

Cytomegalovirus infection in renal transplant recipients diagnosed by nested-PCR

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Abstract

A prospective study of cytomegalovirus (CMV) infection was carried out on 34 renal transplant recipients managed at a General Hospital in Ribeirão Preto, SP, Brazil. Serologic tests showed that all patients were infected with CMV before renal transplantation. Two nested-PCR techniques with primers that recognize sequences of the glycoprotein B (gB) and H (gH) genes were used for CMV detection in blood and urine samples during the post-transplantation period. CMV was detected more frequently in blood samples than in urine samples ($P < 0.001$). Thirty-three patients had CMV detected at least once in blood and/or urine samples. Seven of these patients (21.2%) were diagnosed as having symptomatic CMV infection and showed a worse clinical outcome, with a higher death rate ($P = 0.03$). No association between CMV viremia and graft rejection was observed. Nested-PCR was not useful to identify patients at risk for symptomatic CMV infection since only 21.2% of the patients with CMV infection were symptomatic.

Key words

- Cytomegalovirus
- Polymerase chain reaction
- Kidney transplantation

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Introduction

Cytomegalovirus (CMV) is one of the most common causative agents of infections that affect renal transplant recipients. In countries where diagnosis of active infection and treatment of symptomatic cases are not a routine the patients usually show a worse outcome. The incidence of symptomatic CMV infection during the post-transplant period ranges from 20 to 60% (1,2). Diagnosis of CMV infection in renal transplant recipients should be carried out by detection of the virus. The polymerase chain reaction (PCR) is a highly sensitive technique that

may detect CMV earlier than cell culture or antigenemia determination (3-5). In this study, we used two nested-PCR techniques, recognized as highly sensitive (6), for CMV detection in clinical specimens. The purpose of this study was to examine the relationship of CMV infection with clinical aspects of renal transplant recipients.

Material and Methods

Study population

Thirty-four consecutive patients who underwent renal transplantation at the Ribeirão

Preto General Hospital (RPGH) of São Paulo University between July 1996 and October 1997 were prospectively enrolled in this study.

Immunosuppressive therapy was started on the day of transplantation with oral administration of cyclosporine, prednisolone, and azathioprine. Cyclosporine administration was started at a dose of 8 mg kg⁻¹ day⁻¹, and was reduced to 1 mg kg⁻¹ day⁻¹ weekly until a maintenance dose of 4 mg kg⁻¹ day⁻¹ was reached. Prednisolone administration was started at a dose of 1 mg kg⁻¹ day⁻¹ for 10 days, and was reduced to a dose of 0.75 mg kg⁻¹ day⁻¹ for 20 days, 0.5 mg kg⁻¹ day⁻¹ for 30 days, and finally a maintenance dose of 0.25 mg kg⁻¹ day⁻¹. Azathioprine was always administered at a dose of 2 mg kg⁻¹ day⁻¹.

The medical team diagnosed graft rejection based on the criteria of Hibberd et al. (7).

Whole blood with EDTA as an anticoagulant and urine samples were obtained from patients admitted to the hospital before transplantation and weekly after transplantation. Thereafter, blood and urine samples were collected one to three times a month from outpatients. The patients were observed for at least 3 months except in cases of graft loss or death. A total of 343 blood samples, with an average of 10 per recipient (range 3-20), and 282 urine samples, with an average of 8 (range 1-13), were obtained from the 34 renal transplant recipients for analysis. A lung biopsy was also obtained from one patient.

Clinical and laboratory data were obtained by examination of the patient's records at the RPGH. The patients were divided into two groups, those having symptomatic CMV infection and those having asymptomatic CMV infection, according to the method of Van der Berg et al. (8), with some modifications. Briefly, the symptomatic CMV-infected patients were characterized by the presence of viral DNA detected in at least 2 consecutive samples of peripheral blood leu-

kocytes (PBLs) or urine, and unexplained fever (>37.5°C) for at least 3 days, in combination with at least one of the following features: arthralgia, leukopenia (<3 x 10⁹/l), thrombocytopenia (150 x 10⁹/l), liver enzyme elevation (ALT >50 U/l), pneumonitis or gastrointestinal ulceration without other causes. Asymptomatic CMV-infected patients had viral DNA detected in at least 2 consecutive samples of PBLs or urine, without presenting the signs, symptoms, or laboratory abnormalities stated above. Graft rejection associated with detection of CMV viremia was also analyzed in all patients.

The authors of this paper performed the diagnostic test for CMV infection and the medical team looking after the patients was responsible for treatment.

Serology

Anti-CMV IgG and IgM were detected in serum by indirect immunofluorescence following protocols described by Reynolds et al. (9), using anti-human IgG or IgM immunoglobulin conjugated to fluorescein isothiocyanate (Biomerieux, Lyon, France).

Sample preparation and DNA purification

For PBL separation, 3 to 5 ml of EDTA-treated whole blood samples was processed immediately after collection. Briefly, 1 ml of 1% dextran was added to each blood sample and the mixture was incubated at 37°C for 30 min. Ten milliliters of PBS was added to the supernatant fluids and centrifuged at 300 g for 7 min. The PBL pellets were washed in 10 ml of PBS, centrifuged and suspended in 200 µl of PBS. The lung biopsy sample was prepared for DNA extraction by crushing the tissue in 200 µl of PBS. DNA was extracted from 200 µl of PBLs, from the lung biopsy, or from 200 µl of untreated urine samples using the Qiamp Blood Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's recommendations. The DNA

samples were resuspended in 200 μ l of water.

Nested-PCR

To reduce the risk of false-positive results, each step of the nested-PCR was carried out at different locations with different pipettes, and using tips with filters (Gibco, Gaithersburg, MD, USA). Two nested-PCRs were carried out using 2 primer sets that recognize part of the glycoprotein B (gB) and H (gH) genes. The external primers (gB1604 5'GAAACGCGCGGCAATCGG3' and gB1319 5'TGGAAGTGGAAACGTTTGGC3'; gH172 5'TGGTGTTTTTTCACG CAGGAA3' and gH203 5'CCACCTGGAT CACGCCGCTG3') were those selected by Chou and Dennison (10) and Chou (11), and the internal primers (gBn1 5'GCGCCGTT GATCCACACACC3' and gBn2 5'TACG CCCAGCTGCAGTTCAC3'; gHn1 5'GCG TGAGGGTCCAGCGCTTC3' and gHn2 5'CCTCACTGTCTTCACCGTCT3') were selected from the sequence of the CMV laboratory strain AD169 (12). The reaction mixture of the first round of amplification contained 5 μ l DNA sample, 50 mM KCl, 10 mM Tris-HCl, pH 9, 3 mM MgCl₂, 50 μ M each of the dNTPs, and 0.3 μ M of primers gB1604 and gB1319 or primers gH172 and gH203. The mixture was incubated at 95°C for 3 min in an automated thermal sequencer (Techne, Cambridge, UK), the temperature was reduced to 80°C and 1 U of *Taq* DNA polymerase (Gibco) was added (hot start); the final reaction volume was 50 μ l. The amplification of CMV DNA was carried out with 15 cycles of 60 s at 94°C, 120 s at 65°C, and 120 s at 72°C, followed by 30 cycles of 60 s at 94°C, 90 s at 55°C, and 120 s at 72°C, including a final extension of 3 min at 72°C. As a template in the second round of amplification 2 μ l from the first round was used, including the same components, except for 0.3 μ M of the internal primers gBn1 and gBn2 or gHn1 and gHn2. Amplification was

carried out beginning with the hot start procedure, followed by 30 cycles of 60 s at 95°C and 60 s at 65°C, with a final extension of 3 min at 72°C. Amplification products (95 bp for gB primers and 80 bp for gH primers) were visualized after electrophoresis on 2% agarose gel stained with ethidium bromide.

A PCR with primers for β -globin gene amplification was carried out in blood samples to detect possible false-negative results, which were not included in the study (13).

Nested-PCR sensitivity

A laboratory strain of CMV (AD169) was used to determine the sensitivity of nested-PCR. Ten microliters of decimal dilutions of this virus was added in quadruplicate to a 96-well microplate which contained a monolayer of human fibroblasts. The fibroblast monolayer was observed daily for the cytopathic effect, characterized by the presence of giant rounded cells with increased cytoplasmic granules. The tissue culture infective dose 50 (TCID₅₀) of the virus was calculated by the Reed and Muench method (14). Two microliters of the same dilutions as used above was tested by nested-PCR using both gB and gH primer sets.

Statistical analysis

The MacNemar test was used to analyze the ability of nested-PCR with gB and gH primer sets to detect CMV in blood and urine samples. The Fisher exact test and chi-square test were used to compare the proportions of positive nested-PCR tests in blood and urine, to compare CMV detection in blood samples collected during the first week of transplantation to CMV detection during subsequent periods, and to compare clinical evolution, graft rejection, graft loss, and death between symptomatic and asymptomatic patients.

Results

All patients had anti-CMV IgG but not IgM before transplantation, indicating that all of them were infected with CMV before transplantation.

Both nested-PCRs used for CMV detection were highly sensitive as compared to the observation of cytopathic effects in tissue culture. The CMV AD169 strain titer was $10^{3.5}$ TCID₅₀/ml. Nested-PCR with the gB and gH primer sets was up to 10,000-fold

and 3,162-fold more sensitive than observation of the cytopathic effect, respectively.

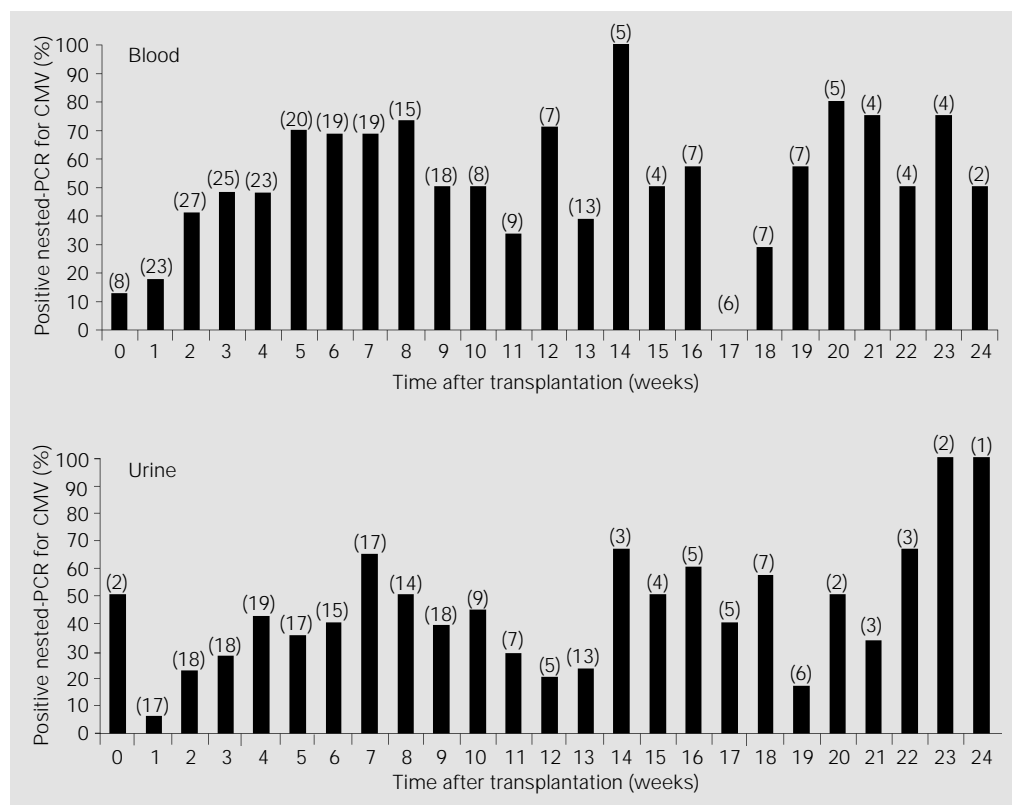
The results of nested-PCR using the gB and gH primer sets in blood and urine samples are shown in Table 1. CMV was detected more frequently in blood samples than in urine samples ($P < 0.001$). Nested-PCR with the gB primer set detected a larger number of positive samples than nested-PCR with the gH primer set ($P < 0.001$).

CMV was detected at least twice in urine or blood samples after renal transplantation

Table 1 - Positive and negative tests for CMV detected in blood and urine samples by nested-PCR using the gB and gH primer sets.

	Blood			Urine		
	Positive	Negative	Total	Positive	Negative	Total
Positive	94	69	163	45	53	98
Negative	20	153	173	16	168	184
Total	114	222	336	61	221	282

Figure 1 - Percentage of patients with CMV detected in blood and urine by nested-PCR with gB or gH primer sets. The number of patients studied is shown in parentheses.



in 32 patients (94.1%). CMV was detected only in a lung biopsy during 13 months of follow-up in one patient. One patient with CMV detected once in urine died 4 weeks after transplantation. Figure 1 shows the percentage of patients with CMV detected in blood and urine during a follow-up of 24 weeks after renal transplantation.

CMV was detected before transplantation and within 1 week after the procedure in blood samples from 5 (16.1%) of 31 patients and in urine samples from 2 (10.5%) of 19 patients. The percentage of patients with CMV detected in blood and urine increased from the second week after transplantation, reaching a maximal level between the 5th and 8th week in blood (70-75%) and between the 4th and 10th week in urine (43-65%).

The number of positive results observed during the period of 0 to 7 days after transplantation was compared with the number of positive results observed during the subsequent periods, as shown in Table 2. A significant increase in CMV detection was observed after the first week of transplantation.

Of the 32 patients with active CMV infection, 6 (18.7%) were classified as having symptomatic CMV infection and 26 (78.8%) as having asymptomatic CMV infection. The clinical outcome and laboratory data for these patients are shown in Figure 2.

Symptoms related to CMV infection appeared between the 2nd and 5th week after renal transplantation in 4 (66.6%) patients. Two patients developed symptoms between the 14th and 15th week. Arthralgia and leukopenia were not observed in any patient.

Table 2 - Comparison of CMV detection in blood samples collected during the first week of transplantation with the subsequent periods.

All comparisons were carried out with the period of 0 to 1 week. *Statistically significant (Fisher's exact test).

Weeks	Number of samples		P value
	Total	Positive	
0-1	31	5	
2-4	75	34	0.005*
5-8	73	51	<0.001*
9-12	42	21	0.002*

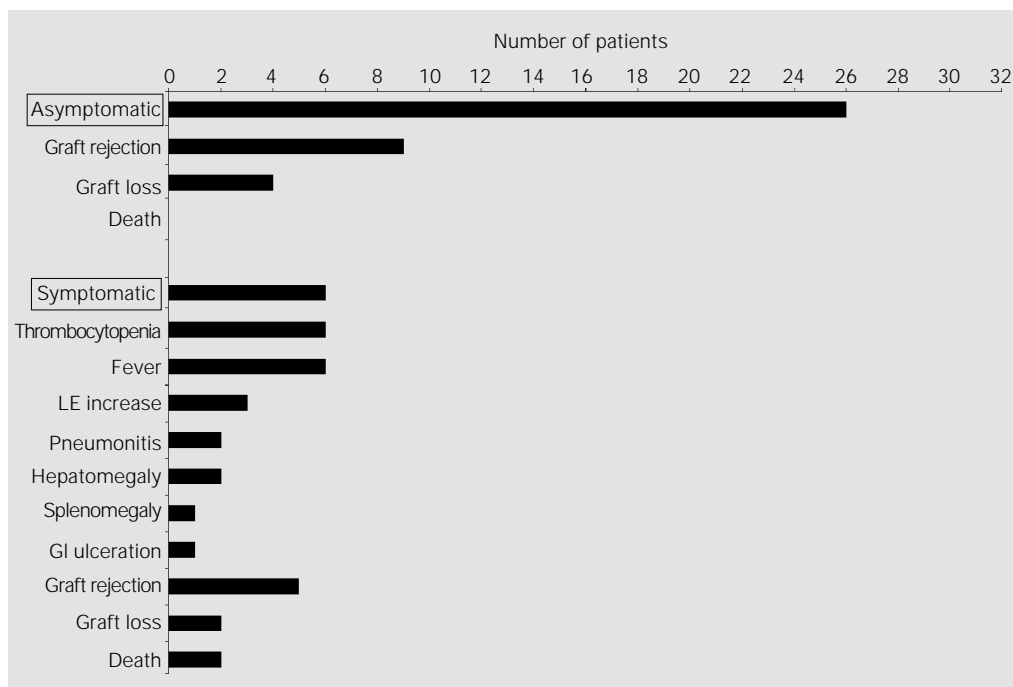


Figure 2 - Clinical and laboratory aspects that may be related to the CMV infection observed in the 33 renal transplant recipients with CMV detected in blood and/or urine. The percentages refer to the total number of symptomatic and asymptomatic patients. LE: Liver enzymes; GI: gastrointestinal.

Five (83.3%) patients had CMV viremia and 3 (50%) had viruria at the time of appearance of symptoms. Three of these patients also had viremia 1 to 2 weeks before the onset of symptoms.

Fourteen patients developed graft rejection and 13 of them showed CMV viremia at least once after renal transplantation. Eight (57.1%) of the latter patients showed CMV viremia within one week of transplantation or before graft rejection and 5 (35.7%) showed CMV viremia only after graft rejection. These data show no association between time of CMV viremia detection and graft rejection.

Graft rejection occurred in 4 (66.6%) of 6 symptomatic patients and in 9 (34.7%) of 26 asymptomatic ones, and graft loss occurred in 3 (50%) of 6 symptomatic patients and in 4 (15.4%) of 26 asymptomatic ones. These differences in frequency were not statistically significant ($P > 0.05$). However, the symptomatic patients showed a significant increase in death rate compared to the asymptomatic ones ($P = 0.03$).

Discussion

All patients were infected with CMV before renal transplantation as shown by the detection of anti-CMV IgG in all of them. The high frequency of CMV infection observed agrees with previous data indicating a prevalence of 90 to 100% anti-CMV antibodies in Brazilian populations (15,16).

The PCR technique has shown higher sensitivity and earlier ability for CMV detection as compared to antigenemia and viral isolation in cell culture (3-5). In the present study we used two highly sensitive nested-PCRs for CMV detection, one with the gB primer set and the other with the gH primer set. Nested-PCR with these primers detected 10,000- and 3,162-fold fewer viruses than the observation of cytopathic effect in cell culture, respectively.

Nested-PCR using gB primers detected

more positive samples than nested-PCR using gH primers ($P < 0.001$). This result agrees with our sensitivity test mentioned above. However, nested-PCR with gB primers did not detect 10.9% of positive blood samples and 14% of positive urine samples that were detected by using gH primers. Therefore, we believe that both gB and gH primers should be used for diagnosis, perhaps in a multiplex fashion.

CMV was detected more frequently in blood than in urine samples ($P < 0.001$). This result is different from that obtained by Hokeberg et al. (17), who found a higher proportion of CMV viruria in kidney transplant recipients. The presence of PCR inhibitors in urine samples may account for a negative result; however, in our study we used a column containing an anion-exchange resin for CMV DNA purification that eliminated possible PCR inhibitors. However, it was not possible to carry out the PCR for β -globin in urine samples due to the low sensitivity of this test and to the low cellular DNA concentration in these samples.

CMV was detected at least twice after transplantation in 32 (94.1%) patients. The high prevalence of CMV DNA detected in this study is in agreement with previous reports showing that, depending on the serologic status of donor/recipient and type of immunosuppression, 60 to 100% of the patients shed CMV (18-20).

Considering that PCR is a highly sensitive technique and that peripheral blood monocytes can harbor CMV in a latent state, a positive PCR result in blood samples can indicate the detection of a latent rather than an active infection (21). The nested-PCR used in this study, although highly sensitive, probably detected only active CMV infection, since a significant increase in the number of positive results was observed starting during the second week after transplantation (Table 2). If nested-PCR had detected latent infections, a larger number of positive tests would have been expected before transplan-

tation since all patients were previously infected with CMV. Other evidence that our nested-PCR detected active CMV infection came from the observation of 4 patients who developed graft rejection. CMV was detected in these patients only after the onset of graft rejection and probably as a consequence of the vigorous immunosuppression (corticoids and OKT3) administered to control graft rejection, which led the latent virus to reactivate. However, it is possible that our nested-PCR detected latent infection in the lungs of one patient. This patient, not included among the CMV active infection cases, never presented viremia or viruria and remained asymptomatic during follow-up.

CMV was detected within the first week of transplantation in 16.1 and 10.5% of the patients when blood and urine samples were analyzed, respectively (Figure 1). The number of patients with CMV viremia and viruria increased in the second week after transplantation and reached the highest level (70-75%) between the 5th and 8th week in blood samples and (43-65%) between the 4th and 10th week in urine samples. Thus, CMV could be detected intermittently for a long period of time. The presence of CMV viremia and viruria within the first week of renal transplantation is in agreement with data reported by Rowley et al. (22). Bitsch et al. (3) and Barber et al. (5) also reported the detection of CMV DNA by PCR for long periods of time after kidney transplantation.

Six (18.7%) patients developed symptomatic CMV infection, a frequency also observed in previous studies indicating the presence of symptomatic infection in 20 to 60% of patients after transplantation (1,2,23). Rubin and Colvin (24) found that the time between the 4th and 12th week after renal transplantation is critical for symptomatic CMV infection. In this study, symptomatic CMV infection appeared between the 2nd and 5th week after renal transplantation in 4 (66.6%) patients. Two patients also devel-

oped symptomatic CMV infection between the 14th and 15th week after transplantation. Hokeberg et al. (17) detected arthralgia and thrombocytopenia in 66% and 7% of patients with CMV disease, respectively, whereas in the present study no patient had arthralgia, and all of them had thrombocytopenia. Leukopenia, commonly found in this kind of patient, was also not observed. CMV viremia, reported as a risk factor for symptomatic infection (25,26), was found in 3 patients (42.9%) starting 1 to 3 weeks before the onset of symptoms and persisted during the disease. Two other cases presented CMV viremia only at the onset of symptoms.

Symptomatic CMV-infected patients showed a higher risk of death compared to asymptomatic ones ($P = 0.03$), in agreement with other reports (27-29).

CMV infection has been associated with decreased graft survival; however, the mechanisms by which the virus induces rejection are not well known (30). Graft rejection of the cellular type, which may be associated with CMV infection (31), was observed in 10 patients, but the association of diagnosis of CMV infection with graft rejection was not detected in any patient.

The use of ganciclovir was not investigated because only 2 of the 6 symptomatic patients were treated.

Although the PCR used in the present study detected active CMV infection, at least in most patients, it was not useful to identify patients at risk of symptomatic CMV infection. Only 6 (18.7%) of 32 patients with active CMV infection developed symptoms. The inefficacy of PCR in identifying patients at risk of symptomatic CMV infection was also observed by other authors (3,32). Quantitative PCR seems to be more effective in identifying patients at risk of symptomatic CMV infection (33,34). We are currently developing a quantitative PCR using a plasmid containing a sequence of the gB gene.

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