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Cytomegalovirus drug resistance mutations in transplant recipients with suspected resistance



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Abstract

Resistant CMV infections are challenging complications after SOT and HSCT. Prompt recognition of ARMs is imperative for appropriate therapy. 108 plasma samples from 96 CMV + transplant recipients with suspected resistance were analysed in CNM in a retrospective nationwide study from January 2018 to July 2022 for resistance genotyping. ARMs in UL97 and UL54 were found in 26.87% (18/67) and 10.60% (7/66) of patients, respectively. Patients' ARM distribution in UL97 was as follows: L595S n=3; L595S/M460I n=1; L595S/N510S n=1; L595W n=1; C603W n=4; A594V n=3; A594E n=1; C607Y n=1; L397R/T409M/H411L/M460I n=1; L397I n=1; H520Q n=1; four patients showed ARMs in UL54 as well (F412C n=1; T503I n=2; P522S n=1), whereas three patients exhibited ARMs in UL54 only (L5011/T5031/L516R/A834P n=1; A987G n=2). L516R in UL54 and L397R/I and H411L in UL97 have been found for the first time in a clinical sample. L595S/W was the most prevalent ARM found to lend resistance to GCV. In UL54 all ARMs lent resistance to GCV and CDV. In addition, A834P, found in one patient, also lent resistance to FOS. CMV load did not differ significantly in patients with or without ARMs, and no differences were found either between patients with ARMs in UL97 or in UL97 and UL54. Despite extensive use of classical antivirals for the treatment of CMV infection after HSCT and SOT, ARMs occurred mainly in viral UL97 kinase, which suggests that CDV and mostly FOS continue to be useful alternatives to nucleoside analogues after genotypic detection of ARMs.

Keywords Cytomegalovirus, *UL54 gene*, *UL56 gene*, *UL97 gene*, Transplant patients, Resistance mutations to antivirals, Letermovir, Ganciclovir, Foscarnet, Solid organ transplant, Hematopoietic stem cells transplant

Introduction

CMV is a herpesvirus with a high worldwide prevalence; it causes serious complications in immunocompromised patients, particularly those who are recipients of hematopoietic progenitors (HSCT) or solid organ (SOT) [1, 2]. The effects of CMV disease in these patients are

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responsible for high morbidity and mortality rates, as well as an increased risk of long-term graft loss [2-4].

The effectiveness of the preventive strategies currently used has managed to limit the incidence of the disease in the months following transplantation [4, 5]. However, prolonged antiviral treatments increase the risk of selecting drug-resistance viral strains [2, 4, 6], which, added to the scarce therapeutic options, becomes challenging for the management of transplant recipients. Drug resistance, defined as a viral genetic alteration that decreases susceptibility to one or more antiviral drugs, should be suspected when CMV viremia fails to improve

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or continues to increase after two weeks of appropriately dosed and delivered antiviral therapy [7]. Consequently, the need for genotypic analysis to detect resistance mutations during therapies is imperative. Prophylaxis with GCV IV or VGCV oral is the treatment of choice. FOS is often the first choice for the treatment of UL97-mutant ganciclovir-resistant CMV. A major concern with FOS is its high nephrotoxicity, as well as the alternative CDV. Approved in 2017 by the US Food and Drug Administration for the prevention of CMV in HSCT recipients [8, 9], a novel therapeutic alternative, such as letermovir, that do not have cross-resistance with current treatments has become a concern due to the rapid development of resistance mutations described recently [10]. Mutations conferring resistance to LET are most commonly mapped to UL56. The rates of ARM in SOT patients is 5-12% depending on the group of patients studied but often is higher than 20% in patients with suspected ARM [11].

This study aimed to analyse the frequency of the appearance of mutations in UL97, UL54 and UL56 associated with antiviral resistance in clinical samples obtained from CMV+transplant recipients with suspected resistant CMV to antivirals.

Materials and methods

Clinical samples and transplant patients

In this retrospective study, 108 plasma samples from 96 transplant patients with suspicion of CMV resistant to antivirals were submitted to National Center for Microbiology (CNM) by hospitals all over the country from January 2018 to July 2022, to undergo genotypic analysis of antiviral resistance through sequencing of *ul54* and ul97 genes. Residual samples were stored at -80 °C until genotypic LET resistance characterization through ul56 gene sequencing was performed. Median age of patients was 56 years-old. 64 SOT patients (39 SOT-K, 11 SOT-H, 7 SOT-C, 7 SOT-L) received prophylaxis and 32 HSCT patients received pre-emptive therapy. Individual therapy, viral load, gender, age and region where patient was living is detailed in Table 1 of supplementary material. Resistant and refractory CMV infection definitions were in agreement with consistent criteria [7]. This study was approved by the Ethics Committee of the "Instituto de Salud Carlos III" (CEI PI 11_2021-v3).

DNA extraction, PCR design and sequencing

DNA extraction was performed from 200 μ M of clinical sample (one sample per patient), using the "QIAamp Min ELUTE Virus Spin" Kit (QIAGEN), as per the manufacturer's instructions. Systematic search and alignment of partial and complete sequences for the genes *ul54*, *ul56* and *ul97* were downloaded from GenBank database. Alignments using SeqMan (DNASTAR, Lasergen INC) and Mega X were performed to obtain the consensus and

majority sequences, which were used as wild sensitive or resistance reference sequences. Three synthesized DNA fragments, containing all consensus resistance mutations described to date for each gene [12, 13] were cloned in E. coli plasmids and used as PCR and sequence-positive controls (Table 1). Three pairs of oligonucleotides were designed for PCR amplification of 990, 2246 and 649 bp fragments from ul97, ul54 and ul56, respectively. In addition, eight for UL54, six for UL56 and six for UL97 oligonucleotides were designed for Sanger sequencing (Table 2). Reactions were performed in Biorad C1000 Touch Thermal Cycler in a volume of 50µL and using Platinum SuperFi II DNA Polymerase (Thermo Fisher, Invitrogen), according to the manufacturer's instructions. The oligonucleotides used to carry out the amplification were at a final concentration of 0.9 µM. PCR conditions for each gene are detailed in Table 3.

A PCR product was considered available for sequencing when a detectable band of appropriate molecular weight was obtained by electrophoresis. Pre-sequencing purification of the PCR product was performed with the ExoProStarTM Enzymatic PCR and Sequence Reaction Clean-Up Kit 500 reactions (IllustraTM, Germany), following the manufacturer's instructions. PCR products were processed for Sanger dideoxy sequencing with BigDye v. 3.1 (Applied Biosystems) in ABI PRISM 3100 sequencer (Applied Biosystems, California, USA).

Multiplex real-time PCR for determination of CMV and EBV viral load and detection of HHV6, HHV7 and HHV8

We developed a 6-plex real-time PCR assay that is currently used in Reference Laboratory for Immune Preventable Diseases of National Centre for Microbiology. It was able to detect HHV6, HHV7 and HHV8 and to detect and quantify CMV and EBV. Quantitation used two sets of quantitative standards (for CMV and EBV) produced as follows: Relevant fragments of DNA (those amplified in real-time PCR) were inserted in a plasmid and cloned in transformed E. coli. Extracted serial dilutions of DNA from culture media were standardized against WHO standards provided by Health Protection Agency (UK) for determination of CMV and EBV viral load. This multiplex assay included plasmid DNA positive control for HHV6, HHV7, HHV8 and an internal control (IC) of amplification. CMV/EBV quantitation demonstrated a sensitivity of 10 IU/mL and a wide dynamic range between 10 and 106 IU/mL for quantification of CMV and EBV in clinical samples and detection of HHV6, HHV7, HHV8 and an IC simultaneously. Quantitation accuracy was assessed with 2013 Cytomegalovirus and Epstein-Barr (DNA) EQA panels of QCMD and it was checked yearly using WHO standards. Primers (Sygma) and probes (Metabion) are in Table 4.

 Table 1
 Previously described mutations associated to resistance to antivirals [13]

U.54D301NCX, CXN408DXCX, CXN408DXCX, CXN408DXCX, CXN412C/SV/VYCX, CXD13AFE/V/YYCX, CXN95KT503CX, CXK512F/V/FCX, CXK512F/V/FCX, CXB514CX, CXD13AFE/V/YYCX, CXK512F/V/FCX, CXK512F/V/FCX, CXB514CX, CXB514CX, CXB514CX, CXB514CX, CXCX, CXCXCX, CX <th>Target</th> <th>Mutation</th> <th>Antiviral</th>	Target	Mutation	Antiviral
M400/KCCC, CDVM410KCCC, CDVP412/CS/WLCCC, CDVP412/CS/WLCCC, CDVM495KISSL501CCC, CDVM495KCCC, CDVL501CCC, CDVL516/P/WLCCC, CDVL516/P/WLCCC, CDVL516/P/WLCCC, CDVL545/SWCCC, CDVL545/SWCCC, CDVL545/SWCCC, CDVL545/SWCCC, CDV, FOSA697/CCC, CDV, FOSA697/CCC, CDV, FOSL700AFOSV715/MLFOSL776MFOSCVT791CS, CCVV781CS, CCVK805QK805QCDVA699/FOS, CCV, CDVK805QFOS, CCV, CDVK805QCDVA699/FOS, CCV, CDVK805QFOS, CCV, CDV </th <th>UL54</th> <th>D301N</th> <th>GCV, CDV</th>	UL54	D301N	GCV, CDV
N10KCCC,CDVP412C,S7V/LCCC,CDVP413A/EV/N/YCSC,CDVP503CCC,CDVT503CCC,CDVF503CCC,CDVF512F/N/RCCC,CDVE521CCC,CDVE521CCC,CDVB521CCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVD588F/NCCC,CDVT700APSCCCV715/MP550/Q/KPSCCCV721P550/Q/KPSCCCV721P550/Q/KPSCCCV721P56CCC,CDVV721PSP56CCC,CDVV311PSCCCPSV2304PSCCCPSV2304PSCCCPSV2304PSCCCPSP374PSP374PSP374PSP374PSP374PSP374PSP374PSP374PSP374PSP374PSP374PSP374PSP374 <th></th> <th>N408D/K</th> <th>GCV, CDV</th>		N408D/K	GCV, CDV
F12CS/W1CCC,CPUDH13A/E/WA/P1CCC,CPUN495KGCLS01GC/,CPUS131GC/,CPUK13E/W.RGC/,CPULS16P/W3GC/,CPULS16P/W3GC/,CPULS16P/W4GC/,CPULS16P/W4GC/,CPULS16P/W5GC/,CPULS16P/W6GC/,CPU<		N410K	GCV, CDV
DistaCCX CDVM95KGCX CDVL5011GCX CDVK312EN/RGCX CDVL504P//GCX CDVB217GCX CDVD22A/SGCX CDVD386/NGCX CDVD586/NGCX CDVD595/NGCX CDV<		F412C/S/V/L	GCV, CDV
M995KF05L5011CCV, CDVK503E/N/RCCV, CDVL516P/WCCV, CDVB217CCV, CDVP522/V5CCV, CDVP522/V5CCV, CDVP525/V5CCV, CDV, F05T6915CCV, CDV, F05S9951CCV, CDV, F05S9951CCV, CDV, F05J700AF05L737MF05L737MF05L737MF05L737MF05L737MF05L737MF05, CCVV7811F05, CCVV7812F05, CCVV7814F05, CCVV7814F05, CCVK805QF05, CCVT6135F05, CCVT638AF05, CCVT638AF05, CCVL1264H214L1274F05, CCVT638AF05, CCVT638AF05, CCVL1264F05, CCVL1274L121PL1264L121PL1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L171L1271L174L1271L174L1271L174L127		D413A/E/V/N/Y	GCV, CDV
LS01GCV,C0VTS03GCV,C0VKS13E/N/RGCV,C0VLS16P/WGCV,C0VBS2L/SGCV,C0VDS8L/NGCV,C0VLS4SSWGCV,C0VDS8L/NGCV,C0V,F0SA697/GCV,C0V,F0SA697/GCV,C0V,F0SLS5SSWGCV,C0V,F0SLS5SSWGCV,C0V,F0SA697/GCV,C0V,F0SLS5SSWGCV,C0V,F0SA697/GCV,C0V,F0SLS5SSWGCV,C0V,F0SLS5GCV,C0V,F0SLS5GCV,C0V,F0SLS5GCV,C0V,F0SLS5GCV,C0VTS7GS,CV,C0VV715/MFOS,CCV,C0VV781FOS,CCV,C0VV781FOS,CCV,C0VTS13FOS,CCV,C0VRS6FOSV781FOS,CCV,C0VTS13FOS,CCV,C0VTS13FOS,CCV,C0VRS8AFOSGG14FOS,CCV,C0VRS8AFOSGG14FOS,CCV,C0VA897GCS,CCV,C0VA987GCSGS41FOS,CCV,C0VA987GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCSGS41GCS		N495K	FOS
F331 GCV CDV K313E/W/R GCV CDV L516P/W GCV CDV B21T GCV CDV P522.v5 GCV CDV D588/N GCV CDV, F05 T6915 GCV CDV, F05 A927 GCV CDV, F05 5957 GCV CDV, F05 5957 GCV CDV, F05 5957 GCV CDV, F05 1790A F05 1737M F05 1838 F05 1839 F05 18315 F05 18316 F05 18317 F05 18318 F05 1931 F14 1941 F14 1941 F14 1941 F14 1941 F14 1941 F14 1941 F14		L501I	GCV, CDV
KisternGCV.COVListernGCV.CDVF5217GCV.CDVF524/5GCV.CDVD585/NGCV.CDV/CDT615GCV.CDV/CDF65GCV.CDV/CDF65GCV.CDV/CDF700AGCV.CDV/CDT700AF05F750/Q/KGCV.CDV/CDF750/Q/KF05F750/Q/KF05F750/Q/KF05F750/Q/KF05F750/Q/KF05F750/Q/KF05F750/Q/KF05F751F05 <th></th> <th>T503I</th> <th>GCV, CDV</th>		T503I	GCV, CDV
LS16P/WCSC (CDVIS1T1CCV (CDVPS22/SCCV (CDVLS4SS/WCCV (CDV/FOSN682/NCCV (CDV/FOSN692/VCCV (CDV/FOSA692/VCCV (CDV/FOSCT70/ACCV (CDV/FOSLT37/MCCV (CDV/FOSLT37/MFOSLT37/MFOSLT37/MFOSCCV (CDV/FOSCCV (CDV/FOSLT37/MFOSLT37FOSLT37FOSLT37FOSLT37FOSLT31FOSLT37FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41FOSLT41 </th <th></th> <th>K513E/N/R</th> <th>GCV, CDV</th>		K513E/N /R	GCV, CDV
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P522/595%95%L55%/V62% CDVD588/N62% CDV, F05701562% CDV, F05999762% CDV, F057700AF051705/MF051705F051705F051706F051707 </th <th></th> <th>I521T</th> <th>GCV, CDV</th>		I521T	GCV, CDV
L5455/W GCV,CDV H5915 GCV,CDV,F0S A692V GCV,CDV,F0S A692V GCV,CDV,F0S J700A F0S L776M F0S V715/VM F0S L776M F0S V715/VM F0S,GCV,CDV L776M F0S,GCV,CDV L776M F0S,GCV,CDV L776M F0S,GCV,CDV L776M F0S,GCV,CDV L776M F0S,GCV,CDV L802M F0S,GCV,CDV H805Q F0S,GCV,CDV H805Q F0S,GCV,CDV B834P F0S,GCV,CDV T834P F0S,GCV,CDV T834P F0S,GCV,CDV B834P F0S,GCV,CDV T63912.del F0S,GCV,CDV B834P F0S,GCV,CDV T63912.del F0S,GCV,CDV L156 G841A F0S,GCV,CDV L156 G241A F0S,GCV,CDV L211P LET LET L237D LET LET L357T LET LET L2		P522A/S	GCV, CDV
D588E/NGCV.CDV.FDSF0F15GCV.CDV.FDSA692VGCV.CDV.FDS5695TGCV.CDV.FDSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL737MFOSL802MFOSK805QCDVH812LFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK805QFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOSK81AFOS <th></th> <th>L545S/W</th> <th>GCV, CDV</th>		L545S/W	GCV, CDV
TestsGCV.CDV,FOSS992VGCV.CDV,FOSS695TGCV.CDV,FOST700AFOSU37MFOSL'37MFOSL'37MFOSF756D/Q/KFOS,GCV,CDVV7811FOS,GCVV7811FOS,GCVV787LFOS,GCVV787LFOS,GCVK805QCDVK805QFOS,GCVK805QFOS,GCVK805QFOS,GCVK805QFOS,GCVK805QFOS,GCVK805QFOS,GCV,CDVT813SFOS,GCV,CDVT813EFOS,GCV,CDVK805QGCVK805QFOS,GCV,CDVK805QFOS,GCV,CDVK805QFOS,GCV,CDVK814AFOS,GCV,CDVK815AFOS,GCV,CDVK815AFOS,GCV,CDVK815AFOS,GCV,CDVK815AFOS,GCV,CDVK815AFOS,GCV,CDVK815AFOSFOSFOS,GCV,CDVK815AFOS <th></th> <th>D588E/N</th> <th>GCV, CDV, FOS</th>		D588E/N	GCV, CDV, FOS
A692V GCV.CDV,FOS SS695T GCV.CDV,FOS T700A FOS V715/VM FOS L737M FOS L737M FOS,GCV.CDV L756D/Q/K FOS,GCV.CDV L776M FOS,GCV.CDV V7811 FOS,GCV V787L FOS,GCV V787L FOS,GCV V787L FOS,GCV V787L FOS,GCV V781L FOS,GCV,CDV R805Q FOS,GCV,CDV R805Q FOS,GCV,CDV R805Q FOS,GCV,CDV R813S FOS,GCV,CDV R813S FOS,GCV,CDV R834P FOS,GCV,CDV R834A FOS,GCV,CDV R834A FOS,GCV,CDV R834A FOS,GCV,CDV R834A FOS,GCV,CDV R834A FOS,GCV,CDV 1835A FOS UL56 V231L L241P LET L241P LET L241P LET L241P LET 1241F LET 1241F LET 1241F LET 1241F LET 1241F LET 1241F LET		T691S	GCV, CDV, FOS
S695TGCV,C0V,F0ST700AF0ST700AF0SL737MF0SE756D/Q/KF0S,GCVT776MF0S,GCVV7811F0S,GCVV7871F0S,GCVB02MF0S,GCVV7871F0S,GCVF0S,GCVF0S,GCVV7871F0S,GCVF0S,GCVF0S,GCVV8121F0S,GCVF0S,GCV,C0VF8135F834F0S,GCV,C0VF8211F0S,GCV,C0VF838AF0S,GCV,C0VP897GGCV,C0VA9872delF0S,GCV,C0V1244KEF1244KEF1244KEF1244KEF1244KEF1244KEF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1247EF1257EF1257EF1247EF1257EF1257EF1257EF1257EF1257EF1257EF1257EF1257EF1257EF1257EF1257EF1257EF1257EF12		A692V	GCV, CDV, FOS
T700AFOSV715A/MFOSV715A/MFOSF75D/Q/KFOSF75D/Q/KFOSK05F75D/Q/KF76MFOSV7811FOSF0SFOSK0SOQFOS <th></th> <th>S695T</th> <th>GCV, CDV, FOS</th>		S695T	GCV, CDV, FOS
V715A/M FOS L737M FOS L737M FOS E756D/Q/K FOS,GCV,CDV V7811 FOS,GCV V7871 FOS,GCV V7871 FOS,GCV V7871 FOS,GCV V7871 FOS,GCV V7871 FOS,GCV V7871 FOS,GCV V8020 FOS,GCV V8121 FOS,GCV,CDV T8135 FOS,GCV,CDV R8080V FOS,GCV,CDV R834P FOS,GCV,CDV R834P FOS,GCV,CDV R841A FOS,GCV,CDV A981-2 del FOS,GCV,CDV V231L LET L241P LET L241P LET L2571 LET K399M LET K399M LET L501 LET L502 J353A LET K397 L405P LET L405P LET L405P LET L405P LET <th></th> <th>T700A</th> <th>FOS</th>		T700A	FOS
L73M F05 F756D/Q/K F05, GCV, CDV L776M F05, GCV V7811 F05, GCV V7812 F05, GCV L802M F05, GCV V802M F05, GCV K805Q CDV R809V F05, GCV, CDV B121 F05, GCV, CDV B135 F05, GCV, CDV B34P F05, GCV, CDV R834P F05, GCV, CDV G841A F05, GCV, CDV B34P F05, GCV, CDV B34P F05, GCV, CDV G841A F05, GCV, CDV L156 G23D UL56 L6T 123D L6T 123T L6T 124F L24IP 123T L6T 123T L6T 124F L24IP 123T L6T 123T L6T 124F L24IP 1257 L6T 1257 L6T </th <th></th> <th>V715A/M</th> <th>FOS</th>		V715A/M	FOS
PSS, GCV, CDV POS, GCV, CDV L776M POS, GCV V781L POS, GCV V787L POS, GCV L802M POS, GCV K805C CDV A809V POS, GCV, CDV T813 POS, GCV, CDV T813 POS, GCV, CDV T821L POS, GCV, CDV T8211 POS, GCV, CDV T8211 POS, GCV, CDV T824A POS, GCV, CDV T824A POS, GCV, CDV T824A POS, GCV, CDV R987C GCU, CDV V236M LET L237D LET L24K LET L2571 LET T244K LET L2571 LET V197 Y321C L2571 LET Y321C LET Y323A MBV Y33A		L737M	FOS
Image:		E756D/Q/K	FOS, GCV, CDV
V781FOS, GCVV787LFOS, GCVB002MFOS, GCVK805QCDVA809VFOS, GCV, CDVT813SFOS, GCV, CDVT813SFOS, GCV, CDVR834PFOS, GCV, CDVB841AFOS, GCV, CDVM87GGCV, CDVV236MLFTL241PLFTL241PLFTL241PLFTL257LFTL257F261CL174KLFTV230LLFTL257LFTL257LFTL257LFTL257LFTL257LFTL257LFTL257LFTL397LFTM329TLFTM329TLFTL397RMBVL405PGCVM8VL405PM8VL405PM80VMBVH411L/V/NMBVM460/V/Y/L/TGCV		L776M	FOS, GCV
V787LFOS, GCVIB02MFOS, GCVK805QGDVA809VFOS, GCV, CDVT813SFOS, GCV, CDVT813TFOS, GCV, CDVT813TFOS, GCV, CDVT813TFOS, GCV, CDVT813SFOSGR41AFOS, GCV, CDVM891-2 delFOS, GCV, CDVV31LEFTF237DEFTE337DEFTE337DEFTE337DEFTE344KEFTE357DEFTE371EFTE372DEFTE372DEFTE372DEFTE372DEFTE373DEFTE372DEFTE373DEFTE364CEFTF361CEFTF361CEFTF362DEFTF362DEFTF362DEFTF362DEFTF362DEFTF364DEFTF364DEFTF364DEFTF364DEFTF364DEFTF374DEFTF364DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFTF374DEFT		V781I	FOS, GCV
L802MF05, GCVK805QCDVK805QF05, GCV, CDVV812LF05, GCV, CDVT813SF05, GCV, CDVT8211F05, GCV, CDVT838APF05G841AF05, GCV, CDVG841AF05, GCV, CDVM981-2 delF05, GCV, CDVV231LLEFUL56V231LL241PLEFL241PLETL241PLETL2571LETL2571LETL2571LETL2571LETL397ALETK397TLETK397TLETL397RLETL397RGCVL397RGCVH405PGCVK405QKBVK405QKBVK405QKBVK405QKBV		V787L	FOS, GCV
K805QCDVA809VF05, GCVK805QF05, GCV, CDVT813LF05, GCV, CDVT813SF05, GCV, CDVT8211F05, GCV, CDVT838AF05G841AF05G841AF05, GCV, CDVA987GGCV, CDVA987L2 delGCV, CDVV231LLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE237DLETE364AGEME44KLETE367LETE367LETE369MLETE374MBVE375AMBVE374MBVE374MBVH11L/VN/LTMBVM660/VUTGCV		L802M	FOS, GCV
A809 Pos, GCV V812L Fos, GCV, CDV T8211 Fos, GCV, CDV R834P Fos, GCV, CDV T838A Fos G841A Fos, GCV, CDV A987-2 del Fos, GCV, CDV V312-2 del Fos, GCV, CDV V391-2 del Fos, GCV, CDV V391-2 del Fos, GCV, CDV V391-2 del Fos, GCV, CDV V230L LET V236M LET E237D LET E237D LET I241P LET I2571 LET V321C LET I257V LET V321C LET I325Y LET UL97 V33A M399T LET I397R MBV L405F GCV I405F GCV I405P GCV I405P GCV I405P GCV		K805Q	CDV
V812LFOS, GCV, CDVT813SFOS, GCV, CDVT8211FOS, GCV, CDVT8211FOS, GCV, CDVT838AFOSG841AFOS, GCV, CDVA981-2 delFOS, GCV, CDVA987GGCV, CDVUL56V231LU230MLET1241PLET1241PLET1244KLET12457LET12470LET12571LET12571LET1232NLET1235YLET1235YLET1237ALET1237ALET1237ALET1237ALET1244LET1257LET1257LET1257LET1257LET1257LET1257LET1257LET1257LET1297MBV1297MBV1411L/VNMBV1400MMBV1400LV/Y/LTGCV1400LV/Y/LTGCV		A809V	FOS, GCV
T813S F0S, GCV, CDV T8211 F0S, GCV A834P F0S, GCV, CDV B38A F0S, GCV, CDV B41A F0S, GCV, CDV A981-2 del F0S, GCV, CDV A987G GCV, CDV V236M LET E237D LET E237D LET L241P LET 1244K LET 12571 LET Y321C LET Y321C LET Y321C LET M329T LET M400/V//LT MBV M400/V/V/LT MBV M400/V/V/LT MEV <th></th> <th>V812L</th> <th>FOS, GCV, CDV</th>		V812L	FOS, GCV, CDV
T8211F0S, GCVA834PF0S, GCV, CDVT838AAF0SG841AF0S, GCV, CDVA981-2 delF0S, GCV, CDVA987GGCV, CDVV231LLET236MLET237DLET1241PLET1244KLET1244KLET1257LET1257LET1257LET1257LET1257LET1257LET1257LET1257LET1257LET1257LET1257LET1260LET127LET1297LET1297MBV1297MSV1405PGCV1409MMBV1411L/YNMBV1411L/YNMSV1466GGV		T813S	FOS, GCV, CDV
A834P FOS, GCV, CDV T838AA FOS B841A FOS, GCV, CDV A981-2 del FOS, GCV, CDV A987G GCV, CDV V230M LET V231L LET V230M LET V321C LET V321C LET V321C LET V330A MBV L405P GV L405P GV L405P GV H4111LV/N MBV M460V/V/LT MSV		T821I	FOS, GCV
T838A FOS G841A FOS, GCV, CDV A981-2 del FOS, GCV, CDV A987-2 del GCV, CDV UL56 V231L GCT V236M LET E237D LET L241P L211 LET L241P LET SQUESO F261C LET LET V1256 V325Y LET LET V1260 L2571 LET LET V127 K325Y LET LET V1297 M329T LET LET L397R MBV LET LET L405P L397R MBV MSV L405P GCV GCV MSV L405P MBV MSV MSV L405P MBV MSV MSV L405P GCV GCV GCV M460/V/V/LT MBV MSV GCV		A834P	FOS, GCV, CDV
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A987G GCV,CIV UL56 V231L LET V236M EZ EZ E237D LET EZ L241P LET EZ L245 EZ ET V236M LET EZ SQUE EZ ET L241P LET EZ L257 EZ ET V2302 ET ET V2312 EZ ET V324K CO ET V2312 EZ ET V3212 EZ ET V3214 EZ ET V3234 EZ EZ V3234 EZ EZ <		A981-2 del	FOS, GCV, CDV
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ND291 EL1 R369M LET UL97 V353A MBV L397R L405P GCV T409M MBV MBV H411L/Y/N MBV MBV V466G GCV GCV		M220T	
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L39/R MBV L405P GCV T409M MBV H411L/Y/N MBV M460/V/Y/L/T GCV V466G GCV		1 307R	MRV
L400F GCV T409M MBV H411L/Y/N MBV M460/V/Y/L/T GCV V466G GCV		L 405P	GCV
H411L/Y/N MBV M460I/V/Y/L/T GCV V466G GCV		TAN9M	MRV
M460 I/ V /Y/L/ T GCV V466G GCV		H4111 /Y/N	MRV
		M460//////T	GCV
V DAAT		V466G	GCV

Table 1 (continued)

Target

Mutation	Antiviral
P468Q	GCV
H520E/Q	GCV
A590V	GCV
A591V	GCV
C592G	GCV
A594V/T/P/E/G	GCV
L595S/W/F	GCV
E596G	GCV
G598S	GCV
K599T	GCV
C603W /R/S	GCV
C607Y/F/L/W	GCV
A619V/G	GCV
*591–594; 591–607; 595, 595–603; 600; 601; 601–603	GCV
	GCV

*In frame codon deletions; MBV (maribavir); GCV (ganciclovir or derivate); Boldface in UL97 indicates the seven most common described (canonical) mutations conferring drug resistance to GCV [13].

 Table 2
 Oligonucleotides designed in the study for PCR and sequencing

Table 3	PCR conditions	for the am	plification	of target genes
	1 Chi contantionis	for the ann	philication	or larget genes

sequencing	
Name	Oligonugleotide 5´-3'
UL54 F	ACTGCGATGTCTCTGACCTG
UL54 R	TCGCTGCTCTTTGAGGATCG
UL54 seq1 F	CGCTATCGATGCCTGTCCTT
UL54 seq2 F	TGGACGTCTACGAGTTCCCT
UL54 seq3 F	CCCTCGGCTTCTCACAACAA
UL54 seq4 R	TCGGCATTAGCCACGAAACA
UL54 seq5 F	TAAAATTCCGTTGCGGCGTG
UL54 seq6 F	AACAGTAGTAGCAGCGTCGG
UL54 seq7 R	TGATTGTTTCGAGCCCCTCC
UL54 seq8 F	TGTCTTTTTGTGGAGCCCGT
UL97 F	GACATGAGCGACGAGAGCTAC
UL97 R	CTGCGAGCATTCGTGGTAGA
UL97 seg1 F	CGTAAGCACAGCGAGACGG

Temperature				Time (min)
	UL54	UL56	UL97	
Denaturation	98 ° C	98 ° C	98 ° C	00:30
Cycling 35x Denaturation	98 ° C	98 ° C	98 ° C	00:10
Annealling	60 ° C	60 ° C	60 ° C	00:15
Extension	68 ° C/1 min	68 ° C/ 00:30	68 ° C/00:30	
Extension	72 ° C	72 ° C	72 ° C	05:00

Table 4 Primers and probes used in Multiplex real time PCR

Name	Oligonucleotide 5'-3'; probes 5`repórter-3`quencher	Target gene
CMV _{probe}	6FAM-TAACAACACATATAAGTATCCGTCCTCCTG-BHQ-1	UL123
CMV F	TCTGTTTGACTGTRGAGGAGG	UL123
CMV R	GGGCATIGAGRTAGCGATAAA	UL123
EBV _{probe}	HEX-ACKTKTAGTAACRCATTCCCTTG-BHQ-1	BZLF1
EBV F	TGTTTCAACTGACTAGGYACC	BZLF1
EBV R	ATTCCTCCAGCTGCGAG	BZLF1
HHV-6 probe	Texas Red-AGATCCGTGGACGGCGG-BHQ-2	HHV6 US22 DR6
HHV-7 _{probe}	Cy5-CAGACCACGATCCCCACACC-BHQ-3	HHV7 gp65
HHV67-F	GGCCAYAABCGRTACTG	HHV6 US22 DR6/ HHV7 gp65
HHV67-R	CTGTGTCAGACKCACRC	HHV6 US22 DR6/ HHV7 gp65
HHV-8 probe	Atto390-TGGAGTGCAGGTAAACGCCA-Eclipse	ORF 23, UL21
HHV8F	TCCGGTAGTATCTGCGTGTC	ORF 23, UL21
HHV8 R	CCTACGCGTTAAGAAGCCAC	ORF 23, UL21
IC _{probe}	IRD700-AATGATTGGGCCACGTCACG BHQ-3	Suid alphaherpesvirus 1
IC-F	CAGATTAGCAATTGGTGCGAA	Suid alphaherpesvirus 1
IC-R	GTGGGCAAATCCGAGGAA	Suid alphaherpesvirus 1

Amplification was carried out in a Rotor Gene thermocycler 6-plex with Quantitect Multiplex PCR kit (Qiagen) with 0,24 μ M of each primer y 0,25 μ M of each probe under the following conditions: Hold 95°C 15 min; 6 cycles of 94°C 30 s, 61°C 30 s; 40 cycles of 95°C 20 s,58° 60 s; hold 40°C 2 min.

Sequence data and statistical analysis

The analysis and editing of the sequences was carried out with the SeqMan (Lasergene) and MegaX software. Amino acid sequences obtained were included in a database with previously created sequences containing all described ARMs for feasible searching of resistance mutations as well as sequences from reference laboratory strains such as Towne and AD169. Statistical analysis was performed using SPSS v28.0 software (SPSS, Chicago, IL). Kruskal Wallis ANOVA test was used to compare the viral load of CMV between clinical samples with and without ARMs and between clinical samples with ARMs in UL97 only and in UL54 plus UL97, as well as with clinical samples which were unable to sequence UL54 and/or UL97. It was followed by Wilcoxon test for pairwise comparisons between medians (SD), 95% CI and p-value ≤ 0.05 .

Results

Analysis of antiviral resistance mutations in UL97, UL54 and UL56

108 CMV positive PCR plasma from 96 transplanted patients yielded sequence data which enabled the analysis of at least one of the three genes of study. Studied genes UL54, UL97 and UL56 were fully characterized in 66, 67 and 96 CMV-positive patients, respectively. In 9 patients UL54 were characterized but not UL97. In other 10 patients UL97 were characterized but not UL54. In 20 patients, only UL56 could be fully analysed.

 Table 5
 ARM and CMV load in 21 SOT and HSCT patients with suspicion of resistance to antivirals

Patient	GenBank∞	Transplant	UL54	ARM	UL56	UL97	ARM	CMV load (IU/mL)	Antiviral*
1	UL54P1 UL97P2	SOT-C	R	F412C	S	R	C603W	9,83 × 10 ³	GCV, FOS
2	UL97P12	SOT-C	S	-	S	R	L397R / T409M / H411L / M460I	1,00×10 ⁵	GCV
3	UL97P13	SOT-K	S	-	S	R	A594V	7,29×10 ⁴	GCV
4	UL97P16	SOT-L	S	-	S	R	C603W	1,53×10 ³	GCV
5	UL97P17	SOT-K	S	-	S	R	L595S/N510S	1,38×10 ⁴	GCV
6	UL97P19	SOT-K	S	-	S	R	L595S	5,92×10 ⁴	GCV
7	UL54P4	HSCT	R	A987G	S	S	-	8,74×10 ³	VGCV, CDV
8	UL97P20	SOT-K	S	-	S	R	L595W	4,12×10 ⁴	GCV
9	UL97P21	SOT-K	S	-	S	R	C607Y	6,83×10 ³	GCV
10	UL97P22	SOT-H	S	-	S	R	H520Q	3,75×10⁵	GCV
11	UL54P6 UL97P8	SOT-L	R	T503I	S	R	C603W	3,75×10⁵	GCV, FOS
12	UL97P23	SOT-L	S	-	S	R	L397I	2,65×10 ³	VGCV
13	UL97P24	SOT-L	S	-	S	R	L595S	2,84×10 ³	GCV
14	UL97P11 UL54P10	SOT-L	R	P522S	S	R	M460I/L595S	6,57×10 ³	GCV, FOS
15	UL97P14	SOT-H	S	-	S	R	A594V	7,85×10 ³	VGCV, FOS
16	UL97P25	SOT-C	S	-	S	R	L595S	7,50×10 ³	GCV
17	UL97P26	HSCT	S	-	S	R	A594E	3,85×10 ³	VGCV
18	UL97P15	SOT-K	S	-	S	R	A594V	$1,45 \times 10^{4}$	VGCV
19	UL54P7 UL97P9	SOT-L	R	T503I	S	R	C603W	3,24×10 ³	VGCV, FOS
20	UL54P3	SOT-K	R	L5011/ T5031/ L516R/ A834P	S	S	-	3,60×10 ³	GCV,FOS
21	UL54P5	SOT-L	R	A987G	S	S	-	$1,21 \times 10^{4}$	GCV, CDV

*Antiviral therapy before ARM testing. In bold red ARMs previously described as selected under drug in vitro [13]. In bold purple ARM not previously described [13]. SOT-C=SOT hearth, SOT-K=SOT Kidney, SOT-L=SOT Lung, SOT-H=SOT Hepatic, R=resistant, S=susceptible wild type. ∞GenBank accession numbers: UL54P1 OQ560469; UL54P3 OQ560470; UL54P4 OQ560471; UL54P5 OQ560472; UL54P6 OQ560473; UL54P7 OQ560474; UL54P10 OQ560475; UL97P2 OQ560451; UL97P8 OQ560452; UL97P9 OQ560453; UL97P11 OQ560454; UL97P12 OQ560455; UL97P13 OQ560456; UL97P14 OQ560457; UL97P15 OQ560458; UL97P16 OQ560459; UL97P17 OQ560460; UL97P19 OQ560461; UL97P20 OQ560462; UL97P21 OQ560463; UL97P22 OQ560464; UL97P23 OQ560465; UL97P24 OQ560466; UL97P25 OQ560467; UL97P26 OQ560468.



Fig. 1 Boxplot that represents the distribution of viral load in each group Non-determined (ND)

ARM was found in 21 transplant patients, 19 of them SOT recipients and 2 HSCT (Table 5). Regarding ARMs in UL97, 3 were cardiac transplant recipients, 2 liver transplant recipients, 6 lung transplant recipients, 6 kidney transplant recipients and 1 HSCT. Regarding ARM in UL54, 1 cardiac transplant recipient, 1 kidney transplant recipient, 4 lung transplant recipients and 1 HSCT. No ARM was found in UL56.

T503I was the most prevalent ARM in UL54 (3/7 patients), followed by A987G (2/7 patients) and L595S in UL97 (5/18 patients), followed by C603W (4/18 patients), A594V (3/18 patients), M460I (2/18 patients). L397I, L397R, T409M, H411L, H520Q, N510S, L595W and C607Y were found in one patient. Moreover, four patients developed ARMs simultaneously in UL54 (F412C 1; T503I 2; P522S 1), and in three patients ARM was detected in UL54 only (L501I; T503I; L516R; A834P). ARMs L397R and H411L in UL97 and L516R in UL54, which were previously described as obtained by drug selection in vitro, were found in two patients. L397I in UL97, which was detected in one cardiac recipient, has not been described before.

Viral load and the presence of ARM

Viral loads for the 96 patients included in the study are shown in Table 1 supplementary material and Table 1 for the 21 patients with ARMs. No significant differences were found between the viral load of the samples with and without ARMs, either with ARMs only in UL97 and UL54-UL97 or UL54 only. On the contrary, significant

Table 6 Viral load comparisons between groups. Willcoxon test

	UL54sUl97s	UL54rUL97s	UL54rUL97r	UL54sUL97r
UL54rUL97s	0.74964	-	-	-
UL54rUL97r	0.93237	1.00000	-	-
UL54sUL97r	0.87874	0.87874	1.00000	-
ND	0.00071	0.74390	0.43513	0.02603

In bold significant p-value ≤ 0.05 .

differences were found for the viral load of the samples with non-determined UL54/UL97 and without ARMs either with ARMs only in UL97 (Fig. 1; Table 6). A viral load threshold of 9.86×10^3 IU/mL was established to be able to analyse complete sequences with enough feasibility and accuracy to characterize ARMs in the three genes. Below this threshold, only UL56 was fully sequenced in all clinical samples.

Polymorphism in UL54 DNA polymerase and UL97 kinase

The occurrence of polymorphism in UL54 is concentrated in specific positions, mostly in S655L and F669L, but other mutations were also found such as T885A, R792C and D898N and a duplication SS in the 585 position. Four patients exhibited D605E mutation in UL97, one of them together with ARM C603W. No polymorphism was found in UL56 sequences.

Discussion

In this study, we developed a genotypic method of amplification through PCR and Sanger sequencing to analyse ARM in the UL54, UL56 and UL97 genes in clinical samples from 96 transplant recipients with suspected resistance to antivirals. To date, this is the study with the highest number of patients conducted in Spain. Moreover, according to a recent review of Chou S [13], we discovered a novel ARM "L397I" in UL97. Additionally, other three ARMs, such as L397R and H411L in UL97 and L516R in UL54, which were previously described as selected under drug in vitro, we detected them directly in clinical samples [13]. Interestingly, mutation at 397 position of UL97 confers resistance to maribavir despite this drug was not used in any patient. In this strain, high level GCV resistance ARM M460I (5-20 fold increase in ganciclovir 50% inhibitory concentrations) was also found, which suggests that GCV therapy could previously selected low level GCV resistance ARMs (<2,2 Foldincrease in ganciclovir 50% inhibitory concentrations) producing cross-resistance to maribavir [13].

ARM was found in 18/67 (26.87%) patients regarding UL97, whereas 5.97% developed combined resistance to UL97 and UL54, and 4.54% to UL54 only. This rate was close to the 27% detected in SOT patients through Sanger sequencing in a previous study conducted in Barcelona [11]. Most ARMs were found in SOT patients, mainly in kidney and lung transplant recipients as described elsewhere [14, 15].

Most ARM was detected only in UL97 (14/21, 66.66%), indicating that the use of classical antivirals such as CDV and FOS, whose action mechanisms do not depend on UL97 kinase, is a reliable therapeutic option despite their wide use in transplant patients as alternative drugs. There was involvement of both UL97 and UL54 in 19.04% (4/21) of patients with ARM. Surprisingly, in three patients ARM was only found in UL54; this fact may be explained by the fact that some ARM in UL97 may have reverted to wild-type after switching therapy to FOS or CDV. In this sense, previous experiments have shown that, fortunately, the most common ARM found, L595S/W, reverts after a while, provided that the selective pressure of GCV is removed [16], suggesting a certain disadvantage of this ARM compared to susceptible wild-type. Of note is the high proportion of patients with treatment failure unrelated to ARM: 72,72% (48/66) and 89,23% (58/65) regarding UL97 and UL54, respectively. Unknown factors probably related to the patient's condition and/or virus virulence may be also responsible for most refractory CMV infections. Therefore, the absence of response to treatment is not decisive to establish a case of antiviral resistance, and confirmation with genotypic methods [11, 13] is required at any rate [17]. Only two HSCT patients had not refractory CMV infections, which is in agreement with previous studies indicating that resistant CMV infections remain a rare complication in HSCT recipients, whereas refractory infections are more commonly found [18].

In this study, we searched for consensus ARM related to the lack of effectiveness of the main antivirals used against CMV (GCV, FOS, CDV, VGCV and LET) (Table 1). The presence of each of the mutations can affect a single drug or several ones simultaneously. Among the mutations found in the UL97 gene, H520Q/E and C603W/R/S were previously associated with high rates of resistance to GCV. However, the role of others, such as D605E, is controversial and, depending on the study, may be regarded as a resistance mutation or a variant of the natural sequence [19]. Recent recombinant phenotypic experiments indicated that this mutation did not confer resistance to GCV [13]. Therefore, we did not consider D605E, found in three patients, as an ARM.

Concerning resistance to LET, previously described ARMs were related to mutations located between amino acids 230 and 370 of UL56 [10, 18]. In vitro and clinical studies showed that ARM developed faster than in UL97 and UL54, which is a reason for increasing concern among clinicians and virologists. Regarding UL56, since two naturally occurring sequence polymorphisms (L241P and R369S) were described to confer 160-fold and 38-fold reduced susceptibility to LET [20], respectively, we decided to study this gene despite only one patient with suspected resistance was treated with LET and, even with treatment failure, no ARM was found in UL56. Although the main target of ARM to LET has been found in UL56, other ARMs in UL51 and UL89 could not be ruled out. Seven patients with ARMs in UL54 were found, four of them with combined ARMs in UL97, which suggests that most of the ARMs were accumulated in UL97 kinase when GCV or a closely related antiviral as VGCV was used. This finding is in agreement with previous studies, in which more than 90% of ARMs occurred in the UL97 gene, specifically between codons 460-520 and 590-607 [3, 6, 13, 15]. Other antivirals, such as FOS and CDV could be used instead in these cases, which highlights the importance of genotypic determination of ARMs for a right therapeutic choice. ARM was also found in UL54 DNA polymerase being T503I the most common (3/7 patients) which has been described as conferring resistance to GCV and CDV as well as A987G (2/7 patients). One patient developed multiple ARMs in UL54, one of which (A834P) is related to the appearance of resistance to FOS [19, 21].

In addition to the above-mentioned ARMs, other mutations compared to reference wild-type strains were found because of a certain polymorphism in UL54. The frequency of some of them is high, as in the case of S655L (51.14%) and F669L (42.86%) located at UL54. However, their consideration as candidate ARMs requires further recombinant phenotypic or marker transfer studies. It should be noted that the occurrence of multiple ARMs, which markedly increases antiviral resistance, thus

complicating prognosis and treatment management [22, 23], was a common event: (8/21) of patients with ARMs.

In the search of ARMs in cohorts of patients with suspected resistance to antivirals, efforts have been made in many laboratories worldwide to develop NGS-based methods due to their ability to multiplex large numbers of samples. However, in our experience, for routine virological screening with few patients, NGS assays are still quite costly and time-consuming compared to PCR and Sanger sequencing. The main advantage of NGS was that ARMs may be characterised in samples with lower viral load [11] or when minor resistant subpopulations exist.

Despite limitations, the findings of this work contribute to reinforce the observation of the presence of mutations associated with drug resistance previously described, while making a case for the discussion on the involvement of new ones in the emergence of antiviral resistance. It is also shown that drug resistance is an important feature of CMV pathogenesis in transplant recipients that may threaten transplant outcomes, while the value of genotypic testing to identify potential antiviral resistance mutations is highlighted, which in turn could contribute to a better virological diagnosis and clinical performance.

Limitations of the study

CNM service portfolio includes characterization of resistance mutations in UL97 and UL54. Treatment with LET was carried out in only one patient. However, due to the rapid emergence of ARMs in UL56, its characterization was included to know if a basal level of ARM occurred. Sanger sequencing is not able to detect subpopulations of CMV below 20-30% of the total, therefore minor subpopulations of CMV with ARMs, if any, were not identified. We established that direct amplification of clinical samples and sequencing required a viral load threshold ranging from 10³ IU/mL to 10⁴ IU/mL in order to obtain high-quality sequences for feasible analysis. In contrast, real-time PCR was able to detect below 10^2 IU/mL. Despite CMV has been previously detected at hospital, in many samples UL54, UL97 and UL56 are unable for feasible analysis because of poor quality of sequences attributable to low viral load and/or repeatedly freezing/melting processes, etc. Therefore, the patient was included in the study only when at least one gene was able to analyse. Moreover, different PCR efficacies result in that nearby 30% of patients only UL56 was able to be analysed.

Some relevant characteristics of patients such as CMV serostatus (D/R) or days after SOT or HSCT were not available.

Abbreviations

ARM	Antiviral resistance mutation
CDV	Cidofovir
CMV+	Positive CMV real-time PCR
CMV	Cytomegalovirus

CNM National Center for Microbiology FBV Fostein-Barr Virus FOS Foscarnet GCV Ganciclovir HHV-6 Human herpesvirus 6 HHV-7 Human herpesvirus 7 HHV-8 Human herpesvirus 8 HSCT Hematopoietic stem cells transplantation LET Letermovir SOT

Solid organ transplantation

VGCV Valganciclovir

Supplementary Information

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Supplementary Material 1

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Authors' contributions

David Tarragó contributed to the study conception and design. Material preparation was performed by Vanessa Recio and Irene González, data collection and analysis were performed by David Tarragó, manuscript was written by David Tarragó.

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Data Availability

The datasets generated and analysed regarding clinical samples and patients are in Table 1 of Supplementary material.

Declarations

Ethics approval and consent to participate

All methods were carried out in accordance with relevant guidelines and UE regulations. All experimental protocols including the use of residual clinical specimens submitted for virological diagnosis and written informed consent from all subjects and/or their legal guardian(s) was approved by the Ethics Committee of the "Instituto de Salud Carlos III" (CEI PI 11_2021-v3).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

- Camargo JF, Komanduri KV. 2017. Emerging concepts in cytomegalovirus infection following hematopoietic stem cell transplantation. Hematol. Oncol Stem Cell Ther 10:233–238. (2) Razonable RR, Humar A. 2013. Cytomegalovirus in solid organ transplantation. Am J Transplant 13:93-106.
- 2. Razonable RR, Humar A. Cytomegalovirus in solid organ transplant recipients-Guidelines of the American Society of Transplantation Infectious Diseases Community of Practice. Clin Transplant. 2019;33(9). https://doi. org/10.1111/ctr.13512.

- Razonable RR. Drug-resistant cytomegalovirus: clinical implications of specific mutations. Curr Opin Organ Transplant. 2018;23:388–94.
- Chan ST, Logan AC. The clinical impact of cytomegalovirus infection following allogeneic hematopoietic cell transplantation: why the quest for meaningful prophylaxis still matters. Blood Rev. 2017;31:173–83.
- Chen K, Cheng MP, Hammond SP, Einsele H, Marty FM. Antiviral prophylaxis for cytomegalovirus infection in allogeneic hematopoietic cell transplantation. Blood Adv. 2018;2:2159–75.
- Fischer L, Imrich E, Sampaio KL, Hofmann J, Jahn G, Hamprecht K, Göhring K. Identification of resistance-associated HCMV UL97- and UL54-mutations and a UL97-polymorphism with impact on phenotypic drug-resistance. Antiviral Res. 2016;131:1–8.
- Chemaly RF, Chou S, Einsele H, Griffiths P, Avery R, Razonable RR, Mullane KM, Kotton C, Lundgren J, Komatsu TE, Lischka P, Josephson F, Douglas CM, Umeh O, Miller V, Ljungman P, Resistant Definitions Working Group of the Cytomegalovirus Drug Development Forum. Definitions of resistant and refractory cytomegalovirus infection and disease in transplant recipients for use in clinical trials. Clin Infect Dis. 2019;68:1420–6.
- Ligat G, Cazal R, Hantz S, Alain S. The human cytomegalovirus terminase complex as an antiviral target: a close-up view. FEMS Microbiol Rev. 2018;42:137–45.
- El Helou G, Razonable RR. Letermovir for the prevention of cytomegalovirus infection and disease in transplant recipients: an evidence-based review. Infect Drug Resist. 2019;12:1481–91.
- Jung S, Michel M, Stamminger T, Michel D. Fast breakthrough of resistant cytomegalovirus during secondary letermovir prophylaxis in a hematopoietic stem cell transplant recipient. BMC Infect Dis. 2019;19(1):388.
- 11. López-Aladid R, Guiu A, Mosquera MM, López-Medrano F, Cofán F, Linares L, Torre-Cisneros J, Vidal E, Moreno A, Aguado JM, Cordero E, Martin-Gandul C, Carratalá J, Sabé N, Niubó J, Cervera C, Capón A, Cervilla A, Santos M, Bodro M, Muñoz P, Fariñas MC, Antón A, Aranzamendi M, Montejo M, Pérez-Romero P, Len O, Marcos M. Improvement in detecting cytomegalovirus drug resistance mutations in solid organ transplant recipients with suspected resistance using next-generation sequencing. PLoS ONE. 2019;14(7):e0219701.
- 12. Lurain NS, Chou S. Antiviral drug resistance of human cytomegalovirus. Clin Microbiol Rev. 2010;23(4):689–712.
- 13. Sunwen Chou S. Advances in the genotypic diagnosis of cytomegalovirus antiviral drug resistance. Antiviral Res. 2020;176:104711.

- Iwasenko JM, Scott GM, Naing Z, Glanville AR, Rawlinson WD. Diversity of antiviral-resistant human cytomegalovirus in heart and lung transplant recipients. Transpl Infect Dis. 2011;13:145–53.
- 15. Hakki M, Chou S. The biology of cytomegalovirus drug resistance. Curr Opin Infect Dis. 2011;24:605–11.
- Sedlak RH, Castor J, Butler-Wu SM, Chan E, Cook L, Limaye AP, Jerome KR. Rapid detection of human cytomegalovirus UL97 and UL54 mutations directly from patient samples. J Clin Microbiol. 2013;51:2354–9.
- Fischer L, Sampaio KL, Jahn G, Hamprecht K, Göhring K. Identification of newly detected, drug-related HCMV UL97- and UL54-mutations using a modified plaque reduction assay. J Clin Virol. 2015;69:150–5.
- Sassine J, Khawaja F, Shigle TL, Handy V, Foolad F, Aitken SL, Jiang Y, Champlin R, Shpall E, Rezvani K, Ariza-Heredia EJ, Chemaly RF. Refractory and resistant Cytomegalovirus after hematopoietic cell transplant in the Letermovir Primary Prophylaxis Era. Clin Infect Dis. 2021;73:1346–54.
- Aslani HR, Ziaie S, Salamzade J, Zaheri S. Incidence of ganciclovir resistance in CMV-positive renal transplant recipients and its association with UL97 gene mutations. Iran J Pharm Res. 2017;16:802–7.
- Goldner T, Zimmermann H, Lischka P. Phenotypic characterization of two naturally occurring human cytomegalovirus sequence polymorphisms located in a distinct region of ORF UL56 known to be involved in in vitro resistance to letermovir. Antiviral Res. 2015;116:48–50.
- 21. Razonable RR. Role of letermovir for prevention of cytomegalovirus infection after allogeneic haematopoietic stem cell transplantation. 2018. Curr Opin Infect Dis. 2018;31:286–91.
- Avery RK, Arav-Boger R, Marr KA, Kraus E, Shoham S, Lees L, Trollinger B, Shah P, Ambinder R, Neofytos D, Ostrander D, Forman M, Valsamakis A. Outcomes in transplant recipients treated with Foscarnet for Ganciclovir-Resistant or refractory cytomegalovirus infection. Transplantation. 2016;100:e74–e80.
- Scott GM, Weinberg A, Rawlinson WD, Chou S. 2007. Multidrug Resistance Conferred by Novel DNA Polymerase Mutations in Human Cytomegalovirus Isolates. *Antimicrob. Agents Chemother* 51:89 LP – 94.

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