Human cytomegalovirus (HCMV), a human beta-herpesvirus, is the leading viral cause of congenital infections and a significant cause of morbidity and mortality in immunosuppressed patients (5). Methods for diagnosis of HCMV infections include serology, antigenemia, shell vial assay, culture, and, more recently, PCR (5, 13). PCR detection of HCMV DNA in clinical specimens is limited chiefly by the sensitivity and specificity of the primers used for amplification. PCR specificity is determined by the uniqueness of HCMV primers, while its sensitivity relies chiefly on the invariance of viral sequences in clinical HCMV strains as well as the method used to detect the PCR products.

Despite the availability of the complete sequence of HCMV strain AD169, primers from only a few (about 10) of the more than 200 genes in the HCMV genome have been tested to date for their suitability in PCR detection of HCMV DNA in clinical specimens (4, 6, 7). Two of the more commonly used primer pairs are derived from the HCMV DNA polymerase gene (UL54) and from the major immediate-early gene 1 (IE1) exon 4 sequences (7). The HCMV UL54 gene varies only slightly (about 2%) in clinical HCMV strains (19) and is well conserved in the genomes of other human herpesviruses (2, 6, 11, 14, 18, 20). Thus, type-specific UL54 primers must be used to improve specificity in the detection of HCMV DNA. IE1 exon 4 sequences are unique to HCMV, but these sequences are known to vary approximately 18% in clinical HCMV strains and result in decreased sensitivity of the primers for the detection of HCMV DNA in clinical specimens (8).

We tested the hypothesis that other immediate-early (IE) gene primers may be as suitable or more suitable for developing sensitive and specific HCMV PCR. We also hypothesized that the parallel use of two IE gene primers would increase the diagnostic performance of HCMV PCR. We focused on the UL37 exon 3 (UL37x3) and US3 IE genes and designed primers (UL37x3 primers 99, 112, 113, and 116 and US3 primers 24, 30, 48, and 49) from the HCMV strain AD169 genome (EMBL accession no. X17403) (Table 1 and Fig. 1). We compared the primers mentioned above to UL54 primers (primers 108, 109, 110 and 147) for the detection of HCMV in clinical specimens. Although homologs of the UL37x3 open reading frame are present in beta-herpesviruses, including HCMV and human herpesviruses types 6 and 7 (14, 20, 21), homology searches for the nucleotide sequences of the UL37x3 primers in the combined (GenBank, EMBL, DDBJ, and PDB) sequence databases using the BLASTN search program (1) confirmed that the designed UL37x3 primers are unique to HCMV (9). The HCMV US3 gene is not conserved in the genomes of other human herpesviruses (2, 6, 11, 14, 18, 20), and the designed primers are also unique to HCMV sequences (9). Thus, the designed UL37x3 and US3 IE gene primers are specific for HCMV.

The present study used nested PCR to test 19 pediatric patient specimens (urine, cerebrospinal fluid, and lung tissue) that had scored positive for HCMV in shell vial assays (Table 2). As controls, 22 shell vial-negative specimens were analyzed by PCR amplification. HCMV (strain AD169) DNA served as a positive control for all PCR amplification experiments. HCMV (strain AD169) DNA was purified from HCMV-infected human diploid fibroblasts lysed in buffer (50 mM Tris-HCl [pH 7.5], 20 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate) and digested with proteinase K (50 μg/ml) at 37°C for 24 h, followed by sequential phenol and chloroform extractions and ethanol precipitation. In addition, a separate PCR mixture containing only water was included in each ex-

### TABLE 1. HCMV DNA primer pairs used for nested PCR and the predicted amplification products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pair</th>
<th>Sequence positions (nt)*</th>
<th>Predicted size fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL37x3</td>
<td>99/112 113/116</td>
<td>50819-50840/49912-49931</td>
<td>929</td>
</tr>
<tr>
<td>US3</td>
<td>48/49 24/30</td>
<td>194748-194767/193889-193908</td>
<td>879</td>
</tr>
<tr>
<td>UL54</td>
<td>108/147 109/110</td>
<td>79965-79988/79332-79355</td>
<td>657</td>
</tr>
</tbody>
</table>

*a The nucleotide numbers are from the HCMV strain AD169 genome sequence in the EMBL data bank (accession no. X17403).
experiment as a negative control for PCR amplification (10, 15, 16). The cerebrospinal fluid was used directly for PCR amplification while the urine specimens were first diluted (1:2 to 1:5) in water or viral transport medium. The lung tissue biopsy specimen was treated with 1% lysis buffer (10 mM Tris HCl [pH 8.3], 50 mM KCl, 2.5 mM MgCl₂, 0.1 mg of gelatin per ml) for 60 min at 65°C and for 10 min at 95°C prior to PCR amplification. The PCR mixtures contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 µM each dATP, dGTP, dCTP, and TTP, 0.25 to 1.0 µM each primer, and 0.625 to 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) per 50 µl of reaction mixture. Templates were denatured at 95°C for 45 s and then subjected to 35 cycles of 95°C for 45 s, 50 to 70°C for 1 min, and 72°C for 2 min. The last cycle consisted of 95°C for 50 s, 50 to 70°C for 1 min, and a final extension at 72°C for 10 min. One (to five) microliters of each amplification product in the first round was further amplified by using nested inner primers for a second round of reactions under the same conditions. Finally, the production and size of PCR products were visualized by ethidium bromide staining following electrophoretic separation in agarose gels in 1× TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). Molecular size DNA markers (123 bp; Gibco-BRL) were used in all gels.

All of the tested primers successfully amplified HCMV DNA by nested PCR and produced fragments of the predicted sizes (Table 1), which were similar to those amplified from HCMV strain AD169 DNA (16). The UL37x3 primers detected HCMV DNA in 15 of 19 (79%) specimens (Table 2). By comparison, the UL54 and US3 primers detected HCMV DNA in 12 (63%) and 11 (58%) of the 19 specimens, respectively. US3 primers gave equivocal results for another two specimens. All shell vial-positive specimens which scored positive with UL54 primers also scored positive with either UL37x3 or US3 primers. There were six shell vial-positive specimens (specimens 3, 11, 16, 22, 23, and 25) that scored negative with UL54 primers but positive with either UL37x3, US3, or both. However, one shell vial-positive specimen (specimen 27) scored negative with primers from all three genes. The viral load in the patient who provided this specimen was probably very low since shell vial assays performed on subsequent specimens from this patient were consistently negative. More recently, we have tested four additional shell vial-positive specimens with the UL37x3 primers and these have scored positive by nested PCR (10).

The specificity of the designed primer sets was verified by PCR analysis of negative clinical specimens. Shell vial-negative specimens were included as negative controls for these experiments. No PCR amplification was observed for 11 shell vial-negative specimens tested with the UL54 primers, for 7 tested with the UL37x3 primers, and for 11 tested with the US3 primers (10, 15, 16).

Using heteroduplex mobility analyses (12), we have found that UL37x3 sequences from nucleotides 50531 to 49961 are invariant (less than 1 to 2% variation) in six of six clinical HCMV strains tested (10). These results suggest that most UL37x3 sequences are well conserved (98 to 99%) in different clinical HCMV strains. This suggestion is further supported by the electrophoretic mobility analysis of the PCR products from HCMV clinical strains. We found no detectable deletions or insertions in UL37x3 DNA sequences amplified from the clinical specimens in comparison to UL37x3 sequences from HCMV strain AD169 DNA.

The observed lack of PCR amplification by UL54 primers in some shell vial-positive specimens is not likely due to mutations or variation in the primer sequences. Mutations in the HCMV UL54 gene are known to confer ganciclovir and foscamet resistance (3, 17). However, the UL54 primers tested herein lie outside of the sites of those mutations. The relatively stringent annealing temperatures (60 to 65°C) used, the lack of extraction methods for clinical specimens, and the use of

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**TABLE 2. PCR detection of HCMV DNA in clinical specimens by using UL37x3, US3, and UL54 primers**

<table>
<thead>
<tr>
<th>PCR detection method</th>
<th>Score for clinical specimen no.:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene primers</td>
<td></td>
</tr>
<tr>
<td>UL37x3</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>UL54</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>US3</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Shell vial assay</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
</tbody>
</table>

* All specimens are urine except for specimens 1 (lung biopsy tissue) and 3 (cerebrospinal fluid). +, positive; −, negative; ∼, equivocal.
* Specimens 12 and 13 are serial diagnostic specimens taken from the same patient 2 days apart.
* This specimen was positive by shell vial assay, but several subsequent specimens from the same patient were shell vial negative.
* Primer pairs 99/112 and 113/116 were used as outer and inner primer pairs, respectively.
* Primer pairs 10/10 and 109/110 were used as outer and inner primer pairs, respectively.
* Primer pairs 48/49 and 24/30 were used as outer and inner primer pairs, respectively.
ethidium bromide staining to detect the size and potential rearrangement of the PCR products predictably reduced the detection by the UL54 primers as well as by the other primers tested. It is likely that the observed sensitivities of the UL37x3, UL54, and US3 primers used are minimal estimates, given that diagnostic laboratories commonly employ more sensitive techniques such as DNA hybridization with subsequent radioactive, electrochemiluminescent, or colorimetric detection of amplified DNA.

In this study, we found that the overall sensitivities of UL37x3, US3, and UL54 primers are comparable. Nonetheless, primers from the conserved UL37x3 region were the most sensitive of those tested in this study. The selection of unique DNA sequences for UL37x3 primers also yielded excellent specificity. Moreover, UL37x3 and US3 primers detected HCMV DNA in some clinical specimens even when UL54 primers did not. For optimal sensitivity of the diagnostic method, the parallel use of primers from the UL37x3 and US3 genes is recommended.

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