TECHNICAL REPORT

Comparison of polymerase chain reaction and pp65 antigen test for early detection of human cytomegalovirus in blood leukocytes of cardiac transplant recipients

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Objective: To establish whether polymerase chain reaction (PCR) for cytomegalovirus deoxyribonucleic acid (DNA) can provide clinical information for the management of the infection.

Methods: Leukocytes in 30 heart transplant recipients were monitored by pp65 antigen testing and PCR for 82 to 365 days after transplantation.

Results: Of the 30 patients, 26 developed cytomegalovirus infection, nine of whom were symptomatic. Altogether, 300 leukocyte samples were examined. The concordance between PCR and pp65 antigen test was 82.6%. In symptomatic patients after surgery, PCR detected cytomegalovirus infection after 38 ± 16 days and the pp65 antigen test, after 48 ± 15 days. Symptomatic infection correlated with a higher number of pp65-positive leukocytes than did asymptomatic infection: 310 ± 356 vs 24 ± 35 ($p < 0.005$)/200,000 examined, respectively. Clearance of virus was observed by PCR after 125 ± 73 days (range 29 to 225) in symptomatic, and after 82 ± 70 days (range 16 to 301) in asymptomatic, cases of infection.

Conclusions: The positive predictive value of PCR for symptomatic infection was 34.6%. Our findings correlate with previous reports and show that the qualitative detection of cytomegalovirus DNA is not associated with overt disease whereas quantitation of pp65-positive leukocytes closely correlate with symptom onset. Insofar as the results are not quantitative, PCR is not a marker of clinically apparent infection. Careful monitoring of cytomegalovirus infection based on quantitative pp65 antigen assay can fulfill all clinical needs for early diagnosis and proper management of the infection

Key words: Heart transplantation, human cytomegalovirus (HCMV), polymerase chain reaction, pp65 antigen test

Human cytomegalovirus (HCMV) infection continues to be a major cause of morbidity and mortality in heart transplant patients [1-8]. Proper management of the infection with antiHCMV therapies, such as ganciclovir and foscarnet [9-11], depends on early diagnosis. Procedures for prompt diagnosis as well as careful monitoring of viral infection have been developed, including direct detection of HCMV lower matrix phosphoprotein (pp65 antigen) [12] in polymorphonuclear leukocytes (PMNLs), the major HCMV carriers in peripheral white blood cells during acute infection [13,14], and polymerase chain reaction (PCR) [15] for HCMV deoxyribonucleic acid (DNA) detection in blood and tissue samples [16-19]. The pp65 antigen test has been shown to correlate well with active infection and clinical disease in transplant patients [20-22]. Moreover, change in the antigenic load, as

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measured by the variation in pp65-positive PMNLs (antigenemia), also correlates with the progression of clinical symptoms [20,23,24]. Although PCR has proved to be a powerful method in diagnostic virology due to its high sensitivity, the clinical relevance of qualitative PCR results in HCMV DNA detection appears to be limited by a low predictive value for HCMV disease [20,25-27].

The present study was designed to contribute to the ongoing analysis of the role of qualitative PCR results for HCMV DNA detection in PMNLs in heart transplant patients. HCMV DNA results by PCR were compared with quantitative detection of HCMV pp65 in the PMNLs of 30 heart transplant recipients during the first year after transplantation, with emphasis on early identification of ongoing HCMV infection, its correlation with the development of clinical disease and monitoring of antiviral treatment.

MATERIAL AND METHODS

Patients

The study involved 30 patients who had undergone orthotopic heart transplantation (HT) and did not require treatment for acute rejection between January 1993 and July 1994 in the Cardiac Surgery Department at the University of Turin, Italy. All patients were administered a triple immunosuppressive regimen of prednisone, cyclosporine and azathioprine. Rabbit antithymocyte globulin was used for the first 10 days after heart transplantation.

Human cytomegalovirus monitoring

Virological monitoring of HCMV infection proceeded as follows: heparinized blood samples for pp65 antigen test and DNA detection were collected weekly during the first month after HT and then every 15 days until the third month. From the third to the twelfth month, blood was examined monthly. During the acute phase of HCMV infection, blood was tested twice a week. Meanwhile, serum samples were collected for HCMV serology.

PMNL sample preparation

PMNLs were separated by sedimentation of heparinized blood samples (3 to 7 mL) in a 6% dextran solution (5 vol blood/1 vol dextran). Leukocytes were centrifuged at 2500 rpm for 10 min at room temperature and the resulting pellet washed in 0.8% ammonium chloride (NH_4Cl) for 1 to 2 min to avoid blood contamination. PMNLs were then centrifuged and resuspended in 1 mL of buffered saline solution (PBS) at pH 7.4. Aliquots of 200,000 PMNLs were used for the pp65 antigen test and for DNA detection by PCR.

pp65 antigen test (antigenemia)

Aliquots of 200,000 PMNLs were prepared for glass slides using a cytocentrifuge (Cytospin 2, Shandon Southern Products, Astmoor, UK). PMNLs were fixed in 5% formaldehyde-PBS for 10 min, and air-dried and stained with a pool of monoclonal antibodies to HCMV pp65 antigen (C10-C11; Biotest, Dreieich, Germany) for 45 min. After the first incubation, the slides were washed twice in PBS supplemented with 1% fetal calf serum, then stained with a fluorescein-conjugated F(ab')2 fragment of rabbit antimouse immunoglobulins (Dako, Denmark). Slides were rinsed twice in PBS, then mounted and examined under a fluorescent microscope. HCMV pp65-positive PMNLs were counted and referred to 200,000 examined cells. Different levels of antigenemia (antigenic load) were defined as low, moderate and high when the number of pp65-positive PMNLs was < 5, 5 to 50 and $> 50/200{,}000$ examined cells, respectively [20].

Polymerase chain reaction for HCMV DNA

Primers chosen for amplification by PCR were located within the translated region of the viral genome (EI1) coding for the major immediate early antigen (sequences 5' to 3': upstream primer AGA CCT TCA TGC AGA TCT CC; downstream primer GGT GCT CAT GCA CAT TGA TC) [28,29]. A 262 base-pair sequence of HCMV DNA was amplified. Aliquots of 1×10^6 PMNLs were incubated in lysis buffer [10 mM tris(hydroxymethyl)aminomethane (TRIS)-hydrochloric acid (HCl), 50 mM potassium chloride (KCl), 2.5 mM magnesium chloride ($MgCl_2$), 0.1 mg/mL gelatine, 0.45% nonidet P40 (NP40), 0.45% polysorbate (Tween) 20] with 0.15 μg/μL proteinase K (International Biotechnologies, New Haven, CT) for 3 h at 37°C. Aliquots of 200,000 lysed PMNLs were subjected to DNA amplification in 100 μL PCR reaction buffer containing 50 mmol/L KCl, 10 mmol/L TRIS-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 20 nmol of deoxynucleoside triphosphates (dNTPs), 50 pmol of each primer and 2 units of AmpliTaq (Perkin-Elmer Cetus, Norwalk, CT).

The PCR reaction was performed in an automated thermal cycler (DNA Thermal Cycler, Perkin-Elmer Cetus) for 40 cycles (denaturation: 94 °C for 40 sec; annealing: 55°C for 1 min; extension: 72 °C for 3 min plus 5 sec autoextension every cycle). Fifteen microlitres of each amplified product were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and photographed. Each amplified sample (20 μL) was subjected to hybridization with an oligonucleotide probe within the amplified region of HCMV DNA by DNA enzyme immunoassay (Sorin Saluggia, Italy) [30].

The sensitivity of the protocol used in this study was determined from experiments on HCMV-infected fibroblasts with AD 169 and Towne strains. The sensitivity was 1 infected cell in 4×10^5 uninfected cells. Primer specificity was established with other related human herpesviruses (Epstein-Barr virus, herpes simplex virus types 1 and 2, varicella-zoster virus).

To prevent carry-over of amplified DNA sequences and false-positive reactions, samples were prepared under a biosafety hood in a room physically isolated from that in which the PCR was performed. Separate sets of supplies and positive pipetting devices were dedicated to sample preparation and to assess reactions. All other recommended precautions for PCR were followed [31]. HCMV-infected human embryonic lung fibroblast cells were used as positive controls. Negative controls (non-infected human embryonic lung fibroblast cells) and a reagent mixture free from DNA template, considered '0' DNA control, were used in each run. As a performance control for the PCR procedure, *B*-globin gene was routinely amplified according to a previously described method [31].

HCMV serology

Antibodies to HCMV were determined in all recipients before HT and in the serum sample of every donor at the time of transplantation and during the follow-up. HCMV-specific immunoglobulin G (IgG) was detected with enzyme-linked immunofluorescent assay (ELFA; VIDAS, bioMérieux, Marcy l'Etoile, France). HCMV-specific IgM was determined by a u-capture enzyme-linked immunosorbent assay (ELISA; Sorin, Saluggia, Italy), using a peroxidase-labelled HCMV antigen from AD 169 strain.

Diagnosis and treatment of HCMV infection

Diagnosis of HCMV infection was determined by a positive pp65 antigen test and/or PCR for HCMV DNA in PMNLs. Diagnosis of HCMV primary infection was based on virus-specific IgG seroconversion from negative to positive in a seronegative recipient before HT. In seropositive patients before HT, infection was recorded as recurrent. Infected patients were treated with 9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG or ganciclovir) at the dosage of 10 mg/kg/day intravenously for 14 to 21 days only when the infection became symptomatic (disease), when fever occurred in combination with any of the following signs: leukopenia, thrombocytopenia, liver enzyme rise and gastroenteric signs. In our series, nine patients experienced HCMV systemic symptomatic infection. The most common clinical symptom was fever associated with leukopenia and/or thrombocytopenia. Two patients had HCMV hepatitis and one had gastroenteric disease. For statistical analysis, Student's *t* test was used.

RESULTS

Follow-up of heart transplant patients

The study covered 82 to 365 days of follow-up after HT (mean 230 days). Twenty-six of the 30 transplant patients developed HCMV infection: nine had symptomatic disease [four primary in seronegative recipients (R–) with seropositive donor (D+), and five recurrent infections in seropositive recipients (R+)], and all were treated with ganciclovir. The most common clinical symptom was fever associated with leukopenia and/or thrombocytopenia. Two primary infections underwent two ganciclovir treatments due to a return of the infection. All patients with symptomatic infection recovered with no complications. Seventeen asymptomatic HCMV-infected patients were not treated. Three seropositive recipients and one seronegative recipient with a seropositive donor had not developed HCMV infection at follow-ups of 188, 179, 94 and 365 days, respectively, after HT.

Table 1 Antigenic load determined by pp65 antigen test compared with human cytomegalovirus (HCMV) DNA detection by polymerase chain reaction (PCR) in polymorphonuclear leukocytes (PMNLs) in 26 heart transplant patients with HCMV infection

	HCMV DNA appearance (days after HT)	pp65 appearance (days after HT)	Antigenic load: pp65+ PMNLs evaluated on 200,000 cells	HCMV DNA persistence (days after HT)	pp65 persistence (days after HT)
HCMV disease (n = 9) (range)	38 ± 16 (12 to 63)	48 ± 15 (29 to 79)	310 ± 356** (1 to 1000)	125 ± 73* (29 to 225)	65 ± 59* (18 to 172)
HCMV asymptomatic (n = 17) (range)	38 ± 41 (8 to 161)	40 ± 40 (8 to 161)	24 ± 35** (1 to 125)	82 ± 70 (16 to 301)	49 ± 32 (15 to 117)

**$p < 0.005$ (Student's t test); *$p < 0.05$ (Student's t test).
HT = heart transplantation.

Of 300 PMNL samples (5 to 22 blood samples for each patient, mean 10), PCR was able to detect HCMV DNA in 169 (56.3%) samples, of which 117 were also pp65 antigen test-positive (69.3%); 52 were pp65-negative, corresponding to early and advanced stages of HCMV infection after ganciclovir treatment in symptomatic patients. Antigenemia and PCR gave negative concordant results in 131 PMNLs. The overall concordance between PCR and antigenemia was 82.6% (248 samples).

HCMV symptomatic infection or HCMV disease

PCR detected HCMV DNA in PMNLs 38 ± 16 days after HT (range 12 to 63) and the pp65 antigen test became positive 48 ± 15 days (range 29 to 79) after HT (Table 1). PCR detected HCMV DNA 18 days (mean) before antigenemia in four patients whereas, in five patients, the two tests proved simultaneously positive. Clinical symptoms appeared 55 ± 15 (range 40 to 82) days after HT. In two patients, both PCR and pp65 antigen test became positive at the onset of symptoms. In the other seven transplant patients, PCR was positive 23 days (mean) before the onset of symptoms whereas the pp65 antigen test showed positive at the onset of fever and leukopenia in one patient and, in the other six, 12 days (mean) before symptoms.

In HCMV disease, the mean number of pp65-positive PMNLs/200,000 during an ongoing infection was 310 ± 356 (range 1 to 1000). This antigenic load was significantly different from that in asymptomatic infection (24 ± 35 pp65-positive PMNLs/200,000; $p < 0.005$). A statistically significant difference was also observed in pp65 levels at the onset of symptoms between primary and reactivated infections: 542 ± 395 pp65-positive cells in primary (four patients, six episodes) infections and 122 ± 157 in recurrent infections (five patients, five episodes; $p = 0.05$). In two of nine patients, mild fever and leukopenia were observed when the number of pp65-positive PMNLs ranged from 20 to 50 PMNLs/200,000. In four patients, the onset of fever and leukopenia correlated with an antigenic load of 50 to 100 positive PMNLs whereas severe clinical symptoms (hepatitis and gastroenteric disease) appeared when levels of antigenemia exceeded 100 pp65-positive PMNLs (three patients; Figure 1).

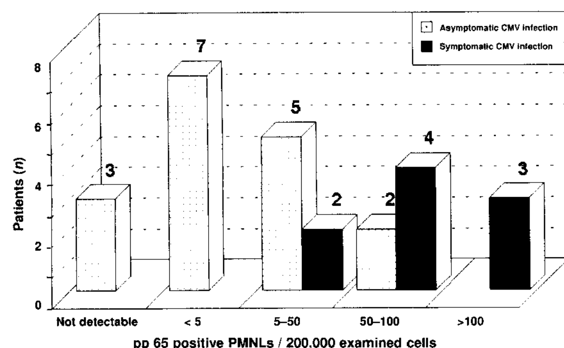

Figure 1 Comparison between pp65 antigen levels at the time of symptom onset in patients with symptomatic HCMV infection and the maximum levels of pp65 antigenemia in asymptomatic patients.

All patients underwent antiviral treatment which was initiated at the onset of fever and leukopenia. Ganciclovir therapy was discontinued when the antigenic load decreased to very low levels (< 10 pp65-positive PMNLs/200,000) and after at least 1 week free of HCMV clinical symptoms (14 to 21 days of therapy). Figure 2 shows the correlation between pp65 antigen test and HCMV disease in two primary infections (patients 1 and 2). Ganciclovir therapy was started on days 40 and 49, respectively. During treatment, the antigenic load significantly decreased (< 25 pp65-positive PMNLs/200,000) with the disappearance of fever. Ganciclovir was discontinued after 21 (patient 1) and 14 (patient 2) days. PCR detected HCMV DNA for 225 and 168 days, respectively and, in patient 1, a relapse of HCMV infection was observed 37 days after ganciclovir was discontinued. The patient was given a second course of therapy which resulted in a complete recovery.

In all symptomatic transplant patients, PCR documented HCMV DNA clearance after 125 ± 73 (range 29 to 225) days whereas the pp65 antigen test became negative after 65 ± 59 (range 18 to 172) days ($p < 0.05$; Table 1). In only two patients, the extended persistence of HCMV DNA in blood correlated with a relapse of HCMV infection. Both relapses were documented by a marked increase in antigenic load (720 and 1000 pp65-positive PMNLs/200,000), as shown in Figure 2 for patient 1. After the second course of ganciclovir, when the antigenic load was no longer detectable, HCMV DNA remained positive for 225 and 167 days.

IgM to HCMV was detected in six of nine (66.6%) patients (three primary and three recurrent infections) after a mean of 55 days from HT and 117 days after the onset of pp65 in PMNLs (data not shown).

HCMV asymptomatic infection

Seventeen patients experienced asymptomatic recurrent infection and did not undergo antiviral treatment. PCR detected HCMV DNA 38 ± 41 (range 8 to 161) days after HT whereas antigenemia was seen after 40 ± 40 (range 8 to 161) days (Table 1).

Figure 1 shows the variations in antigenic load in asymptomatic patients. The mean number of pp65-positive PMNLs was 24 ± 35/200,000 (range 1 to 125). In three PCR-positive patients, the pp65 antigen test was negative during monitoring. One of these pp65-negative patients was a D–/R– recipient who may have experienced HCMV blood transmission either by transfusion during surgery or by graft from a false seronegative donor assessed according to traditional serological methods. PCR detected HCMV DNA in these recipients for 44, 74 and 16 days, respectively. During this period, the three patients were clinically and virologically monitored with pp65 antigen testing (twice a week) to detect the onset of active infection, which was not verified. PCR detected HCMV infection at the same time as the pp65 antigen test in 10 patients and 23 days before antigenemia in four other transplant patients.

Figure 2 shows an example of the virological follow-up with pp65 antigen test and PCR in two asymptomatic patients (patients 3 and 4). Patient 3 experienced high pp65 levels (up to 125 pp65-positive PMNLs/200,000). This recipient was clinically and virologically monitored, but remained symptomless; no preemptive therapy was therefore administered.

PCR detected HCMV DNA for 82 ± 70 (range 16 to 301) days whereas pp65 was detectable for 49 ± 32 (range 15 to 117) days (Table 1). The extended persistence of HCMV DNA was not associated with the onset of HCMV disease, and low levels of antigenemia persisted for extended periods with no signs of disease. IgM to HCMV was positive in four of 17 (23.5%) patients at 62 days from HT and 24 days after the onset of pp65 in PMNLs (data not shown).

DISCUSSION

The present study confirms the usefulness of a close follow-up of heart recipients for HCMV infection [20,24]. Early virological diagnosis of HCMV infection is mandatory and can now be achieved with the pp65

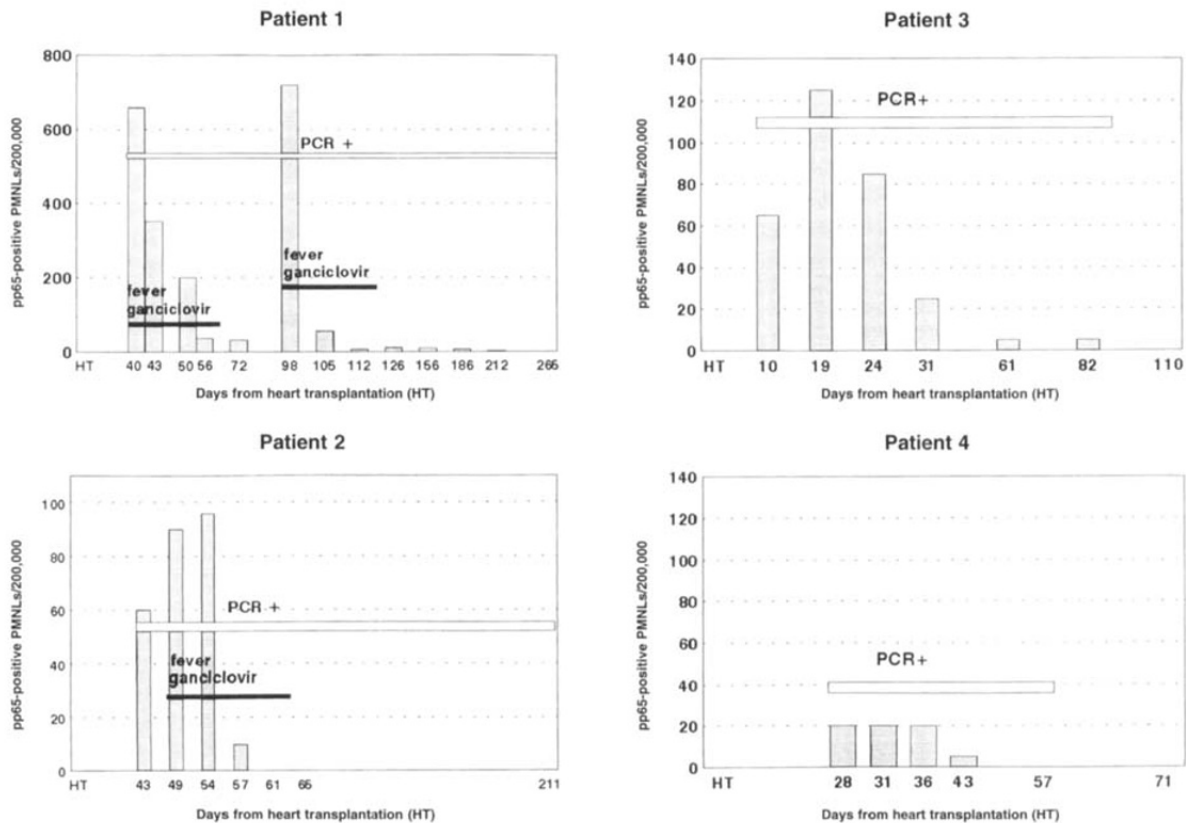

Figure 2 Virological monitoring by pp65 antigen test and PCR for HCMV DNA in two symptomatic HCMV-infected patients who underwent ganciclovir treatment (patients 1 and 2) and in two asymptomatic HCMV-infected patients (patients 3 and 4).

antigen test, allowing the timely detection of HCMV disease developing from HCMV infection and thereby permitting prompt antiviral therapy.

A wide range of individual responses to HCMV infection is seen. One critical factor is HCMV cross-matching between donor and recipient [2,7]. In the present study, four of five HCMV mismatched (D+/R–) patients developed HCMV disease whereas only five of 24 R+ (21%) recipients developed HCMV disease and underwent therapy. A prompt individual recovery of the immune system may be sufficient to control and resolve HCMV infection without antiviral therapy [8,20], as HCMV infection may be completely asymptomatic in some patients.

The evolution of HCMV infection into disease, as seen in our study patients, correlated to pp65-positive PMNLs as demonstrated by a statistically significantly greater and different antigenic load in patients with HCMV disease compared with those with asymptomatic infection, as previously reported [20,21,23,24], In our experience of heart transplant patients, the evaluation of the antigenic load, as expressed by the absolute number of pp65-positive PMNLs, needs to be associated with the clinical status of the patient, which is the rationale for guided antiviral treatment. Indeed, although high antigenic levels (> 100 pp65-positive cells/200,000) are mostly associated with clinical disease, we found that mild symptoms such as fever and leukopenia could be present even when the antigenic load was < 100 pp65-positive PMNLs/200,000 (six of nine patients). On the other hand, moderate-to-high levels of antigenemia may persist for extended periods [33] without symptoms. In these cases, the antigenic load *per se* cannot be sufficient to establish the clinical relevance of HCMVinfection, and strict clinical monitoring of the patient is required for the detection of any variation in clinical status.

The PCR results suggest that the procedure can lead, in most but not all cases, to early detection of viral DNA in PMNLs when the pp65 antigen test is still negative [16,20]. We found no correlation between the onset of clinical symptoms and the presence of HCMV DNA. The positive predictive value of HCMV DNA by PCR for development of HCMV disease was, in fact, low at 34.6%.

According to other PCR analyses for HCMV infection, the results of the present study confirm that quantitation of pp65 antigen provides precise information for the clinician, especially regarding the efficacy of antiviral therapy [20,24,25-27]. The antiviral treatment influences HCMV antigen expression as a result of less viremia whereas, because of its high sensitivity, PCR is able to detect small amounts of DNA even when the infection is latent. On the other hand, sequential negative PCR blood samples provide a strong indication of the absence of HCMV infection. Qualitative PCR as performed in the present study provided no indications concerning the viral loads that might be useful in the treatment and monitoring of HCMV infection.

On the other hand, quantitative PCR for HCMV DNA by coamplification of the target DNA and the same modified sequence as internal standard is, at present, such a highly complex technique as to be unsuitable for diagnostic and clinical purposes [34]. Recent reports on two new techniques, the quantitative branched-DNA amplification assay and the hybridization antibody-capture assay for HCMV DNA detection in PMNLs and other biological samples, suggest that these new procedures may be reliable in monitoring the efficacy of antiHCMV therapies and be suitable for the identification of patients developing resistance [35-38]. New approaches to PCR techniques are required for full comprehension of HCMV infection. Reverse transcription PCR for HCMV messenger ribonucleic acid (mRNA) has been proposed as a highly sensitive technique for an improved understanding of viral replication and for the identification of subjects at higher risk of developing overt disease [39].

The persistence of HCMV DNA by PCR in PMNLs in symptomatic and asymptomatic HT patients is in agreement with previously published reports [20,33]. In some cases, the presence of HCMV DNA in blood may suggest that viral infection is not completely eradicated and that a risk of viral replication resumption may be present. Again, only quantification of pp65 antigen in PMNLs correlates with overt clinical disease as the extended persistence of HCMV DNA can be observed in symptomless patients.

In our experience, careful monitoring of HCMV infection based on the pp65 antigen test associated with a strict clinical evaluation of each patient can satisfy the clinical need for early and prompt diagnosis and management of HCMV infection in heart transplant patients.

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References

1. Kaye MP. The registry of the International Society for Heart and Lung Transplantation tenth official report – 1993. J Heart Lung Transpl 1993; 12: 541-8.
2. Bourge RC, Naftel DC, Costanzo-Nordin MR, et al.

Pretransplantation risk factor for death after heart transplantation: A multi-institutional study. J Heart Lung Transpl 1993; 12: 549-62.
3. Dummer JS, White LT, Ho M, Griffith BP, Hardesty RL, Bahnson HT. Morbidity of cytomegalovirus infection in recipients of heart or heart-lung transplant who received cyclosporine. J Infect Dis 1985,152: 1182-91.
4. Grossi P, De Maria R, Caroli A, Zaina MS, Minoli L. Infections in heart transplant recipients: The experience of the Italian heart transplantation programme. J Heart Lung Transpl 1992; 11: 847-65.
5. Kirklin JK, Naftel DC, Levine TB, et al. Cytomegalovirus after heart transplantation. Risk factors for infection and death: A multi-institutional study. J Heart Lung Transpl 1994; 13: 394-404.
6. Wreight TG, Hakim M, Gray JJ, et al. Cytomegalovirus infections in heart and heart-and-lung transplant recipients. J Clin Pathol 1988; 41: 660-7.
7. Ho M, Dummer JS. Risk factors and approaches to infections in transplant recipients. In: Mandell GJ, Douglas RG, Bennett JE, eds. Principles and practice of infectious disease. New York: Churchill Livingstone 1990: 2284-91.
8. Costanzo-Nordin MR, Swinnen LJ, Fisher SG, et al. Cytomegalovirus infection in heart transplant recipients: Relationship to immunosuppression. J Heart Lung Transpl 1992; 11: 837-46.
9. Collaborative DHPG Treatment Study Group. Treatment of serious cytomegalovirus infections with 9-(1,3-dihydroxy-2-propoxymethyl) guanine in patients with AIDS and other immunodeficiencies. N Engl J Med 1986; 314: 801-5.
10. Erice A, Jordan MC, Chace KBA, Fletcher V, Chinnock BJ, Balfour HH. Ganciclovir treatment of cytomegalovirus disease in transplant recipients and other immunocompromised hosts. JAMA 1987; 257: 3082-7.
11. Walmsley ST, Chew E, Read SE, et al. Treatment of cytomegalovirus retinitis with trisodium phosphonoformate hexahydrate (foscarnet). J Infect Dis 1988; 157: 569-72.
12. The TH, van der Berg AP, van Son WJ, et al. Recent advances in the early and reliable immunodiagnosis of cytomegalovirus infection in immunocompromised hosts. In: Landini MP, ed. Progress in cytomegalovirus research. Amsterdam: Elsevier 1991: 209-20.
13. Dankner WM, McCutchan JA, Richman DD, Hirata K, Spector SA. Localization of human cytomegalovirus in peripheral blood leukocytes by in situ hybridization. J Infect Dis 1990; 161: 31-6.
14. Gerna G, Zipeto D, Percivalle E, et al. Human cytomegalovirus infection of the major leukocyte subpopulations and evidence for initial viral replication in polymorphonuclear leukocytes from viremic patients. J Infect Dis 1992; 166: 1236-44.
15. Saikki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988; 239: 487-91.
16. Jiwa NM, van Gemert GW, Raap AK, et al. Rapid detection of human cytomegalovirus DNA in peripheral blood leukocytes of viremic transplant recipients by the polymerase chain reaction. Transplantation 1989; 48: 72-6.
17. Zipeto D, Revello MG, Silini E, et al. Development and clinical significance of a diagnostic assay based on the polymerase chain reaction for detection of human cytomegalovirus DNA in blood samples from immunocompromised patients. J Clin Microbiol 1992; 30: 527-30.
18. Olive DM, Simsek M, Al-Mufti. Polymerase chain reaction assay for detection of human cytomegalovirus. J Clin Microbiol 1989; 27: 1238-42.
19. Hsia K, Spector DH, Lawrie J, Spector SA. Enzymatic amplification of human cytomegalovirus sequences by polymerase chain reaction. J Clin Microbiol 1989; 27: 1802-9.
20. Gerna G, Zipeto D, Parea M, et al. Monitoring of human cytomegalovirus infections and ganciclovir treatment in heart transplant recipients by determination of viremia, antigenemia and DNAemia. J Infect Dis 1991; 164: 488-98.
21. The TH, van der Bij W, van der Berg AP, et al. Cytomegalovirus antigenemia. Rev Infect Dis 1990; 12(suppl 7): S737-44.
22. van der Berg AP, van der Bij W, van Son WJ, et al. Cytomegalovirus antigenemia as a useful marker of symptomatic cytomegalovirus infection after renal transplantation – a report of 130 consecutive patients. Transplantation 1989; 48: 991-5.
23. Koskinen KP, Niemonen SM, Mattila SP, Hayry PJ, Lautenschlager IT. The correlation between symptomatic CMV infection and CMV antigenemia in heart allograft recipients. Transplantation 1993; 55: 547-51.
24. The TH, van der Berg AP, van Son WJ, et al. Monitoring for cytomegalovirus after organ transplantation: A clinical perspective. Transpl Proc 1993; 25(suppl 4): 5-9.
25. The TH, van der Ploeg M, van der Berg AP, Vlieger AM, van der Giessen M, van Son WJ. Direct detection of cytomegalovirus in peripheral blood leukocytes - a review of the antigenemia assay and polymerase chain reaction. Transplantation 1992; 54: 193-8.
26. Boland GJ, de Weger RA, Tilanus MGJ, Ververs C, Bosboom-Kalsbeek K, de Gast GC. Detection of cytomegalovirus (CMV) in granulocytes by polymerase chain reaction compared with the CMV antigen test. J Clin Microbiol 1992; 30: 1763-7.
27. Delgado R, Lumbreras C, Alba C, et al. Low predictive value of polymerase chain reaction for diagnosis of cytomegalovirus disease in liver transplant recipients. J Clin Microbiol 1992; 30: 1876-8.
28. Zipeto D, Silini E, Parea M, et al. Identification of human cytomegalovirus isolates by the polymerase chain reaction. Microbiologica 1990; 13: 297-304.
29. Gerna G, Revello MG, Percivalle E, Zipeto D, Silini E, Barbarini G, Milanesi G. Human cytomegalovirus viraemia in HIV-1 seropositive patients at various clinical stages of infection. AIDS 1990; 4: 1027-31.
30. Mantero G, Zonaro A, Albertini A, Bertolo P, Primi D. DNA enzyme immunoassay: General method for detecting product of polymerase chain reaction. Clin Chem 1991; 37: 422-9.
31. Saiki RK, Chang CA, Faloona F, et al. Enzymatic amplification of *B*-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985; 230: 1350-4.

32. Kwok S, Higuchi R. Avoiding false positive with PCR. Nature 1989; 339: 237-8.
33. Bitsch A, Kirchner H, Dennin R, et al. The long persistence of CMV DNA in the blood of renal transplant patients after recovery from CMV infection. Transplantation 1993; 56: 108-13.
34. Zipeto D, Baldanti F, Zella D, et al. Quantitation of human cytomegalovirus DNA in peripheral blood polymorphonuclear leukocytes of immunocompromised patients by the polymerase chain reaction. J Virol Meth 1993; 44: 45-56.
35. Miner R, McMullen D, Drew L, et al. Monitoring patients on anti-CMV therapies using a quantitative branched DNA (bDNA) signal amplification assay. In: The second national conference on human retrovirus and related infections, Washington DC, January 29-February 2,1995.
36. Drew L, Miner R, Garvey J, et al. Documentation of in-vivo anti-CMV activity in the cerebrospinal fluid (CSF) by bDNA assay. In: The second national conference on human retrovirus and related infections, Washington DC, January 29-February 2, 1995.
37. Imbert-Marcille BM, Cantanovich D, Boedec S, et al. Evaluation of a new quantification method for cytomegalovirus in leukocytes: Clinical value in renal transplant recipients. The Fifth International Cytomegalovirus Conference, Stockholm, May 21-24, 1995.
38. Gozlan J, Salord JM, Chouaid C, 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; 31: 1943-5.
39. Buffone GJ, Hine E, Demmler GJ. Detection of mRNA from the immediate early gene of human cytomegalovirus in infected cells by in vitro amplification. Mol Cell Probes 1990; 4: 143-51.