Rapid Detection of Human Cytomegalovirus UL97 and UL54 Mutations Directly from Patient Samples

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Running title: Human Cytomegalovirus Drug Resistance Detection

Abstract: Human cytomegalovirus (CMV) is a significant contributor to morbidity and mortality in immunocompromised patients, particularly in the transplant setting. The availability of anti-CMV drugs has improved treatment, but drug resistance is an emerging problem. Here we describe an improved, rapid sequencing-based assay for the two genes in CMV where drug resistance occurs, UL97 and UL54. This assay is performed in 96-well format with a single master mix and provides clinical results within two days. It sequences codons 440-645 in UL97 and codons 255-1028 in UL54 with a limit of detection of 240 IU/ml. With this assay, we tested 43 specimens that had previously been tested for UL97 drug resistance and identified 3 with UL54 mutations. One of these patients had no concurrent UL97 mutation, pointing towards the need for an assay that facilitates dual UL97/UL54 testing for complete resistance profiling.

Introduction: Human cytomegalovirus (CMV) causes morbidity and mortality in immunocompromised patients, including transplant and HIV infected patients (1). The first line
drug therapy for CMV infection is ganciclovir (GCV) or its prodrug valganciclovir (VGCV).

GCV or VGCV must be activated by phosphorylation before they act on HCMV. This phosphorylation is carried out by the viral kinase UL97, and activated GCV subsequently inhibits the viral DNA polymerase UL54. Clinically, GCV resistance usually arises first from a UL97 mutation resulting in decreased accumulation of activated drug. Subsequent UL54 mutations can confer high levels of GCV resistance and variable degrees of cross resistance to the second-line drugs cidofovir (CDV) and foscarnet (FOS) (2).

Our group previously published a rapid PCR- and sequencing-based detection method for UL97 mutations conferring ganciclovir resistance (3). This test, currently used to detect mutations directly from clinical specimens, results in sequence for UL97 codons 440-645. This region covers the known drug resistance mutation sites in UL97. However, because UL54 is the target of all currently marketed anti-CMV drugs, a detection method for UL54 mutations is also needed. Polymerase mutations are more likely to emerge after prolonged GCV treatment and typically add to the level of resistance conferred by UL97 mutations or introduce cross-resistance to CDV or FOS (4).

Despite its predicted utility in treatment management, clinical tests for UL54 drug resistance mutations have been slower to develop for a number of reasons. Firstly, the coding sequence of UL54 is almost twice that of UL97. In addition, the baseline sequences are more variable and the number and variety of resistance mutations is greater in UL54 than in UL97. Polymerase genotypic testing therefore requires more extensive sequencing, typically covering codons 300-1000. To meet the clinical need for complete CMV drug resistance profiles, we adapted our previous UL97 sequencing method (3) to develop a single assay that amplifies six regions (2 in UL97 and 4 in UL54) and is performed rapidly in 96-well format with a single
master mix. While other UL54 sequencing assays have been published (5-9), our method has the advantage of being performed in a 96-well format with a single master mix, without need for multiple rounds of PCR, thus minimizing the potential for error when setting up 6 PCR reactions and 12 sequencing reactions per patient sample.

**Materials and Methods:**

**PCR** Four optimized primer sets were designed to amplify four regions of interest (ROI) within UL54 (Figure 1, Table 1). The forward primer for ROI 3 was adapted from Hantz et al. 2010 (5). The UL97 primer sets published by our laboratory for the previous UL97-only method were used in this protocol (3). Primer sets were aliquoted into separate wells for each region of interest and dried down into plates (10 minutes at 95°C) to facilitate the use of a single master mix for all regions.

Amplification by these primers was performed on a StepOne Plus instrument (Life Technologies, Carlsbad, CA), using the Roche FastStart High-Fidelity PCR system, with the inclusion of SYBR green to allow real-time monitoring of accumulation of PCR product. The UL97 and UL54 ROI 1, 2, and 4 primers were used at a final concentration of 400 nM. The ROI3 primers were used at a concentration of 600 nM. The ROI3 reverse primer was created by combining two primers to achieve a G/A mix at the 17th base of the primer. The program for cycling was as follows: denaturation at 95°C for 2 min, followed by 45 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s. Final elongation was done at 72°C for 4 min, with cooling at 37°C for 1 min prior to a melt curve stage of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The melt curve was included in the cycling conditions as a quality control measure. If a single peak was observed at the appropriate temperature the amplification was considered successful and the PCR product was sequenced. The melting temperatures (°C) for each region are as
Negative reactions resulted in melting temperatures between 63 and 78 °C. Resulting amplicons were sequenced in both the forward and reverse direction, using the same primers used for amplification, utilizing the ABI Prism BigDye v.3.1 terminator cycle sequencing kit (0.5 µl Big Dye, 0.75 µl reaction buffer, 0.12 µl 0.4 µM primer, 2.38 µl dH20, 1.25 µl PCR product cleaned with ExoSap-It enzyme (Life Technologies, Carlsbad, CA) per manufacturer’s directions) on an Applied Biosystems 3730XL DNA analyzer, and the sequence was analyzed using Applied Biosystems SeqScape software.

**Sensitivity and Extraction** The sensitivity of the assay was determined by analyzing an Accrometrix® CMVtc Panel (Life Technologies, Carlsbad, CA) containing intact, encapsulated viral particles of CMV strain AD169. The panel consisted of a normal human plasma (tested non-reactive for CMV DNA) and four viral dilutions over a 10-fold concentration gradient from 240-240,000 IU/ml. For each concentration, 1ml was extracted and eluted to 100µl on a Roche MagnaPure LC using the Large Volume Total Nucleic Acid extraction kit (Roche Diagnostics, Basel, Switzerland). PCR was performed as described above and a 1.5% TBE agarose gel stained with ethidium bromide was loaded with 10µl PCR product and 4 µl loading dye and run at 120 V to assess product formation at different template concentrations. The PCR products from the lowest dilution were also analyzed by sequencing. Additionally, two serially diluted patient samples were analyzed to assess sensitivity on clinical specimens.

**Frequency of UL54 mutations in clinical specimens.** The combined UL97/UL54 method was validated against the previous UL97-only method by testing 43 retrospective patient plasma samples from 41 patients. The UL54 results were compared to previous reference lab test results when available. Mutations were also confirmed by performing sequencing in both the forward
and reverse directions. A sample exchange with an outside reference laboratory (ARUP Laboratories, Salt Lake City, UT) was also performed to validate results for the eight specimens with leftover sample available. These samples were residual after routine clinical testing for UL97 resistance from 2005-2012, stored frozen at -20°C. Clinical data was collected for each of the 41 patients with approval from the University of Washington Internal Review Board.

Results:

Sensitivity: The sensitivity of the combined UL97/UL54 method, amplifying 2 regions of UL97 (3) and 4 regions of UL54 (Figure 1, Table 1), was assessed with an Accrometrix® four-member, 10-fold CMV dilution panel. After PCR amplification, gel electrophoresis showed that the assay successfully amplifies the lowest dilution of CMV (240 IU/ml) in all UL97 and UL54 regions (Figure 2). Furthermore, the PCR products from the lowest dilution were successfully sequenced. Our laboratory’s conversion factor for IU/ml to copies/ml puts the limit of detection of this assay around 1,000 copies/ml or better, similar to the sensitivity of the previous UL97-only method (3). The assay was repeated on 2-fold serial dilutions of two patient samples and sequencing was successfully performed on the PCR product from the 780 or 390 copies/ml dilutions for all 6 amplicon regions on both patient samples (Figure S1).

UL54 mutations in patient specimens: We evaluated residual samples from patients previously tested for mutations in the UL97 region to determine the frequency of UL54 mutations in that population. A total of 43 such residual patient plasma samples from 41 patients were available for testing by our combined UL97/UL54 assay. All six amplicon regions sequenced well for all but three samples, where ROI 3 or 4 failed to amplify due to low viral template. There was good agreement between the previous UL97-only method and the new combined UL97/UL54 method for all patients. Twenty four patients with no UL97 mutations by the UL97-only method were
confirmed to have wild type UL97 sequence with the combined UL97/UL54 method. Six of
these samples were confirmed wild type by ARUP Laboratories. Sixteen patients with UL97
mutations detected by the UL97-only method were confirmed to have the same UL97 mutations
by the combined UL97/UL54 method (Table 2). Two of these samples, including one with both
UL97 and UL54 mutations (patient 14), were confirmed by ARUP Laboratories. Four additional
patients had predominantly wild type UL97 sequences with minor drug-resistant mutant variants
(A594V and A594S) detected by the original UL97-only method. Subsequent testing with the
UL97/UL54 method identified only the majority sequence. This discrepancy is likely due to the
well-known, inherent limitations of detecting minor variants by Sanger sequencing (10,11)
combined with the long term storage of these samples.

Importantly, previously unidentified UL54 mutations were revealed by the new combined
UL97/UL54 method for three patients. For two of these (patients 14 and 38), the UL54 mutations
occurred in the setting of previously-selected UL97 mutations (Table 2). For the final patient
(patient 3), a UL54 mutation was present in the absence of a UL97 mutation.

For these three patients, we reviewed their clinical history pertaining to CMV status for
the period around the sample draw date to investigate how UL97/UL54 mutations affect the
course of CMV disease and to check for concordance of our UL54 results with previous
reference lab results. Patient 14 is a 66 year old male first diagnosed with COPD at age 45. He
received a bilateral lung transplant in February 2007 and was given ganciclovir prophylactically
to prevent CMV infection (CMV serostatus D‘R‘). Upon completion of his treatment, his viral
loads began increasing as shown in Figure 3. During an initial increase in CMV viral load UL97
resistance testing indicated wild type genotype. However, a subsequent increase in viral load was
presumed to result from the L595S UL97 resistance mutation, which confers ganciclovir
resistance (3,12,13). Once resistance was identified, drug treatment was switched from
ganciclovir to foscarnet for an extended period, during which UL54 resistance testing (performed
by an outside reference laboratory) initially revealed a wild type UL54 genotype. However, the
V715M UL54 mutation, conferring foscarnet resistance (14-17), was identified during
subsequent testing by the reference lab. These mutations were confirmed with our laboratory’s
combined UL97/UL54 sequencing method.

During the past nearly four years the patient was on an HSV prophylactic dose of
acyclovir, with a brief treatment of valganciclovir during a CMV viral load increase around 1800
days. Our laboratory obtained residual specimen from quantitation assays performed during this
increase in viral load and tested for UL97 and UL54 resistance mutations. These specimens
revealed that the UL97 mutation, L595S, was lost and the UL54 mutation, V715M, was
maintained years after exposure to foscarnet. Detailed analysis of the sequencing performed
around 870 days revealed that the L595S UL97 mutation was present as a mix of variant and
wild type in one sequencing direction and only variant in the other direction, indicating a small
proportion of UL97 wild type virus present in a background of L595S variant. The UL54
sequencing at this time indicated 100% V715M variant in both sequencing directions.

Patient 38 did not receive their direct clinical care from our institution, so only viral load
and resistance profile data were available for analysis. After multiple instances of UL97
resistance testing that identified wild type CMV during an acute viral load increase, UL97
mutation H520Q, conferring ganciclovir resistance (15,16) was identified (Figure 4). Our
laboratory’s validation testing of the combined UL97/UL54 method identified that this UL97
mutation was accompanied by a UL54 mutation, N408D, conferring cidofovir and ganciclovir
resistance (14,16-18).
Patient 3 also did not receive their direct clinical care from our institution and had only one viral load quantitation and one drug resistance testing performed at our institution. This patient was clinically tested for UL97 resistance mutations and found to have a wild type genotype. Subsequent testing for validation of the combined UL97/UL54 assay confirmed a wild type UL97 genotype and identified a UL54 mutation, N408D.

In addition to the three patients with UL54 mutations, we retrieved viral load data for 33 of the 43 specimens sequenced. Analysis of viral load profiles for these patients failed to reveal any consistent patterns in viral load in patients with or without drug resistance mutations (Supplementary Information, Figure S2), possibly due to the heterogeneous nature of our population and the complicated and unpredictable nature of host immune response to viral reactivation and infection in immunosuppressed individuals. These data point to the difficulty of predicting resistance during CMV infection and account for our laboratory’s typical yield on resistance testing: from 2005-2012 only 31% of specimens ordered for UL97 sequencing carried drug resistance mutations.

Discussion:

Human Cytomegalovirus (CMV) results in serious complications in immunocompromised patients, particularly in a bone-marrow or solid-organ transplantation setting. Tissue-invasive CMV disease, such as pneumonia and hepatitis, is responsible for high morbidity and mortality rates in transplant patients. The use of prophylactic anti-CMV therapy beginning early post-transplant has reduced the incidence of CMV associated morbidity and mortality. However, with the increased administration of anti-CMV drugs, there has been an increased emergence of drug resistant CMV. The incidence of drug resistance varies depending on the organ transplanted and immunosuppressive regimen, but it is commonly seen in 5-10% of transplant patients who are CMV-seronegative receiving a CMV-seropositive organ (D+/R-) (4).
CMV drug resistance arises from mutations in the viral kinase UL97 or the DNA polymerase UL54. Clinical genotypic testing of UL97 and, less frequently, UL54, leads to diagnosis of CMV drug resistance. Current CMV treatment guidelines call for initial genotyping of UL97 when drug resistance is suspected. If a drug resistant variant is identified in UL97, subsequent UL54 sequencing is recommended (19). Many cases of resistance have been documented, both as case reports (6,20-26) and cohort studies (5,15,27). Most of these cases report resistance arising from canonical UL97 ganciclovir resistance mutations. Some also examine the incidence of UL54 mutations providing cross-resistance to cidofovir, foscarnet and ganciclovir.

Here we present a rapid method to sequence the conserved regions of UL97 and UL54 where most resistance mutations occur. As evidenced by the multitude of case studies presenting UL97 and UL54 genotyping data, PCR methods for identifying resistance have been available for quite some time. However, most of these methods rely on nested PCR reactions (5,23) which require multiple rounds of PCR before sequencing, increasing the time to result and risk of contamination. Methods that do not rely on nested PCR utilize a LightCycler instrument (6), requiring PCR set-up in capillaries with subsequent PCR product selection and clean up via agarose gel electrophoresis, all of which can become cumbersome for high-throughput clinical analysis. Our method utilizes dried-down primer sets in a 96-well format so a single master mix can be multi-channel pipetted for PCR amplification and sequencing reactions. Confirmation of PCR product formation is performed with melt-curve analysis abrogating the need for gel electrophoresis, a step that adds time and potential for sample contamination or mix-up. This rapid, combined UL97/UL54 method is highly scalable and results in a two day turn-around time from specimen extraction to resulting.
Using this combined UL97/UL54 sequencing method, we identified 3 patients out of 41 tested who had UL54 resistance mutations. The mutations we identified, V715M (Patient 14) and N408D (Patient 3 and 38), are well-characterized resistance mutations (2, 10, 12, 13, 18). Patient 14 is a lung transplant recipient who developed multiple-drug resistant CMV. Cases of patients with dual UL97 and UL54 mutations are particularly prevalent in lung transplant recipients. A recent 39-patient study showed that lung transplant recipients accounted for 57% of patients with dual UL97 and UL54 mutations, even though that transplant procedure accounted for only 22% of CMV-positive patients (27). Several cases of multi-drug resistant, dual UL97/UL54 mutant CMV viruses have been reported in solid organ and stem-cell transplant recipients (21,24,25). A 2002 cohort study identified a lung transplant recipient with a UL54 V715M associated with the UL97 mutation A594V (15).

Interestingly, in the absence of ganciclovir treatment, Patient 14 lost the UL97 L595S variant but maintained the UL54 V715M mutant over years of treatment with just acyclovir and reduced immunosuppression. It is likely that the L595S mutation causes a fitness disadvantage to CMV such that once the selective pressure of ganciclovir was removed, the small population carrying wild type UL97 (evident in the sequencing performed around 870 days) rebounded. This phenomenon has been previously documented in patients with CMV retinitis when therapy was shifted from ganciclovir to cidofovir (26,28).

Identification of the UL54 mutant in the absence of a UL97 mutation points to the question of whether UL54 mutations can exist by themselves. The current paradigm of treatment calls for identification of a UL97 mutation before testing for a UL54 mutation. However, we have identified two patients, 14 and 3, that at some point during treatment harbored a UL54 mutation but not a UL97 mutation. In patient 14, the presence of just a UL54 mutation is likely
the result of a fitness cost of the prior UL97 mutation. For patient 3 the clinical history is unknown. It is possible that the UL54 mutation N408D arose without a prior UL97 mutation, although no previous samples are available. In a 2010 study of transplant recipients, a kidney transplant recipient was identified as having CMV with UL54 mutation A834P, conferring resistance to ganciclovir, cidofovir, and foscarnet, without a concomitant UL97 mutation 158 days post antiviral exposure (5).

This new rapid method for combined UL97/UL54 drug resistance testing contributes significantly to higher throughput clinical testing for CMV drug resistance diagnosis. It simplifies UL97 and UL54 analysis, allowing for more comprehensive resistance genotyping that is as sensitive as the previous UL97 only method. Although current consensus recommends testing only UL97 initially, we highlight the possibility of UL54 mutations occurring without UL97 mutations. With this new, more efficient assay, routine combined testing can be prospectively studied to define clinical utility.

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**References:**


Figure 1. Schematic of CMV UL97 and UL54 and the regions of interest amplified by this assay. Adapted from Lurain and Chou 2010 (4).

Figure 2. Assay sensitivity assessed by amplification of an Accrometrix® CMVtc Panel
Representative 1.5% TBE gel electrophoresis of ROI1 PCR using an extracted Accrometrix® CMVtc Panel as template. Lanes 1-5 represent panel components as follows: 1- negative control; 2- 240 IU/ml; 3- 2,400 IU/ml; 4- 24,000 IU/ml; 5- 240,000 IU/ml. Lane 6 is a CMV positive control. Equivalent results were obtained for all UL97 and UL54 ROIs.
Table 1. UL54 primers used in this study

<table>
<thead>
<tr>
<th>UL54 Region</th>
<th>Primer sequences</th>
<th>Sequence coverage (codons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROI 1</td>
<td>F primer GGT GCT CCG TGA ATC GTT AC</td>
<td>255-465</td>
</tr>
<tr>
<td></td>
<td>R primer GTG AGA AGC CGA GGG AAA GG</td>
<td></td>
</tr>
<tr>
<td>ROI 2</td>
<td>F primer CGG CCG CCA CCA AGG TGT ATA TTG</td>
<td>471-607</td>
</tr>
<tr>
<td></td>
<td>R primer GCA CCG TCG TAC CTT TGC TGT AG</td>
<td></td>
</tr>
<tr>
<td>ROI 3</td>
<td>F primer CGT TGC TGT GTC ACC TAA CG</td>
<td>612-860</td>
</tr>
<tr>
<td></td>
<td>R primer AAC ACG GCT CTG AAA A G/A TTG</td>
<td></td>
</tr>
<tr>
<td>ROI 4</td>
<td>F primer CGC GGT TCA TCA AAG ACA AC</td>
<td>848-1028</td>
</tr>
<tr>
<td></td>
<td>R primer CAC GCC GTA TTT CTT GAC TT</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Retrospective patient samples tested for UL54 mutations and concordance of UL97 results between the combined UL97/UL54 method and UL97-only method. Dash indicates no mutation present, NA indication no result available.

<table>
<thead>
<tr>
<th>Patient</th>
<th>UL54 mutation</th>
<th>UL97 mutation</th>
<th>Viral quantity* (copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>N408D</td>
<td>-</td>
<td>2,200</td>
</tr>
<tr>
<td>14</td>
<td>V715M</td>
<td>L595S</td>
<td>6,800</td>
</tr>
<tr>
<td>38</td>
<td>N408D</td>
<td>H520Q</td>
<td>1,200</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>M460V</td>
<td>12,000</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>A594V</td>
<td>140,000,000</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>L595S</td>
<td>14,000</td>
</tr>
<tr>
<td>17</td>
<td>-</td>
<td>L595S</td>
<td>300,000</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>Del600-603</td>
<td>200,000</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>Del596-603</td>
<td>470,000</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>M460V</td>
<td>4,600</td>
</tr>
<tr>
<td>27</td>
<td>-</td>
<td>H520Q</td>
<td>20,000</td>
</tr>
<tr>
<td>32</td>
<td>-</td>
<td>H520Q</td>
<td>12,000</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>A594V</td>
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</tr>
<tr>
<td>41</td>
<td>-</td>
<td>L595F</td>
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</tr>
<tr>
<td>42</td>
<td>-</td>
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<td>11,000</td>
</tr>
<tr>
<td>43</td>
<td>-</td>
<td>L595S</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Viral quantity is from original clinical viral load testing prior to sample storage.
Figure 3. CMV viral load, treatment, and mutation time course for patient 14. Blue dots indicate quantitation by qPCR. Tick marks in mutation labels indicate dates of testing. Asterisk indicates resistance testing performed for this study. UL97 results without an asterisk were from clinical testing using the UL97-only method. UL54 results without an asterisk were done by a reference lab.

Figure 4. CMV viral load and mutation profile for patient 38. Blue dots indicate quantitation by qPCR. Tick marks in mutation labels indicate dates of testing. Asterisk indicates resistance testing performed for this study. UL97 results without an asterisk were from clinical testing using the UL97-only method.