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Detection of Cytomegalovirus in Blood Donors by PCR Using the Digene SHARP Signal System Assay: Effects of Sample Preparation and Detection Methodology

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Cytomegalovirus (CMV) is an important cause of transfusion-associated morbidity and mortality; however, only 0.4 to 12% of the blood products obtained from seropositive blood donors transmit infection. The effects of three commercially available whole-blood sample preparation kits on the detection of CMV PCR products by a semiquantitative adaptation of the Digene SHARP Signal System Assay (DSSSA) in samples from volunteer blood donors was assessed. Of 101 samples from seropositive blood donors, CMV was detected in 0 (0%) of the samples extracted with a QIAamp blood kit (QIAGEN), 1 (1%) of the samples extracted with an Amplicor whole-blood specimen preparation kit (Roche), and 8 (8%) of the samples extracted with an Isoquick nucleic acid extraction kit (modified by the addition of carrier tRNA) (Microprobe). CMV DNA was not detected in samples from seronegative blood donors ($n = 13$). Nested PCR of selected samples confirmed the detection of CMV in the sane eight samples extracted with the modified Isoquick nucleic acid extraction kit and detected an additional nine CMV-positive samples ($n = 50$). Samples from volunteer blood donors contain low copy numbers of CMV DNA. PCR amplification of such specimens can result in analytical sampling errors, giving results similar to the variations in titers recognized during determinations of the 50% tissue culture infective dose. The detection of CMV in blood samples from volunteer blood donors by PCR is a function of sample preparation, amplification conditions, and detection methodology. Accurate assessments of the clinical utility of CMV DNA detection by nucleic acid amplification for blood product screening and patients will require highly standardized and quantitative methodology.

Blood products from cytomegalovirus (CMV)-seropositive donors cause significant rates of morbidity and mortality, particularly in transplant recipients and neonates (4). While CMV-seronegative blood products have been shown to reduce CMV transmission, 50 to 80% of blood donors are seropositive, which can pose problems in obtaining sufficient seronegative donations (11, 16). Although the presence of antibody to CMV provides evidence of acute or prior CMV infection, only 0.4 to 12% of blood products obtained from CMV-seropositive donors actually transmit CMV (4, 12, 16, 21). While leukofiltration can decrease transfusion-associated CMV (4, 11), assays capable of detecting potentially infectious donations could increase the availability and safety of blood products.

Although PCR has the analytical sensitivity to detect CMV in samples from blood donors, the results of PCR-based detection vary considerably. Bevan et al. (1) detected CMV in samples from 25 to 79% of seropositive and 19 to 35% of seronegative blood donors, whereas Bitsch et al. (2) and Jiwa et al. (13) were unable to detect CMV by PCR in samples from seropositive and seronegative blood donors. Smith et al. (17) reported that 8% of CMV-seropositive blood donors were PCR positive.

The inconsistent PCR-based detection of CMV in samples from blood donors probably represents the lack of standardization and poor reproducibility of in-house PCR assays (10, 15, 22). This study assessed the effects of three commercially available whole-blood sample preparation kits on semiquantitative detection of CMV PCR products by the Digene SHARP Signal System Assay (DSSSA) in samples from blood donors. The detection of CMV was also confirmed by nested PCR using a noncontiguous region of the CMV genome.

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MATERIALS AND METHODS

Whole-blood samples were obtained from 114 apheresis donors at The Canadian Red Cross Toronto Centre. CMV antibody was detected by CMVSCAN (Becton Dickinson, Cockeysville, Md.). EDTA-anticoagulated whole-blood samples were obtained from approximately one CMV-seronegative donor for every five CMV-seropositive donors. Since the storage of whole-blood samples for 24 h results in the loss of detectable CMV (3), all samples were extracted within 4 h of collection by a technologist blinded to the donor's serostatus.

Nucleic acid extraction. For total nucleic acid extraction with the Isoquick nucleic acid extraction kit (ISO; catalog no. MXT-020-100; Microprobe Corp., Bothell, Wash.), 100 μl of blood was mixed with lysis buffer and incubated with an extraction matrix. Nucleic acids were partitioned into the aqueous phase by centrifugation and precipitated with isopropanol. After being washed with ethanol, the pellet was dried and resuspended in distilled water.

A pilot study of 60 CMV-seropositive donor specimens that used the ISO kit protocol provided by the manufacturer revealed that we were unable to macroscopically visualize a pellet after the final centrifugation step or detect CMV by PCR (data not shown). Because of concerns about insufficient precipitation of target DNA, the manufacturer's protocol was modified to include the addition of 5 μg of carrier tRNA (yeast tRNA [catalog no. 15401-011]; Gibco BRL) prior to isopropanol precipitation for all study samples.

Extraction with a QIAamp blood kit (QIA; QIAGEN Inc., Chatsworth, Calif.) involved the addition of 200 μl of blood to the lysis buffer, incubation, and the subsequent addition of isopropanol. The mixture was centrifuged in a microcentrifuge for 1 min with the supplied spin column, and bound DNA was eluted from the column with distilled water.

Extraction with an Amplicor whole-blood specimen preparation kit (AMPLICOR; Roche Diagnostic Systems Inc., Branchburg, N.J.) involved mixing 500 μl of blood with specimen wash solution. After centrifugation and repeat washing,

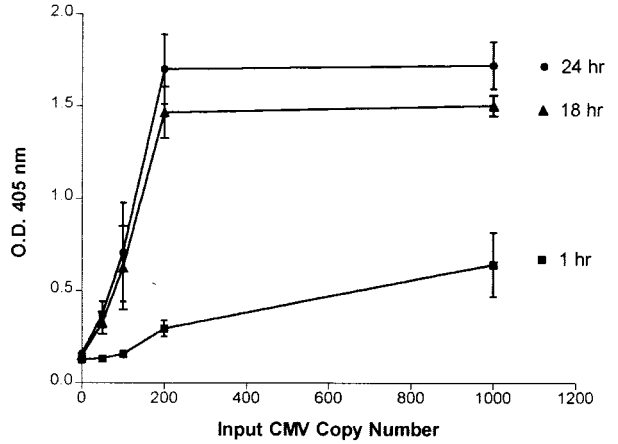

FIG. 1. Relationship between input CMV copy number and output OD after PCR using DSSSA. Error bars indicate standard errors ($n = 5$).

the pellet was incubated with the extraction reagent at 60°C for 30 min and then at 100°C for 30 min. All extracted samples were stored at −20°C until amplified.

Detection of CMV PCR products by using DSSSA. The primer pair of Demmler et al. (9) targeting a 435-bp sequence within the major immediate-early antigen (MIE) of the CMV Towne strain was supplied by Digene Diagnostics Inc. (Silver Spring, Md.), with one primer 5′ labeled with biotin (MIE-4 biotin, 5′-CCA AGC GGC CTC TGA TAA CCA AGC C [GenBank accession no. X17403; nucleotides 171508 to 171484], and MIE-5, 5′-CAG CAC CAT CCT CCT CTT CCT CTG G [nucleotides 171074 to 171098]). PCR buffer mixtures consisted of 5 μl of 10× reaction buffer containing 10 mM Tris; 50 mM KCl (pH 8.3); 1.0 mM $MgCl_2$; a deoxynucleoside triphosphate (dNTP) mix containing 200 μM (each) dATP, dCTP, dGTP, and dTTP; 25 pmol of each primer; 1.25 U of AmpliTaq; and 25 μl of the extracted sample (total volume, 50 μl). Samples were denatured at 94°C for 5 min and subjected to 40 amplification cycles as follows: denaturation for 1 min at 94°C, annealing for 2 min at 55°C, and extension for 3 min at 72°C, with a final extension at 72°C for 7 min in a Perkin-Elmer 480 thermocycler.

PCR products were denatured and hybridized with a CMV-specific RNA probe. RNA-DNA hybrids were transferred to streptavidin-coated capture plates. Alkaline phosphatase-labeled RNA-DNA hybrid antibody was added, and bound antibody was detected by using *p*-nitrophenylphenol. The optical density (OD) at 405 nm was read at 1, 18, and 24 h after substrate addition with a microplate reader. The color intensity was proportional to the amount of captured RNA-DNA hybrids, with a maximum assay sensitivity at 24 h after substrate addition. The cutoff was calculated as twice the mean of negative controls (a minimum of four per assay) plus 0.08.

A titration of 0 to 1,000 copies of a plasmid containing the CMV MIE target region (supplied by Jim Lazar, Digene Diagnostics Inc.) in duplicate was amplified in each run. The OD at 1 h after substrate addition was used to generate a run-specific calibration curve and estimate the input CMV copy number in samples. The sensitivity of DSSSA was compared with that of Southern blotting and shown to be approximately 100- to 1,000-fold more sensitive than the detection of PCR products after agarose gel electrophoresis (data not shown).

Detection of CMV by nested PCR. The outer primer set consisted of upstream primer JC, 5′-CAT AAT CTC ATC AGG GGA GC (GenBank accession no. X17403; nucleotides 171741 to 171760), and downstream primer JD, 5′-TTG GGC TAA CTA TGC AGA GC (nucleotides 172045 to 172026), which generated a 305-bp PCR product flanking the region amplified by the A and B primer pair of Jiwa et al. (13). The inner primer set consisted of upstream primer JB, 5′-GAA GGC TGA GTT CTT GGT AA (GenBank accession no. X17403; nucleotides 171819 to 171838), and downstream primer JA, 5′-AGC TGC ATG ATG TGA GCA AG (nucleotides 171964 to 171945).

Nested PCR was performed by adding 5 μl of 10× reaction buffer containing 10 mM Tris; 50 mM KCl; 1.5 mM $MgCl_2$ (pH 8.5); a dNTP mix containing 250 μM (each) dATP, dCTP, dGTP, and dTTP; 50 ng of each primer; 1.25 U of AmpliTaq; and 25 μl of sample (total volume, 50 μl). Samples were denatured at 94°C for 2 min and subjected to 40 amplification cycles as follows: denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 30 s at 72°C, with a final extension for 4 min at 72°C in a Perkin-Elmer 9600 thermocycler. Three microliters of PCR products from the first PCR was reamplified by using the same amplification protocol with 250 ng of each inner primer.

Nested PCR of CMV AD 169 and the plasmid containing the MIE generated a 146-bp PCR product after agarose gel electrophoresis. Specificity was confirmed by Southern hybridization (13). Multiple negative controls, aerosol-resistant tips, and separate processing areas were used to minimize the risk of amplicon contamination.

RESULTS

Figure 1 illustrates the relationship between the input copy number of the plasmid containing the CMV MIE target region and the output OD after PCR, as detected by DSSSA. PCR was performed in duplicate on five different runs with OD measurements after 1, 18, and 24 h of substrate incubation. The coefficients of variation for the five assay runs with 0, 50, 100, 200, and 1,000 CMV input copies were 6.1, 13.5, 35.3, 62.2, and 61.6% at 1 h; 4.3, 78.5, 81.9, 21.0, and 15% at 18 h; and 3.6, 81.2, 85.3, 22.4, and 17% at 24 h, respectively. On the basis of input CMV plasmid titrations, the DSSSA results were reproducible; DSSSA detected approximately 50 input copies after 24 h of substrate incubation. Crude assessments of the input CMV copy number could be performed only after 1 h of substrate incubation because as few as 200 input copies saturated the assay after 24 h of substrate incubation.

To determine the lower limits of detection for DSSSA and nested CMV PCR, end point titrations of the supplied plasmid were amplified in triplicate on at least three separate occasions. While DSSSA detected approximately 50 input copies after 24 h of substrate incubation, nested CMV PCR detected 1 to 5 input copies. Assay sensitivity was also compared by using serial end point titrations of CMV AD 169. A starting inoculum of CMV AD 169 capable of infecting 50% of MRC-5 cells in shell vials, as detected by fluorescence after staining with anti-CMV immediate-early antibody (Dupont), was further diluted to approximately 10^{-6} to 10^{-7} and remained detectable by DSSSA (Fig. 2). Once again nested CMV PCR was 1 to 2 logs more sensitive, detecting dilutions of 10^{-7} to 10^{-8}.

Of particular importance, when the plasmid containing the CMV target sequence and/or CMV AD 169 had been titrated to their end points, only some of the individually amplified triplicate samples generated PCR products, as detected by both assays (Fig. 2). Figure 3 demonstrates nested CMV PCR products from a serial end point titration of CMV AD 169. In less dilute samples, CMV AD 169 was amplified from all three PCRs, whereas at the end point titration only one or more of the three replicate samples were amplified. With further dilution, no PCR products were detected.

The results of PCR-based detection of CMV using DSSSA in samples from blood donors are summarized in Table 1. Of 114 blood donors, 101 were CMV seropositive and 13 were

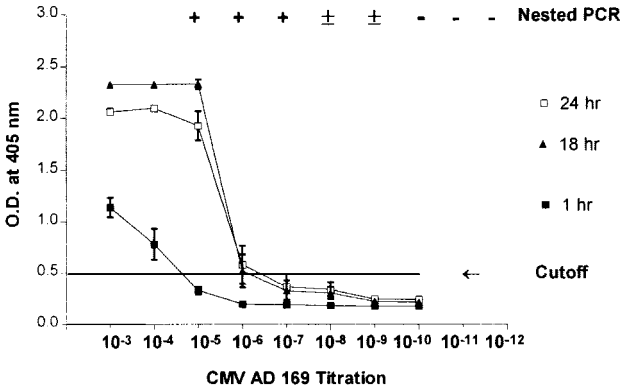

FIG. 2. PCR-based Detection of an end point titration of CMV AD 169 by DSSSA and nested PCR. Error bars indicate standard errors ($n = 3$). The OD was measured after the indicated time of substrate incubation. +, nested CMV PCR positive for three replicates of a sample amplified in triplicate; −, nested CMV PCR negative for three replicates of a sample amplified in triplicate; ±, detection of CMV PCR products in one or two replicates of a sample amplified in triplicate.

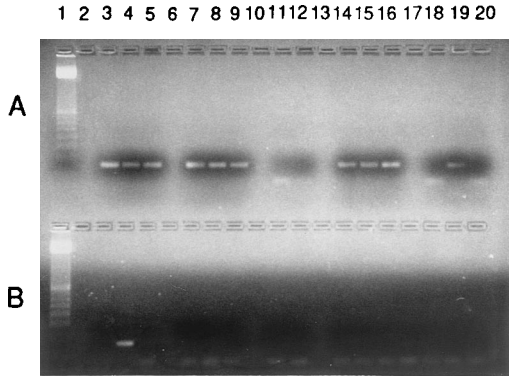

FIG. 3. Nested PCR products after 1.5% agarose gel electrophoresis from an end point titration of CMV AD 169 (performed in triplicate) are shown. Nested PCR generates a 146-bp CMV-specific PCR product. (A) Lanes: 1, molecular weight markers (100-bp ladder [Pharmacia]); 3 to 5 and 7 to 9, titrations of 10^{-5} (3 to 5) and 10^{-6} (7 to 9); 11 to 12, negative PCR controls; 14 to 16 and 18 to 20, titrations of 10^{-7} (14 to 16) and 10^{-8} (18 to 20). (B) Lanes: 1, molecular weight markers (100-bp ladder); 3 to 5 and 7 to 9, titrations of 10^{-9} (3 to 5) and 10^{-10} (7 to 9); 11 to 12, negative PCR controls; 14 to 16, titrations of 10^{-11}; 17, negative control for second PCR; 18 to 20, titrations of 10^{-12}.

seronegative. Each blood sample was independently extracted by using ISO, QIA, and AMPLICOR kits and amplified once (i.e., 342 individual amplifications). Each sample with an OD close to the assay cutoff (± 30%) was reamplified in duplicate and retested. All 13 seronegative blood donor samples were PCR negative by DSSSA. Eight modified ISO-extracted samples were CMV positive (8% of seropositive blood donor samples). In contrast, all samples extracted by using a QIA kit were CMV PCR negative and only one of the samples extracted with an AMPLICOR kit was positive (the ISO-extracted sample from this blood donor was also CMV PCR positive).

All of the specimens detected by DSSSA were negative after 1 h of substrate incubation, suggesting that these samples contained less than 200 copies of CMV (Fig. 1). The OD values of the eight ISO-extracted samples after 24 h of substrate incubation were 1.397, 1.288, 1.010, 0.838, 0.735, 0.613, 0.449, and 0.388, with assay cutoffs ranging between 0.354 and 0.410. Five of these samples were repeatedly positive in duplicate by DSSSA. Two of the samples were repeatedly positive in only one of the duplicate pairs by DSSSA (repeated three times), suggesting a low copy number (see Discussion). For one sample, there was sufficient material only to repeat DSSSA once.

To verify the DSSSA results, ISO-extracted samples from 60 of 114 blood donors (10 seronegative and 50 seropositive blood donor samples, which included the 8 [42 + 8] modified-ISO-extracted samples which were CMV positive by DSSSA) were tested by nested CMV PCR in duplicate. All 10 samples from seronegative blood donors were nested PCR negative. By nested PCR, 17 of the 50 (34%) samples from seropositive donors were PCR positive. All eight of the DSSSA-positive samples were nested PCR positive; five of the eight samples repeatedly positive in duplicate by DSSSA were nested PCR positive in duplicate (repeated three times). Three samples which were close to the cutoff by DSSSA but repeatedly reactive in only one of a duplicate pair were nested PCR positive, but in only one of the duplicate pairs (repeated three times). An additional nine ISO-extracted samples negative by DSSSA were nested PCR positive in duplicate; three of them were PCR positive in only one of the duplicate pairs (repeated three times) (see Discussion).

DISCUSSION

Dramatically different CMV PCR detection rates in samples from blood donors have been documented (1, 2, 17, 24), which probably relates to the lack of standardization of in-house PCR assays (10, 15, 22). We first assessed the sensitivity and reproducibility of DSSSA. Although the coefficient of variation between the input CMV target and the output OD varied between 4 and 85%, the reproducibility from run to run was good. By including a calibration curve with each amplification run and detecting the OD after different intervals of substrate incubation, we were able to utilize DSSSA as a semiquantitative standardized PCR product detection assay. We then demonstrated that sample preparation dramatically altered the detection rate of CMV in samples from blood donors.

We followed the manufacturers' protocols (except for the modified ISO protocol), each of which involved specified blood volumes. Although ISO-extracted samples with carrier tRNA provided the greatest detection sensitivity, this protocol included multiple tedious centrifugation and precipitation steps, increasing the risk of amplicon contamination. While QIA and AMPLICOR kits were easy to use, carrier tRNA might have improved their extraction performances. Although the AMPLICOR kit was not intended to detect CMV in whole-blood samples, by using this kit and DSSSA detection, CMV DNA was detected in 12 of 45 (25%) human immunodeficiency virus-infected individuals (14).

Nested CMV PCR confirmed the detection accuracy of the DSSSA and was 1 to 2 logs more sensitive at detecting serial end point titrations of CMV AD 169, detected the plasmid containing the CMV target insert, and detected CMV in more donor samples. Because nested PCR was performed on samples positive and/or borderline by DSSSA, the prevalence of CMV by nested PCR may not be typical of the overall prevalence of CMV in samples from blood donors. Despite the enhanced sensitivity of nested PCR, such assays are technically difficult, prone to contamination, and poorly reproducible between laboratories (22), precluding their suitability for routine diagnostic use.

The interpretation of PCR results obtained from samples containing small amounts of CMV DNA is important. Serial end point titrations of CMV AD 169 and the plasmid containing the CMV target insert demonstrated that with progressively smaller amounts of target in the sample, one or more of the samples amplified in triplicate became negative before all three replicate samples became negative. Thus, the PCR product(s) or signal(s) obtained after amplifying small amounts of target is probably analogous to a 50% tissue culture infective dose at which only one or more tubes inoculated with terminal titrations of infectious virus will grow virus. This was also

TABLE 1. Detection of CMV DNA in samples from blood donors[a] by PCR using DSSSA

Extraction method	No. of samples with PCR result	
	Positive	Negative[b]
ISO[c]	8	106
QIA	0	114
AMPLICOR	1	113

[a] In terms of CMV antibody status, 101 of the blood donors were positive and 13 were negative.
[b] No CMV-seronegative samples were CMV PCR positive.
[c] Modified by the addition of 5 μg of tRNA prior to the precipitation of nucleic acids.

observed for donor samples. For example, five of eight samples with relatively higher OD values by DSSSA demonstrated complete concordance in the results for all replicates by nested PCR and DSSSA. However, three of the eight DSSSA donors that were positive in only one of the replicate pairs were positive by nested PCR, but in only one of the replicate pairs (repeated three times in duplicate). Similarly, three of nine blood donors that were positive only by nested PCR were repeatedly positive in only one of the duplicate pairs (repeated three times in duplicate). These results are consistent with the theoretical assessments of Coupland (8). In essence, by detecting one target in a sample by an assay capable of detecting one target, a Poisson distribution of the target results in approximately one-third of the test reactions producing negative results (8, 20). Variable amplification from samples containing low copy numbers is an important aspect of PCR assay design that needs to be addressed.

It is possible that the variability in CMV detection by DSSSA and nested PCR was due to amplicon contamination. However, multiple negative controls and samples from seronegative donors were negative by both assays; amplification variability occurred consistently at the titration end points of CMV AD 169 and the plasmid containing the CMV target insert and in donor samples demonstrating low OD values by DSSSA.

While DSSSA provides a semiquantitative estimate of the amount of CMV DNA in a sample, the quantification of input targets by PCR requires that there be a reproducible relationship between the input target and output PCR product. This is difficult because (i) PCR efficiency changes with product synthesis and substrate utilization, (ii) inhibitors and extraction methodology affect target detection, and (iii) primer-target sequence heterogeneity between CMV strains can alter the amount of amplification (6). While coamplification with a positive internal control which has the same primer sequence as the target can correct for inhibitors and variable amplification efficiency (7), coamplified targets compete for substrates and cannot control for primer-target sequence heterogeneity (5, 6). Addressing these problems and developing defined CMV DNA standards are needed to ensure intra- and interassay CMV PCR reproducibility (23).

The detection of CMV DNA in 8% of modified-ISO-extracted samples by DSSSA is consistent with the reported CMV transmission rates of 0.4 to 12% from seropositive blood products (4, 12, 16, 21). While DSSSA has a limited ability to quantify CMV DNA, the fact that none of the samples were positive after 1 h of substrate incubation suggests that the samples contained less than 200 copies of CMV DNA. We also recognize that the detection of DNA does not distinguish replicating virus from latent virus and that clinical studies are required to determine the assay sensitivity required for detecting potentially infectious blood products.

The et al. (19) demonstrated a relationship between the amount of CMV antigenemia and the presence of symptomatic versus asymptomatic disease in transplant recipients, although there was some overlap. Because nucleic acid amplification increases the analytical sensitivity of CMV detection (10, 18), the overlap in the detection of symptomatically and asymptomatically infected patients can be expected to increase (25). Therefore, quantitative and highly standardized PCR assays to accurately assess the relationship between CMV viremia and the clinical manifestations of CMV infection and/or disease will be required.

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