Diagnostic Value of Reverse Transcription-PCR for Detection of Cytomegalovirus pp67 in Samples from Solid-Organ Transplant Recipients

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Received 7 March 2006/Returned for modification 25 April 2006/Accepted 3 July 2006

We evaluated a highly sensitive quantitative real-time one-step reverse transcription-PCR (RT-PCR) for detection of human cytomegalovirus pp67 transcripts in monitoring of solid-organ transplant recipients. Results were compared with those of pp65 antigen testing and quantitative DNA-PCR. Due to a low clinical sensitivity, the pp67 RT-PCR was not able to replace these assays.

The monitoring of immunocompromised patients for infection with the human cytomegalovirus (HCMV) is still a challenge despite the introduction of a variety of new assays. Most of these assays reflect viral replication indirectly. The recently introduced nucleic acid sequence-based amplification assay (NASBA) allows amplification of viral late mRNA and is therefore a direct marker of viral replication (6). However, the commercially available NASBA (NucliSens CMV pp67; Organon Teknika B.V., Boxtel, The Netherlands) is very costly. In order to establish an alternative, a quantitative real-time one-step reverse transcription-PCR (RT-PCR) was generated to detect pp67 transcripts. The results were compared with those of the pp65 antigen test and in-house real-time DNA-PCR for monitoring of solid-organ transplant recipients. For better comparability of results, all assays were performed on identical numbers of PBL.

Plasmid quantification standards. A plasmid standard for pp67 RT-PCR was generated by amplifying the respective gene fragment using the following primers: pp67 FP (forward primer), 5'-AAAAGAGACCGCTCTCTGG-3'; pp67 RP (reverse primer), 5'-AGGGCGCGTCTCTTACGTATC-3'; and pp67 P (probe), 5'-CGCTCTTTTTGTACGTCTGAAATC GACCC-3'. The PCR product was ligated into pCRII (Invitrogen, Groningen, The Netherlands), followed by amplification with M13 primers. Initiating from the SP6 promoter, a positive-sense single-stranded-RNA standard was generated. The RNA was purified and quantified using the RiboGreen kit (Molecular Probes, Oregon) as described previously (16, 17). Ten-fold dilutions (10⁴ to 10⁷ molecules) were prepared in 100 μg of tRNA/ml (Sigma, München, Germany). The detection limit was 10⁴ molecules/reaction, and the linear range was between 10⁵ and 10⁷ molecules (correlation coefficient of the standard curve, 1.0; slope, 3.54; PCR efficiency, 0.91 [E = 10^{slope - 1}]).

The variability of three independent standard curves ranged from 22.08 ± 0.1 for the detection of 10⁵ molecules to 37.86 ± 1.5 for the detection of 10⁷ molecules. For quantification of HCMV DNA, the AD1 PCR was applied as described previously, using plasmid pJW4303gBl (12). The detection limit was 10³ molecules/reaction, and the linear range was between 10¹ and 10⁷ molecules (correlation coefficient of the standard curve, 1.0; slope, 3.72; PCR efficiency, 0.85). No contaminating DNA was found in the RNA preparations.

Patients. A total of 80 samples from 23 transplant recipients (kidney, n = 19; liver, n = 2; kidney and pancreas, n = 2) of the University Clinics Freiburg were included. Patients were monitored weekly by pp65 antigen test and quantitative AD1 HCMV PCR (12). Patients were retrospectively enrolled if a positive test result in one of the routine diagnostic tests was obtained. Three to four samples were collected per patient. Antiviral therapy (AVT) (ganciclovir or foscarnet) was started if active HCMV infection was suspected. HCMV infection and disease were defined according to the 4th International HCMV Workshop with modifications (9, 12). Seven patients had asymptomatic HCMV infection (grade I), and 16 patients developed symptomatic HCMV infection and disease (grades II and III).

Sample preparation and PCR. Peripheral blood lymphocytes (PBL) were isolated from 6 ml blood and quantified using a CASY cell counter (Scharfe System GmbH, Reutlingen, Germany). Cells (2 × 10⁶) were directly used for pp65 antigen testing (13). A 1-ml aliquot of the cell preparation (each) for DNA and RNA extraction was stored in liquid nitrogen. For quantitative AD1 DNA-PCR, the PBL were thawed and washed, and cell pellets were lysed (12). For pp67 one-step RT-PCR, the mRNA was extracted from 1 ml PBL in a final volume of 20 μl using the PolyATtract System 1000 (Promega, Madison, Wis.) and stored at −70°C. The amount of mRNA/μl of the 20-μl RNA extract was calculated in reference to the initial CASY cell quantification of 1 ml PBL. RT-PCR was performed using the LightCycler RNA Master-HybProbe kit (Roche, Mannheim, Germany) on the LightCycler instrument. Reaction mixtures in a total volume of 20 μl contained RNA.
Master HybProbe mix, 500 nM (each) forward and reverse primer (pp67 FP and RP), 200 nM of probe, 3.25 mM of Mn(OAc)₂, and mRNA of 2 × 10⁵ PBL.

RT-PCR conditions were as follows: RT at 61°C for 20 min and 95°C for 2 min, and 60 cycles of PCR with 92°C for 15 s and 55°C for 30 s. No-template controls and DNA of HCMV-infected fibroblasts were included for all amplifications.

**Comparison of diagnostic tests.** The arithmetic means of the quantitative results of the pp65 antigen test, the pp67 RT-PCR, and the AD1 PCR were determined (mean viral loads). Mean viral loads were significantly higher in symptomatic patients than in asymptomatic patients (Table 1) in all three assays (P < 0.0001). In contrast to the pp65 antigen test and the AD1 PCR, only 40% of the samples analyzed by pp67 RT-PCR were found to be positive in both the symptomatic and the asymptomatic groups (Table 1).

Twelve of 16 symptomatic patients tested positive for pp67 RT-PCR in at least one sample, if all samples analyzed during follow-up were considered (clinical sensitivity of 75%). The clinical sensitivities of the pp65 antigen test and of DNA-PCR were 94% and 100%, respectively. Only 7 of 16 patients tested positive by pp67 RT-PCR if the time point of the first appearance of symptoms was considered (clinical sensitivity at beginning of symptoms, 44%). Therefore, pp67 RT-PCR was not useful in early identification of symptomatic patients. All patients of the asymptomatic group tested positive in all three assays for at least one sample (data not shown).

The low clinical sensitivity of the pp67 RT-PCR might be explained in different ways: first, viral mRNA is degraded by DNases in vivo and during transport and storage of samples; second, due to a short period of mRNAemia, the point of positivity might be easily missed in a weekly interval of patient screening; third, the assay detection limits are insufficient to detect an active infection in the early stages.

To our knowledge, no data on pp67 RT-PCR screening of transplant recipients are available in the literature. Therefore, we compared our results with those of the commercially available pp67 NASBA, which is widely used. The clinical sensitivity of the pp67 NASBA ranged between 20% and 80% and was equivalent to or lower than that of pp65 antigen testing (65 to 100%) or quantitative PCR (91 to 100%) (1, 4, 7, 8, 10, 11, 14, 15). Only one author found the pp67 NASBA to be superior in identifying patients with HCMV disease (2, 3). Although the performance of in-house pp67 RT-PCR affords specific experience and equipment—available in most virological laboratories—it has two considerable advantages compared to the pp67 NASBA: time of assay performance and cost. Assay performance needed less than 2 h, and the costs were calculated with approximately 5 EUR per reaction (mRNA extraction and RT-PCR). Performing the NucliSens CMV pp67 takes 4 to 6 h, and costs per reaction (approximately 40 EUR) might exceed the budget of many laboratories.

Additionally, viral load kinetics were analyzed for 17 patients who received AVT (15 symptomatic and 2 asymptomatic), with a minimum of three samples available during follow-up. The mean follow-up time was 29 days (9 to 105 days). Changes in viral load had to be at least 10-fold to be considered in the evaluation. Two main features were observed: either (i) a steady state or decrease of viral load (feature one: direct decrease of viral load, indifference, indifference and then decrease, increase first and then decrease) or (ii) an increasing tendency (feature two: increase of viral load, decrease first and then increase, increase first and then indifference, indifference and then increase). Viral load kinetics followed feature one when measured by AD1 DNA-PCR in 16/17 (94%) of the cases and when measured by the pp65 antigen test or pp67 RT-PCR in 14/17 (82%) of the cases. The median time to negative test results after initiating AVT was 240 days for the pp65 antigen test (n = 1 patient), 79 days for AD1 DNA PCR (n = 7), and 41 days for pp67 RT-PCR (n = 8) for those patients that became negative. Therefore, successful AVT was most frequently indicated by AD1 DNA-PCR. Mean treatment periods monitored by AD1 PCR were longer than mean treatment periods monitored by pp67 RT-PCR.

However, due to the low clinical sensitivity of pp67 RT-PCR, this advantage was of little clinical use. In the literature, the duration of positive test results following antiviral treatment was found to be almost similar for the pp65 antigen test and the pp67 mRNA assay (5, 7).

In conclusion, the pp65 antigen test and quantitative DNA-PCR are both useful tools for monitoring solid-organ transplant recipients for HCMV infections. Although the analytical sensitivity of the pp67 RT-PCR was high, this assay did not have an advantage in terms of early identification of clinically relevant infections. Therefore, it cannot replace the pp65 antigen test or DNA PCR in this clinical context.

### Table 1. Correlation of clinical grade with pp65, DNA, and RNA testing results

<table>
<thead>
<tr>
<th>Method</th>
<th>Clinical grade(s)</th>
<th>No. of pos. samples/no. studied (%)</th>
<th>Range</th>
<th>Mean</th>
<th>Clin. sens. (%)</th>
<th>Clin. sens. at begin. of symptoms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp65 antigen test&lt;br&gt;a</td>
<td>I</td>
<td>24/25 (96)</td>
<td>0–148</td>
<td>21</td>
<td>5.6–36.4</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td></td>
<td>II and III</td>
<td>53/55 (96)</td>
<td>0–2,000</td>
<td>137</td>
<td>43.6–231</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td>AD1 PCR&lt;br&gt;b</td>
<td>I</td>
<td>25/25 (100)</td>
<td>48–221,000</td>
<td>21,423</td>
<td>224–4222</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td></td>
<td>II and III</td>
<td>55/55 (100)</td>
<td>12–397,000</td>
<td>38,331</td>
<td>9135–67527</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td>pp67 RT-PCR&lt;br&gt;c</td>
<td>I</td>
<td>10/25 (40)</td>
<td>0–846</td>
<td>86</td>
<td>1.8–171</td>
<td>12/16 (75)</td>
</tr>
<tr>
<td></td>
<td>II and III</td>
<td>22/55 (40)</td>
<td>0–1,964</td>
<td>130</td>
<td>25.6–235.8</td>
<td>12/16 (75)</td>
</tr>
</tbody>
</table>

a Quantification is given as no. of positive nuclei per 2 × 10⁵ cells.
b Quantification is given as no. of genome copies per 2 × 10⁵ cells.
c pp67 RT-PCR performance needed less than 2 h, and the costs were calculated with approximately 5 EUR per reaction (mRNA extraction and RT-PCR). Performing the NucliSens CMV pp67 takes 4 to 6 h, and costs per reaction (approximately 40 EUR) might exceed the budget of many laboratories.

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This work was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF), FKZ 01 K19951. We thank Renate Mielke for excellent technical assistance. We acknowledge Dieter Neumann-Haefelin for critically reading the manuscript.

REFERENCES


