NOTES

Rapid Detection Directly from Patient Serum Samples of Human Cytomegalovirus UL97 Mutations Conferring Ganciclovir Resistance

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Ganciclovir-resistant cytomegalovirus can cause disease and death in transplant recipients. We describe here a rapid PCR- and sequencing-based assay for ganciclovir resistance that can be performed in 1 to 2 working days directly from patient specimens, without the need for amplification of the virus by cell culture. An evaluation of 120 sequential samples submitted for clinical testing revealed a variety of silent and amino acid mutations.

Human cytomegalovirus (HCMV) is an ubiquitous pathogen that can cause severe or fatal disease in transplant recipients. First-line therapy consists of ganciclovir, and the use of this agent has markedly reduced the morbidity and mortality of HCMV infections (1). However, the development of resistance to ganciclovir has been increasingly recognized (8). About 90% of ganciclovir resistance results from mutations in the HCMV phosphotransferase gene, UL97 (5). Most of these mutations occur within the putative ATP-binding (codons 460 to 520) and substrate recognition (codons 590 to 607) sites of UL97 (4). At least two UL97 mutations associated with ganciclovir resistance outside these regions have been reported (10, 11), but these mutations appear to be rare, and at least one appeared in the context of an accompanying mutation within the substrate recognition site. The availability of clinical testing for mutations conferring ganciclovir resistance may be of prognostic value and could allow the timely choice of alternative antivirals.

To be clinically useful, an assay for ganciclovir resistance must be highly accurate, and detect multiple mutations associated with resistance. Furthermore, it must be performed rapidly, such that the results are available in a clinically relevant time frame. In this regard, the gold standard method for mutation detection, sequencing of the viral genome, has been limited by the need to amplify virus in culture before PCR and sequencing can be performed (9). Since the growth of CMV in culture is extremely slow, this typically results in delays of 3 to 4 weeks until the assay is completed, at which time the clinical utility of the result may be limited.

To address the need for a rapid HCMV resistance assay suitable for use directly on clinical specimens, we first sought to optimize the efficiency of PCR amplification of the UL97 region containing the most common mutations. The most commonly used PCR primers for this region, described by Lurain et al. (9), generally are used for PCR on viral isolates that have been amplified by tissue culture and fail to successfully amplify direct patient samples (unpublished observations). These primers amplify a 921-bp region of UL97 (from codons 400 to 707), and we reasoned that the efficiency of amplification by these primers might be limited by their length. Our hypothesis was supported by the results of Spector and coworkers, who used seven overlapping primer sets to amplify and sequence the relevant region of UL97 directly from plasma from 10 patients with known ganciclovir-resistant CMV infections (10, 11), and by the results of Jabs and coworkers, who used a nested PCR approach to amplify this region for sequencing directly from patient materials (6, 7). Since the use of seven primers sets would be impractical for most clinical laboratories and to avoid the need for nested PCR, we designed two optimized primer sets allowing amplification of this region: forward primer 5'-TGG CCG ACG CTA TCA AAT TT-3' and reverse primer 5'-CCC AGC GCC GAC AGC TCC GAC-3', amplifying codons 439 to 557, and forward primer 5'-ATG CTC GAG GTC ATC TTG-3' and reverse primer 5'-CCC AGC GCC GAC AGC TCC GAC-3', amplifying codons 550 to 645. Amplification by these primers was performed on a Roche LightCycler instrument, 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 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. Both primer sets efficiently amplified HCMV, with an efficiency comparable to our clinical HCMV quantitation assay (2) (data not shown). The resulting amplimers were then sequenced by using the ABI Prism BigDye terminator cycle sequencing kit on an Applied Biosystems 3730
DNA analyzer, and the sequence was analyzed by using Applied Biosystems SeqScape software (Fig. 1). The assay could be completed and reported on the next working day after receipt of the specimen.

Since the major impetus for developing the new assay was to allow analysis from direct patient specimens, we performed probit analysis on serially diluted HCMV to determine the detection limit for the assay. The 95% detection limit for obtaining usable sequence information was <750 copies/ml for both amplicons. Since clinically relevant resistance is unlikely at viral loads below this level, we considered the sensitivity of the assay suitable for clinical use. We therefore performed an initial evaluation of the assay on 31 clinical specimens, consisting of plasma (n = 9), serum (n = 8), blood, (n = 2), cerebrospinal fluid (n = 2), vitreous fluid (n = 1), and fluids not otherwise identified (n = 9). Fourteen specimens had viral loads in excess of 10,000 copies/ml, and all fourteen provided usable sequence with both the 439-557 and 550-645 primer sets. Of 17 specimens with viral loads between 1,000 and 10,000, 16 (94%) and 15 (88%) provided usable sequence with the 439-557 and 550-645 primer sets, respectively. Notably, the assay was able to identify mixed infections with mutant and wild-type strains (Fig. 1), when the minor strain comprised 10 to 20% of total virus.

We next compared the performance of our rapid assay to that of established assays based on analysis of culture-expanded specimens. Stored culture fluid, obtained from CMV-positive clinical samples that had been sent to an outside commercial reference laboratory for phenotypic and/or genotypic drug resistance testing, was recultured and the supernatant tested with our new assay. A subset of these samples had been tested in-house for genotypic drug resistance by the primer set described by Lurain et al. (9). Of 20 samples compared to the commercial test, 18 showed agreement, while for two samples a mutation (Q449K) was identified by our rapid assay that is not reported by the commercial reference laboratory assay. Similarly, of 15 samples compared to culture and the in-house assay using the primer set of Lurain et al., 14 showed agreement. In one specimen, the new method identified one mutation (D605E) that was not detected by the traditional assay. In a recent study evaluating this issue, there was >90% agreement between UL97 sequences directly amplified from clinical specimens and those from culture isolates (7). In the case of discrepancies, it is likely that the results of an assay performed directly on patient specimens more closely reflect the clinically relevant viral species.

Finally, we monitored the performance of our rapid assay in clinical use over the period from July 2004 to December 2006. At our institution, the assay is used mainly in the management of patients receiving solid organ or stem cell transplants. Of 143 specimens submitted for resistance testing, usable sequence was obtained from 120 (84%). The most common cause of assay failure was the submission of samples with very low or undetectable viral levels. Of the six samples that had detectable virus but could not be sequenced, five had viral levels of <200 copies/ml, and the other had 1,300 copies/ml. The 120 samples successfully sequenced were from 71 different individuals. Overall, only 9 of 71 patients had wild-type sequences at the nucleotide level compared to the reference AD169 strain, whereas 25 of 71 had silent nucleotide mutations that did not result in amino acid substitution. The most common mutations seen in the samples were the silent mutations D456D, G557E, G579G, and L634L. Multiple mutations were found in the same sample ca. 30% of the time and probably indicate the presence of distinct CMV genotypes in our

![FIG. 1. Detection of CMV UL97 mutations. The figure shows a typical sequencing result and analysis from a clinical specimen showing infection with a mixture of two viral variants (arrows). seq., sequence.](http://www.asmscience.org/content/jcm/54/7/2682.full.pdf)
samples (3). Of the patients with amino acid mutations, 25 of 37 (68%) had at least one mutation previously reported to be associated with ganciclovir resistance. An additional 12 of 37 patients had a single amino acid mutation with uncertain impact on resistance: D605E, H469Y, and Q449K (8, 3, and 1 patient, respectively). Ten patients (14%) had evidence of mixed infection with more than one sequence. Of the 22 patients with samples from more than one time point, 5 (23%) had changes in the UL97 sequence over time. A single patient had virus with different UL97 sequences detected from urine versus cerebrospinal fluid. The mutations detected in all 120 specimens are described in Table 1.

In summary, our assay allows the rapid determination of genotypic HCMV resistance directly from patient specimens. The timely detection of HCMV resistance has enabled clinicians to tailor antiviral therapy for their patients in a clinically relevant time frame, leading to increased demand for HCMV resistance testing. Further studies will determine the manner in which such testing can best be utilized for the management of patients at risk for HCMV disease.

REFERENCES


