

TRANSFUSION COMPLICATIONS

High prevalence of cytomegalovirus DNA in plasma samples of blood donors in connection with seroconversion

Malte Ziemann, Sabine Krueger, Andrea B. Maier, Alexander Unmack, Siegfried Goerg, and Holger Hennig

BACKGROUND: Human cytomegalovirus (CMV) is considered to latently infect blood cells. Transfusion-transmitted infection (TT-CMV) of immunocompromised patients occurs despite the use of CMV-seronegative or leukoreduced units.

STUDY DESIGN AND METHODS: The prevalence of CMV DNA in plasma was investigated in 82 blood donors who had previously been seronegative for CMV and showed anti-CMV immunoglobulin G for the first time, 598 blood donors who were seropositive for at least 1 year, and 150 seronegative blood donors. In a second part of the study, the overall prevalence of CMV DNA in blood donations was assessed based on 31,745 donations.

RESULTS: CMV DNA was repeatedly detected in plasma samples of 44 percent of newly seropositive donors (12%-62%, depending on the interval to the last seronegative donation). All steadily seropositive or seronegative donors were negative for the presence of CMV DNA. Detection of CMV DNA in connection with seroconversion was accompanied by significantly increased neopterin, increased alanine aminotransferase, and reduced white blood cell counts, but the sensitivity of these surrogate markers was only 71 percent. The overall prevalence of CMV DNA in blood products due to primary CMV infection of donors was at least 0.13 percent.

CONCLUSION: Viremia of newly seropositive donors may be an important reason for the residual risk of TT-CMV despite leukoreduction. Furthermore, transfusion of WBC-reduced blood components from seronegative donors could imply a greater risk of TT-CMV than transfusion of WBC-reduced blood from donors who have been seropositive for at least 1 year, because window-phase donations but no reactivation could be detected in this study.

Human cytomegalovirus (CMV) is an ubiquitous β -herpesvirus causing mostly asymptomatic or mild mononucleosislike infections in immunocompetent subjects¹ with a prevalence of between 40 and 100 percent in adult populations.² Contrarily, infection of immunocompromised patients with CMV is a significant cause of morbidity and mortality. Symptoms of CMV infection in these patients cover a broad range from direct manifestations of viral replication like fever, leukopenia, thrombocytopenia, hepatitis, enteritis, and pneumonia to indirect sequelae like an elevated risk for renal allograft rejection or an impaired cellular immune response.^{3,4}

An important route of infection for risk groups like seronegative recipients of marrow transplants or newborns is assumed to be transmission of CMV by blood products from latently infected blood donors (so called transfusion-transmitted CMV infection [TT-CMV]). Even if the exact sites and mechanisms of latency still remain to be clarified, CMV DNA has repeatedly been found in peripheral blood white blood cells (WBCs) of healthy, CMV-seropositive individuals, especially in cells of the myeloid lineage.^{5,6}

Consequently, leukodepletion of blood products and inventories of seronegative blood donors have been employed to reduce the rates of TT-CMV since the 1980s.^{7,8} Even after implementation of these strategies, however,

ABBREVIATIONS: CRP = C-reactive protein; TT-CMV = transfusion-transmitted cytomegalovirus.

From the Institute of Immunology and Transfusion Medicine, University of Lübeck, Lübeck, Germany.

Address reprint requests to: Holger Hennig, Institute of Immunology and Transfusion Medicine, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany; e-mail: hennig@immu.mu-luebeck.de.

Received for publication February 2, 2007; revision received March 29, 2007, and accepted April 28, 2007.

doi: 10.1111/j.1537-2995.2007.01420.x

TRANSFUSION 2007;47:1972-1983.

"break-through" infections persist with rates as high as 1 to 3 percent of transfused high-risk patients.⁹⁻¹⁵ The impact of active CMV infection of donors (both primary and reactivated) with transmission of infectious virus as a reason for these break-through infections is discussed controversially.¹⁶⁻¹⁸

To our knowledge, infectious virus has never directly been detected in leukodepleted blood components. This may be due to the relatively poor sensitivity of commonly applied viral cultures or shell vial assays¹⁹ even if more sensitive methods have been described recently.²⁰ CMV DNA in serum or plasma, on the other hand, is associated with active CMV infection²¹ and used routinely for diagnosis and monitoring of CMV infections in risk groups like transplant recipients²² or acquired immune deficiency syndrome (AIDS) patients.²³

Drew and coworkers¹⁷ found CMV DNA in the last seronegative sample of 1 of 192 donors (0.5%) and in the first seropositive sample of 2 donors (1.0%), both of whom were excluded from donation because of elevated alanine aminotransferase (ALT). This contrasts with the findings of Zanghellini and colleagues²⁴ who detected CMV DNA in plasma of 4 of 5 adolescents with CMV seroconversion, but so far no further study has addressed the prevalence of CMV DNA associated with seroconversion of donors.

Because the actual date of seroconversion can be any point between the last seronegative and the first seropositive donation, the prevalence of CMV DNA in the plasma of first-time seropositive donors would be expected to be higher if the interval since the last seronegative sample is relatively short. Nevertheless, there are no data about the correlation between interdonation interval and prevalence of CMV DNA in plasma of newly seroconverted donors or the variations in prevalences of CMV DNA between different donor collectives.

Therefore, we conducted a prospective study, grouping newly seropositive donors according to the interval since their last seronegative sample and measuring CMV DNA in plasma samples before and after seroconversion. Another objective of our study was to determine the sensitivity of surrogate markers for viral infections, like neopterin, ALT, or WBC count for the detection of CMV DNA in plasma in connection with seroconversion.

MATERIALS AND METHODS

Blood donors

Between August 2000 and June 2004, approximately 12,800 volunteer regular blood donors (47% female, 53% male) donated approximately 34,000 whole-blood donations per year (41% by female and 59% by male donors). They were between 18 and 67 years old and healthy and gave informed consent before the donation. Out of this blood donor collective, we investigated 82 well-defined CMV seroconversion cases, whereas the total number of

CMV seroconversions during this period has not been determined. Donors were grouped according to the interval since the last seronegative sample, with intervals of less than 120, 120 to 729, and 730 days or more.

Additionally, 598 latently infected blood donors who had been seropositive for at least 1 year were included in this study, 148 of whom had been excluded from donation because of elevated ALT (more than 73 U/L or 112 U/L for female and male donors, respectively). Sampling dates from latently infected donors were distributed evenly throughout the year considering potential seasonal reactivations.¹⁶ A total of 150 CMV-seronegative donors were tested for CMV DNA as controls.

In a further part of the study, all available samples from previously seronegative donors who were repeatedly reactive in the recombinant CMV immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) between January and December 2006 were tested by polymerase chain reaction (PCR) to determine the minimum rate of CMV DNA-positive donations due to primary CMV infection of donors in our donor population.

Blood specimens

Whole-blood samples were collected in 5.5-mL tubes containing potassium-ethylenediaminetetraacetate (EDTA) at a concentration of 1.6 mg EDTA per milliliter of blood (Monovette, Sarstedt, Nümbrecht, Germany). Such samples were centrifuged at $3291 \times g$ for 4 minutes and EDTA plasma was separated within 24 hours. Plasma specimens were stored at 4 to 8°C for no longer than 72 hours or at less than -30°C until further processing.

Because of the impossibility of determining the actual seroconversion date, the date of the first seropositive sample from a previously seronegative donor was assumed to be the date of seroconversion.

Standard and control specimens

Human CMV quantitated viral DNA control, *ad169* strain, Lot 110-018 (Advanced Biotechnologies Inc., Columbia, MD) was used to determine the detection limit of the CMV PCR (TaqMan, Applied Biosystems, Foster City, CA) described below and to quantify CMV DNA-positive samples. Lyophilized CMV DNA-positive cells from an external proficiency testing program (Instand e.V., Düsseldorf, Germany) were used as positive samples for the development and optimization of the TaqMan CMV PCR.

CMV serology

Anti-CMV screening was performed with an automated enzyme immunoassay to detect IgG antibodies against the autologous fusion proteins CG1 and CG2 (Biotest Anti-CMV recombinant IgG ELISA, Biotest AG, Dreieich,

Germany). Reactive samples were retested in duplicate and considered to be repeatedly reactive if at least one of the two repetitions also gave a positive result. In the first part of the study, repeatedly reactive samples were further confirmed by an automated ELISA with AD169-coated microparticles (AxSYM CMV IgG, Abbott GmbH, Wiesbaden, Germany).

Nucleic acid isolation

DNA from 1 mL of EDTA-plasma was prepared using the Extractor (NucliSens™, bioMérieux Deutschland GmbH, Nürtingen, Germany) according to the manufacturer's protocol for pooled plasma or serum samples up to 2.0 mL. To increase the nucleic acid yield, we added a first incubation step of samples together with the lysis buffer, which is based on guanidine thiocyanate at 60°C with horizontal shaking at 110 r.p.m. for 30 minutes. Total nucleic acids from 1 mL of plasma were eluted in 50 µL of elution buffer of which 20 µL was investigated in one PCR experiment to detect CMV DNA.

During the last part of the study between January and December 2006, DNA from 1 mL of EDTA-plasma was isolated with magnetic extraction reagents (NucliSens, bioMérieux, Boxtel, the Netherlands) according to the manufacturer's instructions.

TaqMan PCR

For amplification and simultaneous detection of PCR products, we developed a novel approach based on a quantitative PCR core kit (qPCR, Eurogentec, Seraing, Belgium) on a sequence detection system (ABI Prism 7700 SDS, Applied Biosystems). Primers and fluorogenic TaqMan probe for CMV DNA detection were chosen after comparative analysis of 68 sequences containing the glycoprotein B region of the CMV genome, which were available from the GenBank Nucleotide Database with computer software (OMIGA, Version 2.0, Oxford Molecular, Oxford, UK). In addition, we performed a nucleotide-nucleotide BLAST search via the Internet at <http://www.ncbi.nlm.nih.gov/BLAST/> for the chosen oligonucleotides. For the forward primer, TaqMan probe, and reverse primer, we found 84, 80, and 84 hits, respec-

tively, to CMV sequences that had been submitted to various databases. The sequence alignments ensured that the primers were homologous at the last 12 nucleotides at the 3' end in all of the sequences and showed a maximum of one single-nucleotide polymorphism at the upper sequence. The TaqMan probe showed 100 percent homology or only one mismatch to all of the CMV hits. Seventeen non-CMV BLAST hits each to only one of the three CMV oligonucleotides ensured that no other organism could be detected with this method.

A sequence from the human C-reactive protein (CRP) gene, which was found to be detectable in human plasma was coamplified in each reaction as internal control.²⁵ The CMV probe was labeled with FAM as reporter and TAMRA as quencher and the CRP probe with VIC and TAMRA dyes. The CMV primers and probe were custom-synthesized by Eurogentec (Liege, Belgium), the CRP probe by Applied Biosystems (Weiterstadt, Germany), and the CRP primers by TIB Molbiol (Berlin, Germany). The sequences of all the oligonucleotides are provided in Table 1.

PCR experiments were carried out in special optical tubes (MicroAmp optical tubes/caps, PE Applied Biosystems, Foster City, CA) in a total volume of 50 µL. Concentrations of MgCl₂, CMV probe and primers were optimized by means of chessboard titrations. Final concentrations were 3.5 mmol per L for MgCl₂, 150 nmol per L for CMV forward primer, 300 nmol per L for the respective reverse primer, and 250 nmol per L for the CMV probe. The concentration of the CRP probe was 100 nmol per L, whereas those of the CRP primers were limited to 40 nmol per L each. Thermal cycler conditions were 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Threshold values were calculated as the upper 10-fold standard deviation (SD) of the background fluorescence signal measured over the baseline from Cycle 3 to Cycle 30. Results were interpreted as follows: a C_T of less than 40 is positive; a C_T of equal to 40 is negative.

To determine the 95 percent detection limit of the TaqMan CMV PCR, we investigated semilogarithmic dilutions of the CMV quantitated viral DNA control containing between 10² and 10^{-0.5} genome equivalents per µL (geq/µL) of CMV strain AD169. Twenty-eight samples of each concentration were processed in four consecutive TaqMan PCR procedures according to our protocol for

TABLE 1. Primer and probe sequences

Oligonucleotide	Sequence 5'→3'	Melting temperature (°C)
CMV		
Forward primer	CCCTCAAGTATGGAGATGTGGTG	59
TaqMan probe	FAM-AACACCACCAAGTACCCCTATCGCGTG-TAMRA	69
Reverse primer	AGCGAATAAGATCCGTACCCTG	58
CRP		
Forward primer	CCTGACCAGCCTCTCTCATGC	61
TaqMan probe	VIC-TTTGGCCAGACAGGTAAGGGCCACC-TAMRA	70
Reverse primer	TGCAGTCTTAGACCCACCC	59

plasma samples. The 95 percent detection limit was calculated by means of probit analysis. Quantification of CMV DNA-positive samples was carried out by means of a standard curve derived from these validation experiments.

Diagnosis of CMV DNAemia

All samples were analyzed by TaqMan PCR in duplicate. Samples with invalid internal control or diverging results were retested twice. DNAemia was diagnosed by reproducibly positive results.

Surrogate markers for viral infections

As part of the routine blood donor screening, ALT levels were determined by the standard IFCC method at 37°C (GPT ALAT liquid IFCC, Medizintechnik Guder, Bad Oeynhausen, Germany) with an automated analyzer (COBAS Mira plus CC, Roche Diagnostics Instruments Center, Rotkreuz, Switzerland). ALT screening was mandatory in Germany until 2004 with limits for donor admission of no more than 73 and 112 U per L for female and male donors, respectively. WBC counts were measured with an automated hematology analyzer (Coulter Gen S, Beckman Coulter, Krefeld, Germany).

In a subgroup of 56 samples, the neopterin concentration was analyzed by an ELISA (neopterin ELISA RE59349, IBL Immuno Biological Laboratories, Hamburg, Germany). The cutoff value for elevated neopterin was set at 10 nmol per L representing the 98th percentile of a healthy asymptomatic population.²⁶

Statistical analysis

Unless stated otherwise, means are calculated as arithmetic means ± SD. Confidence intervals (CIs) were calculated with a p value of 0.05. Differences between groups were examined with the U test. Calculations were assisted by database and statistical programs (Excel, Microsoft Corp., Redmond, WA; SPSS, SPSS Inc., Chicago, IL). The probability of appearance of CMV DNA in plasma of latently infected blood donors was calculated with the upper limits of 1 - α confidence intervals of the binomical distribution for an α level of 0.05.

The sensitivity of surrogate markers for detection of CMV DNA-positive donations was calculated as the number of CMV DNA-positive donations with elevated markers related to the total number of CMV DNA-positive donations tested for this marker. For neopterin, for instance, this results in the formula

$$\text{Sensitivity} = \frac{\text{Number of CMV DNA-positive donations with elevated neopterin}}{\text{Number of CMV DNA-positive donations tested for neopterin}}$$

The percentage of patients potentially transfused with CMV DNA-positive blood components due to primary CMV infection of donors was calculated according to the formula

$$\% \text{Patients} = 100 \times [1 - (1 - p)^{\text{number of units transfused}}]$$

In this formula, p denotes the proportion of CMV DNA-positive donations related to all donations. Therefore, (1 - p) is the probability of donations being negative for the presence of CMV DNA, and (1 - p)ⁿ is the probability of n units of blood from different donations all being negative for CMV DNA. So 1 - (1 - p)ⁿ equals the probability of n units blood containing at least one CMV DNA-positive unit.

RESULTS

TaqMan PCR

Of 1055 plasma samples tested by TaqMan PCR, 1042 (98.8%) were clearly positive or negative, whereas only 13 (1.2%) showed ambiguous results. These were due to insufficient sample volume for repeated testing (6 samples) or equivocal results even of repeated testing (7 samples with 2 positive and 2 negative results each). All samples with ambiguous results were excluded from analysis.

The 95 percent detection limit of the TaqMan PCR was calculated to be 4.88 geq per PCR procedure (3.66-8.22 geq/PCR) with semilogarithmic dilutions of CMV quantitated viral DNA control (Table 2). For 1-mL plasma specimens, it would correspond to approximately 13.5 geq per mL, if an efficacy of DNA isolation of 90 percent is assumed. The mean CMV DNA concentration in positive samples was 166 geq per mL (SD, 395 geq/mL), with a maximum of approximately 3200 geq per mL.

CMV DNA in connection with seroconversion of blood donors

Eighty-two blood donors who were previously tested negative for the presence of CMV IgG antibodies at the

TABLE 2. Observed frequencies in TaqMan CMV PCR

Standard (geq/PCR procedure)	Number of subjects	Observed responses	Probit
100	28	28	1.000
31.6	28	28	1.000
10	28	28	0.999
3.16	28	21	0.784
1	28	15	0.384
0.316	27*	4	0.263

* One subject was excluded due to negative results for CRP DNA.

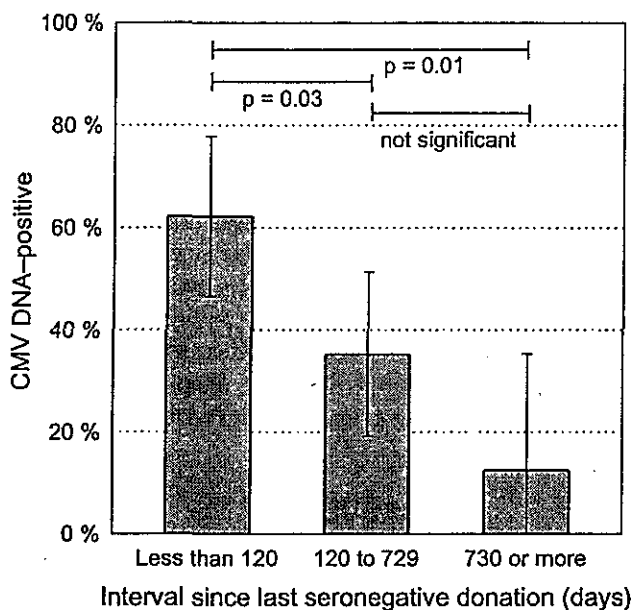


Fig. 1. Prevalence of CMV DNA in plasma of newly seropositive donors ($n = 79$) in dependence on the interval since the last seronegative sample. Prevalences are shown as percentages with 95 percent CI. The percentage of CMV DNA-positive donors was significantly greater after intervals of less than 120 days.

time of their last donation were repeatedly reactive in both anti-CMV assays and further investigated for the presence of CMV DNA. Thirty-six of 82 newly seropositive samples (44%) were repeatedly positive for the presence of CMV DNA by TaqMan PCR. The prevalence of CMV DNA was significantly higher if the interval since the last seronegative donation was less than 120 days ($p < 0.01$), whereas further differences between donors with longer intervals were not significant (Fig. 1).

In 68 (83%) of 82 seroconversion cases, we investigated the last CMV-seronegative donation before seroconversion. This way, we identified 2 reproducibly CMV DNA-positive window-phase donations (2.9%), 68 and 98 days before the first CMV-seropositive donation.

A second seropositive sample was available from 71 (87%) of 82 donors. Sixty-two of these samples (76%) were drawn within 1 year after the first seropositive sample. Samples from 4 donors were reproducibly CMV DNA-positive on Days 3, 5, 20, and 84 after the first seropositive sample, respectively (Table 3). Further plasma samples were available from only 2 of these donors. Both tested CMV DNA-negative on Day 97 and 207, respectively. So CMV DNA-negative samples were available from 59 of 82 donors (72%) within 1 year after the first seropositive sample, whereas no sample tested CMV DNA-positive 1 year or more after the first seropositive sample.

CMV DNA in latently infected and seronegative blood donors

All plasma samples of 150 seronegative and 450 latently infected donors who had been seropositive for at least 1 year were tested negative for the presence of CMV DNA. Additionally, 148 samples of latently infected donors with elevated ALT were available with mean ALT levels of 113 U per L (range, 76-906 U/L). These samples, too, all tested negative for the presence of CMV DNA. Based on the sample size, the proportion of CMV DNA-positive donors related to the total donor population (95% CI) was estimated to be less than 0.5 percent for latently infected donors and no more than 2 percent for latently infected donors with elevated ALT or seronegative donors.

Overall prevalence of CMV DNA in blood donations

In 2006, 102 previously seronegative donors tested repeatedly reactive in the recombinant IgG ELISA. This corresponds to an annual seroconversion rate of 0.8 percent relative to the total donor population.

Thirty-six donations from newly seropositive donors (41% of available samples) tested repeatedly positive for CMV DNA. Assuming the prevalence of CMV DNA in the first seropositive donation to be 41 percent for all 102 seroconversion cases results in a minimum rate of CMV DNA-positive units of 42 of 15,094 seropositive units (0.28%) or 42 of 31,745 units (0.13%), if the CMV serostatus is not taken into consideration (Fig. 2). These rates underestimate the actual prevalence of CMV DNA-positive units, because both window-phase donations and further seropositive donations containing CMV DNA have been ignored.

Surrogate markers for CMV DNAemia

The three common surrogate markers for subclinical viral infections, neopterin, ALT, and WBC count, have been tested in comparison to CMV DNA. Newly seropositive donors with CMV DNAemia had significantly higher neopterin and ALT values, as well as significantly lower WBC counts compared to newly seropositive donors without detectable CMV DNA in plasma. The best sensitivity was achieved by the neopterin ELISA, which detected 61 percent of CMV DNA-positive samples. The sensitivity of ALT was 42 percent if any values outside the normal range were considered. ALT values above the former German national limits for donor admission (>73 or 112 U/L for female and male donors, respectively) were detected in only 4 of 36 CMV DNA-positive donors and in no CMV DNA-negative donor. This equals a sensitivity of 11 percent.

WBC counts were slightly low in 6 of 36 CMV DNA-positive subjects (between 3.4×10^9 and $3.9 \times 10^9/L$),

TABLE 3. CMV DNA in plasma samples*

Donor status	Number of samples	CMV DNA		Excluded
		Positive	Negative	
Seronegative donors	150	0 (0%)	150 (100%)	0 (0%)
Donors with seroconversion				
Last seronegative sample	68	2† (3%)	64‡ (94%)	2§ (3%)
First seropositive sample	82	36 (44%)	43 (52%)	3 (4%)
Second seropositive sample	71	4¶ (6%)	66** (93%)	1†† (1%)
Donors who were seropositive for at least 1 year	450	0 (0%)	450 (100%)	0 (0%)
Donors with elevated ALT‡‡ who were seropositive for at least 1 year	148	0 (0%)	148 (100%)	0 (0%)

* Data are reported as number (%).
 † Drawn 68 and 98 days before the first seropositive sample (median, 83 days).
 ‡ Drawn 15 to 1513 days before the first seropositive sample (median, 192 days).
 § One due to ambiguous PCR results and one due to insufficient sample volume for repeated testing after a positive PCR result. Drawn 35 and 105 days before the first seropositive sample (median, 70 days).
 || One due to ambiguous PCR results and two due to insufficient sample volume for repeated testing after a positive PCR result.
 ¶ Drawn 3 to 84 days after the first seropositive sample (median, 12.5 days).
 ** Drawn 15 to 798 days after the first seropositive sample (median, 131 days). Fifty-seven of 66 samples (86%) were drawn after an interval of no more than 365 days.
 †† Due to insufficient sample volume for repeated testing after a positive PCR result. Drawn 39 days after the first seropositive sample.
 ‡‡ 76 U/L or more.

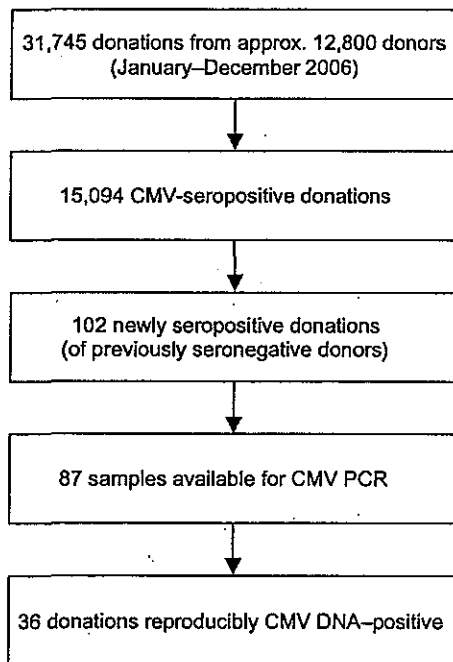


Fig. 2. Prevalence of CMV DNA in plasma of blood donors due to primary CMV-infections. Between January and December 2006, 36 out of 87 donations from newly seropositive donors contained CMV DNA (41%). 44 samples tested DNA-negative and 7 samples were excluded (5 because of ambiguous results and 2 due to insufficient sample volume for repeated testing after a positive PCR result). Assuming the prevalence of CMV DNA in the first seropositive donation to be 41 percent for all 102 seroconversion cases results in a minimum rate of CMV DNA-positive units of 42 out of 15,094 seropositive units (0.28%) or 42 out of 31,745 units (0.13%), if the CMV-serostatus is not considered.

resulting in a sensitivity of 17 percent. A combined screening with neopterin, ALT, and WBC counts would have a sensitivity of 71 percent, if all values outside the normal range are considered (Table 4).

DISCUSSION

The prevalence of CMV DNA in plasma of newly seropositive donors was 44 percent in our study. To our knowledge, the only other study examining CMV DNA in connection with seroconversion of blood donors is the study of Drew and colleagues¹⁷ who detected CMV DNA in only 2 of 192 first-time seropositive donors (1%).

One possible reason for this marked difference is the lower limit of detection of the TaqMan PCR (~13.5 geq/mL) in comparison to the PCR applied by Drew and coworkers (400 geq/mL). In the first part of our study, only 4 of 82 newly seropositive donors (5%) had CMV DNA levels of 400 geq per mL or more in their first seropositive sample. Furthermore, the interval to the last seronegative donation is given as "8 weeks to years" in the study of Drew and coworkers without any mean or medium interval given. A high proportion of donors with long interdonation intervals could have led to a lower number of CMV DNA-positive donors, as the prevalence of CMV DNA in our study was significantly higher after short interdonation intervals. Even an influence of the different target sequence used by Drew and coworkers (pol instead of gB) cannot be excluded.

Zanghellini and colleagues²⁴ reported approximately 45 seronegative adolescents, who were screened for development of CMV antibodies at monthly intervals. They detected 6 seroconversion cases and tested plasma samples from 5 seroconverted adolescents by CMV PCR finding CMV DNA in samples from 4 subjects (80%). This