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New PCR Assay for Rapid and Quantitative Detection of Human Cytomegalovirus in Cerebrospinal Fluid

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Rapid Chelex extraction combined with an automated hybridization assay for the detection of PCR-amplified human cytomegalovirus DNA from cerebrospinal fluid was established. Quantitation of DNA was performed with a plasmid being used as an external standard. The detection limit was 10 copies per μl. Quantitative detection of human cytomegalovirus DNA could be achieved over a range from 10 to 10^4 copies per μl.

The spectrum of neurological manifestations attributable to human cytomegalovirus (HCMV) infection of the central nervous system (CNS) in AIDS patients includes subacute radiculomyelopathy, peripheral neuropathy, and encephalitis. HCMV-caused disorders of the CNS are often masked by the direct effects of human immunodeficiency virus and the presence of other opportunistic pathogens (3, 4).

Amplification of HCMV DNA by PCR permits a noninvasive, sensitive, and specific diagnosis of HCMV CNS disease (1). However, detection of amplified fragments by radioactive or nonradioactive Southern or dot blot hybridization is work- and time-intensive. In the present study, a rapid extraction protocol combined with an automated nonradioactive hybridization assay for the quantitative detection of PCR-amplified HCMV DNA from cerebrospinal fluid (CSF) was established. A total of 111 CSF samples from patients hospitalized at the University of Frankfurt Clinics were obtained from March to November 1994 and stored frozen at −70°C within 1 h after lumbal puncture. Twelve samples were collected from AIDS patients with CNS disease. Five of these samples were follow-up CSF specimens from a patient with meningoencephalitis. HCMV infection of the CNS was diagnosed by rapid virus isolation (6) and confirmed by postmortem histopathologic examination. Another 24 samples were obtained from AIDS patients without clinical and virological signs of CNS disease. The remaining samples were obtained from human immunodeficiency virus-negative patients suffering from CNS disease not attributable to HCMV infection. DNA was extracted from CSF with Chelex 100 (7). HCMV DNA was amplified with 20-base oligonucleotide primers, HCMV-22 and HCMV-24D, which flank a 113-bp segment of the major immediate-early (MIE) sequence of AD169. The HCMV-22 sequence is 5′-ACT AAC CTg CAT ggg ACg Tg (HCMV MIE 1878 to 1897), and that for HCMV-24D is DIG-5′-ATC TCC TCg AAA ggC TCA Tg (HCMV MIE 2171 to 2190). One of the primers was 5′ end labelled with digoxigenin (DIG). The fragments were hybridized with a 5′-end-biotinylated (Bio), 25-bp probe, HCMV-25SB (Bio-5′-AAg ATT AAC TCT TgC ATg TgA gCg g [HCMV MIE 1926 to 1950]). Primers and the biotinylated probe were provided by Dianova (Hamburg, Germany).

PCR was performed in a 50-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each deoxynucleoside triphosphate, 0.5 μM each primer, 5 μl of extraction supernatant, and 2 U of *Taq* polymerase (Boehringer, Mannheim, Germany). Samples were denatured for 1 min at 95°C and amplified for 25 cycles (OmniGene; Hybaid, Teddington, United Kingdom) as follows: 30 s at 94°C for denaturation, 30 s at 48°C for annealing, and 35 s at 72°C for elongation, which was extended to 5 min in the last cycle to ensure completion for the amplified products. To ensure that the reactions occurred within the exponential phase of the amplification curve (25 amplification cycles), the kinetics of our PCR assay was tested. Therefore, after every second cycle, the amplification rate of our PCR assay was determined.

Each PCR run included positive (10^4 AD169 DNA copies per 5 μl) and negative (pooled CSF samples from 20 patients without neurological disease) controls, which were tested in duplicate. For hybridization with the Enzymun-Test DNA detection kit (Boehringer), 40 μl of the amplification product was transferred into sample cups and diluted with 360 μl of denaturation reagent. The hybridization procedure was performed automatically with an ES 300 processor (Boehringer) as described elsewhere (5).

For the quantification of HCMV DNA, an external plasmid standard containing a 701-bp sequence within the coding region of the MIE gene of HCMV was constructed. The MIE gene of AD169 was amplified with primer pair HCMV 18 and HCMV 19 as previously described (8). The amplification product was ligated into vector pGEM-T (Promega, Madison, Wis.) according to the manufacturer's instructions. The resulting 3,702-bp plasmid (pJUV) was extracted from positive clones with the Qiagen purification system (Qiagen GmbH, Hilden, Germany). The concentration of pJUV was measured with a GeneQuant II spectrophotometer (Pharmacia, Freiburg, Germany).

Different concentrations of plasmid pJUV in 5 μl of supernatant were amplified in three independent PCRs. The optical densities at 422 nm (OD_{422}) obtained for each of the pJUV concentrations after hybridization were plotted against the plasmid copy number used as the PCR template to obtain a standard curve, which was constructed with SigmaBlot software (Sigma Diagnostics, Deisenhofen, Germany).

In each PCR, the supernatant of AD169-infected human foreskin fibroblasts containing 10^4 copies per 5 μl of HCMV DNA served as the control for the efficiency of amplification. Quantitation of amplified HCMV DNA with the standard

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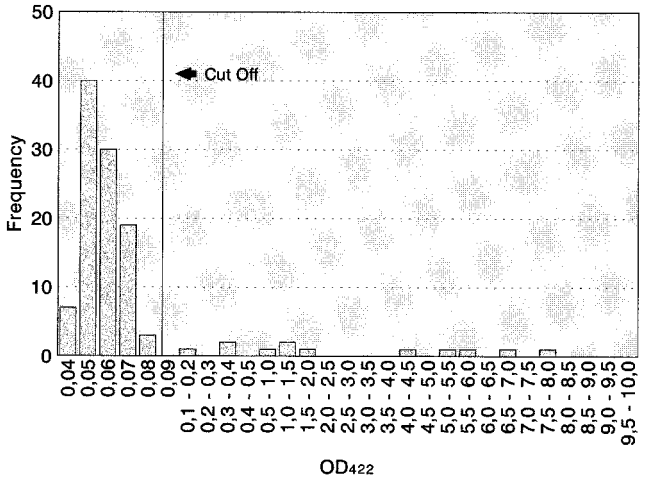

FIG. 1. Distribution of the OD_{422} values after testing of 111 CSF samples by HCMV DNA PCR and hybridization in an ES 300.

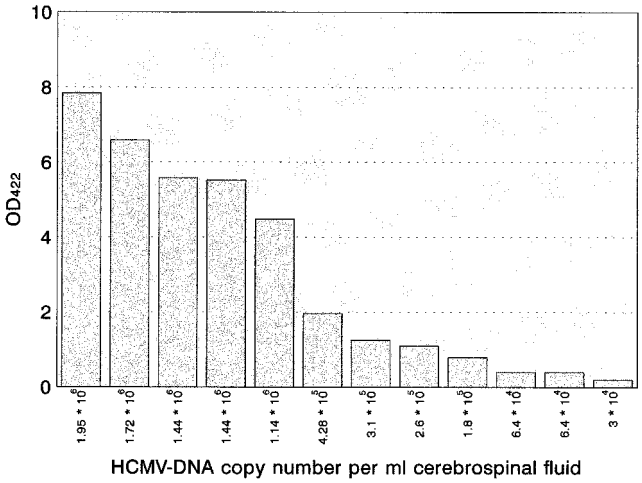

FIG. 2. HCMV DNA copy numbers per milliliter for 12 positive CSF specimens.

curve was validated if the expected OD_{422} of the positive control was between 7.00 and 8.00.

Linear regression analysis of the results of three independent PCR and hybridization assays for pJUV gave an r^2 of 0.998, verifying the reproducibility of the linear relationship between the OD_{422} and the HCMV DNA concentration. Because of the high reproducibility of each assay, the initially established standard curve was used for all PCR assays. A linear increase of the OD_{422} signal in the range of 10 to 10^4 plasmid copies per 5 μl was observed. The detection limit of the PCR was 10 copies of pJUV per μl.

Twelve samples (10.8%) gave a positive signal. A strict discrimination between positive and negative specimens was obtained (Fig. 1). The cutoff OD_{422} value (mean OD_{422} for the negative controls ± two standard deviations) was fixed at 0.09, with an equivocal zone of ±10%. HCMV DNA-positive CSF samples yielded virus concentrations ranging from 3.0×10^4 to 1.95×10^6 DNA copies per ml (Fig. 2).

With the Enzymun test, detection of HCMV-specific DNA in 30 samples can be performed within 3.5 h. Diagnostic results for CSF after pretreatment, amplification, and hybridization can be obtained within 6 h.

Quantification methods based on the coamplification of target DNA and the same modified target sequence as an internal standard have the advantage of detecting potential inhibitory effects present in the samples. Whereas the competitive PCR is the method of choice for the quantitative detection of HCMV DNA in peripheral blood mononuclear cells, it can, because of the competition with the internal standard, yield a reduced sensitivity. This drawback might limit its usefulness for the diagnosis of HCMV encephalitis. As virus is recovered by cell culture from CSF from only about 50% of AIDS patients with HCMV CNS disease (2), it has to be supposed that HCMV DNA is frequently present at low copy numbers in CSF. Therefore, it is of importance to use a highly sensitive PCR assay. A further advantage of our method is cost-effectiveness, since only a sample dilution and one positive control are required for the quantitation of viral DNA.

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