in blood and urine, DNA was extracted with a QIAamp DNA Minikit (Qiagen Corp., Tokyo, Japan) and real-time quantitative PCR was performed as previously described [27]. The results were expressed as the number of CMV-DNA copies per 10^6 WBCs in blood, and per mL in urine. The intra-day coefficients of variation for these assays were 0.76% for total IgM (n=20), 6.1% for CMV-IgM (n=20), 13.6% for CMV antigenemia (n=8), 8.8% for blood CMV-DNA (n=10), and 10.1% for urine CMV-DNA (n=10).

Based on previous reports [24, 29] and the assay manufacturer's criteria, the following threshold values were defined as positive: total IgM ≥20 mg/dL, CMV–IgM ≥0.9 IgM index value (negative 0–0.89, borderline 0.90–1.99, and positive ≥2.0), CMV antigenemia ≥1 CMV antigen-positive cell per 5×10^4 WBCs, CMV-DNA in blood $\geq1\times10^2$ copies per 10^6 WBCs, and CMV-DNA in urine $\geq3\times10^3$ copies per mL.

Statistical analysis

Data are expressed as median (range) or number (%). Univariate analyses were performed using the Mann-Whitney nonparametric rank test or the Fisher exact test as appropriate for between-group comparisons. When blood markers were significantly different between the groups in the quantitative analyses, the cut-off levels associated with the presence of symptoms were determined by ROC analysis [15]. Sensitivity, specificity, negative predictive value, positive predictive value, and the likelihood ratios for positive and negative results were calculated. Linear regression was performed to determine the correlations between the values of each laboratory marker and the gestational ages of the newborns, and correlation coefficients (R^2) were calculated. Differences were considered statistically significant if $P<0.05$.

Results

Patient clinical characteristics

The median gestational age of the symptomatic infants was significantly lower than that of the asymptomatic infants (36 [31–38] vs. 38 [35–41] weeks, $P<0.05$). The median BW also differed significantly between the groups (2188 [1378–3160] vs. 2758 [2060–3840] g for symptomatic and asymptomatic, respectively; $P<0.05$). Furthermore, symptomatic newborns underwent clinical examination earlier than asymptomatic newborns (1 [0–27] vs. 19 [0–28] days of age, $P<0.01$). Ten newborns with congenital CMV

infection had typical manifestations at the time of initial examination; of these, two had SGA, four had hepatosplenomegaly/hepatitis, five were thrombocytopenic, seven had brain abnormalities, seven had ABR abnormalities, and four had chorioretinitis.

Positive rates of laboratory markers in newborns with congenital CMV infection

Of all the newborns with congenital CMV, 78, 52, 44, 96, and 100% tested positive based on total IgM, CMV-IgM, CMV antigenemia, and blood and urine CMV-DNA, respectively. The positive rate for total IgM in the symptomatic group was significantly lower than in the asymptomatic group. There were no significant between-group differences in CMV-IgM, CMV antigenemia, and blood and urine CMV-DNA (Table 1).

Comparison of laboratory values between symptomatic and asymptomatic newborns

Quantitative analysis revealed that the total IgM values were significantly lower and the viral loads in blood were significantly higher in the symptomatic infants than in the asymptomatic infants (P<0.01). The values for CMV-specific antigenemia and IgM, and for urine CMV-DNA, did not differ significantly between the groups (Figure 1).

Total IgM and blood CMV-DNA values associated with symptoms

Total IgM and blood CMV-DNA values in symptomatic or asymptomatic newborns with congenital CMV infection were analyzed by ROC analysis. Cut-off values of 25 mg/dL for total IgM and 1.8×10^2 copies per 10^6 WBCs for blood CMV-DNA were identified as associated with symptomatic status in infected newborns. The areas under the curve for low total IgM and high blood CMV-DNA were 0.854 and 0.842, respectively.

The test performance characteristics were as follows: a cut-off value of 25 mg/dL for total IgM had a sensitivity of 80%, a specificity of 85%, a negative predictive value of 15%, a positive predictive value of 80%, and likelihood ratios for positive and negative results of 5.20 and 0.24, respectively. A cut-off value of 1.8×10^2 copies per 10^6 WBCs for blood CMV-DNA had a sensitivity of 80%, specificity of

Table 1 Positive rates of laboratory markers in congenital cytomegalovirus-infected newborns.

	n	Total IgM	CMV-IgM	CMV antigenemia	Blood CMV-DNA	Urine CMV-DNA
All	23	18 (78%)	12 (52%)	10 (44%)	22 (96%)	23 (100%)
Symptomatic	10	5 (50%)[a]	6 (60%)	4 (40%)	10 (100%)	10 (100%)
Asymptomatic	13	13 (100%)	6 (46%)	6 (46%)	12 (92%)	13 (100%)

Data are expressed as number (%), [a]P<0.01 compared to asymptomatic newborns. CMV=cytomegalovirus, IgM=immunoglobulin M.

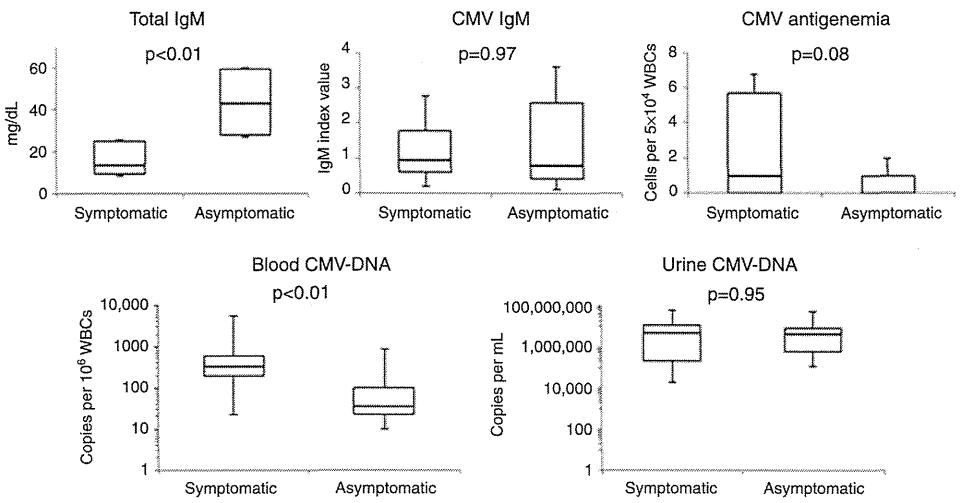

Figure 1 Comparison of laboratory marker values between symptomatic and asymptomatic newborns with congenital cytomegalovirus infection. Data are expressed as median and range. IgM index value: negative 0–0.89, borderline 0.9–1.99, and positive >2. CMV=cytomegalovirus, IgM=immunoglobulin M, WBC=white blood cell.

92%, a negative predictive value of 14%, a positive predictive value of 89%, and likelihood ratios for positive and negative results of 10.4 and 0.22, respectively.

Correlation between values of each laboratory marker and gestational age

No significant correlations were found between the values of each laboratory marker and the gestational age of symptomatic and asymptomatic newborns (total IgM: R^2=0.098, P=0.38 in symptomatic and R^2=0.00091, P=0.92 in asymptomatic newborns, CMV-IgM: R^2=0.0023, P=0.97 in symptomatic and R^2=0.00015, P=0.97 in asymptomatic newborns, CMV antigenemia: R^2=0.12, P=0.32 in symptomatic and R^2=0.072, P=0.86 in asymptomatic newborns, blood CMV-DNA: R^2=0.17, P=0.24 in symptomatic and R^2=0.017, P=0.67 in asymptomatic newborns, and urine CMV-DNA: R^2=0.0019, P=0.90 in symptomatic and R^2=0.059, P=0.43 in asymptomatic newborns).

Discussion

Laboratory techniques for the diagnosis of CMV infection, such as CMV-IgM, CMV antigenemia, and CMV-DNA, have greatly improved during recent years. We assessed these markers concurrently in newborns with congenital CMV infection to evaluate their efficacy for the detection of congenital CMV and prediction of symptomatic status. Low total IgM values and high blood CMV loads were associated with the presence of clinical symptoms in newborns with congenital CMV infection. High CMV-DNA copy numbers in the blood of symptomatic newborns has been reported previously [6, 11, 21]; however, to our knowledge this is the first report of a relationship between low total IgM values and clinical symptoms in newborns with congenital CMV infection.

Interestingly, when total IgM rather than CMV-specific IgM was assessed, we found that the positive rates for total IgM, as well as the total IgM values in the symptomatic group were significantly lower than in the asymptomatic group. Because fetal CMV infection early in pregnancy tends to cause more severe symptoms than infection late in pregnancy [20], we speculate that maternal CMV infection during early pregnancy results in lower total IgM in symptomatic cases. It is plausible that the longer period since infection in symptomatic cases reduces the total IgM value at birth. Alternatively, symptomatic newborn patients who were infected early in pregnancy might have a poor immunological response to infection. Further studies on the mechanisms underlying the low total IgM values at birth in symptomatic newborns are needed.

CMV-IgM had a low positive rate in both symptomatic and asymptomatic newborns with congenital CMV infection. This may have resulted from the insensitivity of the current method for CMV-IgM measurement in newborns. In addition, it may be that not all congenitally CMV-infected fetuses and newborns produce CMV-IgM, such as occurs in immunocompromised or reinfected patients [3, 17, 21].

CMV antigenemia tests, which involve direct detection of antigens in neutrophils using a monoclonal antibody against CMV, are also useful diagnostic tools for CMV infection in patients with immunodeficiencies or after immunosuppressive therapy [2]. However, in our population, a CMV antigenemia test using C7 detection was positive in only 40% and 46% of symptomatic and asymptomatic newborns, respectively; this difference was not significant.

Other studies have also reported high positive rates for CMV-DNA in blood (100% by Revello et al. [22], 95% by Nelson et al. [18], and 91% by Lanari et al. [11]). Thus, blood CMV-DNA may be more useful than CMV-IgM and CMV antigenemia for the diagnosis of congenital CMV infection; however, because blood CMV-DNA is not positive in 100% of congenitally infected newborns, we suggest that blood CMV-DNA should not be used to screen for congenital CMV infection.

A potential limitation of our study was that the gestational age significantly differed between symptomatic and asymptomatic newborns. This occurred because some of the symptomatic newborns were born preterm and subsequently admitted into neonatal intensive care units. However, the lack of correlations between total IgM or blood CMV-DNA values and gestational age suggests that the gestational age was not the factors attributing low total IgM and high blood CMV-DNA values in symptomatic newborns.

In conclusion, we propose that low total IgM values and high blood CMV loads are significant characteristics of newborns with symptomatic congenital CMV infection.

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