

Application of Real Time PCR in post transplant monitoring of cytomegalovirus infection: comparison with other diagnostic approaches

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SUMMARY

Immunosuppressive status in solid organ transplant recipients is often related to the reactivation of Human Cytomegalovirus (HCMV) infection that remains one of the major causes of morbidity and mortality.

Therefore, the early detection of HCMV followed by infection monitoring is important to institute prompt and appropriate treatment.

In recent years good results have been obtained by HCMV DNA amplification methods; qualitative and quantitative approaches have shown good sensitivity and specificity, but they often require post-PCR manipulation that adds time to the analysis and may lead to contamination problems.

Recently, Real Time PCR (RT-PCR) has been proposed in HCMV DNA analysis as a valid method for its good sensitivity and rapidity.

In the present study, twenty-five solid organ transplant recipients were analyzed for HCMV diagnosis; 60 peripheral blood leukocytes and 120 plasma samples were tested by RT-PCR and the results compared to those obtained by a qualitative Nested PCR and a quantitative DNA enzyme immunoassay.

KEY WORDS: Real Time PCR, Cytomegalovirus, transplant

Received January 19, 2006

Accepted May 09, 2006

INTRODUCTION

Human Cytomegalovirus (HCMV) is a ubiquitous member of the Herpesviridae family which affects most of the population and can persist in the host after primary infection. In healthy humans HCMV infection is usually subclinical and occurs asymptotically while in the immuno-

compromised host it can induce serious illness that represents, despite improved treatment, one of the major causes of morbidity and mortality (Ho, 1991).

The wide range of HCMV complications determine the need for specific assays for rapid and reliable diagnosis and for monitoring the status of infection so as to guide specific antiviral therapy.

In recent years a sensitive method has been developed to identify the HCMV-encoded lower matrix protein pp65 in peripheral blood leukocytes and the number of positive cells has been shown to be related to the severity of active infection (Gerna *et al.*, 1991; Landry and Ferguson, 1993).

Moreover HCMV diagnosis has been improved by detection of viral nucleic acid using a molecular approach such as Polymerase Chain

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Reaction (PCR), and in this field several qualitative methods have been proposed (Nazzari *et al.*, 2000; Zipeto *et al.*, 1992). Recently it has been demonstrated that to perform a correct diagnosis of HCMV infection status it is necessary to use a quantitative approach to distinguish productive from latent or non-productive infection. Identification of a cut-off represents a good index to start antiviral treatment and to monitor the infection outcome during therapy (Fox *et al.*, 1995; Martin-Davila *et al.*, 2005; Patel *et al.*, 1995). Several methods have been described for the quantitative analysis of HCMV DNA (Boeckh and Boivin, 1998; Humar *et al.*, 1999; Schafer and Laufs, 1996). In addition, Real Time PCR (RT-PCR), a new rapid and more sensitive system, has been introduced for the analysis of HCMV genoma in cellular and non cellular samples (Gault *et al.*, 2001; Livak *et al.*, 1995; Najioullah *et al.*, 2001).

The present report analyzed RT-PCR for qualitative and quantitative HCMV genomic load evaluation in the infection monitoring of transplanted patients.

RT-PCR results were compared to those obtained in the same samples by a nested qualitative PCR and a quantitative DNA enzyme immunoassay

MATERIALS AND METHODS

Patients and samples

Blood samples were obtained during HCMV infection monitoring after solid organ transplant from January to June 2004.

Samples analyzed in the study were recovered from 25 patients who underwent transplantation at the Transplant Unit of Policlinico Umberto I Hospital in Rome, 17 of those received kidney transplants, 6 liver; 1 kidney-pancreas and 1 lung. The present study analyzed two different blood compartments: the cellular and non cellular fraction; the first included peripheral blood leukocytes cells (PBL) and the second plasma.

PBL 60 PBL samples were isolated from EDTA-treated venous blood by sedimentation. Briefly, 5 ml of blood was allowed to incubate at 37°C for 20 min. The leukocyte-rich upper layer was harvested and the contaminating erythrocytes were lysed with 0.8% NH₄CL. Cells were washed twice in phosphate-buffered saline (PBS), count-

ed, adjusted to 5x10⁶/ml and immediately processed for DNA extraction.

Plasma 120 plasma samples were collected: 5 ml of EDTA-treated venous blood were centrifugated at 3000 g for 10 min, the supernatant was harvested and immediately stored at -20°C until use.

DNA extraction

Cellular samples

DNA from 10⁶ PBL was extracted by alkaline thermolysis by using Extracell Kit (Amplimedical SpA Diagnostic Group-Divisione Bioline, Italy) according to the manufacturer's instructions. Extracted DNA was suspended in 100 µl of specific buffer and immediately used for PCR; an aliquot was stored at -20°C until use for quantitative PCR.

Plasma samples

DNA was extracted from 300 µl of plasma using the Extragen kit (Amplimedical SpA Diagnostic Group-Divisione Bioline, Italy) according to the manufacturer's instructions.

Ten µl of purified DNA containing a 600 bp sequence of human beta globin (HBG) gene were added to plasma samples as internal control. Extracted DNA was suspended in 15 µl of DNase and RNase free ultrapure water and immediately used for PCR; an aliquot was stored at -20°C until use for quantitative PCR.

Nested PCR

DNA extracted from PBL and plasma specimens was analyzed by nested PCR using primers designed for a 110 bp HCMV segment located in the gp58 region.

In PCR reaction mixture a couple of primers for the amplification of a 600 bp sequence of HBG gene was included. The first round of amplification was carried out for 35 cycles (denaturation 95°C for 1 min, annealing 58°C for 1 min, extension 72°C for 1 min) and an additional 5 min at 72°C in an automatic thermal cycler (Hybaid, UK). The total volume of PCR mixture was 50 µl and it contained 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 µM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, dTTP), 0.2 µM of each outer primers and 1.25 U of Taq polymerase. After the first amplification round 1 µl of the amplicons was used for the second round which consisted of 30 cycles

(denaturation 95°C for 1 min, annealing 50°C for 1 min, extension 72°C for 1 min) and an additional 5 min at 72°C, using the inner primers. One positive and one negative control were included in each run. PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualized by U.V. light. The sensitivity of nested PCR corresponded to 10 genomic copies. Gel signal of PCR product was considered acceptable only if on the agarose gel the beta globin line was also disclosed, confirming the efficiency of the extraction and amplification steps; otherwise the procedure was repeated from sample extraction.

Precautions to avoid carryover and to ensure the validity of results were strictly observed (Kwok, 1990).

Quantitative Competitive PCR

HCMV DNA in samples identified as positive was determined using a competitive DNA PCR (QC-PCR). The assay is based on the coamplification of HCMV genomic 260 bp sequence relative to MIEA region with an internal standard molecule in a known concentration. Competitor was constructed from plasmidic DNA containing a 300 bp sequence that was amplified by the same set of HCMV primers.

QC-PCR was carried out in an automatic thermal cycler (Hybaid, UK) for 35 cycles (denaturation 94°C for 30 s, annealing 55°C for 30 s, extension 72°C for 1 min) and an additional 5 min at 72°C. PCR products were quantified by an enzyme immunoassay in a microtitre plate by hybridization with probes specific respectively for HCMV and for competitor sequences according to the manufacturer's instructions (Amplimedical SpA Diagnostic Group-Divisione Bioline, Italy).

The absorbance was measured at 450/630 nm using a photometer (Sorin Biomedica ETI-system reader); for each specimen, the 630 nm was subtracted from the 450 nm absorbance value. The assay could quantify the presence of between 20 and 500.000 genomic copies for 10⁵ PBL or ml of plasma. The low limit for linear range was 100 genomic copies for cellular samples and 500 genomic copies for non cellular samples respectively. So at values lower than the former limits we assigned <100 copies/1x10⁵ and <500 copies/ml respectively.

Real Time PCR

Extracted DNA was also analyzed by RT-PCR. The assay uses fluorescent Taqman methodology and an instrument capable of measuring fluorescence in real time (ABI Prism 7000).

The Taqman reaction requires a hybridization probe labeled with two different fluorescent dyes: a reporter and a quencher. The system used in the present study includes an HCMV MIEA region specific probe labeled with FAM as reporter dye and with a non fluorescent quencher dye (NFQ) to test CMV DNA presence in samples. Moreover a human beta globin specific probe labeled with VIC as reporter dye and with a non fluorescent quencher dye as internal control was added in the same reaction. The PCR reactions were carried out in 96-well plates adding 5 µl of samples DNA extracted to 20 µl of amplification mixture (Q-ampliMIX, Q-ampliMASTER, Q-ampliPROBE). For each work session we introduced four positive (standards) and one negative control. The standard contents were 10², 10³, 10⁴, 10⁵ copies/5 µl of HCMV MIEA gene sequence respectively and were used to construct a curve as active reference to confirm that the generated signals represented the results of PCR amplification and to quantify the HCMV DNA in the unknown samples. The PCR-RT thermal profile consisted of a first stage (one cycle at 50°C for 2 min), a second stage (one cycle at 95°C for 10 min) and third stage which included 45 cycles (95°C for 15 s).

Computer software was designed to examine the fluorescent intensity of both the reporter dye (FAM) and the quenching dye (NFQ) and to compare the fluorescent intensity of both the unknown samples and the positive standards after PCR reaction; the system allows to verify the viral presence in the analyzed samples and to quantify the HCMV DNA in positives cases. The analytical sensitivity of this assay makes it possible to identify the presence of approximately 10 molecules of the target DNA in 5 µl of extracted DNA added to the amplification reaction.

Therefore in samples with target DNA below the above limit no specific fluorescence was detected and they were considered negative (nd = not detected) when the internal controls indicated that amplification occurred correctly.

The low limit for linear measuring range was

respectively 40 copies/10⁵ PBL and 208/ml of plasma; samples with specific fluorescence corresponded to genomic copies lower than the above values were considered positive and reported respectively as <40 copies/10⁵ PBL and <208 copies/ml of plasma.

Statistics

Regression analysis by Passing and Bablok method and Spearman rank correlation were used to compare QC-PCR and Real Time PCR values obtained from cellular and plasma samples.

RESULTS

In our study 180 samples obtained from 25 solid organ transplanted patients during HCMV infection monitoring were tested in two different blood compartments, cellular and non cellular fractions. HCMV DNA presence was determined by the positivity to a standardized Nested PCR and to a Real Time PCR (RT-PCR).

The qualitative study of 60 cellular samples showed the same results by Nested PCR and RT-PCR in 56 (93%) samples, in fact 20 (33%) samples were negative and 36 (60%) positive by both methods. Instead, disagreement was obtained in four (7%) cellular samples in which negative values were observed by RT-PCR and positive values by Nested PCR. Nevertheless quantitative evaluation of those samples by QC-PCR showed values lower than 100 copies/10⁵ PBL.

The analysis of the 36 HCMV positive cellular samples showed non quantified values by both methods (<40 for RT-PCR and <100 for QC-PCR copies/10⁵ PBL) in 15 (42%) specimens; 6 (16%) samples were quantified only by QC-PCR and 15 (42%) were measured by both methods (Table 1) (Fig. 1).

About the 120 non cellular samples our study showed that 36 (30%) were negative for HCMV infection by all analysis systems; 23 samples (19%) were positive by Nested PCR and negative by RT-PCR.

Quantitative analysis by QC-PCR showed values lower than the cut-off limit (500 copies/ml) in all the above Nested PCR positive specimens.

21 (18%) HCMV positive samples had non quantified values, lower than the cut-off limit of the

TABELLA 1 - Results obtained from cellular sample by Real Time (RT-PCR) and Quantitative Competitive Polymerase Chain Reaction (QC PCR) are compared. The values reported represents the number of samples negative or that showed HCMV DNA copies values higher or lower than cut-off limits of each method (40 for RT PCR, 100 for QC PCR).

NESTED + QC-PCR	RT-PCR			
	NEG	<40	>40	TOT
NEG	20	0	0	20
<100	4	15	0	19
>100	0	6	15	21
TOT	24	21	15	60

Concordance: 50/60 = 83%

approaches used (<208 copies/ml for RT-PCR and <500 copies/ml for QC-PCR).

Another 17 (14%) HCMV positive samples could only be quantified by RT-PCR because their values were lower than 500 copies by QC-PCR. Finally 23 plasma samples (19%) were measured by both methods (Table 2) (Fig. 1).

Statistical analysis

Data obtained by employed quantitative methods, RT PCR and QC-PCR, were statistically analyzed. The Spearman rank correlation test was applied to compare values that could be determined by both methods. The r statistic value was 0.85 for cellular samples and 0.89 for non cellular samples respectively both less than 0.95 (linearity limit) (Fig. 2).

DISCUSSION

In recent years PCR has been widely reported as a good tool in the rapid diagnosis of HCMV infection for its high sensitivity and easy performance (Bonon *et al.*, 2005; Fan *et al.*, 2002; Sia and Patel, 2000). The qualitative approach for HCMV infection has shown a low predictive value for the identification of HCMV disease (Barber *et al.*, 2000), so many authors addressed their interest in developing systems for HCMV that allowed

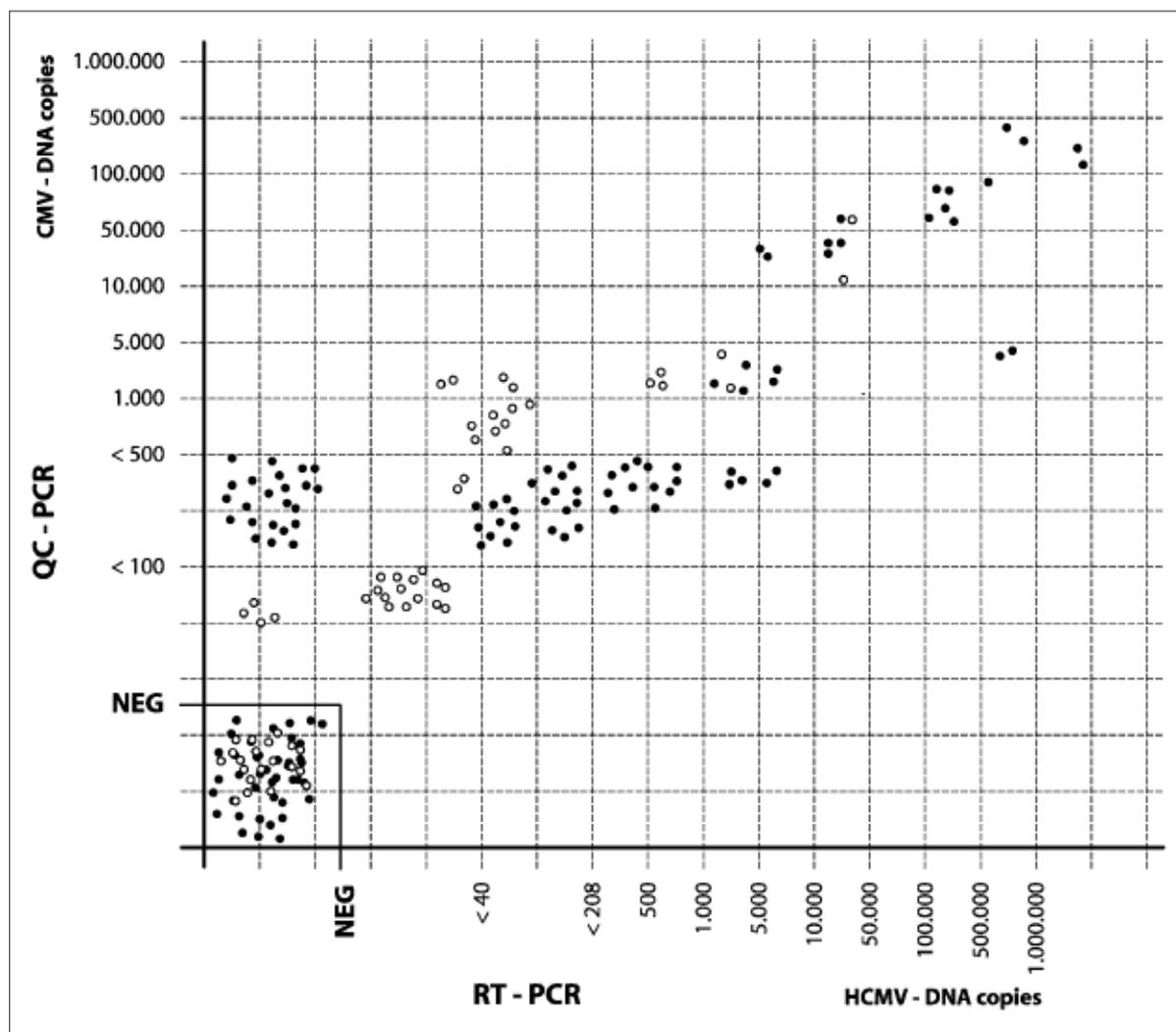


FIGURE 1 - Comparison of HCMV DNA copies collected by Real Time and Quantitative Competitive PCR. Values obtained from peripheral blood leukocytes (○) and plasma (●) samples are respectively reported as copies/ 10^6 cells and copies/ml; the values <40 and <208 on x-axis and those <100 and <500 on y-axis represent the lower limits of used methods.

sensitive detection while improving specificity for disease onset (Sanchez *et al.*, 2001).

Viral load detection proved to be useful at predicting HCMV disease because increased levels in solid organ transplant recipients with HCMV disease have been found (Martin *et al.*, 2005; Piiparinen *et al.*, 2005; Saltzman *et al.*, 1992).

Recently, in this field RT-PCR has been proposed to be helpful in detecting HCMV DNA in different samples: sera, plasma, urine, peripheral blood leukocytes, cerebrospinal fluid (Aquino *et al.*, 2001; Cunningham *et al.*, 1995; Delgado *et al.*, 1992; Hadaya *et al.*, 2003; Tarrago *et al.*, 2004).

RT-PCR advantages include the decrease of potential PCR contamination due to no post-PCR manipulation of samples, the shorter PCR processing time, high reproducibility and good standardization. (Heid *et al.*, 1996; Numata *et al.*, 2005; Sanchez and Storch, 2002; Schvoerer *et al.*, 2005).

We addressed the present study to compare data obtained from Real Time PCR and another analytical system to find the best approach for an early indication of HCMV active infection. We tested two compartments of blood samples because surveillance of HCMV infection in trans-

TABELLA 2 - Results obtained from cellular sample by Real Time (RT-PCR) and Quantitative Competitive Polymerase Chain Reaction (QC-PCR) are compared. The values reported represents the number of samples negative or that showed HCMV DNA copies values higher or lower than cut-off limits of each method (40 for RT-PCR, 100 for QC-PCR)

NESTED + QC-PCR	RT-PCR			TOT
	NEG	<208	>208	
NEG	36	0	0	36
<500	23	21	17	61
>500	0	0	23	23
TOT	59	21	40	120

Concordance: 80/120 = 67%

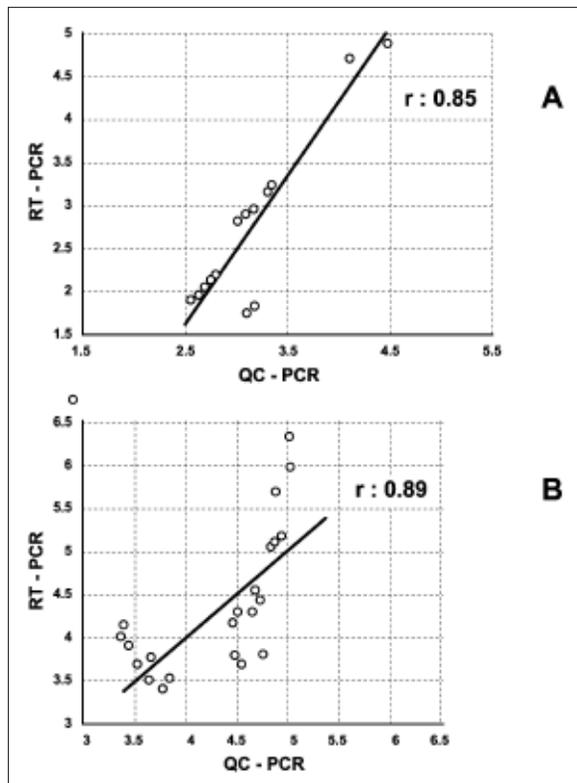


FIGURE 2 - Spearman rank correlation test between data obtained by real time (RT - PCR) and competitive quantitative (QC - PCR) polymerase chain reaction. The test was applied to quantizable results by both methods. **A** Correlation test for peripheral blood leukocytes samples. **B** Correlation test for plasma samples.

plant recipients is usually based on early detection of virus in blood and many authors are in agreement on the significance correlation between HCMV disease and PBL or plasma viral load (Caliendo *et al.*, 2000; Gerna *et al.*, 1998). At present it is well demonstrated that in immunocompromised conditions a generalized infection of endothelial cells occurs and the release of HCMV into plasma or the uptake by leukocytes represent the common feature of all clinical syndromes during disseminated infection (Gerna *et al.*, 2004).

Some authors reported that it is necessary to investigate HCMV DNA research in cellular samples because viral presence in the non-cellular compartment (serum, plasma) is often low and insignificant (Gerna *et al.*, 1994; Manez *et al.*, 1996).

Other authors emphasized that HCMV replication occurs inside the cell but in addition it is a lytic virus. This peculiarity allows viral particles to go outside the cells during an active infection, so HCMV can be found free in blood during the acute phase of infection and high levels of HCMV DNA in plasma are reported to be highly predictive of later development of HCMV disease (Rollag *et al.*, 2002).

The present study shows that RT-PCR results do not always correlate with those obtained by qualitative PCR Nested assays. In fact 7% of cellular and 19% of non cellular samples were positive by Nested assays and negative by RT-PCR; otherwise QC-PCR quantification of all the above samples showed values below the cut-off limit that are not usually associated with active infection but with virus latency (Boeckh and Boivin, 1998).

Moreover the statistical analysis of quantifiable results, in other words above the cut-off limits, displayed a good correlation between QC-PCR and RT-PCR whether in PBL or in plasma samples. Small differences between the two systems employed are perhaps due to the different acid nucleic extraction methods used for cellular or non cellular specimens.

In conclusion, considering the high variability of HCMV viral load among patients who develop disease, as indicated by several authors (Hassan-Walker *et al.*, 1999; Rollag *et al.*, 2002), and the need to monitor post-transplant status to detect HCMV in blood before the onset of dis-

ease, we noted the good capacity of RT-PCR. We suggest that this approach may be helpful in the monitoring of transplanted patients due to its good standardization and sensitivity.

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