Pathol. Oncol. Res. (2008) 14:399–409
DOI 10.1007/s12253-008-9030-3

ORIGINAL PAPER

Monitoring of Cytomegalovirus Reactivation in Bone Marrow Transplant Recipients by Real-time PCR

Seyed H. Ghaffari · Narghes Obeidi · Mehdi Dehghan · Kamran Alimoghaddam · Ahmad Gharehbaghian · Ardashir Ghavamzadeh

Received: 1 March 2008 / Accepted: 5 March 2008 / Published online: 8 April 2008

Abstract Cytomegalovirus (CMV) has been recognized as the most important viral pathogen in persons undergoing bone marrow transplantation (BMT). The aim was to develop a quantitative PCR assay to quantify CMV DNA in peripheral blood leukocytes (PBLs) of bone marrow transplantation (BMT) patients. An in-house real-time PCR assay based on TaqMan technology was developed to monitor the quantity of CMV DNA in PBLs of the BMT recipients. Sequential blood samples (415 specimens) were collected from 43 patients as weekly intervals until day 100 after transplantation. The CMV DNA was quantified in parallel with the pp65 antigenemia assay in PBL samples. Viral reactivation occurred in 51% and 41.8% of the recipients as detected by RQ-PCR and antigenemia assays respectively. There was a significant correlation between both assays ($P<0.0001$); however, the RQ-PCR was more sensitive than the antigenemia. CMV DNA was detected by the RQ-PCR by a median of 14 days earlier than the antigenemia. Preemptive therapy was implemented in the antigenemia positive cases. The administration of ganciclovir led to a rapid decrease in the viral load. After preemptive therapy, the antigenemia achieved a negative result earlier than the RQ-PCR assay (a median of 17.5 days). An increase of viral load in both quantitative assays and of cyclosporine serum level were identified as the most significant risk factors for CMV reactivation. The quantitative CMV PCR might be a useful tool for monitoring the CMV reactivation and guiding the efficacy of the CMV preemptive therapy in BMT recipients.

Keywords Cytomegalovirus · Cyclosporine · pp65 Antigenemia · Real-time PCR · TaqMan

S. H. Ghaffari (✉) · M. Dehghan · K. Alimoghaddam · A. Ghavamzadeh
Hematology, Oncology and BMT Research Center,
Tehran University Medical Sciences,
Shariati Hospital,
Tehran 11411, Iran
e-mail: shghaffari2000@yahoo.com

N. Obeidi · A. Gharehbaghian
Iranian Blood Transfusion Organization Research Center,
Tehran, Iran

Introduction

Human Cytomegalovirus (CMV) causes significant morbidity and mortality in severely immunosuppressed individuals such as bone marrow transplantation (BMT) patients [1]. The prevention of CMV disease is therefore a major goal in the clinical management of these patients. Although, antiviral prophylaxis has led to a reduction in both morbidity and mortality of CMV disease in the past few years, however, the severe marrow toxicity, increased invasive fungal infection [2], and delayed CMV-specific T-cell reconstitution [3] associated with currently available antiviral agents (i.e., ganciclovir, foscarnet, and cidofovir) has remained a significant problem [1]. There are two strategies that are currently being used to prevent the development of CMV disease in bone marrow transplant recipients. First, the universal prophylaxis consists of effective viral therapy is given to all recipients at risk of CMV reactivation [4, 5]. Second, the preemptive therapy consists of selective viral therapy is given only to patients with proven CMV reactivation [6, 7]. Preemptive therapy reduces the incidence and the severity of the CMV disease; however, it depends on early laboratory identification of those at a high risk of disease [8]. The key to efficient and effective management of CMV infection in these patients is to develop a highly sensitive and quantitative detection

method capable of quantifying the CMV viral load and rapidly identifying patients at high risk of developing CMV disease, and monitoring the preemptive antiviral therapeutic strategies [9].

Several techniques are presently available for detection of CMV include shell viral culture [10], the CMV antigenemia assay [11], hybrid capture assay [12], and qualitative [13] and quantitative PCR assays [14–23]. At present, the CMV antigenemia assay is widely used to monitor BMT recipients. This method aims to detect pp65 antigen expressed in CMV-infected all nucleated cells (ANC) using a monoclonal antibody [24]. Although, a correlation was found between the number of pp65-positive PBLs and the development of clinical symptoms, this method poses a number of problems. It is difficult to perform before engraftment because the number of leukocytes is limited and false-negative results are obtained due to the poor sensitivity of the technique and the weak expression of the pp65 antigen in white blood cells in some patients who develop CMV disease [25]. Alternatively, a PCR method using CMV-specific primers has been used to diagnose CMV reactivation early after BMT. In addition to its high sensitivity and specificity for detecting CMV, this test is highly advantageous in that it is not influenced by the white blood cell count in peripheral blood or by pp65 antigen expression. PCR is definitely a useful diagnostic method for detecting CMV reactivation, but it may be too sensitive for clinical use. That is, when the results of CMV PCR are positive, they do not necessarily indicate an imminent risk of CMV disease and the results obtained are frequently overestimated [15, 26]. Recently, quantitative PCRs based on TaqMan technologies for detection of CMV reactivation after BMT have been investigated. This method measures PCR product accumulation by means of a dual-labeled fluorogenic probe and provides a very accurate and reproducible measure of gene copies [9, 15, 21, 23].

The aim of our study was to develop an in-house quantitative TaqMan-based PCR assay capable of quantifying the CMV load in PBLs with a high precision and reproducibility and to evaluate the feasibility and advantages of this real-time PCR assay to monitor CMV infection in allogeneic bone marrow recipients in comparison with the pp65 antigenemia reference method.

Materials and Methods

Patients and Samples

A total of 415 samples from 43 patients who underwent related allogeneic BMT between April 2004 and February 2005 were enrolled in this study. All patients gave their written informed consent. The blood samples for the CMV antigenemia assay and CMV DNA detection were drawn weekly from the day prior to the initiation of conditioning regimen until day +100 post-transplantation. CMV PP65 antigenemia test and CMV DNA were prospectively quantified in PBLs samples. The patients were evaluated weekly for development of any signs or symptoms of CMV infection, WBC, acute graft versus host disease (GVHD), cyclosporine level, and number of platelet and packed RBC transfusions. GVHD prophylaxis consisted of cyclosporine-A (CSA) 3 mg/kg I.V. from day −2 to +6 and changed to oral CSA 12 mg/kg until day +60. The cyclosporine serum level was checked weekly and tried to maintain it between 100–300 ng/ml. The drug tapered off by 5% weekly from day +60 to 180 in cases with no GVHD.

Monitoring and Preemptive Therapy of CMV Viremia

The patients were monitored weekly by antigenemia and RQ-PCR assays for evidence of CMV viremia. The result of antigenemia was used to determine each patient's preemptive treatment. Preemptive ganciclovir therapy was initiated when more than five antigen positive cells/5×10^4 PBL were found to be positive by the antigenemia assay. Preemptive therapy consisted of the I.V. administration of ganciclovir at 5 mg/kg two times daily for at least 19 days or until the patient was negative by antigenemia, followed by 1 month of maintenance therapy (ganciclovir at 5 mg/kg/day for 5 days/week).

Samples Preparation

About 5 ml of an EDTA-treated peripheral blood (PB) samples were drawn from The BMT recipients and from the healthy volunteers once a week from the time of admission until the 100 days posttransplant. About 2 ml of whole blood was used for the CMV antigenemia assay and the remaining blood was used for DNA extraction. Nucleated cells were obtained by centrifugation of whole blood, and red blood cells were destroyed with a hypotonic solution (0.2% NaCl). About 5×10^6 nucleated cells were lysed and DNA was extracted. The extracted DNA was then dissolved in 100 μl of distilled water and the concentration of DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm.

CMV pp65 Antigenemia Assays

The CMV antigenemia assays were performed by indirect immunofluorescence detection of pp65 (65 to 68 kDa) in PBLs, by the standard procedures using CMV Brite Turbo Kit (Argene Biosoft, Varilhes, France). Briefly, cytospin slides with 200,000 cells per glass slide were prepared, fixed and permeabilized. The presence of the CMV pp65

antigen was detected with monoclonal antibody against the pp65 antigen of CMV and was visualized with a specific secondary antibody. The numbers of CMV antigen-positive cells were counted and the results were expressed as the number of positively staining cells per 50,000 leukocytes.

Real-time Quantification PCR

For generation of a standard curve for the routine TaqMan runs, a plasmid containing the 435 bp region of UL83 gene was constructed. The corresponding sequence of the 450 bp gene region was inserted into pTZ57R/T vector using a In T/A cloning Kit (Fermentas UAB, Lithuania) and termed pTZ-UL83. The ligated product was transformed into DH5α bacterial strain. The colonies that were obtained were prescreened by PCR to confirm the size of the insert. After plasmid preparation, linearization with restriction enzyme, and purification from agarose gel, the DNA concentration was determined with a spectrophotometer and the corresponding copy number was then calculated. A standard graph of the Ct values obtained from serially diluted pTZ-UL83 (10 to 10^7 copies per capillary) was constructed. Using a Roche LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany), the Ct values from clinical samples were plotted on the standard curve, and the copy numbers were automatically calculated. A sample consisting of distilled water was used as a negative control.

The sequences of the PCR primers and the probe used to quantify CMV were selected from the phosphorylated matrix protein (pp65) gene (UL83 region; locus HSPPBC; GenBank) of CMV as described by Griscelli et al. [9]. The sequences of the forward and reverse primers were 5′-GCA GCC ACG GGA TCG TAC T-3′ and 5′-GGC TTT TAC CTC ACA CGA GCA TT-3′, respectively. The TaqMan probe (5′-FAM-CGC GAG ACC GTG GAA CTG CG-TAMRA-3′) selected between both primers was fluorescence labeled with 6-carboxy fluorescein (FAM) at the 5′ end as the reporter dye and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end as the quencher. All PCR reactions were preformed in a total volume of 20 μl containing 1× Taq Polymerase buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 200 nM each primer, 100 nM TaqMan probe, 1 U Taq DNA Polymerase and 100 ng of DNA. The thermocycler condition was 1 cycle at 95°C for 4 min; followed by 45 cycles, each at 95°C for 10 s, 60°C for 45 s; and 1 cycle at 40°C for 1 min.

Criteria for Diagnosis of CMV-reactivation, Infection and CMV Disease

A patient was considered to have CMV-reactivation when CMV was detected in his blood by antigenemia and/or PCR assays. A patient was classified as symptomatic if he had one or more of the symptoms defined by the International CMV work shop [27]. A patient was considered to have CMV-disease when CMV was demonstrated in biopsy specimens by immunohistochemical analysis and this was accompanied by clinical signs and symptoms.

Data Analysis

Comparison of data was performed by the nonparametric Spearman correlation coefficients and Mann–Whitney *U* test with the assistance of SPSS 13 software (SPSS, Chicago, IL). By this test, the average ranks of two independent samples are statistically compared. The Wilcoxon test was used to compare the value of matching samples. Two-tailed *P* values of <0.05 were considered to be of statistical significance.

Results

Patient Characteristics

The patient's characteristics are summarized in Table 1. Forty three bone narrow transplants recipients were enrolled in this study. There were 11 females and 32 males, with a median age of 22 years (age range, 9 to 51 years). All of the recipient patients were seropositive for CMV before BMT, and they received a graft either from seronegative donors ($n=2$) or from seropositive donors ($n=41$). Acute graft versus host disease (GVHD) grading was based on criteria defined by Przepiorka et al. [28].

Establishment of a Real-time PCR Assay for Quantifying CMV-DNA

A recombinant plasmid (pTZ-UL83) containing 450-bp region of a sequence located in the UL83 gene, which codes for the lower matrix protein detected in the pp65 antigenemia test, was constructed. A serially diluted pTZ-UL83 plasmid was then tested by the real-time PCR assay, and a standard curve of the Ct values was constructed (Fig. 1a). A wide linear range from 10 to 10^7 copies of the control plasmid was established (Fig. 1b). A minimum of five copies of the plasmid could be detected by this system. In order to confirm the specificity of this assay, a CMV-negative cell line, PBLs from CMV-seronegative patients, and several virus strains were tested by this system and all were negative for CMV. No cross-reactivity between CMV and herpes simplex viruses or Epstein–Barr virus was observed.

In order to determine the CMV viral load in blood of the BMT recipients by real-time PCR, we first tested for viral DNA in the PB samples taken from ten healthy volunteers. The prevalence of CMV in the PB samples was 60%; however, the CMV number was less than 10^3 copies/assay.

Table 1 Patient's characteristics

Characteristic	Value
No. of patients	43
Median age (range) in years	22 (9–51)
Sex	
Male	32 (74%)
Female	11 (26%)
Underlying disease	
Acute myeloid leukemia (AML)	17 (39.5%)
Acute lymphoblastic leukemia (ALL)	10 (23.3%)
Chronic myelogenous leukemia (CML)	10 (23.3%)
Myelodisplastic syndrome (MDS)	4 (9.3%)
Aplastic anemia (AA)	1 (2.3%)
Major thallassemia (MT)	1 (2.3%)
Pretransplantation WBC	6,290 (840–87,700)
Pretransplantation CMV IgG titer	15 (1.5–258)
Pretransplantation CMV serology (recipient/donor)	
D+/R+	41 (95.3)
D−/R+	2 (4.7)
Type of transplant	
Matched related	43 (100)
Conditioning regimen	
Busulfan + Endoxan (AML, ALL, CML)	32 (49)
Fludarabin + Busulfan (M.T)	33 (51)
ATG + Endoxan (AA)	26 (40)
GVHD prophylaxis	
Cyclosporine + methotrexate	43 (100)
Acute GVHD grade	
None	8 (18.6%)
Grade 1	10 (23.3%)
Grade 2	19 (44.2%)
Grade 3	5 (11.6%)
Grade 4	1 (2.3%)
Symptomatic CMV infection	6 (14%)
Preemptive treatment	7 (16.3%)
Episodes of CMV reactivation/patients	
Total	29/23
By real-time PCR assay	28/22
By pp65 antigenemia assay	21/18
Median days (range) of first positive results	
By RQ-PCR	21 (7–49)
By pp65 antigenemia	35 (14–49)
Median days (range) of first negative results after ganciclovir treatment	
By RQ-PCR	17.5 (7–28)
By pp65 antigenemia	7 (7–28)

Therefore, we considered the normal cut-off level of CMV in the PB of healthy subjects to be 10^3 copies/2×10^5 PBL.

Detection of CMV Viremia by CMV Real-time PCR and pp65 Antigenemia Assays

We examined the kinetics of the CMV viral load in patients who underwent allogeneic bone marrow transplantation. Peripheral blood was collected once before transplantation and once per week after transplantation for 100 days. A total of 415 sequential peripheral blood samples from 43 patients were collected and analyzed simultaneously by the real-time PCR and the pp65 antigenemia assays. As shown in Table 2, 93 samples (22.4%) were positive by the real-time PCR assay and 38 samples (9.1%) were positive by pp65 antigenemia assay. About 36 of the 93 samples were positive by both CMV DNA and pp65 antigenemia, while 320 of the 415 samples were negative by both assays. Fifty seven PCR positive samples were pp65 antigen-negative. The result obtained for the two diagnostic methods with the 415 samples were significantly correlated (n=415; r=0.295; P<0.0001 by the Spearman rank test). We also studied correlation between the CMV DNA copy number and the number of pp65-positive cells in PBLs on the basis of the results for 93 samples which were positive by the PCR assay (Fig. 2). A statistically significant correlation was observed between the CMV DNA copy number and the number of pp65-positive cells, as examined by the Spearman rank test (n=93; r=0.369; P<0.0001).

A total of 28 episodes of CMV reactivation were detected by PCR in 22 patients during the follow-up (16 patients had one and six patients had two episodes). A total of 20 episodes of CMV reactivation were detected by pp65 antigenemia in 18 patients (16 patients had one and two patients had two episodes). In 19 episodes of CMV reactivation both the CMV real-time PCR and pp65 antigenemia assays were found to be positive. In four of these 19 episodes, the PCR test and antigenemia became positive simultaneously, while in remaining 15 episodes the real-time PCR test preceded the antigenemia assay by a median of 14 days (ranged, 7–35) (P=0.001 by the Wilcoxon test).

Preemptive Therapy

Seven of the 22 CMV PCR positive patients received preemptive ganciclovir therapy because of the antigenemia positive evidence of CMV viremia (>5 antigen positive cell/50,000) on the day that positivity was documented. The treatment led to a marked decrease in both the CMV DNA copy number and he number of the CMV antigen-positive cells. The median time interval necessary to obtain a negative results after implementation of preemptive treatment by CMV DNA PCR assay was 17.5 days (range, 7–28 days) and by the pp65 antigenemia assay was 7 days (range, 7–28 days). The antigenemia achieved a negative result earlier than the real-time PCR test (a median of 7 days, ranged, 0 to 14) which was moderately significant (P=0.038 by the Wilcoxon test).

In 15 of the 22 CMV PCR positive patients, preemptive treatment was not implemented either because of a low (ten

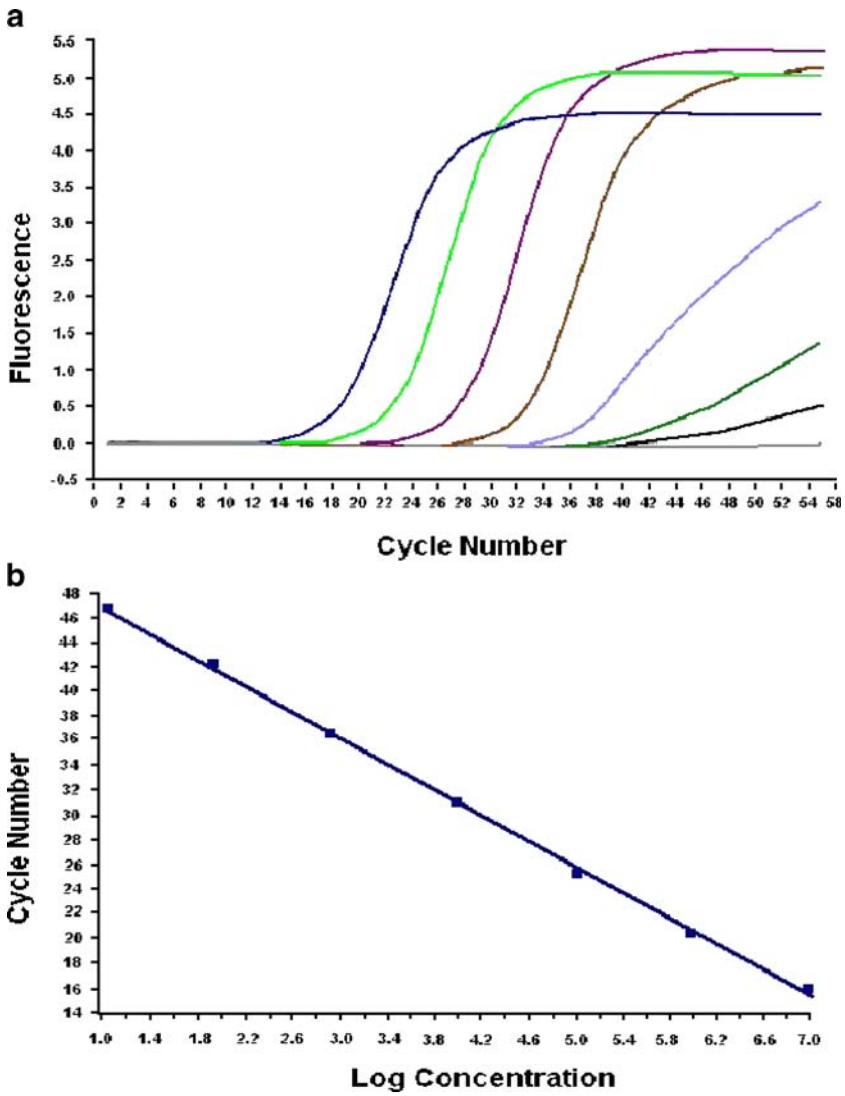

Fig. 1 **a** Amplification profile of standard CMV real-time PCR. Serial 10-fold dilutions with 10^4 to 10 copies of CMV-plasmid per reaction (capillary) were amplified for 45 cycles. **b** Standard curves for CMV real-time PCR. Ct values were plotted against various numbers of copies of the standard CMV-recombinant plasmid. The correlation coefficient was 0.9968, and the slopes were −3.7

patients) or negative (five patients) antigenemia assay. In these cases, the CMV DNA copy number was significantly lower than the preemptive treated patients (with median of 3.6×10^4 vs 5.5×10^5 copies; $P=0.003$ by Mann–Whitney test) (Fig. 3a). In 12 of these 15 cases, after 3 weeks the CMV PCR result became spontaneously negative and the patients did not develop CMV disease. However, 3/15 patients who had CMV antigenemia <5 later became CMV symptomatic, one died from pulmonary infection. It appears that a threshold level of less than five antigen positive cell/50,000 PBLs should be used as an indicator of risk to guide the preemptive therapy in haematopoietic stem cell transplant recipients.

Figure 4 illustrates the clinical courses of four patients (P8, 19, 31, and 40). Three of the patients had one episode and patient 19 had two episodes of CMV reactivation as detected by both CMV real-time PCR and pp65 antigenemia assays. Three patients were administered with ganciclovir starting on the day they became positive for CMV in the antigenemia assay, as shown by an arrow.

Symptomatic/Asymptomatic Patients

In this study, six patients were diagnosed as having CMV-related symptoms. As shown in Fig. 3b, the maximum CMV DNA viral loads were higher in the patients with

Table 2 Results of CMV real-time and CMV pp65 antigenemia assays in PBLs of BMT recipients

		pp65 Antigenemia assay		
		Positive	Negative	Total
Real-time PCR assay	Positive	36	57	93
	Negative	2	320	322
	Total	38	377	415

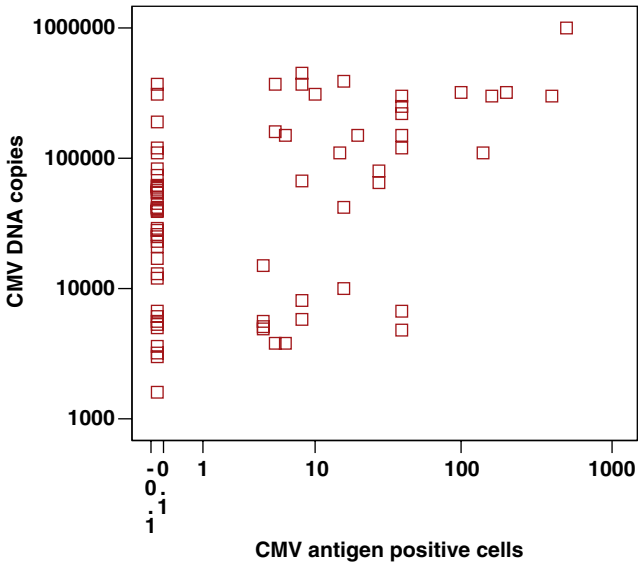

Fig. 2 Correlation between CMV DNA copy number and the number of pp65-antigen positive cells in PBLs samples of 93 PCR-positive recipients. (Spearman rank test: n=93, r=0.369, P<0.0001)

CMV symptomatic infection (six patients) than in the asymptomatic patients (16 patients). The median peak DNA viral load in the blood of the symptomatic patients was 3.7×10^5 copies/ml (range, 5.6×10^4 to 4.5×10^5), which was significantly higher than that observed in the asymptomatic group (median, 4.1×10^4 copies/ml; range, 3.8×10^3 to 1.5×10^6; P<0.01 by Mann–Whitney test).

Multivariate Analysis

The distribution of age, sex, WBC counts, platelet counts, Hb levels, recipient–donor serostatus, and acute GVHD did not differ significantly between the group of patients who had symptomatic CMV infection and those who remained asymptomatic (Fisher's exact and Mann–Whitney tests; P> 0.05). In this study, increasing the viral load in both of the quantitative assays emerged from multivariate regression analysis as the most significant risk factor for symptomatic CMV infection. Immunosuppressive regimen was also identified as a risk factor in univariate analysis. The CMV reactivation was significantly related to the increase of cyclosporine level.

As illustrates in Fig. 5, the maximum cyclosporine levels were significantly higher in CMV DNA reactivated patients (22 patients) than in the unreactivated patients (21 patients). The median peak cyclosporine levels in the blood of the CMV DNA reactivated patients was 571 ng/ml (range, 285–990), which was significantly higher than that observed in the CMV DNA negative patients (median, 440.4 ng/ml; range, 80–448; P<0.0001 by Mann–Whitney test and Wilcoxon test).

Discussion

We established a real-time PCR assay for quantifying CMV-DNA based on TaqMan technology. The CMV TaqMan PCR was based on the amplification of a 159-bp region of a sequence located in the UL83 gene which codes for the lower matrix protein detected in the pp65 antigenemia assay [9]. The RQ-PCR technique established in this study allowed the quantification of CMV DNA over a wide dynamic range for CMV gene amplification (10 to 10^7 copies of plasmid). We could diagnose CMV infection in the clinical samples and detect as few as five copies of

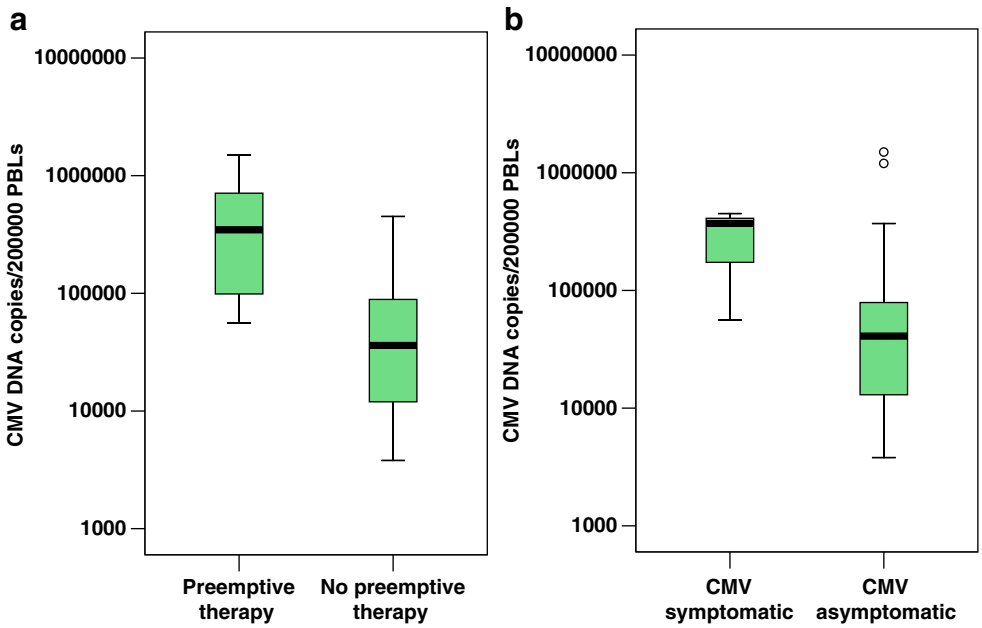

Fig. 3 Comparison of peak CMV DNA viral loads. **a** The median peak CMV DNA viral load was significantly higher in patients who received preemptive therapy (n=7) than in untreated patients (n=15) (with median of 3.5×10^5 vs 3.6×10^4; P=0.003 by Mann–Whitney test). **b** The median peak CMV DNA viral load was significantly higher in patients with CMV symptomatic infection (n=6) than in asymptomatic patients (n=16) (with median of 3.7×10^5 vs 4.1×10^4 copies; P=0.01 by Mann–Whitney test)

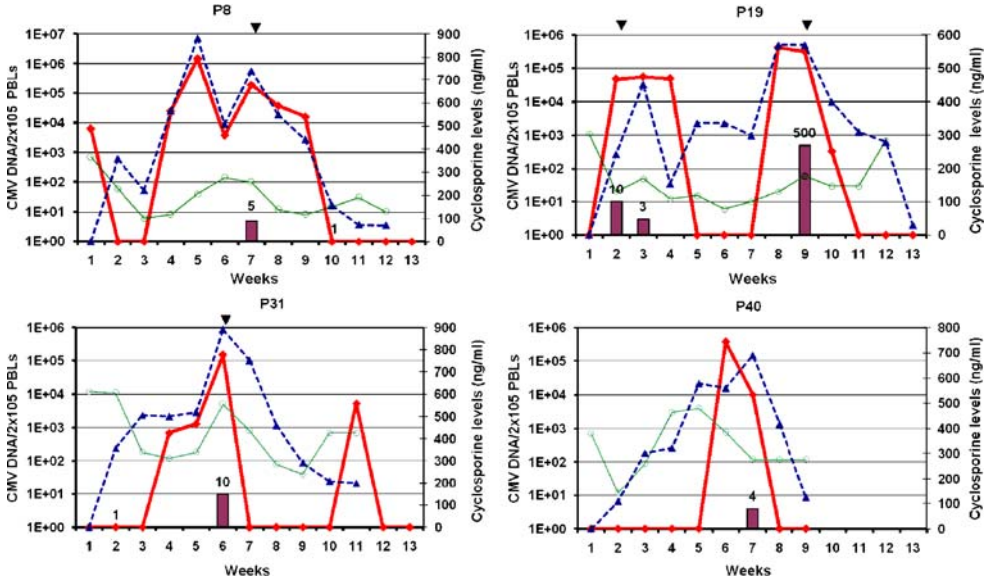

Fig. 4 Clinical course of CMV reactivation in BMT patients. Sequential samples from patients were analyzed by both real-time PCR and pp65 antigenemia assays. CMV viral load profiles were plotted for four patients who had elevated viral loads. Preemptive treatment with ganciclovir was implemented in three patients based upon antigenemia assay positivity (as shown by an *arrow head*). *Solid line*, the number of CMV DNA copy number detected by the real-time TaqMan assay; *bars and numbers*, the number of CMV antigen-positive cells/5×10^4 PBLs detected by the pp65 antigenemia assay; *hatched line*, serum cyclosporine level reported as nanogram per milliliter; *shaded line with open circles*, white blood cells (WBC)

CMV DNA per 2×10^5 PBL of the patient's samples. Using this technique, 415 PBL samples from the 43 BMT recipients were evaluated in parallel with the pp65 antigenemia assay. We used PBL since these cells are the main CMV carriers during the active CMV infection. The detection of CMV antigenemia in PBLs has been shown to be an early marker of CMV infection [9]. Some groups have also found that detection and/or quantitation of DNA in PBL provides better clinical correlation than detection and/or quantitation of DNA in plasma [29–33].

In the present study, viral reactivations occurred in 51% and 41.8% of allogeneic bone marrow recipients as detected

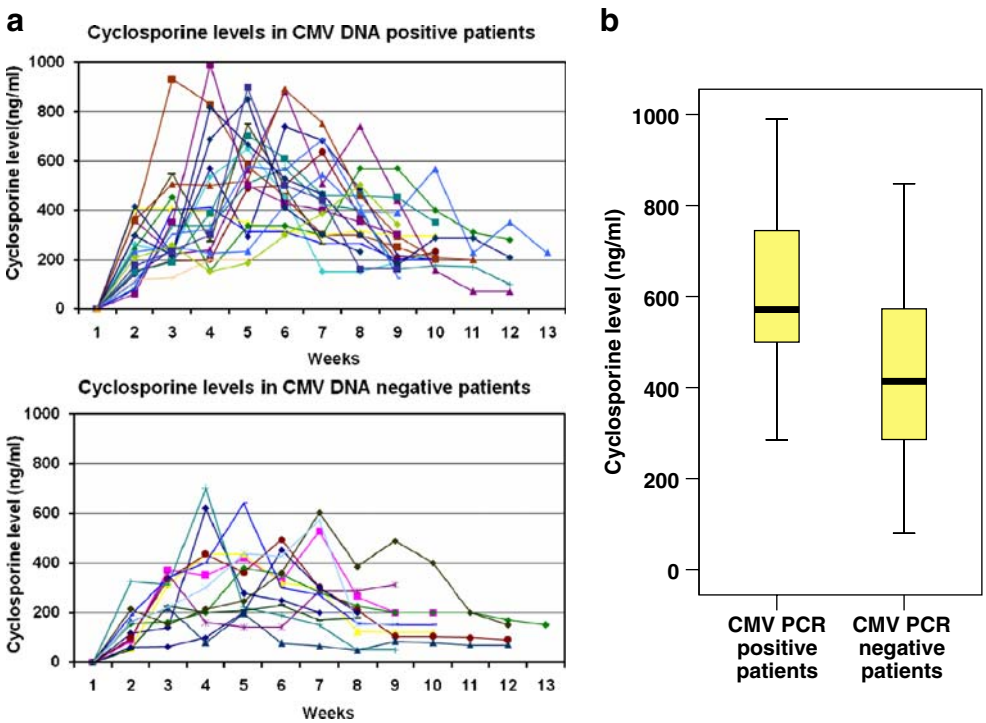

Fig. 5 The serum cyclosporine levels in the allogeneic post-BMT recipients during the 100 days post-BMT. **a** Graphic representation of cyclosporine levels in serum of the CMV DNA positive and negative patients. **b** Comparison of the peak cyclosporine levels in patients with CMV DNA positive and negative. The median peak cyclosporine level was significantly higher in the CMV reactivated patients (n=22) than in unreactivated patients (n=21). (Wilcoxon and Mann–Whitney test: P<0.0001)

by RQ-PCR and antigenemia assays, respectively. We found a significant correlation between the results of the CMV RQ-PCR and the antigenemia assays in PBL samples (P=0.0001). This is in agreement with the results of other studies that have used real-time PCR assays [9, 14, 15, 20, 22, 23, 33–35]. Both assays were concordant for 85% of the patients and 86% of the specimens. However, the PCR quantification of CMV DNA was more sensitive than the antigenemia assay for the detection and monitoring of CMV reactivation in BMT patients. The sensitivities of the PCR assay and the antigenemia were found to be 96.5% and 69%, respectively. By the PCR assay, 28 of 29 episodes of CMV viremia could be diagnosed, while the antigenemia test was able to detect only 20 episodes. The real-time PCR produced a 1.4% increase in the rate of detection of CMV. Also, as shown in Fig. 2, there were samples with a negative or a low level of antigenemia with a large number of CMV DNA copies. Similar differences between the results of an antigenemia assay and quantitative PCR have been reported by others [9, 14, 15, 20, 22, 23, 33–36].

While detection of CMV pp65-antigen is still widely used for monitoring CMV infection, real-time PCR assays have been recently developed for routine quantitation of CMV DNA. However, correlations are lacking between results of pp65 antigenemia and quantitative PCR assays [34]. Identification of a cutoff level for RQ-PCR assay would be an important indicator of time to initiate an anti-CMV treatment [15] and would therefore reduce the number of patients treated with preemptive therapy who are not destined to develop CMV disease [8]. Several studies have attempted to determine a CMV DNA copy number equivalent to the levels of antigenemia and to implement CMV PCR as pp65 antigenemia in clinical practice. Martin-Davila et al. [37] found an antigenemia of ten positive cells/2×10^5 PMNs equated with a plasma CMV DNA of 1,330 copies/ml or a PMN cutoff of 713/5×10^6 cells. For an antigenemia level of 20 positive cells/2×10^5, the comparable copy numbers for plasma was again 1,330 copies/ml, but increased to 4,755 copies/5×10^6 PMN cells. Mhiri et al. [35] defined a positive cut-off value equivalent to 2,150 CMV DNA copies/ml of white blood cell samples by hybrid capture assay and a viral load higher than 400 copies/ml of plasma samples by RQ-PCR. Garrigue et al. [34] reported thresholds of ten and 50 positive cells/2×10^5 cells were equivalent to 3.3 log10 copies/ml (2,000 copies/ml) and 3.8 log10 copies/ml (6,300 copies/ml), respectively. Three additional studies also reported the threshold of 50 pp65 antigen-positive cells/2×10^5 PBLs were correlated to ~4 log10 genome copies/ml of whole blood [38–40]. Guiver et al. [41] found a higher threshold (4.6 log10 copies/ml), possibly because of a different technology and DNA input. In our study, we defined a positive cut-off value higher than 1,000 CMV DNA copies/2×10^5 of PBL by RQ-PCR; however, the establishment of a threshold to initiate an anti-CMV treatment was not possible and needed confirmation by testing a larger number of patients. All these results shows that studies evaluating these tests are highly heterogeneous, there is no available international standard for CMV PCR, and each study determined its own assays characteristics for their own setup. There is also no consensus as the optimal blood compartment (whole blood, Peripheral blood leukocytes, plasma) as of yet [34]. Most reports on detecting CMV DNA by PCR have utilized 'in-house' assays with variable primers for the same gene or different genes (e.g. the immediate early CMV gene or the CMV DNA polymerase gene) making it difficult to extrapolate results from one institution to another [42]. Therefore, each clinical laboratory needs to determine its own assays characteristics for an appropriate patients' monitoring [34]. There is some commercially available assay that would allow laboratories to generate data with more widespread applicability. Lengerke et al. [43] found 89–92% concordance between an in-house quantitative PCR (amplified a portion of the immediate early CMV gene) on whole blood and CACM (commercially available assay amplifies a 365 bp region of the CMV DNA polymerase gene) on plasma. Allice et al. [44] found a 98% correlation between a TaqMan real time PCR amplifying the immediate early gene and CACM, both assays using PBL. The authors conclude that the real time PCR assay offers several advantages, including speed and precision versus CACM. Pumannova et al. [45] also concluded that the light cycler real time quantitative PCR was superior to an ELISA-PCR, both amplified the same segment of CMV genome, the UL 83 gene encoding pp65. These authors felt that the light cycler was superior in performance and rapidity and it was more suitable for routine diagnosis.

The analysis of CMV viremic episodes in which the results of both tests eventually turned positive revealed that a detection of CMV DNA by real-time PCR in PBL allowed for an early diagnosis of CMV replication after transplantation. CMV DNA was detected by PCR in 15 of the 19 episodes by a median of 14 days prior to the detection of an antigenemia result which indicates that a positive PCR test result is an earlier marker of CMV viremia than a positive antigenemia result (P=0.001 by the Wilcoxon test). Similar results have been reported by others. Griscelli et al. [9] found that CMV replication in blood leukocytes was detectable by PCR with a median of 15 days prior to antigenemia. Leruez-Ville et al. [23] also reported that Real-time CMV PCR in blood plasma allowed for an early diagnosis of CMV replication after transplantation, with a positive CMV PCR result occurring before a positive CMV antigenemia result by a median of 8 days. Schvoerer et al. [36] results showed that the PCR was

regularly positive before a antigenemia assay (4 to 52 days before). Mori et al. [46] studied the clinical significance of early diagnosis of CMV in 19 allogeneic stem cell transplanted patients. They concluded that CMV antigenemia had a limited value in prediction of early diagnosis of CMV gastro-intestinal disease and suggested that PCR could have a more diagnostic significance. Also, it has been reported that the use of antigenemia assay will not give an accurate indication of a viral load or viral load rate increase since it counts the number of infected cells only. On the other hand, a viral dynamics study in CMV showed that the initial CMV load and the rate of viral load increase are significantly associated with the development of disease [35].

The treatment induced a rapid decrease in both the number of viral DNA copies and the number of CMV antigen-positive cells. The first negative antigenemia results preceded the first negative PCR results by a median of 7 days (range, 0 to 14). It has been suggested that the CMV DNA may represent the true viral copy number in the specimens; a negative PCR result after treatment might be a better indicator of a completely successful treatment than the pp65 antigemia [23]. Therefore, continuing anti-CMV therapy until the PBL CMV PCR becomes negative might prevent a recurrence of CMV disease. By contrast, discontinuation based on the first negative antigenemia results has led to a significant number of CMV pneumonia [11]. It is also shown, by Weinberg et al. [47], that the patients who developed a recurrent CMV disease were still positive by PCR at the time the therapy was discontinued for the preceding episode; whereas, the patients who did not developed a recurrent disease tended to clear the CMV DNA from their blood faster. These observations suggest that quantitative CMV PCR might be a useful tool to monitor the efficacy of anti-CMV therapy in bone marrow recipients and the discontinuation of CMV preemptive therapy based on quantitative CMV PCR might prevent a recurrent CMV disease. Ikewaki et al. [48] observed that real-time PCR was more suitable for monitoring CMV reactivation in adult T-cell leukemia–lymphoma patients than the antigenemia assay. Ksouri et al. [33] suggests that the CMV-DNA assay is better assay for monitoring patients receiving preemptive therapy, especially in CMV-GI disease, and that after CMV-infected cell destruction, the genome of defective virus could still be remaining and detected by PCR.

In this study, increasing the viral load in both of the quantitative assays emerged from multivariate regression analysis as the most significant risk factor for CMV infection. Also, the immunosuppressive regimen was identified as a risk factor in univariate analysis. Increasing cyclosporine serum level was significantly related to the CMV reactivation. In general, the maximum cyclosporine levels were significantly ($P<0.0001$) higher in the CMV reactivated patients than in the PCR negative patients (Fig. 5). We examined the patient's cyclosporine level that was performed for all the patients, and attempted to correlate changes in the cyclosporine level with variations in the CMV viral load in PBL. As shown in Fig. 3, as the cyclosporine level rises to the maximum, there is a concomitant rise in the CMV viral load to the highest level. More than 81% of the patients who had a viral reactivation showed a very close correlation between the cyclosporine level and the viral load, where the patients began very low in cyclosporine, and as the cyclosporine levels increase, there was a concomitant rise in the viral load. In some cases, the cyclosporine levels can rise with no change in the viral load, and in others, changes in the viral load were not read out as a change in the cyclosporine level. This information should be helpful in developing future therapeutic approaches including decreasing the immune suppression and the use of CMV-specific T cells immunotherapy. Our results suggest that with more frequent and careful monitoring of cyclosporine serum levels in BMT patients, some of the CMV reactivations may be prevented.

In conclusion, the increases of the viral load in both quantitative assays and of cyclosporine serum levels were identified as the most significant risk factors for CMV reactivation. The results of both quantitative assays were significantly correlated; however, the RQ-PCR assay was more sensitive than the pp65 antigenemia assay. The quantitative CMV PCR might be a useful tool for monitoring the CMV reactivation and the patient's response to antiviral therapy in BMT recipients.

Acknowledgement This work was supported by grants from Hematology, Oncology and BMT Research Center, Tehran University Medical Sciences. We would like to thank Mr. Chahardouli for assistance in lab and Dr. Shamshiri for assistance in the statistical analysis. We would like to thank all clinicians and nurses who participated and provided material for this study. In particular we acknowledge Drs. Jahani, Mosavi, Bibordi, Bahar, Irvani.

References

1. Boeckh M, Boivin G (1998) Quantitation of cytomegalovirus: methodologic aspects and clinical applications. Clin Microbiol Rev 11:533–554
2. Salzberger B, Bowden RA, Hackman R et al (1997) Neutropenia in allogeneic marrow transplant recipients receiving ganciclovirfor prevention of CMV disease: risk factors and outcome. Blood 90: 2502–2508
3. Li CR, Greenberg PD, Gilbert MJ et al (1994) Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood 83:1971–1979
4. Goodrich JM, Bowden RA, Fisher L et al (1993) Ganciclovir prophylaxis to prevent cytomegalovirus disease after allogeneic marrow transplant. Ann Intern Med 118:173–178

5. Winston DJ, Ho WG, Bartoni K et al (1999) Ganciclovir prophylaxis of cytomegalovirus infection and disease in allogeneic bone marrow transplant recipients. Results of a placebo-controlled, double-blind trial. Ann Intern Med 118:179–184
6. Goodrich JM, Mori M, Gleaves CA et al (1991) Early treatment with ganciclovir to prevent cytomegalovirus disease after allogeneic bone marrow transplantation. N Engl J Med 325:1601–1607
7. Schmidt GM, Horak DA, Niland JC et al (1991) A randomized controlled trial of prophylactic ganciclovir for cytomegalovirus pulmonary infection in recipients of allogeneic bone marrow transplants. N Engl J Med 324:1005–1011
8. Aitken CW, Barrett-Muir C, Millar K et al (1999) Use of molecular assays in diagnosis and monitoring of cytomegalovirus disease following renal transplantation. J Clin Microbiol 37:2804–2807
9. Griscelli F, Barrois M, Chauvin S et al (2001) Quantification of human cytomegalovirus DNA in bone marrow transplant recipients by real-time PCR. J Clin Microbiol 39:4362–4369
10. Gleaves CA, Smith TH, Shuster EA et al (1984) Rapid detection of cytomegalovirus in MRC-5 cells inoculated with urine specimens by using low-speed centrifugation and monoclonal antibody to an early antigen. J Clin Microbiol 19:917–919
11. Boeckh M, Gooley TA, Myerson D et al (1996) Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir at engraftment after allogeneic marrow transplantation: a randomized double-blind study. Blood 10:4063–4071
12. Hebart H, Gamer D, Loeffer J et al (1998) Evaluation of Murex CMV DNA hybrid capture assay for the detection and quantification of cytomegalovirus infection in patients following allogeneic stem cell transplantation. J Clin Microbiol 36:1333–1337
13. Einsele H, Steidle M, Vallbracht A et al (1991) Early occurrence of human cytomegalovirus infection after bone marrow transplantation as demonstrated by the polymerase chain reaction technique. Blood 77:1104–1110
14. Gault E, Miche Y, Dehe'e A et al (2001) Quantification of human cytomegalovirus DNA by real-time PCR. J Clin Microbiol 39: 772–775
15. Machida U, Kami M, Fukui T et al (2000) Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation. J Clin Microbiol 38: 2536–2542
16. Mori T, Okamoto S, Matsuoka S et al (2000) Risk-adapted preemptive therapy for cytomegalovirus disease in patients undergoing allogeneic bone marrow transplantation. Bone Marrow Transplant 25:765–769
17. Nitsche A, Steuer N, Schmidt CA et al (2000) Detection of human cytomegalovirus DNA by real-time quantitative PCR. J Clin Microbiol 38:2734–2737
18. Razonable RR, Brown RA, Espy MJ et al (2001) Comparative quantification of cytomegalovirus (CMV) DNA in solid-organ transplant recipients with CMV infection by using two high-throughput automated systems. J Clin Microbiol 39:4472–4476
19. Schaade L, Kockelkorn P, Ritter K et al (2000) Detection of cytomegalovirus DNA in human specimens by lightCycler PCR. J Clin Microbiol 38:4006–4009
20. Tanaka N, Kimura H, Iida K et al (2000) Quantitative analysis of cytomegalovirus load using a real-time PCR assay. J Med Virol 60:455–462
21. Yun Z, Lewensohn-Fuchs I, Ljungman P et al (2000) Real-time monitoring of cytomegalovirus infections after stem cell transplantation using the TaqMan polymerase chain reaction assays. Transplantation 69:1733–1736
22. Yakushiji K, Gondo H, Kamezaki K et al (2002) Monitoring of cytomegalovirus reactivation after allogeneic stem cell transplantation: comparison of an antigenemia assay and quantitative real-time polymerase chain reaction. Bone Marrow Transplant 29:599–606
23. Leruez-Ville M, Ouachee M, Delarue R et al (2003) Monitoring cytomegalovirus infection in adult and pediatric bone marrow transplant recipients by a real-time PCR assay performed with blood plasma. J Clin Microbiol 41:2040–2046
24. Tanabe K, Tokumoto T, Ishikawa N et al (1997) Comparative study of cytomegalovirus (CMV) antigenemia assay, polymerase chain reaction, serology, and shell vial assay in the early diagnosis and monitoring of CMV infection after renal transplantation. Transplantation 64:1721–1725
25. Seropian S, Ferguson D, Salloum E et al (1998) Lack of reactivity to CMV pp65 antigenemia testing in a patient with CMV disease following allogeneic bone marrow transplant. Bone Marrow Transplant 22:507–509
26. Solano C, Munoz I, Gutierrez A et al (2001) Qualitative plasma PCR Assay (AMPLICOR CMV Test) versus pp65 antigenemia assay for monitoring cytomegalovirus viremia and guiding preemptive ganciclovir therapy in allogeneic stem cell transplantation. J Clin Microbiol 39:3938–3941
27. Ljungman P, Plotkin S (1995) Workshop on CMV disease—definitions, clinical severity scores and new syndromes. Scand J Infect Dis Suppl 99:87–89
28. Przepiorka D, Weisdorf D, Martin P et al (1994) Consensus conference on Acute GVHD grading. Bone Marrow Transplant 15:825–828
29. Jabs DA, Forman M, Enger C et al (1999) Comparison of cytomegalovirus loads in plasma and leukocytes of patients with cytomegalovirus retinitis. J Clin Microbiol 37:1431–1435
30. Pellegrin I, Garrigue I, Ekouevi D et al (2000) New molecular assays to predict occurrence of cytomegalovirus disease in renal transplant recipients. J Infect Dis 182:36–42
31. Schafer P, Tenschert W, Cremaschi L, et al (2001) Area under the viraemia curve versus absolute viral load: utility for predicting symptomatic cytomegalovirus infections in kidney transplant patients. J Med Virol 65:85–89
32. Boeckh M, Hawkins G, Myerson D et al (1997) Plasma PCR for cytomegalovirus DNA after allogeneic marrow transplantation: comparison with PCR using peripheral blood leukocytes, pp65 antigenemia and viral culture. Transplantation 64:108–113
33. Ksouri H, Eljed H, Greco A et al (2007) Analysis of cytomegalovirus (CMV) viremia using the pp65 antigenemia assay, the amplicor CMV test, and a semi- quantitative polymerase chain reaction test after allogenic marrow transplantation. Transpl Infect Dis 9:16–21
34. Garrigue I, Boucher S, Couzi L et al (2006) Whole blood real-time quantitative PCR for cytomegalovirus infection follow-up in transplant recipients. J Clin Virol 36:72–75
35. Mhiri L, Kaabi B, Houimel M, Arrouji Z, Slim A (2007) Comparison of pp65 antigenemia, quantitative PCR and DNA hybrid capture for detection of cytomegalovirus in transplant recipients and AIDS patients. J Virol Methods 143:23–28
36. Schvoerer E, Henriot S, Zachary P et al (2005) Monitoring low cytomegalovirus viremia in transplanted patients by a real-time PCR on plasma. J Med Virol 76:76–81
37. Martin-Davila P, Fortun J, Gutierrez C et al (2005) Analysis of a quantitative PCR assay for CMV infection in liver transplant recipients: an intent to find the optimal cut-off value. J Clin Virol 33: 138–144
38. Gouarin S, Vabret A, Gault E et al (2004) Quantitative analysis of HCMV DNA load in whole blood of renal transplant patients using real-time PCR assay. J Clin Virol 29:194–201
39. Li H, Dummer S, Estes WR, et al (2003) Measurement of human cytomegalovirus loads by quantitative real-time PCR for monitoring clinical intervention in transplant recipients. J Clin Microbiol 41:187–191
40. Mengelle C, Sandres-Saun'e K, Pasquier C et al (2003) Automated extraction and quantification of human cytomegalovirus

DNA in whole blood by real-time PCR assay. J Clin Microbiol 41:3840–3845
41. Guiver M, Fox AJ, Mutton K, Mogulkoc N, Egan J (2001) Evaluation of CMV viral load using TaqMan CMV quantitative PCR and comparison with CMV antigenemia in heart and lung transplant recipients. Transplant 71:1609–1615
42. Drew WL (2007) Laboratory diagnosis of cytomegalovirus infection and disease in immunocompromised patients. Curr Opin Infect Dis 20:408–411
43. Lengerke C, Ljubicic T, Meisner C et al (2006) Evaluation of the COBAS Amplicor HCMV Monitor for early detection and monitoring of human cytomegalovirus infection after allogenic stem cell transplantation. Bone Marrow Transplant 38:53–60
44. Allice T, Enrietto M, Pittaluga F et al (2006) Quantitation of cytomegalovirus DNA by real-time polymerase chain reaction in peripheral blood specimens of patients with solid organ transplants: comparison with end-point PCR and pp65 antigen test. J Med Virol 78:915–922
45. Pumannova M, Roubalova K, Vitek A, Sajdova J (2006) Comparison of quantitative competitive polymerase chain reaction-enzyme-linked immunosorbent assay with LightCycler-based polymerase chain reaction for measuring cytomegalovirus DNA in patients after hematopoietic stem cell transplantation. Diagn Microbiol Infect Dis 54:115–120
46. Mori T, Mori S, Kanda Y et al (2004) Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant 33:431–434
47. Weinberg A, Hodge TN, Li S et al (2000) Comparison of PCR, antigenemia assay, and rapid blood culture for detection and prevention of cytomegalovirus disease after lung transplantation. J Clin Microbiol 38(2):768–772
48. Ikewaki J, Ohtsuka E, Kawano R et al (2003) Real-time PCR assay compared to nested PCR and antigenemia assays for detecting cytomegalovirus reactivation in adult T-cell leukemia-lymphoma patients. J Clin Microbiol 41:4382–4387