CYTOMEGALOVIRUS DIAGNOSIS – STILL AN OPEN QUESTION

ABSTRACT

Cytomegalovirus is widely spread in the population, but most carriers are asymptomatic. The increased number of immunocompromised patients is at high risk of reactivating or acquiring CMV disease, which shows high mortality rates among this population. CMV diagnosis that can distinguish ongoing infection from latency is therefore of utmost importance. In this review we present different approaches for CMV diagnosis and discuss the critical parameters involved.

INTRODUCTION.

During the last decades, improvements in medicine procedures and treatment have raised the number of immunocompromised patients. Immunosuppressive treatments are used in transplant and oncological patients, lowering their leukocyte counts. Neutropenic patients lack the main host defense against viruses, the cell-mediated immunity.

Cytomegalovirus (CMV) is an opportunistic virus with a complex infection pattern, primary infection being followed by persistent or latent infection. CMV primary infection might occur during pregnancy and since the virus is present in body secretions including urine, saliva and milk, its seroprevalence in the population reaches 70 to 100% (Ho 1991; Marin et al. 2004). The efficient CMV transmission can explain its widespread distribution. Although most carriers are asymptomatic, this ubiquitous virus became a common complication for immunocompromised patients. There are evidences that CMV replicates at low level or persists by integration into host cell chromosome (Jarvis & Nelson 2002). Mechanisms of latency of the virus, reactivation and other pathogenic aspects are presently under investigation. These data are important for accurate diagnosis and vaccine development, and for identification of potential targets for chemotherapy. The aim of this review is to discuss the applicability of different methods for the diagnosis of CMV infection, presenting general concerns and a few examples of its use.

Biology and pathogenicity of CMV.

CMV is a ã-herpesvirus with a double-stranded linear DNA genome (~230kbp), encoding for more than 200 proteins. It has three temporally regulated families of genes: ã (alpha) or IE genes are expressed in an immediate early phase of virus
replication, α (beta) or E genes show a delayed early expression, and α (gamma) or L genes are expressed in a later phase of the replication cycle in infected cells (Spear & Longnecker 2003). Transcription of IE genes is controlled by multiple factors that bind to a promoter region and multiple enhancer sites. Most IE genes transcripts have regulatory functions positively or negatively acting on E genes promoter. Actually, some IE and E genes exhibit a cooperative interaction in controlling replication gene promoters. L genes encode viral structural proteins involved in the formation of mature viral particles (Sweet 1999).

CMV spreads via bloodstream and can invade various organ systems, causing different levels of damage and symptoms (Siniger & Jahn 1996). Pneumonitis, for instance, is frequent in recipients of allogeneic renal, liver, or heart transplants. However, there is low incidence of lung damage in syngeneic bone marrow recipients or in AIDS patients (Henry & Hamilton 1993).

During viremia, CMV can be detected in monocytes, lymphocytes, neutrophils and in the acellular fractions of blood (Bruggeman 1993). Differential tropism and CMV replication in endothelial, as well as in white blood cells, are regulated by a number of viral genes that might be used as targets for antiviral therapy (Siniger et al. 1999; Hanson et al. 2001). Endothelial and blood cells are thought to be the major reservoirs in life-long persistence of the virus (Grefte et al. 1993; Jarvis & Nelson 2002; Compton 2004). Inside the cell, CMV accumulates in large cytoplasmic vacuoles that fail to enter the lysosomal pathway and do not fuse to the plasma membrane (Fish et al. 1996).

It is suggested that reactivation of the latency state is achieved by up-regulation of IE gene expression to a threshold level that allows for E gene products to be synthesized and then lead to completeness of virus replication (Kramer & Coen 1995; Isomura & Stinski 2003).

Viral glycoproteins are thought to have important role in virus attachment and subsequent entry into human cells. The gene encoding for the structural glycoprotein B is used to classify CMV in at least five genotypes, gB1 through 5, which correlate with cell tropism and virulence (Chou & Dennison 1991, Brown 1995, Sheph et al. 1998).

Seronegative transplant recipient of solid organs from seropositive donor has the worst prognosis if CMV infection is not prevented (Razonable et al. 2002). Without the constraints of a normal immune system, CMV disseminates very fast. In these cases, qualitative assays may help on prevention and management of post-transplantation. Preemptive therapy for immunocompromised patients has resulted in reduced incidences of CMV disease (Razonable et al 2002, Hong et al 2004). Nevertheless, the usual drugs, ganciclovir, cidofovir and fosarnet are either myelotoxic or nephrotoxic (Michiel & Mertens 2004).

Non-molecular based diagnosis of CMV.

Non-molecular methods for CMV diagnosis are either based on patient's humoral response, on viral antigens detection in leukocytes or on virus cultivation.

There is no indication for CMV serology of immunocompromised patients. Although it can identify infected recipient and/or donor, a pertinent issue for transplantation prognosis, the immune failure of recipients and the wide prevalence of CMV significantly reduce its clinical value (Halling et al. 2001).

The antigenemia assay quantitatively detects CMV lower matrix protein pp65 by monoclonal antibody and directly immunoassaying of polymorphonuclear leukocytes (PMN). The assay is highly specific and has been adopted by many laboratories, although the subjective interpretation of results is considered a drawback. Another shortcoming of the test is the high rate of false-negative results due to the low number of leukocytes of transplant patients. The number of infected PMN has different relevance depending on the type of pathology. Although thresholds have been recommended, there is still lack of standardization. Nevertheless, new diagnostic methods are frequently compared to antigenemia because of its good quantitative correlation with the symptoms development (Griffiths & Whitley 2000).

Recovery of CMV by cell culture methods, even using the faster shell vial approach (turn around time of 16 to 48 h), has low sensitivity and is cumbersome, not suited for large number of samples (Razonable et al 2002).

Molecular-based CMV diagnosis.

The use of molecular-based approaches to detect CMV infection is a significant innovation, showing reduced turnaround time, high sensitivity, high specificity, and easy of manipulation. In-house PCR-based methods can be used as an alternative
approach to the expensive commercial kits. Nevertheless, lack of standardization precludes comparison of results obtained from different studies. There is no consensus of which is the better method.


Shibata et al. (1988) described the use of a PCR protocol to detect CMV in peripheral blood of patients infected with human immunodeficiency virus (HIV). A pair of primers and a probe was used to amplify and detect CMV immediate early gene with good sensitivity (10 copies of recombinant plasmids per assay). Adding another set of primers, directed to the late gp64 gene, resulted in detection of all tested wild-type CMV isolates and could discriminate infective from non-infective CMV DNA-containing particles. While 49% of HIV-infected blood was positive for CMV in PCR, only 24% was positive in culture, indicating that culture was far less sensitive than PCR. The same approach was used by Demmler et al. (1988) who detected CMV with high sensitivity, 93% after gel electrophoresis and 100% after dot-blot. PCR showed to be more sensitive than enzyme-like immunoabsorbent assay (ELISA), non-radioactive DNA hybridization assay or virus culture in urine from renal transplanted patients with very low viral load (Olive et al. 1989). In the same year, Cassol et al. (1989) reported the use of PCR to detect CMV DNA in a variety of tissues of infected marrow transplant recipients. PCR could not detect CMV DNA in peripheral blood of healthy volunteers, independently of their CMV serological status, or in blood of sero-positive-transplanted patients without evidence of active CMV disease, pointing to its potential clinical utility (Rowley et al. 1991).

Later PCR technology was used as a tool for epidemiologic studies. Restriction analysis of the repetitive sequences amplified from the heterogeneous L-S junction region showed its usefulness for epidemiological purposes (Zaia et al. 1990; Sokol et al. 1992; Bale et al. 1993). Amplification and subsequent restriction analysis of other regions (CMV DNA polymerase, glycoproteins B and H) resulted in profiles that could differentiate all strains studied (Chou 1990).

Quantitative in-house PCR.

The importance of quantitative assays has been recognized because of the high prevalence of CMV in health individuals. Efficient and effective management of CMV infection must distinguish CMV reactivation from latency, which is possible through quantitative assays performed longitudinally. Moreover, monitoring viral load can indicate the best treatment, suggesting lower or increased doses, or even changing the antiviral drug (in the case of resistance), without exposing patients to unnecessary risks to their health. Treatment length and reactivation of a latency state can also be determined by following viral load along the time.

In-house competitive PCR assays.

Viral load can be estimated from a standard curve, generated by plotting logarithmic values of PCR products (densitometric data) against logarithmic values of known quantities of target DNA. Interpolating the values of PCR products from the clinical sample into the standard curve determines the concentration of target DNA in this sample. Generally, amplified products are quantified by densitometric evaluation after gel electrophoresis or hybridization. To be a reliable approach, quantitative PCR must consider data from the exponential phase of the reaction. After reaching the plateau or saturation, PCR products concentration is independent of the original number of targets. In conventional PCR, where amplicons are visualized after gel electrophoresis, care should be taken since samples with higher viral load will reach the plateau before those with a lower number of viruses.

The efficiency of two duplex-PCR assays for quantitation of CMV was compared. One assay used a 435 bp fragment of the major immediate early gene of CMV (MIE-globin assay) whereas the second assay used a 200 bp fragment of the late antigen CMV gene (LA-globin assay) as specific target. A 536 bp fragment of the human globin gene was co-amplified in both assays. Co-amplification of CMV DNA and the human gene were compared to a standard curve construct with end-point titration of known amounts of recombinant plasmid containing CMV DNA insert and human placental DNA. The limit of detection of LA-globin was 100 copies of CMV, a log lower than the MIE-globin assay (Kulski 1994).

Another approach for viral load determination is the use of competitive PCR. In this method an internal control competes with the target DNA for
the same primer pair. Any variable affecting the reaction has the same effect on competitor (internal control) and target DNA present in the sample. In theory, there is no variation in primer efficiency and the relative product ratio of competitor and target templates is proportional to the ratio of the initial amount of each of them (Clementi et al. 1993). It is important to keep in mind that amplification efficiency is inversely proportional to the size of the amplified product (Chelly et al. 1990).

In competitive PCR, homologous competitors have the same sequence as the target, except for a difference in the length, either a deletion, an insertion, or a mutation that creates or abolishes a restriction site in the control DNA. Evans et al. (1999) described the use of a competitive PCR for detection of CMV DNA in serially collected serum samples from three bone marrow transplant recipients and from 15 patients with severe CMV disease. A 239 bp region of CMV major immediate early gene was used as a target and a plasmid where the same fragment was cloned with a 50 bp deletion was used as competitor. Amplification products were distinguished by their size upon gel electrophoresis and their relative amount were compared and plotted after regression analysis. Some discrepancies were reported because of saturation of the PCR reaction (Evans et al. 1999).

Homologous competitors do avoid differences in primer efficiency, but they can form heteroduplexes that might interfere with the reaction. Heterologous internal competitors, on the other hand, are recognized by the same primer as the target, but contain a completely different intervening sequence. Although not forming heteroduplexes, the size and G+C content of the competitor must be similar to that of the target to avoid differences in amplification efficiency. The effect of antiviral therapy in AIDS patients with CMV retinitis was in fact analyzed using heterologous competitive PCR assay (Gerna et al. 1994).

A combination of competitive PCR and sandwich hybridization was used to determine the CMV viral load in culture samples (Alexandre et al. 1998). The method used the same primer pair to amplify CMV target DNA and the internal standard. Both 313 bp products were captured on a solid support by a probe that recognizes identical sequences. Specific detection sequence of 40 bp, different in the target and the competitor template, were used in parallel hybridization experiments with specific biotinylated probes. A streptavidin conjugated-enzyme was used to perform a colorimetric detection allowing for quantification on a long concentration range (3 logs), leading to very reproducible results.

A complication in a quantitative approach is the accurate measurement of amplification products in competitive PCR, when competitor and target are present in very different relative amounts. Therefore, it is desirable to carry out competitive PCR with at least three serial ten-fold dilution of competitor against a fixed volume of sample (Diviacco et al. 1992). Another drawback is that samples with different viral load achieve the plateau after different number of PCR cycles (Evans et al. 1999). Fixing the amount of competitor and serially diluting the sample is also a possibility to avoid such discrepant results.

**Real-time PCR.**

Generally, in-house competitive PCR has not been useful for clinical laboratories because of cumbersome procedures, limited potential for batch testing and lack of standardization. Real-time PCR technology is a promising improvement for the quantification of CMV DNA, with potential to high-throughput usage in clinical settings. The technology is based on the use of fluorescent probes; Molecular Beacons (MB) and TaqMan probes are widely used. TaqMan are linear dual-labeled probes with a reporter fluorescent dye at the 5' end and a quencher at the 3' end. The proximity with the quencher precludes the fluorescence of the 5' dye. Cleavage by the 5'nuclease activity of Taq polymerase physically separates the reporter from the quencher and the sequence-specific signal is generated (Livak et al. 1995).

Molecular beacons are single-stranded oligonucleotide probes that have a stem-loop structure. The loop portion of the molecule is a probe sequence that is complementary to the target nucleic acid sequence. The stem is formed by the annealing of two complementary arm sequences, unrelated to the target, at either side of the probe. A fluorescent dye is attached to the 5' end of the probe and a quencher is attached to the other end (3'). The stem keeps these two moieties in close proximity to each other, causing the fluorescence to be quenched by resonance energy transfer in the absence of the target. When the probe anneals to a target molecule, it undergoes a conformational change that forces the arms apart, separating the fluorophore and the quencher, allowing the probe to fluoresce (Tyagi & Kramer 1996). As
with TaqMan probes, after each cycle, additional reporter dye molecules are separated from the quencher and the fluorescence intensity is continuously monitored during the PCR cycling.


The choice of the target genes was mainly determined by searches in GeneBank, looking for conserved regions (Leruez-Ville et al. 2003). Different sets of primers and TaqMan probes directed to the same region of the immediate-early gene locus of CMV were tested by Nitsche et al. (2000). Nevertheless, using BLAST analysis to compare different CMV targets, we found a more specific CMV region, which has also been widely used for molecular-based diagnosis, the glycoprotein B gene (Azevedo et al. 2003).

**Commercial kits for CMV diagnosis.**

**AMPLICOR CMV (Roche Diagnostics)** - Roche Molecular Systems offers a qualitative (AMPLICOR CMV Test) and a quantitative (COBAS AMPLICOR CMV MONITOR Test) assay. The quantitative reactions are performed on the semiautomated COBAS system and the lower limit sensitivity of the test is 400 CMV DNA copies per milliliter of plasma. The test amplifies by PCR a 365 bp sequence of CMV DNA polymerase gene UL54 using nonisotopic hybridization for detection in a microplate assay (Sia et al. 2000). The quantitative test is more sensitive than the qualitative AMPLICOR and showed good reproducibility between three laboratories. COBAS AMPLICOR CMV MONITOR was linear to 50,000 CMV DNA copies/ml, showing a greater variation at the low copy number (Caliendo et al. 2001). Although the test has a rapid turnaround time (4 h) it is performed in the expensive COBAS AMPLICOR instrument not available in most laboratories.

**Digene Hybrid Capture CMV DNA assay (Digene Corporation)** - The test is a solution hybridization where CMV DNA is recognized by a complementary RNA probe of approximately 40,000 bp. The tube inner surface is coated with antibodies that recognize and capture the RNA-DNA hybrids. Alkaline phosphatase-conjugated antibodies, which are also hybrid specific, are used in a chemoluminescence detection system. Because of the hybrid large size, approximately 1,000 conjugated antibodies binds each immobilized hybrid molecule, amplifying the signal. The procedure can be accomplished in 6 h but the assay allows for delayed processing. External standards are provided to build a calibration curve used to determine the sample viral load. The second-generation assay is claimed to detect 700 CMV copies per ml of whole blood. Comparison showed good correlation and similar sensitivity and specificity between pp65 antigenemia levels and the second-generation hybrid-capture assay (Mazzulli et al., 1999). Although the amount of emitted light, measured in a luminometer, is proportional to the amount of target DNA in the sample, the clinical significance of a positive result in this system is still not clear.

**Branched-DNA assay (Chiron Corporation)** - This assay relies on signal amplification rather than DNA amplification, which makes it less susceptible to contamination. CMV DNA present in the sample hybridizes to a series of capture probes bound to a solid surface and to target probes that allow for further hybridization of branched-DNA (b-DNA) amplifier molecules. Alkaline phosphatase-labeled probes bound to the b-DNA and light emission occurs after cleavage of a chemiluminescent substrate. Quantification is determined by comparison with a standard curve assuming the proportionality of light emission and the amount of virus DNA present in the sample. The procedure starts with DNA extraction from at least 2 x 10^6 PBL, which is a limitation for neutropenic patients. A second-generation b-DNA assay for CMV showed similar sensitivity when compared to antigenemia (Myerow et al. 1997, Boeck & Boivin, 1998).

**NucliSens assay (Organon Teknika Diagno-**
tics) — This nucleic acid sequence based amplification (NASBA) assay performs an isothermal amplification of pp67 mRNA. Although qualitative, the detection of this late transcript of CMV has been described as a marker for CMV ongoing infection (Witt et al. 2000). Nevertheless, NucliSens showed low sensitivity failing to detect pp67 mRNA in 4 of 11 patients who developed CMV infection (Pellegrin et al. 2000).

Methodological aspects of CMV diagnosis.

Appropriate blood compartment, method of extraction, primer set sensitivity and the viral target gene are technical features that have been pointed as influencing a reliable diagnosis of CMV disease (Hamprecht et al. 1997, Razonable et al. 2002, Gault et al. 2001, Yun et al. 2000). Since there is no standardization, studies that compare different assays are welcome.

CMV is a cell-associated virus, but detection and quantification has also been performed in acellular fractions of the blood such as plasma and serum (Li et al. 2003). The optimal specimen depends on the methodological approach and on patient’s pathology. Using the COBAS AMPLICOR CMV Monitor, viral load was determined in 319 samples from 16 solid organ transplant patients and 1 blood stem cell transplant using, in each assay, whole blood (WB), plasma (PL), peripheral blood leukocytes (PBL) and peripheral blood mononuclear cells (PBMC). The study demonstrated simultaneous existence of CMV DNA in the different blood compartments, but WB yielded higher viral loads (Razonable et al. 2002). Quantification of CMV using an in-house real-time PCR assay directed to the UL83 region in WB, PL and PBL also showed that WB is the most sensitive specimen for CMV detection (Mengelle et al. 2003). Whole blood allows for CMV recovery from both cellular and acellular fractions, what could explain the better results obtained (Razonable et al. 2002, Mengelle et al. 2003).

Monitoring ganciclovir therapy for CMV infection in patients with AIDS, bone marrow and solid organ transplants patients, Boivin et al. (1997) showed that viral load was significantly different among patients before therapy. Ganciclovir decreased viral load in both polymorphonuclear and mononuclear leukocytes populations, but in bone marrow transplant patients the viral load reduction was less impressive, providing evidence that different pathologies present variation in CMV infection behaviour. A number of studies show that the quantity of viral DNA in leukocytes is generally greater than in plasma for both transplant recipients and AIDS patients (Gerna et al. 1994). Five European virological laboratories analyzed 2,718 blood samples and detection of CMV DNA in leukocytes was more sensitive than in plasma (von Müller et al. 2002). Nevertheless, another clinical study has shown that CMV DNA viral load in plasma is an independent marker of CMV disease and survival in AIDS patients (Caliendo et al. 2001).

Boon et al. (2002) investigated the nature of viral DNA present in plasma and serum of renal transplant patients. DNA extracted from serum or plasma specimens were fractionated by size, indicating that amplification occurred from fragments smaller than 2 kb. Primers sets that amplified smaller fragments consistently presented a higher viral load. They suggested that CMV DNA present in the acellular fractions of blood is highly fragmented and did not represent the actual viral load in bloodstream.

A German group demonstrated that different DNA extraction methods influence the detection limit of a plasmid containing CMV sequences from serum or plasma (Hamprecht et al. 1997). In their experience, a proteinase K treatment followed by phenol/chloroform extraction was more sensitive (3 x 10^9) than a single tube method that uses a chaotropic reagent (3 x 10^9). The worst detection limit method used proteinase K without the purification step, detecting 10^5 genome-equivalents per ml of sample. Others described a better performance of commercial kits for DNA extraction from blood (Razonable et al. 2002).

Reactivation or relapse of CMV disease is best evaluated in longitudinal studies when a raise in systemic viral load is related to the onset of symptoms. Patients with CMV disease that have received a solid organ transplant usually show a higher level of antigenemia when compared to bone marrow transplant (Boeckh & Boivin 1998). Nevertheless, when comparing quantitative PCR data, there is a bias toward the chosen specimens. Many studies referred to volumes of whole blood and non-normalized samples, which makes comparison quite complicated for neutropenic patients. Even considering this drawback, it is accepted that DNA levels differ among patient populations (Boeckh & Boivin 1998). Kidney transplant patients have a lower systemic viral load than other solid organ recipients,
but CMV DNA can be detected in the urine of renal patients (Toyoda et al. 1997). Heart and liver transplant recipients have higher viral load detected in blood than marrow transplant patients (Ziaia et al. 1997; Cope et al. 1997). A threshold in viral load that could indicate a higher risk of CMV disease is still a conflicting issue. Low CMV load, for instance, has been associated with disease in rapidly progressing patients (Gor et al. 1998).

The lower limit of detection, inter- and intra-assay variability, linear range and upper limit of quantification of commercial kits and in-house tests have been analyzed. The in-house competitive test used a homologous competitor with a 20-nucleotide insertion. Primers amplified a fragment of the D region of strain AD169 and a 32P-labelled probe was used. The in-house PCR test analyzed remained linear to a higher viral load (1,000,000 CMV DNA copies/ml) when compared to COBAS AMPLICOR CMV MONITOR test (Caliendo et al. 2001).

The comparison of a Light-Cycler assay and COBAS AMPLICOR CMV MONITOR to quantitatively detect CMV in solid organ transplant recipients showed good correlation in 19 infection episodes. Light-Cycler can analyze more samples per run and is faster, an advantage to reference laboratories, aiming high-throughput systems (Razonable et al. 2001).

Two real-time technologies for CMV diagnosis, using TaqMan and MB probes directed to the same region amplified by the same set of primers, have been compared. Both assays were highly reproducible and reliable, but designing MB was considered a more time-consuming activity (Jeubink et al. 2003).

A real-time PCR directed to the glycoprotein B was more sensitive than COBAS AMPLICOR CMV Monitor and the classical pp65 antigenemia when parallel comparison was undergone with 404 plasma samples from 66 transplant patients. The in-house approach also showed the widest dynamic range, from 250 to 10^6 DNA copies/ml (Pang et al. 2003).

When comparing different results, not only the specimen for CMV molecular-based diagnosis is important, but also the amount of starting material should be taken into account. Griscelli quantify CMV by PCR-TaqMan technology using a 10^6 cells assay and pp65 gene as a target (Griscelli et al. 2001). Careful inter-experiment variability assays were performed achieving good correlation coefficients for both PCRs (> 0.99). The assay could detect 10 plasmids using separated amplification reactions for CMV pp65 gene and a human housekeeping gene, the GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Nevertheless, the assay started with five times more leukocytes than used in a standard pp65 antigenemia test, surely a drawback for neutropenic patients.

Efficiency of distinct primers set can account for observed differences in assays' sensitivity and it is also an important topic to be considered when choosing an in-house approach for CMV diagnosis (Shibata et al. 1988, Kulski 1994, Nitsche et al. 2000).

In a quantification assay, normalization of samples is thought to be of utmost importance. Nevertheless, a few publications addressed this question (Griscelli et al. 2001, Azevedo et al. 2003, Hänfler et al. 2003, Hong et al. 2004). Amplification of a control gene, such as a housekeeping gene (goblin, actin or glyceraldehyde phosphate dehydrogenase, for instance), allows for the correction of variation in sample preparation or in tube-to-tube amplification efficiency. Specially when dealing with immunocompromised patients, specimen volumes poorly represent the neutropenic status of patients. There is a big difference in harbouring 10^6 CMV genomes in 2 x 10^6 leukocytes or 10^6 CMV genomes in 2 x 10^4 leukocytes. There is also a big difference in presenting a high viral load that might be your particular default viral load and showing a raise or a fall in your viral load longitudinally. There is no extensive study addressing the CMV viral load of health population. In a clinical study, two volunteers, who were to be enrolled in the health control group but presented light symptoms of viral respiratory infections, showed a higher CMV viral load than the average viral load found in ICU patients (our unpublished results). How can CMV viral load cutoff for reactivation of disease be delineated if there is not enough data concerning "normal" viral load?

**Final remarks.**

There are successful examples of quantitative methods for CMV diagnosis in the literature. The major problems are lack of standardization or expensive commercial kits. The European Group for Blood and marrow Transplantation Infectious Disease Working Party pointed some critical parameters for a reliable CMV diagnosis: (i) blood samples should be collected with EDTA; (ii) standard amounts of DNA should be added per PCR reaction to normalize assays; (iii) a human cellular
gene should be included as positive control; (iv) a specific hybridization step should be included to ensure specificity; (v) primers should be directed to a conserved region of CMV; (vi) a standard number of leukocytes should be used from the beginning (Grundy et al. 1996). The last parameter, a clear concern for samples normalization, could be difficult to achieve considering that immunosuppressed patients have usually small amounts of leukocytes.

Real-time PCR is a potent new approach for quantitative CMV diagnosis. Obtaining data without opening the tube has the advantage of avoiding post-PCR handling, a possible source of DNA carryover. Its clinical applicability can be improved, complying with the critical parameters presented above. The most appropriate region of viral genome to be used as a target remains to be determined. Since there is still no consensus, the choice of the better method to be used, either in-house or commercial, should rely on specific laboratory’s characteristics.

CMV DNA levels per a given number of cells (PBL or PBMC) cannot be compared to CMV DNA levels per volume of blood, plasma or serum. Concerning neutropenic patients, for instance, the number of leukocytes per volume of blood is highly variable. It is, therefore, important, for these patients, to express viral copies per number of leukocytes, using a human gene to normalize results.

In some diagnostic tests, as those for residual disease detection, the sensitivity is the main issue. Theoretically, since CMV latency or asymptptomatically persistence is widespread, a very sensitive assay has no higher value. In a context where 10^4 copies/perm is considered a normal viral load to health individuals with latent CMV, the main question is the accuracy of the quantitative PCR to detect reactivation (Leuerez-Ville et al. 2003). Detection of sub-clinical low level of CMV has no proven clinical impact, but increase or decrease in viral load longitudinally can be used to guide therapy. Correlation of viral load and the risk of CMV disease is still a matter of intense discussion. Although a substantial amount of data suggests that viral load measurements in a weekly base is useful to predict CMV disease in transplant patients, randomized trials are still required to evaluate its clinical value.

REFERENCES


Caliendo AM, Schuurman R, Yen-Lieberman B, Spector SA, Andersen J, Manjiry R, Crumpacker


Exner MM, Lewinski MA 2002. Sensitivity of multiplex real-time PCR reactions; using the LighTcycler and the ABI PRISM 7700 sequence Detection system; is dependent on the concentration of DNA polymerase. Molecular and Cellular Probes 16: 351-357.


Kulski JK 1994 Quantitation of human cytomegalovirus DNA in leukocytes by end-point titration and duplex polymerase chain reaction.


