Journal of Medical Virology 74:78–84 (2004)

Assessment of CMV Load in Solid Organ Transplant Recipients by pp65 Antigenemia and Real-Time Quantitative DNA PCR Assay: Correlation With pp67 RNA Detection

Carlo Mengoli,[1,2]* Riccardo Cusinato,[2] Maria A. Biasolo,[1,2] Simone Cesaro,[3] Cristina Parolin,[1] and Giorgio Palù[1,2]*

[1]*Dipartimento di Istologia, Microbiologia e Biotecnologie Mediche, Università di Padova, Padova, Italy*
[2]*Servizio di Microbiologia e Virologia dell'Azienda Ospedaliera di Padova, Padova, Italy*
[3]*Clinica di Oncoematologia Pediatrica, Dipartimento di Pediatria, Università di Padova, Padova, Italy*

After bone marrow (BM) or solid-organ (SO) transplantation viremic *Cytomegalovirus* (CMV) infection is observed frequently. Quantitative assay of CMV in blood helps the management of this clinical condition. In the present report, 83 samples from 39 solid organ recipients, three CMV assays were compared simultaneously for the first time: the Nuclisens CMV pp67 assay (nucleic acid sequence-based amplification, NASBA), an ''in-house'' quantitative real-time PCR assay (TaqMan) for CMV DNA, and pp65 antigenemia. The relation between CMV DNA and pp65 antigenemia, the quantitative assays, was evaluated on a larger group including 251 blood samples from 118 solid organ recipients. Real-time PCR provided the best results; $\geq$130 CMV DNA copies/2×10^5 peripheral blood leukocytes (PBLs) predicted $\geq$1 pp65 antigen positive (Ag+) cell/2×10^5 PBLs. By taking pp65 antigenemia as the ''gold standard,'' the sensitivity of CMV DNA quantitation and of the pp67 RNA assay were 0.95 and 0.20, respectively, while the corresponding specificity values were 0.50 and 0.93. When real-time PCR was considered as the ''gold standard,'' the sensitivity and specificity of the pp65 antigenemia were 0.65 and 0.91, respectively. Among the three tests examined, the sensitivity of the pp67 RNA assay was the lowest. On the other hand, the pp67 RNA assay was highly specific and effective in pinpointing high viremia patients. The present report, by providing predictive values for all three diagnostic profiles, DNA load, antigenemia, and pp67RNA, is a contribution for validation of real-time PCR as a new standard for quantitative assessment of CMV viremia in clinical settings. ***J. Med. Virol. 74:78–84, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: CMV; solid organ transplant; real-time PCR; pp67 RNA assay; pp65 antigenemia; predictive value

INTRODUCTION

Cytomegalovirus (CMV) is an opportunistic pathogen that complicates the care of immunocompromised patients. CMV infection is common because the virus can infect patients de novo, by reactivating from latency, or by reinfection events after a primary episode. After bone marrow (BM) or solid-organ (SO) transplantation, viremic CMV infection is frequently observed. Ganciclovir (GCV) prophylactic treatment will reduce the incidence of CMV disease during the first 100 days after transplantation. Disadvantages of chemoprophylaxis are GCV toxicity, selection of resistant viral variants, and possible late recurrence of CMV disease after treatment interruption [Pass, 2001]. An alternative approach to prevent CMV disease is pre-emptive GCV therapy, based on sentinel monitoring of CMV replication. Quantitative detection of CMV in blood

Grant sponsor: ISS; Grant number: OAG/F19; Grant sponsor: ISS; Grant number: 40D.64-30C.57; Grant sponsor: PRIN 2002; Grant sponsor: FIRB 2001.

*Correspondence to: Carlo Mengoli and Giorgio Palù, Dipartimento di Istologia, Microbiologia e Biotecnologie Mediche, Università di Padova, Via A. Gabelli 63, 35121 Padova, Italy.
E-mail: carlo.mengoli@unipd.it; giorgio.palu@unipd.it

Accepted 30 March 2004
DOI 10.1002/jmv.20149
Published online in Wiley InterScience (www.interscience.wiley.com)

helps in managing pre-emptive therapy [Emery et al., 2000]. Until now, this measurement has been performed by pp65 antigen immunofluorescent assay (pp65 antigenemia) on peripheral blood leukocytes (PBLs). In order to start the pre-emptive treatment, accepted thresholds are 10 pp65 antigen positive (Ag+) cells/2 × 10^5 PBLs after solid organ transplantation, 1–2 pp65 Ag+ cells after BM transplantation [Halwachs et al., 1993; Locatelli et al., 1994]. A higher threshold of 50 pp65 Ag+ cells was also advocated to instigate pre-emptive antiviral treatment of solid organ transplant recipients [Gerna et al., 1991].

A more recent approach to measure CMV load consists in PCR detection and quantitation of the CMV genome. Thresholds, which predict CMV disease, have still to be defined for these assays [Emery et al., 2000]. Nevertheless, a reduction in CMV disease, lesser use of antiviral drugs, and an improved survival were obtained with pre-emptive therapy guided by PCR screening [Einsele et al., 1995]. A significant advance in PCR was the development of homogeneous assays for real-time fluorescence detection of PCR-amplified products in a closed-tube format. An example is TaqMan; whose diagnostic application has been expanding rapidly [Schweitzer and Kingsmore, 2001].

Viral antigen detection and molecular DNA assays, however, are not entirely satisfactory for assessing CMV replication [Gerna et al., 1998] and for predicting the onset of CMV disease. The detection of RNA transcripts of viral origin has the potential of discriminating between latent and replicating/cytolytic CMV pool in vivo. Indeed, late transcripts might reflect objectively active CMV replication with clinical and prognostic involvement. Detection of late mRNA by reverse transcription polymerase chain reaction (RT-PCR) proved to be more specific, although less sensitive, than DNA PCR for the diagnosis of acute CMV disease [Gotzlan et al., 1993]. Recently, a technique for the detection of late pp67 mRNA by direct isothermal nucleic acid sequence-based amplification (NASBA) was developed. NASBA screening was shown as an efficient tool both for predicting development of CMV disease [Blok et al., 1998] and for deciding when to start or terminate pre-emptive therapy in BM recipients [Gerna et al., 1999]. In this context, however, whereas pp65 antigenemia (sensitivity 50%, specificity 89%) and DNA blood PCR (sensitivity 69%, specificity 75%) were accurate, late pp67 mRNA NASBA showed high specificity (90%) but low sensitivity (13%) in human immunodeficiency virus (HIV)-infected patients [Blank et al., 2000].

In the present study, an assessment was made of the diagnostic power of a home-made quantitative real-time PCR assay of CMV DNA in comparison and correlation with pp67 mRNA assay and pp65 antigenemia on a group of blood samples from solid organ transplant recipients. The sample collection was extended further for a greater accuracy in the analysis of the relation between the two quantitative assays, CMV DNA and pp65 antigenemia.

MATERIALS AND METHODS

Patients and Samples

Blood samples were collected from SO transplant recipients during the period January 2000–December 2001. The first 83 samples obtained from 39 patients were submitted to quantitative real-time PCR assay of CMV DNA, pp67 mRNA assay, and pp65 antigenemia since we wanted to have a preliminary appreciation of the discriminating diagnostic power of the three tests. These patients had a liver transplant in 7 instances, heart in 7, kidney in 22, lung in 3.

For a greater accuracy, the relation between the two quantitative tests, CMV DNA viral load and pp65 antigenemia, was evaluated on a larger group of data. This was done by enrolling the following 79 SO transplant recipients (16 liver, 12 heart, 48 kidney, and 3 lung recipients), thus obtaining additional 168 samples. Therefore, a total of 251 samples from 118 patients were included for this purpose.

CMV DNA Assay

Leukocytes (10^6), obtained from peripheral blood of patients, were incubated in 250 μl lysate buffer (0.1% Triton X-100, 0.1% SDS, 20 mg/ml proteinase K, Tris-HCl 10 mM-EDTA 1 mM) at 56°C for 1 hr and boiled for 10 min. Quantitative PCR testing was performed on 5 μl aliquots.

CMV DNA was assayed by an original "in-house" quantitative real-time PCR. The method was based on real-time detection of accumulated fluorescence (TaqMan). The target gene was UL122 (IE2 exon 4). Real-time PCR primers and probe for CMV were selected using Primer-Express software (Applied Biosystems, Foster City, CA). The forward (CM-5T) and reverse (CM-3T) primer sequences were TCATCCACACT-AGGAGAGCAGACT and GCCAAGCGGCCTCTGAT, respectively. The probe ACTGGGCAAAGACCTTCAT-GCAGATCTC contains a fluorescent reporter dye (6-carboxyfluorescein, FAM) at the 5′-end and a fluorescent quencher dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3′-end. A 137-bp fragment was amplified. The extracted DNA was assayed with the sequence detector system (ABI PRISM 7700) in 50 μl of PCR mixture containing 25 μl TaqMan Universal Master Mix, 15 pmol of each primer and 10 pmol of probe. Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 sec and 60°C for 1 min.

A 137-bp PCR fragment, amplified from a CMV positive specimen with CM-5T and CM-3T primers, was ligated into the pCR2.1 vector (TA-cloning Kit, Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The resulting plasmid, pTA-CMV, was quantified accurately by evaluation with a fluorimetric Picogreen method and a serial dilution was used as a reference for CMV quantification. A six-point standard curve was made by serial dilution (from 5×10^6 to 50 copies) of pTA-CMV into a genomic DNA

solution (0.05 μg/μl) derived from a CMV-seronegative donor. This standard curve showed a linear course throughout the mentioned limits. The detection threshold was 10 genomic copies/reaction. All standard curve points were done as duplicate assays. For each couple of observed readings *O1* and *O2*, the ratio (*O1-O2*) / *E* was calculated, where *E* was the expected reading. Intra-test variability was defined as the standard deviation of ratio (*O1-O2*)/*E*, and never exceeded 10%. The sensitivity was evaluated by comparison with an "in house" quantitative competitive PCR. The specificity was 100%, with no crossreaction being observed with other human *Herpesviridae*.

The CMV genomic copy number of the clinical samples was calculated automatically by 7700 ABI PRISM SDS software and then expressed as copy number/ 2×10^5 PBLs. The number of cells present in the PCR mix, predetermined by cell counting, was confirmed by real-time quantitative PCR amplification of a β-globin sequence gene (81-bp amplicon) by forward (AGGGCCTCACCACCAACTT) and reverse (GCACCTGACTCCTGAGGAGAA) primers and 6-FAM-ATCCACGTTCACCTTGCCCCACA-TAMRA as probe. A standard curve for the β-globin gene amplicon was also made in the same way as for the CMV target gene. A 192-bp PCR product, generated by the forward primer ACACAACTGTGTTCACTAGC (named pCO3) and the reverse primer GGAAAATAGACCAATAGGCAG (named pGH-21), was ligated into the pCR2.1 vector. The resulting construct was named pBGL and was used as positive standard.

The human genomic β-globin DNA amplification, run in parallel for each sample with the same amount of extracted DNA (5 μl) and identical thermal cycling conditions, assessed the absence of PCR inhibitors as well. Each sample was processed and assayed in duplicate.

pp65 Antigenemia and NASBA pp67 RNA Assay

The pp65 antigenemia assays were done by standard procedures and expressed as number of pp65 Ag+ cells/ 2×10^5 PBLs. The reagents for indirect immunofluorescence were monoclonal blend anti-Human Cytomegalovirus pp65 CINApool 1C3 AYM-1 (Argene Biosoft, Varilhes, France) and sheep anti-mouse IgG-FITC (Bio-Rad, Marnes-la-Coquettes, France).

The Nuclisens CMV pp67 assay (NASBA method) was carried out according to the manufacturer's instruction (Organon Teknika, Boxtel, The Netherlands).

Statistical Analysis

The intrinsic accuracy of the standard curve performed routinely in each session of real-time CMV assay was evaluated with the parametric Lin's test [Lin, 2000]. The diagnostic accuracy of the three diagnostic tests was assessed by calculating proper diagnostic accuracy indexes; the data were evaluated as categorical. The ROC method was applied using pp65 antigenemia as a gold standard. The correlation between the three tests was evaluated by multiple regression.

In order to obtain a surrogate of a clinical end point, a latent class cluster analysis was made. This method identifies the clusters of cases sharing similar clinical characteristics, by performing a probability-based classification. The package Latent GOLD 2.0 (Statistical Innovations, Inc., Belmont, MA) was used. For this purpose, the size rank of the quantitative data (pp65 antigenemia, CMV DNA) was used after $\log_{10}$ transformation, and by truncating the decimal digits. Negative results (absent signal) were arbitrarily set to −1. Nuclisens pp67 RNA assay outcome was set to "0" (negative) or "1" (positive).

RESULTS

The internal accuracy of CMV DNA assay was confirmed routinely by the Lin's test. Indeed, the concordance correlation coefficient was always close to the ideal value of 1.

The correlation between CMV DNA viral load, assessed by the real-time PCR method, and the present-time quantitative gold standard, pp65 antigenemia, was evaluated by the ROC curve method on 251 blood samples. In detail, ≥130 DNA copies/ 2×10^5 PBLs predicted ≥1 pp65 Ag+ cell/2×10^5 PBLs, ≥799 DNA copies predicted ≥10 pp65 Ag+ cells, ≥7,872 DNA copies predicted ≥50 pp65 Ag+ cells. These data are plotted in Figure 1.

The diagnostic accuracy indexes of both CMV DNA assay and Nuclisens CMV pp67 assay are shown in Table I, where the data are evaluated as categorical. In the upper part of the table, pp65 antigenemia is adopted as the gold standard. The specificity of pp67 RNA is good (0.93), but the sensitivity is very low (0.20). The sensitivity of real-time PCR is high (0.95). The apparent low specificity (0.50) of this assay can be explained presumably by its sensitivity, intrinsically higher than that of the reference test (i.e., most apparent false positives could be true positives). As a beyond chance measure

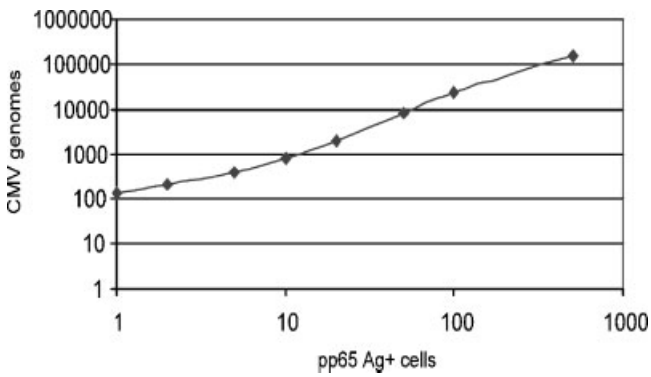

Fig. 1. Relation between pp65 antigenemia and quantitative CMV DNA assay. Since the relation was not linear, the points were calculated by means of a non-parametric, distribution-independent approach, the ROC curve method. This was obtained by setting various breakpoints for the pp65 Ag+ cell/2×10^5 peripheral blood leukocytes: 1, 2, 5, 10, 20, 50, 100, and 500.

TABLE I. Diagnostic Accuracy Indexes

	Sensitivity	Specificity	Kappa	*P*-value
Gold standard: pp65 antigenemia				
Real-time PCR	0.95	0.50	0.45	0.0000
pp67 RNA	0.20	0.93	0.12	0.0483
Gold standard: real-time PCR				
pp65 antigenemia	0.65	0.91	0.45	0.0000
pp67 RNA	0.18	1.00	0.11	0.0137

Upper part of the table: Diagnostic accuracy indexes of CMV quantitation by real-time PCR and of Nuclisens CMV pp67 assay if pp65 antigenemia is used as reference test (gold standard). Lower part of the table: Diagnostic accuracy indexes of pp65 antigenemia and of Nuclisens CMV pp67 assay if CMV genomic quantitation by real-time PCR is adopted as gold standard. Kappa is the measure of agreement of each test with the gold standard; *P*-value refers to kappa (null hypothesis: $k = 0$).

of agreement, the Cohen's kappa coefficient is used [Cohen, 1960]. For comparison, the same indexes are also evaluated on pp65 antigenemia and Nuclisens CMV pp67 assay by taking the real-time CMV DNA assay as the gold standard, instead of pp65 antigenemia (lower part of the table).

The ROC method was also used to compare the diagnostic accuracy of quantitative CMV DNA to that of pp67 RNA detection. Again, the "gold standard" was pp65 antigenemia. When the break-point for this test was set to ≥ 1 pp65 Ag+ cell/2×10^5 PBLs, the areas under the curve were 0.87 for CMV DNA and 0.54 for pp67 RNA (P-value $< 10^{-6}$). The difference in diagnostic accuracy was evident (Fig. 2). However, when viremia was high (in cases with ≥ 50 pp65 Ag+ cells/2×10^5 PBLs), the accuracy of pp67 RNA was much better (Fig. 3). At this level, no significant difference in accuracy between quantitative CMV DNA and pp67 RNA assay was detectable.

When the cases were grouped according to the pp67 RNA detection, the presence of pp67 RNA predicted higher pp65 expression and higher CMV genomic copy levels in a significant way (MANOVA, P-value 6.29×10^{-9}).

The multiple regression analysis demonstrated a significant correlation among the three tests (P-value $<$ 0.000001, R-squared = 0.5090). However, the Ramsey regression specification error test (RESET) for omitted variables [Ramsey, 1969] was significant (P-value = 0.0009). Moreover, an interaction between pp67 RNA and genomic viral load (P-value = 0.001) was detected. This interaction implies a different slope for the regression line between pp65 antigenemia and CMV DNA in relation to the outcome of the pp67 RNA test. Namely, when pp67 was positive, the line was steeper (Fig. 4). The difference between the slopes was significant (P-value = 0.0014). As a consequence, when the pp67 RNA test was positive, the ratio between CMV DNA copy number and pp65 Ag+ cell number was, on the average, 89:1 (95% CI 23–155:1), whereas, when the pp67 RNA test was negative, this value was 209:1 (95% CI 173–244:1).

A latent class cluster analysis was made. A three-cluster model was found suitable for data fitting. Cluster 1 included CMV non-viremic or very low-viremic cases, cluster 2 contained low–moderate level viremia patients, and cluster 3 referred to high viral load infections

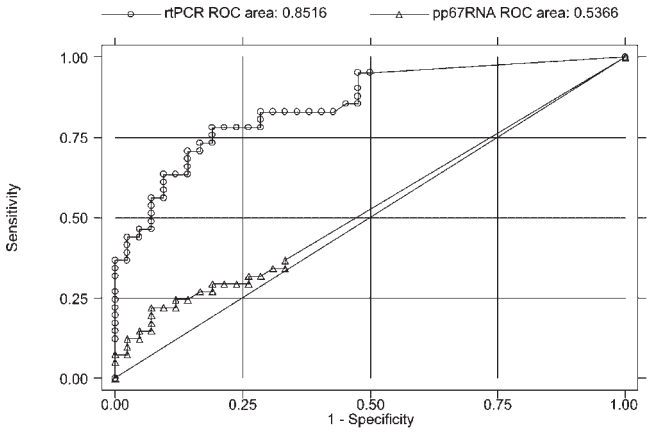

Fig. 2. Comparative evaluation of real-time PCR for CMV DNA and of pp67 RNA assay by the ROC curve method. The gold standard is the pp65 antigenemia (≥ 1 pp65 antigen positive cell/2×10^5 PBLs). The area under the curve provides an estimation of the diagnostic accuracy. Real-time PCR (circle) performs better than the other method (triangle) in predicting the outcome of the pp65 antigenemia when the breakpoint is ≥ 1 positive cell/2×10^5 PBLs (P-value $< 10^{-6}$).

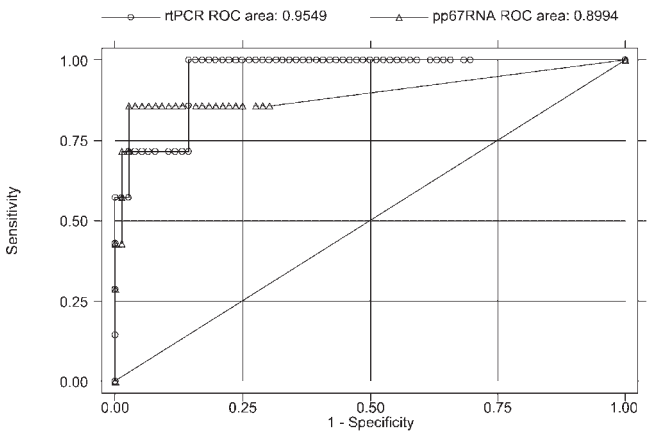

Fig. 3. Comparative evaluation of real-time PCR for CMV DNA and of pp67 RNA assay by the ROC curve method. By setting the breakpoint for pp65 at ≥ 50 positive cells/2×10^5 PBLs, the accuracy of pp67 RNA assay proves comparable to that of real-time PCR.

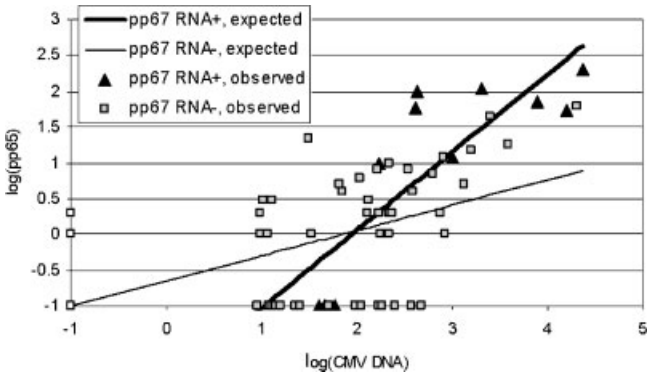

Fig. 4. Linear regression test between pp65 antigenemia (y-line) and CMV DNA (x-line). Data are $\log_{10}$-transformed. If the pp67 RNA test is positive, the line is steeper. The difference between the slopes is significant (P-value = 0.0014).

(Fig. 5). In Table II, the combined predictive value of the three tests is shown as probability for each diagnostic profile to belong to a definite cluster. The modal column indicates the mode, that is, the cluster with the highest probability (1 for cluster 1, and so on). Clearly, cluster 1 (44 cases) includes patients with no infection or very low CMV viremia, cluster 2 (24 cases) includes patients with low to intermediate viremia, and cluster 3 (15 cases) includes highly viremic patients.

DISCUSSION

After BM or solid organ transplantation, viremic CMV infection is observed frequently. Quantitative detection

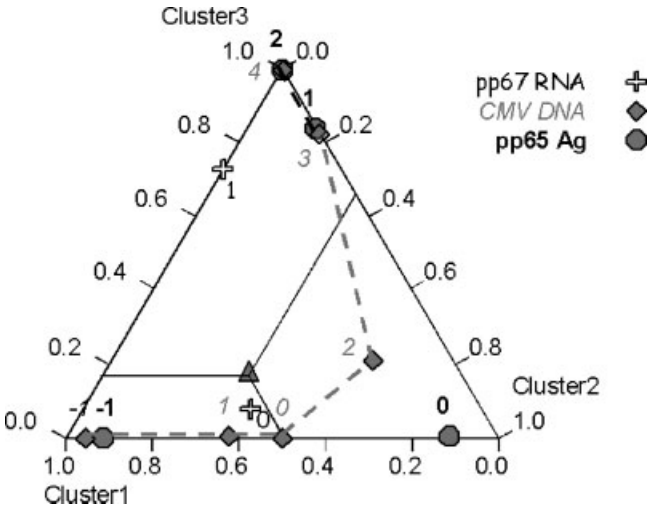

Fig. 5. Tri-plot of the three-cluster model. Quantitative data (pp65 antigenemia, CMV DNA) have been transformed to $\log_{10}$ and rounded by truncating the decimal digits; negative results (zero results) have been arbitrarily set to −1. Circles indicate pp65 antigenemia, where "−1" means negative result, "0" refers to the range 1–9, "1" refers to 10–99, "2" refers to 100–999. The scale of CMV DNA (diamonds) extends to level "3" (1,000–9,999) and "4" (10,000–99,999). A broken line connects CMV DNA levels. Nuclisens pp67 RNA assay is indicated by crosses (negative = "0", positive = "1"). The small inner triangle indicates the overall probabilities for the three clusters associated with the vertices of the outer triangle. It represents the centroid of the latter.

of CMV in blood is mandatory in managing the patient. To date, this task has been fulfilled by pp65 antigenemia. The pp65 antigen is a structural (late) protein, the lower matrix phosphoprotein. It is detected by a monoclonal antibody in the PBLs. This method is time-consuming, labor-intensive and requires skilled operators. The interval between sampling and processing must be <3 hr. False negative results may occur in neutropenic patients. False positive results, in some cases, can reflect late phagocytosis by PBLs.

Real-time PCR has practical advantages. The interval between sampling and processing may be long. The cost of each PCR is low because consumables are relatively cheap. After DNA extraction, results are obtained in 1–2 hr. The use of a closed system implies no chance of cross contamination with previous PCR products. The method is non-subjective in reading and automated. The dynamic range of quantitation is wide (7–8 logarithmic decades), the technical sensitivity is high (<5 copies) as it is the quantitative precision. Together with low turn-around times and decreased costs, these aspects have revolutionized the field of molecular diagnostics, enabling a shift toward a high-throughput and automated technology [Bustin, 2000; Klein, 2002]. Quantitative real-time PCR is probably superior to pp65 antigenemia, and its use in this field could represent a progress, particularly in a setting where immunosuppression is severe, as in BM transplant.

The present report is a contribution to validation of real-time PCR as a new standard for quantitative assessment of CMV viremia in a clinical setting. This was accomplished through a correlative investigation involving pp65 antigenemia and pp67 RNA assay. As a surrogate of a clinical end point, the latent class analysis provided clear predictive values for the combination of the three tests. Every threefold eventual outcome of the diagnostic triad was linked to a probability to belong to a definite cluster (highly viremic, low viremic, or non-viremic) of implicit clinical relevance.

Extending a preliminary report [Mengoli et al., 2001], a threshold of 1 pp65 antigen positive cell/2×10^5 PBLs appeared to correspond to 130 CMV genomes/2×10^5 PBLs, as detected by real-time PCR. This result can be compared to that obtained by Li et al. [2003], although different biological materials (whole blood instead of PBLs) were used. Indeed, Li et al. proposed the CMV viral load of 1,000 copies/ml of blood as corresponding to 1 to 2 pp65 Ag+/2×10^5 PBLs. The superior sensitivity of real-time CMV quantitation with respect to pp65 Ag assay is evident from our data (Fig. 1, Table I) confirming a recent report [Leruez-Ville et al., 2003].

Assessment of CMV mRNA represents the biological link between presence of CMV genome and CMV gene expression. A diagnostic approach at this level has the potential of better distinguishing between active viral replication leading to disease and latent infection. Several recent studies report comparative evaluation of pp65 antigenemia, molecular detection of CMV genome, and pp67 RNA by NASBA in a clinical environ-

TABLE II. Predictive Values of all Diagnostic Profiles

pp67 RNA	CMV DNA	pp65	Modal	Cluster 1	Cluster 2	Cluster 3
0	−1	−1	1	0.9890	0.0110	0
0	−1	0	1	0.5622	0.4378	0
0	0	−1	1	0.9626	0.0374	0
0	0	0	2	0.2684	0.7315	0.0001
0	1	−1	1	0.8802	0.1198	0
0	1	0	2	0.0948	0.9043	0.0009
0	1	1	2	0.0013	0.8802	0.1185
0	2	−1	1	0.6774	0.3225	0
0	2	0	2	0.0288	0.9607	0.0105
0	2	1	3	0.0002	0.3972	0.6026
0	3	0	2	0.0076	0.8840	0.1084
0	3	1	3	0	0.0553	0.9447
0	4	1	3	0	0.0052	0.9948
1	1	−1	1	0.9964	0.0036	0
1	2	1	3	0	0.0011	0.9989
1	3	1	3	0	0.0001	0.9999
1	3	2	3	0	0	1
1	4	1	3	0	0	1
1	4	2	3	0	0	1

Combined predictive value of the three diagnostic tests as probability for each diagnostic profile to belong to a definite cluster. The profile is defined by the outcome of the three diagnostic tests as reported in the first three cells of each row. The probability for every profile to belong to each of the three clusters is reported in the last three cells. Quantitative data (pp65 antigenemia, CMV DNA) are transformed to log10 and rounded by truncating the decimal digits; negative results (absence of signal) are arbitrarily set to −1. Nuclisens pp67 RNA assay outcome is indicated by "0"(negative) or "1" (positive). The modal column indicates the cluster with the highest probability.

ment (transplant recipients) [Pellegrin et al., 2000; Amorim et al., 2001; Kulkarni et al., 2001; Hebart et al., 2002]. However, the present report is the first concurrent quantitative real-time detection of CMV DNA by TaqMan method, pp65 antigenemia, and pp67 RNA assay. On the sensitivity side, the performance of pp67 RNA assay was the lowest among the three diagnostic tests. However, the specificity was very high (100%). Further, pp67 RNA was positive only in cases with high viral load, thus predicting a severe CMV infection (Table II, Fig. 5). Indeed, recent studies with Nuclisens assay for CMV pp67 mRNA in blood have confirmed the high clinical specificity but have questioned the sensitivity of the assay [Blok et al., 1998; Caliendo et al., 2002]. On the other hand, this is not the full picture. The existence of an interaction between genomic viral load and pp67 RNA assay suggests the latter exerts a positive modifier effect as far as pp65 antigen expression is concerned. When the pp67 RNA test was positive, on the average 89 CMV DNA copies were found for every pp65 Ag+ cell, whereas, when the pp67 RNA test was negative, this value was 209. This suggests that higher efficiency in late (lytic) gene expression, as a measure of productive infection, can occur with lower genomic copies per infected cell. Besides, the ratio between CMV DNA copies and the pp65 antigen positive cell count may be viewed as a surrogate marker for the pp67 RNA assay. Thus, information provided by the pp67 RNA assay is not redundant with respect to either pp65 antigenemia or CMV DNA.

The multiple regression, albeit significant, had R-squared = 0.5090, indicating the proportion of the total variance of pp65 antigenemia that could be explained by the other two variables. This value and the outcome of the RESET test suggest the existence of at least one further predictor, which, although not considered, must substantially impact on viral gene expression. This hidden variable should be detected and monitored in order to predict the outcome of CMV infection with greater accuracy, and to help clinical decisions. The nature of this indicator is a matter of conjecture. It might be CMV related, as the presence of other RNA transcripts, or host related, as immunological aspects and genetic determinants. In this regard, immunological parameters recently involved in protective antiviral immunity have been expansion of $\gamma\delta$ T-cells after kidney allograft transplantation [Lafarge et al., 2001] and counts of CMV-specific $CD8^+$ T-cell [Aubert et al., 2001]. Pharmacodynamic aspects could provide additional prognostic information in solid organ recipients with CMV disease. In fact, under standard antiviral therapy, viral load decay was slower (half-life 8.8 days) among patients with CMV recurrence than among patients without recurrence (half-life 3.2 days) [Humar et al., 2002]. Expression of immune evasion genes US3, US6, and US11 could also play a role [Greijer et al., 2001a]. Moreover, the study of immediate-early (IE) mRNA [Gerna et al., 2000, 2003; Revello et al., 2001; Goossens et al., 2004] appeared to be a valuable tool for early diagnosis of CMV infection. Indeed, additional insight is expected by studying profiles of CMV gene transcription.

At present, there is a need for a more sensitive assay for pp67 RNA along with a quantitative version of the test, as proposed recently [Greijer et al., 2001b]. For the time being, real-time PCR that measures blood CMV

DNA load represents a sensitive and specific test to assist clinicians in preventing CMV disease.

REFERENCES

Amorim ML, Cabeda JM, Seca R, Mendes AC, Castro AP, Amorim JM. 2001. CMV infection of liver transplant recipients: Comparison of antigenemia and molecular biology assays. BMC Infect Dis 1:2.

Aubert G, Hassan-Walker AF, Madrigal JA, Emery VC, Morte C, Grace S, Koh MB, Potter M, Prentice HG, Dodi IA, Travers PJ. 2001. Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. J Infect Dis 184:955–963.

Blank BSN, Meenhorst PL, Mulder JW, Weverling GJ, Putter H, Pauw W, van Dijk WC, Smits P, Lie-A-Ling S, Reiss P, Lange JMA. 2000. Value of different assays for detection of human cytomegalovirus (HCMV) in predicting the development of HCMV disease in human immunodeficiency virus-infected patients. J Clin Microbiol 38: 563–569.

Blok MJ, Goossens VJ, Vanherie SJV, Top B, Tacken N, Middeldorp JM, Christiaans MH, van Hoof JP, Bruggeman CA. 1998. Diagnostic value of monitoring human cytomegalovirus late pp67 mRNA expression in renal allograft recipients by nucleic acid sequence-based amplification. J Clin Microbiol 36:1341–1346.

Bustin SA. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193.

Caliendo AM, St George K, Allega J, Bullotta AC, Gilbane L, Rinaldo CR. 2002. Distinguishing cytomegalovirus (CMV) infection and disease with CMV nucleic acid assays. J Clin Microbiol 40:1581–1586.

Cohen J. 1960. A coefficient of agreement for nominal scales. Educ Psychol Meas 20:37–46.

Einsele H, Ehninger G, Hebart H, Wittkowski KM, Schuler U, Jahn G, Mackes P, Herter M, Klingebiel T, Loffler J. 1995. Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. Blood 86:2815–2820.

Emery V, Sabin CA, Cope AV, Gor D, Hassan-Walker AF, Griffiths PD. 2000. Application of viral load kinetics to identify patients who develop cytomegalovirus disease after transplantation. Lancet 355:2032–2036.

Gerna G, Zipeto D, Parea M, Percivalle E, Zavattoni M, Gaballo A, Milanesi G. 1991. Early virus isolation, early structural antigen detection and DNA amplification by the polymerase chain reaction in polymorphonuclear leukocytes from AIDS patients with human cytomegalovirus viraemia. Mol Cell Probes 5:365–374.

Gerna G, Zavattoni M, Baldanti F, Sarasini A, Chezzi L, Grossi P, Revello MG. 1998. Human cytomegalovirus (HCMV) leukodnaemia correlates more closely with clinical symptoms than antigenemia and viremia in heart and heart-lung transplant recipients with primary HCMV infection. Transplantation 65:1378–1385.

Gerna G, Baldanti F, Middeldorp JM, Furione M, Zavattoni M, Lilleri D, Revello MG. 1999. Clinical significance of expression of human cytomegalovirus pp67 late transcript in heart, lung, and bone marrow transplant recipients as determined by nucleic acid sequence-based amplification. J Clin Microbiol 37:902–911.

Gerna G, Baldanti F, Lilleri D, Parea M, Alessandrino E, Pagani A, Locatelli F, Middeldorp J, Revello MG. 2000. Human cytomegalovirus immediate-early mRNA detection by nucleic acid sequence-based amplification as a new parameter for pre-emptive therapy in bone marrow transplant recipients. J Clin Microbiol 38: 1845–1853.

Gerna G, Lilleri D, Baldanti F, Torsellini M, Giorgiani G, Zecca M, De Stefano P, Middeldorp J, Locatelli F, Revello MG. 2003. Human cytomegalovirus immediate-early mRNAemia versus pp65 antigenemia for guiding pre-emptive therapy in children and young adults undergoing hematopoietic stem cell transplantation: A prospective, randomized, open-label trial. Blood 101:5053–5060.

Goossens VJ, Christiaans MH, Blok MJ, Terporten PH, Sillekens P, Lukacsi A, Van Hooff JP, Bruggeman CA. 2004. Onset and duration of cytomegalovirus immediate early 1 mRNA expression in the blood of renal transplant recipients. J Med Virol 72:94–101.

Gotzlan J, Salord JM, Chouaid C, Duvivier C, Picar O, Meyohas MC, Petit JC. 1993. Human cytomegalovirus (HCMV) late-mRNA detection in peripheral blood of AIDS patients: Diagnostic value for HCMV disease compared with those of viral culture and HCMV DNA detection. J Clin Microbiol 31:1943–1945.

Greijer AE, Verschuuren EA, Dekkers CA, Adriaanse HM, van der Bij W, The TH, Middeldorp JM. 2001a. Expression dynamics of human cytomegalovirus immune evasion genes US3, US6, and US11 in the blood of lung transplant recipients. J Infect Dis 184:247–255.

Greijer AE, Verschuuren EA, Harmsen MC, Dekkers CA, Adriaanse HM, The TH, Middeldorp JM. 2001b. Direct quantification of human cytomegalovirus immediate-early and late mRNA levels in blood of lung transplant recipients by competitive nucleic acid sequence-based amplification. J Clin Microbiol 39:251–259.

Halwachs G, Zach R, Pogglitsch H, Holzer H, Tiran A, Iberer F, Wasler A, Tscheliessnigg HP, Lanzer G, Folsch B. 1993. A rapid immunocytochemical assay for CMV detection in peripheral blood of organ-transplanted patients in clinical practice. Transplantation 56:338–342.

Hebart H, Rudolph T, Loeffler J, Middeldorp J, Ljubicic T, Jahn G, Einsele H. 2002. Evaluation of the NucliSens CMV pp67 assay for detection and monitoring of human cytomegalovirus infection after allogeneic stem cell transplantation. Bone Marrow Transplant 30: 181–187.

Humar A, Kumar D, Boivin G, Caliendo AM. 2002. Cytomegalovirus (CMV) virus load kinetics to predict recurrent disease in solid-organ transplant patients with CMV disease. J Infect Dis 186:829–833.

Klein D. 2002. Quantification using real-time PCR technology: Applications and limitations. Trends Mol Med 8:257–260.

Kulkarni A, Westmoreland D, Fox JD. 2001. Molecular-based strategies for assessment of CMV infection and disease in immunosuppressed transplant recipients. Clin Microbiol Infect 7:179–186.

Lafarge X, Merville P, Cazin MC, Bergé F, Potaux L, Moreau JF, Déchanet-Merville J. 2001. Cytomegalovirus infection in transplant recipients resolves when circulating $\gamma\delta$ lymphocytes expands, suggesting a protective antiviral role. J Infect Dis 184:533–541.

Leruez-Ville M, Ouachèe M, Delarue R, Sauget AS, Blanche S, Buzyn A, Rouzioux C. 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.

Li H, Dummer JS, Estes WR, Meng S, Wright PF, Tang YW. 2003. Measurement of human cytomegalovirus loads by quantitative real-time PCR for monitoring clinical intervention in transplant recipients. J Clin Microbiol 41:187–191.

Lin LIK. 2000. A note on the concordance correlation coefficient. Biometrics 56:324–325.

Locatelli F, Percivalle E, Comoli P, Maccario R, Zecca M, Giorgiani G, De Stefano P, Gerna G. 1994. Human cytomegalovirus (HCMV) infection in paediatric patients given allogeneic bone marrow transplantation: Role of early antiviral treatment for HCMV antigenaemia on patients' outcome. Br J Haematol 88:64–71.

Mengoli C, Biasolo MA, Cusinato R, Palù G. 2001. Cytomegalovirus viremia assessment by quantitative real time PCR. Abstracts of the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy (Chicago). Washington, D.C.: American Society for Microbiology. 305p.

Pass RF. 2001. Cytomegalovirus. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, editors. Fields virology. Vol. 2, 4th edition. Philadelphia, PA: Lippincott Williams & Wilkins. pp 2675–2705.

Pellegrin I, Garrigue I, Ekouevi D, Couzi L, Merville P, Merel P, Chene G, Schrive MH, Trimoulet P, Lafon ME, Fleury H. 2000. New molecular assays to predict occurrence of cytomegalovirus disease in renal transplant recipients. J Infect Dis 182:36–42.

Ramsey JB. 1969. Tests for specification errors in classical linear least squares regression analysis. J Royal Statist Soc Ser B 31:350–371.

Revello MG, Lilleri D, Zavattoni M, Stronati M, Bollani L, Middeldorp JM, Gerna G. 2001. Human cytomegalovirus immediate-early messenger RNA in blood of pregnant women with primary infection and of congenitally infected newborns. J Infect Dis 184:1078–1081.

Schweitzer B, Kingsmore S. 2001. Combining nucleic acid amplification and detection. Curr Opin Biotechnol 12:21–27.