Table 2. Comparisons of Clinical Features between Chronic High Epstein-Barr Virus (EBV) Load Carriers and Control Recipients

Feature	Chronic high EBV load carriers (n = 11)	Control recipients (n = 20)	P[a]
Sex			.45
Male	6	7	
Female	5	13	
Age at transplant, years	1.5 ± 1.3	5.9 ± 5.4	.13
Follow-up period after transplant, years	4.0 ± 2.0	4.9 ± 2.5	.43
EBV serostatus before transplant			**.02**
Positive	1	11	
Negative	10	9	
Rejection episodes			.13
Yes	5	15	
No	6	5	
Trough levels of tacrolimus, ng/mL			
1 month after transplant	9.2 ± 1.4	9.0 ± 1.6	.96
3 months after transplant	6.2 ± 1.8	6.1 ± 1.0	.94
6 months after transplant	5.2 ± 1.3	6.6 ± 1.5	**.03**
12 months after transplant	4.0 ± 1.5	4.7 ± 1.7	.32
18 months after transplant	3.4 ± 1.4	4.7 ± 2.6	.27
Features at the time of blood sampling for EBV monitoring			
Time after transplant, years	2.9 ± 1.8	4.3 ± 2.3	.14
EBV load in whole blood, copies/mL	23,800 ± 31,600	100 ± 200	**.01**
Trough levels of tacrolimus, ng/mL	2.5 ± 1.2	2.6 ± 1.4	.64
Percentage of $CD4^+$ lymphocytes within the lymphocyte population	38.9 ± 8.7	35.5 ± 4.2	.71
Percentage of $CD8^+$ lymphocytes within the lymphocyte population	20.9 ± 9.1	24.1 ± 6.5	.31

NOTE. Data are means ± standard deviations, unless otherwise indicated. Boldface type indicates statistically significant differences.

[a] The Fisher exact test or the Mann-Whitney U test was used to compare factors.

from each chronic high EBV load carrier is shown in Figure 1. EBV DNA was not detected in most of the selected plasma samples from chronic high EBV load carriers throughout the chronic high EBV load state (data not shown). In some carriers, viral loads gradually decreased, whereas they continued to be elevated in others. For comparison, the median EBV load was 2.5×10^4 copies/mL (range, 1.9×10^3–3.1×10^6 copies/mL) of whole blood for 15 patients with IM and 4.0×10^5 copies/mL of whole blood for 2 patients who developed PTLD after a bone marrow transplant. EBV DNA was detected in 4 of 25 healthy control subjects (range, 0–2.7×10^2 copies/mL).

Next, we compared clinical features between the chronic high EBV load carriers and control recipients (Table 2). Most of the chronic high EBV load carriers were seronegative for EBV before transplant, indicating that primary infection was a risk factor for the chronic high EBV load carrier state. Trough levels of tacrolimus were not significantly different, except 6 months after transplant, at which point the dose of tacrolimus was reduced because of the high EBV load.

Dominant EBV-infected cell compartments. Viral DNA was dominantly found only in a population of $CD19^+$ cells of the peripheral blood from all chronic high EBV load carriers (Figure 2).

Measurement of IL-10 concentration. Because several reports have demonstrated that levels of IL-10 might be predictive of the development of PTLD [18], plasma concentrations of IL-10 were compared among chronic high EBV load carriers (n = 11), control recipients (n = 20), and patients with IM (n = 14). The mean levels of IL-10 in both chronic high EBV patients and control recipients were not elevated (mean ± SD, 2.1 ± 2.7 and 2.7 ± 5.7 pg/mL, respectively), and no significant difference was found between the 2 groups, although the levels in both groups were significantly lower than that in patients with IM (mean ± SD, 10.1 ± 6.5 pg/mL).

Precursor frequency of EBV-specific $CD8^+$ T cells in peripheral blood. HLA-A2 and HLA-A24 tetramers were used to analyze the precursor frequency of EBV- and cytomegalovirus (CMV)–specific $CD8^+$ T cells in the 4 groups of subjects who had HLA-A2 or HLA-A24: chronic high EBV load carriers (A24, n = 8; A2, n = 4), control recipients (A24, n = 13; A2, n = 10), healthy control subjects (A24, n = 10), and patients with IM (A24, n = 6). EBV-specific $CD8^+$ T cells were detected

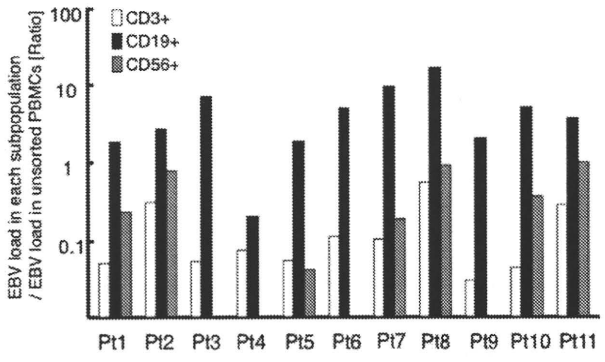

Figure 2. Epstein-Barr virus (EBV) load in each subpopulation of peripheral blood mononuclear cells (PBMCs) from chronic high EBV load carriers. Each subpopulation was positively selected with antibody-coated magnetic beads from PBMCs obtained from chronic high EBV load carriers. Viral load in each compartment was quantified by real-time polymerase chain reaction.

with 1 or more of the relevant tetramers in 9 of 10 chronic high EBV load carriers, in 18 of 19 control recipients, and in all subjects in the other groups. The frequency of EBV- and CMV-specific $CD8^+$ T cells in the 4 groups are summarized in Figure 3*A* and 3*B*. The frequency of $CD8^+$ T cells specific for lytic-cycle antigens (*Bam*HI R leftward reading frame 1 [BRLF1] and *Bam*HI M leftward reading frame 1 [BMLF1]) in patients with IM was significantly higher than in other groups. Regarding the 3 latent antigens (EBNA3A, EBNA3B, and LMP2), no statistical difference was found in the frequencies of EBV-specific $CD8^+$ T cells among the groups, although the frequency of LMP2-specific $CD8^+$ T cells were lower than those for the other epitopes in all groups. CMV pp65–specific $CD8^+$ T cells were measured for comparison, and no difference was found in the frequencies among the groups.

Patterns of EBV-related gene expression in PBMCs. To investigate the levels and patterns of EBV gene expression, 6 latent and 2 lytic genes were quantified by multiplex real-time RT-PCR in all study groups. Representative quantitative results for chronic high EBV load carriers for each gene are shown in Figure 4*A*. EBER1 and BARTs were detected in all samples, and LMP2 was detected in 6 samples. EBER1 had the highest relative expression level, followed by BARTs and LMP2. The mean expression levels ± SD of EBER1, BARTs, and LMP2 were $10^{-4.0\pm0.9}$, $10^{-4.5\pm1.7}$, and $10^{-6.4\pm1.6}$, respectively. In contrast, more EBV-related genes were detected in patients with IM ($n = 15$) and PTLD ($n = 2$) (Figure 4*B* and 4*C*). In patients with IM, the mean expression levels ± SD of EBNA1, EBNA2, LMP1, LMP2, EBER1, BARTs, and BZLF1 were $10^{-7.0\pm1.6}$, $10^{-5.8\pm2.0}$, $10^{-6.7\pm1.1}$, $10^{-5.6\pm1.9}$, $10^{-4.2\pm1.3}$, $10^{-4.7\pm2.0}$, and $10^{-7.8\pm0.6}$, respectively (Figure 4*D*). Additionally, EBER1 and BARTs with or without LMP2 were detected in only 2 of 20 control recipients, an expression pattern similar to that observed in the chronic high EBV load carriers (data not shown). With regard to healthy control subjects, EBER1 was detected in 2 of 23, and no other gene was detected in any of them.

DISCUSSION

Serial monitoring of EBV load for early diagnosis has become standard practice in the management of transplant recipients [5, 19–21]. This monitoring has led to the identification of a group of asymptomatic patients with chronic high EBV loads over long periods [10–12]. The incidence of chronic high EBV load, clinical features, and the risk of late-onset PTLD in liver transplantation have been reported by a few groups. D'Antiga et al [10] stated that 14 (41%) of 34 pediatric liver transplant recipients showed positive RT-PCR results for viral capsid antigen immunoglobulin M or early antigen immunoglobulin G lasting >6 months. Viral loads >500 copies/10^5 PBMCs occurred in most of them. All 14 of these patients were seronegative for immunoglobulin G before transplant. Three patients developed late-onset PTLD. Green et al [12] reported that 36 (18%) of 196 children who had undergone liver transplant had >16,000 copies/mL of whole blood or 200 copies/10^5 PBMCs in at least 50% of samples over a minimum period of 6 months after EBV infection. Three-quarters of these children were negative for EBV before transplant. Only 1 patient developed PTLD. In the present study, 11 (35%) of 31 patients were found to be chronic high EBV carriers, and 10 of these 11 patients were seronegative for EBV before liver transplant, consistent with previous reports. No recipient with chronic high EBV loads developed late-onset PTLD. Because the majority of chronic high EBV load carriers were negative for EBV before transplant, primary EBV infection while receiving immunosuppressive drugs is key to understanding this chronic high EBV load carrier state.

Bingler et al [11] reported that 9 (45%) of 20 pediatric heart transplant recipients with chronic high EBV loads (defined as the presence of >16,000 copies/mL or 200 copies/10^5 PBMCs in at least 50% of samples over a minimum period of 6 months, developed late-onset PTLD. The incidence among heart recipients in this report was higher than those among liver recipients stated above. This difference may result from the aggressiveness of immunosuppression during the posttransplant period. The incidence of PTLD varies significantly between different types of organ transplantation [22, 23]. This variation is thought to be related to the degree and duration of immunosuppression and the number of EBV-positive donor lymphocytes in the graft. Additional studies are required to examine the association between the incidence of late-onset PTLD among those with chronic high EBV loads and the method of immunosuppression.

In the present study, a chronic high EBV load carrier state was defined as the continuous presence of EBV loads >5000 copies/mL of whole blood over a minimum period of 6 months

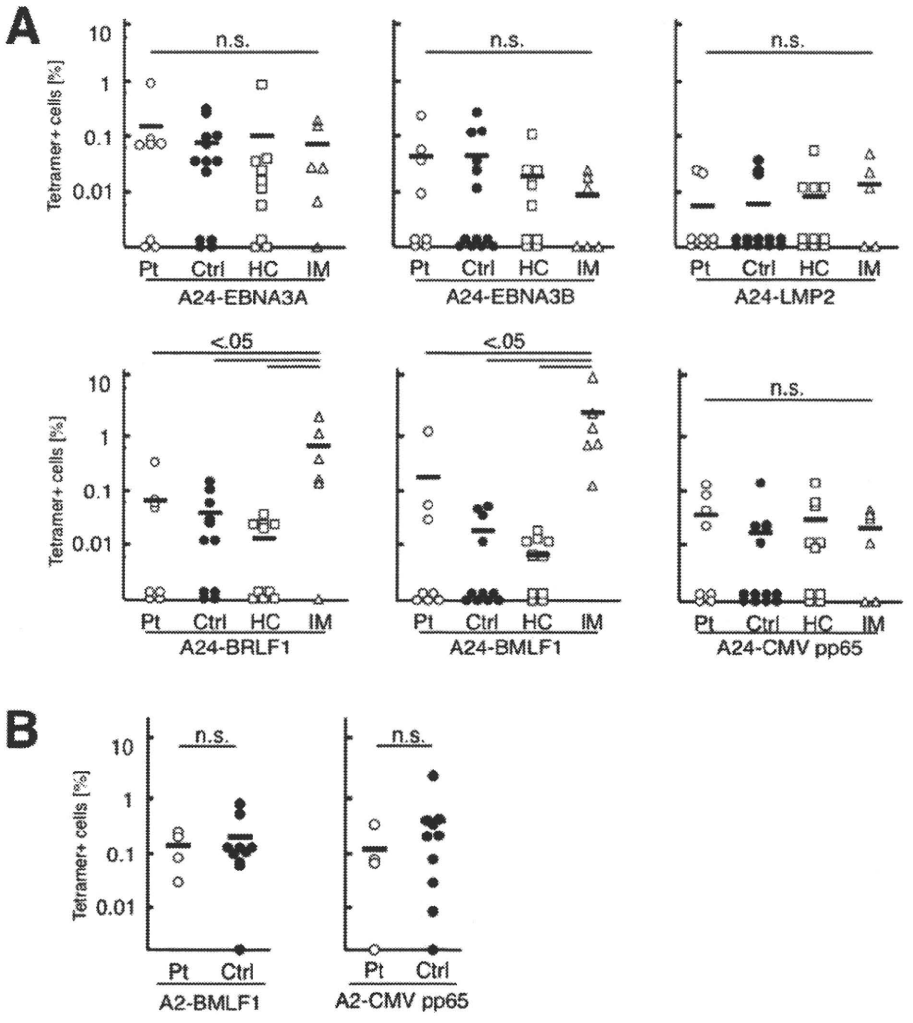

Figure 3. Comparison of the frequency of Epstein-Barr virus (EBV)–specific $CD8^+$ T cells among peripheral blood mononuclear cells (PBMCs). The precursor frequency of EBV-specific $CD8^+$ T cells in each study group was measured using major histocompatibility complex (MHC) peptide tetramers. EBV tetramer–positive cells are shown as a percentage of total $CD8^+$ T cells. *A*, HLA-A24 tetramer–positive cells among total $CD8^+$ cells in PBMCs from chronic high EBV load carriers (Pt [*white circles*]; $n = 8$), control recipients (Ctrl [*black circles*]; $n = 13$), healthy EBV-positive control subjects (HC [*squares*]; $n = 10$), and patients with infectious mononucleosis (IM [*triangles*]; $n = 6$). Horizontal bars denote the mean value in each group of subjects. *B*, HLA-A2 tetramer–positive $CD8^+$ cells among PBMCs from chronic high EBV load carriers (Pt [*white circles*]; $n = 4$) or control recipients (Ctrl [*black circles*]; $n = 10$).

after asymptomatic infection or after complete resolution of symptomatic EBV disease. Previously, we reported that, in hematopoietic stem cell transplantation, 90.6% (3/32) of all asymptomatic patients had an EBV load $<10^{2.5}$ copies/μg of DNA (82.4% [14/17] of asymptomatic patients with a positive EBV load had $<10^{2.5}$ copies/μg of DNA), 83% (5/6) of the patients with clinical symptoms had between $10^{2.5}$ and 10^4 copies/μg of DNA, and 100% (5/5) of patients who developed PTLD had $>10^4$ copies/μg of DNA [24]. Recently, we measured the EBV load in whole blood in order to monitor liver transplant patients, and our conversion factor from copies per microgram of DNA to copies per milliliter of whole blood was 10–20 (authors' unpublished data). This conversion factor is close to that reported in another review [25]. Using this factor, $10^{2.5}$ (equal to 316) copies/μg of DNA is similar to 3160–6320 copies/mL of whole blood, and we decided that 5000 copies/mL of whole blood was the value expected to produce EBV-related clinical symptoms but not PTLD in our system for quantifying the EBV load.

$CD8^+$ cytotoxic T cells are known to be important in controlling EBV-associated PTLD [4]. The combination of increased EBV load and the absence of EBV-specific $CD8^+$ T cells can predict EBV lymphoproliferative disease [26]. Regarding the frequency of $CD8^+$ T cells specific for 2 lytic antigens

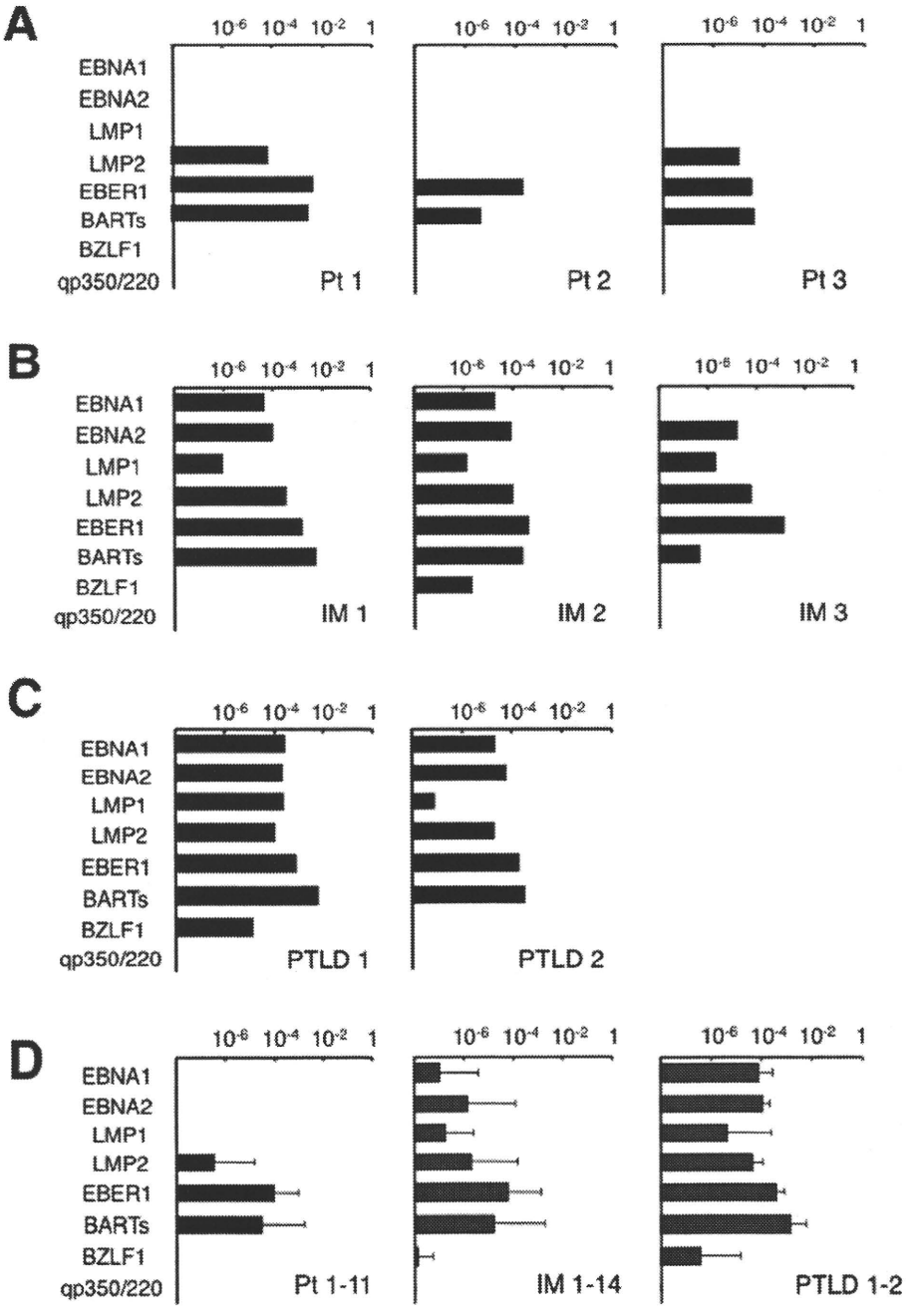

Figure 4. Comparison of the expression patterns of Epstein-Barr virus (EBV)–related genes in peripheral blood mononuclear cells (PBMCs). Relative quantification of EBV genes was performed in each study group by multiplex real-time reverse-transcription polymerase chain reaction. *A*, Representative data from chronic high EBV load carriers (Pt 1, 2, and 3). *B*, Representative data from 3 patients with infectious mononucleosis (IM 1, 2, and 3). *C*, Relative quantification of EBV gene expression in 2 patients with posttransplant lymphoproliferative disorder (PTLD 1 and 2). *D*, Levels of EBV gene expression in 11 chronic high EBV load carriers, 15 patients with IM, and 2 patients with PTLD. Data are means ± standard deviations.

(BRLF1 and BMLF1) and 3 latent antigens (EBNA3A, EBNA3B, and LMP2), we found no significant difference between chronic high EBV load carriers and control recipients. Macedo et al [27] reported that the frequency of EBV-specific $CD8^+$ T cells in "stable" (quiescent) transplant recipients was equal to or higher than that in healthy control subjects, which may agree with our results. In solid-organ transplant recipients, chronic administration of immunosuppressive drugs causes the impairment of cellular immune surveillance and allows EBV-infected B cells to proliferate. Smets et al [28] reported that

recipients with high EBV loads showing low activity of specific $CD8^+$ T were at high risk for PTLD, using an enzyme-linked immunospot assay in pediatric solid-organ transplant recipients. Such a functional impairment may be present and influence the chronic high EBV load state.

Analyzing the expression profile of EBV-related genes is useful in clarifying the pathogenesis of EBV-associated diseases [16]. In each chronic high EBV load carrier, EBER1 and BARTs were abundantly detected, and LMP2 was found in half of the carriers. The mean expression level of LMP2 was low, compared with those of EBER1 and BARTs. No transcript specific for latency type 3 was detected in chronic high EBV load carriers. This restricted pattern is latency type 0, which is found in EBV-infected memory B cells [8, 29, 30], suggesting that the pattern of infected B cells in those with chronic high EBV loads may be the same as that in healthy EBV carriers. Qu et al [31] reported that peripheral blood lymphocytes in those with chronic high EBV loads after solid-organ transplant were divided into 2 types of infected cells: lymphocytes expressing LMP2 and carrying a low number of copies of EBV, and others coexpressing LMP1 and LMP2 and having a high number of copies of EBV. In the present study, expression of LMP1 was not observed in all chronic high EBV load carriers. Quantification in the blood sample may not have been sufficiently sensitive to detect a small population of infected cells expressing the LMP1 gene. Additionally, the expression pattern may vary with the time at which the blood sample is obtained. Transcripts specific for latency type 3 were detected in some samples from chronic high EBV load carriers obtained within 4 months after EBV infection (data not shown), and the pattern of EBV gene expression subsequently changed to latency type 0. Dynamic changes in the immune response, such as an increased precursor frequency of EBV-specific $CD8^+$ T cells after reduced immune suppression, may influence expression patterns in chronic high EBV load carriers; however, this change was not evaluated in the present study.

After primary infection, EBV persists as a latent infection in memory B cells. Then, memory B cells occasionally differentiate into plasma cells that undergo lytic infection and produce new virus. Newly infected naive B cells become transformed but are controlled by $CD8^+$ T cells specific for EBV. This cycle is necessary to maintain a latent infection in vivo [21, 32]. Thus, we suggest a novel mechanism for the maintenance of high viral loads in the blood of chronic high EBV load carriers. First, a large number of memory B cells latently infected with EBV survive after the primary or reactivated EBV infection occurring after transplant. Second, the latently infected memory B cells readily differentiate into plasma cells to produce the virus. Immunosuppression may influence these processes. Treatment with acyclovir or ganciclovir for a relatively prolonged period may modulate the chronic high EBV load state. However, our results did not provide mechanistic or therapeutic information.

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ORIGINAL ARTICLE

Multicenter evaluation of prototype real-time PCR assays for Epstein-Barr virus and cytomegalovirus DNA in whole blood samples from transplant recipients

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ABSTRACT

Quantitative PCR is becoming widespread for diagnosing and monitoring post-transplantation diseases associated with EBV and CMV. These assays need to be standardized to manage patients in different facilities. Five independent laboratories in Japan compared home-brew assays and a prototype assay system to establish a standard quantitative procedure for measuring EBV and CMV. Reference standards and a total of 816 (642 EBV and 174 CMV) whole blood samples from post-transplantation recipients were used for this multicenter evaluation. The prototype reference standard for EBV was compared to a panel of samples, with a theoretical expected value made using EBV-positive cells containing two virus genome copies per cell. The mean ratio of the reference standard at each site to the standard of the prototype assay was ≤4.15 for EBV among three different sites and ≤3.0 for CMV between two laboratories. The mean of the theoretical expected number of the EBV genome : prototype reference was close to 1.0. The correlation coefficients between the viral copy numbers determined using the prototype assay and those using each home-brew assay were high (EBV, 0.73–0.83, median = 0.78; CMV, 0.54–0.60, median = 0.57). The dynamics of the EBV and CMV loads in transplant recipients were similar between the assay types. There was an inter-laboratory difference among the quantification results, indicating that a unified protocol and kit are favorable for standardizing the quantification of EBV and CMV. Such standardization will help to standardize the diagnosis and monitoring of diseases associated with EBV and CMV.

Key words cytomegalovirus, Epstein–Barr virus, real-time PCR, standardization, viral load.

Herpes viruses are widespread pathogens in the human population and often become reactivated in latently infected immunocompromised patients. These viruses thus frequently occur after hematopoietic stem cell and solid organ transplantation, and occasionally result in symptomatically severe disease (1, 2). EBV and CMV are representative viruses causing disease after transplant recipients. Life-threatening PTLD is known to be caused by the EBV virus (3). Frequent clinical manifestations of CMV are pneumonia and gastrointestinal disease (4). Because these viruses replicate without any clinical symptoms, quantitative methods are required to distinguish asymptomatic infection from impending diseases. Routine monitoring of these viruses and pre-emptive

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List of Abbreviations: EBV, Epstein-Barr virus; CMV, cytomegalovirus; PTLD, post-transplant lymphoproliferative disorder.

intervention for virus-associated diseases are therefore important (5, 6).

Recently, quantitative real-time PCR assays have become widespread methods for diagnosing and monitoring EBV-associated diseases after transplantation (5, 7). The CMV pp65 antigenemia assay has been widely used to evaluate the viral load of CMV-associated diseases and is considered the gold standard. However, quantitative PCR is increasingly used in diagnosing and monitoring transplant recipients because of its speed, reproducibility, and ease of use (6, 8). Currently, laboratories rely on their own home-brew quantitative PCR assay system. These home-brew assay systems need to be standardized because discrepancies existing between laboratories lead to site-specific patient management algorithms.

Five independent laboratories comprised the working group in this study and compared the quantitative results of each home-brew assay and a prototype assay system to establish a standardized quantitative procedure for measuring EBV and CMV. Distributed reference standards and whole blood samples from solid organ transplantation and hematopoietic stem cell transplantation recipients were used in this multicenter evaluation.

MATERIALS AND METHODS

Study group and samples

Five independent laboratories comprised the working group of the Japan Molecular Center of Excellence sponsored by Roche Diagnostics K.K, each using a different quantitative home-brew EBV PCR assay. Each laboratory provided details of its home-brew testing procedure (Table 1). The prototype assay kit (JMCoE EBV primer probe standard set, CMV primer probe standard set, DNA master mix set; Nihon Gene Research Laboratories, Sendai, Japan) and the reference standard for EBV and CMV were developed by Roche Diagnostics K.K. (Tokyo, Japan) and distributed among the participating laboratories. In total, 642 (EBV) and 174 (CMV) whole blood samples from solid organ and hematopoietic stem cell transplantation recipients as part of routine follow up after transplantation were studied retrospectively. The sample set for comparison was different among the participating sites: for EBV, 100 samples were used in site A, 100 in B, 240 in C, 72 in D, and 130 in E; for CMV, 103 in A and 71 in E. No samples were redundant among the participating sites. Each site carried out quantitative EBV and CMV testing on all reference standards and clinical samples using both their own home-brew procedure and the prototype test. The study design and purpose, approved by the institutional review board of each university hospital, were fully explained to all of the patients and/or guardians, and informed consent was obtained.

Preparation of the reference standard for the prototype assay

As a reference standard for the prototype assay, a plasmid that contained the EBV *BALF5* gene and one containing CMV *IE* gene were constructed from pGEM-T vector (Promega, Madison, WI, USA) (9, 10). The copy number of the plasmids was calculated on the basis of its absorbance at 260 nm.

Preparation of the panel samples with the EBV-positive cell line

To evaluate the value of the reference standard plasmid for the prototype assay, EBV-positive samples in which the actual EBV copy number could be estimated were prepared. Namalwa cells containing two EBV genome copies per cell were used as a source of EBV DNA. BJAB cells, known to be EBV negative, were used to prepare a background cellular matrix. Three types of sample were constructed: 5×10^6 Namalwa cells (defined as Namalwa 100%); 5×10^5 Namalwa cells with 4.5×10^6 BJAB cells (defined as Namalwa 10%); and 5×10^4 Namalwa cells with 4.95×10^6 BJAB cells (defined as Namalwa 1%). The theoretical expected value of the whole Namalwa 100% sample was 1×10^7 copies. When DNA was extracted from the Namalwa 100% sample, 58.4 μg/200 μl distilled water was obtained. In

Table 1. Comparison of procedural characteristics among different laboratories

Laboratory	Target gene		Sample volume (μl)	Extraction kit	Elution volume (μl)	DNA input (μl)	Reaction volume (μl)	Platform Platform
	EBV	CMV						
A	BALF5	IE	Whole blood (200)	QIAamp	50	5	25	Mx3000P
B	BNFR1	–	Whole blood (200)	QIAamp	200	5	25	ABI 7700
C	BALF5	–	Whole blood (200)	QIAamp	150	5	25	ABI 7700
D	BXLF1	–	Whole blood (200)	QIAamp	50	5	25	LightCycler 2.0
E	BALF5	IE	Whole blood (200)	QIAamp or MagNA Pure	100	10	50	ABI 7700
Prototype	BALF5	IE	Whole blood (200)	QIAamp	200	50	100	Cobas Taqman48

the case of the prototype assay, 2 μg extracted DNA from 200 μl whole blood was transferred to a single assay well. Therefore, 2 μg of 58.4 μg of DNA was used as a sample to evaluate the value of the reference standard. Two micrograms of DNA from Namalwa 100% were expected to contain 3.42×10^5 ($1 \times 10^7 \times 2/58.4$) copies of the EBV genome. To evaluate different concentrations of DNA as an assay template, 0.2 μg of 58.4 μg was also measured in the prototype assay. The results from other Namalwa constructs were assessed in the same way.

Prototype assay system

Viral DNA was extracted from 200 μl whole blood using QIAamp DNA blood kits (Qiagen, Hilden, Germany) and eluted in 200 μl distilled water. The specific primers and fluorogenic probes for EBV and CMV were as follows: EBV forward: CGGAAGCCCTCTGGACTTC, EBV reverse: CCCTGTTTATCCGATGGAATG, EBV probe: FAM-TGTACACGCACGAGAAATGCGCC-TAMRA (9); CMV forward: GACTAGTGTGATGCTGGCCAAG, CMV reverse: GCTACAATAGCCTCTTCCTCATCTG, CMV probe-1: FAM-AGCCTGAGGTTATCAGTGTAATGAA GCGCC-TAMRA (10), CMV probe-2: FAM-AGCCTGAGGTTATCAATATCATGAAGCGCC-TAMRA. Because a variation was reported within the sequence that would be amplified with the CMV-specific primers (11), two different probes were mixed and used for CMV quantification. Fifty microliters of a 200-μl DNA extraction solution was added as a reaction mixture containing the master mix reagent, specific primers, and probes. A real-time PCR reaction was carried out with a model Cobas TaqMan 48 (Roche Diagnostics K.K., Tokyo, Japan). All samples and standards were run in duplicate. Regarding the prototypic assay for EBV, the standard curves obtained were linear from 10 to 10^5 copies/reaction with an average slope of -3.50. The standard curves of the CMV assay were also linear from 10 to 10^5 copies/reaction with an average slope of -3.87.

Statistical analysis

The concordance was analyzed by kappa statistics. The number (copies/ml) was converted to its logarithmic value. Regression analysis was carried out by simple regression on the home-brew assay to the prototype test.

Results

Procedural characteristics of home-brew assays among the five laboratories

Specific primers and probes, DNA extraction kit, DNA elution volume, real-time PCR reaction volume, and the real-time PCR platform were varied among participating laboratories (Table 1). The sequences of the primers and the probe for EBV were identical at sites A, C and E. The sequences of the primers and the probe for CMV at sites A and E were consistent. A reference standard for the home-brew assay was prepared in each laboratory.

Evaluation of reference standards for EBV and CMV

The copy numbers of the standards in three (for EBV) or two (for CMV) home-brew systems using the same primer and probe set were measured based on the copy number of the reference standards for the prototype assays. The ratios of the reference standard in each site to the prototype assay standard at different copy numbers are shown in Table 2. The mean ratio was ≤ 4.15 for EBV among three different sites and ≤ 3.0 for CMV between two laboratories.

To evaluate the value of the EBV reference standard plasmid for the prototype assay, EBV-positive samples with an expected theoretical value were prepared using Namalwa cells known to contain two EBV genome copies per cell. When the prototype real-time PCR assay was carried out with 2 μg DNA extracts from these samples per reaction mixture, the mean of the theoretical expected number of EBV genome : quantitative result ratio was 0.62. In the

Table 2. Quantitative result of each home-brew reference standard based on the value of the prototype reference

Laboratory		Value of each home-brew assay reference standard (ratio)					Ratio (mean)
		10^2	10^3	10^4	10^5	10^6	
EBV	A	ND	9.9×10^2 (0.99)	9.3×10^3 (0.93)	8.8×10^4 (0.88)	6.4×10^5 (0.64)	0.86
	C	ND	3.4×10^3 (3.4)	4.9×10^4 (4.9)	4.2×10^5 (4.2)	4.2×10^6 (4.2)	4.15
	E	ND	7.9×10^2 (0.79)	6.3×10^3 (0.63)	6.4×10^4 (0.64)	6.7×10^5 (0.67)	0.68
CMV	A	1.7×10^2 (1.7)	2.0×10^3 (2.0)	2.2×10^4 (2.2)	2.1×10^5 (2.1)	ND	2.00
	E	4.0×10^2 (4.0)	3.3×10^3 (3.3)	3.0×10^4 (3.0)	1.8×10^5 (1.8)	ND	3.03

The average copy numbers were calculated from 12 wells at each concentration.

Table 3. Quantitative result of the prototype reference standard based on the value of the constructed samples with a theoretical expected value

Constructed sample	Expected value of EBV in sample (copies)	Expected value of EBV in DNA extract (copies/reaction)	Actual value (copies/reaction, mean)	Expected value/Actual value
Namalwa 100%	1.0×10^7	342 460†	524 200	0.65
		34 246‡	38 205	0.90
Namalwa 10%	1.0×10^6	41 967†	65 210	0.64
		4197‡	4893	0.86
Namalwa 1%	1.0×10^5	4073†	7113	0.57
		407‡	312	1.31

†Real-time PCR assay was carried out with 2 μg DNA extract per reaction.
‡Real-time PCR assay was carried out with 0.2 μg DNA extract per reaction.

case of the 0.2-μg DNA extracts, the mean ratio was 1.0 (Table 3).

Quantitative results for patient samples for EBV

Some samples were positive by one assay but negative by the other. The concordance rates between each home-brew assay and the prototype assay were 88% (88/100) (site A *vs* the prototype assay, $P < 0.001$), 86% (86/100) (site B *vs* the prototype assay, $P < 0.001$), 93% (222/240) (site C *vs* the prototype assay, $P < 0.001$), 93% (67/72) (site D *vs* the prototype assay, $P < 0.001$), and 97% (126/130) (site E *vs* the prototype assay, $P < 0.001$). The viral loads of almost all of these discordant samples were low copy numbers. Indeed, complete concordance was observed in the quantitative results for samples with results of ≥696 copies/ml for the prototype assay. The viral DNA copy numbers were compared using all samples determined to be positive according to both the prototype assay and each home-brew assay. A strong correlation was detected between the viral copy numbers determined by the prototype assay and those of each home-brew assay (Fig. 1). Longitudinal monitoring of nine representative individual transplant recipients is shown in Figure 2. The dynamics of the EBV load in all patients were similar, although some discrepancies were observed within the follow-up period.

Quantitative results for patient samples for CMV

Some samples were positive by one assay but negative by the other. The number of these discordant samples was larger than that in the comparisons for EBV. The concordance rates between each home-brew assay and the prototype assay were 59% (59/100) (site A *vs* the prototype assay, $P < 0.001$) and 86% (61/71) (site E *vs* the prototype assay, $P < 0.001$). The viral loads of all of these discordant samples were low copy numbers. Indeed, complete concordance was observed in the quantitative results for the samples with ≥36 copies/ml in the prototype assay.

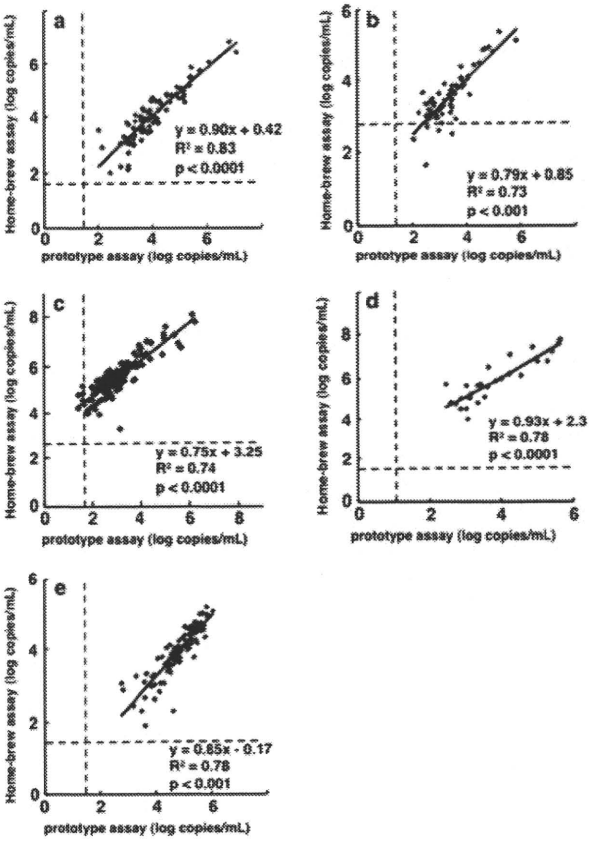

Fig. 1. Comparison of EBV viral loads determined by the prototype and each home-brew assay. (a) Correlation between the prototype assay and home-brew assay A ($n = 100$). (b) Correlation between the prototype assay and the home-brew assay B ($n = 100$). (c) Correlation between the prototype assay and the home-brew assay C ($n = 240$). (d) Correlation between the prototype assay and the home-brew assay D ($n = 72$). (e) Correlation between the prototype assay and the home-brew assay E ($n = 130$). Dotted lines show the detection limits indicating one copy per reaction for each assay.

Comparison of the prototype assay and each home-brew assay for all positive samples according to both assays had a high degree of correlation (Fig. 3). Longitudinal monitoring of five representative individual transplant recipients is

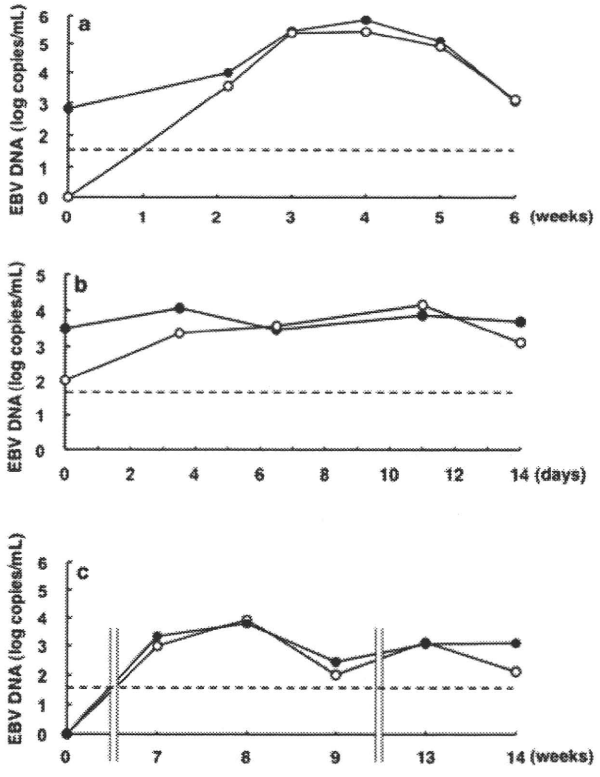

Fig. 2. Results of monitoring EBV viral loads in 12 individual patients after transplantation as measured by the prototype assay and a home-brew assay. (a–c) Dynamics of EBV load in three patients at site A. (d–f) Dynamics of EBV load in three patients at site B. (g–i) Dynamics of EBV load in three patients at site E. The date of the first of the consecutive samples from one patient was defined as day 0. ○, prototype assay; •, home-brew assay. Dotted lines show the detection limits indicating one copy per reaction for each home-brew assay.

demonstrated in Figure 4. The dynamics of the CMV load in all patients were similar, although some discrepancies were observed within the follow-up period.

DISCUSSION

Standardized calibration materials and commercially available assays have been developed for standardized quantification for specific viruses, such as HIV and hepatitis C virus (12–14). Standardization is necessary for consensus guidelines in patient management. Hayden *et al.* (7) reported a multicenter comparison of different real-time PCR assays for EBV. This study was carried out at eight sites using three panels consisting of serial dilutions of commercially available EBV DNA and extracts from 19 whole blood specimens. Strong concordance among laboratories was observed with respect to the qualitative results, whereas quantitative discordance was seen at a maximum of 4 log-units. This discrepancy decreased when a common

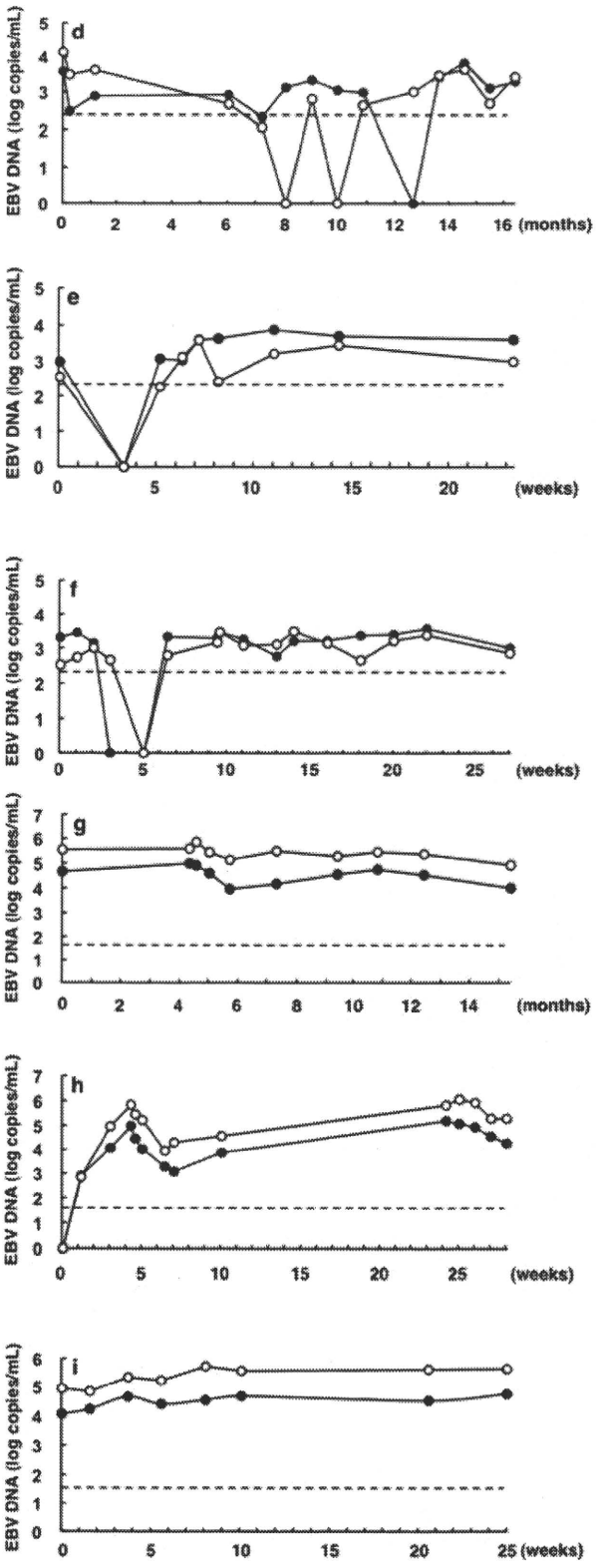

Fig. 2. Continued.

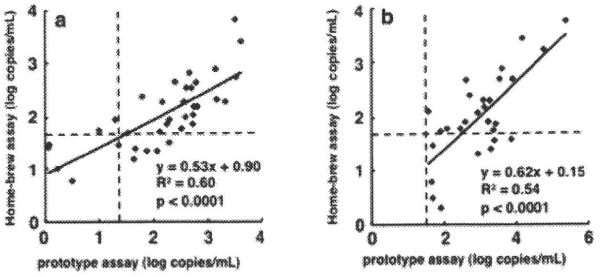

Fig. 3. Comparison of CMV viral loads as determined by the prototype and each home-brew assay. (a) Correlation between the prototype assay and home-brew assay A ($n = 103$). (b) Correlation between the prototype assay and home-brew assay E ($n = 71$). Dotted lines show the detection limits indicating one copy per reaction for each assay.

reference standard was used to obtain quantitative results. Preiksaitis *et al.* (15) reported an international comparison of EBV DNA quantitative assays. They distributed a panel of samples to 28 laboratories. The panel of samples consisted of seven constructs using EBV-positive cell lines and three clinical plasma samples. Half of the quantitative results were within ±0.5 log-units, whereas the maximum variation was approximately 4 log-units. With regard to CMV quantification, Pang *et al.* (16) recently reported an international comparison of CMV viral load assays. They distributed a panel of samples to 33 laboratories. The panel of samples consisted of seven constructs using purified CMV stock and three clinical plasma samples. Fifty-eight percent of the quantitative results were within ±0.5 log-units whereas the maximum variation was approximately 4 log-units. In the present study, five independent laboratories were involved in comparing the quantitative values for EBV and CMV from each home-brew assay and the prototype assay. The maximum variations were 4.15 for EBV and 3.03 for CMV, which is acceptable in comparison with previous reports (7, 15, 16). Additionally, the dynamics of the EBV load in 12 patients and the CMV load in five patients were found to be similar, and this comparison may be unique. Even the inter-laboratory variation appears to be small; however, it is uncertain whether this variation is a problem for treating patients. The development of a prototype assay may help eliminate concern related to variability. Our next goal is to determine the clinical cut-off value for each virus and to conduct a large, prospective study.

Controversy exists as to which blood compartment should be used for measuring EBV. Whole blood, peripheral blood mononuclear cells, plasma, and serum have been used as samples from patients. To diagnose EBV-associated PTLD, earlier studies used peripheral blood mononuclear cells because EBV infection occurs in this cell compartment (17–19). Plasma or serum samples are

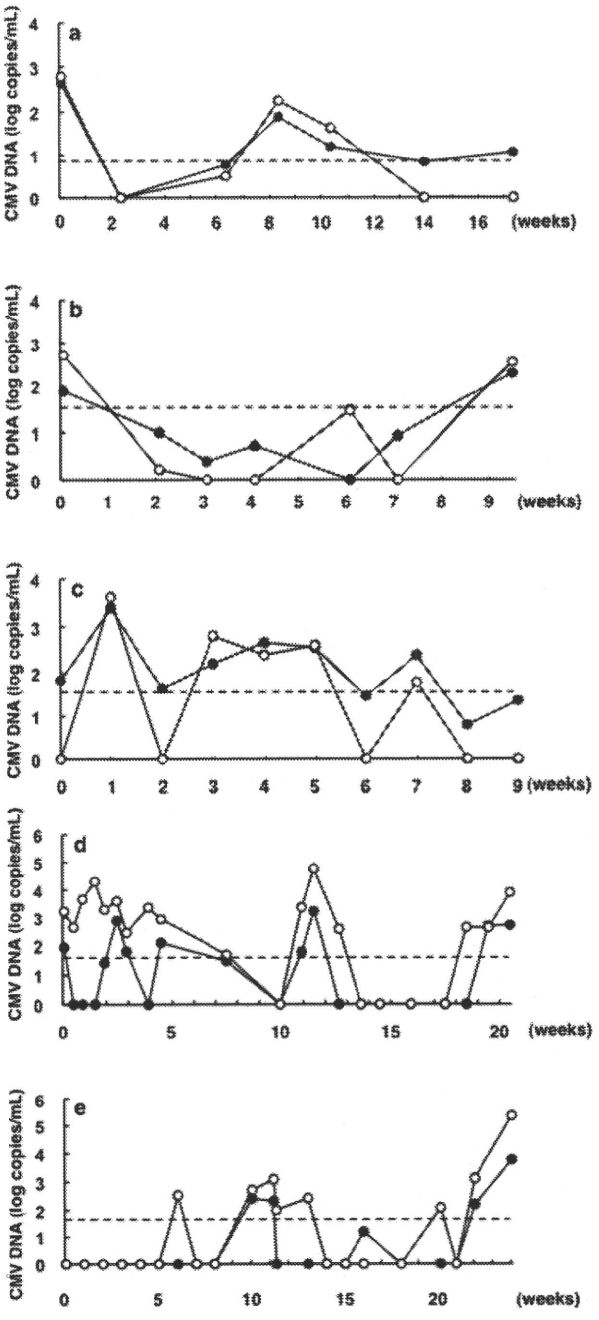

Fig. 4. Results of monitoring CMV viral load in five individual patients after transplantation as measured by the prototype assay and a home-brew assay. (a–c) Dynamics of CMV load in three patients at site A. (b) Dynamics of CMV load in two patients at site E. The date of the first of consecutive samples from one patient was defined as day 0. ○, prototype assay; ●, home-brew assay. Dotted lines show the detection limits indicating one copy per reaction for each home-brew assay.

readily obtained and widely used for diagnosing EBV-associated PTLD; however, the sensitivity appeared to be low (20, 21). Several reports have revealed that whole blood, containing all blood compartments, is better than

plasma/serum when testing patients with PTLD (22–24). Additionally, serum or plasma is reported to be suitable for EBV-associated infectious mononucleosis (19, 25). Discussion regarding which blood compartment should be used for measuring CMV has been ongoing. CMV latently infects a variety of leukocytes, but predominantly cells of the monocyte/macrophage lineage. CMV quantification can be carried out with serum or plasma, but the sensitivity is greater in whole blood and leukocytes than in acellular fractions of the blood (26, 27).

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