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Enhanced Analytical Sensitivity of a Quantitative PCR for CMV Using a Modified Nucleic-Acid Extraction Procedure

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Accurate and rapid diagnosis of CMV disease in immunocompromised individuals remains a challenge. Quantitative polymerase chain reaction (QPCR) methods for detection of CMV in peripheral blood mononuclear cells (PBMC) have improved the positive and negative predictive value of PCR for diagnosis of CMV disease. However, detection of CMV in plasma has demonstrated a lower negative predictive value for plasma as compared with PBMC. To enhance the sensitivity of the QPCR assay for plasma specimens, plasma samples were centrifuged before nucleic-acid extraction and the extracted DNA resolubilized in reduced volume. Optimization of the nucleic-acid extraction focused on decreasing or eliminating the presence of inhibitors in the pelleted plasma. Quantitation was achieved by co-amplifying an internal quantitative standard (IS) with the same primer sequences as CMV. PCR products were detected by hybridization in a 96-well microtiter plate coated with a CMV or IS specific probe. The precision of the QPCR assay for samples prepared from untreated and from pelleted plasma was then assessed. The coefficient of variation for both types of samples was almost identical and the magnitude of the coefficient of variations was reduced by a factor of ten if the data were log transformed. Linearity of the QPCR assay extended over a 3.3-log range for both types of samples but the range of linearity for pelleted plasma was 20 to 40,000 viral copies/ml (vc/ml) in contrast to 300 to 400,000 vc/ml for plasma. Thus, centrifugation of plasma before nucleic-acid extraction and resuspension of extracted CMV DNA in reduced volume enhanced the analytical sensitivity approximately tenfold over the dynamic range of the assay. J. Clin. Lab. Anal. 14:32–37, 2000. © 2000 Wiley-Liss, Inc.

Key words: CMV; quantitative PCR; CMV viral load; plasma

INTRODUCTION

CMV infection as reflected by the presence of CMV viremia occurs in 20 to 60% of immunocompromised patients but only a fraction of those will ultimately develop CMV disease (1,2). Though progress in the prophylaxis of CMV disease has been made, CMV remains a major cause of morbidity and mortality in immunocompromised patients (1,2). Distinguishing between CMV infection and CMV disease is still a challenge. After primary infection, the virus may remain latent and become an opportunistic pathogen in hosts with impaired cellular immunity (3–8).

Viremia as detected by conventional culture (CC) is still the most reliable predictor of CMV disease, but this method is very labor intensive and can take up to 6 weeks for results (9,10). The introduction of a rapid-centrifugation shell-vial culture method reduced the time for virus detection down to 24–72 hr, although specimens with a low viral load can be a problem (11,12). The CMV antigenimia assay provides a more rapid result by directly detecting CMV antigens in white blood cells (13). Even though this assay is more sensitive than CC or shell vial (11,14), the antigenimia assay is labor intensive and its sensitivity varies greatly among different laboratories (15,16).

A number of nucleic-acid amplification methods have been developed for detection of CMV for diagnosis of CMV disease (17–21), and these methods have been applied to a wide

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variety of specimens with varying results (17–21). Most of these methods show greater analytical sensitivity than CC, shell-vial culture, and the antigenemia assay. Perhaps because of the higher analytical sensitivity, PCR shows a lower positive predictive value in seropositive patients (17,19). To overcome this problem, a number of studies have evaluated changes in the viral load as measured by QPCR to diagnose CMV disease (22–27). All of these reports demonstrated a high positive predictive value for diagnosis of CMV disease for PBMC and plasma. However, when PBMC were compared to plasma as the source of specimen, plasma samples were negative for CMV in patients with disease more often than for PBMC samples, resulting in a lower negative predictive value of the assay with plasma specimens. However, PBMC are more difficult and laborious to work with than plasma. We undertook to overcome this problem by improving the analytical sensitivity of QPCR using plasma as the specimen source.

In this report we describe a modification of a commercially available DNA-extraction procedure and QPCR assay that enhances the analytical sensitivity of the assay using plasma as the specimen source. Enhancement of sensitivity was achieved by introducing a simple and rapid centrifugation step before nucleic-acid extraction from plasma specimens and concentration of the nucleic acid at the resolubilization step. In addition, we compared the analytical performance of the enhanced QPCR method to that of the method recommended by the manufacturer.

MATERIALS AND METHODS

Sample Preparation

Peripheral blood from healthy volunteers was drawn using standard venipuncture technique with EDTA anticoagulated tubes. Plasma was separated within 2 hr of collection by centrifuging at 2,000*g* for 10 min at room temperature. After processing, plasma was pipetted into 200-µl or 500-µl aliquots and stored at –80°C until tested.

DNA Extraction From Plasma (Standard Extraction Procedure)

DNA was extracted from 200 µl of plasma using the QiAmp Tissue Extraction kit (Qiagen, Boston, MA) according to the manufacturer's recommendations. The extracted material was eluted from the columns using 200 µl of Dnase- and RNAse-free H_2O (Gibco-BRL, Gaithersburg, MD) pre-warmed to 75°C. Extracted material was immediately used for PCR analysis.

DNA Extraction From Pelleted Plasma (Modified Extraction Procedure)

Five hundred microliters of plasma were aliquoted into a microcentrifuge tube and centrifuged at 15,000*g* in a microcentrifuge (Eppendorf, Westbury, NY) at room temperature for 15 min. The supernatant was discarded and the pellet was resuspended in 200 µl of sterile Phosphate Buffered Saline (PBS, 0.138 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, and 0.01 M KH_2PO_4, pH:7.4; SIGMA, St. Louis, MO). DNA was extracted from the pellet using a modification of the QiAmp Tissue DNA Extraction kit. The steps were taken as described by the manufacturer except that after the sample was applied to the column and washed twice with 400 µl of AL buffer (provided by manufacturer, Qiagen, Boston, MA), two additional washes with AW buffer (provided by manufacturer) occurred. In addition, DNA was eluted from the columns using 50 µl of Dnase- and RNAse-free H_2O (Gibco-BRL, Gaithersburg, MD) pre-warmed to 75°C.

CMV Quantitative PCR (QPCR)

Quantification of CMV was performed using a modification of the CMV Viral Quant test (Biosource International, Camarillo, CA) as previously described (28). Denatured amplicon and fivefold serial dilutions were detected by hybridization with either CMV or IS probes followed by the addition of horseradish peroxidase and substrate. The colorimetric reaction was read at 450 nm. An optical density reading of $\geq$ 0.300 was considered positive. Calculation of the CMV titer was performed by multiplying the OD value of the CMV by the respective highest dilution that gave an OD between 0.3 and 1.5, and then dividing by the product of the OD value of the IS and respective highest dilution that gave an OD between 0.3 and 1.5. This value was multiplied by 100 for the number of IS molecules and by the sample dilution factor.

CMV DNA and Purified CMV

Purified CMV DNA and CMV were obtained from a commercial source (ABI Columbia, MD). The number of viral particles present in the purified CMV had been counted by electron microscopy by the commercial source. The purified CMV preparation had a nominal titer of 9.0×10^9 CMV viral particles per ml. In order to confirm the amount of CMV viral particles present in the reference material, we split the sample in half and extracted the DNA using a standard Phenol/Chloroform method including glycogen as a carrier during the precipitation step (29). After DNA extraction we calculated a titer of 4.5×10^9 per ml for the purified CMV sample, which confirmed the amount of virus present in the reference sample.

Statistical Evaluation

Calculations were performed using the Stata v 5.0 statistical software package (Stata, Computing Resources Center, Santa Monica, CA).

RESULTS

Analytical Sensitivity and Specificity of the QPCR Method for Purified CMV

The analytical sensitivity of an assay is measured by the lowest concentration (the highest dilution) of a particular target analyte that can be accurately and reproducible detected by the test. In order to determine the analytical sensitivity of the QPCR, we prepared a single dilution where we alternate a two- with a fivefold dilution of the purified CMV DNA. The concentration of CMV DNA was confirmed by UV spectrophotometry and fluorometry as already described (29). The dilutions that contained purified DNA comparable to a range of 0.1 to 1,000 viral copies (vc) of CMV in 10 µl of PBS buffer were tested in triplicate over three different experiments. Using the PCR conditions described we were able to detect DNA comparable to 1–5 vc of CMV in the PCR reaction (Table 1).

Analytical specificity of the QPCR was assessed by testing cross-reactivity with a panel of closely related viral DNAs obtained from the same commercial source. Serial dilutions of purified DNA from HSV type1 and type 2, and Epstein-Barr virus were prepared and tested in triplicate over three different experiments. Up to a concentration of 1,000 copies of viral DNA into the PCR reaction, no cross-reactivity with any of the DNA tested was observed (Table 2).

Nucleic-Acid Extraction From Plasma and Pelleted Plasma

We evaluated the yield of nucleic-acid extraction for CMV from plasma and pelleted plasma using the Qiagen Tissue DNA extraction kit. Two reference samples were prepared that contained purified CMV DNA equivalent to 500 vc/ml and 5,000 vc/ml in PBS in sufficient quantity to perform the entire experiment. Recovery of purified CMV DNA after the standard DNA extraction procedure was measured by QPCR. Two hundred microliter samples containing DNA equivalent to either 500 or 5,000 vc/ml were extracted according to manufacturer's recommendation. The nucleic acid was eluted from the column in the same volume as the original sample. QPCR was performed using 10 µl of extracted material and compared to the results obtained using 10 µl from each sample without nucleic-acid extraction. Both samples were tested in duplicate and in three different experiments. As shown in Table 2, recovery was nearly 100%. There was almost no difference for the calculated amount of virus for the samples that undergo nucleic-acid extraction compared to the samples that did not. Under the conditions tested, the extraction procedure showed a high recovery of nucleic acid.

Next, we examined both the effect of plasma and the effect of pelleted plasma combine with a tenfold concentration of plasma on the performance of the PCR. Plasma samples were aliquoted into 200- and 500-µl aliquots and frozen until tested. The 500-µl aliquots were thawed and centrifuged as already described and resuspended in 200 µl of PBS. Interestingly, the approximate volume of the pelleted material from the centrifuged plasma was less than 10 µl. Prior to the extraction of these samples, CMV DNA comparable to either 500 vc or 5,000 vc was added to each 200 µl. The 200-µl aliquots of plasma and resuspended pelleted material were extracted according to manufacturer's recommendations but the sample from the pelleted material was eluted from the column in 50-µl volume. As shown in Table 2, the spiked plasma samples showed very similar results to the control samples that did not undergo nucleic-acid extraction. In contrast, the samples extracted in the presence of pelleted plasma showed a tenfold decrease in titer compared to the control samples that did not undergo nucleic-acid extraction. This result showed that the centrifugation step and/or the concentration of original plasma sample to one-tenth of the original volume introduced inhibitors into the sample that were not removed by the extraction procedure. In order to remove the inhibitors present in the pelleted plasma material, we added two extra washes with AL buffer after the samples were applied to the columns. Again both samples were tested in duplicate and in three different experiments. As seen in Table 2, the introduction of two extra washes to the extraction procedure substantially decreased the amount of inhibitors that were introduced during the centrifugation step and/or the concentration of the original plasma volume.

In order to determine if the centrifugation step indeed aids in pelleting intact virus, we prepared 2 samples that contained 500 and 5,000 viral particles vc/ml plasma. These samples were prepared by spiking fresh plasma, previously tested for

TABLE 1. Analytical sensitivity and specificity of QPCR using purified viral DNA

Purified	Equivalents of viral copies into the QPCR								
viral DNA	1,000[a]	500	100	50	10	5	1	0.5	0.1
CMV	1237[b] ± 377	476 ± 127	113 ± 36	48 ± 19	15 ± 5	6 ± 3	2 ± 2	ND	ND
HSV-1	ND	ND	ND	ND	ND	ND	ND	ND	ND
HSV-2	ND	ND	ND	ND	ND	ND	ND	ND	ND
EBS	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]The values represent the calculated equivalent viral copies into the QPCR.
[b]The values represent the mean value of equivalent viral copies into the QPCR ± the standard deviation of samples tested in triplicate and in three different experiments.

TABLE 2. Validation of DNA extraction procedure for QPCR for CMV

CMV sample	Sample preparation	500 CMV copies/ml[a]	5,000 CMV copies/ml[a]
DNA	None	550 ± 106	5560 ± 1406
DNA	Extraction	489 ± 108	4796 ± 900
DNA – pelleted plasma	Extraction	38 ± 18	538 ± 145
DNA + pelleted plasma	Extraction + 2 washes	497 ± 165	4780 ± 1160
Virus	Extraction	515.4 ± 95.5	5158 ± 1584
Virus/pelleted plasma	Extraction + 2 washes	416.4 ± 98.4	4864 ± 1560

[a]The values represent the mean value ± standard deviation of samples tested in duplicate and in three different experiments.

TABLE 3. Precision studies for QPCR for CMV

Controls	Mean value[a]	Standard dev.[a]	Coefficient of variation (%)
High control			
Plasma	19,433	6227	32
Pelleted plasma	20,494	6502	31.7
Log plasma	4.288	0.154	3.6
Log pelleted plasma	4.311	0.151	3.5
Low control			
Plasma	1,867	709	38
Pelleted plasma	2,100	743	35
Log plasma	3.271	0.179	5.5
Log pelleted plasma	3.322	0.160	4.8

[a]vc/ml plasma.

CMV, with purified virus and frozen at –80°C until tested. Samples were analyzed in duplicate and in three different experiments. Extraction and analysis of 200 µl of spiked plasma produced signals equivalent to the theoretical number of added viral particles (see Virus and Extraction, Table 2). Similarly, extraction of the pellet resulting from centrifuging the 500 µl of the spiked plasma resulted in signals that did not differ significantly from the analysis of the entire 500-µl sample (see Virus/ pelleted plasma, Table 2). The pelleted plasma samples did include the two additional washes similar to the studies with purified DNA. Thus, using the centrifugation step and a tenfold concentration of the original plasma sample in combination with the two extra washes, we were able to pellet down the virus and at the same time remove the inhibitors present in the final extracted material.

Comparison of Performance Characteristics for QPCR Using Plasma and Pelleted Plasma for CMV

The precision of the QPCR method was therefore determined in similar fashion to that described for a quantitative HCV test (30). First, high and low control samples were prepared by spiking fresh plasma with two different amounts of purified CMV. The samples were aliquoted into single-use 500-µl or 200-µl aliquots and stored at –80°C until tested. All samples were continuously mixed and maintained on ice during mixing and aliquoting. Again, nucleic acid from the 200-µl aliquots were extracted according to manufacturer's recommendation and the 500 µl aliquots were centrifuged before nucleic-acid extraction and processed as described above. Samples from each control were analyzed in triplicate and the experiments were repeated five times for pelleted and uncentrifuged aliquots. The titers and log titers for both the high and low controls of both plasma and pelleted plasma showed normal distributions (data not shown). Having determined that the error of the mean for both linear and log-transformed CMV concentrations as determined by the QPCR method were normally distributed, the precision of the assay was evaluated using parametric calculations. Table 3 shows the results from the precision studies for the two extraction procedures. Both extraction procedures showed very similar coefficients of variation. Use of log-transformed data reduced the coefficient of variation approximately tenfold as previously observed with QPCR for HCV (30).

Sensitivity and Linear Dynamic Range for Plasma and Pelleted Plasma for CMV QPCR

In order to determine the sensitivity and linear dynamic range of both extraction procedures we prepared a dilution of purified CMV spiked into plasma. Alternate twofold and fivefold dilution was prepared using previously tested CMV negative plasma. Each dilution was aliquoted into 200 and 500 µl and the samples were frozen at –80°C until tested. Each dilution was analyzed in duplicate for each extraction procedure and the experiment was repeated three times. Figure 1 shows the plot of the measured log-transformed concentrations against the log-transformed input value. As clearly seen in Figure 1, the QPCR was linear over a 500-fold range regardless of the extraction procedure performed. Moreover, there was complete overlap for both extraction procedure curves between 500vc/ml plasma and 10,000vc/ml plasma. The correlation coefficients for both plasma and pelleted plasma were close to 1 for both transformed and untransformed data. Even though both extraction procedures showed linearity over a 500-fold range, the extraction procedure using pelleted plasma was linear between 20 and 40,000 vc/ml plasma while the standard extraction-procedure linearity was achieved between 300 and 400,000 vc/ml plasma. As anticipated, pelleting the plasma and concentrating the extracted CMV DNA allowed the detection of virus at lower concentrations and down-shifted the dynamic range of the assay approximately tenfold.

DISCUSSION

Diagnosis of CMV disease in immunocompromised individuals remains a challenge due to the difficulty in differentiating CMV viremia from CMV disease (1–4). A number of molecular quantitative methods have been described (17–21,31–34). It has been suggested that CMV viral load corre-

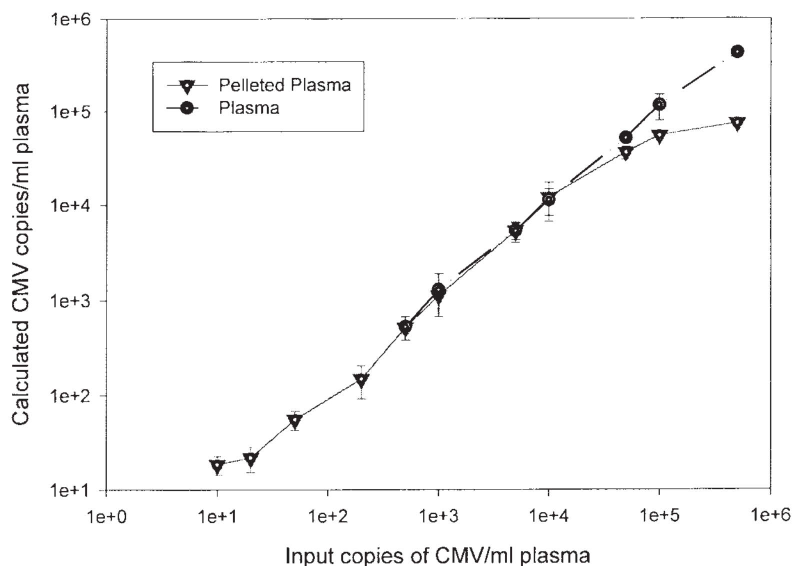

Fig. 1. Linearity of the QPCR method for plasma and pelleted plasma was assessed by testing a serial dilution of purified HCMV spiked into plasma. Alternate twofold and fivefold serial dilutions were analyzed in duplicate and repeated three times. The log-transformed values for the measured concentrations were plotted against the log-transformed values of the input values for CMV.

lates with CMV disease and could be used as a marker for diagnosis, risk assessment, and response to therapy for CMV disease (17–21,31–34). A number of these reports have shown a high positive predictive value for diagnosis and risk assessment of CMV disease when using PBMC and plasma in immunocompromised individuals. Although plasma specimens are easier to manipulate and their integrity is easier to maintain in the clinical laboratory than are PBMC, the clinical sensitivity of the assays was lower for plasma than PBMC, thus lowering the negative predictive value of the assay. Interestingly, plasma was found to have a lower negative predicitve value regardless of the patient population studied (i.e., HIV-infected, bone marrow, and solid organ transplant patients) (17–21,31–34).

In the present study we have described the enhancement of the analytical sensitivity of a rapid QPCR assay for quantitation of CMV in plasma. By introducing a simple centrifugation step before nucleic-acid extraction and resolubilization of extracted CMV DNA into a reduced volume, we were able to enhance the analytical sensitivity of the QPCR on plasma approximately tenfold. This was accomplished with no adverse effect on the assay's precision or the magnitude of the linear dynamic range (Table 3 and Fig. 1). The QPCR assay displayed a linear dynamic range of 3.3 logs regardless of the extraction procedure. Using a standard microcentrifuge we were able to pellet down essentially 100% of CMV virions spiked into plasma (see Table 2). The centrifugation step allowed us to increase the volume of plasma evaluated from 200 µl to 500 µl. Resuspending the final DNA extracted material in a volume that was one-tenth the original sample also enhanced sensitivity. Initially, 500 µl of plasma was centrifuged and the extracted CMV DNA was eluted in 50 µl. However, we found that the concentration process introduced inhibitors of the PCR reaction into the extracted DNA. This problem was overcome by simply increasing the number of washes applied to the column used to bind the deproteinized DNA during the extraction procedure. The introduction of these two wash steps had little impact in the overall time spent by the technologist extracting the nucleic-acid specimens (data not shown).

The increased sensitivity due to concentration of the virus in the plasma sample was also reflected when we compared the sensitivity of our QPCR with others (31–34). A number of QPCR assays that used plasma as the specimen source have stated sensitivities between 200 to 2,500 vc/ml plasma (31–34). In contrast, the sensitivity of our QPCR was 20 vc/ml plasma. Thus, the sensitivity of our QPCR assay was approximately 10–100 times greater than most of other plasma QPCR assays. One point of interest is the difference in the amount of original plasma material tested in other QPCR assays compared to ours. The majority of the extraction procedures in those assays reconstituted the extracted DNA back to the original volume of plasma used for the extraction. Because these assays used 5–10 µl of extracted sample in their PCR reactions, this translated into testing an equivalent of 5 to 10 µl of original plasma for virus. In contrast, we reconstituted the extracted DNA in a volume that was one-tenth the volume of the original plasma sample and used 10 µl of extracted sample in the PCR. This translated into testing an equivalent of 100 µl of original plasma sample. Thus, the difference in the amount of the equivalent original plasma tested presumably accounts for much of the difference in the enhanced sensitiv-

ity of our QPCR compared to others. It is possible that the increased sensitivity of the modified QPCR assay might improve the clinical sensitivity of QPCR from plasma samples.

In conclusion, the introduction of a simple centrifugation step using a conventional microcentrifuge, coupled with concentration of the extracted CMV DNA at the resolubilization step, demonstrated an improvement in the analytical sensitivity of the QPCR using plasma as the specimen source. We increased the analytical sensitivity of the assay approximately tenfold without affecting the precision and dynamic range of the overall assay.

REFERENCES

1. Rubin RH. Infection in the organ transplant recipients. In: Rubin RH, Young LS, editors. Clinical approach to infection in the compromised host, 3rd ed. New York: Plenum Medical Book Company; 1994.
2. Ho M. Epidemiology of cytomegalovirus infections. Rev Infect Dis 1990;12(Suppl 7):701–710.
3. Zaia JA. Cytomegalovirus infection. In: Forman SJ, Blume KG, Thomas ED, editors. Bone marrow transplantation. Boston: Blackwell Scientific Publications; 1994.
4. Zaia JA. Prophylaxis and treatment of CMV infections in transplantation. In: Mills J,Volberding P, Carey L, editors. Fourth triennial symposium on new directions in antiviral chemotherapy. New York: Plenum Medical Book Company; 1995.
5. Rubin RH. Impact of cytomegalovirus infection on organ transplants recipients. Rev Infect Dis 1990;12(Suppl 7):754–766.
6. Stratta RJ, Shaefer MS, Markin RS, et al. Clinical patterns of cytomegalovirus disease after liver transplantation. Arch Surg 1989;124:1443–1449.
7. Singh N, Mieles L, Yu VL, et al. Decreased incidence of viral infections in liver transplant recipients. Possible effects of FK 506? Dig Dis Sci1994;39:15–18.
8. Farrugía E, Schwab TR. Management and prevention of cytomegalovirus infection after renal transplantation. Mayo Clin Proc 1992;67:879–890.
9. Wiesner RH, Marin E, Porayko MK, et al. Advances in the diagnosis, treatment, and prevention of cytomegalovirus infections after liver transplantation. Gastroenterol Clin North Am 1993;72:351–366.
10. Gleaves CA, Smith, TF, Shuster E, et al. Comparison of standard tube and shell vial culture techniques for the detection of cytomegalovirus in clinical specimens. J Clin Microbiol 1985;21:217–221.
11. Brumback B, Bolejack S, Morris MV, et al. Comparison of culture and the antigenemia assay for detection of cytomegalovirus in blood specimens submitted to a reference laboratory. J Clin Microbiol 1997; 35:1819–1821.
12. Bitsch A, Kirchner H, Dennin R, et al. The long persistence of CMV DNA in the blood of renal transplants patients after recovery from CMV infection. Transplantation 1993;56:108–113.
13. Halwachs G, Zach R, Holzer H, et al. A rapid immunocytochemical assay for CMV detection in peripheral blood of organ transplanted patients in clinical practice. Transplantation 1993;56:338–342.
14. Bein G, Bitsch J, Hoyer J, et al. The detection of human cytomegalovirus immediate early antigen in peripheral blood leukocytes. J Immunology Methods 1991;137:175–180.
15. Boeckh B, Wooghd P, Stevers-Ayers T, et al. Factors influencing detection of quantitative cytomegalovirus antigenemia. J Clin Microbiol 1994;32:832–834.
16. Kidd M, Fox J, Pillay D, et al. Provision of prognostic information in immunocompromised patients by routine application of polymerase chain reaction for cytomegalovirus. Transplantation 1993;56:867–871.
17. Brytting M, Jazi M, Bostrom L, et al. Cytomegalovirus DNA in peripheral bloods leukocytes and plasma from bone marrow transplant recipients. Transplantation 1995;60:961–965.
18. Abecassis M, Koffron A, Kaplan B, et al. The role of PCR in the diagnosis and management of CMV infection in organ recipients. Transplantation 1997;63:275–279.
19. Gozlan J, Salord JM, Choua D, et al. 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 1993;33:1943–1945.
20. Schofer P, Tenschert W, Gutensahn K, et al. Minimal effect of delayed sample processing on result of quantitative PCR for cytomegalovirus DNA in leukocytes compared to results of antigenemia assay. J Clin Microbiol 1997;35:741–744.
21. Gallez-Hawkins M, Tegtmeier B, Veer A, et al. Evaluation of quantitative plasma PCR plate assay for detecting cytomegalovirus infection in marrow transplant recipients. J Clin Microbiol 1997;35:788–790.
22. Multimer D, Matyi-Toth A, Shaw J, et al. Patterns of viremia in liver transplant recipients with symptomatic cytomegalovirus infection. Transplantation 1997;63(1):68–73.
23. Schafer P, Braun RW, Mohring K, et al. Quantitative determination of human cytomegalovirus target sequences in peripheral blood leukocytes by nested PCR and temperature gradient gel electrophoresis. J Gen Virol 1993;74:2699–2707.
24. Zipeto D, Revello E, Silini M, et al. Quantitation of human cytomegalovirus DNA in peripheral blood polymorphonuclear leukocytes of immunocompromised patients by polymerase chain reaction. J Virol Methods 1993;44:45–56.
25. Zipeto D, Morris S, Hong C, et al. Human cytomegalovirus (CMV) DNA in plasma reflects quantity of CMV DNA present in leukocytes. J Clin Microbiol 1995;33:2607–2611.
26. Mendez J, Espy M, Smith TF, Wilson J, et al. Clinical significance of viral load in the diagnosis of Cytomegalovirus disease after liver transplantation. Transplant 1998;65:1477–1481.
27. Matchotka SV, Garrett CT, Schwartz AM, Callahan, R. Amplification of the proto-oncogene int-2, c-erb B2 and c-myc in human breast cancer. Clin Chim Acta 1898;184(3):207–217.
28. Ferreira-Gonzalez A, Fisher RA, Weymouth LA, et al. Clinical utility of a quantitative PCR for diagnosis of cytomegalovirus disease in solid organ trasnplant patients. Transplant, 1999;68:991–996.
29. Sambrook J, Fritsch EF and Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1898.
30. Ferreira-Gonzalez A, Shiffman ML, Lofland DH, et al. Assessing clinical utility of hepatits C virus quantitative RT-PCR data: implications for identification of responders among α-interferon-treated patients. Mol Diagnosis 1996;1:109–120.
31. Imbert-Marcille BM, Cantarovich D, Ferre-Aubine V, et al. Usefulness of DNA viral load quantification for cytomegalovirus disease monitoring in renal and pancreas/renal transplant recipients. Transplant 1997;63:1476–1481.
32. Mutimer D, Matyi-Toth A, Shaw J, et al. Patterns of viremia in liver transplant recipients with symptomatic cytomegalovirus infection. Transplant 1997;63:68–73.
33. Hebart H, Muller C, Loffler J, et al. Monitoring of CMV infection: a comparison of PCR from whole blood, plasma-PCR, pp65-antigenimia and virus culture in patients after bone marrow transplantation. Bone Marrow Transplant 1996;17:861–868.
34. Boivin G, Handfiels J, Toma E, et al. Compartive evaluation of the cytomegalovirus DNA load in polymorphonuclear leukocytes and plasma of human immunodeficiency virus-infected subjects. J Infect Dis 1998;177:355–360.