In the name of God

S.U.M.S.

Department of Internal Medicine

Shiraz E-Medical Journal

Vol. 7, No. 3, July 2006

http://semj.sums.ac.ir/vol7/jul2006/pcr.htm

Development and Evaluation of a Real-Time TaqMan-PCR for the Detection of Human Cytomegalovirus DNA in Bone Marrow Transplant Recipients.

Ghaffari H*, Obeidi O**, Dehghan M*, Chahardouli B*, Alimoghaddam K*, Gharebaghian A**, Shamshiri AR*, Ghavamzadeh A*.

* Assistant Professor, , Hematology, Oncology and Bone Marrow Transplantation Research Center; Tehran University of Medical Sciences, Tehran, Iran., ** Research Assistant, Iran Blood Transfusion Organization Research Center, Tehran, Iran.

Correspondence: Dr Seyed H. Ghaffari, Faculty member at Hematology, Oncology and BMT Research Center, Tehran University Medical Sciences, Shariati Hospital, Tehran, Iran, 11411, Telephone: +98 21 84902638, Fax: +98 21 88004140, Email: shghaffari2000@yahoo.com.

Abstract:

Introduction: Cytomegalovirus (CMV) has been recognized as the most important viral pathogen in persons undergoing bone marrow transplantation (BMT). In this study, we present the development of a TaqMan-based real-time PCR assay to quantify human cytomegalovirus (CMV) DNA in peripheral blood leukocytes (PBLs) of bone marrow transplantation patients.

Materials and Methods: A plasmid containing the target sequence from the pp65 region (UL83) of CMV was constructed as a positive control template. Serial dilutions of 10^7 to 10^1 plasmids per assay were prepared. Peripheral blood samples were collected from patients after transplantation. CMV DNA was quantified by RQ-PCR in parallel with the pp65 antigenemia assay in PBL samples.

Results: The real-time PCR assay could detect CMV DNA in patient's samples with a wide linear range, from 10 to over 10^7 copies of CMV. Real-time PCR assay results correlated with those of the CMV pp65 antigenemia assay ($P < 0.0001$).

Discussion: The TaqMan assay may be a useful tool for rapid quantification of CMV infection and for monitoring of CMV reactivation in bone marrow transplantation recipients. The results of both quantitative assays were significantly correlated; however, the RQ-PCR assay was more sensitive than the pp65 antigenemia assay.

Key Words: Cytomegalovirus, TaqMan PCR, pp65 Antigenemia, Bone Marrow Transplantation.

Introduction:

Cytomegalovirus (CMV) is the most important viral pathogen in persons undergoing bone marrow transplantation (BMT).[1] The CMV has been associated with an immunosuppressive state, super infection with other opportunistic pathogens, and allograft rejection, causing significant morbidity and mortality in transplant recipients.[2,3] Two different strategies, prophylaxis and preemptive therapy, are used for the prevention of symptomatic CMV infection after transplantation. The former consists of the administration of an effective antiviral agent, such as gancicovire to all recipients at risk of CMV reaction, whereas the latter strategy uses antiviral only in persons with proven viral reaction before the state of the occurrence of disease emerges.[1,2] As antiviral therapy is myelotoxic and nephrotoxic, full treatment is often preemptive therapy. This therapy should rely on the use of an early and sensitive marker of CMV reactivation.[1,4,5]

Preemptive therapy based on pp65 antigenemia has been widely used to monitor CMV infection in BMT recipients. However, this method has some disadvantages. This technique requires immediate processing, is time-consuming, and cannot be automated. It has a low sensitivity for detecting early infection or disease that may occur before engraftment due to the lack of leukocytes readily examinable at this period. It has also a low predictive value for occurrence of CMV-related gastroenteritis.[6,7]

There are several requirements for an optimal assay for CMV monitoring. PCR has revolutionized diagnostic virology by providing a powerful tool to detect and quantify viral DNA and RNA in various clinical specimens.[7]

Recently, quantitative PCRs based on TaqMan technologies have been investigated for detection of CMV reactivation after BMT. This method measures the PCR product accumulation by means of a dual-labeled fluorogenic probe and provides a very accurate and reproducible measure of gene copies.[4, 5, 6, 8]

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 sensitivity and to evaluate the use 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:

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 post-transplant. 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). The nucleated cells were lysed and DNA was recovered by a boiling method and stored at -20^{o}C until use. DNA was dissolved in 100 ml of distilled water and the concentration of the extracted 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 us-

ing 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.

Development of Real-time PCR:

Cloning of CMV gene: 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 (Figure 1). 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.

Real-time assay: The sequences of the PCR primers and the probe used to quantify CMV were selected from the phophorylated matrix protein (pp65) gene (UL83 region; locus HSPPBC; GenBank) of CMV.[4] 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 ml containing 1X Taq Polymerase buffer, 1.5 mM $MgCl_2$, 200 mM dNTP, 200 nM each primer, 100 nM TaqMan probe, 1U Taq DNA Polymerase and 100 ng ml of DNA. The thermocycler condition was one cycle at 95 ^oC for 4 min; followed by 45 cycles, each at 95^oC for 10 S, 60^oC for 45 S; and one cycle at 40^oC for 1 min.

Data analysis.

Comparison of data was performed by the non-parametric 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.

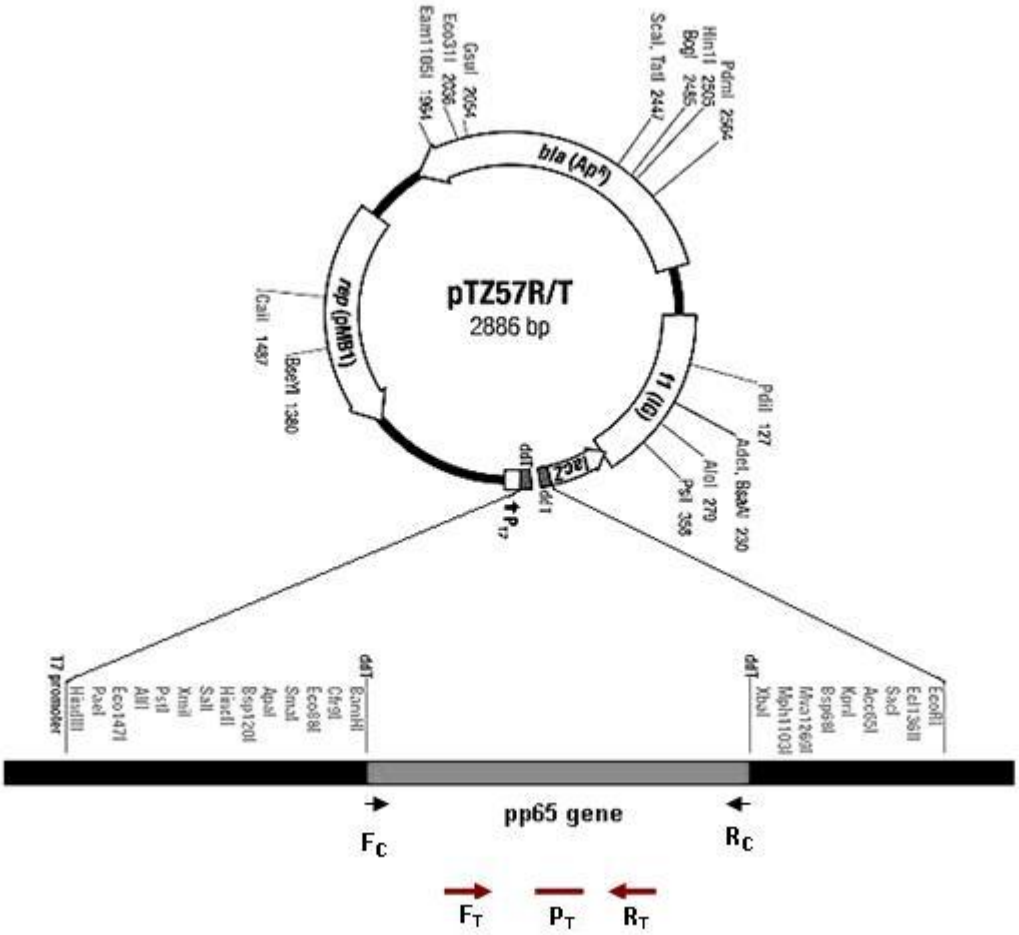

Figure 1. Construction of a CMV-plasmid recombinant. A recombinant plasmid (pTZ-UL83) containing 450-bp region of a sequence located in the UL83 gene, which codes for the lower matrix protein detected in the pp65 antigenemia test, was constructed. Fc and Rc;forward and reverse primers used for cloning of UL83 gene. FT and RT; forward and reverse primers used for real-time assay. PT; TaqMan probe.

Results:

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 (Figure 1). 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 (Figure 2A). A wide linear range from 10 to 10^7 copies of the control plasmid was established (Figure 2B). 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 10 healthy volunteers. The prevalence of CMV in the PB samples was 60%; however, the CMV number was less than 10^3 copies /assay. Therefore, we considered the normal cut-off level of CMV in the PB of healthy subjects to be 10^3 copies/$2x10^5$ 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 1, 93 samples (22.4%) were positive by the real-time PCR assay and 41 samples (9.9%) were positive by pp65 antigenemia assay. About 36 of the 93 samples were positive by both CMV DNA and pp65 antigenemia, while 317 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 (Figure 3). 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 ($n = 93$; $r = 0.369$; $P < 0.0001$).

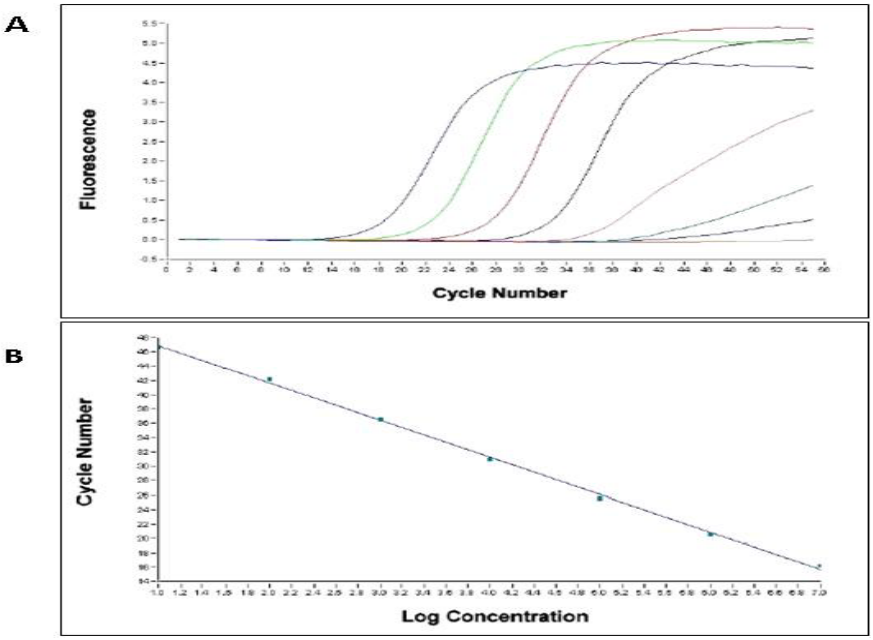

Figure 2. Amplification profile of standard CMV real-time PCR. Serial 10-fold dilutions with 10^4 to 10 copies of CMV-plasmid per reaction (capillary) were amplified for 45 cycles. (B) Standard curves for CMV real-time PCR. Ct values were plotted against various numbers of copies of the standard CMV-recombinant plasmid. The correlation coefficient was 0.9968, and the slopes were -3.7.

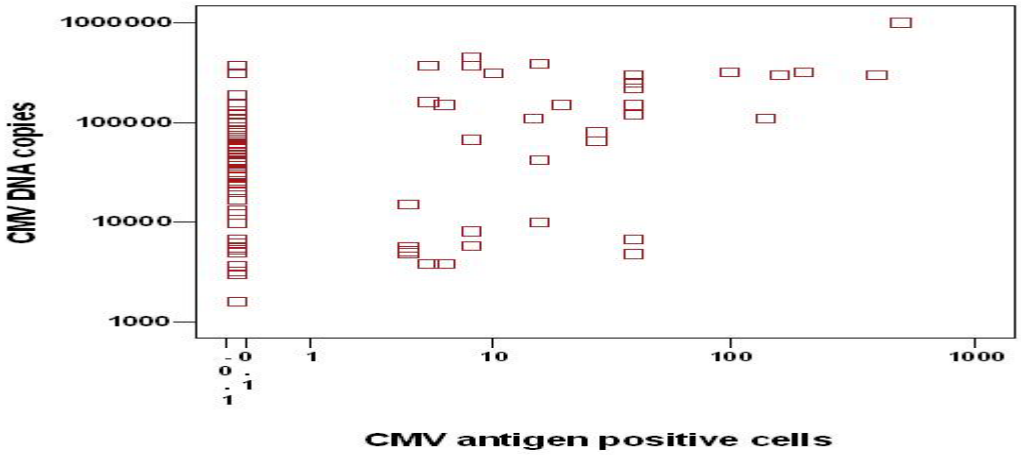

Figure 3. Correlation between CMV DNA copy number and the number of pp65-antigen positive cells in PBLs samples of 93 PCR-positive recipients. (Spearman rank test: n= 93, r= 0.369, P<0.0001).

Table 1. 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	5	317	322
	Total	41	374	415

Discussion:

The aim of this study was to develop a quantitative real-time PCR-based assay capable of quantifying the human CMV viral load in allogeneic bone marrow recipients and then to compare this optimized RQ-PCR assay with the pp65 antigenemia assay in PBLs of the same patients. 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.[4]

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 5 copies of CMV DNA per $2x10^5$ 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.[4] 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.[9,10,11,12]

In the present study, viral reactivation was occurred in 51% and 41.8% of allogeneic bone marrow recipients as detected 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.[4,5,6,14,15] 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 Figure 3, 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.[4,5,6,14,15,16,17]

In conclusion, 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 also like to thank 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, and Irvani.

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