



Research paper

Persistent primary cytomegalovirus infection in a kidney transplant recipient: Multi-drug resistant and compartmentalized infection leading to graft loss



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ABSTRACT

Cytomegalovirus (CMV) is one of the most common opportunistic infections after transplantation. To prevent CMV infections, universal prophylaxis and pre-emptive therapy with ganciclovir or its prodrug valganciclovir is applied. However, prolonged antiviral therapy may result in drug-resistance emergence. We describe a case of a 43-year-old CMV-seronegative patient who underwent kidney transplantation from a CMV-seropositive donor and developed CMV disease despite valganciclovir prophylaxis. CMV viral load increased even though valganciclovir dose was augmented and immunosuppressive therapy reduced. CMV genotyping revealed mutations in the viral UL97 protein kinase, explaining ganciclovir-resistant CMV infection. The viral load failed to respond to foscavir, cidofovir and CMV-neutralizing immunoglobulins. Kidney allograft dysfunction developed 3 months post-transplantation with a histopathologic diagnosis of CMV nephropathy and potentially concomitant T-cell mediated rejection. A transplantectomy was performed on day 164 post-transplantation since the patient had uncontrollable CMV disease associated with a circulating multidrug-resistant DNA polymerase-mutant virus. Detailed monitoring in this patient demonstrated hallmarks of complicated CMV disease: (i) relatively rapid evolution of drug-resistant CMV mutants in the setting of persistent high blood viral loads, (ii) emergence of viral drug-resistance linked to acute graft rejection, (iii) transient and, thereafter, lack of response to various anti-CMV treatments, (iv) compartmentalization and heterogeneity of CMV viral populations, (v) possible differential ability of viral mutants to cause disease in the graft, and (vi) detection of minor viral variants by next generation sequencing. Translational research platforms that provide rapid molecular genotyping for detection of CMV drug-resistance are essential in guiding CMV disease management in high-risk transplant recipients.

1. Introduction

Kidney transplantation remains the optimal treatment for end-stage renal disease. In the last decades, early outcome after renal transplantation improved significantly with the implementation of potent immunosuppressive regimens leading to impaired immunity and the consequent increased risk for microbial infections (Haidar and Singh, 2017; Kotton and Fishman, 2005).

Cytomegalovirus (CMV), the single most important pathogen in transplant recipients due to both direct (related to viral replication) and

indirect (as a consequence of the induced immune suppression) effects, increases post-transplant morbidity and mortality (Kotton and Fishman, 2005; Stern et al., 2014). To prevent CMV infections, prophylaxis or pre-emptive therapy with ganciclovir or its prodrug valganciclovir is given to patients at risk (Kotton et al., 2018). However, prolonged therapy with anti-CMV drugs may result in emergence of drug-resistant viruses (Cherrier et al., 2018; Lurain and Chou, 2010; Springer et al., 2005). Monitoring CMV drug-resistance when clinically suspected enables rationally guided adaptation of antiviral treatment. In this respect, our translational research platform RegaVir (Research Group for

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Antiviral Resistance, www.regavir.org) was set up to provide rapid herpesvirus drug-resistance diagnosis and typing among immunocompromised hosts that fail antiviral therapy.

CMV genotyping relies on PCR amplification and sequencing of the UL97 protein kinase gene, whose product catalyses the initial phosphorylation of ganciclovir, and of the UL54 gene, coding for the DNA polymerase [the target of nucleoside (ganciclovir and valganciclovir), nucleotide (cidofovir) and pyrophosphate (foscarnet) analogues] (James and Prichard, 2011; Lurain et al., 2002). The standard method used for sequencing these genes is the Sanger dideoxy method with a detection limit of 20–30%. With next generation sequencing (NGS), improved detection of emerging drug-resistant CMV subpopulations can be achieved (Chou et al., 2014; Sahoo et al., 2013).

We report here the case of a renal transplant recipient who developed a life-threatening multi-drug resistant CMV infection that failed to respond to various alternative antiviral treatments, finally leading to graft loss. The objective of this work is to highlight the usefulness of rapid CMV genotyping for implementation of precision medicine and the advantage of NGS over conventional sequencing to detect CMV subpopulations.

2. Materials and methods

2.1. CMV viral load determination

CMV viral load was determined following an in-house PCR assay based on a quantitative in-house real-time PCR with continuous TaqMan-probe detection on an ABI 7900 real-time thermocycler (Applied Biosystems) (Goegebuuer et al., 2009).

2.2. Antiviral resistance assay for CMV (genotyping by sanger sequencing)

DNA from the clinical samples was extracted with a Qiaamp[®] DNA Blood Mini Kit (Qiagen). Three (UL97) and five (UL54) overlapping primer sets, 5'-end flanked with universal M13 sequences (to be able to sequence the amplicons with universal M13-primers), covering the regions of the viral genes where drug-resistance associated mutations map (nucleotides 990–2124 and 800–3260, respectively) were designed. PCR amplifications were carried out using the Faststart High Fidelity PCR System kit (Roche) and amplification products were purified [QIAquick PCR Purification Kit (Qiagen)]. All Sanger sequencing assays were performed bidirectionally using M13 primers. Gene amplicons were directly sequenced with the BigDye Terminator v3.1 sequencing kit (ThermoFischer Scientific).

Sequencing kit (ThermoFischer Scientific). Sequencing reactions were purified using gel filtration spin columns (Centri-Sep[™] 96-Well Plates, ThermoFischer Scientific) and analyzed with the automated sequencer ABI 3730 genetic analyzer (Applied Biosystems). The sequencing results were computer assembled and compared with UL97 protein kinase and DNA polymerase sequences from the reference AD-169 CMV strain (Genbank accession number [X17403](https://www.ncbi.nlm.nih.gov/nuccore/X17403)) using software SeqScape, version 2.7 (Applied Biosystems).

2.3. Detection of minor variants by next generation sequencing (NGS)

The Expand[™] Long Template PCR System kit (Roche) was used to amplify ~2-kb and ~4-kb targets for UL97 and UL54, respectively. PCR products were purified [QIAquick PCR Purification Kit (Qiagen)], quantified [Qubit dsDNA HS Assay Kit on the Qubit[®] 2.0 fluorometer (ThermoFischer Scientific)] and used to prepare DNA libraries (one library per sample) using 1 ng DNA with Nextera XT DNA Sample Preparation Kit and Nextera XT DNA Sample Preparation Index Kit (Illumina)]. The resulting dsDNA products were purified with Agencourt[®] AMPure[®] XP beads. The quality, fragment length distribution and concentration of the libraries were assessed (Qubit[®] fluorometer and Qubit[®] dsDNA HS Assay Kit). All libraries for NGS were

diluted to a 2 nM concentration and equal volumes of 5 µl of each library were pooled. After the normalization step, spiking of the libraries with a 5% PhiX Control v3 library (12.5pM; Illumina) was done. The library pool was sequenced with paired-end (2 × 150bp) reads on the Miseq v.2 system (Illumina). Primary data analysis was done with “Sequencing Analysis Viewer” software v1.8.11 (Illumina) and secondary data analysis with CLC bio Genomics Workbench version 8.0.2 software (Qiagen). Mapping was performed with local alignment of the reads to the reference sequences and variants were called with the Quality based variant detection tool to detect minor viral variants. Deep-sequencing data were confirmed by analyzing two independent PCR target amplifications for each viral gene.

3. Case report and evaluation of emergence of CMV drug-resistance

A 43-year old female patient (CMV seronegative) with end-stage renal failure due to obstructive renal disease received a kidney transplant from a 36-year old CMV-seropositive deceased donor (with CMV IgM and IgG positive). The immunosuppressive regimen consisted of tacrolimus, mycophenolate mofetil, corticosteroids and induction with basiliximab. Valganciclovir for CMV prophylaxis was initiated on day 5 post-transplantation at 450 mg once a day.

One week after transplantation, CMV PCR was negative in blood and the patient had excellent kidney function (serum creatinine 1.0 mg/dL; eGFR 58 mL/min/1.73 m²) but on day 29 post-transplantation CMV PCR became positive (Fig. 1). CMV viral load increased and she developed epigastric pain and diarrhea without fever. The dose of valganciclovir was then augmented to 2 × 450 mg/d (day 43 post-transplantation). Epigastric pain disappeared but diarrhea persisted and she presented with progressive adynamia. The viral load rose despite the increased valganciclovir dosing and at day 67 post-transplantation, a blood sample (RV-1050) was evaluated for drug-resistance by genotyping of UL97 and UL54 CMV genes. This specimen could be considered as a baseline viral genotype since no mutations linked to drug-resistance were detected (Table 1). At day 71 post-transplantation, she was hospitalized for mild liver dysfunction, anorexia, and acute renal functional deterioration that partly recovered (serum creatinine 1.3–1.4 mg/dL; eGFR 40–50 mL/min/1.73 m²). The CMV viral load increased to 6.4 log copies/mL. Because RV-1050 presented a wild type CMV genotype, valganciclovir was continued at a dosage of 2 × 450 mg/day (Table 1).

At day 78 post-transplantation, liver function tests deteriorated and she developed mild diarrhea. Mycophenolate mofetil dosage was decreased to 2 × 500 mg/day. On day 85 post-transplantation, she still had mild diarrhea, persistent mild liver dysfunction, and persisting high CMV viral load. Valganciclovir was discontinued and ganciclovir (dose 2 × 2.5 mg/kg/day) was started. At day 87 post-transplantation, she had no other symptoms than persistent adynamia and diarrhea. A kidney transplant biopsy per protocol (CS_3_2) was essentially normal without inflammation, in which wild type CMV was retrospectively found. At this occasion, a blood sample (RV-1057) showed a persisting high CMV viral load of 6.5 log/mL. At day 91 post-transplantation, liver function tests improved and diarrhea decreased. Ganciclovir therapy was halted after 7 days (day 94) when RV-1057 CMV genotyping was available, demonstrating a heterogeneous population of the UL97 C592G mutant, known to confer 2.5x ganciclovir resistance. As subsequent lab tests showed progressive thrombocytopenia and increased C-reactive protein with high CMV viral load, foscarnet was started at an initial dose of 2 × 2800 mg/day (2 × 50 mg/kg, adjusted for renal function fluctuating between 40 and 50 mL/min/2.73 m²). This dose was lowered after 6 days treatment due to further renal function decline. The patient developed progressive renal dysfunction and creatinine levels rose to 6.27 mg/dL (eGFR 7 mL/min/1.73 m²). At day 101 post-transplantation, mycophenolate mofetil was stopped. Foscarnet therapy was discontinued at day 108 post-transplantation due to renal

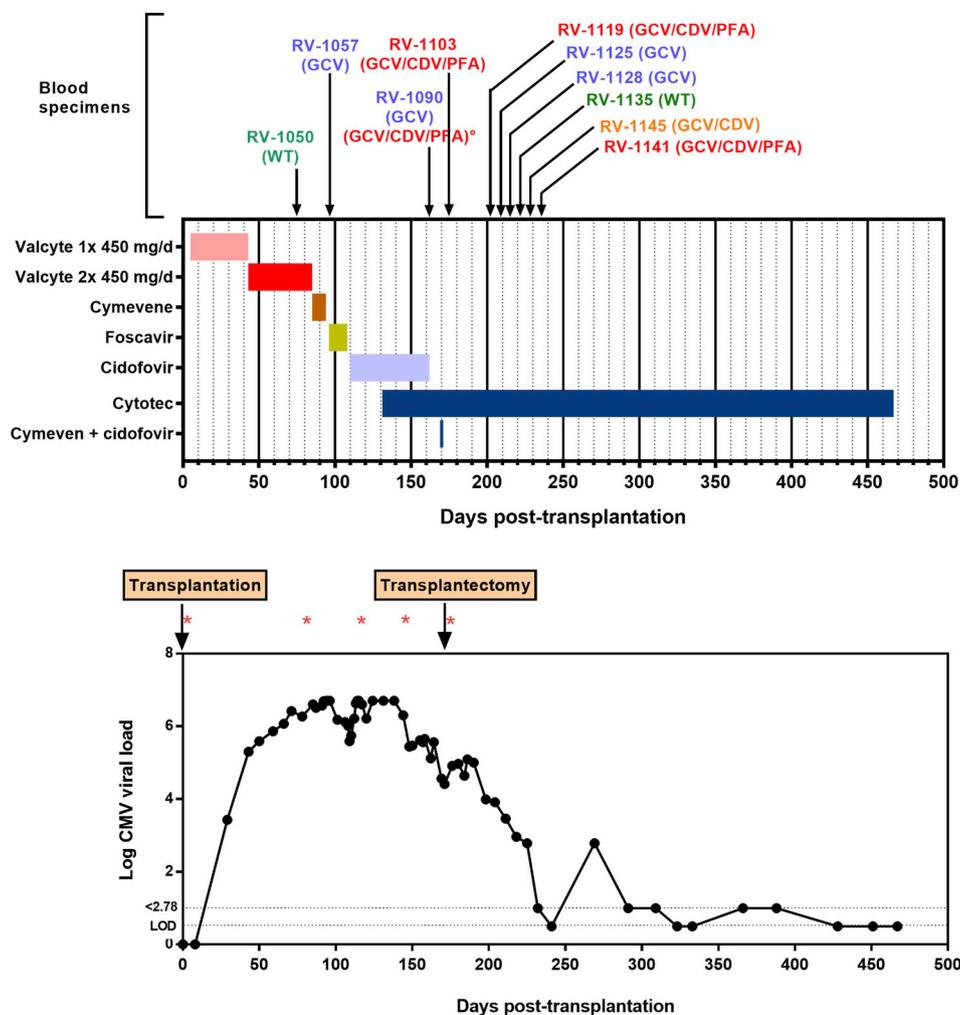


Fig. 1. Overview of CMV-DNA PCR in relation to time after kidney transplantation. Anti-CMV therapy and samples were genotypically analyzed prospectively (by Sanger sequencing) or retrospectively (by NGS). Abbreviations: WT: wild-type, GCV: ganciclovir, CDV: cidofovir, PFA: foscarnet. Between brackets are indicated the drugs against which resistance was determined following conventional CMV genotyping. Indicates resistance as determined retrospectively by NGS differing from Sanger sequencing.

* Transplant biopsy performed. Cytotec: CMV Ig.

impairment and as thrombocyte counts had normalized. C-reactive protein levels remained mildly increased.

A kidney allograft biopsy for graft dysfunction performed at day 109 post-transplantation showed clear T-cell mediated rejection with tubulitis grade 2 and interstitial inflammation grade 3. Anti-rejection treatment with high dose corticosteroids was started. The kidney biopsy (CS_3_3) showed renal tubular epithelial cells with nuclear inclusions that stained positive for CMV confirming the diagnosis of CMV nephropathy (Fig. 2). Retrospective genotyping by Sanger sequencing indicated a mix of wild type and A594V UL97 mutant (known to be associated with 5–15x ganciclovir resistance) in the kidney biopsy while the UL97 A594V and C592G mutations were detected as heterogeneous populations in the blood (CS_3_8). At day 110 after transplantation, cidofovir was initiated with weekly infusion (1st infusion 1 mg/kg, then 2.5 mg/kg and thereafter 5 mg/kg weekly dosage).

The renal function and diarrhea improved but liver functional tests worsened by day 117 post-transplantation. CMV viral load persisted (6.6 log copies/mL) but the creatinine level improved (1.3 mg/dL; eGFR 45 mL/min/1.73 m²). Recovery of liver function tests but recurrent mild thrombocytopenia were seen at day 124 post-transplantation. She was then again hospitalized at day 131 post-transplantation for fever and flu-like symptoms and CMV Ig (100 IU CMV antibodies/kg weekly)

was started in addition to the weekly cidofovir infusions. Seven days later, kidney function deteriorated to creatinine levels of 3.3 mg/dL and tacrolimus levels were also minimized. CMV viral load continued to be high (5.6 log/mL) and at day 152 post-transplantation a blood sample (RV-1090), genotyped by Sanger sequencing, showed a heterogeneous UL97 A594V population. A kidney transplant biopsy at day 157 post-transplantation (CS_3_4) showed minimal tubulitis and some glomerulitis, extensive interstitial fibrosis, clear nuclear inclusions and anisokaryosis, all suggestive of CMV inclusions, but CMV stain and PCR were negative in this biopsy. She developed progressive liver dysfunction and progressive epigastric pain with visualization of an erosive gastropathy at endoscopic evaluation. Two biopsies were taken, staining negative at the stomach level but positive for CMV at the duodenum. At this time, the patient had a circulating (CS_3_9) CMV infection associated with a mix of wild type and UL97 A594V viruses.

At day 162 post-transplantation, the patient reported eye symptoms and anterior uveitis was diagnosed. An intra-ocular sampling showed weakly positive CMV PCR (i.e. CMV uveitis), suggesting a possible contribution of cidofovir in the development of uveitis. Cidofovir was discontinued. Finally, at day 165 post-transplantation, it was decided to carry out a transplantectomy to enable cessation of immunosuppression tacrolimus. Monotherapy with low-dose methylprednisolone was

Table 1
Genotyping variants detected by standard sequencing (Sanger) and next generation sequencing (Illumina platform).

Day	PT	Sample ID	CMV DNA (log)	Specimen type	Analysis performed	Amino acid changes related to resistance in: UL97 (protein kinase)	UL54 (DNA polymerase)	Amino acid changes related to natural genetic polymorphisms in:	Resistance to:
0		CS_3.1 (B-1825736)	NA***	Transplant kidney biopsy	Retrospective (Sanger)	Not amplifiable		Not amplifiable	/
67		RV-1050	6.07	Blood	Prospective (Sanger)	None	None	S897L	Wild-type
87		CS_3.2 (B-1836685)	NA	Kidney transplant protocol biopsy	Retrospective (NGS)	None	None	S897L	Wild-type
88		RV-1057	6.51	Blood	Prospective (Sanger)	C592G ^b	None	S897L	GCV
109		CS_3.3 (B-1839887)	NA	Kidney biopsy for graft dysfunction	Retrospective (NGS)	C592G (10.97) ^a A594V (10.75)	None	S897L (99.60)	GCV
109		CS_3.8	NA	Blood	Retrospective (Sanger)	A594V ^b C592G ^b	None	S897L	GCV
152		RV-1090	5.47	Blood	Prospective (Sanger)	A594V ^b	None	S897L	GCV
157		CS_3.4 (B-1846802)	NA	Kidney biopsy for graft dysfunction	Retrospective (NGS)	C592G (2.09) A594V (68.44)	T503A (1.59) P522S (1.73) Δ 981–982 (3.03)	S897L (99.72)	GCV, CDV, PFA
158		CS_3.9	NA	Blood	Retrospective (Sanger)	Not amplifiable			/
165		RV-1103	5.56	Blood	Retrospective (Sanger)	A594V ^b	None	S897L	GCV
165		CS_3.7 (B-1847724-01-03)	NA	Transplantectomy kidney biopsy	Prospective (Sanger)	A594V ^b	Δ 981–982 ^b P522S (1.27) Δ 981–982 (37.37) T503A ^b	S897L (99.87)	GCV, CDV, PFA
192		RV-1119	5.0	blood	Retrospective (Sanger)	A594V ^b	None	S897L	GCV, CDV
198		RV-1125	3.99	blood	Prospective (Sanger)	A594V ^b	Δ 981–982 ^a	S897L	GCV, CDV, PFA
204		RV-1128	3.91	blood	Prospective (Sanger)	A594V ^b	None	S897L	GCV
211		RV-1135	3.46	blood	Prospective (Sanger)	None	None	S897L	Wild-type
218		RV-1145	2.96	blood	Prospective (Sanger)	A594V	P522S ^b	S897L	GCV, CDV
225		RV-1141	2.78	blood	Prospective (Sanger)	A594V ^b	Δ 981–982	S897L	GCV, CDV, PFA

NA: not available.

^b Heterogeneous population of mutant and wild-type virus detected by Sanger sequencing.

^a Percentage of subpopulation variants detected by deep sequencing (Illumina platform).

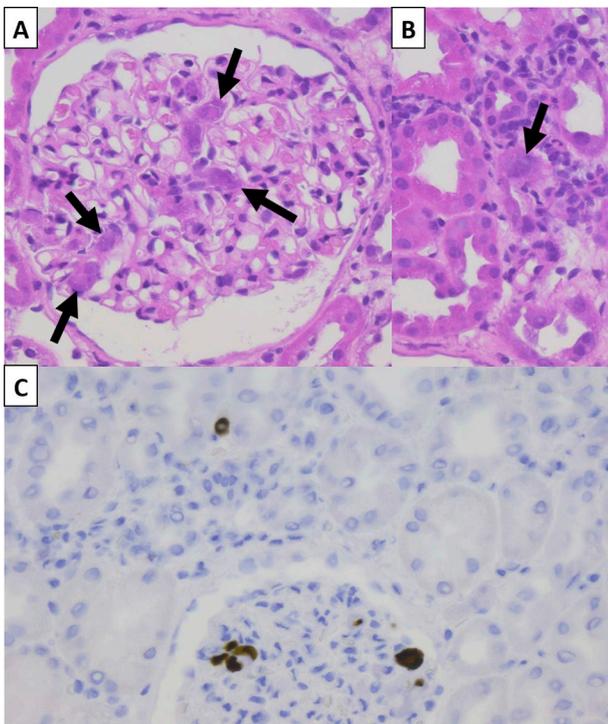


Fig. 2. The kidney biopsy showed several glomerular (A) and tubular (B) cells with enlarged nuclei, containing inclusions (arrows). These showed immune reactivity for CMV (C), confirming the diagnosis of CMV nephropathy.

continued. Four days after the transplant nephrectomy, liver function tests improved but CMV viral load remained high at 4.56 logs. Therefore, ganciclovir in combination with foscarnet and CMV Ig were restarted. Histopathological evaluation of the explanted kidney graft (CS_3_7) demonstrated T-cell mediated rejection type 2 (intimal arteritis, although in the absence of tubulitis or interstitial inflammation), and was suggestive of glomerular CMV localization (Fig. 2). This biopsy (CS_3_7) presented the A594V UL97 and the novel T503A UL54 changes, detected retrospectively as heterogeneous populations by Sanger sequencing. The resistance phenotype of the T503A UL54 substitution can be inferred from the properties of known mutant T503I (linked to ganciclovir and cidofovir resistance) (Chou, 2008), though the level of resistance conferred by different amino acid substitutions at the same codon may vary. The blood sample obtained at the time of the transplant nephrectomy (RV-1103) harbored the UL97 A594V mutation together with the DNA polymerase 981–982 deletion, both detected as mixed populations by conventional sequencing. Since the DNA polymerase 981–982 deletion is known to confer multi-drug resistance, antiviral therapy was halted and CMV Ig was continued from day 171 post-transplantation onwards.

As the CMV viral load decreased very slowly, several blood samples were genotyped between days 192 and 225 post-transplantation. Except for RV-1135 that had a wild-type CMV genotype, they all carried the A594V UL97 change with no changes in the DNA polymerase (RV-1125 and RV-1128) or with the P522S substitution (RV-1145), or the deletion 981–982 (RV-1141). By day 322 after transplantation, CMV PCR became negative.

4. Retrospective deep sequencing

Because data obtained by conventional genotyping (Table 1) indicated the presence of heterogeneous populations in several specimens, four samples obtained before transplantectomy were analyzed retrospectively for quantification of the viral variants by deep sequencing using the Illumina platform (Table 1). Results for RV-1050

(obtained during valganciclovir treatment) were consistent with conventional sequencing in showing a 99.55% population with the UL97 natural polymorphism S897L relative to the reference CMV strain. At day 88 post-transplantation, resistance-related subpopulations with the UL97 mutations C592G (10.97%) and the A594V (10.75%) were detected in RV-1057 (obtained when valganciclovir therapy was switched to ganciclovir). With the Sanger sequencing method, we successfully detected the C592G change, but not the A594V mutation. At day 152 post-transplantation, after the patient had been treated with valganciclovir, ganciclovir, and foscarnet and when she was under cidofovir and CMV Ig therapy, the same UL97 resistance-subpopulations were detected but with increased proportion of the A594V (68.44%) relative to the C592G (2.09%) (RV-1090). Three UL54 resistant-related variants were detected at low abundance in the RV-1090: T503A (1.59%), P522S (1.73%), together with the 981–982 deletion (3.03%), that similar to the UL97 C592G variant, were not identified by Sanger sequencing. Thirteen days later (RV-1103), the UL97 resistance-subpopulation decreased [A594V (22.86%)] or became undetectable (C592G) but the UL54 deletion 981–982, increased in abundance, which could be expected with discontinuation of ganciclovir and use of foscarnet and cidofovir during the preceding few weeks.

5. Discussion

This case report illustrates the potentially devastating course of CMV infection after solid organ transplantation and focuses on the importance of monitoring CMV load, awareness of antiviral resistance and hints towards potential clinical implications of NGS for diagnosis and treatment decisions.

This case report is important in several aspects. First, our report clearly illustrates the importance of drug-resistance monitoring over time for tailored anti-viral therapy. The case is marked by the swift evolution of drug-resistant CMV mutants related to persistently high blood viral loads. Second, next to the direct consequences of CMV infection also indirect consequences are present in this case report, as the patient developed acute graft rejection. Third, this case also demonstrates the transient and incomplete response to various anti-CMV treatments, the possibility of dissimilar CMV viral populations in different body compartments and the increased ability of certain viral mutants to replicate in the graft. Finally, by using NGS, the limitations of Sanger sequencing to detect minor viral subpopulation are put forward. Careful molecular diagnostics in our patient allowed optimal treatment options and support in the difficult but eventually life-saving decision to perform transplantectomy.

CMV increases the risk of acute allograft rejection and interstitial fibrosis with tubular atrophy in the kidney graft by inducing an excessive immune reaction due to upregulation of cell adhesion molecules, increased expression of human leukocyte antigens and activation of cytotoxic T cells (Opelz and Dohler, 2015; Reischig et al., 2006). The risk of CMV infection is highly dependent on the donor and recipient serological status. Besides the donor/recipient serological status, short prophylaxis duration and higher levels of immunosuppressive therapy are risk factors for developing CMV disease (Hasegawa et al., 2017). Universal prophylaxis (administration of antivirals to all patients at high risk for CMV, i.e. donor-positive, recipient-negative) is recommended in the early post-transplantation period up to 6 months to increase graft survival in patients lacking CMV-specific immunity (Komorowska-Jagielska et al., 2018; Kuo et al., 2010).

Valganciclovir is currently the most commonly used drug for CMV prophylaxis because of its improved bioavailability relative to ganciclovir (Kotton et al., 2018). The usual dose of valganciclovir for prophylaxis is 900 mg daily versus treatment dose (900 mg twice daily), although this needs to be adjusted for the variable kidney function, as was done in the case of our patient. Valganciclovir 450 mg daily is also effective for CMV prophylaxis and is associated with lower risk for hematological side effects than the high dose (Gabardi et al., 2015;

Halim et al., 2016; Heldenbrand et al., 2016; Stevens et al., 2015). According to a systematic review and meta-analysis, valganciclovir 900 mg and 450 mg daily dosing are equipotent for CMV prophylaxis in all-risk renal transplant recipients at least within the first year of transplantation with no differences regarding acute rejection, allograft loss, mortality, opportunistic infections, premature discontinuation of valganciclovir treatment and leukopenia (Xin et al., 2017). However, major concerns with the 450 mg daily dose are higher risk of ganciclovir resistance and breakthrough infection among CMV donor-positive/recipient-negative (D+/R-) kidney transplant recipients (Gabardi et al., 2015; Stevens et al., 2015).

Renal transplant recipients with both early-onset (< 3 months) and late-onset (> 3 months) CMV DNAemia with ≥ 2000 copies/ml are at increased risk for graft loss (Reischig et al., 2017). In our case, persistent high viral replication in blood occurred despite implementation of a double valganciclovir dose and reduction of immunosuppression. Failure to achieve significant viral load reduction or persisting symptomatic disease beyond 2 weeks of antiviral therapy should be interpreted as an inadequate response. As these clinical features by themselves do not imply that viral drug-resistance is present, genotypic assays for viral drug-resistance mutations should be performed. Among solid organ transplant patients, the median duration of valganciclovir therapy prior to drug-resistance emergence is approximately 22 weeks (Cherrier et al., 2018) and foscarnet has been used successfully to manage CMV infections due to UL 97-resistant mutations in D+/R-transplant recipients (Myhre et al., 2011). “In our patient, at day 157–158 post-transplantation, the UL97 mutant virus could be cleared from the allograft (CS_3.4) but not from the blood (CS_3.9) following cidofovir and foscarnet treatment and reduction of immunosuppressive treatment, as the A594V UL97 mutant was detected in the blood. High levels of CMV replication, unresponsive to different anti-CMV agents, CMV Ig and reduced immune suppression finally lead to the emergence of a multidrug-resistant circulating CMV infection and graft loss (day 165 post-transplantation).

Our patient was at risk for development of CMV drug-resistance with prolonged antiviral drug exposure, ongoing active replication, lack of prior CMV immunity, strong immune suppression and potentially insufficient drug delivery (Kotton et al., 2018). Further, the lack of CMV specific immunity, which is known to play a critical role in the development and severity of CMV disease, could also be responsible for the failure to clear the virus before transplantectomy. Analysis of CMV-specific T-cell frequencies and function is being considered as a potential biomarker to predict the patient's ability to control CMV disease (Egli et al., 2012). In CMV-naive solid organ transplant recipients, primary CMV infection usually occurs following reactivation of the latent virus carried in the graft. Considering that, the donor had CMV IgM and IgG, indicative of a recent primary infection with a possibly ongoing asymptomatic low viremia, the donor might have transmitted an active CMV infection to our patient. CMV transmission has been reported from a CMV IgM positive donor only a few days after transplantation (Gangopadhyay et al., 2016), similar to our patient (CMV PCR positive at day 29 post-transplantation).

Discordant genotypes between a blood specimen and the grafted kidney (compartmentalization) were noticed in our case. At three months post-transplantation, a wild-type CMV was replicating in the kidney (CS_3.2) while the circulating virus (RV-1057) bore changes in the UL97 protein kinase. NGS showed a clear advantage over conventional genotyping in detecting UL97 variants in RV-1057: C592G and A594V (by NGS) versus C592G (by Sanger sequencing). It can be hypothesized that drug-resistant CMV was selected first in blood and then invaded the graft since at day 109 post-transplantation, the A594V mutant was found in blood (CS_3.8) and kidney (CS_3.3). An even more striking superiority of NGS over Sanger sequencing is exemplified by sample RV-1090, as three different DNA polymerase mutations, including the multi-drug resistance Δ 981–982 were solely detected by NGS. Thus, resistance to ganciclovir, cidofovir and foscarnet could have

been predicted almost 2 weeks in advance if NGS was performed prospectively. Another remarkable example of viral compartmentalization was found at transplantectomy. Although the A594V UL97 was found in blood (RV-1103) and kidney (CS_3.7), the Δ 981–982 and P522S DNA polymerase mutants were present in blood and the T503A mutation in the kidney, indicating that the T503A, detected as a minor population in blood at day 152 by NGS had the ability to infect and replicate in the kidney. Our findings clearly indicate a compartmentalized evolution of the viral subpopulations as highlighted in our previous studies (Bache et al., 2014; Bauters et al., 2016), which warrants genotyping of tissue-specific specimens together with blood in patients unresponsive to antiviral therapy. Because different viral mutants can be selected at relatively low amounts in blood where high levels of viral replication take place and some minor viral variants have the ability to invade the graft causing disease, their rapid detection by NGS should be beneficial. Unlike the C592G UL97, P522S and Δ 981–982 DNA polymerase mutants, the UL97 A594V and DNA polymerase T503A mutants were able to infect the graft. This could be explained by a difference in tissue-specific virulence of the viral mutants or a sampling or local drug concentration issue. Analyzing viral evolution of compartmentalized CMV subpopulations is limited by the practical difficulty of obtaining tissue-specific samples. However, compartmentalized subpopulations should be sought in cases of CMV life-threatening disease when organ biopsy is performed for graft dysfunction.

Although NGS provides a more detailed diagnosis of viral mutant subpopulations, the few studies that have analyzed herpesvirus subpopulations by deep sequencing were done retrospectively because specimens are currently processed in batch (Chou, 2015; Chou et al., 2014; Garrigue et al., 2016). NGS is becoming more accessible but automation and streamlined processing of samples is still required for *ex tempore* diagnosis of viral drug-resistance to deliver results in a short time.

In conclusion, this case report offers a detailed outlook of the relevance of rapid molecular modern techniques, in particular NGS, for detection of compartmentalized CMV drug-resistance in the guidance of CMV disease management in high-risk transplant recipients. In-depth studies on viral evolution of compartmentalized CMV subpopulations will shed light on the differential capacity of viral mutants to cause tissue-specific disease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2019.06.004>.

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