Evaluation of a Quantitative Plasma PCR Plate Assay for Detecting Cytomegalovirus Infection in Marrow Transplant Recipients

GHISLAINE M. GALLEZ-HAWKINS,¹ BERNARD R. TEGTMEIER,² ANNA TER VEER,³
JOYCE C. NILAND,³ STEPHEN J. FORMAN,⁴ AND JOHN A. ZAIA^{1*}

Departments of Pediatrics,¹ Infectious Diseases/Clinical Pathology,² Biostatistics,³ and Hematology/Bone Marrow Transplantation,⁴ City of Hope National Medical Center, Duarte, California 91010

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A plasma PCR test, using a nonradioactive PCR plate assay, was evaluated for detection of human cytomegalovirus reactivation. This assay was compared to Southern blotting and found to perform well. As a noncompetitive method of quantitation, it was similar to a competitive method for detecting the number of genome copies per milliliter of plasma in marrow transplant recipients. This is a technically simplified assay with potential for adaptation to automation.

Historically, transplantation patients have been monitored for the presence of cytomegalovirus (CMV) in blood and lungs by culture (5, 13, 16) or, more recently, by antigen detection in peripheral blood leukocytes and CMV DNA detection by PCR (2–4, 9, 11, 18, 21, 22). As methods of detection become more sensitive, the need to quantify CMV becomes important in assessing their relevance to the prediction and management of CMV disease (6, 7, 15, 17).

Plasma PCR (P-PCR) has been used as a clinical marker for detecting CMV in marrow transplant recipients with early asymptomatic pulmonary CMV infection (1, 4, 22). Amplification of CMV DNA is traditionally detected by Southern blotting, a method not easily adaptable to high-volume sample analyses. For this study, we adopted a 96-well plate assay based on enzyme-linked immunosorbent assay technology to detect and measure the amount of PCR product. Here, we show that the plate assay is useful for reporting CMV reactivation in bone marrow transplant (BMT) recipients and that quantitation of CMV DNA in plasma by a noncompetitive method with this assay is valid.

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Plasma was collected from heparinized blood by centrifugation for 10 min at $700 \times g$. The supernatant was collected, filtered through a 0.45- μ m-pore-size filter, and stored at -20°C until processed. The plasma DNA was isolated as described previously (1). Briefly, it was mixed 1:1 with proteinase K-containing buffer (120 $\mu\text{g}/\text{ml}$) (11), incubated at 55°C for 60 min and then at 95°C for 15 min, and spun at $700 \times g$ for 15 min. Ten microliters of digested plasma was added to 40 μl of PCR master mix. The primers chosen for this study amplify a 160-bp region of U_L83 encoding the pp65 matrix protein (23). The primer generating a strand complementary to the probe was biotinylated at the 5' end (Genosys Biotechnologies, Inc., The Woodlands, Tex.), and the probe was 5' GTTCTCCATG GAGCAAACGACTCGTGCGC. The PCR conditions were as described by Aspin et al. (1), except that the number of cycles was increased to 35 and uracil-*N*-glycosylase (Perkin-

Elmer Cetus, Norwalk, Conn.) was used routinely at 1 U per PCR to avoid carryover contamination. The plate assay method for PCR product detection was as described by Rasmussen et al. (15), with the following modifications: 200 pmol of probe per ml was added to the well, the hybridization temperature was 55°C , and tetramethylbenzidine (0.1 mg/ml) was used for peroxidase detection. Optical density (OD) values were read spectrophotometrically at 450 nm. The mean OD of negative P-PCRs was determined to be 0.055 ± 0.051 U in a panel of 800 specimens from patients known to have never shed virus after receipt of a BMT, as determined by conventional blood and bronchoalveolar lavage fluid cultures (16). We therefore defined as negative any sample with an OD equal to or less than 0.2, the mean OD from the negative panel plus 3 standard deviations.

To compare the results obtained by plate assay to those obtained by Southern blot detection, we ran the same amplified plasma samples on a 1.8% agarose gel, transferred them by alkaline vacuum onto a Zeta-Probe membrane, and hybridized them with the above-described probe 3' labeled with α - ^{32}P . The results were as follows: number of samples Southern blot and plate assay positive, 62; number of samples Southern blot positive and plate assay negative, 8; number of samples Southern blot negative and plate assay positive, 9; number negative by both assays, 391. With the Southern blot as the comparative standard for 470 samples, the P-PCR assay had a sensitivity of 0.89, a specificity of 0.98, a positive predictive value of 0.87, and a negative predictive value of 0.98. Discrepancies between the two methods of detection occurred only at very low levels of CMV DNA. We concluded that the plate assay is as efficient as Southern blotting for detection of P-PCR products.

To set up the noncompetitive method of PCR quantitation, the *Bam*HI P fragment (about 6.5 kb) of Towne CMV DNA was inserted into pSP64 in the *Bam*HI and *Eco*RI sites. This plasmid, called p22E, contains the coding region for pp65(U_L83) and pp71(U_L82). By amplifying serial dilutions (10^6 to 1 genome copy) of p22E diluted in seronegative plasma, a standard curve was obtained representing an average OD of six independent experiments (Fig. 1A). The numbers of genome copies (gc) from unknown samples were derived from the corresponding OD values. Appropriate dilutions of plasma were made when the OD values were above 2.0, the level at

* Corresponding author. Mailing address: Department of Pediatrics, City of Hope National Medical Center, 1500 E. Duarte Rd., Duarte, CA 91010. Phone: (818) 359-8111. Fax: (818) 301-8458.

FIG. 1. Comparison of noncompetitive and competitive methods of quantitation for a representative plasma sample. (A) Noncompetitive quantitation. Serial dilutions of amplified p22E were plotted and fitted to a logarithmic curve from which unknown samples were extrapolated. An EXCEL (Microsoft) growth-fitting program was used. The dotted line shows the equivalent number of gc derived from a sample with an OD of 1.7. (B) Competitive quantitation. Serial dilutions of pPCRmimic were mixed with a constant amount of sample plasma (OD, 1.7) and amplified. The results were plotted, and the intersection of the two curves, from which the number of gc was derived (shown by the dotted line), was calculated as described in the PCRmimic protocol.

which there are more than 1,000 gc in the PCR. Dilution experiments revealed that 1 to 10 gc can be detected by this assay. In this assay, a clinical sample with an OD of 1.7 yields 352 gc/5 μ l (70,400 gc/ml; dotted line in Fig. 1A).

In contrast, with a competitive method of quantitation, a control plasmid DNA construct must be available (20). We made this using the PCR Mimic Construction Kit (Clontech, Palo Alto, Calif.), by amplifying a heterologous DNA fragment using two primers consisting of the two 25-bp sequences from human CMV U_L83 (23) and 20-bp sequences homologous with the PCR mimic fragment. The resulting 250-bp hybrid PCR product (pPCRmimic) was cloned into Bluescript KS+ (Stratagene, La Jolla, Calif.) by the method of Marchuk et al. (12). The following probe for the mimic DNA was tested by plate assay and did not cross-react with the amplification product of human CMV U_L83: 5' TTCTCCAAAATGGCTCGTGACCC TCCCCGC 3'. Moreover, in the plate assay described above, serial dilutions of pPCRmimic yielded the same OD as p22E (Fig. 1A and data not shown). In the competitive method, a

FIG. 2. PCR-plate assay results showing the history of a BMT recipient with a positive bronchoalveolar lavage (BAL) result. P-PCR and a shell vial blood culture were done weekly from day (d) -8 to day +69 post-BMT receipt. P-PCR was repeated three times, and the mean OD $\pm$ the standard deviation is shown. The Southern blot results correspond to the plate assay results, and the latter are represented as the number of gc per milliliter.

constant amount of a digested plasma sample was added to the different reaction mixtures containing known serial dilutions of pPCRmimic (8). pPCRmimic competes for primer binding with the CMV DNA present in the reaction mixture. Ten-microliter aliquots of the amplification reaction mixture were hybridized in two 96-well plates, one coated with the U_L83 probe and the other coated with the PCRmimic probe. The resulting ODs were plotted, and the crossover of the two curves was calculated to give the number of CMV gc present in the reaction mixture (Fig. 1B). For a representative specimen exhibiting an OD of 1.7, 264 gc were determined in 5 μ l of plasma by using the competitive PCR (calculations were done according to the instructions included with PCR Mimic Construction Kit [Clontech]).

Both methods gave similar numbers of gc (7.04×10^4 /ml versus 5.3×10^4 /ml), an observation which has been made by others (19). The noncompetitive method has been adopted by many investigators, since it is particularly suitable for use in clinical laboratories (10, 14, 15, 24). In addition to simpler calculations, the latter method requires only one reaction tube per sample, permitting an increased number of samples to be tested at one time and use of less specimen sample per assay. We decided to adopt the noncompetitive method for later studies.

The pPCRmimic construct was also used to detect nonspecific inhibitors present in digested plasma. Fifty to 1,000 gc of pPCRmimic were introduced into 144 amplified plasma samples, and inhibition was found in 2.7% of the samples. The presence of ganciclovir in the plasma did not inhibit the PCR (data not shown). The nature of this inhibition is not known, but it appeared sporadically and was not found in multiple specimens from the same subject. A representative serial analysis of CMV reactivation in a BMT recipient (Fig. 2) showed plasma PCR positivity 1 week prior to bronchoalveolar lavage and blood culture positivity, and the P-PCR stayed positive for 3 weeks after ganciclovir therapy was started. Figure 2 indicates the usefulness of the quantitative plate assay by showing the presence of CMV DNA in plasma from the first shedding to the peak of infection and from the start of antiviral treatment to clearance of the virus from blood.

We concluded that the plate assay for PCR is useful for early detection of CMV and that its ease of use for high throughput

and nonradioactive detection may be useful in laboratories where automation is required. The advantages of this method are that the assay does not require radioactivity. By passive labeling of the probe applied to the 96-well plates, batch testing of banked specimens can be rapid and more cost efficient. Moreover, this method of detection permits analysis of both competitive and noncompetitive methods of DNA quantitation. We show that quantitation of CMV DNA in plasma by a noncompetitive method is valid and that the plate assay is useful for reporting CMV reactivation in BMT recipients. The quantitative results could define a population at risk for CMV disease.

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