

# Comparison of PCR, Antigenemia Assay, and Rapid Blood Culture for Detection and Prevention of Cytomegalovirus Disease after Lung Transplantation

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**The goal of this study was to evaluate serial cytomegalovirus (CMV) blood culture, antigenemia testing, and qualitative and quantitative plasma CMV PCR for their ability to predict CMV disease and thus to direct preemptive therapy after lung transplantation. Forty-one patients provided 414 samples for blood culture, 290 samples for antigenemia testing, and 432 samples for PCR. Seven patients developed 11 episodes of CMV disease. CMV PCR had sensitivity, specificity, and positive predictive and negative predictive values of 79, 99, 84, and 99%, respectively, compared with 48, 99, 85, and 98%, respectively, for antigenemia testing, and 8, 100, 100, and 97%, respectively, for culture. Only quantitative CMV PCR correlated with disease stage: asymptomatic patients had a mean of 1,500 CMV DNA copies/ml, whereas patients who developed CMV disease had 5,087 copies/ml 12 to 4 weeks before symptoms and 32,000 copies/ml at diagnosis. Furthermore, CMV PCR-measured DNA increased 5- to 10-fold immediately preceding symptoms. PCR and antigenemia test values decreased with anti-CMV therapy. CMV DNA (as detected by PCR), but not antigenemia, persisted in patients who later developed recurrent CMV disease. The data indicate that lung transplant recipients will benefit from monitoring of CMV disease by plasma CMV PCR.**

Cytomegalovirus (CMV) infection is a major cause of morbidity and mortality in lung transplant recipients. Among solid-organ transplant patients, lung recipients have the highest incidences of CMV disease, 39 to 41% compared with 8 to 32% for other solid-organ recipients (20). The large amount of virus present in the lung may be an important contributor to this effect (7). Additional factors that predispose lung transplant recipients to CMV disease include high acute rejection rates and resulting aggressive immunosuppressive therapy.

CMV manifestations can be very severe when they involve a transplanted lung, a phenomenon that occurs in 13 to 28% of all patients (6, 9, 12). CMV pneumonitis directly compromises graft function, and mortality is 2 to 14%. Other CMV-related syndromes in lung transplant patients include viral syndrome, colitis, and, less frequently, encephalitis, retinitis, myocarditis, hepatitis, pancreatitis, and urogenital and skin infections. CMV infection in transplant recipients also predisposes the patient to secondary fungal and bacterial infections (23). In addition, CMV infection, whether symptomatic or not, is associated with an increased risk of chronic rejection, which in lung transplant patients manifests itself as bronchiolitis obliterans (17).

The incidence of CMV disease in lung transplant recipients typically peaks between 3 and 24 weeks after transplantation but may occur later, as a relapse or in association with antirejection therapy.

Several prophylactic strategies, such as administration of ganciclovir and/or CMV hyperimmune globulin (CMVIG), have been evaluated for lung transplant patients with varied success (2). The role of preemptive therapy has also been

examined for high-risk CMV disease transplant recipients (10). Preemptive treatment is usually offered to lung transplant recipients during periods of intensive immunosuppression, i.e., in the immediate posttransplant period and in association with treatment of acute rejection. Despite these intensive efforts to prevent CMV disease, breakthrough CMV pneumonitis frequently occurs. Increasing the success of preemptive therapy requires good markers of CMV replication. CMV culture from multiple sites and detection of CMV DNA or antigen in the blood have been evaluated as putative predictors of CMV disease in various organ transplant settings (8, 11, 13, 14, 22). Both culture and testing for antigenemia have shown varied results in predicting CMV disease in lung transplant patients (1). CMV PCR, in a small number of lung transplant recipients, discriminated between patients with and without CMV pneumonitis (4, 5). CMV PCR on bronchoalveolar lavages or transbronchial biopsies was also useful for diagnosis of CMV pneumonitis and assessment of response to therapy (18).

In this study, we compare serial CMV blood culture, testing for antigenemia, and qualitative and quantitative CMV PCR for the ability to predict CMV disease in lung transplant patients.

## MATERIALS AND METHODS

**Study design.** Between May 1996 and April 1998, all lung transplant recipients less than 1 year posttransplantation were enrolled in this prospective study. Their immunosuppressive regimen included cyclosporine, azathioprine, and steroids. All patients received CMVIG and intravenous ganciclovir (5 mg/kg of body weight/day) during the first 28 days after transplantation, followed by a high dose of acyclovir (800 mg three times a day) for up to 6 months posttransplantation and a low dose of acyclovir (200 mg twice a day) from 6 months to 1 year. CMV-seronegative recipients ( $R^-$ ) who had a seropositive donor ( $D^+$ ) received intravenous ganciclovir for the first 90 days after transplantation and seven doses of CMVIG. Patients also received CMVIG and 5 days of ganciclovir in association with the use of OKT3 for acute rejection. For treatment of CMV disease, patients received ganciclovir and three doses of 100 mg each of CMVIG per kg every three to four days. Blood surveillance for CMV comprised rapid shell vial culture, antigenemia testing, and plasma PCR performed weekly between day 28

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TABLE 1. Demographic characteristics of 41 lung transplant recipients enrolled in the study of early diagnosis of active CMV infection

Characteristic	Value	No. of patients who developed CMV disease
Sex of patients		
Male	22	
Female	19	
Age		
Median	50 yr	
Range	20–68 yr	
CMV profile		
R <sup>+</sup> and D <sup>+</sup>	17	4
R <sup>+</sup> and D <sup>-</sup>	13	1
R <sup>-</sup> and D <sup>+</sup>	7	2
R <sup>-</sup> and D <sup>-</sup>	4	0

and day 90 posttransplantation, every two weeks from day 91 to day 180, and monthly from day 181 to day 360. Clinical management was not affected by results of the CMV blood surveillance.

**Definitions.** CMV end-organ disease was defined as isolation or immunohistochemical detection of CMV in bronchoalveolar lavage, lung biopsy, or other tissue biopsy specimens associated with compatible clinical signs and symptoms. CMV viral syndrome was defined as otherwise unexplained fever in a patient with a positive CMV culture from any site. CMV infection was defined as detection of CMV replication.

A true-positive assay was defined by the presence of CMV end-organ disease or viral syndrome at the time the blood was obtained or if the sample was part of a sequence of positive samples preceding or following the diagnosis of CMV disease.

**CMV culture.** Blood cultures were performed using the rapid shell vial technique. Human lung embryonic cells grown on coverslips were inoculated in duplicate with 0.3 ml of white blood cell (WBC) suspension from EDTA-anticoagulated blood (15). The coverslips were stained at 48 h with anti-pp72 mouse monoclonal antibody (Dupont) followed by an anti-mouse fluorescein isothiocyanate-conjugated antiserum (Baxter). Two or more positive fluorescent nuclei indicated a positive result.

**CMV antigenemia.** The antigenemia assay was performed using a CMV Light kit (Chemicon) according to the manufacturer's instructions. Specimens were processed when the laboratory received them within 6 h of collection (3, 19). A positive result was defined as at least two positive cells in duplicate wells containing  $2 \times 10^5$  WBC.

**Qualitative CMV plasma PCR.** This test was performed as previously described (25) using a single amplification method followed by Southern blot detection of a CMV fragment located in the *EcoRI* fragment D region. DNA, extracted from 5  $\mu$ l of plasma with an InstaGene kit (Bio-Rad) or from 2  $\mu$ l of heat-inactivated plasma (100°C for 50 to 55 s), was amplified with 1 U of *Pyrococcus furiosus* (Pfu) polymerase (Stratagene), 0.5  $\mu$ M concentrations (each) of primers 625 and 461 (26), and 50  $\mu$ M concentrations of each deoxynucleoside triphosphate in 50  $\mu$ l of Pfu buffer (50 mM KCl, 10 mM Tris-Cl, 5 mM MgCl<sub>2</sub>). The amplification was performed in a Perkin-Elmer thermal cycler using 45 cycles of DNA denaturation at 95°C for 15 s, primer annealing at 61°C for 15 s, and DNA elongation at 72°C for 30 s. Following PCR, the amplicon was separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with digoxigenin (Boehringer Mannheim)-labeled probe 628 (25). Bound probe was detected with antidigoxigenin antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) and CDP-star chemiluminescent substrate (Tropix). This method consistently detected one copy of DNA per reaction. Each run included two positive viral controls (CMV strain AD 169) and two negative controls. Patient samples were run in duplicate. The test was valid if the controls yielded the expected results and the patient replicates were in agreement.

**Quantitative CMV PCR.** This test was performed using a competitive method. The internal standard (IS) for this assay was synthesized by the following procedure. A 335-bp fragment in the CMV region MIE2783 to MIE3117 was amplified by PCR; a 92-bp internal segment was deleted from the above-mentioned fragment, resulting in a 243-bp fragment, the IS, which shared primer sites with the parent fragment (MIE2783-MIE3117). The 243-bp IS was amplified by PCR and inserted in the vector PCR-Script SK (+) (Stratagene). *Escherichia coli* strain XL1-blue (Stratagene) transformed with the IS-containing plasmid was grown in Luria-Bertani medium (Gibco BRL) and selected with an ampicillin marker. The plasmid DNA was purified with a 500- $\mu$ l ion-exchange resin column (Qiagen) and quantitated with a spectrophotometer (model DU 62; Beckman). The number of IS copies was confirmed by amplification as described above with known amounts of CMV DNA quantitative standards (ABI). The PCR products

were separated by agarose gel electrophoresis, stained with Vista Green, scanned with a Storm instrument (Molecular Dynamics), and quantitated using Image-Quant software. Using 1,000 IS copies/reaction tube and serial dilutions of the ABI quantitative standards in CMV-seronegative plasma, a linear relationship between input and measured CMV DNA was identified between 1,000 to 1,000,000 copies of CMV DNA/ml of plasma. DNA, extracted from clinical specimens as described above, was coamplified with 1,000 copies of IS/reaction tube and quantitated with the Storm software. Positive and negative controls were run with each clinical specimen.

**Statistical analysis.** Paired and unpaired two-tailed *t* tests were performed as specified in Results using Statview 512 software. Differences were considered significant with a *P* of  $\leq 0.05$ .

## RESULTS

**Patient demographics and clinical outcomes.** The study enrolled 22 males and 19 females with a median age of 50 years (range, 20 to 68 years) (Table 1). Seventeen recipient-donor pairs were R<sup>+</sup> and D<sup>+</sup>, 13 pairs were R<sup>+</sup> and D<sup>-</sup>, seven pairs were R<sup>-</sup> D<sup>+</sup>, and four pairs were R<sup>-</sup> and D<sup>-</sup>. Patients were monitored for 4 to 12 months, with mean and median times of 10.4 and 12 months, respectively.

Seven patients developed 11 episodes of CMV disease, which included seven cases of pneumonitis and four CMV viral syndromes. Of the seven patients who developed CMV disease, two were R<sup>-</sup> and D<sup>+</sup>, four were R<sup>+</sup> and D<sup>+</sup>, and one was R<sup>+</sup> and D<sup>-</sup>. The average time of the first episode of CMV disease was 131 days (median, 128 days) posttransplantation.

**Laboratory results. (i) CMV blood culture.** The sole positive culture from 414 samples came from a symptomatic patient. Thus, the specificity of culture was 100%, but this test failed to detect the remaining 10 CMV disease episodes of patients from whom specimens were available at the time symptoms began, indicating a low sensitivity of 8% (Table 2). The positive blood culture specimen was also positive by PCR and antigenemia testing.

**(ii) CMV antigenemia.** Antigenemia assays were performed on 290 acceptable specimens. Fourteen specimens obtained from five patients were positive, 6 specimens were indeterminate, and the remaining 270 specimens were negative. Antigenemia testing detected four out of the nine CMV disease episodes of patients from whom specimens were available (sensitivity of 48%). In these four symptomatic patients, antigenemia was detected up to 6 weeks before the beginning of clinical symptoms. Four samples obtained during the week immediately preceding the initiation of CMV-associated disease varied from 2 to 1,400 positive cells/ $2 \times 10^5$  WBC. Among three patients who had serial specimens preceding the beginning of CMV disease, two showed  $\geq 4$ -fold increases in the number of positive cells immediately before the beginning of symptomatic CMV infection and one did not. The number of

TABLE 2. Performance characteristics of CMV rapid culture, antigenemia testing, and plasma PCR for lung transplant recipients

Characteristic	% of results of indicated nature by:		
	Rapid culture	Antigenemia assay	Qualitative PCR
Sensitivity	8 (1/13) <sup>a</sup>	48 (12/25)	79 (27/34)
Specificity	100 (401/401) <sup>b</sup>	99 (263/265)	99 (393/398)
PPV	100 (1/1) <sup>c</sup>	85 (12/14)	84 (27/32)
NPV	96 (389/413) <sup>d</sup>	98 (263/268)	99 (393/395)

<sup>a</sup> Number of true-positive assays/total number of assays performed in the presence of CMV disease.

<sup>b</sup> Number of true-negative assays/total number of assays performed in the absence of CMV disease.

<sup>c</sup> Number of true-positive assays/total number of positive assays.

<sup>d</sup> Number of true-negative assays/total number of negative assays.

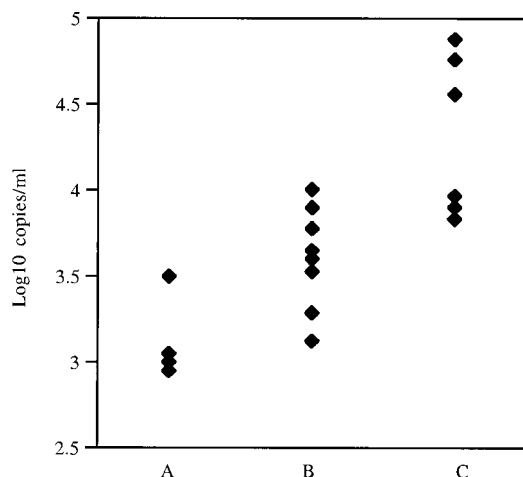


FIG. 1. Distribution of CMV viral burdens in lung transplant recipients. Column A, levels of CMV DNA in the plasma of patients who remained asymptomatic; column B, levels 12 to 4 weeks prior to development of symptoms; column C, levels at clinical diagnosis.

positive cells rapidly decreased on therapy in all four symptomatic patients, and the antigenemia test result became negative within 2 to 4 weeks. However, five episodes of confirmed CMV disease, three of which were de novo and two were recurrences, were not detected by antigenemia testing. There were two positive antigenemia test results, with 2 and 56 cells/ $10^5$  WBC, for patients who did not develop CMV disease (specificity of 99%). Twelve of 14 positive antigenemia specimens were also positive by PCR, including one not associated with CMV disease.

(iii) **Qualitative CMV PCR.** CMV DNA detection was attempted with 432 plasma specimens, 32 of which had positive results. PCR was positive in 9 out of 11 CMV disease episodes. There were no positive PCR results with  $R^-D^-$  patients. In the remaining recipient-donor categories there were five positive PCRs unrelated to CMV (specificity of qualitative CMV PCR of 99%). One of these occurred with a patient who was receiving ganciclovir preemptive therapy in association with treatment of acute rejection; two occurred with patients who had previously had CMV disease, and two occurred with patients who never developed CMV disease. In the patients who developed CMV disease, a positive PCR result preceded the beginning of symptoms by 1 to 12 weeks and results remained positive for 1 to 5 weeks during therapy. Furthermore, for the three patients who developed the four episodes of recurrent CMV disease, plasma CMV PCR was still positive after each course of ganciclovir therapy was completed.

(iv) **Quantitative CMV PCR.** Circulating CMV DNA was measured by quantitative PCR in 36 plasma samples positive by qualitative PCR. Levels between 6,800 and 75,886 copies/ml (mean  $\pm$  standard error [SE] =  $32,130 \pm 12,111$  copies/ml) were detected in specimens from six patients with CMV disease from whom plasma samples were submitted during the week prior to the initiation of symptoms (Fig. 1). These CMV DNA levels were significantly higher (analysis of variance, significant at 95%) than the CMV DNA burdens measured in four patients who did not develop symptomatic disease (range of <1,000 to 3,002 copies/ml; mean  $\pm$  SE =  $1,500 \pm 500$  copies/ml) and in eight patients 12 to 4 weeks prior to development of symptoms (range of 1,333 to 10,100 copies/ml; mean  $\pm$  SE =  $5,087 \pm 1,060$ ). Four patients had sequential specimens from 4 weeks before CMV disease declared itself and through anti-CMV-specific therapy (Fig. 2). There was a 5-

to 10-fold increase in the CMV DNA titers prior to disease development. In addition, during the first four weeks of ganciclovir therapy, plasma CMV DNA levels decreased by  $\geq 10$ -fold in the four patients from whom samples were available. CMV plasma DNA persisted at low but detectable levels in the patients who developed recurrent disease.

## DISCUSSION

This longitudinal prospective study comparing the results of CMV plasma PCR, antigenemia testing, and rapid blood culture for early preclinical diagnosis of CMV disease analyzed, over a period of 2 years, samples from 41 patients, 7 of whom developed 11 episodes of symptomatic CMV disease. The incidence of de novo CMV disease in this population was 17%. The incidence of CMV infection was 30% (data not shown), which is considerably lower than the 89% reported by other lung transplantation centers (9). This difference may be ascribed to a diverse approach to CMV prophylaxis among transplantation centers.

Plasma CMV PCR outperformed the other methods as a predictor of CMV disease. Qualitative plasma PCR had the highest sensitivity and negative predictive value (NPV) (79 and 99%, respectively) and a good specificity and positive predictive value (PPV) (99 and 84%, respectively). The addition of the quantitative PCR step further improved the discriminative power of the PCR assay. Levels of CMV DNA were significantly higher at the time of CMV disease than levels 4 to 12 weeks prior to initiation of symptoms or than levels associated with asymptomatic CMV. However, in patients who developed CMV disease, the quantitative plasma CMV PCR detected a 5- to 10-fold increase in the number of CMV DNA copies/ml of plasma before or at the onset of clinical symptoms. Thus, a single plasma CMV PCR level of  $\geq 10,000$  copies/ml or a  $\geq 5$ -fold increase between two sequential measurements seemed to indicate high risk of CMV disease and could be used to initiate anti-CMV preemptive therapy. Although the number of specimens available at the time CMV disease declared itself was limited, our findings are strengthened by agreement with data from previous studies of other solid-organ and bone marrow transplant recipients (13, 24).

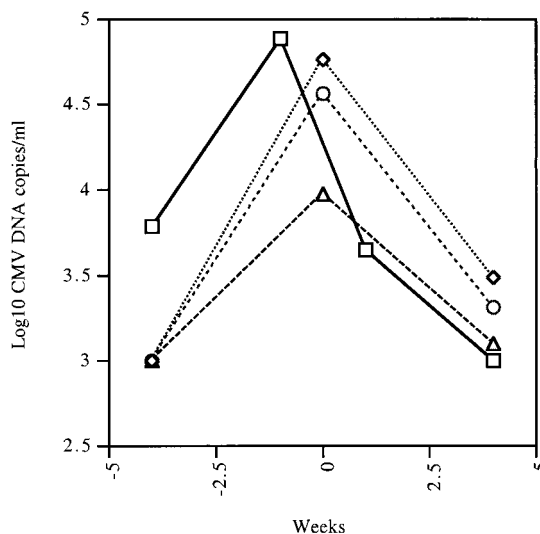


FIG. 2. Kinetics of plasma CMV viral burdens in four lung transplant recipients who developed CMV disease. Week 0 indicates diagnosis and initiation of treatment.



In this study, the number of CMV DNA copies/ml underwent a 10-fold reduction in the first month of therapy in successfully treated patients. This observation suggests that quantitative plasma CMV PCR might be a useful tool to monitor the efficacy of anti-CMV therapy in lung transplant recipients. In addition, patients who developed recurrent CMV disease were still positive by PCR at the time therapy for the preceding episode was discontinued whereas patients who did not develop recurrent disease tended to clear the CMV DNA from their blood faster. This result suggests that continuing anti-CMV therapy until the plasma CMV PCR becomes negative might prevent recurrent CMV disease. Further studies are necessary to confirm this observation.

CMV blood culture was insensitive although very specific for the diagnosis of CMV disease. These results were unique to our study population, who received intensive anti-CMV prophylaxis. CMV blood culture tends to be more sensitive and less specific with samples from untreated patients.

CMV antigenemia has been previously shown to predict CMV disease in lung transplant recipients (10). In our patient population, which received intensive ganciclovir and CMVIG prophylaxis when CMV disease was anticipated, antigenemia testing had a sensitivity, specificity, PPV, and NPV of 48, 99, 85, and 98%, respectively. These results are in contrast with those reported by Egan et al. (10), who studied nine lung transplant patients, among others, and found that antigenemia testing had a 100% sensitivity and 93.7% specificity. The differences between the two studies with respect to the number of patients (41 versus 9), posttransplantation antiviral management (intense prophylaxis against CMV versus no prophylaxis at all), and duration of the observation period (12 months versus 8 months) might have contributed to these differences. In our study, CMV disease occurred in association with low and high numbers of positive cells, which implies that preemptive anti-CMV therapy needs to be entertained with any positive antigenemia test result. On treatment, the number of positive cells rapidly decreased and the antigenemia test result became negative after 1 month of therapy for all patients, including those who later experienced a recurrence of the disease. Unlike PCR, antigenemia testing did not identify the patients at high risk of recurrent CMV.

A difficulty associated with antigenemia testing is the interpretation of indeterminate results. At the time this study was initiated, the Chemicon kit provided the best level of discriminating fluorescence when compared with those of other commercially available kits, namely, Biotest and Incstar (data not shown). However, 6 out of 290 antigenemia test results were uninterpretable. Although the uninterpretable assays represented only 2% of the total number of tests, this same number seems high considering that only 5% (16 tests) had positive results.

Besides performance characteristics, there are other important issues to consider before setting up a laboratory monitor, such as cost, reproducibility, ease of processing, and quality assurance of the assays. Both the antigenemia assay and PCR can contribute to identification of patients who are on the verge of developing CMV disease. PCR is currently more expensive than the antigenemia test. PCR prices have been declining in the last couple of years due to increased test utilization and are expected to further decrease as commercial kits become available. With respect to reproducibility, the antigenemia test is marketed in a kit format but the readout is subjective and the interlaboratory variability can be very wide (A. Erice, C. Crumpacker, W. Britt, L. Drew, R. Hillam, S. Kao, J. Kohlberg, M. Landry, N. Lurain, J. Manishewitz, M. Nokta, S. Spector, A. Weinberg, B. Yen-Lieberman, D. Brambilla, and

P. Reichelderfer, Presented at the Third Conf. Retrovir. Opportunistic Infect., Washington, D.C., 29 to 31 January 1996). PCR is currently performed in many laboratories by in-house methods, and there is no information on how these assays compare. The availability of high-performance CMV PCR kits (16) and semiautomated platforms is expected to increase the reproducibility of results. In addition, the recent introduction of proficiency panels for CMV PCR offered by clinical laboratory accreditation agencies, such as the College of American Pathologists, will help to homogenize and control the quality of results of CMV PCR. In contrast, there are no quality assurance programs for antigenemia testing, and the technical difficulties for setting up proficiency panels for this test might be insurmountable. Finally, the utility of antigenemia testing is limited by the rapid loss of signal during storage because specimens have to be processed within 6 h of collection (3, 19). In contrast, the results of quantitative CMV PCR are stable for at least 1 week at refrigerator temperature (21, 24).

CMV pneumonitis has a major impact on the success of lung transplantation. Thus, many centers preemptively treat these patients with antivirals and/or CMVIG at times of high risk of CMV disease, such as in the immediate posttransplantation period and during treatment of rejection. However, even when preemptive therapy is used as described here, there is breakthrough disease. Monitors using quantitative plasma CMV PCR can further decrease the incidence of CMV disease. Further studies are needed to determine if such monitors can replace the broad use of preemptive anti-CMV therapy with a more focused approach.

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