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# 5

# Mass spectrometry-based comparative sequencing to detect ganciclovir resistance in the UL97 gene of human cytomegalovirus

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## Abstract

*Background:* Persistent infections with herpesviruses such as human cytomegalovirus (HCMV) frequently occur after solid organ or stem cell transplantation, and are due to either failure of the host to immunologically control the virus or emerging resistance of the virus to the antiviral drug(s) used. Antiviral therapy can be guided by viral drug susceptibility testing based on screening for known resistance-inducing mutations in the viral genome. Mass spectrometry-based comparative sequence analysis (MSCSA) might be advantageous for this purpose because of its suitability for semi-automation.
*Objectives:* The applicability of MSCSA to detect sequence polymorphisms and drug resistance-inducing mutations in the HCMV genome was investigated.
*Study design:* We analyzed the 3′ part of the HCMV UL97 gene, which encodes the kinase that is activated by the commonly used anti-HCMV drug ganciclovir. Sequences obtained by MSCSA of material from HCMV-infected patients (43 samples) and the HCMV type strain were compared to conventional cycle sequencing results.
Results: In 94.1% of all samples the results obtained by MSCSA of the UL97 gene were identical to those from conventional cycle sequencing. The threshold to detect mutant sequences in a mixture with wildtype material was 20% using either technique. Furthermore, MSCSA was successfully applied to study the development of drug resistance in a patient who developed encephalitis due to ganciclovir-resistant HCMV.
*Conclusions:* MSCSA was found to be equally accurate compared to conventional cycle sequencing in the analysis of UL97 of HCMV.

## BACKGROUND

Human cytomegalovirus (HCMV) is a beta-herpesvirus with an approximately 235-kbp double-stranded (ds) DNA genome that encodes at least 165 gene products.[1] Primary infection in adult, immunocompetent individuals is mostly mild or asymptomatic.[2] However, as with other herpesviruses, infections with HCMV can cause major problems after solid organ or stem cell transplantation (SCT). When the host fails to immunologically control the virus infection, HCMV can persist for weeks and cause severe morbidity and mortality. Antiviral drug resistance is relatively rare,[3-5] but can occur in immunocompromised patients after prolonged antiviral therapy.[6-11] HCMV infections are commonly treated with ganciclovir (GCV) or its orally administered prodrug valganciclovir (vGCV).[12;13] Activation of this deoxyguanisone analogue requires three successive phosphorylation steps carried out by the kinase encoded by the viral UL97 gene and two cellular kinases.[14] This yields the active triphosphate form of the drug that selectively inhibits the viral DNA polymerase UL54 by disrupting viral DNA synthesis.[15] GCV resistance mutations in clinical isolates mainly map to the viral kinase gene UL97,[16-19] but after prolonged treatment mutations in the viral polymerase gene UL54 can also emerge.[19;20] In the case of a persistent infection, drug susceptibility testing of the virus can support antiviral therapy management.

## OBJECTIVES

We have evaluated the application of a recently developed method for mass spectrometry-based comparative sequence analysis (MSCSA) to the identification of drug resistance-associated mutations in HCMV. The results were systematically compared to those obtained using a conventional cycle sequencing method. Furthermore, we studied the development of antiviral drug resistance in a SCT patient who developed encephalitis caused by a GCV resistant HCMV mutant.

## STUDY DESIGN

***Virus strains and patient samples***

Forty-three CMV DNA positive clinical samples (EDTA-plasma) from forty-three different (immunocompromised) patients with HCMV infection were selected for comparison of single nucleotide polymorphism (SNP) detection. Clinical and antiviral treatment

*Table 1. List of amplicons and primers used for MSCSA and cycle sequencing analysis of UL97 of HCMV.*

| Application | Forward primer[a] | HCMV-specific sequence | Reverse primer[b] | HCMV-specific sequence | Amplified region[c] |
|---|---|---|---|---|---|
| MSCSA Amplicon 97AB | CMV97A F | gtgctcacggtctggatgtcg | CMV97B SP6R | ggtaacattcgcgcagacggtg | 1090–1558 |
| MSCSA Amplicon 97CD | CMV97C F | gcgcgccgcatccccaactg | CMV97D SP6R | catggtctgcgagcattcgtg | 1513–1980 |
| Cycle sequencing analysis | 1125CMVRs | gtgctcacggtctggatgt | 1135CMVRas | cggtgggtttgtaccttctc | 1090 ± 65 |

[a] Forward primers for MSCSA PCR were tagged with T7 promoter: 5′-cagtaatacgactcactatagggagaaggct-3′.
[b] Reverse primers for MSCSA PCR were tagged with SP6 promoter: 5′-cgatttaggtgacactatagaagagaggct-3′.
[c] Numbers indicate most 5′ or 3′ nucleotide in UL97 of the AD169 HCMV type strain (GenBank accession number X17403) that anneals with the forward or reverse primer, respectively.

*Table 2. Comparison of the performance of MSCSA and cycle sequencing analysis of UL97 of HCMV.*

| | Total | Sequence match[a] | SNP in MSCSA[b] | SNP in cycle sequencing[c] | Amplicons in duplicate (MSCSA) | Identical duplicate MSCSA sequences |
|---|---|---|---|---|---|---|
| Samples analyzed | 44 | n.a. | n.a. | n.a. | n.a. | n.a. |
| MCSCA reactions (97AB + 97CD) | 136 | 128 (94.1%) | 1 (0.7%) | 7 (5.1%) | 58 | 56 (96.6%)[d] |
| SNPs detected | 289 | 281 (97.2%) | 1 (0.3%) | 7 (2.4%) | n.a. | n.a. |
| Nucleotides sequenced | 58,015 | 58,007 (99.99%) | n.a. | n.a. | n.a. | n.a. |

n.a. not applicable.
[a] Identical between MSCSA and cycle sequencing results.
[b] SNPs (mutations compared to the AD169 reference sequence) found by MSCSA, but not by cycle sequencing.
[c] SNPs found by cycle sequencing, but not by MSCSA.
[d] The non-matching duplicate MSCSA sequences were identical to the cycle sequence results of the individual PCR products that were used for MSCSA analysis.

data were derived from patient charts (see Table S1).[5] Additionally, nine plasma-EDTA and four cerebrospinal fluid (CSF) samples from a case patient with HCMV encephalitis (see below) were included for analysis of GCV resistance. DNA was isolated from all samples with the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics) using a MagNA Pure LC Instrument (Roche Diagnostics). HCMV strain AD169 was obtained from American Type Culture Collection, Manassas, VA, USA. HCMV DNA loads were determined by real-time PCR.[21]

***Mass spectrometry-based comparative sequence analysis (MSCSA) and cycle sequencing***

The MassARRAY® /iSEQTM – comparative sequence analysis technique (Sequenom, San Diego, USA;[22] ) uses PCR amplification of a target and its subsequent in vitro transcription to produce RNA strands that are cleaved to produce a sequence-specific set of fragments for analysis by mass spectrometry. The obtained spectra are then compared to theoretical spectra derived from a database with reference sequences.

A schematic representation of the MSCSA and cycle sequencing approaches for HCMV UL97 analysis is provided in Figure 1. The 3′ region of the HCMV UL97 gene was PCR amplified in two amplicons, UL97AB and UL97CD, of 469 and 468 bp, respectively, by use of T7 promoter-tagged forward primers and SP6-tagged reverse primers (see Table 1). PCR reactions were performed in a 10-ml volume in 384-well microtiter plates, as previously described.[5]

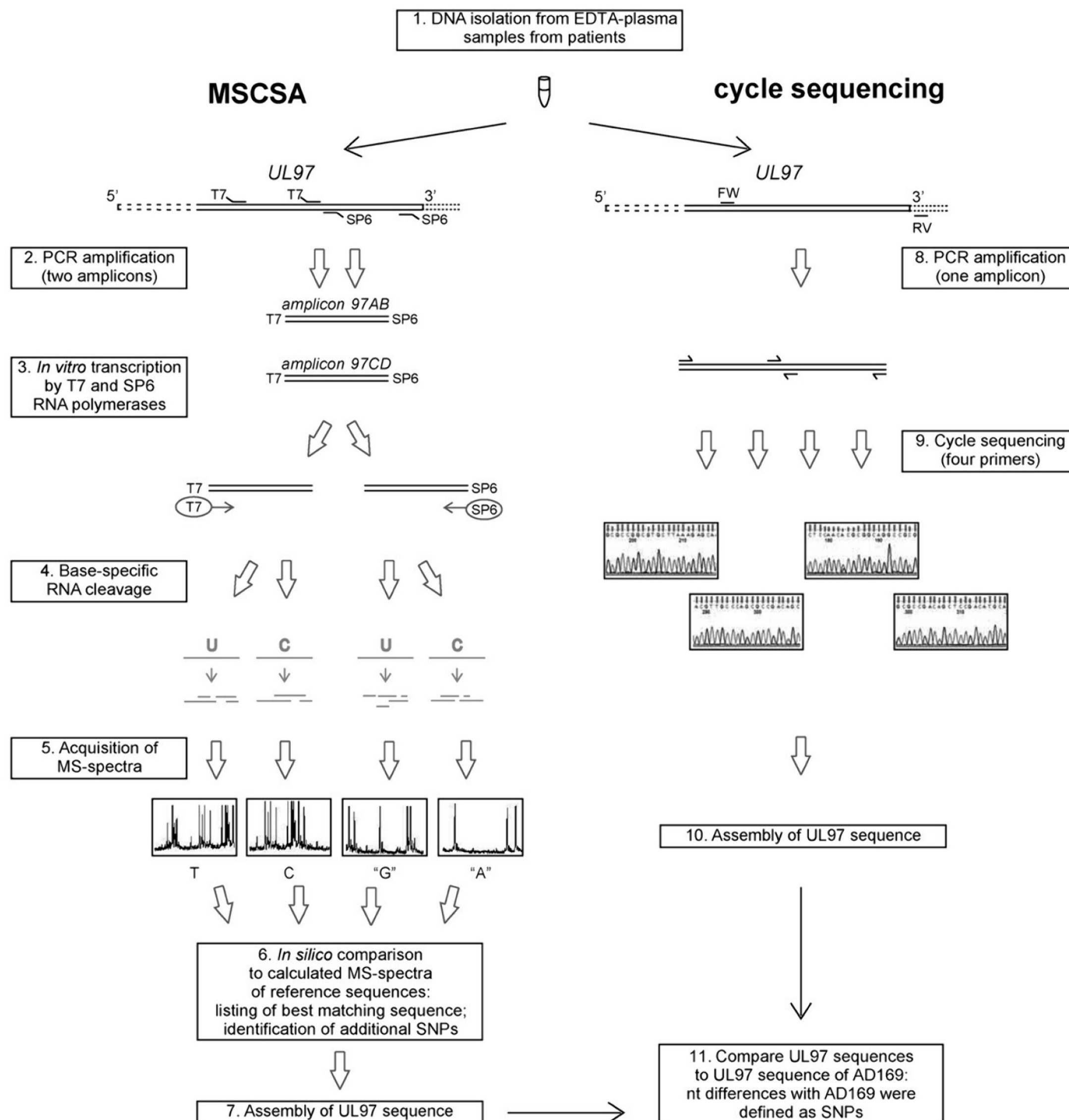
The sample was further processed by shrimp alkaline phosphatase treatment, in vitro transcription, and Cor U-specific RNaseA cleavage, according to the manufacturer's instructions and using a MassARRAY® Liquid Handler (Matrix + FusioTM Chip Module; Sequenom, San Diego). The fragments resulting from RNA cleavage were diluted in double-distilled water and desalted with clean resin (Sequenom), transferred to a SpectroCHIP array (Sequenom), and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MassARRAY Compact Analyzer, Sequenom). The acquired spectra were analyzed using iSEQTM Software Version 1.0.0.2.

A set of reference sequences was created for both the 97AB and the 97CD amplicon, based on previously published UL97 sequences from HCMV clinical isolates that were GCV-sensitive in phenotypic assays.[23] The set was later supplemented with three and nine sequences for amplicons 97AB and 97CD, respectively, derived from clinical isolates from Leiden University Medical Center (GenBank accession numbers GU992367–GU992375).

***Cycle sequencing reactions were performed on an ABI Prism***

3100 Genetic Analyzer (Applied Biosystems) after PCR amplification of the 3′ region of the UL97 gene from all samples, as previously described (see Figure 1).[5] Assembled

*Figure. 1. Schematic overview of HCMV UL97 genotyping by MSCSA and cycle sequencing.*




**Step 1.** After isolation of CMV DNA from patient samples, each sample was analyzed by either method. **Step 2**. (MSCSA) Amplification of the 3′ part of the HCMV UL97 gene in two amplicons (UL97AB and UL97CD) by use of T7 promoter-tagged forward primers and SP6-tagged reverse primers. **Step 3**. In vitro transcription of both amplicons by T7 and SP6 RNA polymerase (two reactions with each enzyme). **Step 4.** C- or U-specific RNaseA cleavage of plus and minus sense RNA transcripts. **Step 5**. Acquisition of MS-spectra of the resulting RNA cleavage products. Using these four base-specific cleavage reactions per sample each nucleotide in the sequence is specifically probed. **Step 6**. Comparison of the acquired MS spectra using iSEQTM Software Version 1.0.0.2, which lists the best-matching sequence from a database of reference sequences and any additional sequence variations for each target region. **Step 7**. Assembly of the amplicon 97AB and 97CD sequences. **Step 8**. (cycle sequencing) Amplification of the 3′ part of the HCMV UL97 gene in one amplicon. **Step 9**. Four cycle sequencing reactions were performed per PCR product. **Step 10**. Assembly of the four obtained sequences. **Step 11.** Comparison of MSCSA and cycle sequencing results. Nucleotide differences compared to the UL97 sequence of AD169 were defined as SNPs.

UL97 sequences were aligned to the sequence of the AD169 strain (GenBank accession number X17403) and SNPs were defined as nucleotide variations compared to this reference sequence. Data from both methods were compared (Figure 1, Step 11).

***Analysis of mixed wild-type and mutant sequences***

HCMV gene UL97 and a region of gene UL96 were cloned by inserting a 2.9 kb BglII-XhoI restriction fragment from construct pHB5[24] (kindly provided by Dr. Albert Zimmerman and Dr. Hartmut Hengel, Düsseldorf, Germany) into a pBluescript KS-derived (Stratagene) shuttle vector with the appropriate restriction sites. A 434-base pair fragment from this plasmid (corresponding to position 1587–2021 in UL97) was replaced by a PCR-amplified UL97 gene product from an HCMV isolate of the encephalitis patient described below, introducing a C1781T mutation (encoding a A594V amino acid substitution) and three translationally silent mutations. Wild-type and mutant constructs were mixed in various ratios to give a final plasmid DNA concentration of 1 ng/ml.

## RESULTS

***Comparing the accuracy of MSCSA and cycle sequencing***

To assess the accuracy of MSCSA, we analyzed amplicons 97AB and 97CD derived from 43 clinical samples and from the HCMV type strain AD169 in duplicate. This set included plasma samples from 43 immunocompromised patients containing GCV-sensitive and GCV-resistant HCMV. The sequences obtained were verified by conventional cycle sequencing and every mismatch with the AD169 sequence was defined as a SNP. The 97AB or 97CD PCR amplification needed for MSCSA analysis failed for some of these samples (in 40 of 176 reactions). As a result, amplicon 97AB could be analyzed in 35 samples and amplicon 97CD in 43 samples, and duplicate reactions were available for 22 and 36 samples, respectively. The detection limit for clinical samples was comparable for both techniques (10 log3.6 copies/ml), although especially the amplification of the 97AB amplicon regularly failed for samples with lower viral loads (see Table S2).

After optimization of the reference database, 128 MSCSA sequences (94.1%) were identical to the cycle sequencing data of the same sample. Discrepancies were found in 8 samples, with one sequence containing a SNP recognized by MCSCA and seven by cycle sequencing only (Table 2). Accordingly, 97.2% of the SNPs were recognized by both methods. Overall more than 58 kb of UL97 sequence were analyzed by both methods, with as few as eight (0.01%) nucleotides differing between the MSCSA and cycle sequencing-derived sequences.

Fifty-eight reactions were performed in duplicate, which revealed that the variability between independent MSCSA experiments was low: for 56 samples (96.6%) identical MSCSA results were obtained. Discrepancies between duplicate MSCSA sequences or between MSCSA and cycle sequencing results were confirmed in two thirds of samples by cycle sequencing of the MSCSA PCR product (see Table S2).

Seven of the analyzed patient samples contained GCV resistance associated mutations.[16;25-28] Of these, four samples containing A594V; C592G, A591V and L595F substitutions in UL97 were identified by cycle sequencing and subsequently confirmed by MSCSA analysis. Three other resistance-associated SNPs could not be verified by MSCSA as PCR reactions failed due to low viral loads (in two samples; M460I and M460V) or probably due to the presence of mixed viral populations resulting in discrepancies between MSCSA and cycle sequencing results (C603W; see above and Table S2).

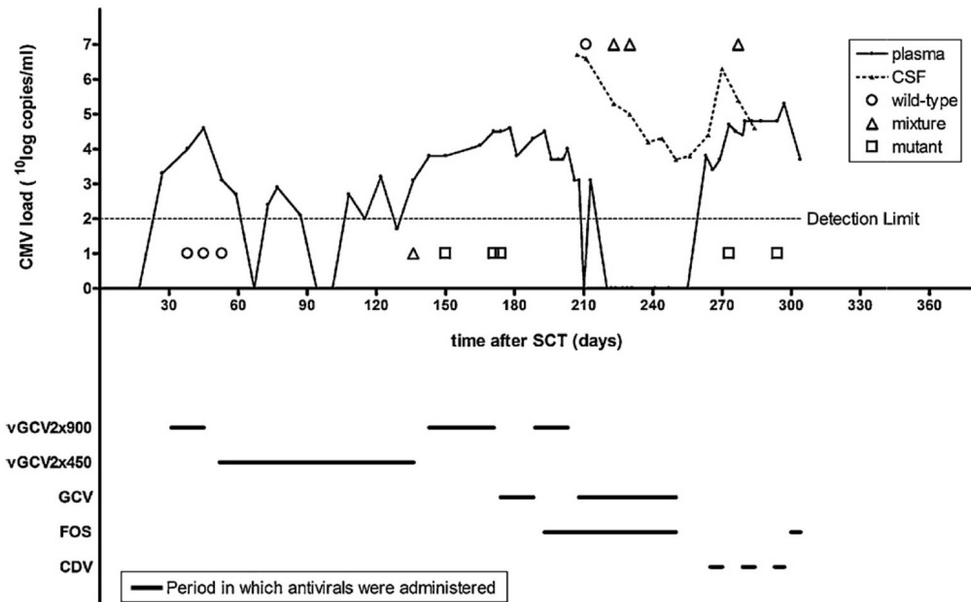
To mimic the clinical situation of a patient in which a drug resistant viral subpopulation begins to emerge due to the selection pressure of antiviral treatment, samples containing a mixture of wild type and mutant (C1781T) UL97 plasmids were analyzed. Using either method, the mutation and three translationally silent SNPs could be detected in mixtures containing 20% or more of the mutant sequence.

***Application of MSCSA to a case of HCMV encephalitis after SCT***

A 52-year old female with relapsed acute myeloid leukemia, secondary to myelodysplastic syndrome, received a haplo-identical T-cell depleted peripheral blood SCT after a myeloablative conditioning regimen. The patient was seropositive for HCMV, whereas the donor was seronegative. The patient was treated according to a preemptive HCMV treatment protocol, which was guided by HCMV DNA loads in plasma.[21] The patient's plasma became positive for HCMV DNA at 27 days after transplantation and she was treated with oral vGCV, 900 mg twice daily for two weeks. Because of persistent low levels of HCMV DNA and a lack of T cell recovery, treatment with a reduced vGCV dose was started at day 45 after transplantation (450 mg twice daily) and continued for 4 months. However, during the last month of treatment, HCMV DNA loads steadily increased and resistance to GCV was suspected. This was treated with intravenous GCV (5 mg/kg twice daily) for two weeks and, subsequently, with valganciclovir (900 mg twice daily) and foscarnet (60 mg/kg three times daily) for two weeks.[29]

Because the patient developed cognitive and neurological impairments, CSF was analyzed, which revealed a HCMV DNA load of 10 log 6.7 copies/ml. Other causes of encephalitis were excluded and MRI of the brain showed diffuse non-specific white matter abnormalities, supporting the diagnosis of HCMV encephalitis. Combination treatment with foscarnet (60 mg/kg three times daily) and GCV (5 mg/kg twice daily)

*Figure 2. Case report.*




In a patient with a persistent HCMV DNA load, despite antiviral treatment with valganciclovir (vGCV) and ganciclovir (GCV), encephalitis developed and high levels of HCMV DNA were found in the cerebrospinal fluid (CSF). Sequence analysis of the HCMV UL97 gene, derived from nine plasma and four CSF samples, was performed in duplicate using cycle sequencing and MSCSA and revealed consistently wild-type isolates (circles), mixed sequences (wild-type UL97 and the mutant encoding the A594V substitution; triangles) or exclusively mutant virus (squares). The results between both methods matched for all samples tested. vGCV2 × 900: twice a day 900 mg vGCV; vGCV2 × 450: twice a day 450 mg vGCV.

was started, followed after two months by cidofovir (5 mg/kg once weekly for three weeks). Unfortunately, the patient died from urosepsis.

Using MSCSA and cycle sequencing, plasma samples taken between days 38 and 53 showed no mutations, but later a mixture of wild-type and C1781T mutant UL97 sequences was detected (Figure 2). This mutation, specifying a A594V mutation in the UL97encoded kinase is known to be associated with resistance to GCV.[25;30] It remained present in subsequent DNA samples isolated from plasma. The HCMV DNA extracted from CSF samples also contained mixed sequences with this mutation.

## DISCUSSION

In immunocompromised patients, information on the presence or absence of drug resistance-associated mutations may help to guide treatment of persistent or recurrent

HCMV infections. In such situations, virus characterization needs to be sensitive, accurate, and rapid in order to be of clinical relevance. We investigated the applicability of an MSCSA method for automated high-throughput DNA sequence analysis for the detection of sequence polymorphisms and drug resistance-inducing mutations in the 3′ region of the HCMV UL97 gene, and compared the results to cycle sequencing analysis of the same genome region. This method was previously developed by Sequenom and validated for genotyping of Neisseria meningitidis. The method was presented as a highly reproducible alternative to sequence analysis methods relying on chain termination by dideoxynucleotide incorporation.[22] Recently the first reports on the use of this technique for viral genotyping were published.[31;32]

We conclude that Sequenom's MassARRAY protocol, in combination with the iSEQ software, can be equally accurate compared to conventional sequencing techniques (see Table 2). The presence of mixed virus populations in patient samples may have contributed to the differences between duplicate MSCSA experiments and between MSCSA and cycle sequencing experiments, as the presence or absence of the discrepant SNPs in the MSCSA sequences were confirmed in many of the samples by cycle sequencing of the MSCSA PCR product. The use of two amplicons to cover the UL97 region of interest resulted in a clinically relevant detection limit of 10 log3.6 HCMV copies per ml. The sensitivity of mutation detection in the mixed plasmid DNA samples was comparable to cycle sequencing as well. We would like to stress that the accuracy of SNP detection by MSCSA is largely dependent on the quality (and quantity) of the sequences in the reference database, as performance improved considerably when the databases were supplemented with new sequences (data not shown).

Various genotypic screening methods have been developed for mutation detection in viral genomes, including that of HCMV, each with its own advantages and limitations.[33-35] Some methods allow rapid screening, but only of fixed genome positions known to be involved in antiviral drug resistance. MSCSA combines the possibility of detection of all nucleotide variations within a designated region of a viral genome with reduced hands on time due to the automation of post-PCR processing and analysis. Recently developed deep-sequencing methods allow for detection of minor variants of HCMV in patients[36], but are laborious and may be less suitable in a clinical setting. MSCSA is performed in a 96-well format and the experimental processing of one plate (48 samples, as the UL97 gene was amplified in two amplicons) is comparable to the workload of conventional cycle sequencing of about 32 samples: results are obtained in about twelve hours, of which four hours are hands-on time. The costs for the analysis

of one sample by MSCSA (two amplicons) are estimated to be twice as high as compared to cycle sequencing (one amplicon): the needed chemicals and disposables were estimated to cost around twelve to fourteen euro (excl VAT). Optimization of the PCR step in MSCSA (e.g., PCR amplification of the 3′ end of UL97 using a single amplicon, instead of the current two) would further reduce the average processing time and cost needed for MSCSA.

The MSCSA method evaluated here was successfully applied to a case of HCMV encephalitis, a rare complication after SCT that is attributed to antiviral drug resistance.[37-40] In agreement with previous cases, the prolonged use of GCV in combination with failing T-cell immune recovery likely contributed to the progression of infection in this patient. The association in this case between the treatment with a low dose of vGCV, motivated by possible myelotoxicity of the drug, and the subsequent development of resistance and viral encephalitis is compelling although not completely proven. Based on the results presented in this study, we conclude that MS-based comparative sequence analysis constitutes a reliable medium to high-throughput screening method that can be applied to detect resistance markers and other point mutations in viral genomes.

## COMPETING INTERESTS

None declared.

## FUNDING

None.

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*Table S1. Details about the patient samples that were analyzed by MSCSA and cycle sequencing.*

| Patient | underlying disease[1)] | antiviral treatment (days) | CMV load ($^{10}$log copies/ml) | polymorphisms/ mutations[2)] |
|---|---|---|---|---|
| AD169 | ATCC strain | N.A. | N.A. | none |
| 1 | RT | GCV 8d | 5,2 | none |
| 2 | myc fun | none | 6,3 | none |
| 3 | SCT | vGCV 2d FOS 6d | 3,6 | none |
| 4 | NHL | GCV 4d | 4,3 | none |
| 5 | SCT | GCV 6d | 4,3 | **C592G** |
| 6 | RT | none | 5,9 | D605E |
| 7 | RT | vGCV 14d | 4,2 | R686Q |
| 8 | RT | none | 4,8 | none |
| 9 | RT | none | 3,5 | H469Y |
| 10 | RT | none | 4,8 | none |
| 11 | RT | vGCV 15d | 3,8 | N510S, D441N |
| 12 | RT | vGCV 50d | 4,9 | N510S; **A594V** |
| 13 | RT | vGCV 70d | 4,0 | A478T; N510S; **A594V** |
| 14 | SCT | vGCV 3d | 4,5 | none |
| 15 | RT | none | 5,0 | none |
| 16 | RT | none | 3,6 | N467S |
| 17 | RT | none | 5,3 | none |
| 18 | RT | none | 5,6 | none |
| 19 | RT | vGCV 60d GCV 13d | 3,3 | H469Y; **C603W** |
| 20 | RT | vGCV 28d | 3,5 | E575K |
| 21 | SCT | none | 4,0 | none |
| 22 | SCT | vGCV 14d | 4,5 | H469Y |
| 23 | SCT | vGCV 48d GCV 22d FOS 14d | 4,6 | none |
| 24 | SCT | none | 3,4 | none |
| 25 | SCT | vGCV 14d | 4,5 | none |
| 26 | RT | GCV 13d | 4,8 | none |
| 27 | SCT | vGCV 4d GCV 2d | 4,5 | none |
| 28 | post partum hepatitis | unknown | 4,0 | none |
| 29 | renal disease | unknown | 5,8 | none |
| 30 | unknown | unknown | 4,9 | H469Y |
| 31 | RT | GCV 2d | 3,9 | none |
| 32 | RT | none | 4,9 | none |
| 33 | SCT | none | 4,3 | none |
| 34 | SCT | vGCV 66d GCV 25d CDV 3 doses | 3,9 | **A591V** or **L595F** |
| 35 | OLT | GCV 18d | 3,4 | none |
| 36 | HIV | none | 4,6 | none |
| 37 | SCT | vGCV 3d | 4,3 | none |
| 38 | SCT | none | 4,9 | none |
| 39 | RT | vGCV 65d GCV 39d | 3,1 | **M460V** |
| 40 | SCT | vGCV 26d | 6,5 | none |
| 41 | RT | vGCV 45d GCV 39d | 2,3 | **M460I** |
| 42 | SCT | none | 5,1 | none |
| 43 | OLT | GCV 25d | 4,5 | none |

1) CDV = cidofovir; d = days; FOS = foscarnet; GCV = ganciclovir; MSCSA = mass spectrometry-based comparative sequence analysis; myc fun = mycosis fungoides; N.A. = not applicable; NHL = non-Hodgkin lymphoma; OLT = orthotopic liver transplantation; RT = renal transplantation; SCT = stem cell transplantation; vGCV = valganciclovir.
2) resistance associated mutations in bold.[16;25-28] Polymorphisms/mutations were identified by cycle sequencing of the samples.

*Table S2. Detailed comparison of results obtained by MSCSA and cycle sequencing.*

| Patient | results cycle sequencing[1)] | | results MSCSA[2)] | | | concordance MSCSA and cycle sequencing | comment |
|---|---|---|---|---|---|---|---|
| | **nt changes in amplicon (1110-2124)** | **Polymorphisms/ Mutations** | **nt changes in amplicon 97AB (1090-1558)** | **nt changes in amplicon 97CD (1513-1980)** | **Polymorphisms/ Mutations** | | |
| AD169 | none | none | no SNPs | no SNPs | none | yes | |
| 1 | c/T@1287; t/C@1509; t/C@1794 | none | c/T@1287; t/C@1509 | t/C@1794* | none | yes | |
| 2 | t/C@1509; t/C@1671; t/C@1794; c/A@1854; c/T@2064 | none | t/C@1509 | t/C@1671; t/C@1794; C/A@1854 | none | yes | |
| 3 | c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1368; t/C@1509* | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | yes | |
| 4 | c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902; c/A@2076; c/T@2106 | none | c/T@1368; t/C@1509 | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | yes | |
| 5 | c/T@1122; c/T@1203; t/C@1509; t/C@1671; c/A@1773; **t/G@1774**; t/C@1794 | **C592G** | c/T@1122; c/T@1203; t/C@1509 | t/C@1671; c/A@1773; **t/G@1774**; t/C@1794 | **C592G** | yes | |
| 6 | t/C@1509; c/T@1575; t/C@1794; c/G@1815 | D605E | t/C@1509 | c/T@1575; t/C@1794; c/G@1815 | D605E | yes | |

*Table S2. Continued.*

| Patient | results cycle sequencing[1] | | results MSCSA[2] | | | concordance MSCSA and cycle sequencing | comment |
|---|---|---|---|---|---|---|---|
| | **nt changes in amplicon (1110-2124)** | **Polymorphisms/ Mutations** | **nt changes in amplicon 97AB (1090-1558)** | **nt changes in amplicon 97CD (1513-1980)** | **Polymorphisms/ Mutations** | | |
| 7 | g/A@2057 | R686Q | no SNPs* | no SNPs | R686Q | yes | |
| 8 | c/T@1368; t/C@1509; t/C@1794; c/A@1869 | none | c/T@1368; t/C@1509 | t/C@1794; c/A@1869 | none | yes | |
| 9 | c/T@1287; c/T@1405; t/C@1509; t/C@1794; c/T@2106 | H469Y | failed | t/C@1794* | H469Y | yes | |
| 10 | c/T@1242; c/T@1368; g/A@1467; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1242; c/T@1368; g/A@1467; t/C@1509 | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | yes | |
| 11 | g/A@1321; c/T@1368; t/C@1509; a/G@1529; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | N510S, D441N | c/T@1368; t/C@1509; a/G@1529* | c/T@1657; c/T@1737; t/C@1794; g/A@1902* | N510S | no; see comment | g/A@1321 discrepancy with original cycle sequencing result was confirmed by sequencing of MSCSA PCR products |
| 12 | c/T@1368; t/C@1509; a/G@1529; c/T@1657; c/T@1737; **c/T@1781**; t/C@1794; g/A@1902 | N510S; **A594V** | c/T@1368; t/C@1509; a/G@1529 | c/T@1657; c/T@1737; **(c/T@1781)**; t/C@1794; g/A@1902 | N510S; **(A594V)** | yes; see comment | c/T@1781 discrepancy between duplicates were confirmed by cycle sequencing of MSCSA PCR products |

| Patient | results cycle sequencing[1] | | results MSCSA[2] | | | concordance MSCSA and cycle sequencing | comment |
|---|---|---|---|---|---|---|---|
| | nt changes in amplicon (1110-2124) | Polymorphisms/ Mutations | nt changes in amplicon 97AB (1090-1558) | nt changes in amplicon 97CD (1513-1980) | Polymorphisms/ Mutations | | |
| 13 | c/T@1368; g/A@1432; t/C@1509; a/G@1529; c/T@1657; c/T@1737; **c/T@1781**; t/C@1794; g/A@1902; c/T@2106 | A478T; N510S; **A594V** | failed | c/T@1657; c/T@1737; **c/T@1781**; t/C@1794; g/A@1902 | A478T; N510S; **A594V** | yes | |
| 14 | c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1368; t/C@1509 | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | yes | |
| 15 | none | none | no SNPs | no SNPs | none | yes | |
| 16 | c/T@1242; a/G@1400; g/A@1467 | N467S | failed | no SNPs | none | yes | polymorphism was not identified because MSCSA of amplicon 97AB failed |
| 17 | c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1368; t/C@1509 | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | yes | |
| 18 | none | none | no SNPs | no SNPs | none | yes | |
| 19 | c/T@1287; c/T@1405; t/C@1509; t/C@1794; **c/T@1809** | H469Y; **C603W** | c/T@1287; c/T@1405; t/C@1509* | t/C@1794* | H469Y | no; see comment | c/T@1809 discrepancy with original cycle sequencing result was confirmed by sequencing of MSCSA PCR products |

*Table S2. Continued.*

| Patient | results cycle sequencing[1] | | results MSCSA[2] | | | concordance MSCSA and cycle sequencing | comment |
|---|---|---|---|---|---|---|---|
| | nt changes in amplicon (1110-2124) | Polymorphisms/ Mutations | nt changes in amplicon 97AB (1090-1558) | nt changes in amplicon 97CD (1513-1980) | Polymorphisms/ Mutations | | |
| 20 | t/C@1509; g/A@1723; t/C@1794 | E575K | failed | t/C@1794 | none | no; see comment | g/A@1723 discrepancy with original cycle sequencing result was confirmed by sequencing of MSCSA PCR products |
| 21 | t/C@1509; t/C@1794 | none | t/C@1509* | t/C@1794* | none | yes | |
| 22 | c/T@1287; c/T@1405; t/C@1509; t/C@1794 | H469Y | c/T@1287; c/T@1405; t/C@1509* | t/C@1794 | H469Y | yes | |
| 23 | t/C@1509; t/C@1794 | none | t/C@1509* | t/C@1794 | none | yes | |
| 24 | none | none | failed | no SNPs | none | yes | |
| 25 | g/A@1188; c/T@1287; c/T@1368; t/C@1509; a/G@1529; c/T@1657; c/T@1737; t/C@1794; g/A@1902; c/T@1959 | none | a/G@1188; c/T@1287; c/T@1368; t/C@1509; a/G@1529 | c/T@1657; c/T@1737; t/C@1794; g/A@1902; c/T@1959 | none | yes | |
| 26 | c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1368; t/C@1509 | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | yes | |
| 27 | c/T@1119; t/C@1509; t/C@1671; t/C@1794 | none | c/T@1119; t/C@1509 | t/C@1671; t/C@1794 | none | yes | |
| 28 | none | none | no SNPs | no SNPs | none | yes | |
| 29 | c/T@1245; t/C@1509; t/C@1794 | none | t/C@1509 | t/C@1794 | none | no | c/T@1245 only found by cycle sequencing |

| Patient | results cycle sequencing[1)] | | results MSCSA[2)] | | | concordance MSCSA and cycle sequencing | comment |
|---|---|---|---|---|---|---|---|
| | nt changes in amplicon (1110-2124) | Polymorphisms/ Mutations | nt changes in amplicon 97AB (1090-1558) | nt changes in amplicon 97CD (1513-1980) | Polymorphisms/ Mutations | | |
| 30 | c/T@1287; c/T@1405; t/C@1509; t/C@1794 | H469Y | c/T@1287; c/T@1405; t/C@1509 | t/C@1794 | H469Y | yes | |
| 31 | c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1368; t/C@1509* | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | yes | |
| 32 | t/C@1509; t/C@1671; t/C@1794 | none | c/T@1287; t/C@1509* | t/C@1671; t/C@1794 | none | no | c/T@1287 only found by MSCSA |
| 33 | g/A@1227; c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1368; t/C@1509* | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | no | g/A@1227 only found by cycle sequencing |
| 34 | t/C@1509; **c/T@1772**; t/C@1794 or t/C@1509; **g/T@1785**; t/C@1794 | **A591V** or **L595F** | t/C@1509* | (**c/T@1772**); (**g/T@1785**); t/C@1794 | **(A591V); (L595F)** | yes; see comment | c/T@1772 and g/T@1785 discrepancies between duplicates were confirmed by cycle sequencing of MSCSA PCR products |
| 35 | t/C@1671; t/C@1794 | none | failed | t/C@1671; t/C@1794 | none | yes | |
| 36 | c/T@1368; t/C@1509; c/T@1657; g/A@1719; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1368; t/C@1509* | c/T@1657; g/A@1719; c/T@1737; t/C@1794; g/A@1902 | none | yes | |

*Table S2. Continued.*

| Patient | results cycle sequencing[1)] | | results MSCSA[2)] | | | concordance MSCSA and cycle sequencing | comment |
|---|---|---|---|---|---|---|---|
| | **nt changes in amplicon (1110-2124)** | **Polymorphisms/ Mutations** | **nt changes in amplicon 97AB (1090-1558)** | **nt changes in amplicon 97CD (1513-1980)** | **Polymorphisms/ Mutations** | | |
| 37 | c/T@1657; c/T@1737; c/T@1752; t/C@1794; g/A@1902 | none | failed | c/T@1657; c/T@1737; c/T@1752; t/C@1794; g/A@1902* | none | yes | |
| 38 | c/T@1368; t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | c/T@1368; t/C@1509* | c/T@1657; c/T@1737; t/C@1794; g/A@1902 | none | yes | |
| 39 | c/T@1368; **a/G@1378;** t/C@1509; c/T@1657; c/T@1737; t/C@1794; g/A@1902; c/T@2106 | **M460V** | failed | c/T@1657; c/T@1737; t/C@1794; g/A@1902* | none | yes | Mutation was not identified because MSCSA of amplicon 97AB failed |
| 40 | g/A@1467 | none | g/A@1467 | no SNPs | none | yes | |
| 41 | c/T@1368; **g/T@1380**; t/C@1509; c/T@1657; c/T@1737 | **M460I** | failed | failed | none | n.a. | Mutation was not identified because MSCSA failed |
| 42 | none | none | no SNPs | no SNPs | none | yes | |
| 43 | c/T@1287; c/T@1405; t/C@1509; t/C@1794 | none | c/T@1287; c/T@1405; t/C@1509 | t/C@1794 | none | yes | |

[1)] SNPs in bold mark resistance associated mutations; SNPs outside MSCSA amplicon underlined. [2)] SNPs between brackets were found in one of two duplicate experiments. * unable to amplify duplicate product. n.a. not applicable