Detection and Monitoring of Cytomegalovirus Infection in Renal Transplant Patients by Quantitative Real-Time PCR

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\textbf{Key Words}
Cytomegalovirus · Transplantation · Quantitative real-time PCR · Cytomegalovirus antigenemia

\textbf{Abstract}

\textbf{Objectives:} To establish a sensitive and specific real-time PCR for quantitation of cytomegalovirus (CMV) DNA in clinical specimens. \textbf{Subjects and Methods:} In a prospective study, CMV DNA was quantified in blood samples of 255 kidney recipients with and without CMV-related symptoms between the years 2000 and 2005 in Kuwait. In a selected group of patients, the effect of anti-CMV chemotherapy was monitored by quantitative real-time PCR (qRT-PCR). \textbf{Results:} The established qRT-PCR assay had a sensitivity to detect 30 CMV DNA copies. CMV DNA was detected in 54/255 (24\%) patients; of these, 17 (31.5\%) were asymptomatic, and 37 patients (68.5\%) had symptomatic CMV infection. Sequential blood specimens were collected from all CMV-positive patients and tested by CMV pp65 antigenemia and qRT-PCR assays. There was a moderate positive correlation between the two assays (Pearson’s correlation = 0.52). The median CMV viral load measured by qRT-PCR was higher in symptomatic ($6.5 \times 10^4$ copies/ml) than in asymptomatic (185 copies/ml) patients ($p = 0.001$). The estimated cut-off value of CMV DNA for CMV symptoms/disease was $\geq 800$ copies/ml of blood. Testing of sequential samples from patients treated with symptomatic CMV infection showed that the viral load was significantly reduced after 3 weeks of anti-CMV chemotherapy ($p = 0.001$). \textbf{Conclusion:} The reported qRT-PCR is a sensitive method for quantitation of CMV DNA in the blood of kidney recipients and can be useful in monitoring the efficacy of anti-CMV therapy.

\textbf{Introduction}

The human cytomegalovirus (CMV) is a common pathogen responsible for asymptomatic and persistent infections in healthy individuals [1]. However, CMV infections are a major cause of morbidity and mortality in immunocompromised patients, especially in recipients of solid-organ transplants and AIDS patients [2–4]. In transplant recipients, CMV infection can result in prolonged fever, leukopenia, hepatitis, colitis, retinitis, allograft injury, and increased susceptibility to opportunistic infections [5, 6]. The prevention of CMV disease is a major goal in the management of kidney transplant patients. Ganciclovir has been the drug most frequently used in the prophylaxis, pre-emptive therapy, and treatment of CMV infections in renal transplant patients [7, 8]. Reliable and sensitive laboratory techniques to initiate pre-emptive therapy require the detection of CMV infection before the onset of symptoms. Introduction of CMV antigenemia (Ag) and PCR assays marked a new era in the early detec-
tion of CMV infection [2]. The detection of the pp65 antigen in leukocytes is a sensitive and widely used method, but it is labor-intensive, requires immediate processing of samples, and relies on subjective interpretation of results [9, 10]. To overcome the problems associated with pp65 Ag assay, qualitative PCR have been established for detection of CMV infections [1]. The main drawback of qualitative PCR assays is their inability to differentiate between latency and higher levels of replication [11–13]. In contrast, quantitative methods may be more clinically useful, as higher CMV DNA load predictably correlates with CMV disease [14, 15]. Several quantitative PCR assays such as the AMPLICOR CMV test, quantitative competitive PCR ELISA, and the COBAS AMPLICOR CMV MONITOR test are available commercially. However, one of the disadvantages of these assays is low sensitivity, where the lowest limit of detection of CMV DNA by the AMPLICOR CMV test is approximately 1,000 copies/ml of plasma [2, 16]. In addition, these tests are expensive, time-consuming and labor-intensive, which diminishes their utility in routine diagnostic laboratories [12, 17]. During the last 6 years, commercial quantitative PCR assays have been available such as Perkin-Elmer Applied Biosystem (PE-ABI, Foster City, Calif., USA) 7700 Sequence Detection System TaqMan™ or the LightCycler™ (Roche Molecular Biochemicals, Indianapolis, Ind., USA) that are less laborious for laboratory personnel than the rest of the quantitative techniques, but none of these assays was developed to detect CMV infection significantly earlier than the late pp65 gene or glycoprotein B gene [11, 13, 18–21]. Therefore in this study, our aim was to develop an in-house quantitative real-time PCR (qRT-PCR) assay using the LightCycler instrument that was optimized for the detection and quantitation of CMV DNA using primers and probes directed at the major immediate-early gene. In this assay, a CMV plasmid standard was constructed in the laboratory to quantitate CMV DNA. Furthermore, we evaluated this qRT-PCR to determine the viral load of CMV in kidney recipients in comparison with pp65 Ag assay, and we also report here the application of this method in monitoring the viral load in patients receiving anti-CMV chemotherapy treatment.

Subjects and Methods

Study Population
A total of 255 patients (143 male and 112 female; median age 41 years) who received kidney transplants between the years 2000 and 2005 at the Organ Transplant Center, Ministry of Health, Kuwait, were enrolled in this study. Blood samples were collected every 3 weeks from week 1 to 14 months after transplantation and tested for CMV infection by either CMV Ag or qualitative PCR assays. Among the CMV-positive infected patients, 22 were male and 32 female with a median age of 43 years. CMV-positive infected patients were divided into two groups, asymptomatic and symptomatic. Asymptomatic infection was defined by the presence of detectable CMV DNA in the blood of patients by qualitative PCR and/or by a positive CMV pp65 Ag test without any clinical manifestations. Symptomatic infection was defined as the presence of detectable CMV DNA in the blood of patients by qualitative PCR and/or by a positive CMV pp65 Ag test with one or more of the following symptoms: prolonged fever, leukopenia (white blood cells <3.5 × 10⁹/µl), thrombocytopenia, elevated liver enzymes, arthralgias, malaise, pneumonitis, gastrointestinal ulceration, or graft rejection [22].

CMV Ag Assay
Peripheral blood (10 ml) was collected from each patient in EDTA-containing tubes and transported to the laboratory within 2–3 h. Samples were processed for antigen detection. CMV pp65 Ag assay was performed using the CMV-vu™ CMV antigen detection kit according to the procedure described by the manufacturer (DiaSorin, Stillwater, Minn., USA). The numbers of cells containing the CMV-specific pp65 antigen were counted and the results expressed as the number of CMV antigen-positive cells per 50,000 leukocytes [23]. The tested samples were assigned to one of four groups according to the number of CMV pp65-positive cells: group 1, pp65 Ag negative; group 2, pp65 Ag positive with 5–10 cells; group 3, pp65 Ag positive with 10–40 cells; group 4, pp65 Ag positive with more than 40 cells.

DNA Extraction
Plasma was separated from the whole blood, and DNA was extracted from 140 µl of plasma using the QIAamp viral DNA extraction mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA from CMV reference strain AD169 (American Type Culture Collection, Rockville, Md., USA) was also extracted by the same extraction procedure. The extracted DNA was eluted with 60 µl of elution buffer.

Qualitative PCR
CMV DNA in the plasma was detected by amplifying a region of the CMV immediate early gene, exon 4. LC-1 and LC-2 primers were used to amplify a 235-bp fragment of the gene. Five µl of the extracted DNA was mixed with 20 µl PCR mix that consisted of 50 mM KCl, 10 mM Tris-HCl, 3 mM MgCl₂, 200 µM of each deoxynucleotide triphosphate, 10 pmol of each primer and 1.25 U of Gold Taq DNA polymerase (Roche, Applied Biosystems, USA). A total of 40 amplification cycles (each cycle consisted of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min) were carried out. The amplified DNA (235 bp) was characterized by agarose gel electrophoresis.

CMV Quantitative Real-Time PCR
Primers and Probes
CMV primers and probes for qRT-PCR were designed using the LightCycler primers and probes design software (Roche). The primers (LC-1: 5’GGATAACGGGGAGATGT3’, and LC-2: 5’GAGGGAAATTTCTGCAAT3’) were expected to amplify a 235-bp DNA fragment between position 40 to 275 of the CMV immedi-
ate early gene and the probes (LC probe 1: TGTGCTACAG-GAATATAGGTCTTTACCTTCAAC-phosphate) were expected to bind between positions 177 and 236 of the same gene. CMV DNA Quantitation

qRT-PCR amplification was carried out using FastStart DNA Master Hybridization Probes Kit (RocheDiagnostics, Mannheim, Germany) and the parameters were adjusted according to the LightCycler operation manual. For quantitation of viral DNA, recombinant DNA plasmid was constructed by cloning the amplified fragment of the immediate early gene, exon 4 (235 bp), of CMV AD169 strain into pGEM-T Easy Vector (Promega, USA) using standard procedures [24]. The identity of the cloned CMV IE gene was confirmed by DNA sequencing using CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, Calif., USA) with universal sequencing primer (40M13; Beckman Coulter). A standard curve for the quantitation of CMV DNA was constructed using tenfold dilutions of the recombinant plasmid DNA.

qRT-PCR was used to measure the viral load in the plasma samples of CMV-infected transplant patients. Seventy-six samples collected from the 54 PCR-positive patients were tested by the qRT-PCR and CMV pp65 Ag assay. From each of the 37 symptomatic patients at least two samples were tested, while one sample was tested from the 17 asymptomatic patients. qRT-PCR was also used to monitor the efficacy of antiviral treatment. Viral load was determined in the plasma of 15 symptomatic patients treated either with ganciclovir or valganciclovir for 2 weeks. The samples were collected at the beginning and after treatment.

Statistical Analysis

Data were statistically analyzed using the SPSS v12 statistics software package (SPSS Inc., Chicago, Ill., USA). Bivariate correlation test was used to compute Pearson’s correlation coefficient. Mann-Whitney standard nonparametric test was used to calculate the p values. A p value less than 0.05 was considered statistically significant. For disease prediction, the positive predictive value and negative predictive value were calculated as previously described [25].

Results

Analysis of Patients

Of the 255 kidney transplant patients, 54 (21.2%) were positive for CMV infection by qualitative PCR or pp65 Ag assay after a period of 1–14 months after transplantation. Among the positive patients, 22 were male and 32 female with a median age of 43 years. Two hundred and one (78.8%) patients remained negative by PCR and pp65 Ag assay after a period of 1–14 months after transplantation. Among the positive patients, 22 were male and 32 female with a median age of 43 years. Two hundred and one (78.8%) patients remained negative by PCR and pp65 Ag assay during the study period. Two hundred and fifty blood samples were collected from the 54 CMV-infected patients with a median follow-up of 120 days. Among the CMV-infected patients, 17 (31.5%) were asymptomatic and 37 (68.5%) had symptomatic CMV infection.

Sensitivity and Specificity of qRT-PCR

Based on the constructed standard curve, the lowest limit of detection of the established method was 30 CMV DNA copies/PCR reaction. No cross-reactivity was observed when the specificity of the assay was tested for other human herpes viruses (herpes simplex virus type 1 and 2, varicella-zoster virus, human herpes virus 6, and Epstein-Barr virus; data not shown).

Reproducibility of qRT-PCR in Measuring CMV Load in Clinical Samples

The reproducibility of the qRT-PCR in measuring CMV viral load accurately in the clinical samples from kidney transplant patients was investigated. Accordingly, intra-assay variability was evaluated using 3 replicates of CMV control dilutions containing $3 \times 10^6$, $3 \times 10^5$, $3 \times 10^4$, $3 \times 10^3$, and $3 \times 10^2$ copies of CMV DNA. The results showed that the detection limit was 30 copies of CMV DNA. A narrow range of standard deviations (SD) characterized the assay at dilutions $3 \times 10^3$ to $3 \times 10^6$ (6.69 ± 1.18 to 3.06 ± 0.21; values are means ± SDs), while there was a wider range of SDs at dilutions 30 and $3 \times 10^2$ (1.77 ± 1.53, 0.67 ± 1.16, respectively). This indicates that at very low DNA concentrations (30–300 copies) a precise quantitation of the CMV DNA cannot be achieved.

Correlation between CMV Ag Assay and qRT-PCR

Based on the categories of CMV pp65 Ag assay results, the 76 samples which were collected from 54 PCR-positive transplant patients and tested by the qRT-PCR and pp65 Ag assay were assigned as follows: group 1, n = 41; group 2, n = 6; group 3, n = 13, and group 4, n = 16. The mean DNA log_{10} genome copy number in groups 1, 2, 3, and 4 were 2.86, 3.7, 4, and 5, respectively. Copy number of CMV DNA increased with the number of CMV pp65-positive cells. The samples in group 4 contained significantly more CMV DNA log_{10} genome than those in group 3 (p = 0.02), group 2 (p = 0.01) or group 1 (p = 0.001; fig. 1). There was a moderate positive correlation between the two assays (Pearson’s correlation coefficient = 0.52; fig. 2).

Measuring CMV DNA Load in Patients with and without Symptoms

Results showed a significant difference in the viral load (median DNA copies/ml of CMV DNA) between symptomatic (6.5 × 10^4 copies/ml) and asymptomatic (185 copies/ml) patients (p = 0.001). Individual DNA values for asymptomatic patients ranged between 1.9 and 4.2 log_{10} copies/ml, whereas for symptomatic patients they ranged from 2.8 to 6.9 log_{10} copies/ml. An arbitrary cut-off level.
of 800 copies/ml was selected to determine the positive predictive value and negative predictive value of the qRT-PCR for a group of symptomatic and asymptomatic patients. Using this cut-off level, the assay had a high positive predictive value (95%) for symptomatic patients; however, its negative predictive value was rather low (52%).

**Monitoring of Anti-CMV Therapy**

Results showed that the level of viral load in the plasma samples of symptomatic transplant patients varied considerably at the beginning of treatment (from 2.8 to 6.8 log_{10}/ml; table 1). After treatment, the CMV-DNA load decreased steadily. After 2 weeks of treatment, no virus could be detected in 7 patients, and in the remaining patients the viral load decreased by 1–5 log (p = 0.001).

**Discussion**

It has been demonstrated that generally active CMV infections occur in 30–70% of transplant recipients with a mortality rate of 5% [26]. Since effective anti-CMV treatment is available, early detection of CMV DNA in the blood is of great importance to identify those patients who are at risk of infection and disease. Recent techniques such as qRT-PCR have improved the monitoring of CMV infection after kidney transplantation. The measurement of viral load by qRT-PCR appears to be an important tool in the prediction/diagnosis of CMV disease, and for differentiating latent from active infection and also for monitoring anti-CMV therapy. The major advantage of the qRT-PCR using the LightCycler system described here is that the primers and fluorescent probes were designed specifically to target the immediate early gene, which is considered to be related to early stages of CMV replication. Therefore, it has a particular advantage over other qRT-PCR LightCycler systems, where the target genes are the late gene of pp65 and glycoprotein B gene [18, 15, 27]. Although CMV was quantified using serial dilutions of plasmid containing the target sequence instead of commercial kits, the assay was found to be reproducible, the inter- and intra-assay standard deviations were good. There were no significant differences in the percentage of coefficient of variation between the inter- and intra-assays (p = 0.99). Furthermore, the assay was sensitive as the lowest limit of detection was 30 CMV DNA copies. This is within the range published in other studies [2, 9, 15, 28].

Our results showed that the mean CMV DNA copy numbers in plasma increased proportionally with the number of CMV antigen-positive cells in leukocytes, suggesting a good general correlation with the Ag assay and are in agreement with previous studies [9, 12, 29]. It was observed that a high number of samples (n = 41) were pp65 Ag-negative or weakly positive (<5 cells/5 × 10^4 leukocytes) but had a mean CMV DNA log_{10} genome of 2.86. Several factors may give rise to discrepancies be-
between pp65 Ag results and qRT-PCR [27]. First, the interval between sampling and processing must be <3 h because a longer delay may reduce the sensitivity of the test [30]. Second, false-negative results may occur in neutropenic patients. Third, pp65 Ag assay relies on the detection of structural protein, the lower matrix phosphoproteins, while qRT-PCR quantifies the virus nucleic acid, specially the early gene, which reflects the beginning of the viral replication.

The qRT-PCR assay can accurately identify patients at risk of developing CMV-related symptoms/disease [9, 31–34]. In our study with a cut-off value of 800 copies/ml, the qRT-PCR had a very high positive predictive value (95%). However, in 6 out of 37 patients with CMV-related symptoms the viral load remained below the cut-off level. This may explain to some extent the relatively low negative predictive value (52%) of the assay. Though the median level of viral load was higher in patients with symptomatic CMV infection than in those without symptoms (p = 0.001), the severity of infection did not correlate well with the viral load. For example, 4 patients with mild symptoms (prolonged fever and leukopenia) had a relatively high viral load (between 10^{4.5} and 10^{6.8} DNA copies). On the other hand, in 2 patients with severe CMV symptoms (renal failure, bleeding, and diarrhea) the DNA copy numbers were at a low level (10^{2.8} DNA copy). Despite this variation, the viral load generally accurately predicted the development of symptomatic infections, confirming previous reports [14, 21, 33]. This discrepancy may be explained by several factors such as the source of donor kidney, nature of immunosuppressive therapy, genotypes of the virus, etc.

In this study, qRT-PCR allowed effective monitoring of patients who were on antiviral treatment. Since this assay is very sensitive for the detection of CMV replication, a negative PCR might be an indicator of a completely successful treatment. However, it was not possible to follow up 8 patients further; it is therefore not known whether a complete clearance of the virus occurred or not in these patients.

**Conclusion**

The qRT-PCR described in this study is a very useful method in detecting CMV DNA early in the course of infection, monitoring patients during CMV infection and disease, and in monitoring of the success of anti-CMV chemotherapy in these patients.

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References