Development of CMV Quantitative Real-Time PCR Assay Validated on White Blood Cells and Plasma Samples from Kidney Transplant Recipients

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ABSTRACT

Cytomegalovirus (CMV) has been recognized as the most important viral pathogen in persons undergoing bone marrow and solid organ transplantation. Rapid diagnosis of CMV infection is critical in the management of the disease so that anti-viral therapy can be started early. An in-house quantitative real-time PCR assay based on TaqMan technology was developed and validated using PBLs and plasma samples from kidney transplant recipients with negative and positive antigenemia assay results.
In the group of renal transplants with negative antigenemia assay, CMV DNA could be detected in 3 PBLs and one plasma sample, indicating that CMV PCR is more sensitive than antigenemia assay in detection of early CMV reactivation..
In the group of patients with positive antigenemia assay, PBLs PCR exhibited equal sensitivity to that of antigenemia assay, furthermore, PBLs were clearly more sensitive and showed higher CMV viral load results compared to plasma samples from patients in the same group. However, none of the plasma specimens with undetectable CMV DNA levels were from patients that later developed active CMV disease, indicating the clinical specificity of plasma samples in determination of active viral replication.
We conclude that PBLs based methods increase sensitivity of detection of CMV DNA, whilst those using plasma increase clinical specificity. The developed assay in this study, inclusive of the results of both PBLs and plasma, provides a test of high sensitivity with the quantitative results

INTRODUCTION

Cytomegalovirus (CMV) continues to be a major cause of morbidity and this occasionally leads to death of patients after organ and bone marrow transplantation (1-3).

Preemptive therapy is a strategy of preventing CMV disease in transplant recipients. It is based on the administration of antiviral agents after transplantation to individuals with microbiological markers that predict CMV disease (4). Highly predictive tests for CMV disease are required when preemptive therapy strategies are to be implemented. Success of preemptive therapy requires good markers for CMV replication (5).

CMV antigenemia assay is a reference test for monitoring CMV viremia and has been validated in clinical trials with immunosuppressed patients (6). However, the assay has several limitations, including, the need for immediate processing (within 6 h) of specimens to achieve optimal sensitivity (7), the interassay variability and the requirement of a sufficient number of leukocytes for performance of the assay (8).

The direct detection of CMV DNA based on PCR has been investigated as an alternative measure for monitoring CMV infection (9, 10), but qualitative PCR may not be able to discriminate between clinically significant and insignificant reactivation in immuocompromised individuals. On the other hand, studies using quantitative PCR have been shown to be useful for detecting patients at high risk of developing CMV disease (11, 12). Real time PCR, one modality of quantitative PCR, is a simple, reliable, cost effective and time saving alternative strategy. In many laboratories, CMV infection diagnosis now relies on real-time PCR assays (13- 16), The question of which type of blood fraction (PBL, plasma, or whole blood) is best for monitoring CMV DNA in blood is still unresolved.

In this study, we have described the development of in-house CMV quantitative real-time PCR using the ABI 7000 Sequence Detection System (Applied Biosystems) and its validation using two different specimen types, plasma and peripheral blood leucocytes (PBLs) from renal transplant patients with negative and positive antigenemia assay results.

MATERIALS & METHODS

Patients and specimens. We tested 65 EDTA blood samples obtained from 40 patients who underwent kidney transplantation and achieved sustained engraftment. EDTA blood specimens were collected as part of routine postoperative management, after obtaining informed consents from the patients, and aliquots of white blood cells and plasma were stored to be used as validation specimens in our study. Of the 40 patients, 13 developed CMV infection as evidenced by positive antigenemia (based on detection of 10 or more positive cells per 50.000 leukocytes). Of these 13 positive antigenemia patients, four patients developed active CMV disease. The

remaining 27 patients never exhibited a positive antigenemia test during the post transplant surveillance period. Additional demographic information is given in table (2)

Infection was defined by positive antigenemia status during posttransplant monitoring, and no infection was defined by negative antigenemia status during posttransplant monitoring. Active CMV disease was defined as a positive CMV antigenemia assay and any of the following: the presence of appropriate symptoms (fever, malaise, and diarrhea) or signs (leukopenia), the presence of retinitis on ophthalmologic exam, or a tissue biopsy CMV positive by either culture or immunohistochemical staining (17).

CMV antigenemia assay. The CMV antigenemia assays were done by standard procedures in our laboratory (8). Results were reported as the number of positive staining cells per 50,000 leukocytes.

Preparation of White Blood Cells and Plasma. EDTA blood specimens were collected and spun in plastic conical centrifuge tubes (Nunc, Kamstrup, Denmark) at 330g for 5 minutes using a MSE Mistral 3000i benchtop centrifuge, (Fisons Instruments, Cranley, England) and the plasma separated. The cell pellet was made up to 10 ml with a red cell lysis buffer containing 0.15M NH_4Cl (Sigma, Poole, England), 0.01M $NaHCO_3$ (Sigma, England), 1mM EDTA (Sigma, England), left at room temperature for 15 minutes and spun at 330g for 5 minutes. The supernatant was replaced with 10 ml phosphate buffered saline and the tube inverted gently 4 times. After a second spin at 330g for 5 minutes the supernatant was removed and the cells were counted in a Kova Glasstic 10 well grid (Hycor Biomedical Inc., California, USA) and made up to a final concentration of 1 x 10^7 cells/ml in lysis buffer constituted from 4M guanidium isothiocyanate in 3M sodium acetate (Sigma, Poole, England) at pH 6.0. A volume of 200 µls of plasma and cells (equivalent to $2x10^6$ cells) were extracted using the QIAamp Blood Kit (Qiagen Ltd., Crawley, England).

Quantitative nested real-time PCR. Real-time PCR was carried out using a nested primer set (18, 19) and CMV specific probe designed in house using Lasergene software program, version 5 (DNAstar), targeting the CMV glycoprotein B (gpB) gene (table 1). The outer and inner products were 326 and 301 base pairs (bp) respectively (figure 1). The taqman probe was labeled with FAM at the 5' end and TAMRA at the 3' end. First round amplification was performed on 2µl of extract added to 8 µl of the first round mastermix. Amplification was carried out on a DNA Engine Tetrad PTC225 (MJ Research, USA). The 20 cycles of denaturation (94°C -10 s), annealing (58°C- 10s) and extension (72°C-30s) were preceded by a 3 min denaturation step of 94°C , to facilitate hot start transfer from ice, and followed by an extension step of 72°C for 5 min.

Table. 1: Primers and probe for quantitative nested CMV real-time PCR assay

Designation	Sequence 5' - 3'	Position*		Product size
CMV F1	5'-TCA TGA GGT CGT CCA GA-3'	2133	-	326 bp
CMV R1	5′- TGA GGA ATG TCA GCT TC- 3′	2149		
		1824	-	
		1840		
CMV F2	5'-TCG TCC AGA CCC TTG AGG TA-3'	2122	–	301 bp
CMV R2	5′- CCA GCC TCA AGA TCT TCA TA-3′	2141		
		1841	-	
		1859		
CMV Probe	FAM-5'-CCC TGG ATA TCG ACC CGC TGG AAA AT-3'-TAMRA			

* Nucleotides are numbered with reference to the GenBank CMV gpB sequence U66425

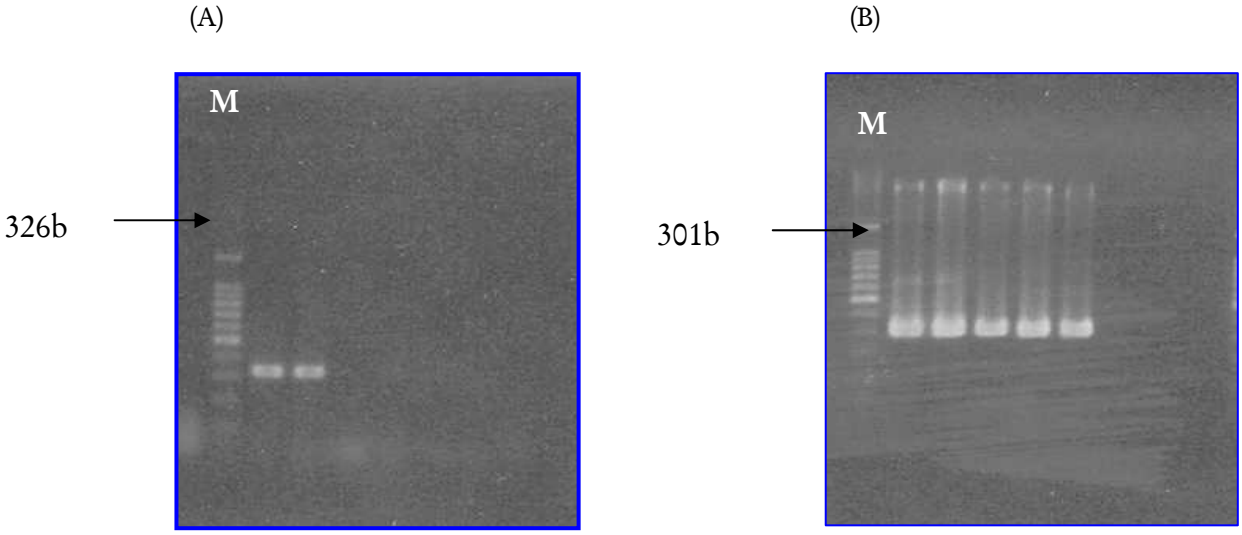

Figure 1 : First and second CMV PCR products

Figure : (A) First round amplification of 326 bp from CMV DNA and (B) second round amplification of 301bp from CMV DNA.. A 100 bp DNA ladder was used for size comparison (M). Products were separated on 2% agarose gel.

Second round amplification was performed with 1µl of the first round products in a total reaction volume of 19 µl containing 1x PCR buffer (Promega), 3.5 mM MgCl2 0.2 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 0.25 U /µl Taq DNA polymerase (Promega), 0.2 mM of primers for the gpB gene, 0.2 mM TaqMan probe, 1 mM 5-carboxy-X-rhodamine (ROX) passive reference dye (Invitrogen) and 2 mg BSA /µl (Sigma). The ABI 7000 Sequence Detection System was switched on 30 min prior to use. Each sample was run in duplicate in standard PCR strips but with optical PCR lids using the following PCR cycling conditions: after an initial denaturation step of 3 min at 95 ° C, a two step PCR procedure was used consisting of 30 s at 95 °C and 1 min at 60 °C for 40 cycles.

A negative control consisting of the reaction mixture and water (in place of template DNA) was added in each run.

Data were obtained during the annealing period. Fluorescence was measured once every cycle immediately after the 60 °C incubation (extension step). Fluorescence curves were analyzed with the ABI 7000 Sequence Detection System software and results were expressed by determination of the threshold of detection, C_T, which marked the cycle at which the fluorescence of the sample became significantly different from the baseline signal. A sample was regarded as positive when the ABI 7000 software determined a C_T in the quantification analysis screen (figure 2).

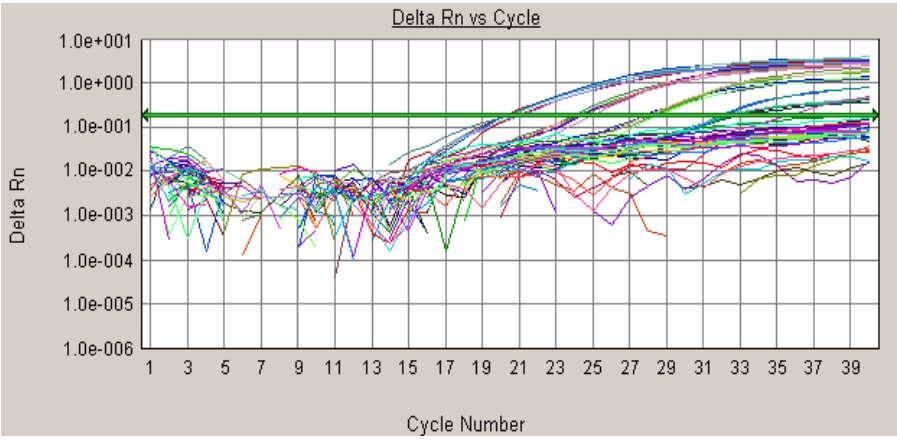

Figure 2: amplification screen of ABI 7000 SDS, show amplification plots of 10 fold serial dilutions of CMV DNA

Quantitative standards. To establish the quantitative assay, we created a plasmid containing CMV DNA amplicon by using the TOPO TA cloning procedures with pCR4-TOPO vector (Invitrogen, Carlsbad, Calif.). Insertion of the correct amplicon was confirmed by nucleotide sequencing using the Thermo Sequenase fluorescently labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech), and the purified recombinant plasmid was quantified by spectrophotometry. Quantification of DNA in clinical samples was achieved by using serial 10-fold dilutions of the previously quantified plasmid standards. Plasmid standards and test samples were run in duplicate, and the average values were used for calculation of the viral load. The prequantitated plasmid standards were diluted in 10 mM Tris (pH 9) and stored at -20°C.

Specificity of the assays. The analytical specificity of the assay was determined by analyzing DNAs extracted from other herpes viruses and DNA from other DNA and RNA viruses routinely tested in the laboratory.

Analytical sensitivity. Analytical sensitivity was determined using serial dilutions of the plasmids DNA, resulting in a log titration ranging from 10 to 10^7 copies/ml. These were then used for PCR for both endpoint gel based and real-time PCR assays and the end-point copy number recorded.

Statistical analysis. Data were entered into Microsoft Excel and exported to Statistical Package for the Social Sciences (SPSS) for Windows (version 9.0; SPSS Inc, Chicago, Ill) for analysis. Student's *t* test was used for the comparison of the mean copy numbers of CMV DNA in the two specimen types from the renal transplant patients

RESULTS & DISCUSSION

Assay development and quality control data. CMV DNA PCR is a highly sensitive, rapid (~ 6 hrs) technique based on selective amplification of specific nucleic acid sequences. The PCR method is used either qualitatively (diagnostic PCR) or quantitatively to measure the viral load, which is proportional to the level of CMV DNA. Ljungman and coworkers (20) demonstrated a direct relationship between the viral load as estimated by PCR and the risk for subsequent development of CMV disease. With the PCR method CMV infection may be detected as early as two weeks before the onset of symptomatic infection. This advantage has given impetus to the preemptive therapy strategy for the prevention of CMV infection and disease in high-risk patients.

In this study, an in-house real-time PCR assay based on TaqMan technology was developed to monitor the quantity of CMV DNA in PBLs and plasma of renal transplant recipients.

Analytical sensitivity of CMV nested real-time PCR was determined using serial dilutions of CMV plasmid DNA. The detection end point was10^{-10}. End point copy number was calculated using the following mathematical formula

End point copy number
= concentration (copies/ml) x input in PCR (2 x 10^{-3}) x Detection end point in PCR

CMV DNA end point copy number =
2.7×10^{12}(copies /ml) x 2 x10^{-3} x 10^{-10}
=
~0.5 copies/ 2 µl (reaction) = 250 copies/ml

The quantitative PCR assay studied here was designed with a reduced level of sensitivity (250copies/ml or 10^6 of PBLS) in an effort to improve the disease specificity. This approach appears to have been effective, since only one plasma and three PBLs out of 40 specimens from 13 transplant recipients that did not have a positive antigenemia or develop active CMV disease post-transplantation were found to have detectable CMV DNA levels by quantitative PCR.

Linearity and specificity of the quantitative real-time PCR assay

A quantitative standard curve was achieved by using a 10-fold dilution series of the pCR4-TOPO plasmid covering plasmid copies from 10 to 10^7 per reaction.

To determine whether there is cross-reactivity with the other viruses, viruses routinely tested in the laboratory were subjected to the real-time PCR using CMV primers and probe. The assay was found to detect their specific viral target and did not cross-react with the other viruses (data not shown).

Assay validation using clinical specimens. To demonstrate that the developed quantitative assay could be used to detect CMV DNA in clinical samples and to compare two specimen types for monitoring CMV reactivation, the assay was validated on specimens from two cohorts of renal transplant recipients, the first cohort consists of postransplant patients who had antigenemia negative results during postoperative screening (40 EDTA specimens from 27 patients), these were found to be also negative for CMV PCR except for one plasma specimen and three PBLs specimens, these data suggest that CMV PCR

performed on EDTA blood specimens is probably more sensitive than antigenemia assay for monitoring CMV reactivation, these results are consistent with previous results showing that CMV PCR was more sensitive than the antigenemia assay which can give frequent false negatives (17, 21) , however, further monitoring of these patients is needed to confirm PCR results.

The patients that had CMV infection (positive antigenemia assay) were similar to those who did not have infection with regard to age and sex, (Table 2); as expected, there were differences in the CMV serostatus of the recipients (R) and donors (D) in the two groups. In the group with infection, 77% (10 of 13) were D+/R-, that is, the donor was seropositive for CMV and the recipient was seronegative for CMV prior to transplant. In the group without infection, 44% (12of 27) were D+/R-.

Table 2: Demographic information

Patient parameter	Patient group	
	No infection	Infection
Number of patients	27	13
Age (yr)		
Mean	45	39
Range	5-63	2-65
Sex (male/female)	17/10	8/5
No. donor seropositive	15	11
No. recipient seropositive	0	0
No. D+/R-*	12	10

* D+/R-, donor CMV seropositive and recipient CMV seronegative.

In case of patients diagnosed with CMV infection (no= 13, of them four developed active CMV disease), testing PBLs were clearly more sensitive than plasma. As shown in table (3), all PBLs (n= 25) specimens were positive by PCR, in contrast to 19 plasma specimens from the same patient category. , concurring with published findings (22). Interestingly, none of these negative plasma specimens were from the four patients that developed active CMV disease later in the study indicating that CMV disease is associated with increased plasma positivity.

Here we confirm the findings of previous studies that plasma-based PCR assays result in delayed detection of CMV DNA due to lower sensitivity of these assays compared to cell- based assays (23, 22, 24). However plasma doesn't require lengthy cell separation procedures and offers a much better opportunity to detect CMV viremia during periods of severe cytopenia when cell based assays perform poorly. Moreover, the frequency of detecting CMV DNA in plasma in patients with active CMV disease is high, making the specificity of plasma DNA testing high (25).

Regarding CMV DNA levels, the viral load in PBLs from patients with CMV infection was generally higher than CMV load in three PBLs samples from patients with no infection; as shown in table (3). The antigenemia positive group had a mean value of 66.027$\pm$ 123 10^6 leucocytes of CMV DNA in their PBLs, in contrast to patients with negative antigenemia who had a mean value of 9587 $\pm$ 76.6 copies/10^6 leucocytes of CMV DNA .

Table 3 : Results of quantitative CMV real-time PCR assay

Specimen category	Parameter	Value for	
		Plasma	Leucocytes
No infection	Number of specimens	40	es40
	Mean viral load ± SD	1200 copies/ml	9587 ± 76.6 copies/10^6 leukocytes
	Maximum viral load	-	12.565
	No. of undetectable *	39	37
Infection	Number of specimens	25	25
	Mean viral load ± SD	21,442 ± 52,135 copies/ml	66.027± 123copies/10^6 leukocytes
	Maximum viral load	38,000 copies/ml	539,000 copies/10^6 leukocytes
	No. of undetectable *	6	0

* (<250 copies)

Moreover, in the group with positive antigenmia results, the PBLs had a mean value 66.037±123 copies /10^6 leucocytes of CMV DNA compared to plasma samples who had a mean value of 21.442±52.135 of CMV DNA, this difference was statistically non significant . (p= 0.59). These results were expected as CMV replication start in cells and is followed by the release of viral particles into plasma.

Some authors have found plasma viral load monitoring of transplant recipients suitable (26, 27). Boeckh et al. (28) concluded that even though the sensitivity of plasma PCR was significantly lower than that of PBLs PCR, plasma PCR could be particularly useful when leukocyte counts were inadequate for the performance of cell-based assays. For others, the higher sensitivity of PBL PCR and its higher yield of CMV DNA make it an optimal sample for monitoring the CMV DNA load during CMV disease in immunocompromised patients (29, 30).

We conclude that CMV loads are higher in PBLs than in plasma. Plasma viral load monitoring is of modest clinical utility for prediction of CMV disease and delays the detection of CMV DNA. On the other hand, when CMV is detected in the plasma fraction, it reflects active viral replication with virus release into plasma from multiple pools, including endothelial cells and the reticuloendothelial system, in addition to circulating leukocytes. As molecular based assays become the norm for routine viral diagnosis, this quantitative nPCR assay offers a highly sensitive test for pre-emptive patient monitoring and a quantitative approach for diagnosing clinical infection; WBCs is more sensitive than plasma, however, testing the plasma fraction is optional.

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