

**ANALYSIS OF CYTOMEGALOVIRUS UL97 DRUG
RESISTANCE MUTATIONS IN PATIENTS
RECEIVING GANCICLOVIR**

**NOKWAZI PEARL NKOSI
NKSNOK009**

**SUBMITTED TO THE UNIVERSITY OF CAPE TOWN
In partial fulfilment of the requirements for the degree
MMED VIROL**

**FACULTY OF HEALTH SCIENCES
UNIVERSITY OF CAPE TOWN**

**Date of Submission: November 2017
Supervisor(s): Dr. N. Hsiao
Dr. S. Korsman
Dr. H. Smuts**

Department of Medical Virology, University of Cape Town

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

DECLARATION

I, ...Nokwazi Pearl Nkosi....., hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature:

Signed by candidate

Date: 01/11/2017.....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people without whom this work would not have been possible.

Prof. D.R. Hardie, C18 NHLS Virology Laboratory Groote Schuur Hospital, for providing effective leadership and for patient referral.

My supervisors: Dr. N. Hsiao; Dr. S. Korsman and Dr. H. Smuts for the conception of the project, technical guidance, troubleshooting and invaluable expertise at various phases of the project. I have acquired numerous skills during the course of the project.

The C18 NHLS Manual Molecular Virology staff for amicably accommodating me by sharing your workspace, thermocyclers, reagents and effective resolution of queries. J. Parsons, V. Waka, N. Mguzulwa, N. Booie, A. Steneveld, N. Jappie.

Dr. H. Rabie at Tygerberg Hospital for patient referral, provision of clinical information and facilitating access to patient folders.

Prof. B. Eley, Sr. P. Apolles, Prof. A. Davidson at Red Cross War Memorial Children's Hospital for patient referral and facilitating access to patient folder and clinical information. Dr. F. du Toit at Groote Schuur Hospital Haematology department and Dr. D. Reddy at Red Cross War Memorial Children's Hospital for patient referral.

The National Health Laboratory Service (NHLS) for funding of the sequencing costs, reagent costs and attendance of the PathRed Conference.

Prof. C. Williamson, Head of Division, UCT Medical Virology, for sponsoring attendance to the Virology Africa Conference.

The Poliomyelitis Research Foundation for funding the Virology Africa poster.

Table of Contents

Abbreviations	1
List of Tables	2
List of Figures.....	2
Abstract	3
Chapter 1.Literature Review	5
Chapter 2.Methods.....	23
2.1. Introduction	23
2.2. Aims and Objectives	24
2.3 Clinical Materials and Methods.....	24
2.3.1 Ethics.....	24
2.3.2 Samples and Source Population.....	24
2.3.3 Assay validation.....	25
2.3.4 Assay.....	25
2.3.5 Sequencing and Sequence Analysis.....	26
2.3.6 Interpretation and Analysis	27
Chapter 3.Results of the Study	29
3.1. Patients	29
3.2 Results.....	31
3.3 Case Reports and Discussion	38
Patient A	38
Patient B	40
Patient C	43
3.4 Other UL97 Drug Resistance Mutations	44
3.5 Sequence Variants/Genetic Polymorphisms	44
3.6 Limitations	45
3.6.1 Sample Size	45
3.6.2 Lack of Clinical Correlation	45
3.6.3 Sequencing.....	46
3.6.4 Bias.....	47
Selection Bias	47
PCR Amplification, Sequencing and Sequence Analysis Bias	48

Chapter 4. Conclusion and Recommendations	52
References	54
Appendices.....	64

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
bp	Base pair
BLAST	Basic local alignment search tool
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
CMVVL	Cytomegalovirus Viral Load
Cps/ml	Copies per millilitre
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribose Nucleoside Triphosphate
FDA	Food and Drug Administration
gB	Glycoprotein B
GC	Guanosine- Cytosine
gH	Glycoprotein H
gN	Glycoprotein N
HAEMAT/ONCOL	Haematological Oncology
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
Kbp	Kilobase pairs
LIS	Laboratory Information System
PCR	Polymerase Chain Reaction
pUL97	Protein 97 in unique long region
NCBI	National Centre of Bio-Informatics
NHLS	National Health Laboratory Services
QCMD	Quality Control of Molecular Diagnosis
SOT	Solid organ transplant
<i>Taq</i> Polymerase	<i>Thermus aquaticus</i> polymerase
UL55	Unique Long Region 55
UL73	Unique Long Region 73
UL75	Unique Long Region 75
UL97	Unique Long region 97
UV	Ultraviolet

List of Tables

Table Number	Table Heading	Page Number
Table 1	Summary of risk factors associated with development of CMV drug resistance mutations.	12
Table 2	Patient demographics and baseline characteristics.	30
Table 3	Patient clinical parameters and CMV UL97 mutations identified.	33
Table 3a	Drug sensitive and resistant CMV UL97 mutations detected.	34
Table 3b	Frequency of CMV UL97 mutations detected.	34

List of Figures

Figure Number	Figure Heading	Page Number
Figure 1	Map of UL97 gene.	14
Figure 2	CMV Genotypic testing workflow.	28
Figure 3	Percentage CMV UL97 mutations detected per diagnostic category.	35
Figure 4	Nucleotide highlighter plot.	36
Figure 5	Amino acid highlighter plot.	37
Figure 6	CMV viral load trend Patient A.	40
Figure 7	CMV viral load trend Patient B.	42
Figure 8	CMV viral load trend Patient C.	44

Abstract

Introduction

Cytomegalovirus (CMV) drug resistance mutations, because of the widespread use of ganciclovir, have been widely reported in international literature, particularly in the post-transplant setting. However, a genotypic assay to detect CMV drug resistance is not available in South Africa and the prevalence of these mutations is therefore unknown.

We aimed to document the prevalence and types of CMV UL97 mutations following exposure to ganciclovir in adult and paediatric oncology patients, transplant recipients and HIV-infected patients in the local tertiary level hospitals: Red Cross War Memorial Children's Hospital, Groote Schuur Hospital and Tygerberg Hospital.

Methods

The study had two components, the first component being a retrospective cross-sectional study using stored extracted DNA from patients with serially elevated CMV viral load levels. Thirty-three samples were tested for this component.

The second component was a prospective case series on patients who were referred by clinicians for genotypic testing in whom CMV drug resistance was suspected. Eight samples were tested for this component. The CMV UL97 gene was amplified by conventional nested polymerase chain reaction (PCR) and Sanger sequencing performed.

Results

CMV UL97 mutations were identified in five of thirty-three (15%) retrospectively screened samples while the prospective testing of eight

patient samples identified drug resistance mutations in three patients (38%). Overall 8/41 (20%) patients had CMV UL97 mutations. A trend of higher risk for development of drug resistance mutations among haematological oncology patients 7/23 (30%) compared to solid organ transplant recipients 1/10 (10%) was observed, however, this difference was not statistically significant ($P=0.306$).

Conclusion

This study, the first of its nature in South Africa, identified the presence of CMV UL97 mutations conferring resistance to ganciclovir in the haematological oncology, primary immunodeficiency and solid organ transplant patients in the Western Cape. The assay successfully detected CMV UL97 drug resistance mutations in whole blood and cerebrospinal fluid clinical samples. Ongoing viral replication in the background of intensive immunosuppression and prolonged antiviral therapy selects for the emergence of CMV UL97 drug resistance mutations.

Chapter 1.Literature Review

Objectives of the Literature Review

Ganciclovir (and its orally bio-available prodrug valganciclovir) is the only antiviral drug available in South Africa for the treatment of cytomegalovirus (CMV) disease since its FDA approval in 1981. The antiviral is widely used in the haematopoietic stem cell and solid organ transplant setting for the treatment and prevention of CMV disease. It has been well documented in international literature that prolonged use of ganciclovir is associated with development of drug resistance mutations either in the UL97 kinase region or in the UL54 DNA polymerase region, necessitating switching of therapy to alternate antivirals, namely, foscarnet and cidofovir (Chou, 1999, Strasfeld and Chou, 2010, Hall Sedlak et al., 2013, Gohring et al., 2015, Campos et al., 2016).

The objective of the literature search was to review the published articles for data of CMV drug resistance mutations and identify suitable molecular genotypic methods, to allow screening of the local at-risk population groups, for CMV UL97 mutations.

Literature Search Strategy

A search was conducted on the electronic database Pubmed, Clinical Key, Scopus and Google Scholar. The search terms used were cytomegalovirus, human cytomegalovirus, human herpesvirus 5, UL97 drug resistance mutations, cytomegalovirus UL97 phosphotransferase mutations, cytomegalovirus drug resistance mutations, detection of cytomegalovirus UL97 drug resistance mutations, antiviral resistance mechanisms, phenotypic UL97 assays, genotypic UL97 assays, CMV in stem cell transplant recipients and cytomegalovirus retinitis.

Review articles and book chapters were selected to provide a comprehensive understanding of the pathogenesis, diagnosis, treatment approaches and

antiviral resistance mechanisms of CMV. Original research articles were selected to provide an understanding of prevalence of CMV drug resistance mutations, historical testing platforms and advances in rapid molecular diagnosis. Articles from 1995 to 2017 were reviewed for this work. International data was used for the review to provide a reference. No local data was available. References from citations of major publications in the field of CMV drug resistance were used to identify relevant articles.

Summary and Interpretation of Literature and its Implications for the Research

Genomic Organisation of Cytomegalovirus and Replication

CMV is a 230kbp enveloped double-stranded Beta herpesvirus in the *Herpesviridae* family. The linear genome is enclosed within an icosahedral capsid. The proteinaceous tegument is found between the capsid and the lipid envelope (Gandhi and Khanna, 2004).

During replication, the immediate early (IE) proteins are expressed on entry of the virus into the nucleus. The IE genes are responsible for transcription regulation. Early genes (E) are subsequently transcribed and are responsible for DNA replication. Late genes that encode for viral structural and assembly proteins are then expressed from the replicated genomes (Lurain and Chou, 2010).

The UL97 kinase (pUL97) is a protein product of gene 97 in the unique long region, expressed during early and late CMV replication kinetics. It is responsible for the initial phosphorylation of ganciclovir into ganciclovir triphosphate, which competitively inhibits viral DNA polymerase (Lurain et al., 2001, Scott et al., 2004, Drew, 2010).

The normal functions of protein UL97 have not been defined; however, deletion of this gene has resulted in a reduced viral yield and severe defects in virion morphogenesis and maturation (Lurain and Chou, 2010). These

findings suggest that the UL97 protein has a role in regulation of viral DNA synthesis. The UL97 protein is also responsible for phosphorylating the retinoblastoma protein (pRb), releasing control of cell cycle progression, thus creating a favourable environment for viral replication. The nuclear lamina component, lamin A/C protein, is also phosphorylated by pUL97, producing breaks in the nuclear lamina that are predicted to aid in viral nuclear egress (Lurain and Chou, 2010).

Another key protein involved in viral replication and development of CMV antiviral resistance is the UL54 protein (DNA polymerase), encoded by UL54 gene, pUL54 (protein 54 in the unique long region). It is expressed during early viral replication, and provides the key functions of DNA polymerisation and exonuclease activity (proofreading capabilities) which ensures high fidelity replication of the CMV genome and a low mutation rate (Lurain and Chou, 2010).

Epidemiology of Cytomegalovirus

CMV is a ubiquitous pathogen. The seroprevalence in developing populations is approximately 90% where acquisition occurs predominantly early on in life in comparison to developed countries where the seroprevalence rate is approximately 40% in adolescents (Griffiths, 2009). The mode of transmission is through saliva, sexual contact, in-utero vertical transmission (transplacentally), perinatally during delivery, postnatally through breastfeeding and during blood transfusion and organ transplantation.

CMV has tissue tropism for a wide variety of cells such as fibroblasts, epithelial cells, endothelial cells, monocytes and leukocytes (Sinzger et al., 2008, Renzette et al., 2014).

Following infection, the virus remains latent in episomal form largely in cells of the myeloid lineage and reactivates after periods of immunosuppression resulting in a productive lytic infection causing CMV end-organ disease. The

populations at high risk of CMV disease include solid organ transplant recipients, haematopoietic stem cell transplant recipients, HIV-infected patients and infants with congenital CMV infection. End-organ disease manifests as CMV retinitis, encephalitis, pneumonitis, hepatitis and ulcerations in the gastrointestinal tract. Bone marrow involvement is indicated by anaemia, neutropenia and thrombocytopenia. Graft rejection and susceptibility to bacterial and fungal infections are indirect effects of CMV infection post-transplantation (Marr et al., 2002, Nichols et al., 2002, Garcia-Vidal et al., 2008, Boeckh, 2009, Ariza-Heredia et al., 2014, de la Camara, 2016).

Antiviral Drugs to treat CMV Disease

There are currently three FDA approved antiviral drugs for the treatment of CMV i.e. ganciclovir, cidofovir and foscarnet (Smith et al., 1997b, Strasfeld and Chou, 2010). Ganciclovir is a nucleoside analogue of guanosine monophosphorylated by the virally encoded kinase pUL97. Host cellular enzymes undertake subsequent triphosphorylation into ganciclovir triphosphate. Ganciclovir triphosphate inhibits viral DNA polymerase. Ganciclovir is not an obligate chain terminator; however, it causes a slowing and ultimately cessation of viral DNA chain elongation (Gilbert et al., 2002). Ganciclovir is also available in oral form. Its L-valyl ester prodrug valganciclovir has a bioavailability of approximately 60% (Pescovitz et al., 2000).

Ganciclovir use is associated with development of myelotoxicity which is reflected by the development of anaemia, leukopenia, thrombocytopenia and bone marrow hypoplasia (Strasfeld and Chou, 2010). Cidofovir and foscarnet do not require prior phosphorylation. Cidofovir is a phosphonmethoxy analogue of cytosine that is converted into the active form by host cellular enzymes (Lischka and Zimmermann, 2008, Lurain and Chou, 2010, Strasfeld and Chou, 2010, Campos et al., 2016).

Foscarnet is a pyrophosphate analogue that binds to and blocks the pyrophosphate active site of viral DNA polymerase blocking cleavage of the pyrophosphate moiety from deoxynucleotide triphosphates, resulting in termination of DNA chain elongation (Lischka and Zimmermann, 2008, Strasfeld and Chou, 2010, Campos et al., 2016).

Foscarnet and cidofovir are therefore effective therapies where UL97 mutations are present; however, their use is limited by their potential to cause nephrotoxicity and electrolyte abnormalities (Ljungman et al., 2001, Lurain and Chou, 2010, Strasfeld and Chou, 2010, Campos et al., 2016).

Foscarnet and cidofovir are not routinely available in South Africa except through Section 5 approval by the Medicines Control Council and therefore, not widely used.

Maribavir is an investigational benzimidazole derivative directly targeting UL97 kinase, with a favourable side effect profile, in Phase III studies. UL97 mutations conferring high-level resistance to foscarnet have been demonstrated in the kinase ATP binding domain (Chou, 2008, Haidar and Singh, 2017).

CMV Infection Management Strategies

There are currently two strategies employed in the transplant setting for management of CMV infection, namely the pre-emptive and prophylactic approaches. The prophylactic approach involves initiating ganciclovir post transplantation prior to reactivation of CMV and development of viraemia. This approach is targeted at high-risk patients and aims to reduce the direct and indirect effects of CMV. There is, however, a high cost of therapy, drug side effects, development of drug resistance and late onset CMV disease associated with this approach (Boppana and Britt, 2013, Kir et al., 2017).

Fayek et al (2016) document further reduction of late onset CMV disease in donor positive (D⁺) / recipient negative (R⁻) kidney transplant recipients by

prolonged use of low dose (400mg) valganciclovir for more than 12 months without increasing antiviral resistance.

The pre-emptive approach involves initiating therapy after demonstration of early CMV viraemia on serial monitoring, thereby reducing unnecessary drug treatment. The major disadvantage is the frequent sampling required in these patients, the associated costs and inadequacy in patients with asymptomatic CMV replication. Good synchronisation and follow-up of results is required in this setting. This approach is preferred in haematopoietic stem cell transplant recipients to avoid delayed engraftment and bone marrow suppression associated with ganciclovir use (Boppana and Britt, 2013, Miller, 2016).

Risk Factors for Emergence of CMV Drug Resistance Mutations in Transplant Recipients

The highest risk of CMV disease occurs at 1-3 months post transplantation. Additional factors that place transplant recipients at risk of CMV disease include donor-recipient serostatus, type and dose of immunosuppression, HLA status and ongoing viral replication largely driven by ongoing immune suppression.

The type of organ transplanted contributes to risk of developing disease with lung and small bowel transplant recipients carrying the highest risk, as a result of intensity of immunosuppressive regimens and high amount of lymphoid tissue in these organs being transplanted (Gordon et al., 2011, Razonable and Humar, 2013, Ramanan and Razonable, 2013, Gohring et al., 2015).

Late CMV disease post transplantation occurs after 3 months as a result of prolonged defects in cell-mediated immunity (Krause et al., 1997, Boeckh et al., 2003, Ozdemir et al., 2007, Boppana and Britt, 2013).

Risk factors for emergence of drug resistance mutations are multifactorial, relating to ongoing viral replication in the presence of non-suppressive drug

therapy due to viral, host and immune factors (Table 1). In the haematopoietic stem cell transplant setting pre-transplantation conditioning regimens are used that can be myeloablative or nonmyeloablative, resulting in severe immunocompromised states post-transplantation. Solid organ transplant recipients also undergo immunosuppressive conditioning regimens. Patients are subsequently placed on immunosuppressive drugs to prevent graft rejection such as methotrexate, steroids, calcineurin inhibitors, tumour necrosis factor inhibitors, monoclonal antibodies and cytotoxic T- cell depleting regimens post-transplantation. These immunosuppressant agents result in depressed cell mediated immunity, blunted antibody responses and leukopenia (Pergam and Jerome, 2010).

Mechanisms of CMV Drug Resistance

Ganciclovir is initially monophosphorylated by pUL97 and subsequent triphosphorylation is undertaken by host cellular enzymes into active form, ganciclovir triphosphate; which subsequently acts as a competitive substrate for DNA polymerase resulting in chain termination of the growing viral strand during replication (Strasfeld and Chou, 2010).

Nucleotide sequence changes in UL97 that result in an impairment of phosphotransferase activity (initial phosphorylation of ganciclovir) or impaired substrate recognition of ganciclovir will result in resistance to ganciclovir (Figure 1) (Lurain and Chou, 2010). Isolated UL97 mutations will remain susceptible to foscarnet and cidofovir. This is because of differences in drug design and mechanism of action - foscarnet is a pyrophosphate analogue which does not require prior phosphorylation, whereas cidofovir is a monophosphate analogue which only requires cellular kinases for diphosphorylation into active form (Lurain and Chou, 2010, Drew, 2010).

The UL54 gene encodes for DNA polymerase, and mutations in this region result in reduced antiviral affinity, reduced DNA chain incorporation and increased antiviral excision out of the DNA chain (Lurain and Chou, 2010, Le Page et al., 2013).

Table 1: Summary of risk factors associated with development of CMV drug resistance mutations.

Host Factors	<ul style="list-style-type: none"> • Impaired host immunity • Donor Recipient serostatus D⁺ R⁻ Solid organ transplant recipients D⁻ R⁺ HSCT recipients • T- cell depletion • Antithymocytes (ATG, OKT3, Alemtuzumab) • Steroid administration • Type of transplant e.g. lung transplant and small bowel have higher risk (Ramanan and Razonable, 2013) • Underlying co-morbidities
Viral Factors	<ul style="list-style-type: none"> • Ongoing viral replication due to impaired host immunity in presence of non-suppressive drug regimens • Compartmentalisation in CNS, eye and kidney • Viral quasispecies
Drug Factors	<ul style="list-style-type: none"> • Prolonged duration of drug exposure • Inadequate dosing • Drug potency • Bioavailability • Poor drug absorption • Drug delivery • Poor compliance

D⁺ R⁻ indicates donor positive recipient negative CMV serostatus; D⁻ R⁺ indicates donor negative recipient positive CMV serostatus. ATG denotes antithymocyte globulin. OKT3 denotes anti CD3 antibody. HSCT denotes Haematopoietic Stem Cell Transplant. CNS denotes central nervous system.

Presence of mutations in UL54 can confer cross-resistance to ganciclovir, foscarnet and cidofovir (Lurain and Chou, 2010, Drew, 2010, Gohring et al., 2015). Isolated UL54 mutations conferring resistance to ganciclovir are rare in the absence of UL97 mutations and have only been reported infrequently

(Green et al., 2016). UL54 mutations characteristically arise following evolution of UL97 mutations.

Mutations secondary to ganciclovir arise in the presence of drug exposure and risk factors mentioned above (Table 1). These mutations can accumulate over time conferring different levels of resistance. Higher levels of resistance are conferred by increasing accumulation of mutations. The clinical associations are broad, some mutations have been identified in asymptomatic subjects, whereas some have been identified in patients with life threatening and severe disease, and some in subjects who have subsequently become virologically suppressed on treatment (Lurain and Chou, 2010).

Diagnosis of CMV UL97 and UL54 Mutations

The presence of CMV drug resistance mutations can be suspected in patients who show an increase or plateauing of CMV viral load levels while being exposed to adequate and regular doses of ganciclovir. A lack of clinical response of CMV disease is also suggestive of the presence of drug resistance mutations (Drew, 2010). Genotypic assays are preferred due to expedited result availability.

Phenotypic and marker transfer studies are traditional methods utilised to confirm novel mutations detected genotypically and these methods will be discussed below.

Phenotypic Assays

Phenotypic assays measure the concentration of drug required to reduce viral growth in cell culture systems by 50% (IC₅₀) or alternatively by 90% (IC₉₀) in comparison to control cell culture systems without drug exposure.

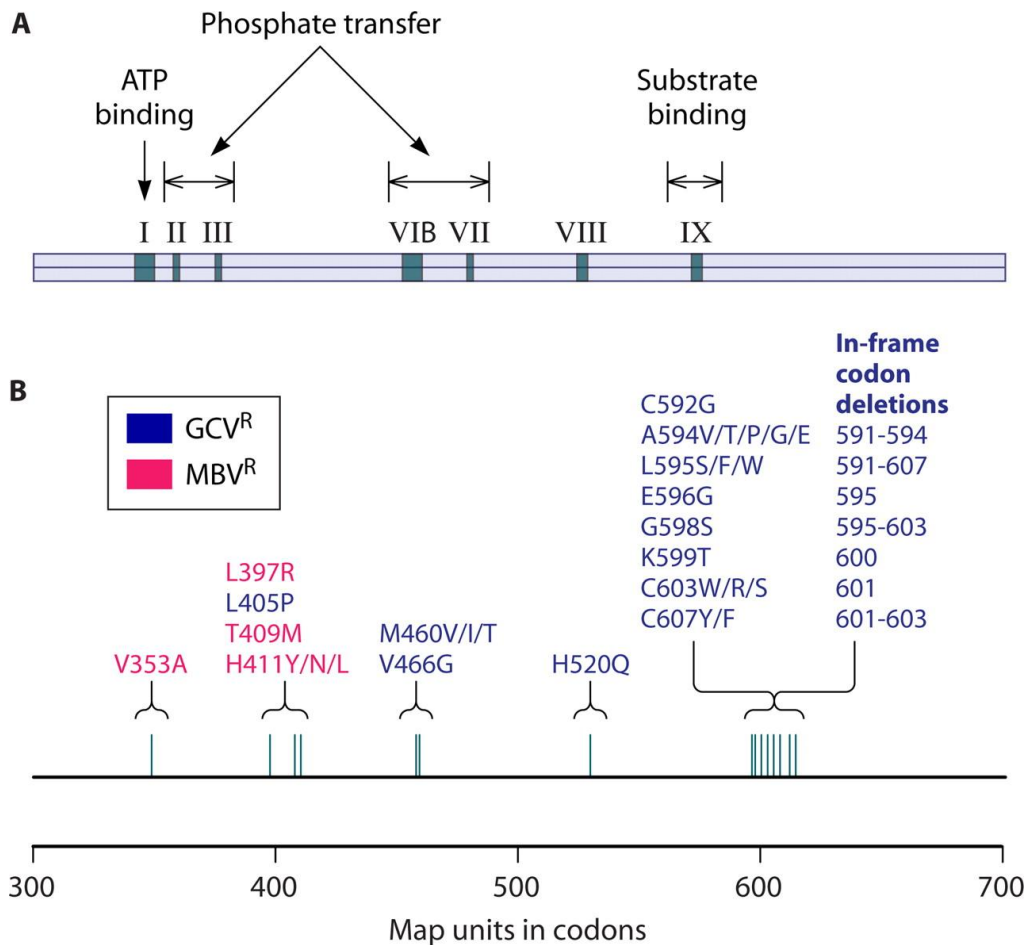


Figure 1. Map of UL97 gene. Ganciclovir resistance mutations resulting in impaired phosphate transfer or substrate recognition are indicated in blue. Maribavir resistant mutations indicated in red. GCV indicates ganciclovir. MBV indicates maribavir. Used with permission from Lurain et al (Lurain and Chou, 2010).

The former is preferred due to better reproducibility (Eckle et al., 2004, Strasfeld and Chou, 2010).

The gold standard for diagnosis of CMV drug resistance mutations is the plaque reduction assay (PRA). This involves inoculating virus into cell culture and introducing drug at varying concentrations. Viral growth is measured after a fixed incubation period as the number of visible plaques in cell culture monolayers.

The plaque reduction assay is limited by its labour intensiveness, poor standardisation in terms of culture conditions, viral plaque definition and enumeration of plaques. These factors are directly influenced by viral inoculum and drug concentration range. Laboratory strains of CMV have different growth characteristics to wild type patient strains i.e. show a faster replication rate due to mutations generated during serial passage, therefore, they may not be effective when used as control strains for these assays. The plaques generated by mutant viruses develop slowly and are usually smaller than plaques from control virus cell counterparts due to extracellular high passage laboratory strains having different replication kinetics compared to cell associated clinical strains (Lurain and Chou, 2010).

The time to result of plaque reduction assays can take more than four weeks, resulting in delays in patient management and clinical decision-making. There are a few laboratory facilities equipped to run and maintain cell culture facilities. This facility is only found at research laboratories. In South Africa, Western Cape, there is one laboratory with cell culture facilities for academic and diagnostic services. Phenotypic drug resistance testing is not offered.

Genotypic Assays

Genotypic assays involve utilising polymerase chain reaction (PCR) to amplify the region of interest (UL97 kinase/UL54 DNA polymerase) where mutations are known to occur from various samples such as tissue, blood, plasma, vitreous fluid and cerebrospinal fluid (CSF). Various PCR design platforms are used ranging from conventional PCR (one step, nested) (Lurain et al., 2001, Hu et al., 2002, Jabs et al., 2006, Castor et al., 2007), real time PCR (Gohring et al., 2006, Castor et al., 2007, Volfova et al., 2014) detection using hybridisation probes, or melting temperature analysis (Chen et al., 2015).

Sanger sequencing or next generation sequencing methods determine the nucleotide sequence. Sanger sequencing is limited in that viral populations

representing more than ten to twenty percent of the viral population in the sample can be detected (Chou et al., 2014).

Improved detection of minority variants can be attained through next generation sequencing (Kampmann et al., 2011, Benzi et al., 2012, Sahoo et al., 2013, Chou et al., 2014).

The nucleotide sequence is then analysed for the presence of nucleotide differences at key codons of interest to identify single or multiple mutations. The level of resistance, cross-resistance or sensitivity is then assessed using the Ulm University Mutation Resistance Analyzer database and published findings (Chevillotte et al., 2010).

Interpretation of genotypic assays is challenging with regard to correlation with clinical outcomes (Boivin et al., 1996, Perez, 1997, Eckle et al., 2000), effect of single versus multiple mutations, presence of mixed viral populations and assay sensitivity in detecting minority viral variants (Chou, 2001, Jabs et al., 2003, Gilbert and Boivin, 2003, Ducancelle et al., 2004, Jabs et al., 2006).

Higher viral load levels, delayed viral clearance requiring prolonged treatment duration and severe clinical outcomes are associated with mixed viral populations in solid-organ and allogeneic stem cell transplant recipients (Humar et al., 2003, Coaquette et al., 2004, Manuel et al., 2009, Vinuesa et al., 2017).

Presence of multiple low-level variants can contribute to a drug-resistant phenotype (Chou et al., 2014). Detection of these low-level mixed drug resistant mutations is associated with poorer outcomes (Houldcroft et al., 2016).

Next generation sequencing methods reproducibly detect low abundance resistance mutations not reported by conventional sequencing (Sahoo et al., 2013). Additional advantages include earlier detection of drug resistant

mutations, monitoring the complex evolution of mutations missed by Sanger sequencing and increased sequencing depth that allows for detection of mutant viral subpopulations that evolve with changing antiviral therapy (Chou et al., 2014).

Marker Transfer Studies

Marker transfer studies, also known as recombinant transfer studies, are employed to phenotypically confirm genotypic mutations. Mutations detected genotypically are introduced into cell culture control laboratory strains through either homologous recombination by co-transfection, recombination of cosmic clones or genetically modified control strains to demonstrate the effect on viral growth. Growth capacity and drug susceptibility of these recombinant strains is then assessed (Chou, 2010, Drouot et al., 2014). Some published mutations detected genotypically have not been characterised phenotypically or by marker transfer studies. (Boivin et al., 2009).

Identification of Gaps or Needs for Further Research

Ganciclovir has been unequivocally documented to be associated with development of drug resistance mutations, which can adversely affect clinical outcomes, not only due to presence of mutations and response to treatment, but also - due to underlying risk factors for CMV replication and disease. A large body of work in this arena has been generated from HIV/AIDS infected patients with CMV retinitis (Jabs et al., 2006, Strasfeld and Chou, 2010), stem cell transplant recipients (Schnepf et al., 2013, Choi et al., 2014, Gohring et al., 2015) and solid organ transplant recipients particularly kidney, lung, renal and cardiac transplant recipients (Boivin et al., 2005, Nogueira et al., 2006, Reddy et al., 2007, Garcia-Martinez et al., 2008, Iwasenko et al., 2011, Yost et al., 2014, Minces et al., 2014).

Since the introduction of highly active antiretroviral therapy (HAART) the prevalence of CMV UL97 drug resistance mutations in HIV-infected patients has declined from more than 20% to approximately 5% (Jabs et al., 1998, Martin et al., 2007, Lurain and Chou, 2010).

The solid organ transplant population has been documented to have a higher prevalence of drug resistance mutations ranging between 5-10% (Lurain and Chou, 2010), depending on various risk factors such as donor–recipient CMV serostatus, duration on ganciclovir, prophylactic or pre-emptive approach employed and type of immunosuppressants prescribed. Stem cell transplant recipients in contrast have been shown to have a 0-4% risk of developing CMV UL97 drug resistance mutations (Boivin et al., 2004, Boivin et al., 2005, Boivin et al., 2009).

Genetic polymorphisms of unknown clinical significance as well as UL97 mutations associated with hypersusceptibility to ganciclovir have also been identified, particularly in stem cell transplant patients (Tanaka et al., 2011, Boutolleau et al., 2011). Some UL97 mutations are described as genetic markers in certain Asian countries, suggesting that these mutations are wild type sequence variants (Tanaka et al., 2011, Sun et al., 2012, Zhang et al., 2013, Lee et al., 2013).

There is no local data on the magnitude of CMV drug resistance mutations in South Africa despite the extensive use of ganciclovir. Prevalence of CMV UL97 drug resistance mutations in the local risk groups needs to be established to elucidate whether the epidemiology is similar to that in international literature. The prevalence of these mutations could be utilised indirectly as an indication of prescribing practices in the different sectors, i.e. therapeutic versus pre-emptive and extent of use of ganciclovir in different transplant settings. Additional factors that may contribute to the lack of significant decline in CMV viral load levels include drug bioavailability, inadequate dosing or poor compliance. Hence, clinical profiles and outcomes need to be documented.

UL97 mutations have been detected in different body compartments such as vitreous fluid and CSF, affecting clinical outcome in patients with CMV retinitis and transplant recipients with CMV encephalitis (Boivin et al., 1997, Julin et al., 2002, Jabs et al., 2003, Kuo et al., 2003, Imai et al., 2004, Jabs et al., 2006, Miller et al., 2006, Hoang et al., 2010, Arslan et al., 2010).

The presence of drug resistance mutations in certain tissue compartments, but not in blood, as demonstrated in the literature, may suggest better penetration of ganciclovir in certain tissue compartments. The implications that this may have on disease outcomes and response to treatment needs to be further explored, particularly to address sufficient drug dosing trends and duration of therapy to avoid selecting for drug resistance mutations (Frange et al., 2013, Gohring et al., 2015).

There are no studies in our setting on patients with retinitis as treatment outcome is usually largely graded on clinical appearance. In severely immunocompromised HIV-infected patients with CMV retinitis who have delayed immune reconstitution, and have been treated with ganciclovir for prolonged periods, there could potentially be an undiagnosed pool of patients who develop CMV UL97 drug resistance mutations. Despite the new change of CD4 threshold with initiation of antiretroviral therapy at higher CD4 levels, this scenario may theoretically apply to severely immunocompromised

patients with delayed antiretroviral treatment initiation, who are on prolonged treatment for CMV retinitis.

The local cohort of transplant recipients remains a high-risk category for development of drug resistance mutations, hence, it is important to establish a baseline of prevalence and document clinical outcomes where possible. This may be even more relevant in the future, depending on evolution of mutations and their impact on clinical outcomes and therapeutic options available. Ganciclovir is currently not a restricted drug because its use is confined to specific risk groups where treatment is indicated. There are no established local studies documenting the extent of CMV drug resistance mutations. Personal communication with Prof. Alan Davidson and Dr. P. Nourse (Red Cross War Memorial Children's Hospital, Haematology-Oncology Department) suggested that the presence of these mutations is an insignificant problem.

This raises the question of whether, in the setting of high prevalence of highly resistant mutations, this agent should be restricted. Alternatively, its use closely monitored for development of mutations in high-risk populations with antiviral substitution where applicable. This would, however, require open dialogue amongst relevant stakeholders, effective cost management, appropriate testing strategies, effective use of limited resources, skilled laboratory personnel to perform the test and skilled clinical interpretation of genotypic results within the background of an appreciation of the role of underlying clinical disease driving CMV replication and impaired host immunity.

A large amount of resources is invested into the transplant setting to ensure graft survival and improved morbidity. The overall cost-benefit analysis would have to be weighed carefully and tailored to our unique setting based on extensive systematic data collation.

With this study, we aim to provide a brief preliminary assessment of the presence of these mutations in our population, which has never been

documented locally, identify the clinical groups at highest risk for development of drug resistance mutations and provide a platform for further studies on this subject.

Future studies could additionally explore the prevalence and evolution of UL97 mutations in patients with previous acyclovir exposure followed by ganciclovir exposure. These would include haematopoietic stem cell transplant and solid organ transplant recipients who receive acyclovir for prophylaxis against Herpes Simplex Virus and CMV followed by ganciclovir routinely in the post-transplant period. *In vivo* and *in vitro* evidence is provided by several studies.

Erice et al (1998) demonstrated the presence of CMV UL97 mutations in four isolates of haematopoietic stem cell transplant recipients due to exposure to acyclovir followed by ganciclovir and absence of mutations in those with exposure only to acyclovir. This suggests rapid evolution and possible predisposition due to using acyclovir prior to ganciclovir. Acyclovir has minimal activity against CMV and this may lead to emergence of mutations because of partially suppressive drug pressure.

The emergence of CMV drug resistance mutations has been documented in solid organ transplant recipients following exposure to valaciclovir and ganciclovir (Alain et al., 2004, Hantz et al., 2005).

Michel et al (2001) have further provided *in vitro* evidence substantiating the phenomenon of acyclovir selecting out mutations to ganciclovir.

Knowledge of the local epidemiology of CMV drug resistance mutations through documentation of case series and descriptive research studies is essential towards informing on current clinical practices and improving patient outcomes. This can be achieved by improving access to alternative therapies and improved diagnostic services portfolios, which are largely driven, amongst other factors, by burden of disease, clinical utility in diagnosis and prognostic value of diagnostics tests within a cost-effective

framework. Towards this end, baseline prevalence needs to be accurately established and documented.

Future studies could additionally attempt to better document clinical outcomes, ganciclovir prescribing practices in the various sectors; explore the impact of ganciclovir pharmacokinetics, drug bioavailability and host risk factors on the development of CMV UL97 drug resistance mutations.

Chapter 2.Methods

2.1. Introduction

The development of drug resistance mutations is a well-described phenomenon in the treatment of cytomegalovirus (CMV) infection in stem cell transplant and solid organ transplant recipients. The selection of drug resistance mutations is multifactorial, considerably driven by host and viral factors such as viral replication in the presence of severe immunosuppression and prolonged drug exposure.

CMV drug resistance mutations are localized to the UL97 (kinase) gene and the UL54 (DNA polymerase) gene. Mutations occur in response to treatment with ganciclovir, a nucleoside analogue of guanosine and its oral prodrug valganciclovir. Prolonged exposure to cidofovir - a monophosphate nucleotide analogue and foscarnet - a pyrophosphate analogue, results in mutations in the UL54 gene. These drug resistance mutations are thought to evolve over time with progressive accumulation, initially in the UL97 gene and subsequently in the UL54 gene. Cases of UL54 drug resistance mutations in the absence of UL97 mutations are rare. These mutations either result in impaired phosphorylation of ganciclovir, changes in the substrate recognition sites or exonuclease activity and thus a continuous rise or plateau in the CMV viral load consequent to continued viral replication despite adequate drug therapy.

Clinical implications vary from asymptomatic disease to progressive disease and adverse outcomes due to CMV disease progression. This paper aims to demonstrate the prevalence of CMV UL97 drug resistance mutations in the local cohort of at-risk groups treated with ganciclovir.

The motivation behind the study is lack of data in most low and middle-income countries where CMV is prevalent at a young age.

2.2. Aims and Objectives

This study aimed to document and characterise the prevalence of CMV drug resistance mutations in the targeted population groups at risk of CMV disease and the development of CMV UL97 drug resistance mutations i.e. HIV- infected patients, solid organ transplant patients and haematopoietic stem cell transplant recipients in Cape Town, South Africa.

The objectives of the study were to:

1. Identify retrospective patient samples and prospective clinician-referred patients with elevated CMV viral load levels, suggestive of possible ganciclovir resistance.
2. Amplify and sequence CMV UL97 gene to identify UL97 drug resistance mutations.
3. Describe the prevalence of CMV UL97 mutations in the population sampled.
4. Describe the clinical history and outcomes in prospective clinician-referred patients with CMV UL97 mutations.

2.3 Clinical Materials and Methods

2.3.1 Ethics

Ethical approval for this study was obtained from the Human Research Ethics Committee University of Cape Town (HREC REF: 655/2015).

2.3.2 Samples and Source Population

The study was a descriptive study, which had two components. The first component being a retrospective cross-sectional study to investigate the prevalence of CMV UL97 mutations in transplant, oncology and HIV- infected patients treated with ganciclovir and have shown elevated serial CMV viral load levels. Extracted DNA previously used for CMV viral load testing, stored at -80°C, was used for this component.

Thirty-three (33) samples were identified through a search of the archived CMV viral load results on the National Health Laboratory Service (NHLS) laboratory information system (LIS) at Groote Schuur Hospital.

The Groote Schuur Hospital NHLS laboratory provides a secondary level of service to surrounding secondary and tertiary hospitals in the Metro-East District in the Western Cape.

Data from 2010-2016 was used to identify trends in CMV viral load levels. Serial CMV viral load results were analysed for increasing or plateauing trends. Clinical parameters such as diagnosis, sample type and location of patient were reviewed during the selection of samples (Table 2).

The second component was a case-series utilising data from patients on whom CMV drug resistance was suspected who were referred by clinicians for testing based on CMV infection, risk factors, treatment with ganciclovir and poorly responsive CMV viral load levels despite adequate ganciclovir dosing. This data was collected prospectively. Eight (8) patients were enrolled for this prospective component.

2.3.3 Assay validation

Prior to testing of patient samples, the assay was optimised on extracted DNA that had been tested for CMV viral load, as well as on samples from an external quality assurance body, Quality Control of Molecular Diagnostics (QCMD), with detection of 100% of UL97 mutations that were present in the external quality assurance samples in 2014, 2015, 2016 and 2017.

2.3.4 Assay

Following retrieval of the extracted nucleic acid, conventional nested PCR amplification of the UL97 gene of CMV was performed using a set of published primers and thermocycling reaction conditions which amplifies codons 433 to 683 of the 2124 bp UL97 gene (Hu et al., 2002). Although all reported CMV UL97 mutations occur between codon 400 to codon 707,

clinically relevant mutations occur between codon 460 and codon 607 of CMV UL97 gene. This region was sufficiently covered by the assay (Lurain et al., 2001). This method was selected on the basis of being shown to effectively amplify the CMV UL97 gene on a variety of samples (cell culture, urine, plasma, CSF and vitreous fluid). The nested design allows for improved sensitivity.

Verification of a 707 bp PCR product was performed on 1% agarose gel electrophoresis stained with ethidium bromide and visualised under UV illumination in the presence of a molecular weight marker to confirm correct band size and quality prior to sequencing. One negative and one positive control was included in every batch of PCR reactions. The negative control served to monitor and identify potential contamination. The positive control was not sequenced.

2.3.5 Sequencing and Sequence Analysis

Sample clean up and Sanger sequencing was performed at an external facility, Inqaba Biotechnologies, using the inner PCR primers. The samples were sequenced bidirectionally using both forward and reverse primers. Sequence quality was analysed by assessing electropherogram peaks, signal strength, level of background noise and presence of artefacts. Poor quality sequences were rejected for analysis.

Sequences were edited using Finch TV for base calling (Finch TV 1.4.0, Geospiza, Inc.; Perkin Elmer Informatics, Seattle, WA, USA; <http://www.geospiza.com>). Bio-Edit Sequence Alignment Editor version 7.1.3.0 was used for manual sequence editing (Hall, 1999) and alignment using ClustalW version 1.4 (Thompson et al., 1994). Subsequently, the sequences were submitted in FASTA format to the Ulm University MRA-mutation resistance analyzer online database (AG Bioinformatics and Systems Biology, Institute of Neural Information Processing Institute of Virology) for presence of CMV UL97 mutations (Chevillotte et al., 2010). This online tool aligns the sequence to the gene of interest and provides a report

on published mutations at key codons of UL97 where mutations are known to occur; with reference to the TB40-BAC4 CMV strain, a bacterial artificial clone of the TB40E lab adapted endotheliotropic CMV strain (Murrell et al., 2016).

Sequences were submitted to the NCBI Basic Local Alignment Search tool (BLAST) for comparison with other published sequences and to ensure that the desired gene sequence product was isolated. The percentage of sequence identity of the submitted sequence against other CMV sequences in the database, the closest match and areas of nucleotide differences were assessed.

2.3.6 Interpretation and Analysis

The Ulm University MRA-Mutation Resistance Analyzer database provides a report detailing the alignment of the submitted CMV UL97 sequence, the region covered as well as presence of insertions and deletions.(Chevillotte et al., 2010). See Appendix V for patient reports.

Mutations associated with ganciclovir resistance at key CMV UL97 codons were analysed. The IC₅₀ ratios based on cell culture experiments were reported for ganciclovir resistant strains. Wild type strains were reported as having no mutations identified.

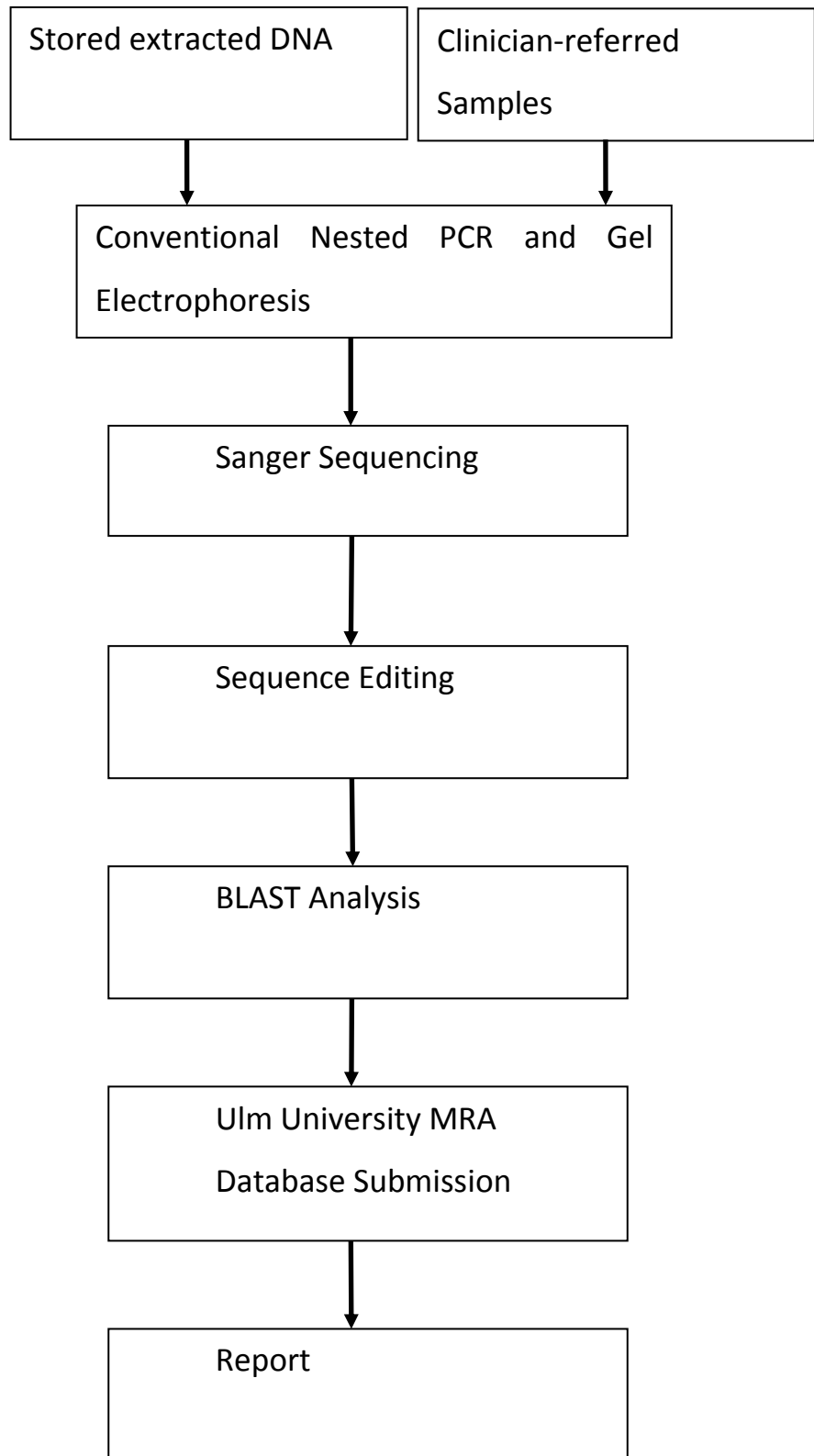


Figure 2 CMV genotypic testing workflow. Schematic diagram representing sample identification, testing and analysis to result of CMV drug resistance mutations. PCR indicates polymerase chain reaction.

Chapter 3. Results of the Study

3.1. Patients

33/41 (80%) of these samples were retrospective samples from patients who were tested based on sequentially elevated CMV viral load results as per information obtained from the laboratory information system (LIS) database and 8/41 (20%) of patients were from patients referred by clinicians on suspicion of presence of possible CMV UL97 drug resistance mutations. The median age of all patients tested in the study was 19 years (IQR 1-65); 22 (54%) were female and 19 (46%) male.

The median viral load of all samples tested was 5600 copies per millilitre (cps/ml), median log value 4.12. Of 9805, CMV viral load records reviewed between 2009 and 2015, 318 (3%) records had consecutive viral load results that were elevated. The patients were separated into categories based on their clinical diagnosis as captured on the NHLS laboratory request form. There were four categories, namely HIV-infected, Solid Organ Transplant, Haematological Oncology and Unknown Diagnosis.

23/41 (56%) patients were grouped in the Haematological Oncology category, 10/41 (24%) patients in the Solid Organ Transplant category, 6/41 (15%) patients were HIV-infected and 2/41 (5%) patients had no clinical diagnosis recorded on the form and therefore in the "Unknown Diagnosis" category (Table 2).

Detailed description on the characteristics of patient categories, patient sample characteristics and source of referral is provided in Table 2.

Table 2 Demographics and baseline characteristics of patients and samples tested.

	All Number (%)	Retrospective Samples	Clinical Patients
Number	41	33	8
Age –Mean Median (Range)	21.7 19 (1-65)	25.9 25 (1-65)	5 2.5 (1-19)
Male-no. (%)	19 (46)	16 (48)	3 (38)
Female-no. (%)	22 (54)	17 (52)	5 (62)
Sample Type Cerebrospinal fluid Whole blood	2 (5) 39 (95)	2 (6) 31 (94)	8 (100)
Diagnostic Categories-no. (%) Solid Organ Transplant Haemat-oncology HIV Infection Unknown Diagnosis	10 (24) 23 (56) 6 (15) 2 (5)	9 (27) 19 (58) 3 (9) 2 (6)	1 (12) 4 (50) 3 (38)
CMV Viral Load (Mean) Copies/ml Log value	411 809 4.12	107 465 3.79	166 7225 5.48
Hospitals: no. (%) Red Cross Children's Hospital Groote Schuur Hospital UCT Private Hospital Tygerberg Hospital Other	17 (41) 9 (22) 8 (20) 2 (5) 5 (12)	12 (36) 8 (24) 8 (24) 5 (15)	5 (63) 1 (12) 2 (25)

Haemat-oncology indicates haematological oncology.

3.2 Results

Of the 41 patient samples amplified and sequenced, 8 patients (20%) had documented CMV UL97 mutations (Table 3). 3/8 (38%) of samples in the clinician referred group had documented CMV UL97 drug resistance mutations, namely H520Q, L595S, C592G and C603W. The C592G and C603W mutations were identified in one patient (Patient C and Table 3).

5/8 (63%) mutations were detected in the retrospective patient group. Amongst the retrospective samples, 5/33 (15%) had documented CMV UL97 drug resistance mutations. The mutations identified were M460V, C592G, Q449K and D605E.

In total, seven mutations were identified, namely H520Q, L595S, C592G, C603W, M460V, D605E and Q449K (Table 3a).

The C592G mutation was identified in two patients, one in the retrospective patient group and another in the clinician referred group. The D605E mutation, detected in two stem cell transplant recipients in the retrospective patient group, is a genetic polymorphism of unclear phenotype. This mutation constituted 1/7 (14%) of all mutations (Table 3 and Table 3b).

According to diagnostic category, the majority of CMV UL97 mutations, 7/41 (17%) were found in patients in the Haematological Oncology category and 1/41 (2.43%) of patients in the Solid Organ Transplant category (Table 2).

These findings suggest the presence of CMV UL97 mutations, not only as sequence variants, but also as resulting in resistance to ganciclovir in the local adult and paediatric setting. There was a higher prevalence in patients with haematological malignancies and patients with primary immunodeficiency disorders.

Haematological oncology patients seem to be at a higher risk of CMV UL97 drug resistance mutations in proportion to solid organ transplant recipients

and HIV infected patients, although this risk is not statistically significant (P=0.306).

Phylogenetic analysis of the UL97 gene sequences showed high conservation in this area, confirming the findings of Lurain et al (2001). Genotypic variation of CMV requires analysis of UL55, UL73 and UL75 genes (Chou, 1992, Tarrago et al., 2003, Pignatelli et al., 2003). Therefore, the nucleotide base and amino acid differences between the sequences were better represented in the highlighter plot (Figure 4 and Figure 5). The highlighter plot was constructed by submitting aligned sequences to an online service available at www.hiv.lanl.gov . The sequences were compared to UL97 of CMV AD169 strain as the master sequence (UL97AD169m).

Table 3 Patient clinical parameters and CMV UL97 mutations identified.

	AGE	DIAGNOSIS	CMV VIRAL LOAD (copies/millilitre)	UL97 MUTATION	GANCICLOVIR	SAMPLE TYPE
PATIENT A	3	Myelodysplasia	271 900(log 5.43)	L595S	Resistant 4.9-8.5x	Whole blood
PATIENT B	2	Pre-B Acute Lymphoblastic Leukaemia	877 6224 (log 6.70)	H520Q	Resistant 5.2-10x	Whole blood
PATIENT C	3	Wiskott-Aldrich Syndrome	8114 (log 3.9)	C592G C603W	Resistant:2.3- 3.2x Resistant:7.9-8.3x	Whole Blood
PATIENT D	32	Multiple Myeloma	4100 (log 3.61)	M460V	Resistant 5.5-9.8x	Whole blood
PATIENT E	38	Acute Myeloid Leukaemia	11 950 (log 4.08)	D605E	Sensitive 0.6-0.9x	Cerebrospinal fluid
PATIENT F	46	Chronic Myeloid Leukaemia	12 650 (log 4.10)	Q449K	Sequence variant	Whole blood
PATIENT G	40	Acute Myeloid Leukaemia	1064 (log 3.00)	D605E	Sensitive 0.6-0.9x	Whole blood
PATIENT H	23	Renal Transplant	32 350 (log 4.51)	C592G	Resistant 2.3-3.9x	Whole blood

Table 3a Drug sensitive and resistant CMV UL97 mutations detected.

Resistant Variants	Sensitive Variants	Wild Type Amino Acid	Mutation Amino Acid
H520Q		Histidine	Glutamine
L595S		Leucine	Serine
C592G		Cysteine	Glycine
C603W		Cysteine	Tryptophan
M460V		Methionine	Valine
	D605E	Aspartic Acid	Glutamic Acid
	Q449K	Glutamine	Lysine

Table 3b Frequency of CMV UL97 mutations detected.

Patient	A	B	C	D	E	F	G	H
Mutation								
L595S	✓							
H520Q		✓						
C592G			✓					✓
C603W			✓					
M460V				✓				
D605E					✓		✓	
Q449K						✓		

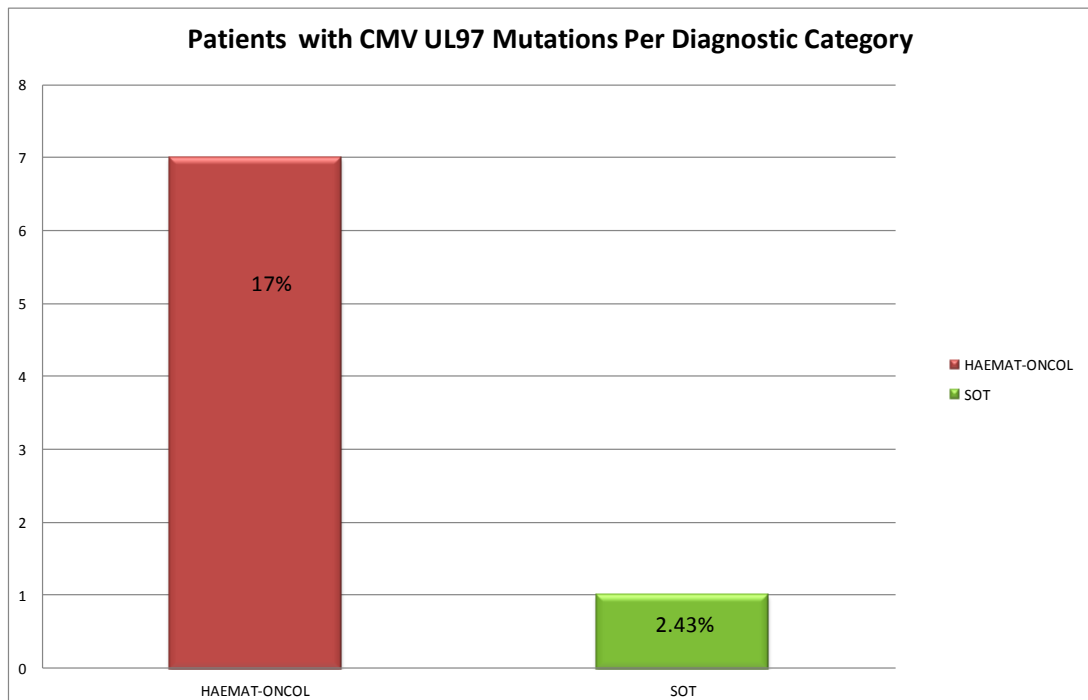


Figure 3 CMV UL97 mutations detected as per diagnostic category in 41 samples tested. HAEMAT-ONCOL indicates Haematological Oncology , SOT indicates Solid Organ Transplant diagnostic category.

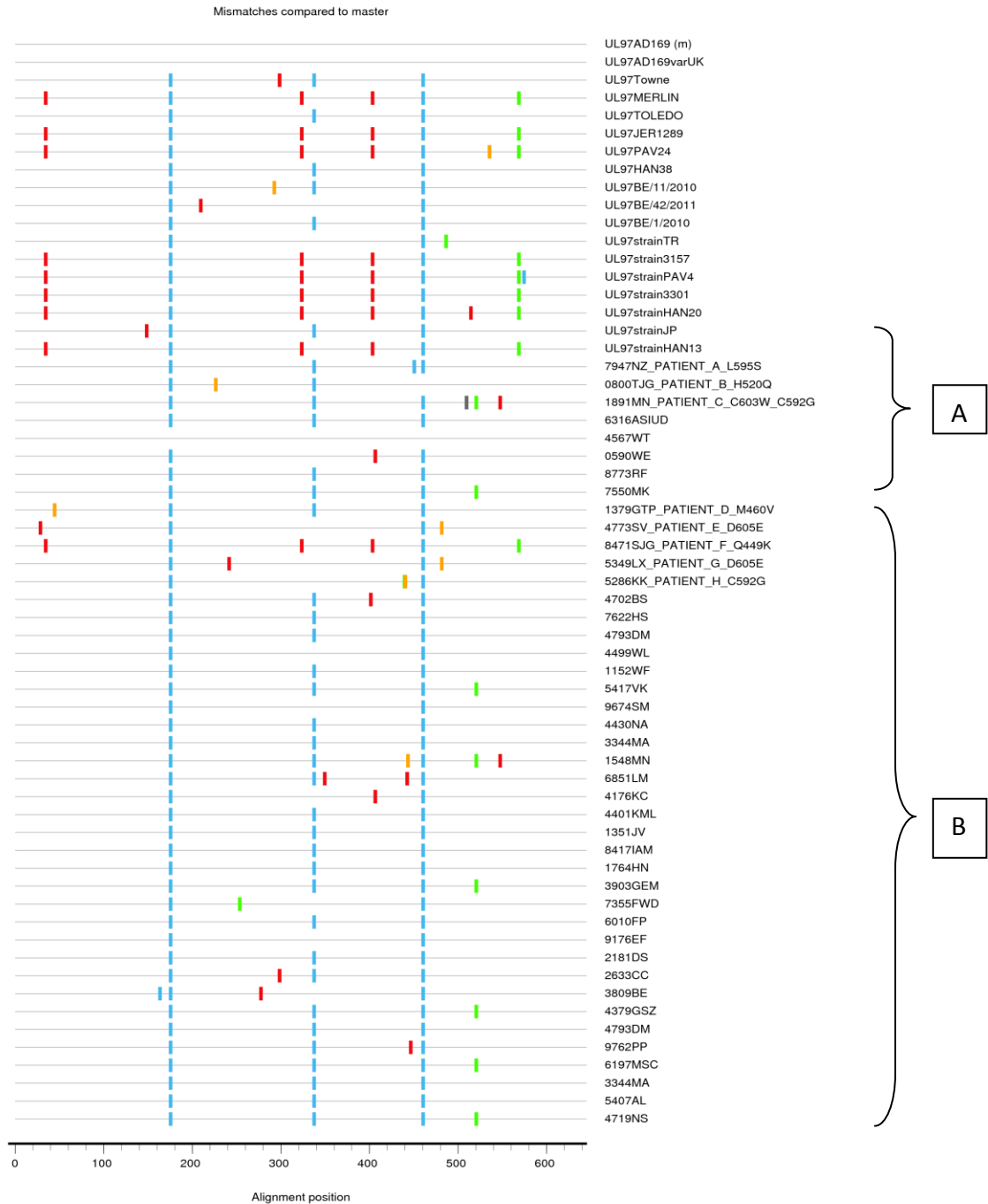


Figure 4: Nucleotide highlighter plot indicating nucleotide base differences in comparison to master sequence CMV AD169 (CMVAD169m). Nucleotide differences are indicated by different colours. Green bar indicates adenine, blue indicates cytosine, orange indicates guanine, red indicates thiamine and royal blue indicates IUPAC mixtures. A indicates the clinician referred group and B indicates the retrospective patient group. www.hiv.lanl.gov

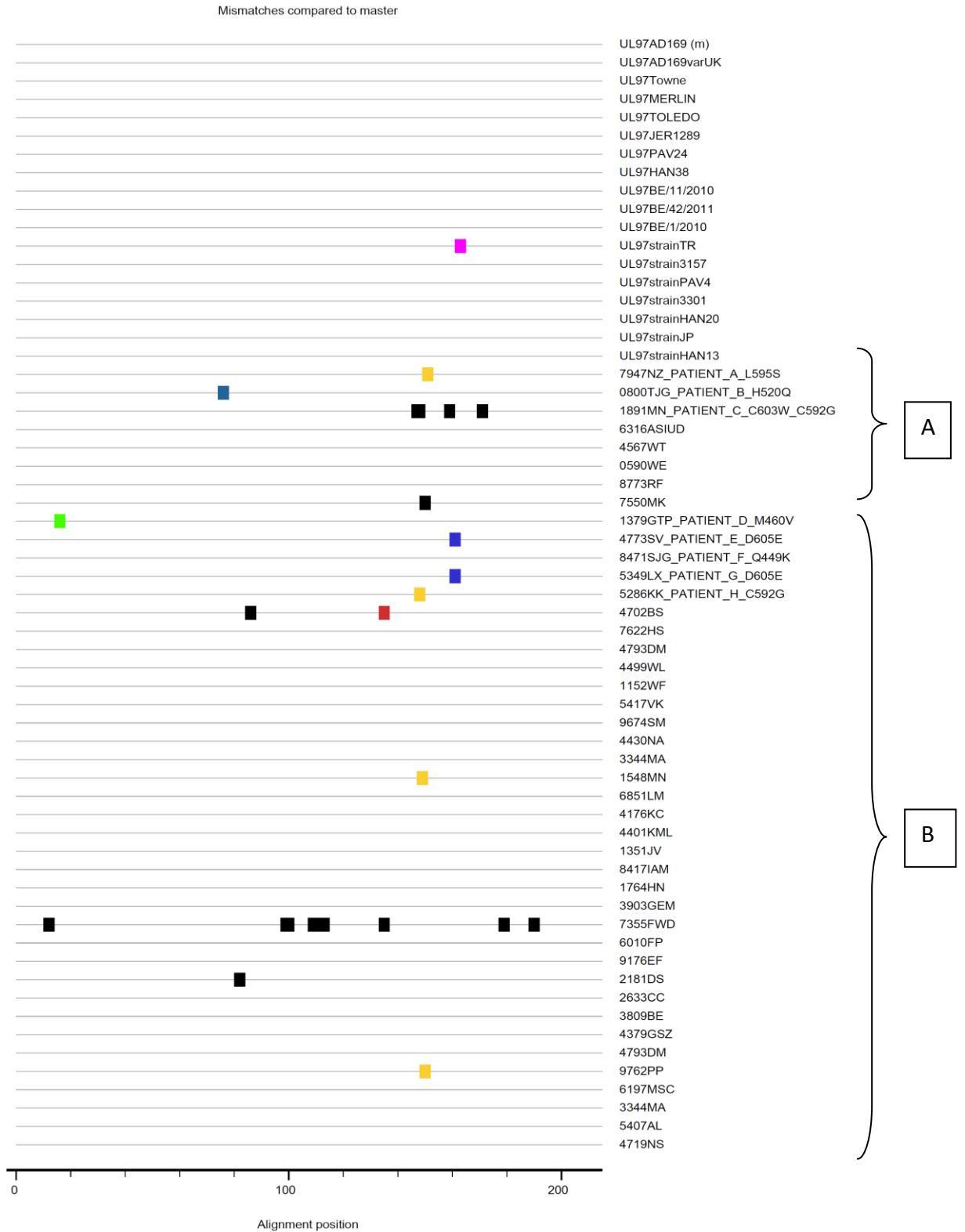


Figure 5 Amino acid highlighter plot indicating amino acid differences in comparison to master sequence CMV AD169 (CMVAD169m). A indicates the clinician referred group. B indicates the retrospective patient group.

Ambiguous nucleotide bases indicated in black Patient C sequence contained ambiguous nucleotide bases indicated in black. www.hiv.lanl.gov

Key (www.hiv.lanl.gov):

His(H)	Asp(D) Glu(E)	Lys(K) Asn(N) Gln(Q) Arg(R)
Met(M)	Ile(I) Leu(L) Val(V)	Phe(F) Trp(W) Tyr(Y)
Cys(C)	Ala(A) Gly(G) Ser(S) Thr(T)	Pro(P)
Other	Gap	

3.3 Case Reports and Discussion

Three paediatric patients in the clinician-referred group are presented in detail to highlight the individual underlying host risk factors for CMV replication necessitating prolonged ganciclovir/valganciclovir treatment and to contrast their distinctive outcomes, largely influenced by underlying co-morbidities.

Patient A

Non-fatal CMV Reactivation in a Post-Haematopoietic Stem Cell Transplant Recipient

Patient A was an HIV-negative 3-year-old child at time of transplantation in March 2014. He was diagnosed with congenital myelodysplasia a few months after birth. The donor was CMV serostatus IgG negative, recipient CMV serostatus IgG positive. The recipient's conditioning regimen prior to transplantation included etoposide and dexamethasone. Valaciclovir prophylaxis and cyclosporin A was initiated in the immediate post-transplant period. He had post-operative complications of Gram-negative sepsis secondary to *Acinetobacter jejunii* and CMV reactivation with possible CMV pneumonia. Successful engraftment occurred on day 11 post transplantation.

Ganciclovir was commenced on day 19 post transplantation in response to CMV viremia. He was re-admitted for intravenous ganciclovir on multiple occasions following elevated CMV viral load levels interspersed with oral

courses of valganciclovir. The total duration of ganciclovir and valganciclovir received was seven months post-operatively.

Genotypic testing performed on day 70 post transplantation, at a viral load of 271 900 cps/ml (log value 5.43) revealed the L595S (leucine to serine at codon 595) mutation. This mutation is associated with high resistance (2.3-3.9x) to ganciclovir compared to wild type strains (Boivin et al., 2009). However, subsequent CMV viral load levels declined (Table 3, Figure 4, Figure 5 and Figure 6). This co-occurred with the reduction in dosage of cyclosporin. He is currently thriving, off immunosuppressant drugs and receiving his routine childhood immunisations.

It has been shown that drug resistance mutations can disappear as a result of cessation of drug pressure removing selection for these mutations or as a result of decreasing immunosuppressant drugs i.e. strengthened host immunity (Boivin et al., 2012, Volfova et al., 2014). The presence of quasi-species could also be a contributing factor (Iwasenko et al., 2007, Renzette et al., 2013, Renzette et al., 2014).

CMV displays significant human inter-host variability and intra-host diversity, with as many as six genotypes observed within one single patient on ultra-deep pyrosequencing (Gorzer et al., 2010, Renzette et al., 2014). Various mechanisms are proposed. These include generation of *de novo* mutations during replication following primary infection, although the DNA polymerase proofreading function should limit these mutations.

Another theory relates to the introduction of new strains through re-infection; however; this theory does not explain the diversity in congenitally infected neonates (Renzette et al., 2014).

During dissemination to distal compartments, the viral population can rapidly evolve, either due to natural selection or population bottlenecks, leading to populations in the distal tissues or organs being genetically differentiated from the peripheral blood compartment. This phenomenon is known as

compartmentalization. The populations in the distal compartments are also less genetically diverse than those in the peripheral blood. Once CMV has disseminated to compartments or if the virus remains in the peripheral blood, the populations become relatively stable and many mutations remain at similar frequencies over time (Renzette et al., 2014). Compartmentalization can be reflected by differences in the distribution of drug resistance-genotypes observed in some patients within the different compartments (Prix et al., 1999, Eckle et al., 2000, Hamprecht et al., 2003, Frange et al., 2013, Gohring et al., 2015).

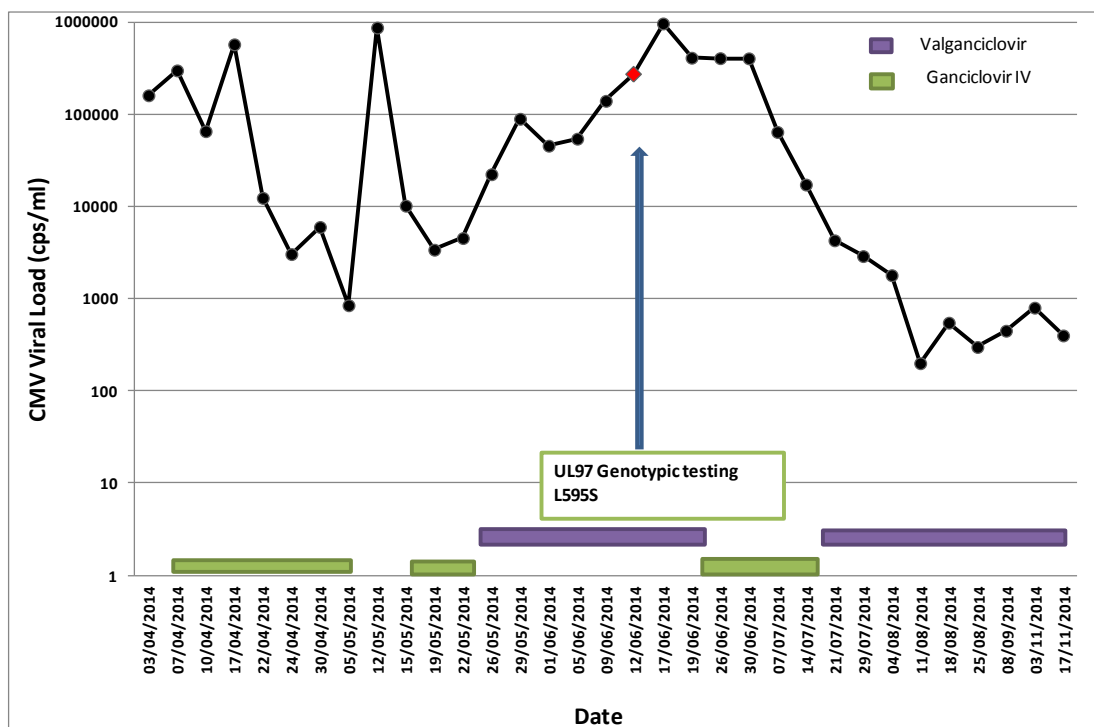


Figure 6 Timeline illustrating CMV viral load trend and detection of UL97 mutations in Patient A over a seven-month period of ganciclovir/valganciclovir post-transplantation. IV indicates intravenous.

Patient B

Fatal Outcome in a Patient with Pre-B Acute Lymphoblastic Leukemia with CMV Infection

Patient B was a 2-year-old patient with pre-B acute lymphocytic leukemia diagnosed at the age of 3 months in 2013. He underwent chemotherapy

successfully and went into remission. Whilst on maintenance chemotherapy, started in February 2014, he developed recurrent infections, persistent severe lymphopenia, intermittent thrombocytopenia, absent immunoglobulins and CMV viremia. He received polyvalent human normal immunoglobulin infusions (Polygam®). Intravenous ganciclovir was initiated followed by oral valganciclovir for three weeks. Ganciclovir was re-initiated following a rebound in CMV viral load on 7/01/2015. There was no evidence of CMV end organ disease on colon biopsy sample and ophthalmoscopy.

Patient B was classified by attending medical personnel as having a poor prognosis for his underlying condition based on age at diagnosis of acute lymphoblastic leukemia, a high white cell count at diagnosis, a severe immunodeficiency and the t (4; 11) translocation.

He had persistently elevated CMV viral load levels ranging between 794 500 cps/ml (5.9 log) and 66363888 cps/ml (log 7.8) over a period of five months. Genotypic testing on day 104 of ganciclovir, at a viral load of 11 950 cps/ml (log 4.08) revealed the H520Q mutation (histidine to glutamine at codon 520) associated with 5.2-10x resistance to ganciclovir (Table 3, Figure 4, Figure 5 and Figure 7).

Attempts to attain Foscarnet were initiated through the Medicines Control Council; however; he demised following the development of neutropenic enterocolitis, encephalitis secondary to methotrexate, neutropenia and *Stenotrophomonas* sepsis.

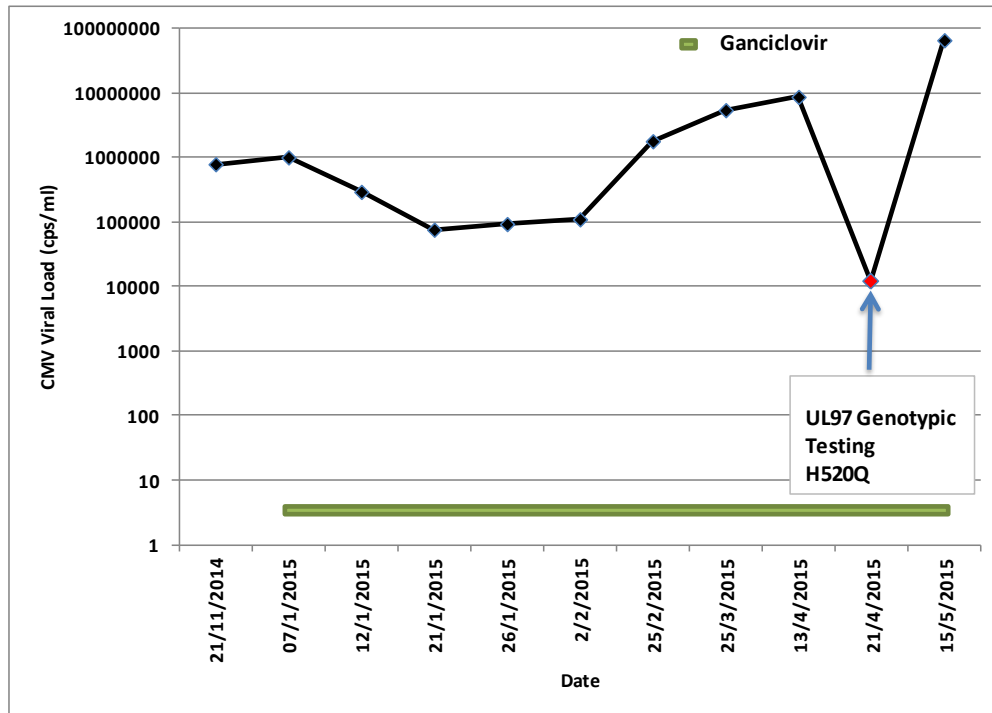


Figure 7 Timeline illustrating CMV viral load trend in Patient B. CMV viral load value at time of genotypic testing reflects interlaboratory and inter-assay variation.

Patient C

Asymptomatic Patient with an X-linked Primary Immunodeficiency and Multiple UL97 Drug Resistance Mutations

Patient C was a 3-year-old HIV-negative patient diagnosed at four months of age with Wiskott-Aldrich syndrome, an X-linked autosomal recessive primary immunodeficiency disease characterised by recurrent bacterial infections eczema and bleeding tendencies caused by thrombocytopenia and platelet dysfunction. He received ganciclovir for more than a year for CMV viremia following ICU admission for presumed CMV pneumonia. Initial genotypic testing for UL97 mutations after a period of 5 months on ganciclovir showed the absence of UL97 mutations i.e. wild type CMV (Figure 8). Biopsy samples of duodenum, stomach and oesophagus revealed eosinophilic gastritis.

Subsequent testing 19 months after the first genotypic test, 22 months on valganciclovir, revealed two mutations in the UL97 gene namely, C592G (cysteine to glycine at codon 592) and C603W (cysteine to tryptophan at codon 603).

This patient experienced multiple episodes of dysentery, dermatitis, upper and lower respiratory tract infections. There was no evidence of CMV end-organ disease. At the time of publication, he had not undergone stem-cell transplantation due to parental refusal. He continued receiving regular clinical follow-up visits, polyvalent immunoglobulin injections (Beriglobin®), prednisone and fluconazole and co-trimoxazole prophylaxis. Following detection of CMV UL97 mutations ganciclovir and prednisone was stopped (Table 3, Figure 4, Figure 5 and Figure 8).

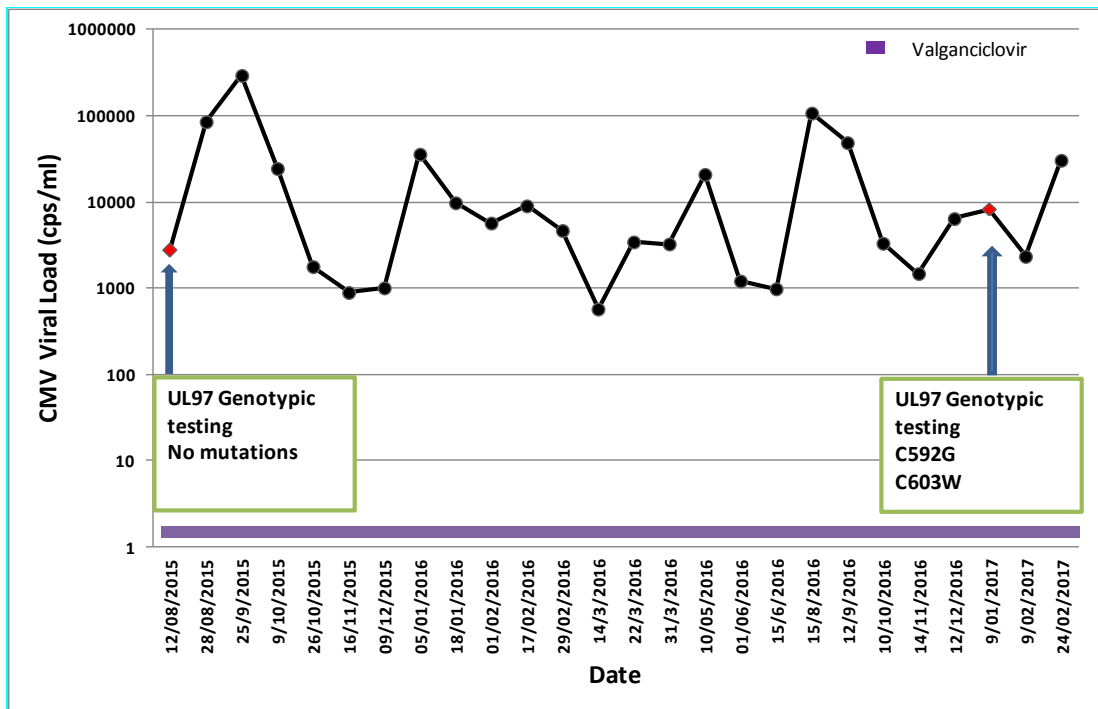


Figure 8 Timeline illustrating CMV viral load trend and evolution of UL97 mutations in Patient C over an eighteen month period of valganciclovir.

Patient D - Patient H were in the retrospective patient group, no clinical history was available other than diagnosis provided on laboratory test request form (Table 3, Figure 4 and Figure 5).

3.4 Other UL97 Drug Resistance Mutations

The C592G (cysteine to glycine at codon 592) and M460V (methionine to valine at codon 460) are ganciclovir resistant mutations that were detected in retrospective patient samples on which clinical history was not available, the former being a renal transplant candidate and the latter a patient with multiple myeloma (Table 3, Figure 4 and Figure 5).

3.5 Sequence Variants/Genetic Polymorphisms

The D605E mutation sequence polymorphism (aspartic acid to glutamic acid at codon 605) confers sensitivity to ganciclovir. It has been described in treatment naïve and treatment exposed persons. In some countries, such as

Asia, a higher prevalence was found compared to European countries (Chou, 2008, Sun et al., 2012, Sung et al., 2012).

The Q449K (glutamine to lysine at codon 449) mutation has only been identified as a sensitive genetic polymorphism of uncertain clinical significance. In our study, this mutation was identified in a patient with chronic myeloid leukaemia. According to the University of Ulm MRA database this mutation has been isolated in a patient previously treated with acyclovir without any exposure to ganciclovir, suggesting that this mutation also occurs naturally among clinical CMV isolates (Erice et al., 1998, Lurain et al., 2001, Hu et al., 2002, Chou, 2008).

3.6 Limitations

3.6.1 Sample Size

There were limited samples available in the prospective clinician referred group of the study. The current impression (personal communication) among clinicians in the transplant setting, is that CMV UL97 drug resistance mutations are not a significant problem, hence referral was poor. The historical lack of diagnostic assays to detect CMV UL97 mutations contributes to limited sample referral.

3.6.2 Lack of Clinical Correlation

The majority of retrospective samples in which mutations were identified do not have a clear, concise clinical history, risk factors and outcomes. Phenotypic correlation could not be assessed in these samples. Subsequent declines in CMV viral load levels to clinically acceptable levels, in the setting of ongoing immune suppression and adequate ganciclovir dosing, could be indirectly used to infer phenotypic sensitivity. The reverse scenario also holds true.

The inability to test multiple samples at different timepoints, in different compartments based on clinical history where indicated further limits the interpretation of results.

3.6.3 Sequencing

Sanger sequencing is known to detect viral populations present more than 20% of the total viral population in the sample (Chou et al., 2014). We cannot entirely exclude the presence of minority variant subpopulations with drug resistance mutations. Detection of wild type sequences does not exclude presence of drug resistant minority variants.

Deep sequencing has been utilised to detect mutations not only sooner than Sanger sequencing, but also in samples where Sanger sequencing had initially failed to detect mutations. (Sahoo et al., 2013, Chou et al., 2014). Sanger sequencing, however, has the advantage of being less prone to artificial mutagenesis than next generation sequencing (Capobianchi et al., 2013, Barzon et al., 2013, Gargis et al., 2016).

The UL54 DNA polymerase region was not analysed. UL54 mutations characteristically evolve after UL97 mutations. Detection of UL54 mutations in the absence of UL97 mutations, although rare, may be associated with resistance to ganciclovir. (Green et al., 2016). Presence of UL54 mutations is associated with high level of resistance to ganciclovir, foscarnet and cidofovir. Their presence should be assessed where treatment with foscarnet and cidofovir is considered.

Unusual patterns of CMV drug resistance mutation emergence have been described. Iwasenko et al (2007) describe a fascinating case of the emergence of a foscarnet-resistant / ganciclovir-sensitive UL54 drug resistance mutation (T700A) one year after the disappearance of a UL97 ganciclovir resistant mutation (L595S). The detection of the T700A UL54 mutation occurred in the absence of concurrent foscarnet use, under

ganciclovir selective pressure in a bone marrow recipient with X-linked severe combined immunodeficiency.

This case report further highlights the influence of quasispecies, sampling bias in relation to sample type and compartmentalisation, and the influence of underlying host factors in detection and interpretation of CMV drug resistance mutations (Iwasenko et al., 2007).

3.6.4 Bias

Research bias can be briefly defined as any systematic error introduced into sampling or testing by selecting or encouraging one outcome or answer over others.

Bias can arise from the study design, population selection, sampling and data analysis, causing interference with the interpretation and significance of study findings. The nature of PCR, PCR reaction conditions and sequencing reactions are additional sources of bias and these are discussed briefly in this section.

The following areas were identified as possible sources of bias in this study.

Selection Bias

The study was designed to detect CMV UL97 drug resistance mutations in the retrospective patient group and the clinician referred group of patients with serially elevated viral loads who may be at risk for presence of CMV drug resistance mutations, as well as those with documented exposure to ganciclovir who prospectively did not suppress their CMV viral loads.

Findings of this study should be interpreted in this context, which may overinflate the magnitude of the problem of CMV drug resistance mutations in the source population. This selection bias is inherent of this particular study design, as risk of developing mutations cannot be measured without knowledge of CMV viral load levels, knowledge of risk groups and exposure

to ganciclovir or valganciclovir. Clinical interpretation of data and administration of ganciclovir are essential prerequisites towards assessment of outcomes.

Due to the limited resources available for this study as well as the fact that it was largely a proof-of-concept study we had limited control over this selection bias, to be able to detect CMV UL97 drug resistance mutations exposure to ganciclovir is a prerequisite (Gerhard, 2008, Pannucci and Wilkins, 2010).

There was no knowledge by the investigators of prior ganciclovir use, duration or dosage in the retrospective patient category. Exposure to ganciclovir was assumed based on serial screening of CMV viral load values, current local practices of ganciclovir initiation above recommended threshold and diagnoses provided on the laboratory request form. This may underestimate the prevalence of CMV UL97 mutations. The prospective category of patients in whom mutations were detected was largely paediatric patients. In this group, there was a higher level of willingness by the paediatricians to refer patients for drug resistance testing.

This may skew the association of incidence of CMV UL97 mutations towards a higher magnitude than that which exists in this sample population.

PCR Amplification, Sequencing and Sequence Analysis Bias

Acinas et al (2005) distinguishes PCR artefacts in laboratory studies into two categories, namely those related to sequence artefacts (PCR errors i.e. formation of chimeric molecules, formation of heteroduplexes and *Taq* DNA polymerase errors) and those resulting in an abnormal distribution of PCR products as a result of unequal PCR amplification (PCR bias) (Acinas et al., 2005).

Wagner et al (1994) and Bracho et al (2004) further characterise these effects to occur because of PCR selection and drift. They describe PCR

selection to occur because of mechanisms such as preferential denaturing due to guanine-cytosine (GC) content in the primer and template, differential efficiency of primer hybridisation or differential DNA polymerase extension rates because of secondary structures of DNA. This may lead to preferential amplification of certain templates resulting in their overrepresentation in the final PCR mixture. They further attribute PCR drift as being related to random variation in the early cycles of the reaction which is not repeatable in subsequent PCR amplification experiments of the same gene family (Wagner et al., 1994, Bracho et al., 2004).

Although these publications referred to amplification of the 16sRNA gene, the concept is well described in virological studies. Yao et al (2005) described three major sources of sequencing error in their work on amplification of the Hepatitis C virus (HCV) Genotype 1 open reading frame –a highly genotypically variable virus. These included, sequencing errors, enzymatic errors during reverse transcription PCR (RT-PCR) and primer bias during PCR (Yao and Tavis, 2005). Reverse transcription, a prerequisite step in the amplification of RNA viruses to generate complementary DNA (cDNA), may also contribute towards the formation of primer-dimers or truncated products that can interfere with sequencing. This paper additionally highlights the importance of careful primer design, particularly for genetically heterogeneous templates, direct sequencing of the amplicons, adequate sequencing depth and bidirectional sequencing to reduce the bias.

PCR amplification cycles, primer annealing temperatures and amplification of multiple templates from a single reaction are additional factors that can affect PCR reaction amplification bias (Suzuki and Giovannoni, 1996, Polz and Cavanaugh, 1998, Sipos et al., 2007).

Thermus aquaticus polymerase (*Taq* polymerase) is a thermostable DNA polymerase enzyme used in PCR reactions to amplify DNA. Enzymatic errors in sequencing, particularly for RNA viruses, have been documented to be approximately $0.2-2 \times 10^{-4}$ errors per base pair per cycle (Eckert and Kunkel, 1990, Smith et al., 1997a, Bracho et al., 1998).

Tindall and Kunkel (1988) simplify these *Taq* Polymerase error rates as 1 nucleotide substitution in every 9000 bases polymerised and 1 frameshift error in every 41 000 bases polymerized (Tindall and Kunkel, 1988). Eckert and Kunkel (1990) suggest that despite these *Taq* polymerase error rates, within a well -defined PCR reaction (adequate pH, dNTP and MgCl₂ concentrations), *Taq* polymerase can still generate highly accurate DNA synthesis (Eckert and Kunkel, 1990). A high fidelity DNA polymerase, which has a hundred times lower error rates than *Taq* polymerase, is preferred for experiments requiring cloning, amplification of large fragments above 1000 base pairs and next generation sequencing.

Smith et al (1997) further suggest that avoiding analysis of cloned sequences and sequencing directly from PCR products derived by amplification of a single target region is beneficial in reducing these sources of error, a finding supported by Simmonds et al (1990) in their work on amplification of the HIV provirus (Simmonds et al., 1990, Smith et al., 1997a).

Within the context of the aforementioned factors, we amplified a single region of the CMV genome, targeting a gene not responsible for genotypic variation. CMV genotyping requires amplification of glycoprotein B (UL55), glycoprotein N (UL73) or glycoprotein H (UL75) (Chou, 1992, Tarrago et al., 2003, Pignatelli et al., 2003, Mujtaba et al., 2016).

Bidirectional sequencing was performed directly from the PCR products without prior cloning. Two independent observers to confirm sequence findings performed the sequence analysis.

The theoretical bias of database analysis against available published sequences is a factor outside of our control. Databases (such as the NCBI BLAST and University of Ulm Mutation Resistance Analyzer, which were used in this study, tend to publish sequences based on findings pertaining to particular geographical locations or pathogens of interest at particular timepoints, which may bias findings.

The University of Ulm database is the only freely available online tool for the analysis of CMV drug resistance mutations. The NCBI BLAST database, although limited by geographical bias towards sequences from first world countries (mainly Britain and America), provides all currently published sequences from groups working on CMV and hence provides the best benchmark of our findings. This bias is predominantly a reflection of limited work done in this arena from developing countries.

Chapter 4. Conclusion and Recommendations

In conclusion, these findings demonstrate that CMV UL97 mutations occur in the local solid organ, stem cell transplant population and patients with primary immunodeficiencies following prolonged treatment with ganciclovir. Not only were sequence variants detected, but also significant drug resistance mutations conferring varying levels of resistance to ganciclovir. This is the first study of this nature in South Africa that attempts to describe the existence and nature of CMV UL97 drug resistance mutations.

The findings of this study demonstrate a higher prevalence of CMV UL97 drug resistance mutations (20%) compared to that described in international literature (0-10%). The prevalence of CMV UL97 mutations in the retrospective group was 5/33 (15%) and 3/8 (38%) in the clinician referred group of patients with elevated CMV viral load on ganciclovir.

There were no CMV UL97 mutations detected in the HIV-infected patients we tested. This may be a reflection on shorter duration of treatment in these patients, which is usually for six weeks according to current local practice.

The haematological oncology patient category displayed the presence of clinically non-significant CMV UL97 sequence polymorphisms, which do not affect response to antiviral treatment, hence should not be reported to clinicians.

Our findings suggest that patients with primary immunodeficiency disorders or post stem-cell transplantation were at a higher risk of developing CMV UL97 mutations. Transplant recipients who undergo extensive myelo-ablation and immunosuppression, in particular, are at the most increased risk of developing drug resistance mutations. These findings should be interpreted within the context of the study design, sample size and limited referral of adult patients in the prospective category for drug resistance testing.

The introduction of CMV genotypic resistance testing provided important insight into the development of ganciclovir mutations in special at-risk populations. Among our cases, we found the use of acyclovir prophylaxis might play a role in the evolution of mutations, as both Patient F and Patient G (Table 3) were adult stem cell transplant recipients who were on acyclovir prophylaxis at the time of UL97 genotypic testing.

Presence of viral quasispecies, underlying host factors promoting CMV replication, drug exposure history, compartment sampled and sequencing platform need to be considered when interpreting results. Testing at a singular timepoint may fail to detect CMV UL97 drug resistance mutations.

These findings suggest a need for the detailed assessment of the presence of CMV UL97 drug resistance mutations in clinically relevant settings. Accurate documentation of ganciclovir dosage, duration, host factors favouring CMV replication and clinical outcomes should be undertaken. The potential role of acyclovir exposure preceding ganciclovir use requires further exploration.

Genotypic testing in appropriate clinical scenarios stands to provide early detection of drug resistant mutations, eliminate the need for ongoing inadequate treatment with ganciclovir, reduction of ganciclovir associated adverse events and alert clinicians towards adequate tailoring of therapy such as reduction of immunosuppressants. Most importantly, assessment for CMV drug resistant mutations would provide essential baseline data towards assessing the need for introduction of Foscarnet and Cidofovir.

References

- ACINAS, S. G., SARMA-RUPAVTARM, R., KLEPAC-CERAJ, V. & POLZ, M. F. 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl Environ Microbiol*, 71, 8966-9.
- ALAIN, S., HANTZ, S., SCIEUX, C., KARRAS, A., MAZERON, M. C., SZELAG, J. C., IMBERT, B. M., FILLET, A. M., GOUARIN, S., MENGELLE, C., DE WILDE, A., COGNE, N., CHAMPIER, G., ROGEZ, S., LEGENDRE, C. & DENIS, F. 2004. Detection of ganciclovir resistance after valacyclovir-prophylaxis in renal transplant recipients with active cytomegalovirus infection. *J Med Virol*, 73, 566-73.
- ARIZA-HEREDIA, E. J., NESHER, L. & CHEMALY, R. F. 2014. Cytomegalovirus diseases after hematopoietic stem cell transplantation: a mini-review. *Cancer Lett*, 342, 1-8.
- ARSLAN, F., TABAK, F., AVSAR, E., MIDILLI, K., MERT, A., OZARAS, R., SOYSAL, T., OZTURK, R. & FERHANOGU, B. 2010. Ganciclovir-resistant cytomegalovirus encephalitis in a hematopoietic stem cell transplant recipient. *J Neurovirol*, 16, 174-8.
- BARZON, L., LAVEZZO, E., COSTANZI, G., FRANCHIN, E., TOPPO, S. & PALU, G. 2013. Next-generation sequencing technologies in diagnostic virology. *J Clin Virol*, 58, 346-50.
- BENZI, F., VANNI, I., CASSINA, G., UGOLOTTI, E., DI MARCO, E., CIRILLO, C., CRISTINA, E., MORREALE, G., MELIOLI, G., MALNATI, M. & BIASSONI, R. 2012. Detection of ganciclovir resistance mutations by pyrosequencing in HCMV-infected pediatric patients. *J Clin Virol*, 54, 48-55.
- BOECKH, M. 2009. How we treat cytomegalovirus in hematopoietic cell transplant recipients. *Blood*, 113, 5711-5719.
- BOECKH, M., LEISENRING, W., RIDDELL, S. R., BOWDEN, R. A., HUANG, M. L., MYERSON, D., STEVENS-AYERS, T., FLOWERS, M. E., CUNNINGHAM, T. & COREY, L. 2003. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. *Blood*, 101, 407-14.
- BOIVIN, G., CHOU, S., QUIRK, M. R., ERICE, A. & JORDAN, M. C. 1996. Detection of ganciclovir resistance mutations quantitation of cytomegalovirus (CMV) DNA in leukocytes of patients with fatal disseminated CMV disease. *J Infect Dis*, 173, 523-8.
- BOIVIN, G., GILBERT, C., MORISSETTE, M., HANDFIELD, J., GOYETTE, N. & BERGERON, M. G. 1997. A case of ganciclovir-resistant cytomegalovirus (CMV) retinitis in a patient with AIDS: longitudinal molecular analysis of the CMV viral load and viral mutations in blood compartments. *AIDS*, 11, 867-73.
- BOIVIN, G., GOYETTE, N., FARHAN, M., IVES, J. & ELSTON, R. 2012. Incidence of cytomegalovirus UL97 and UL54 amino acid substitutions detected after 100 or 200 days of valganciclovir prophylaxis. *J Clin Virol*, 53, 208-13.

- BOIVIN, G., GOYETTE, N., GILBERT, C. & COVINGTON, E. 2005. Analysis of cytomegalovirus DNA polymerase (UL54) mutations in solid organ transplant patients receiving valganciclovir or ganciclovir prophylaxis. *J Med Virol*, 77, 425-9.**
- BOIVIN, G., GOYETTE, N., GILBERT, C., ROBERTS, N., MACEY, K., PAYA, C., PESCOVITZ, M. D., HUMAR, A., DOMINGUEZ, E., WASHBURN, K., BLUMBERG, E., ALEXANDER, B., FREEMAN, R., HEATON, N. & COVINGTON, E. 2004. Absence of cytomegalovirus-resistance mutations after valganciclovir prophylaxis, in a prospective multicenter study of solid-organ transplant recipients. *J Infect Dis*, 189, 1615-8.**
- BOIVIN, G., GOYETTE, N., ROLLAG, H., JARDINE, A. G., PESCOVITZ, M. D., ASBERG, A., IVES, J., HARTMANN, A. & HUMAR, A. 2009. Cytomegalovirus resistance in solid organ transplant recipients treated with intravenous ganciclovir or oral valganciclovir. *Antivir Ther*, 14, 697-704.**
- BOPPANA, S. B. & BRITT, W. J. 2013. Synopsis of Clinical Aspects of Human Cytomegalovirus Disease. In: REDDEHASE, M. (ed.) *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*.**
- BOUTOLLEAU, D., BURREL, S. & AGUT, H. 2011. Genotypic characterization of human cytomegalovirus UL97 phosphotransferase natural polymorphism in the era of ganciclovir and maribavir. *Antiviral Res*, 91, 32-5.**
- BRACHO, M. A., GARCIA-ROBLES, I., JIMENEZ, N., TORRES-PUENTE, M., MOYA, A. & GONZALEZ-CANDELAS, F. 2004. Effect of oligonucleotide primers in determining viral variability within hosts. *Virology*, 1, 13.**
- BRACHO, M. A., MOYA, A. & BARRIO, E. 1998. Contribution of Taq polymerase-induced errors to the estimation of RNA virus diversity. *J Gen Virol*, 79 (Pt 12), 2921-8.**
- CAMPOS, A. B., RIBEIRO, J., BOUTOLLEAU, D. & SOUSA, H. 2016. Human cytomegalovirus antiviral drug resistance in hematopoietic stem cell transplantation: current state of the art. *Rev Med Virol*, 26, 161-82.**
- CAPOBIANCHI, M. R., GIOMBINI, E. & ROZERA, G. 2013. Next-generation sequencing technology in clinical virology. *Clin Microbiol Infect*, 19, 15-22.**
- CASTOR, J., COOK, L., COREY, L. & JEROME, K. R. 2007. Rapid detection directly from patient serum samples of human cytomegalovirus UL97 mutations conferring ganciclovir resistance. *J Clin Microbiol*, 45, 2681-3.**
- CHEN, X. F., LI, T. R., YANG, H., SHAO, Y., ZHANG, J., ZHANG, W., YU, B., WEI, Z., WU, B. & YU, L. 2015. Detection of Two Drug-Resistance Mutants of the Cytomegalovirus by High-Resolution Melting Analysis. *J Clin Lab Anal*.**
- CHEVILLOTTE, M., VON EINEM, J., MEIER, B. M., LIN, F. M., KESTLER, H. A. & MERTENS, T. 2010. A new tool linking human cytomegalovirus drug resistance mutations to resistance phenotypes. *Antiviral Res*, 85, 318-27.**
- CHOI, S. H., HWANG, J. Y., PARK, K. S., KIM, Y., LEE, S. H., YOO, K. H., KANG, E. S., AHN, J. H., SUNG, K. W., KOO, H. H. & KIM, Y. J. 2014. The impact of drug-resistant cytomegalovirus in pediatric allogeneic hematopoietic cell transplant recipients: a**

- prospective monitoring of UL97 and UL54 gene mutations. Transpl Infect Dis, 16, 919-29.*
- CHOU, S. 1992. Molecular epidemiology of envelope glycoprotein H of human cytomegalovirus. J Infect Dis, 166, 604-7.**
- CHOU, S. 1999. Antiviral drug resistance in human cytomegalovirus. Transpl Infect Dis, 1, 105-14.**
- CHOU, S. 2008. Cytomegalovirus UL97 mutations in the era of ganciclovir and maribavir. Rev Med Virol, 18, 233-46.**
- CHOU, S. 2010. Recombinant phenotyping of cytomegalovirus UL97 kinase sequence variants for ganciclovir resistance. Antimicrob Agents Chemother, 54, 2371-8.**
- CHOU, S., ERCOLANI, R. J., SAHOO, M. K., LEFTEROVA, M. I., STRASFELD, L. M. & PINSKY, B. A. 2014. Improved detection of emerging drug-resistant mutant cytomegalovirus subpopulations by deep sequencing. Antimicrob Agents Chemother, 58, 4697-702.**
- CHOU, S. W. 2001. Cytomegalovirus drug resistance and clinical implications. Transpl Infect Dis, 3 Suppl 2, 20-4.**
- COAQUETTE, A., BOURGEOIS, A., DIRAND, C., VARIN, A., CHEN, W. & HERBEIN, G. 2004. Mixed cytomegalovirus glycoprotein B genotypes in immunocompromised patients. Clin Infect Dis, 39, 155-61.**
- DE LA CAMARA, R. 2016. CMV in Hematopoietic Stem Cell Transplantation. Mediterr J Hematol Infect Dis, 8, e2016031.**
- DREW, W. L. 2010. Cytomegalovirus resistance testing: pitfalls and problems for the clinician. Clin Infect Dis, 50, 733-6.**
- DROUOT, E., PIRET, J., LEBEL, M. H. & BOIVIN, G. 2014. Characterization of multiple cytomegalovirus drug resistance mutations detected in a hematopoietic stem cell transplant recipient by recombinant phenotyping. J Clin Microbiol, 52, 4043-6.**
- DUCANCELLE, A., BELLOC, S., ALAIN, S., SCIEUX, C., MALPHETTES, M., PETIT, F., BROUET, J. C., SANSON LE PORS, M. J. & MAZERON, M. C. 2004. Comparison of sequential cytomegalovirus isolates in a patient with lymphoma and failing antiviral therapy. J Clin Virol, 29, 241-7.**
- ECKERT, K. A. & KUNKEL, T. A. 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. Nucleic Acids Res, 18, 3739-44.**
- ECKLE, T., JAHN, G. & HAMPRECHT, K. 2004. The influence of mixed HCMV UL97 wildtype and mutant strains on ganciclovir susceptibility in a cell associated plaque reduction assay. J Clin Virol, 30, 50-6.**
- ECKLE, T., PRIX, L., JAHN, G., KLINGEBIEL, T., HANDGRETINGER, R., SELLE, B. & HAMPRECHT, K. 2000. Drug-resistant human cytomegalovirus infection in children after allogeneic stem cell transplantation may have different clinical outcomes. Blood, 96, 3286-9.**

- ERICE, A., BORRELL, N., LI, W., MILLER, W. J. & BALFOUR, H. H., JR. 1998. *Ganciclovir susceptibilities and analysis of UL97 region in cytomegalovirus (CMV) isolates from bone marrow recipients with CMV disease after antiviral prophylaxis. J Infect Dis, 178, 531-4.*
- FRANGE, P., BOUTOLLEAU, D., LERUEZ-VILLE, M., TOUZOT, F., CROS, G., HERITIER, S., MOSHOUS, D., NEVEN, B., FISCHER, A. & BLANCHE, S. 2013. *Temporal and spatial compartmentalization of drug-resistant cytomegalovirus (CMV) in a child with CMV meningoencephalitis: implications for sampling in molecular diagnosis. J Clin Microbiol, 51, 4266-9.*
- GANDHI, M. K. & KHANNA, R. 2004. *Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. Lancet Infect Dis, 4, 725-38.*
- GARCIA-MARTINEZ, J., FOLGUEIRA, L., DELGADO, R., HERNANDO, S., PRIETO, C., AGUADO, J. M. & OTERO, J. R. 2008. *Viral monitoring and successful treatment of a ganciclovir-resistant cytomegalovirus infection in a heart transplant recipient. Transpl Infect Dis, 10, 123-8.*
- GARCIA-VIDAL, C., UPTON, A., KIRBY, K. A. & MARR, K. A. 2008. *Epidemiology of invasive mold infections in allogeneic stem cell transplant recipients: biological risk factors for infection according to time after transplantation. Clin Infect Dis, 47, 1041-50.*
- GARGIS, A. S., KALMAN, L. & LUBIN, I. M. 2016. *Assuring the Quality of Next-Generation Sequencing in Clinical Microbiology and Public Health Laboratories. J Clin Microbiol, 54, 2857-2865.*
- GERHARD, T. 2008. *Bias: considerations for research practice. Am J Health Syst Pharm, 65, 2159-68.*
- GILBERT, C., BESTMAN-SMITH, J. & BOIVIN, G. 2002. *Resistance of herpesviruses to antiviral drugs: clinical impacts and molecular mechanisms. Drug Resist Updat, 5, 88-114.*
- GILBERT, C. & BOIVIN, G. 2003. *Discordant phenotypes and genotypes of cytomegalovirus (CMV) in patients with AIDS and relapsing CMV retinitis. AIDS, 17, 337-41.*
- GOHRING, K., HAMPRECHT, K. & JAHN, G. 2015. *Antiviral Drug- and Multidrug Resistance in Cytomegalovirus Infected SCT Patients. Comput Struct Biotechnol J, 13, 153-9.*
- GOHRING, K., MIKELER, E., JAHN, G. & HAMPRECHT, K. 2006. *Rapid simultaneous detection by real-time PCR of cytomegalovirus UL97 mutations in codons 460 and 520 conferring ganciclovir resistance. J Clin Microbiol, 44, 4541-4.*
- GORDON, C. R., AVERY, R. K., ABOUHASSAN, W. & SIEMIONOW, M. 2011. *Cytomegalovirus and other infectious issues related to face transplantation: specific considerations, lessons learned, and future recommendations. Plast Reconstr Surg, 127, 1515-23.*
- GORZER, I., GUELLY, C., TRAJANOSKI, S. & PUCHHAMMER-STOCKL, E. 2010. *Deep sequencing reveals highly complex dynamics of human cytomegalovirus genotypes in transplant patients over time. J Virol, 84, 7195-203.*

- GREEN, C. B., O'RIORDAN, A., GRIFFITHS, P. & HAQUE, T. 2016. A deletion at CMV UL54 codon 524 without co-existing resistance-associated mutation at UL97 confers resistance to ganciclovir: A case report. *J Clin Virol*, 80, 24-6.**
- GRIFFITHS, P. D. 2009. Cytomegalovirus. In: ZUCKERMAN A J, B. J., SCHOUB BD, GRIFFITHS PD, MORTIMER P. (ed.) *Principles and Practice of Clinical Virology*. 6th ed. Chichester: Wiley-Blackwell.**
- HAIKAR, G. & SINGH, N. 2017. Viral infections in solid organ transplant recipients: novel updates and a review of the classics. *Curr Opin Infect Dis*, 30, 579-588.**
- HALL SEDLAK, R., CASTOR, J., BUTLER-WU, S. M., CHAN, E., COOK, L., LIMAYE, A. P. & JEROME, K. R. 2013. Rapid detection of human cytomegalovirus UL97 and UL54 mutations directly from patient samples. *J Clin Microbiol*, 51, 2354-9.**
- HALL, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser*, 95-98.**
- HAMPRECHT, K., ECKLE, T., PRIX, L., FAUL, C., EINSELE, H. & JAHN, G. 2003. Ganciclovir-resistant cytomegalovirus disease after allogeneic stem cell transplantation: pitfalls of phenotypic diagnosis by in vitro selection of an UL97 mutant strain. *J Infect Dis*, 187, 139-43.**
- HANTZ, S., MICHEL, D., FILLET, A. M., GUIGONIS, V., CHAMPIER, G., MAZERON, M. C., BENSMAN, A., DENIS, F., MERTENS, T., DEHEE, A. & ALAIN, S. 2005. Early selection of a new UL97 mutant with a severe defect of ganciclovir phosphorylation after valaciclovir prophylaxis and short-term ganciclovir therapy in a renal transplant recipient. *Antimicrob Agents Chemother*, 49, 1580-3.**
- HOANG, Q. V., SIMON, D. M., KUMAR, G. N., OH, F. & GOLDSTEIN, D. A. 2010. Recurrent CMV retinitis in a non-HIV patient with drug-resistant CMV. *Graefes Arch Clin Exp Ophthalmol*, 248, 737-40.**
- HOULDCROFT, C. J., BRYANT, J. M., DEPLEDGE, D. P., MARGETTS, B. K., SIMMONDS, J., NICOLAOU, S., TUTILL, H. J., WILLIAMS, R., WORTH, A. J., MARKS, S. D., VEYS, P., WHITTAKER, E. & BREUER, J. 2016. Detection of Low Frequency Multi-Drug Resistance and Novel Putative Maribavir Resistance in Immunocompromised Pediatric Patients with Cytomegalovirus. *Front Microbiol*, 7, 1317.**
- HU, H., JABS, D. A., FORMAN, M. S., MARTIN, B. K., DUNN, J. P., WEINBERG, D. V. & DAVIS, J. L. 2002. Comparison of cytomegalovirus (CMV) UL97 gene sequences in the blood and vitreous of patients with acquired immunodeficiency syndrome and CMV retinitis. *J Infect Dis*, 185, 861-7.**
- HUMAR, A., KUMAR, D., GILBERT, C. & BOIVIN, G. 2003. Cytomegalovirus (CMV) glycoprotein B genotypes and response to antiviral therapy, in solid-organ-transplant recipients with CMV disease. *J Infect Dis*, 188, 581-4.**
- IMAI, Y., SHUM, C., MARTIN, D. F., KUPPERMANN, B. D., DREW, W. L. & MARGOLIS, T. P. 2004. Emergence of drug-resistant cytomegalovirus retinitis in the contralateral eyes of patients with AIDS treated with ganciclovir. *J Infect Dis*, 189, 611-5.**

- IWASENKO, J. M., SCOTT, G. M., NAING, Z., GLANVILLE, A. R. & RAWLINSON, W. D. 2011. Diversity of antiviral-resistant human cytomegalovirus in heart and lung transplant recipients. *Transpl Infect Dis*, 13, 145-53.**
- IWASENKO, J. M., SCOTT, G. M., ZIEGLER, J. B. & RAWLINSON, W. D. 2007. Emergence and persistence of multiple antiviral-resistant CMV strains in a highly immunocompromised child. *J Clin Virol*, 40, 152-5.**
- JABS, D. A., ENGER, C., DUNN, J. P., FORMAN, M. & HUBBARD, L. 1998. Cytomegalovirus retinitis and viral resistance: 3. Culture results. *CMV Retinitis and Viral Resistance Study Group. Am J Ophthalmol*, 126, 543-9.**
- JABS, D. A., MARTIN, B. K., FORMAN, M. S., HUBBARD, L., DUNN, J. P., KEMPEN, J. H., DAVIS, J. L. & WEINBERG, D. V. 2003. Cytomegalovirus resistance to ganciclovir and clinical outcomes of patients with cytomegalovirus retinitis. *Am J Ophthalmol*, 135, 26-34.**
- JABS, D. A., MARTIN, B. K., RICKS, M. O. & FORMAN, M. S. 2006. Detection of ganciclovir resistance in patients with AIDS and cytomegalovirus retinitis: correlation of genotypic methods with viral phenotype and clinical outcome. *J Infect Dis*, 193, 1728-37.**
- JULIN, J. E., VAN BURIK, J. H., KRIVIT, W., WEBB, C., HOLMAN, C. J., CLARK, H. B. & BALFOUR, H. H., JR. 2002. Ganciclovir-resistant cytomegalovirus encephalitis in a bone marrow transplant recipient. *Transpl Infect Dis*, 4, 201-6.**
- KAMPMANN, S. E., SCHINDELE, B., APELT, L., BUHRER, C., GARTEN, L., WEIZSAECKER, K., KRUGER, D. H., EHLERS, B. & HOFMANN, J. 2011. Pyrosequencing allows the detection of emergent ganciclovir resistance mutations after HCMV infection. *Med Microbiol Immunol*, 200, 109-13.**
- KIR, O., ZEYINOGLU, A., ARDA, B., YILMAZ, M., ASCI, G. & TOZ, H. 2017. Impact of Prophylaxis vs Pre-emptive Approach for Cytomegalovirus Infection in Kidney Transplant Recipients. *Transplant Proc*, 49, 537-540.**
- KRAUSE, H., HEBART, H., JAHN, G., MULLER, C. A. & EINSELE, H. 1997. Screening for CMV-specific T cell proliferation to identify patients at risk of developing late onset CMV disease. *Bone Marrow Transplant*, 19, 1111-6.**
- KUO, I. C., IMAI, Y., SHUM, C., MARTIN, D. F., KUPPERMANN, B. D. & MARGOLIS, T. P. 2003. Genotypic analysis of cytomegalovirus retinitis poorly responsive to intravenous ganciclovir but responsive to the ganciclovir implant. *Am J Ophthalmol*, 135, 20-5.**
- LE PAGE, A. K., JAGER, M. M., IWASENKO, J. M., SCOTT, G. M., ALAIN, S. & RAWLINSON, W. D. 2013. Clinical aspects of cytomegalovirus antiviral resistance in solid organ transplant recipients. *Clin Infect Dis*, 56, 1018-29.**
- LEE, G. C., CHOI, S. M., LEE, C. H., LEE, D. G., CHOI, J. H. & YOO, J. H. 2013. Detection of human cytomegalovirus UL97 D605E mutation in Korean stem cell transplantation recipients and donors. *J Microbiol Biotechnol*, 23, 1154-8.**

- LISCHKA, P. & ZIMMERMANN, H. 2008. Antiviral strategies to combat cytomegalovirus infections in transplant recipients. *Curr Opin Pharmacol*, 8, 541-8.
- LJUNGMAN, P., DELILIERS, G. L., PLATZBECKER, U., MATTHES-MARTIN, S., BACIGALUPO, A., EINSELE, H., ULLMANN, J., MUSSO, M., TRENSCHEL, R., RIBAUD, P., BORNHAUSER, M., CESARO, S., CROOKS, B., DEKKER, A., GRATECOS, N., KLINGEBIEL, T., TAGLIAFERRI, E., ULLMANN, A. J., WACKER, P. & CORDONNIER, C. 2001. Cidofovir for cytomegalovirus infection and disease in allogeneic stem cell transplant recipients. *The Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Blood*, 97, 388-92.
- LURAIN, N. S. & CHOU, S. 2010. Antiviral drug resistance of human cytomegalovirus. *Clin Microbiol Rev*, 23, 689-712.
- LURAIN, N. S., WEINBERG, A., CRUMPACKER, C. S. & CHOU, S. 2001. Sequencing of cytomegalovirus UL97 gene for genotypic antiviral resistance testing. *Antimicrob Agents Chemother*, 45, 2775-80.
- MANUEL, O., ASBERG, A., PANG, X., ROLLAG, H., EMERY, V. C., PREIKSAITIS, J. K., KUMAR, D., PESCOVITZ, M. D., BIGNAMINI, A. A., HARTMANN, A., JARDINE, A. G. & HUMAR, A. 2009. Impact of genetic polymorphisms in cytomegalovirus glycoprotein B on outcomes in solid-organ transplant recipients with cytomegalovirus disease. *Clin Infect Dis*, 49, 1160-6.
- MARR, K. A., CARTER, R. A., BOECKH, M., MARTIN, P. & COREY, L. 2002. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. *Blood*, 100, 4358-66.
- MARTIN, B. K., RICKS, M. O., FORMAN, M. S. & JABS, D. A. 2007. Change over time in incidence of ganciclovir resistance in patients with cytomegalovirus retinitis. *Clin Infect Dis*, 44, 1001-8.
- MILLER, G. G., BOIVIN, G., DUMMER, J. S., MCCONNELL, T., BECHER, M. W., KASSIM, A. & TANG, Y. W. 2006. Cytomegalovirus ventriculoencephalitis in a peripheral blood stem cell transplant recipient. *Clin Infect Dis*, 42, e26-9.
- MILLER, S. 2016. Monitoring for Viral Infections in Transplant Patients. *Clinical Microbiology Newsletter*, 38, 129-134.
- MINCES, L. R., NGUYEN, M. H., MITSANI, D., SHIELDS, R. K., KWAK, E. J., SILVEIRA, F. P., ABDEL-MASSIH, R., PILEWSKI, J. M., CRESPO, M. M., BERMUDEZ, C., BHAMA, J. K., TOYODA, Y. & CLANCY, C. J. 2014. Ganciclovir-resistant cytomegalovirus infections among lung transplant recipients are associated with poor outcomes despite treatment with foscarnet-containing regimens. *Antimicrob Agents Chemother*, 58, 128-35.
- MUJTABA, G., KHURSHID, A., SHARIF, S., ALAM, M. M., AAMIR, U. B., SHAUKAT, S., ANGEZ, M., RANA, M. S., UMAIR, M., SHAH, A. A. & ZAIDI, S. S. 2016. Distribution of Cytomegalovirus Genotypes among Neonates Born to Infected Mothers in Islamabad, Pakistan. *PLoS One*, 11, e0156049.
- MURRELL, I., WILKIE, G. S., DAVISON, A. J., STATKUTE, E., FIELDING, C. A., TOMASEC, P., WILKINSON, G. W. & STANTON, R. J. 2016. Genetic Stability of Bacterial Artificial

Chromosome-Derived Human Cytomegalovirus during Culture In Vitro. J Virol, 90, 3929-43.

NICHOLS, W. G., COREY, L., GOOLEY, T., DAVIS, C. & BOECKH, M. 2002. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. J Infect Dis, 185, 273-82.

NOGUEIRA, E., OZAKI, K. S., TOMIYAMA, H., GRANATO, C. F., CAMARA, N. O. & PACHECO-SILVA, A. 2006. The emergence of cytomegalovirus resistance to ganciclovir therapy in kidney transplant recipients. Int Immunopharmacol, 6, 2031-7.

OZDEMIR, E., SALIBA, R. M., CHAMPLIN, R. E., COURIEL, D. R., GIRALT, S. A., DE LIMA, M., KHOURI, I. F., HOSING, C., KORNBLOU, S. M., ANDERLINI, P., SHPALL, E. J., QAZILBASH, M. H., MOLLIDREM, J. J., CHEMALY, R. F. & KOMANDURI, K. V. 2007. Risk factors associated with late cytomegalovirus reactivation after allogeneic stem cell transplantation for hematological malignancies. Bone Marrow Transplant, 40, 125-36.

PANNUCCI, C. J. & WILKINS, E. G. 2010. Identifying and avoiding bias in research. Plast Reconstr Surg, 126, 619-25.

PEREZ, J. L. 1997. Resistance to antivirals in human cytomegalovirus: mechanisms and clinical significance. Microbiologia, 13, 343-52.

PERGAM, S. A. & JEROME, K. R. 2010. Viral Infections in the Immunocompromised Host. In: KR, J. (ed.) Lennette's Laboratory Diagnosis of Viral Infections. Informa Healthcare.

PESCOVITZ, M. D., RABKIN, J., MERION, R. M., PAYA, C. V., PIRSCH, J., FREEMAN, R. B., O'GRADY, J., ROBINSON, C., TO, Z., WREN, K., BANKEN, L., BUHLES, W. & BROWN, F. 2000. Valganciclovir results in improved oral absorption of ganciclovir in liver transplant recipients. Antimicrob Agents Chemother, 44, 2811-5.

PIGNATELLI, S., DAL MONTE, P., ROSSINI, G., CHOU, S., GOJOBORI, T., HANADA, K., GUO, J. J., RAWLINSON, W., BRITT, W., MACH, M. & LANDINI, M. P. 2003. Human cytomegalovirus glycoprotein N (gpUL73-gN) genomic variants: identification of a novel subgroup, geographical distribution and evidence of positive selective pressure. J Gen Virol, 84, 647-55.

POLZ, M. F. & CAVANAUGH, C. M. 1998. Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol, 64, 3724-30.

PRIX, L., HAMPRECHT, K., HOLZHUTER, B., HANDGRETINGER, R., KLINGEBIEL, T. & JAHN, G. 1999. Comprehensive restriction analysis of the UL97 region allows early detection of ganciclovir-resistant human cytomegalovirus in an immunocompromised child. J Infect Dis, 180, 491-5.

RAMANAN, P. & RAZONABLE, R. R. 2013. Cytomegalovirus infections in solid organ transplantation: a review. Infect Chemother, 45, 260-71.

RAZONABLE, R. R. & HUMAR, A. 2013. Cytomegalovirus in solid organ transplantation. Am J Transplant, 13 Suppl 4, 93-106.

- REDDY, A. J., ZAAS, A. K., HANSON, K. E. & PALMER, S. M. 2007. A single-center experience with ganciclovir-resistant cytomegalovirus in lung transplant recipients: treatment and outcome. *J Heart Lung Transplant*, 26, 1286-92.
- RENZETTE, N., GIBSON, L., BHATTACHARJEE, B., FISHER, D., SCHLEISS, M. R., JENSEN, J. D. & KOWALIK, T. F. 2013. Rapid intrahost evolution of human cytomegalovirus is shaped by demography and positive selection. *PLoS Genet*, 9, e1003735.
- RENZETTE, N., GIBSON, L., JENSEN, J. D. & KOWALIK, T. F. 2014. Human cytomegalovirus intrahost evolution—a new avenue for understanding and controlling herpesvirus infections. *Curr Opin Virol*, 8, 109-15.
- SAHOO, M. K., LEFTEROVA, M. I., YAMAMOTO, F., WAGGONER, J. J., CHOU, S., HOLMES, S. P., ANDERSON, M. W. & PINSKY, B. A. 2013. Detection of cytomegalovirus drug resistance mutations by next-generation sequencing. *J Clin Microbiol*, 51, 3700-10.
- SCHNEPF, N., DHEDIN, N., MERCIER-DELARUE, S., ANDREOLI, A., MAMEZ, A. C., FERRY, C., DEBACK, C., RIBAUD, P., ROBIN, M., SOCIE, G., SIMON, F. & MAZERON, M. C. 2013. Dynamics of cytomegalovirus populations harbouring mutations in genes UL54 and UL97 in a haematopoietic stem cell transplant recipient. *J Clin Virol*, 58, 733-6.
- SCOTT, G. M., ISAACS, M. A., ZENG, F., KESSON, A. M. & RAWLINSON, W. D. 2004. Cytomegalovirus antiviral resistance associated with treatment induced UL97 (protein kinase) and UL54 (DNA polymerase) mutations. *J Med Virol*, 74, 85-93.
- SIMMONDS, P., BALFE, P., PEUTHERER, J. F., LUDLAM, C. A., BISHOP, J. O. & BROWN, A. J. 1990. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. *J Virol*, 64, 864-72.
- SINZGER, C., DIGEL, M. & JAHN, G. 2008. Cytomegalovirus cell tropism. *Curr Top Microbiol Immunol*, 325, 63-83.
- SIPOS, R., SZEKELY, A. J., PALATINSZKY, M., REVESZ, S., MARIALIGETI, K. & NIKOLAUSZ, M. 2007. Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targeting bacterial community analysis. *FEMS Microbiol Ecol*, 60, 341-50.
- SMITH, D. B., MCALLISTER, J., CASINO, C. & SIMMONDS, P. 1997a. Virus 'quasispecies': making a mountain out of a molehill? *J Gen Virol*, 78 (Pt 7), 1511-9.
- SMITH, I. L., CHERRINGTON, J. M., JILES, R. E., FULLER, M. D., FREEMAN, W. R. & SPECTOR, S. A. 1997b. High-level resistance of cytomegalovirus to ganciclovir is associated with alterations in both the UL97 and DNA polymerase genes. *J Infect Dis*, 176, 69-77.
- STRASFELD, L. & CHOU, S. 2010. Antiviral drug resistance: mechanisms and clinical implications. *Infect Dis Clin North Am*, 24, 413-37.
- SUN, J., CAO, G., ZHANG, L., ZHANG, Y., ZHAO, Z., HU, J. & JI, Y. 2012. Human cytomegalovirus (CMV) UL97 D605E mutation has a higher prevalence in infants with primary CMV infection compared with transplant recipients with CMV recurrence. *Transplant Proc*, 44, 3022-5.

- SUNG, H., AN, D., LEE, S. O., CHOI, S. H., KIM, S. H., CHI, H. S. & KIM, M. N. 2012. Detection of a UL97 gene mutation conferring ganciclovir resistance in human cytomegalovirus: prevalence of the D605E polymorphism in Korean immunocompromised patients. *Ann Clin Lab Sci*, 42, 429-34.
- SUZUKI, M. T. & GIOVANNONI, S. J. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol*, 62, 625-30.
- TANAKA, K., HORI, T., YOTO, Y., HATAKEYAMA, N., YAMAMOTO, M., SUZUKI, N. & TSUTSUMI, H. 2011. Human cytomegalovirus UL97 D605E polymorphism has a high prevalence in immunocompetent Japanese infants and children. *Microbiol Immunol*, 55, 328-30.
- TARRAGO, D., QUEREDA, C. & TENORIO, A. 2003. Different cytomegalovirus glycoprotein B genotype distribution in serum and cerebrospinal fluid specimens determined by a novel multiplex nested PCR. *J Clin Microbiol*, 41, 2872-7.
- THOMPSON, J. D., HIGGINS, D. G. & GIBSON, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673-4680.
- TINDALL, K. R. & KUNKEL, T. A. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry*, 27, 6008-13.
- VINUESA, V., BRACHO, M. A., ALBERT, E., SOLANO, C., TORRES-PUENTE, M., GIMENEZ, E., GONZALEZ-CANDELAS, F. & NAVARRO, D. 2017. The impact of virus population diversity on the dynamics of cytomegalovirus DNAemia in allogeneic stem cell transplant recipients. *J Gen Virol*, 98, 2530-2542.
- VOLFOVA, P., LENGEROVA, M., LOCHMANOVA, J., DVORAKOVA, D., RICNA, D., PALACKOVA, M., WEINBERGEROVA, B., MAYER, J. & RACIL, Z. 2014. Detecting human cytomegalovirus drug resistant mutations and monitoring the emergence of resistant strains using real-time PCR. *J Clin Virol*, 61, 270-4.
- WAGNER, A., BLACKSTONE, N., CARTWRIGHT, P., DICK, M., MISOF, B., SNOW, P., WAGNER, G. P., BARTELS, J., MURTHA, M. & PENDLETON, J. 1994. Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. *Systematic Biology*, 43, 250-261.
- YAO, E. & TAVIS, J. E. 2005. A general method for nested RT-PCR amplification and sequencing the complete HCV genotype 1 open reading frame. *Virology*, 337, 88-94.
- YOST, S. E., ECHEVERRIA, A., JIE, T. & KAPLAN, B. 2014. Panresistant cytomegalovirus in a kidney transplant recipient. *Pharmacotherapy*, 34, e1-3.
- ZHANG, Y., ZHAO, Z., SUN, J., CAO, G., ZHAO, F., HU, J., LIU, L. & JI, Y. 2013. A new mutation in the human cytomegalovirus UL97 gene may confer ganciclovir resistance in Chinese kidney transplant recipients. *Arch Virol*, 158, 247-50.

Appendices

I: Ethics Approval

II: PathRed Conference Abstract-Oral Presentation

III: Virology Africa Conference Abstract

IV: Academic Poster Virology Africa 2015

V: University of Ulm, Mutation Resistance Analyzer Database Reports

I:



UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee



Room B52-24 Old Main Building
Groot-Schuur Hospital
Observatory 7925
Telephone (021) 406 6338 • Facsimile (021) 406 6411
Email: nosi.bsama@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

07 September 2015

HREC REF: 655/2015

Dr N Hsiao
Medical Virology
C18 NHLS Virology Lab
NGSH

Dear Dr Hsiao

PROJECT TITLE: ANALYSIS OF CYTOMEGALOVIRUS UL97 DRUG RESISTANCE MUTATIONS IN PATIENTS RECEIVING GANCICLOVIR (FCPath SA MMed-N Nkosi)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th September 2016.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

We also acknowledge that the student Nokwazi Nkosi will be involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH

HREC 655/2015

II: **SETTING UP A CYTOMEGALOVIRUS (CMV) UL 97 RESISTANCE** **MUTATION ASSAY**

Nokwazi Nkosi ¹, Nei-yuan Hsiao ¹

Division of Medical Virology, NHLS Groote Schuur Hospital, University of Cape Town, South Africa

BACKGROUND

Cytomegalovirus (CMV) drug resistance mutations through widespread use of ganciclovir have been widely reported in international literature, particularly in the post- transplant setting. However, a genotypic assay to detect CMV drug resistance is not available in South Africa and the prevalence of these mutations is therefore unknown.

OBJECTIVES/AIMS

We aimed to set up and evaluate an assay to detect CMV UL 97 mutations by analysing External Quality Assurance (EQA) panel and patients referred by clinicians with possible ganciclovir resistance.

METHODS

A literature search was conducted to obtain published primers and a nested Polymerase Chain Reaction protocol for a 707bp region of the UL97 gene for sequencing. Three whole blood samples from patients with a clinical history suggestive of ganciclovir resistance as well as 5 EQA samples from Quality Control for Molecular Diagnostics panel were tested in the evaluation.

RESULTS:

UL97 mutations were identified in 5/5 (100%) EQA samples. Six different UL97 mutations (100%): A594V, H520Q, L595S, M460V, C603W, G598S and a polymorphism associated with hyper susceptibility to ganciclovir (D605E) were detected. The G598S mutation (a vaccinia virus recombinant) was present in all EQA samples.

The L 595S mutation was identified in a 3-year-old child with myelodysplasia post –haematopoietic stem cell transplant who had persistently elevated CMV Viral Load despite being on ganciclovir. However, despite the presence of the mutation he subsequently suppressed on ganciclovir.

CONCLUSIONS

Ganciclovir is used on a large scale in transplant settings. This preliminary work has shown that resistance mutations to ganciclovir may be more prevalent than initially suspected. It has also highlighted the need for documenting the prevalence, nature and effect on clinical outcomes of patients with these mutations; which demands more work to be conducted in this arena.

III:

ANALYSIS OF CYTOMEGALOVIRUS UL97 DRUG RESISTANCE

MUTATIONS

Nokwazi Nkosi, Stephen Korsman, Heidi Smuts, Diana Hardie, Nei-yuan Hsiao

Division of Medical Virology, NHLS Groote Schuur Hospital, University of Cape Town, South Africa

BACKGROUND

Ganciclovir Cytomegalovirus (CMV) drug resistance mutations (DRM) have been widely reported in international literature, particularly in the post-transplant setting. However; without access to genotyping assays; the prevalence of these mutations is unknown in South Africa. We aimed to set up an in-house assay to document the prevalence of CMV UL97 DRM amongst immune-suppressed populations in Cape Town.

METHODS

A literature search was conducted to obtain published primers and a nested Polymerase Chain Reaction protocol for a 707bp region of the UL97 gene for sequencing. A Quality Control for Molecular Diagnostics (QCMD) CMV DRM panel was used for validation. Subsequently, a further 37 samples from transplant, haematological oncology, HIV- infected and clinician-referred patients with possible ganciclovir resistance were evaluated.

RESULTS:

6/6 UL97 mutations were correctly identified in 5/5(100%) QCMD samples, 3/30 (10%) of transplant/haematological oncology and 2/7 (28.57%) of clinician-referred patients. Five mutations from 5/37(13.52%) individuals were identified, namely L595S, H520Q, C592G, M460V and D605E. Noteworthy, the H520Q mutation was detected in a 2-year-old child with pre-B Acute Lymphocytic Leukemia who demised. However, despite the presence of L595S mutation another 3-year-old child with myelodysplasia post-stem cell transplantation subsequently suppressed on ganciclovir.

CONCLUSIONS

CMV DRM to ganciclovir may be more prevalent than initially suspected. The prevalence and clinical outcomes of these mutations needs to be better documented.

IV:

ANALYSIS OF CYTOMEGALOVIRUS UL97 DRUG RESISTANCE MUTATIONS



Nokwazi Nkosi, Heidi Smuts, Stephen Korsman, Diana Hardie, Nei-yuan Hsiao
 Division of Medical Virology, NHLS Groote Schuur Hospital, University of Cape Town, South Africa

Introduction

The development of Cytomegalovirus (CMV) drug resistance mutations (DRM) as a result of ganciclovir use, have been widely reported in the international literature, particularly in the post-transplant setting. The incidence of CMV drug resistance mutations based on international publications have been estimated at 5-10% in Solid Organ transplant recipients, 0-4% in haematopoietic stem cell transplant recipients after 3 months of drug exposure.¹ In HIV/AIDS patients the incidence has declined from >20% to <5% in the post antiretroviral era.² Ongoing viral replication in the presence of impaired host defences, inadequate antiviral drug activity and prolonged drug exposure lead to evolution of mutations over time eventually rendering antiviral therapy ineffective.

Due to the lack of access to established assays locally, the prevalence of CMV DRM in South Africa is currently unknown. In this study, we set up an in-house assay to document the prevalence of CMV UL97 DRM amongst immune-suppressed populations in South Africa.

Objectives

- Describe the prevalence of CMV UL97 DRM in at-risk populations i.e. transplant recipients, HIV infected patients who have been treated with Ganciclovir and have unsuppressed CMV Viral Load (CMVVL).
- Describe the clinical, laboratory profiles and where possible clinical outcomes of patients with CMV UL97 DRM.

Methods

A published set of primers was used to amplify a 707 bp region of UL97 gene for Sanger sequencing.³

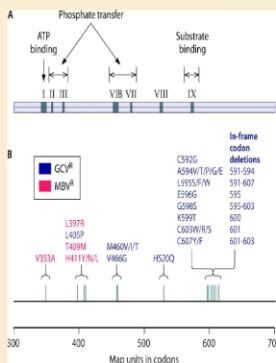


Fig. 1 Map of the kinase subdomain of the UL97 gene.
 (A) Conserved functional regions of the UL97 kinase (pUL97). Codons defined for each region are as follows: region I, 335 to 345; region II, catalytic lysine 355; region III, glutamate 380; region VIB, 453 to 462; region VII, 481 to 483; region VIII, 520 to 527; and region IX, 574 to 579 (34, 89). (B) Confirmed mutations are color coded by resistance profile. Multiple mutations at a single codon are listed in order of frequency. Map units correspond to codons of UL97.¹

A Quality Control for Molecular Diagnostics (QCMD) CMV DRM panel was used for validation. Subsequently, routine CMV viral load samples with detectable viral load were screened for CMV DRM. Further testing was done on patients who failed to respond to intravenous ganciclovir therapy. They will be described separately in the results. The types of CMV DRM will be described jointly. 37 samples from at-risk populations were tested. Sequence analysis for detection of UL97 mutations was performed on an online database available at: <http://www.informatik.uniulm.de/nim/mtarbeiter/HKestler/hcmv/>

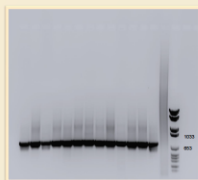


Fig. 2 Agarose gel electrophoresis to confirm adequate PCR product prior to Sanger sequencing. Standard quality control measures undertaken.

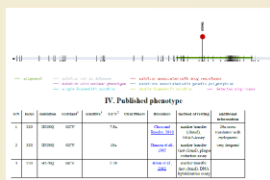


Fig.3 Mutation Resistance Analyzer database result output for Sample 0800. The report indicates alignment of sequence with TB40-BAC 4 UL97 gene. Mutations are identified and details on published phenotypes are provided.

Results

9/9 (100%) UL97 mutations from the 5 samples in the QCMD panel were identified (Fig. 7). Overall, 30 routine CMV viral load samples and 7 samples from suspected GCV resistant patients were tested. They yielded 3 (10%) and 2 (28.57%) UL97 DRM respectively. The majority of samples were from the haematological oncology group (Fig. 4). Overall mutations detected by category are demonstrated in Figure 5.

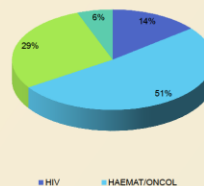


Fig. 4 Sample representation by Risk /Diagnostic Category. SOT indicates Solid Organ Transplant.

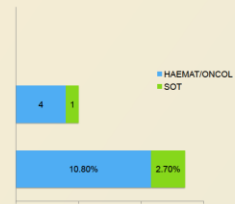


Fig. 5 Proportion of UL97 Mutations detected by Risk/Diagnostic Category

5/37 (13.52%) CMV UL97 mutations were identified in the tested samples. The L595S mutation was identified in a 3 year old child who subsequently suppressed on Ganciclovir. The H520Q mutation was identified in a 2 year old child diagnosed with pre-B Acute Lymphocytic Leukemia and increasing CMVVL who demised.

Table 1. UL97 Mutations Detected and effects on ganciclovir* (based on report from University of Ulm HCMV online database)

IDENTIFIER	AGE	DIAGNOSIS	CMVVL (cps/ml)	UL97 MUTATION	GCV
0800	2	PRE-B ALL	11 950(4.08)	H520Q	RESISTANT
7947	3	MYELODYSPLASIA STEM-CELL TRANSPLANT	271 900 (5.43)	L595S	RESISTANT 4.9-8.5x
5286	23	RENAL TRANSPLANT	32 350 (4.51)	C592G	RESISTANT 2.3-3.9x
1379	42	MULTIPLE MYELOMA	4100 (3.61)	M460V	RESISTANT 5.5-9.8x
4773	38	AML	11 950 (4.08)	D605E	SENSITIVE 0.6-0.9x

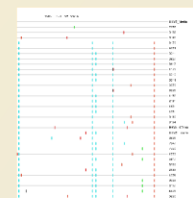


Fig. 6 Highlighter plot of external quality assurance panel and patient sequences indicating base mismatches in comparison to wild type (WT) HHV5 AD169. The consensus sequence (CS) was generated from all participants. An empty box in the consensus sequence column indicates agreement with WT HHV5 AD169.

Fig. 7 QCMD report indicating Kinase (UL97) drug mutation codons identified. In comparison to Wild type (WT) HHV5 AD169. The consensus sequence (CS) was generated from all participants. An empty box in the consensus sequence column indicates agreement with WT HHV5 AD169.

Conclusion

Our assay was able to detect various UL97 DRM among QCMD panel and clinical samples. The prevalence of CMV UL97 Drug resistance mutations in the haematological/oncology patients was 10.8%(4/37) and 2.7%(1/37) in the solid organ transplant patients. No mutations were detected in the HIV infected patients possibly due to shorter treatment durations. The prevalence of these mutations needs to be further documented on a wider scale. Preliminary findings suggest that haematopoietic stem cell transplant patients have a higher risk/prevalence. This may also reflect varying ganciclovir prescribing clinical practices in the different risk groups. Clinical outcomes in the presence of these mutations is dependent on multiple variables and needs to be documented.

References

- Antiviral Drug Resistance of Human Cytomegalovirus. Lurain NS. Chou S. Clin Micro Rev Oct 2010;689-712
- Change over time in Incidence of Ganciclovir Resistance in Patients with CMV Retinitis. Martin BK et al. Clin Infect Dis 2007;44,1001-1008
- Comparison of CMV UL97 Gene Sequences in the Blood and Vitreous of Patients with AIDS and CMV retinitis. Huijing Hu et al. JID2002;185,861-867.

Acknowledgements: We would like to thank the staff in the C16 Virology Manual Molecular Laboratory Department, Dr. H Rabie, Prof. W Preiser, Dr. J Maritz, Prof. B Eley and the Red Cross Children's Hospital IDC Unit for their contribution. Contact details: nokwazi.nkosi@nhls.ac.za, marvin.hsiao@nhls.ac.za

V:

MRA drug resistance mutations



I. General information

Report date: 2017-11-01	
Patient: 7947NZ PATIENT A	Study ID: 7947NZ PATIENT A
Birth date:	Viral load: 271 900(log 5.43)
Sample received:	Sample collected:
ID: 7947NZ	Sample type: Whole Blood
Current Therapy: Valganciclovir	Manifestation: Myelodysplasia
Physician:	Reported by: N. Nkosi

II. Mutations (Substitutions, Insertions and Deletions)

UL97: L595S

III. Alignmentgraph



IV. Published phenotype

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
1	595	L595S	GCV		8.5x		Chou and Bowlin, 2010	marker transfer (clonal); SEAP-Assay	no cross resistance with cyclopravir
2	595	L595S	GCV		8.6x		Drew et al., 2006	marker transfer (not clonal); SEAP assay	
3	595	L595S	GCV		9.2x		Chou et al., 2005	marker transfer (not clonal); SEAP assay	very frequent
4	595	L595S	GCV		4.9x		Chou et al., 2002	marker transfer (not clonal); plaque reduction assay	rather rare
5	595	L595S	GCV		EC50 32.9 µM		Limaye et al., 2000	clinical isolate; plaque reduction assay	isolate contained also UL97 mutation H469T; no EC50 ratios available
6	595	L595S	GCV		EC50 60.3 µM		Limaye et al.,	clinical isolate;	isolate contained

MRA drug resistance mutations

<u>S/N</u>	<u>locus</u>	<u>mutation</u>	<u>resistant as stated by the respective authors</u>	<u>sensitive as stated by the respective authors</u>	<u>Ganciclovir (GCV)</u>	<u>viral fitness</u>	<u>literature</u>	<u>method of testing</u>	<u>additional information</u>
							2000	plaque reduction assay	also UL97 mutation G623S no EC50 ratios available
7	595	L595S	GCV		EC50 29 µM / 25 µM		Wolf et al., 1995	clinical isolate; plaque reduction assay	no EC50 ratios available
8	595	L595S	GCV		EC50 8 µM		Eckle et al., 2000	clinical isolate; plaque reduction assay	no EC50 ratios available
9	595	L595S	GCV		EC50 14.0 µM		Limaye et al., 2000	clinical isolate; plaque reduction assay	isolate contained also UL97 mutation A588P; no EC50 ratios available
10	595	L595S	GCV		EC50 14.4µM		Smith et al., 1997	clinical isolate; plaque reduction assay	no EC50 ratios available

01/11/2017
date

N. Nkosi
signature

MRA drug resistance mutations

I. General information

Report date: 2017-11-01	
Patient: 0800 TJG PATIENT B	Study ID: 0800 TJG PATIENT B
Birth date:	Viral load: 11950 cps/ml (log 4.08)
Sample received:	Sample collected:
ID: 0800TJG	Sample type: Whole Blood
Current Therapy: Ganciclovir	Manifestation: Pre-B Acute Lymphocytic Leukemia
Physician:	Reported by: N. Nkosi

II. Mutations (Substitutions, Insertions and Deletions)

UL97: H520Q

III. Alignmentgraph



IV. Published phenotype

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
1	520	H520Q	GCV		7.8x		Chou and Bowlin, 2010	marker transfer (clonal); SEAP-Assay	20x cross resistance with cyclopravir
2	520	H520Q	GCV		10x		Hanson et al., 1995	marker transfer (not clonal); plaque reduction assay	very frequent
3	520	H520Q	GCV		5.2x		Biron et al., 2002	marker transfer (not clonal); DNA hybridisation assay	

01/11/2017

date

N. Nkosi

signature

MRA drug resistance mutations

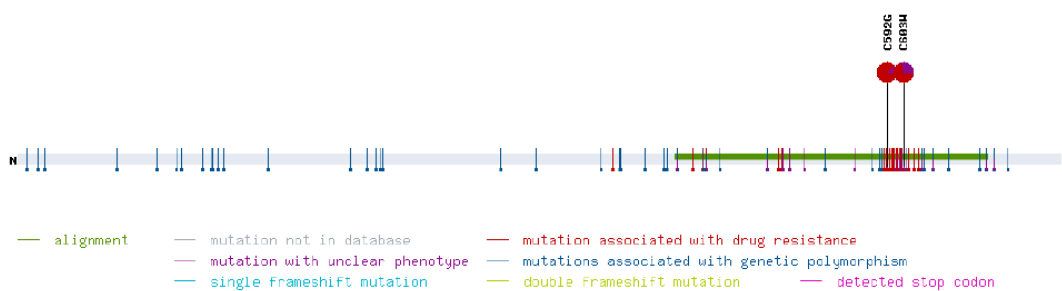
I. General information

Report date: 2017-11-01	
Patient: 1891MN PATIENT C	Study ID: 1891MN PATIENT C
Birth date:	Viral load: 8114 (log 3.9)
Sample received:	Sample collected:
ID: 1891MN	Sample type: Whole blood
Current Therapy: Valganciclovir	Manifestation: Wiskott Aldrich Syndrome
Physician:	Reported by: N. Nkosi

II. Mutations (Substitutions, Insertions and Deletions)

UL97: C592G, C603W

III. Alignmentgraph



IV. Published phenotype

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
1	592	C592G	x		2.9x		Chou et al. 2017	reporter (SEAP)-based yield reduction assay (clonal)	
2	592	C592G	GCV		3.2x		Chou et al. 2010	marker transfer (clonal); SEAP-Assay	
3	592	C592G	GCV		3.0x		Chou et al. 2010	marker transfer (clonal); SEAP-Assay	
4	592	C592G	GCV		3x		Chou and Bowlin. 2010	marker transfer (clonal); SEAP-Assay	no cross resistance with cyclopravir
5	592	C592G	GCV		2.9x		Chou et al., 2005	marker transfer (not clonal); SEAP assay	very frequent; selected when virus exposed to low GCV concentrations; continuation of

MRA drug resistance mutations

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
									GCV therapy is discouraged by the authors
6	592	C592G	GCV		3.9x		Drew et al., 2006	marker transfer (not clonal); SEAP assay	
7	592	C592G	GCV		2.3x		Iwasenko et al., 2009	marker transfer (not clonal); SEAP assay	
8	592	C592G	GCV		2.5x		Chou et al., 2002	marker transfer (not clonal); plaque reduction assay	
9	592	C592G			EC50 10.7µM		Smith et al., 1997	clinical isolate; plaque reduction assay	only found in GCV resistant isolates; isolate contained also UL54 mutations P628L, S655L, A885T; no EC50 ratios available
10	603	C603W	GCV		7.9x		Chou and Bowlin, 2010	marker transfer (clonal); SEAP-Assay	no cross resistance with cyclopravir
11	603	C603W	GCV		8.3x		Chou et al., 2010	marker transfer (clonal); SEAP-Assay	
12	603	C603W	GCV		7.9x		Chou et al., 2010	marker transfer (clonal); SEAP-Assay	
13	603	C603W	GCV		8x		Chou et al., 1997	marker transfer (not clonal); plaque reduction assay	very frequent
14	603	C603W	GCV		EC50 13 / 9.8 µM		Eckle et al., 2000	clinical isolate; plaque reduction assay	isolate contained also UL97 A594V; no information on ratio
15	603	C603W			EC50 9.3 µM		Smith et al., 1997	clinical isolate; plaque reduction assay	only found in GCV resistant isolates; no EC50 ratios available
16	603	C603W			EC50 16.6 µM		Smith et al., 1997	clinical isolate; plaque reduction assay	only found in GCV resistant isolates; no EC50 ratios available

01/11/2017
date

N. Nkosi
signature

MRA drug resistance mutations

I. General information

Report date: 2017-11-01	
Patient: 1379GTP PATIENT D	Study ID: 1379GTP PATIENT D
Birth date:	Viral load: 4100 (log 3.61)
Sample received:	Sample collected:
ID: 1379GTP	Sample type: Whole blood
Current Therapy:	Manifestation: Multiple Myeloma
Physician:	Reported by: N. Nkosi

II. Mutations (Substitutions, Insertions and Deletions)

UL97: M460V

III. Alignmentgraph



IV. Published phenotype

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
1	460	M460V	GCV		9.3x		Chou et al. 2010	marker transfer (clonal); SEAP-Assay	
2	460	M460V	GCV		8.3x		Chou and Bowlin, 2010	marker transfer (clonal); SEAP-Assay	no cross resistance with cyclopravir
3	460	M460V	GCV		8.6x		Chou et al. 2010	marker transfer (clonal); SEAP-Assay	
4	460	M460V	GCV		9.8x		Chou et al. 2005	marker transfer (not clonal); SEAP assay	very frequent; together with D605E 9.8x
5	460	M460V	GCV		8.2x		Drew et al. 2006	marker transfer (not clonal); SEAP assay	
6	460	M460V	GCV		7.5x		Marfori et al. 2007	marker transfer (not clonal); SEAP assay	

MRA drug resistance mutations

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
7	460	M460V	GCV		5.5x		Biron et al., 2002	marker transfer (not clonal); DNA hybridisation assay	
8	460	M460V	GCV		EC50 14.2 µM		Smith et al., 1997	clinical isolate; plaque reduction assay	no EC50 ratios available
9	460	M460V	GCV		EC50 12.4 µM		Smith et al., 1997	clinical isolate; plaque reduction assay	isolate also contained also UL54 mutation S655L, N685S, G874R, A885T, N898D; no EC50 ratios available

01/11/2017
date

N. Nkosi
signature

MRA drug resistance mutations



I. General information

Report date: 2017-11-01	
Patient: 4733SV PATIENT E	Study ID: 4733SV PATIENT E
Birth date:	Viral load: 11950(log 4.08)
Sample received:	Sample collected:
ID: 4773SV	Sample type: Cerebrospinal Fluid
Current Therapy:	Manifestation: Acute Myeloid Leukemia
Physician:	Reported by: N. Nkosi

II. Mutations (Substitutions, Insertions and Deletions)

UL97: D605E

III. Alignmentgraph



IV. Published phenotype

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
1	605	D605E		GCV	0.9x		Chou et al., 2005	marker transfer (not clonal); SEAP assay	
2	605	D605E		GCV	0.6x		Ijichi et al., 2002	marker transfer (vaccinia system, not clonal), plaque reduction assay	confers hypersusceptibility; partially reconstitutes for GCV resistance mutation A594P
3	605	D605E		GCV			Tanaka et al., 2011	sequence data	polymorphism; possible genetic marker for MRA in East Asian countries
4	605	D605E					Reddy et al., 2007	sequence data	
5	605	D605E					Hu et al., 2002	sequence data	

01/11/2017
date

N. Nkosi
signature

MRA drug resistance mutations



I. General information

Report date: 2017-11-01	
Patient: 8471SJG PATIENT F	Study ID: 8471SJG PATIENT F
Birth date:	Viral load: 12650 (log 4.1)
Sample received:	Sample collected:
ID: 8471SJG	Sample type: Whole blood
Current Therapy:	Manifestation: Chronic Myeloid Leukemia
Physician:	Reported by: N. Nkosi

II. Mutations (Substitutions, Insertions and Deletions)

UL97: Q449K

III. Alignmentgraph



IV. Published phenotype

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
1	449	Q449K		GCV			Lurain et al., 2001	sequence data	
2	449	Q449K					Hu et al., 2002	sequence data	phenotype unclear

01/11/2017
date

N. Nkosi
signature

MRA drug resistance mutations

I. General information

Report date: 2017-11-01	
Patient: 5349LX PATIENT G	Study ID: 5349LX PATIENT G
Birth date:	Viral load: 1064(log 3.00)
Sample received:	Sample collected:
ID: 5349LX	Sample type: Whole Blood
Current Therapy:	Manifestation: Acute Myeloid Leukemia
Physician:	Reported by: N. Nkosi

II. Mutations (Substitutions, Insertions and Deletions)

UL97: D605E

III. Alignmentgraph



IV. Published phenotype

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
1	605	D605E		GCV	0.9x		Chou et al., 2005	marker transfer (not clonal); SEAP assay	
2	605	D605E		GCV	0.6x		Ijichi et al., 2002	marker transfer (vaccinia system, not clonal), plaque reduction assay	confers hypersusceptibility; partially reconstitutes for GCV resistance mutation A594P
3	605	D605E		GCV			Tanaka et al., 2011	sequence data	polymorphism; possible genetic marker for MRA in East Asian countries
4	605	D605E					Reddy et al., 2007	sequence data	
5	605	D605E					Hu et al., 2002	sequence data	

01/11/2017
date

N. Nkosi
signature

MRA drug resistance mutations

I. General information

Report date: 2017-11-01	
Patient: 5286KK PATIENT H	Study ID: 5286KK PATIENT H
Birth date:	Viral load: 32 350 (log 4.51)
Sample received:	Sample collected:
ID: 5286KK	Sample type: Whole blood
Current Therapy:	Manifestation: Renal Transplant
Physician:	Reported by: N. Nkosi

II. Mutations (Substitutions, Insertions and Deletions)

UL97: C592G

III. Alignmentgraph



IV. Published phenotype

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
1	592	C592G	x		2.9x		Chou et al. 2017	reporter (SEAP)-based yield reduction assay (clonal)	
2	592	C592G	GCV		3x		Chou and Bowlin, 2010	marker transfer (clonal); SEAP-Assay	no cross resistance with cyclopravir
3	592	C592G	GCV		3.2x		Chou et al. 2010	marker transfer (clonal); SEAP-Assay	
4	592	C592G	GCV		3.0x		Chou et al. 2010	marker transfer (clonal); SEAP-Assay	
5	592	C592G	GCV		2.3x		Iwasenko et al. 2009	marker transfer (not clonal); SEAP assay	
6	592	C592G	GCV		3.9x		Drew et al. 2006	marker transfer (not clonal); SEAP	

MRA drug resistance mutations

S/N	locus	mutation	resistant as stated by the respective authors	sensitive as stated by the respective authors	Ganciclovir (GCV)	viral fitness	literature	method of testing	additional information
								assay	
7	592	C592G	GCV		2.9x		Chou et al., 2005	marker transfer (not clonal); SEAP assay	very frequent; selected when virus exposed to low GCV concentrations; continuation of GCV therapy is discouraged by the authors
8	592	C592G	GCV		2.5x		Chou et al., 2002	marker transfer (not clonal); plaque reduction assay	
9	592	C592G			EC50 10.7µM		Smith et al., 1997	clinical isolate; plaque reduction assay	only found in GCV resistant isolates; isolate contained also UL54 mutations P628L, S655L, A885T; no EC50 ratios available

01/11/2017

date

N. Nkosi

signature

