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Effects of Storage Temperature and Time on Qualitative and Quantitative Detection of Cytomegalovirus in Blood Specimens by Shell Vial Culture and PCR

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Cytomegalovirus (CMV) infectious titers and DNA levels were determined by quantitative shell vial culture and quantitative-competitive PCR with blood samples from 10 renal transplant recipients with active CMV infection. Blood samples were stored at either room temperature or 4°C and were processed at intervals of 0, 6, 24, 48, and 72 h. All samples were culture and PCR positive at baseline. Whereas the sensitivity of shell vial culture progressively declined, with only 55% positive at 24 h and 10% positive at 48 h, all samples remained PCR positive at all time points. Furthermore, the infectious titer diminished by 83 to 91% by 24 h compared to that at baseline ($P < 0.0001$), but quantitative DNA levels did not decline over time. Storage temperature had no significant effect on either infectious titer or DNA levels.

Cytomegalovirus (CMV) is a major cause of morbidity and mortality in immunocompromised hosts (11). Several techniques are used to detect and quantitate CMV from blood to diagnose and predict the presence of clinical disease. Blood samples, however, are often stored or transported to a reference virology laboratory, resulting in a delay of 1 to 2 days or more prior to specimen processing and testing. It has been shown that storage time and temperature affect the measurements of antigenemia levels and infectivity to various degrees (1, 7, 14–16, 18). This study was performed to examine the effects that storage temperature and sample processing time have on the detection of CMV in blood samples by shell vial culture (SVC) and PCR.

MATERIALS AND METHODS

Specimen processing. This study was approved by the Human Studies Committee at Washington University. Blood samples were collected from 10 renal transplant recipients who were CMV seropositive and who were suspected of having CMV viremia. From each patient, 20 ml of whole blood was collected in EDTA-containing tubes (Vacutainer; Becton Dickinson, Rutherford, N.J.) at Washington University Medical Center, and the tubes were transported immediately to the Virology Laboratory, where each sample was divided into 2-ml aliquots. One aliquot was processed immediately (time zero), and the other aliquots were stored at either 4°C or room temperature (RT) prior to processing. Two aliquots, one stored at 4°C and one stored at RT, were then processed after 6, 24, 48, or 72 h of storage. Sample processing involved leukocyte isolation by dextran sedimentation, erythrocyte lysis, and washing of cells as described previously (4). The leukocyte concentration from the preparations was adjusted to between 1.2×10^6 and 2×10^6 cells per ml. From each cell suspension, a lysate was made by centrifuging 1.0×10^6 cells at $14{,}000 \times g$ for 1 min, resuspending the pellet in 40 μl of 0.04 M NaOH, and boiling for 10 min, followed by the addition of 40 μl of 0.1 M Tris (pH 7.6).

Quantitative SVC. From each leukocyte suspension, 0.25 ml was inoculated into each of two SVCs containing MRC-5 human embryonic fibroblasts (ViroMed, Minnetonka, Minn.). The shell vial assay was performed as described previously (4), except that immunofluorescent-antibody staining was performed with a different monoclonal antibody specific for the CMV immediate-early antigen (Chemicon International, Temecula, Calif.) because it gave a stronger signal and an approximately 10% higher sensitivity (unpublished data). The number of infectious centers from each pair of cultures was normalized to 10^5 leukocytes and averaged, and the results were expressed as infectious centers per 10^5 leukocytes.

Plasmid construction. External target (ET) and internal standard (IS) sequences were derived from the sequence of the human CMV major immediate-early 1 gene (IE-1) (5) and were subcloned into plasmids (pET and pIS, respectively). pET was constructed by amplification of a 147-bp product by the assay described by Jiwa et al. (12) and was inserted into the *lacZ* region of the pGEM-5z vector (Promega, Madison, Wis.) according to the instructions of the manufacturer (TA Cloning Kit; Invitrogen, San Diego, Calif.). The IS was a 96-bp construct with G+C content similar to that of the ET. The IS was constructed by using primers whose 5′ ends matched the primers used to amplify the ET and whose 3′ ends spanned a separate 56 bp segment of the IE-1 gene (Fig. 1). The sequences of these composite primers were 5′-AGCTGCATGATGTGAGCAAGCCTGAGGTTATCAGTGTAA-3′ and 5′-GAAGGCTGAGTTCTTGGTAAAGACCTTCATGCAGATCTCC-3′, with the 3′ ends (underlined) corresponding to the regions at positions 2758 to 2777 and 2813 to 2794 of the IE-1 gene (GenBank accession no. HS5MIEG), respectively. Amplification of the IS product was performed with a 100-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 0.2 mM (each) deoxynucleoside triphosphate, 50 pmol of each primer, and 2 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Branchburg, N.J.). The PCR protocol consisted of denaturation for 5 min at 94°C, followed by 32 cycles of denaturation at 94°C for 30 s, primer annealing at 46°C for 30 s, and extension at 65°C for 30 s, followed by a final extension for 10 min at 65°C. The product was subcloned into the pGEM-5z vector as described above. Both pET and pIS were transformed into competent DH5α cells, purified by a silica column process according to the instructions of the manufacturer (Qiagen Midi-Plasmid Purification Kit; Qiagen, Chatsworth, Calif.), and quantitated by spectrophotometry (DU650; Beckman Instruments, Fullerton, Calif.).

DNA amplification and quantitation. CMV DNA levels were measured by a quantitative-competitive PCR assay (QC-PCR) similar to those described previously (2, 9, 20, 23). QC-PCR was performed with lysates of 10^6 cell equivalents from each processed blood sample. A master mixture containing all of the reagents except the patient sample was made prior to distribution to the individual PCR tubes. Each PCR tube contained a 100-μl volume including 8 μl of lysate (10^5 cell equivalents), 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 0.2 mM (each) deoxynucleoside triphosphate, 75 pmol of each primer (12), 750 copies of pIS, and 2 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus). Amplification was performed by using the following conditions: enzyme heat activation for 10 min at 94°C, followed by 37 cycles of denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s, and extension at 72°C for 30 s. The reaction was completed with a 10-min extension at 72°C. Each run contained a negative control plus the internal standard, which served as a control for the absence of PCR inhibition. The techniques suggested by Kwok and Higuchi (13) were followed to avoid false-positive results.

Standard curve. To quantitate the viral load, a standard curve was generated for each amplification run in a way similar to that described previously (2, 9, 20,

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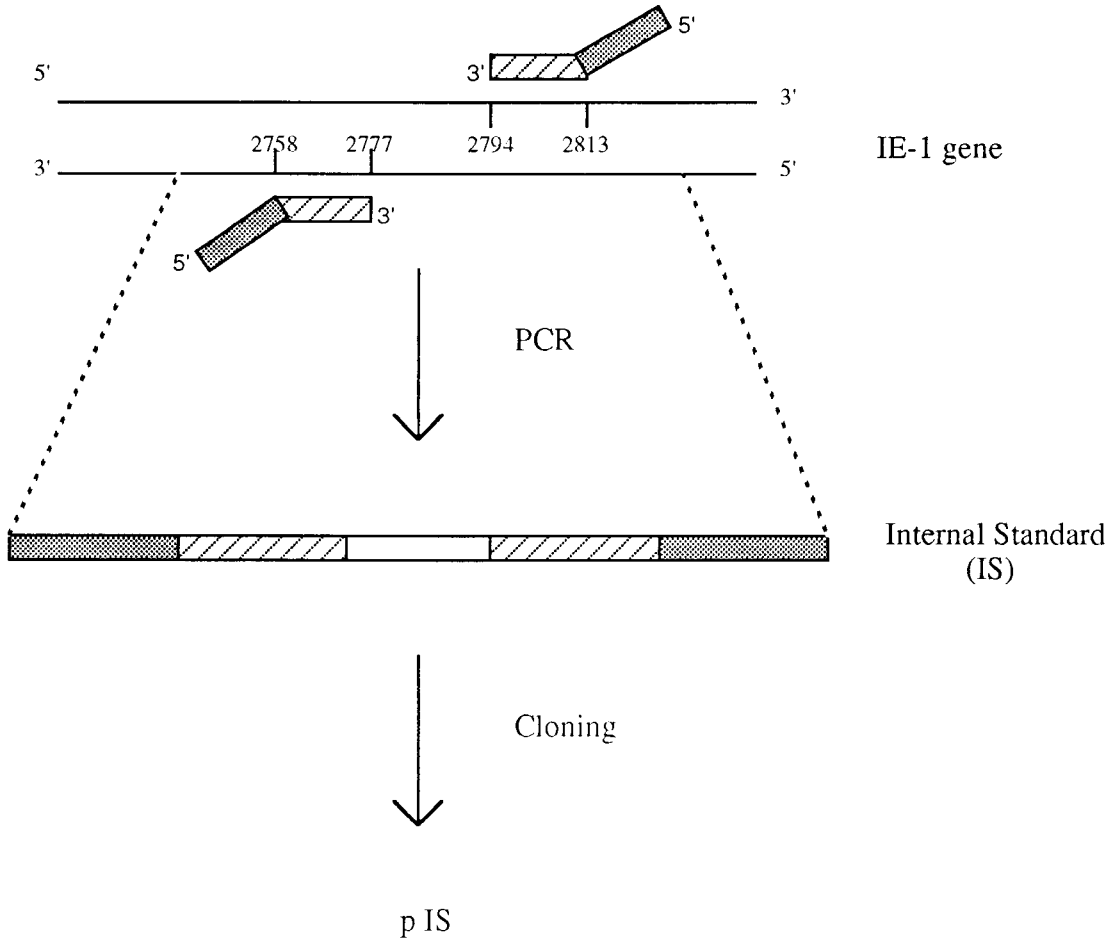

FIG. 1. Illustration of pIS construction. The primers were used to amplify the 96-bp IS. The 3′ ends of the primers (dashed bars) correspond to bases 2758 to 2777 and 2794 to 2813 of the IE-1 gene. The 5′ ends of the primers (solid bars) have the same sequences as the primers that are used to amplify the external target.

23). The standard curves were produced by amplifying 50, 100, 500, 1,000, 5,000, 10,000, and 50,000 copies of pET, each with 750 copies of pIS. pIS was included in the master mixture to ensure a homogeneous distribution in each PCR tube. Additionally, leukocyte DNA from a CMV-seronegative person was added to the standard curve master mixture at a concentration that yielded 10^5 cell equivalents per reaction tube. After amplification as described above, the products were electrophoresed at 200 V for 1 h through a 3% agarose gel containing 0.5 μg of ethidium bromide per ml and were visualized under UV illumination with a digital imaging system (IS-1000; Alpha Innotech Co., San Leandro, Calif.) which provided a measure of the fluorescence intensities of the bands representing the amplified target and standard.

A standard curve was constructed for each amplification run by plotting the log of the ratio of the pET copy number/pIS copy number versus the log of the input pET copy number (Fig. 2). The QC-PCR assay reliably detected as few as 50 copies, and as many as 50,000 copies of the CMV IE-1 gene coamplified with 750 copies of pIS in the presence of DNA from 10^5 leukocytes. Detection of fewer than 50 copies or more than 50,000 copies of target DNA was unreliable and would result in values outside the linear range of the assay. If a copy number greater than 50,000 was obtained, the sample was diluted 1:10 and was retested to fit the linear range of the curve. On the basis of a total of 18 separate standard curve runs, the assay yielded a total variability of 38% for a target copy number of 10,000 and 68% for a copy number of 100. All of the samples from each patient were batched together in the same run to minimize interassay variability.

Statistics. Data were analyzed by Student's two-tailed paired *t* test by using Statview, version 4.01 (Abacus, Berkely, Calif.). Data are expressed as means ± standard errors of the means.

RESULTS

Qualitative results. All 10 specimens were positive by SVC and PCR at baseline (Table 1). After storage at 4°C, six of eight aliquots (75%) were still SVC positive at 6 h, 5 of 10 (50%) were positive at 24 h, 1 of 10 (10%) was positive at 48 h, and 1 of 10 (10%) was positive at 72 h. After storage at RT, 6 of 8 (75%) were still SVC positive at 6 h, 6 of 10 (60%) were positive at 24 h, 1 of 10 (10%) was positive at 48 h, and 0 of 10 (0%) was positive at 72 h. There was no significant difference in detection between specimens stored at 4°C and RT. In contrast, all aliquots remained PCR positive at all time points after storage at either temperature. PCR and SVC results for two of the patients at the 6-h time point were unavailable because the samples were inadvertently processed at the wrong time.

Quantitative results. At baseline there was a mean of 1.54 ± 0.50 infectious centers/10^5 leukocytes by quantitative SVC (QSVC) and a mean DNA copy number of 26,807 ± 18,978 CMV genomes/10^5 leukocytes by QC-PCR. The number of infectious centers and the copy number at each time point were expressed as the mean percentage of baseline value and are presented in Fig. 3. Storage temperature had no significant effect on viral load, as measured by either QSVC or QC-PCR at any of the time points. Compared to the titer at baseline, stored samples had an 83 to 91% reduction in viral titer at 24 h, as measured by QSVC ($P < 0.0001$), and a 100% decrease by 48 h ($P < 0.0001$). In contrast, no significant differences in the mean change from baseline DNA levels were detected by QC-PCR at any time point (Fig. 3B). Interestingly, all mean DNA values were higher than the baseline values for both temperatures. These differences from the baseline were not statisti-

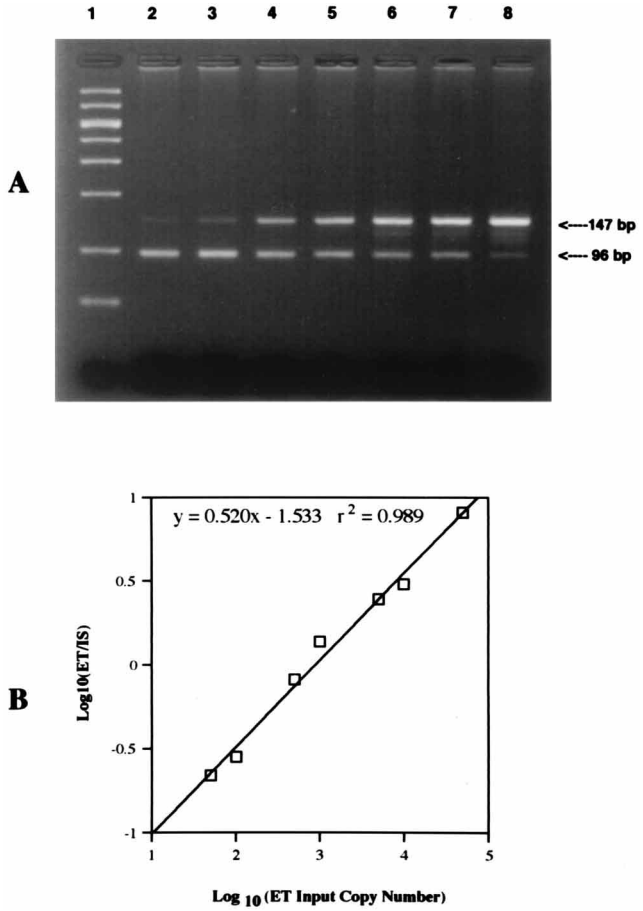

FIG. 2. Example of a standard curve for QC-PCR assay for CMV. (A) Agarose gel electrophoresis of QC-PCR amplification products stained with ethidium bromide and visualized under UV illumination. Lane 1, molecular weight markers; lanes 2 to 8, input number of 750 copies of the plasmid containing the IS and increasing input numbers of the plasmid containing the ET DNA: lane 2, 50 ET copies; lane 3, 100 ET copies; lane 4, 500 ET copies; lane 5, 1,000 ET copies; lane 6, 5,000 ET copies; lane 7, 10,000 ET copies; and lane 8, 50,000 ET copies. (B) A standard curve was constructed for each amplification run by measuring the fluorescence intensity of the amplified ET and IS bands and plotting the log of the ratio of the ET copy number/IS copy number versus the log of the input ET copy number.

cally significant, although the difference between 6 h and the baseline was of borderline significance ($P = 0.07$ for RT; $P = 0.18$ for 4°C).

DISCUSSION

This study has important implications for diagnosis and monitoring of CMV infection in the clinical setting. Following organ transplantation, patients are often far from reference virology laboratories, and consequently, blood specimens submitted for monitoring of CMV infection must be transported prior to specimen processing. In a recent study of CMV infection after renal transplantation (3), we discovered that fewer than 1% of specimens submitted were positive for CMV by shell vial or conventional tube culture when there was a delay in specimen processing of 24 h or more, whereas 46% were positive when the delay was less than 24 h. In contrast, 91 to 100% of samples remained PCR positive regardless of whether they were processed before or after 24 h. In the present study we have confirmed that the CMV infectious titer declines

TABLE 1. Qualitative SVC and PCR results after storage at 4°C and RT

Test	No. of samples positive								
	Baseline (time zero)	6 h[a]		24 h		48 h		72 h	
		RT	4°C	RT	4°C	RT	4°C	RT	4°C
SVC	10	6	6	6	5	1	1	0	1
PCR	10	8	8	10	10	10	10	10	10

[a] Results for two of the specimens are not available.

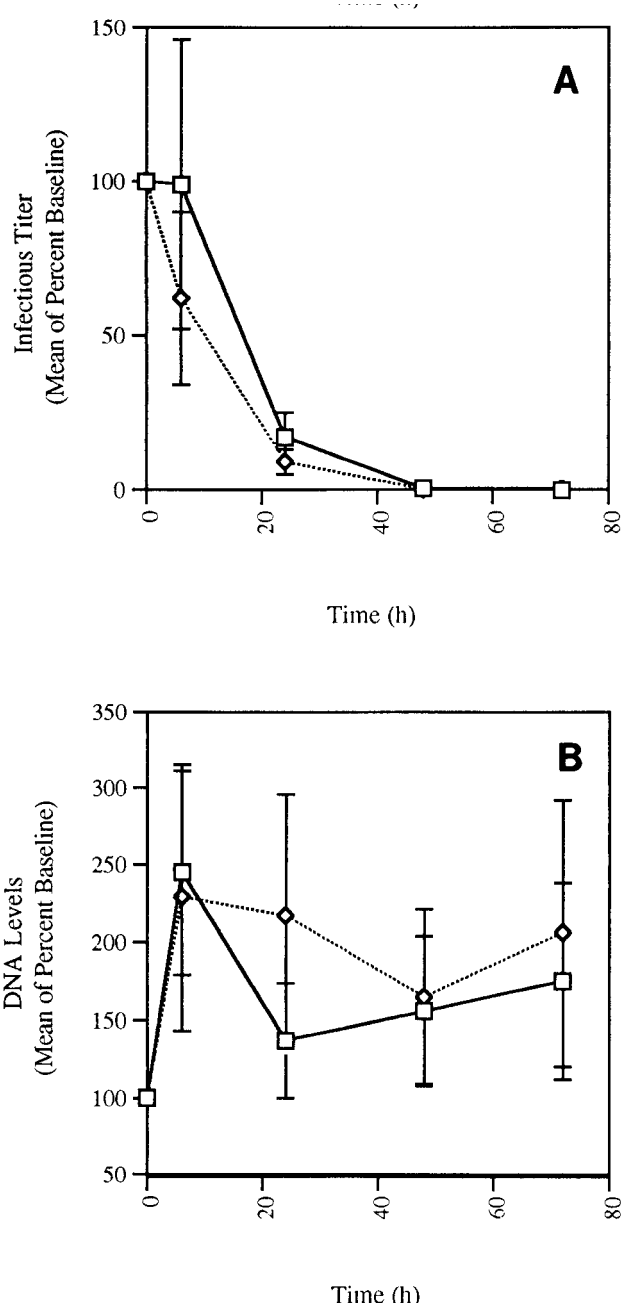

FIG. 3. Mean ± standard error of the mean percent baseline of CMV infectious titer (A) and DNA levels (B) for samples stored at RT (□) and 4°C (◇).

markedly by 24 h in samples stored at either RT or 4°C, and consequently, the ability to detect CMV by SVC declines dramatically when sample processing is delayed. In contrast, PCR results were unaffected by delays in processing for up to 72 h.

Although the presence of CMV in blood can be detected by culture, antigenemia assays, or PCR, the relative roles of these techniques are still being defined. We suggest that when there is a delay in specimen processing, the stability of the analyte is an important parameter for judging the relative merits of the assays. Few previous studies have examined the effects of storage time and temperature on the ability to detect CMV by culture from blood samples. Dworkin et al. (7) found that blood samples stored at 4°C under blood bank conditions had a marked decline in culture positivity after 5 days of storage. Analysis of a small number of samples indicated that two of five samples that were positive at baseline were negative after 2 days of storage and that four of six were negative after 3 days. Likewise, Landry et al. (14) found that infectivity declined after 1 day of storage at 4°C.

The stability of pp65 antigenemia has been addressed in several studies (1, 14, 16, 18). Boeckh et al. (1) studied the effect of storing blood samples for 24 h at either RT or 4°C and found mean reductions of CMV antigen-positive cells from the baseline of 44 and 62%, respectively. The difference in results between the two temperatures was not significant. In another study by Landry et al. (16), blood samples were stored at either RT or 4°C and were processed at 1- and 2-day intervals. There was a 52 to 55% decline in the number of CMV antigen-positive neutrophils at day 1 for both temperatures. Samples processed on day 2 and stored at RT showed a significantly greater decline in terms of the numbers of antigen-positive cells (81% reduction from the baseline), but samples stored at 4°C showed no further reduction. In another study (14), blood samples were stored at 4°C and were processed on days 0, 1, and 2. The qualitative sensitivity of CMV detection by antigenemia remained stable after 2 days, but quantitative levels declined over the 2-day period. In contrast, Niubo et al. (18) found that the quantitative results of the antigenemia assay did not decline significantly after 24 h of storage. It should be noted that none of these studies compared quantitative culture to the antigenemia assay.

The ability to clone DNA from extinct animals (10) and Egyptian mummies (19) suggests that DNA may be stable over extended periods under adverse conditions. The relationship between these findings and the ability to detect specific PCR targets after storage is variable. In recent studies DNA was qualitatively detectable from either cerebrospinal fluid (22) or peripheral blood leukocytes (8) for up to 30 days of storage at room, refrigerator, and freezing temperatures. However, other studies have found decreased DNA yields from blood samples stored at RT or higher temperatures (6, 17, 21). In the present study, we found no decrease in the ability to detect a specific PCR target in samples stored for up to 72 h at either RT or 4°C, and the quantitative assay indicated no decline in the level of the specific PCR target.

Should quantitative testing of CMV become necessary in clinical practice, the stability of the analyte would become even more important than its importance for qualitative assays. From previous studies it appears that, on the basis of specimen stability, the antigenemia assay is preferable to culture, although storage may also compromise quantitative pp65 detection. We have shown, however, that DNA levels remain stable through various storage times and temperatures, suggesting an advantage in stability for assays that measure DNA rather than either pp65 antigenemia or infectivity. Future studies will need to be performed to compare directly the use of PCR against the antigenemia assay to confirm this conclusion.

In summary, we recommend that if a delay of 24 h or more prior to specimen processing is anticipated, culture should not be used to detect or quantitate CMV. PCR is much less affected by a delay in specimen processing, and it appears that samples can be transported or stored at RT without adversely affecting the results of PCR. However, further studies should be performed with a larger number of clinical specimens under conditions which more closely stimulate those of actual specimen transport before a final recommendation can be made concerning optimal conditions of transport of blood samples used for PCR assays.

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REFERENCES

1. **Boeckh, M., P. M. Woogerd, T. Stevens-Ayers, C. G. Ray, and R. A. Bowden.** 1994. Factors influencing detection of quantitative cytomegalovirus antigenemia. J. Clin. Microbiol. **32:**832–834.
2. **Boivin, G., C. A. Olson, M. R. Quirk, S. M. St-Cyr, and M. C. Jordan.** 1995. Quantitation of human cytomegalovirus glycoprotein H in cells using competitive PCR and a rapid fluorescence-based detection system. J. Virol. Methods **51:**329–342.
3. **Brennan, D. C., K. A. Garlock, B. A. Lippmann, R. S. Buller, M. Gaudreault-Keener, J. A. Lowell, S. B. Miller, S. Shenoy, T. K. Howard, and G. A. Storch.** 1997. Control of cytomegalovirus-associated morbidity in renal transplant patients using intensive monitoring and preemptive or deferred therapy. J. Am. Soc. Nephrol. **8:**118–125.
4. **Buller, R. S., T. C. Bailey, N. A. Ettinger, M. Keener, T. Langlois, J. P. Miller, and G. A. Storch.** 1992. Use of a modified shell vial technique to quantitate cytomegalovirus viremia in a population of solid-organ transplant recipients. J. Clin. Microbiol. **30:**2620–2624.
5. **Chou, S.** 1992. Effect of interstrain variation on diagnostic DNA amplification of the cytomegalovirus major immediate-early gene region. J. Clin. Microbiol. **30:**2307–2310.
6. **Cushwas, W. T., and J. F. Medrano.** 1993. Effects of time and temperature on DNA yield and quality. BioTechniques **14:**204–207.
7. **Dworkin, R. J., W. L. Drew, R. C. Miner, S. Mehalko, C. Evans, and R. Baxter.** 1990. Survival of cytomegalovirus in viremic blood under blood bank storage conditions. J. Infect. Dis. **161:**1310–1311.
8. **Farkas, D. H., A. M. Drevon, F. L. Kiechle, R. G. Dicarlo, E. M. Heath, and D. Crisan.** 1996. Specimen stability of DNA-based diagnostic testing. Diagn. Mol. Pathol. **5:**227–235.
9. **Gerna, G., M. Furione, F. Baldanti, and A. Sarasini.** 1994. Comparative quantitation of human cytomegalovirus DNA in blood leukocytes and plasma of transplant and AIDS patients. J. Clin. Microbiol. **32:**2709–2717.
10. **Higuchi, R., B. Bowman, M. Freiberg, O. A. Ryder, and A. C. Wilson.** 1984. DNA sequences from the quagga, an extinct member of the horse family. Nature **312:**282–284.
11. **Ho, M.** 1991. Cytomegalovirus infection and indirect sequelae in the immunocompromised transplant patient. Transplant. Proc. **23:**2–7.
12. **Jiwa, N. M., G. W. Van Gemert, A. K. Raap, F. M. Van de Rijke, A. Mulder, P. F. Lens, M. M. Salimans, F. E. Zwaan, W. Van Dorp, and M. Van der Ploeg.** 1989. Rapid detection of human cytomegalovirus DNA in peripheral blood leukocytes of viremic transplant recipients by the polymerase chain reaction. Transplantation **48:**72–76.
13. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positive results with PCR. Nature **339:**237–238.
14. **Landry, M. L., S. Cohen, and K. Huber.** 1997. Comparison of EDTA and acid-citrate-dextrose collection tubes for detection of cytomegalovirus antigenemia and infectivity in leukocytes before and after storage. J. Clin. Microbiol. **35:**305–306.
15. **Landry, M. L., and D. Ferguson.** 1993. Comparison of quantitative cytomegalovirus antigenemia assay with culture methods and correlation with clinical disease. J. Clin. Microbiol. **31:**2851–2856.
16. **Landry, M. L., D. Ferguson, S. Cohen, K. Huber, and P. Wetherill.** 1995. Effect of delayed specimen processing on cytomegalovirus antigenemia results. J. Clin. Microbiol. **33:**257–259.
17. **Madisen, L., D. I. Hoar, C. D. Holroyd, M. Crisp, and M. E. Hodes.** 1987. DNA banking: the effects of storage of blood and isolated DNA on the integrity of DNA. Am. J. Med. Genet. **27:**379–390.

18. **Niubo, J., J. L. Perez, A. Carvajal, C. Ardanuy, and R. Martin.** 1994. Effect of delayed processing of blood samples on performance of cytomegalovirus antigenemia assay. J. Clin. Microbiol. **32:**1119–1120.
19. **Paabo, S.** 1985. Molecular cloning of ancient Egyptian mummy DNA. Nature **314:**644–645.
20. **Secchiero, P., D. Zella, R. W. Crowley, R. C. Gallo, and P. Lusso.** 1995. Quantitative PCR for human herpesviruses 6 and 7. J. Clin. Microbiol. **33:**2124–2130.
21. **Towne, B., and E. J. Devor.** 1990. Effect of storage time and temperature on DNA extracted from whole blood samples. Hum. Biol. **62:**301–306.
22. **Wiedbrauk, D. L., and W. Cunningham.** 1996. Stability of herpes simplex virus DNA in cerebrospinal fluid specimens. Diagn. Mol. Pathol. **5:**249–252.
23. **Zipeto, D., S. Morris, C. Hong, A. Dowling, R. Wolitz, T. C. Merigan, and L. Rasmussen.** 1995. Human cytomegalovirus (CMV) DNA in plasma reflects quantity of CMV DNA present in leukocytes. J. Clin. Microbiol. **33:**2607–2611.