Detection of human cytomegalovirus DNA in paraffin sections of human brain by polymerase chain reaction and the occurrence of false negative results


Abstract
Paraffin-embedded necropsy material from 6 patients with human cytomegalovirus encephalitis (HCMVE) corroborated by immunocytochemistry and 11 control cases were examined for the presence of human cytomegalovirus (HCMV) DNA by a nested polymerase chain reaction (nPCR). A characteristic 183 base pair (bp) fragment of the HCMV genome could readily be amplified in 4 cases of HCMVE. In 2 cases of HCMVE, viral DNA could be demonstrated only sporadically by PCR, due most likely to inefficient DNA extraction or DNA degradation. All control cases remained negative. The nPCR provides a specific method for detecting HCMV DNA in routinely processed biopsy and necropsy material and may be used in archival tissues for the diagnosis of infection. Fixation of samples and DNA extraction are, however, crucial steps and require careful control if PCR is used for detection of HCMV, to avoid false negative results.

Material and methods
We examined six cases of HCMV (table, cases 1–4 from our Institute, cases 5–6 from the Department of Neuropathology, University of Oxford, UK). All demonstrated typical histological features such as inclusion-bearing cells of Cowdry type A, microglial proliferation, focal parenchymal necroses and lymphocytic infiltration. Six cases with HSV-1 encephalitis and five cases without neuropathological changes were used as controls. Diagnosis was made by routine histological examination and corroborated by immunocytochemistry with a monoclonal antibody (anticytomegalovirus, dilution 1:25, DAKO, Hamburg, Germany) in all cases. Necropsy

Table: Clinical and laboratory data of 6 patients with HCMV encephalitis

<table>
<thead>
<tr>
<th>Number</th>
<th>Age/Sex</th>
<th>AIDS</th>
<th>Histology diagnosis</th>
<th>Immunocytochemistry</th>
<th>HCM-PCR</th>
<th>β-globin-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HCMV</td>
<td>HCMV-pos</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Case 1</td>
<td>44/Male</td>
<td>+</td>
<td>HCMV</td>
<td>HCMV-pos</td>
<td>6/6</td>
<td>nd</td>
</tr>
<tr>
<td>Case 2</td>
<td>47/Male</td>
<td>+</td>
<td>HCMV</td>
<td>HCMV-pos</td>
<td>5/5</td>
<td>&gt;10²</td>
</tr>
<tr>
<td>Case 3</td>
<td>49/Male</td>
<td>+</td>
<td>HCMV</td>
<td>HCMV-pos</td>
<td>5/5</td>
<td>&gt;10⁴</td>
</tr>
<tr>
<td>Case 4</td>
<td>2m/Male</td>
<td>-</td>
<td>HCMV</td>
<td>HCMV-pos</td>
<td>8/8</td>
<td>&gt;10⁴</td>
</tr>
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<td>Case 5</td>
<td>1d/Male</td>
<td>-</td>
<td>HCMV</td>
<td>HCMV-pos</td>
<td>0/7</td>
<td>&lt;10⁴</td>
</tr>
<tr>
<td>Case 6</td>
<td>50/Male</td>
<td>+</td>
<td>HCMV</td>
<td>HCMV-pos</td>
<td>1/7</td>
<td>&lt;10⁴</td>
</tr>
</tbody>
</table>

Age in years, case 4 in months, case 5 in days; HCMV = human cytomegalovirus encephalitis; id = indeterminated; nd = not done; a = PCR result; b = number of positive results/number of sections; c = estimated copy number of target sequences per 5 μl extract.
brains from our laboratory had been routinely fixed in 6% buffered formalin for up to three weeks post mortem before dissection and paraffin-embedding. Samples from the Department of Neuropathology, University of Oxford, had been fixed in 10% buffered formalin. For PCR, paraffin blocks from areas with histologically proven inflammatory changes as well as from remote areas without histological evidence of infection were selected from HCMV and HSV-1 cases. Samples from the periventricular area, which is a predilection site of HCMV, were chosen for PCR from the control cases.

Sections of paraffin-embedded tissue were processed for PCR according to Wright and Manos. Samples were placed in a 1·5 ml reaction tube and treated twice with n-octane and twice with absolute ethanol. Remaining ethanol was evaporated completely under vacuum and 200 μl per sample of proteinase digestion buffer was added containing (final concentrations) 200 μg/ml Proteinase K (Boehringer Mannheim, Mannheim, Germany), 50 mM Tris/Cl pH 8·5, 1 mM EDTA and 1% Triton X-100. Proteinase digestion was carried out for three hours at 55°C, the enzyme was inactivated by boiling the samples for five minutes, and insoluble material was pelleted at 10 000 g for 10 minutes. Five μl of the supernatants were used in PCR. All samples investigated were extracted and tested by nPCR at least in duplicate.

PCR was performed as previously described. A DNA fragment from the coding region of the essential HCMV glycoprotein B (gB) was amplified using two sets of nested primers (E, primer I, and E, primer I). 5'-TCC ACC ACC CAC AGT ACC CGT-3', E, primer I: 5'-CGG AAA CGA TGG TGT AGT TCG-3', I, 5'-GTC AAG GAT CAG TGG CAC AGC-3', I, 5'-GTA GCT GGC ATT CGC ATT GGT-3').

Primers E, and I, created a 268 bp amplifier, primers I, and I, allowed the amplification of a 183 bp fragment located within the external amplifier. PCR was carried out for 30 cycles with the primer pair E, and I, Subsequently, 5 μl of the reaction mix was transferred to a second reaction mix containing the primer pair I, and PCR was continued for another 30 cycles. The final concentrations of reagents were 67 mM Tris/Cl pH 8·8, 16·6 mM (NH₄)₂SO₄, 3 mM MgCl₂, 0·7 mM β-mercaptoethanol, 20 μg/ml gelatine, 0·1 μM external or 1 μM internal primers, respectively, 400 μM of each dNTP, and 1·25 units tag polymerase (Promega, Heidelberg, Germany). The final reaction volume was adjusted to 50 μl. Primer annealing occurred at 60°C (45 s), DNA synthesis at 72°C (45 s), and denaturation at 94°C (60 s). After PCR samples were separated on 4% agarose gels and PCR amplifiers were visualised by ethidium bromide staining and UV-illumination. The specificity of PCR was controlled by Southern blotting using a digoxigenin-labelled 109 bp DNA probe located within the internal 183 bp PCR amplifier. This DNA probe was obtained with a third set of primers (P, primer P, 5'-CTG GCT GTC TCG TGA GAC CTG-3', P, 5'-GTA AAC CAC ATC ACC CGT GGA-3') and directly labelled in PCR by adding digoxigenin-11-dUTP (Boehringer Mannheim) to the reaction mix. Specifically hybridised DNA probe was visualised by chemoluminescence using an alkaline phosphatase-conjugated rabbit anti-digoxigenin antiserum and AMPPD as substrate (Dig Luminense Detection Kit, Boehringer Mannheim, Germany).

To control the efficiency of DNA extraction, a 105 bp amplifier of the human β-globin gene was amplified using the primers la and lb (1a 5'-CTG CGG TTC TGG CCC TGT GG-3', 1b 5'-CTA TTT GTC TCC TTA AAC CTG-3'). PCR was performed as described above.

**Results**

In four cases of HCMVE DNA fragments of the predicted size were consistently amplified by nPCR (fig A, table). The specificity of the amplified DNA fragments could be demonstrated in Southern blots using a digoxigenin-labelled DNA probe (fig B). Only tissues with histological features of inflammation yielded an amplification of HCMV specific DNA sequences, whereas tissues from remote regions without histological evidence of infection remained negative. In two cases of histologically proven HCMV, viral DNA could either not be demonstrated by nPCR (patient 5, table) or was detected only in one of seven extracts tested (patient 6, table). To investigate these two false negative PCR results further, a number of control experiments were performed. Thus all HCMV-PCR negative extracts were tested for the presence of non-specific inhibitors by adding 10", 10", 10", and 10" copies of linearised, purified plasmid DNA containing the entire HCMV gB sequence before amplification by PCR. A non-specific inhibition could not be demonstrated. The detection limit of PCR was estimated to be approximately 10² copies of the HCMV gB target sequence. Knowing the approximate detection limit, PCR positive samples were serially diluted to determine the number of target sequences present. The calculated number of HCMV target sequences in nPCR positive samples reached from >10⁷ (patient 3) to >10⁴ (patient 4, table). To exclude false negative PCR results due to HCMV strain variations and resulting primer mismatches, we tested extracts of patients 4, 5, and 6 in nPCR using all possible combinations of primers 1E, 1L, 1I, P1 and P2. Only sporadic positive results were obtained with extracts of patients 5 (2/9) and 6 (1/9), whereas extracts of patient 4 gave positive results with all primer combinations tested (9/9). No influence of specific primer combinations on the outcome of nPCR could be demonstrated (data not shown).

Finally, to control DNA extraction from paraffin-embedded tissues, all samples were tested for the presence of the β-globin target sequences, which is a single copy gene present in all human cells. Using purified plasmid DNA as control, the sensitivity (approximately 10² copies) and the number of β-globin target...
sequences per sample were estimated (as given above). It could be consistently demonstrated, that extracts of HCMVE patients 5 and 6 contained <10^2 β-globin copies per 5 μl extract. In contrast, the amount of β-globin sequences in HCMV-PCR positive samples ranged between 10^3 and 10^10 copies per 5 μl extract.

None of the control tissues from patients with HSV-1 encephalitis (n = 6) or without pathological findings (n = 5) contained detectable amounts of HCMV DNA (data not shown).

Discussion
PCR has been used to detect HCMV DNA in a variety of clinical specimens, such as peripheral blood, urine, saliva, and formalin-fixed paraffin-embedded surgical biopsies or necropsy samples. Our present findings confirm that PCR can also be used to detect HCMV virus in paraffin sections from human necropsy brains. Necropsy material stored for several years still seems to be suitable for detection of viral genomic DNA sequences. The use of a nested PCR protocol offers maximum sensitivity and specificity. In four cases of HCMVE from our laboratory, we could readily amplify HCMV specific sequences (fig, table). We could, however, only detect CMV specific DNA sequences in tissues with histological features of inflammation or infection, not in areas remote from inflammatory foci. It seems to be mandatory therefore to use samples from regions with inflammatory changes, especially if the sample is small, for example in a stereotactic biopsy.

In two cases of immunocytochemically proven HCMVE from a different Institute, HCMV specific DNA sequences could only sporadically be amplified (fig, table). However, immunocytochemical investigations clearly demonstrated, that these tissues contained viral antigens. Since a non-specific inhibition could be excluded and both samples contained significantly lower amounts of β-globin sequences compared with HCMV-PCR positive samples, the obviously false negative results were most likely due to inefficient DNA extraction or degradation of viral DNA templates. Recently, it has been shown that the fixation protocol for materials used in PCR is a crucial step, since some fixation methods damage DNA and thus deleteriously affect subsequent PCR analysis. An appreciable degradation of longer DNA templates occurs in formalin fixed materials. Therefore short amplimers <300 bp are desirable.

As HCMV is an important clinical pathogen in the immunocompromised host causing significant morbidity and mortality, the development of new diagnostic procedures is urgently needed for the early detection of opportunistic infection. HCMV visceral infection is a major cause of morbidity in AIDS patients and HCMVE has been found in 15–30% of HIV-patients in several large necropsy series. Investigation of prevalence and incidence of HCMV infection of the CNS is important, because patients with HCMVE may benefit from a therapy with ganciclovir or foscarnet. In AIDS patients HCMV infection may mimic HIV-encephalitis or may be masked by concurrent opportunistic viral and non-viral CNS lesions. Therefore PCR may offer an additional diagnostic tool, especially if only small samples (for example, by stereotactic biopsy) are available. Our results confirm that PCR can be used as a specific diagnostic method to detect...
HCMV DNA sequences in formalin-fixed, paraffin-embedded materials. Critical steps are fixation of specimens and subsequent DNA extraction that require careful control, if PCR is used for the detection of HCMV in biopsy or necropsy materials.

Samples from two cases of HCMVE (Cases 5 and 6, Table) were kindly provided by Dr Margaret Eustri, Department of Neuropathology, University of Oxford, UK.


