CYTOMEGALOVIRUS INFECTIONS IN SOLID ORGAN TRANSPLANT RECIPIENTS

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To my family – here and there – this is for you.

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LIST OF ABBREVIATIONS

AECA	anti-endothelial cell antibodies
AIDS	acquired immunodeficiency syndrome
ATG	ani-thymocyte globulin
BMT	bone marrow transplantation
Bp	base pair(s)
CAN	chronic allograft nephropathy
CI	confidence interval
CMV	cytomegalovirus
CNS	central nervous system
CTL	cytotoxic T lymphocyte
DABCYL	4-[4'-dimethylaminophenylazo]benzoic acid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EBV	Epstein-Barr virus
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FAM	carboxy-fluorescein
gB	glycoprotein B
GCV	ganciclovir
HAART	highly active antiretroviral therapy
HEX	hexachlorofluorescein
HHV	human herpesvirus(es)
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV	herpes simplex virus
IC_{50}	50% inhibitory concentration

ICAM-1	intercellular adhesion molecule-1
IE	immediate early
IFN	interferon
IL	interleukin
kbp	kilobase pair(s)
MFF	mycophenolate mofetil
MHC	major histocompatability complex
mL	milliliter
mM	millimolar
mRNA	messenger RNA
NASBA	nucleic acid sequence-based amplification
NCBI	National Center for Biotechnology Information
nm	nanometer
NUH	National University Hospital
NUS	National University of Singapore
OKT3	anti-CD3 monoclonal antibodies
OR	odds ratio
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pp65	phosphoprotein pp65 (UL83)
PRA	plaque reduction assay
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RR	risk ratio
RT-PCR	reverse transcriptase PCR
SGH	Singapore General Hospital
TGF	transforming growth factor
TNF	tumor necrosis factor

UL	unique long
VCAM-1	vascular cell adhesion molecule-1
µL	microtiter
µM	micromolar
US	unique short
VZV	varicella zoster virus

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SUMMARY

Human cytomegalovirus (CMV) is associated with serious infections in immunocompromised hosts such as organ transplant recipients. Prolonged ganciclovir therapy for CMV infection and disease often results in the development of ganciclovir-resistant strains. This thesis has addressed several issues relating to the clinical challenges that CMV infections pose to paediatric solid organ transplant recipients from a Singapore perspective.

We hypothesized that that the high incidence of CMV diseases (28.6%) in the National University Hospital's paediatric solid organ transplant recipients – despite the use of ganciclovir prophylaxis – was due to ganciclovir-resistant CMV mutant strains. We sought to determine the frequency of CMV mutant strains recovered in both transplant and non-transplant populations.

As antiviral susceptibility testing was not performed in Singapore before, we assessed the feasibility of available laboratory techniques; using PCR-based restriction analysis, DNA sequencing and plaque reduction assay, we screened both clinical isolates of CMV and samples obtained from pediatric renal and liver transplant

recipients for ganciclovir-resistance related mutations in the CMV viral genome. Ganciclovir resistance was detected in 25% of renal and 40% of liver transplant recipients. We were also the first to document a case of laboratory confirmed ganciclovir resistant CMV infection in Singapore – in a paediatric renal transplant recipient with clinically confirmed CMV disease and allograft dysfunction.

Our data indicate that laboratory screening for antiviral resistance is warranted. We fine-tuned current genotypic screening methods by developing several PCR-based assays (PCR-RFLP, discriminatory PCR and probe-based real time PCR) for detection of specific mutations in both the UL97 and UL54 genes of CMV that were rapid and which could be utilized directly on clinical samples. Patients who harbored ganciclovir-resistant CMV strains may also contain wild type viruses so these new assays were designed to simultaneously detect wild type and mutant sequences.

Risks and trends in CMV infections have implications for patient management strategies and treatment outcomes. We sought to define the role of CMV infection in chronic allograft dysfunction in prospective study of 119 consecutive renal transplant patients at the two major transplant centers (National University Hospital and Singapore General Hospital) in Singapore. Both univariate and

multivariate analyses of clinical data revealed a significant correlation between allograft dysfunction and presence of CMV DNAemia at five months post-transplant ($P< 0.01$; OR 3.578, 95% CI 1.417-0.031). Our data provides argument that current strategies to improve long term outcomes after renal transplantation should also include serial post-transplant assessment of CMV infection using PCR amplification of viral DNA in sera. We also discovered that at five months post-transplant, presence of IgG specific anti-endothelial cell antibody (AECA) activity was significantly associated with CMV DNAemia ($P < 0.01$) although there was no direct correlation between AECA activity and abnormal renal function at one year post-transplant. We recommend further studies via histopathological assessment of donor kidneys to establish whether or not CMV-induced endothelial damage leads to the production of AECA and subsequent immune injury to the renal allograft.

1 INTRODUCTION AND OBJECTIVES OF THE THESIS

The human cytomegaloviruses (CMV) are ubiquitous herpesviruses, found universally throughout all geographic locations and socioeconomic groups. CMV resides in the host throughout life without causing any symptoms in healthy, immunocompetent individuals, and 50–90% of the population have become seropositive by adulthood (Scholz et al, 2003).

They are responsible for generally asymptomatic and persistent infections in healthy people. While inapparent infection is common during childhood and adolescence, severe disease frequently occurs in the absence of an effective immune response, as in immunologically immature and immunocompromised individuals.

CMV has significant impact on certain high-risk groups. Of concern is the risk of infection to the unborn baby during pregnancy and the risk of infection to immunocompromised persons, such as organ transplant recipients and persons infected with human immunodeficiency virus (HIV).

This chapter provides an overview of the general characteristics of the virus, as well as the pathogenesis, epidemiology, and diagnosis of infections associated with human CMV.

1.1 Properties of the virus

1.1.1 Virus structure and genome

CMV is designated human herpesvirus 5 (HHV-5) in the herpesviridae family, which includes herpes simplex virus types (HSV) 1 and 2, Epstein-Barr virus, varicella-zoster virus (VZV), and human herpesvirus 5, 6, 7 and 8.

Its virion structure, kinetics of viral gene expression, and persistence of the lifetime of their host are typical of the herpesviruses. As a member of the betaherpesvirinae subfamily, it is slow growing, has a propensity for massive enlargement of infected cells and becomes latent in secretory glands and kidneys.

The virion consists of a 100 nm diameter icosahedral nucleocapsid containing a 230 kbp, double-stranded linear DNA genome, surrounded by an envelope that is derived from the nuclear membrane

of the infected cell and contains viral glycoproteins. An amorphous proteinaceous layer between the capsid and envelope is termed the tegument or matrix. The enveloped form measures 150-200 nm.

The CMV genome is the largest of all herpesviruses, and, as with all herpesvirus DNAs, possesses unique terminal and internal repeated sequences. The laboratory CMV AD169 strain has been the best studied and was the first strain to be completely sequenced CMV. Analysis of its genome has shown that it encodes 225 Open Reading Frames (ORFs) of approximately 100 or more amino acids (Chee et al, 1990; Novotny et al, 2001). The Towne and Toledo laboratory strains also contain additional ORFs (Cha et al, 1996). Sequence homology searches and experimental biochemical and/or genetic studies have assigned functional roles to only some of the more than 200 CMV ORFs (Novotny et al, 2001). Many ORFs await functional characterization, and their role in infection, including dissemination, growth in target tissues and pathogenesis, and in counteracting host immune response is yet to be elucidated (Mocarski and Courcelle, 2001).

More than 200 proteins are produced in three overlapping phases (immediate early (IE), early, and late). The predominant proteins critical for virion production are envelope proteins gB, gH, gM, and

gL and the matrix proteins pp65/pp150/pp71 and pp28 (Landolfo et al, 2003).

The human CMV genome contains a single origin of replication and encodes a DNA polymerase gene and a complete set of genes needed for its own DNA replication. Current therapies for CMV disease inhibit viral DNA polymerase as the final target. CMV DNA polymerase is encoded by a CMV ORF designated UL54.

The CMV genome also encodes a protein phosphotransferase enzyme, the product of UL97, but its role in CMV DNA replication is not well elucidated (Chee et al, 1989). Recent work has shown that this phosphotransferase enzyme is able to phosphorylate serine residues (He et al, 1997), as well as to phosphorylate ganciclovir to form ganciclovir monophosphate, necessary for the drug to become an effective inhibitor of CMV DNA replication (Sullivan et al, 1992; Littler et al, 1992). Krosky et al (2003b) have also identified UL44 as the natural substrate of UL97 in infected cells. Genetic and pharmacological evidence, provided by experiments using a novel antiviral drug (maribavir), indicate that UL97 is required at the stage of infection when nucleocapsids exit from the nucleus (nuclear egress) (Krosky et al, 2003a).

1.1.2 Virus growth cycle

CMV is very species-specific and cell type-specific. While a number of animal CMVs exist, all of them are species-specific; likewise, all attempts to infect animals with human CMV have failed.

Laboratory strains of CMV replicates *in vitro* only in human skin or lung fibroblasts, whereas clinical isolates replicate preferentially on endothelial cell cultures. CMV replicates very slowly in cultured cells, with growth proceeding more slowly than that of herpesvirus such as HSV or VZV. Very little virus becomes cell-free; infection is spread primarily cell-to-cell. It usually takes several weeks for an entire monolayer to become entirely infected.

CMV produces a characteristic cytopathic effect marked by cell rounding and enlargement with pronuclear cytoplasmic inclusions in addition to the intranuclear inclusions typical of herpesviruses. The presence of this cytomegalic inclusion cell in clinical specimens is one of the classic hallmarks of CMV infection. These massively enlarged cells (the property of cytomegaly from which CMV acquires its name) contain intranuclear inclusions, which histopathologically have the appearance of owl's eyes. The presence of these cells

indicates productive infection, although they may be absent even in actively infected tissues.

The virus, however, is often isolated from a wide range of epithelial cells of the host. The ductal epithelial cell is most frequently infected, and develops a typical cytopathology (Pass, 2001). During natural infection, CMV replicates productively, in addition to epithelial cells, in endothelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, granulocytes, and monocyte-derived macrophages (Pass, 2001; Landolfo et al, 2003). Tissue types from which CMV has been isolated include the parenchymal organs, salivary glands, eye, gastrointestinal and genitourinary tract (Pass, 2001). The cellular receptor of CMV has yet to be identified but is thought to be widely distributed owing to the wide range of cells that the virus is able to infect (Mocarski and Courcelle, 2001). Candidate receptors include CD13 surface molecules found on peripheral blood mononuclear cells (Larsson et al, 1998), cellular integrins (Feire et al, 2004) and epidermal growth factor receptor (Wang et al, 2003)

Infection leads to a co-coordinated sequence of events which results in to the synthesis of IE, early and late viral proteins (Mocarski and Courcelle, 2001). After primary infection CMV establishes lifelong latency or persistence within the person, in which cells of the myeloid

lineage are an important reservoir. Presence of the virus in a subset of CD34+ myeloid progenitor cells in bone marrow has been established, with a small proportion of these cells containing CMV genomic DNA without detectable viral IE gene expression, termed latent infections (Mocarski and Courcelle, 2001). In healthy carriers, viral DNA is also present in a small proportion of CD14+ monocytes and in dendritic cells and megakaryocytes (Taylor-Wiedeman et al, 1991; Crapnell et al, 2000).

1.2 Pathogenesis and pathology

1.2.1 Immunocompetent hosts – infections and immune responses

CMV may be transmitted person-to-person in several different ways, all requiring close contact with virus-bearing material. There is a four to eight week incubation period in normal older children and adults following viral exposure. The virus causes a systemic infection – it has been isolated from lung, liver, esophagus, colon, kidneys, monocytes, and lymphocytes. The disease is an infectious mononucleosis-like syndrome, although most infections are asymptomatic. The unsuspecting host is thus able to spread the virus both vertically and horizontally. For example, asymptomatic infected

children excrete CMV in their urine for several months and, from this source, the virus is able to spread rapidly in environments such as day-care centers (Weller, 2000).

Virus can appear following primary infection, reinfection, or reactivation. Following infection, the virus is excreted in body fluids (urine, saliva, tears, semen, breast milk and cervical secretions) for months to years, probably due to virus replication in glandular epithelial cells, accompanied by virus release into excretions. However, the levels of shedding and reactivation of the virus vary among individuals (Ling et al, 2003).

Like all herpesviruses, CMV establishes lifelong latent infections. Cells in bone marrow and peripheral blood are the chief reservoirs for latent CMV infection. CMV DNA is found in a small percentage of peripheral blood monocytes, and gene expression is limited to the early or E genes. It has been proposed that bone marrow precursors of blood monocytes are the site of viral latency and provide a means of dissemination upon differentiation into circulating monocytes (Pass, 2001). Differentiation of latently infected monocytes in macrophages leads to reactivation and productive infection.

Recurrent infections may consist of either reactivation of the virus strain causing primary infection or reinfection by a new virus strain. Recurrent infection can be defined as indefinite, but intermittent, excretion of the virus from single or multiple sites. It should be distinguished from prolonged excretion typical of primary infection and also infection in the immunocompromised host (Pass 2001), where productive infection, as measured by viral excretion, is markedly increased.

Cell mediated immunity is depressed with primary infections, and this may contribute to the persistence of viral infection. It may take several months for cellular responses to recover.

CMV cellular reservoirs are leukocytes, epithelial cells of salivary glands, and cervix. Infectious CMV may be shed in body fluids of infected persons, and may be detected in urine, saliva, blood, tears, semen, and breast milk. Examination of organ tissues and of peripheral blood obtained from patients with CMV disease has suggested that peripheral blood mononuclear cells (PBMC) are also a viral reservoir, and further analyses of PBMC revealed monocytes as the predominant infected cell type (Sholz et al, 2003).

T cells are crucial for the control of CMV in infected individuals. More specifically, CMV is thought to be controlled by antigen-specific antiviral CD8 T cells. However, reactivation of CMV occurs often in certain high-risk groups such as immunocompromised hosts, and also in asymptomatically healthy individuals. A series of mechanisms have been proposed to be responsible for CMV reactivation. These include stress (through catecholamine using the cAMP system), inflammation (through tumor necrosis factor (TNF)-α using nuclear factor κβ or through prostaglandins using the cAMP pathway), and some cAMP-elevating drugs. This results in the activation of the CMV IE enhancer/promoter, which is responsible for initiation of virus replication (Reinke et al, 1999).

The high frequency of CMV-specific effector CD8 T cells found in healthy individuals indicates that CMV is more frequently reactivated than previously expected (Reinke et al, 1999) but reactivation remains unnoticed and asymptomatic. This is in contrast to most of the situations of reactivation associated with clinical CMV diseases which occur after transplantation or in immunocompromised hosts. Reactivation of CMV from latency results in serious morbidity and mortality in immunocompromised transplant recipients or immunodeficient individuals and has both direct and indirect effects.

The loss of immune control of CMV that is evidenced by the detection of antigenemia is closely associated with an impaired function of CMV-specific CD8 T cells. In fact, it is the reduced cytokine production rather than a lower frequency or absolute number of CMV-specific CD4 or CD8 T cells that is thought to be responsible for the loss of immune control. Reduced numbers of cytokine-producing CMV-specific CD8 T cells were found in individuals with a higher risk of CMV reactivation. The highest frequencies and absolute numbers of CMV-specific CD8 T cells were noted in those subjects who experienced early or late CMV reactivation. CMV-specific cytotoxic T lymphocyte (CTL) responses are generally lost in subjects undergoing allogeneic bone marrow transplantation (BMT) and restoration of those responses requires an extended period (Reusser et al, 1991).

A series of genes are directly involved in these mechanisms of immune evasion (Ploegh, 1998). The primary target of the proteins encoded by these genes is the class I antigen processing pathway: the unique short (US) region 3, which is expressed in the IE phase binds to and retains major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum (ER). US2 and US11, both early gene products, cause the translocation of MHC class I to the cytosol where it is degraded. US6 blocks the transport of antigen-

peptide into the ER. CMV protein US2 contributes to the degradation of human leukocyte antigen (HLA)-Drα and HLA-Dmα through the inhibition of class II transactivator. The inhibitory effects on T-cell antigen recognition is active when viral or self-antigens are synthesized within the cells, but not if the specific epitope is given as a peptide (Ploegh, 1998). These strategies may explain the immunodominance of CTL directed against viral antigens that can be presented before expression of the US genes. In CMV infected cells, the expression of the viral phosphoprotein pp65 (pp65) inhibits the generation of CMV-specific T-cell epitopes (Ploegh, 1998).

1.2.2 Congenital and perinatal infections

Congenital CMV infection remains a major problem worldwide. The virus can be transmitted *in utero* as the consequence of either a primary or recurrent maternal infection (Stagno et al, 1980). The incidence of symptomatic congenital CMV infections in immune mothers has also been shown to be similar in primary and recurrent maternal infections (Boppana et al, 1999). In addition, symptomatic congenital infections appear to be mostly caused by reinfection of immune mothers during pregnancy by a new CMV strain (Boppana, et al, 2001). On the other hand, congenital infections following

reactivated maternal infection are mostly asymptomatic (Stagno et al, 1982).

CMV can also be acquired by the infant from exposure to virus in the mother's genital tract during delivery, and from maternal breast milk (Stagno, 2001). In these cases, the infants usually have received some maternal antibody, and the perinatally acquired CMV infections tend to be asymptomatic. Transfusion-acquired CMV infections in newborns will vary, depending on the amount of virus received and the serological status of the blood donor.

Fetal and newborn infections with CMV may be severe. Congenital intrauterine infections have been associated with congenital abnormalities, intrauterine growth deficiency and intrauterine death of the fetus, in addition to developmental delay, blindness and deafness of the infected child. The incidence of congenital infection has been estimated to be between 0.2 to 2% of all live births in different regions of the world. In a 1994 study of 1,688 infants with congenital abnormalities in Malaysia, 11.4% showed evidence of congenital CMV infections, higher than congenital toxoplasma (1%) or congenital rubella infection (3.7%) (Balasubramaniam et al, 1994).

1.2.3 Immunosuppressed hosts

Primary CMV infection is always followed by a prolonged, inapparent infection during which the virus remains alive but usually dormant and resides in cells without causing detectable damage or clinical illness. The occurrence of CMV disease is almost exclusively restricted to immunocompromised hosts.

Primary CMV infection in immunosuppressed or immunodeficient hosts is much more severe than in normal hosts. Individuals at greater risk for CMV disease are those receiving organ transplants, those with malignant tumors who are receiving chemotherapy, and those who are HIV-infected or with acquired immunodeficiency syndrome (AIDS). Viral excretion is increased and prolonged, and the infection is more apt to become disseminated. Pneumonia is the most common complication.

The host immune response presumably maintains the virus in a latent state in seropositive individuals. Reactivated infections are associated with disease much more often in the immunosuppressed patients than in normal hosts. Although usually less severe,

reactivated infections may be as virulent as primary infections. Long term immunosuppression can lead to uncontrolled replication and serious disease (Reinke et al, 1999).

In the immunocompetent host, the virus remains efficiently controlled and several components of the immune system are shown to play a role. In mice it has been demonstrated that both T and B cells play a role in the control of CMV (Scholz et al, 2003; Van Lier, 2003). In HIV and CMV co-infected patients, elevated and very stable CD4 and CD8 T-cell responses to CMV have been observed (Harari et al, 2003; Van Lier, 2003). The majority of CMV-specific CD8 T cells in peripheral blood were able to produce a range of antiviral factors after stimulation with specific antigens (IFN-γ, macrophage inflammatory protein-1β, TNF-α) (Van Lier et al, 2003). With regard to CD4 T cells, CMV-specific lymphoproliferation and interleukin (IL)-2 secreting CD4 T-cell responses were present in healthy subjects (Harari et al, 2003); however, deficient CD4 T-cell responses were reported in BMT recipients (Scholz et al, 2003).

1.3 Clinical findings associated with human cytomegalovirus infections

1.3.1 Infections in immunocompetent hosts

CMV infection in normal immunocompetent hosts is usually asymptomatic but occasionally causes a spontaneous infectious mononucleosis syndrome. CMV is estimated to cause 20-50% of non-Epstein-Barr virus (EBV) associated mononucleosis cases (Klemola and von Essen, 1970). The disease is characterized by malaise, myalgia, protracted fever, liver function abnormalities and non-specific constitutional symptoms, which may persist for weeks. The hematological hallmark is a relative lymphocytosis, in which greater than 50% of the peripheral white blood cell differential is composed of lymphocytes, of which, 10% or more are atypical lymphocytes. At the time of occurrence of the mononucleosis syndrome, a variety of cutaneous manifestations also occur. All these clinical symptoms are not the direct consequence of proliferation of CMV in given tissues but indicative of the immunological response toward CMV (Kano et al, 2000).

Complications are rare and include pneumonia, myocarditis, hemolytic anemia, retinitis, gastrointestinal ulceration, hepatitis, central nervous system (CNS) involvement (Guillain-Barre syndrome) and peripheral neuropathy.

1.3.2 Infections in the immunosuppressed host

CMV is an important opportunistic pathogen in immunocompromised patients or immunologically immature hosts. Primary infection, reactivation of latent virus, and reinfection are possible, and they cause a wide range of clinical manifestations, from asymptomatic infection to severe, potentially lethal disease. In organ transplant patients, three potential mechanisms of CMV infection have been recognized: transmission by the donor organ, blood products or reactivation of latent virus in the recipient (Zamora, 2004). Presence of antibody to CMV in the recipient, whether endogenous or passively transferred, provides partial protection against the development of serious and sometimes fatal disease. Absent endogenous antibody protection in the recipient results in primary CMV infections, particularly CMV pneumonitis or gastrointestinal disease, which may be quite severe with mortality rates of 2–20% (Valentine, 1995).

The major clinical diseases related to the types of immunocompromised host are summarized in Table 1.1.

Table 1.1 Clinical syndromes associated with cytomegalovirus infection in the immunocompromised host (adapted from Griffiths and Emery, 2002)

	Solid organ transplant	Bone marrow transplant	AIDS
Hepatitis	++	+	+
Retinitis	+	+	+
Pneumonitis	+	++	
Myelosuppression		+	
Encephalopathy			+
Polyradiculopathy			+
Immunosuppression	+		
Rejection/Graft versus host disease	+		
Atherosclerosis	+		
Death		+	+

+, disease occurs; ++ disease most common

1.3.2.1 Cytomegalovirus infections in transplant patients

All major transplantations (including kidney, liver, heart, heart-lung and bone marrow transplantation) are associated with an increased risk of CMV infection. A survey of 16 studies of 1276 patients showed that the rate of infection after renal transplantation had a range of 59 to 70% (Ho, 1991). The most important source for many patients is the transplanted organ or transfused blood. Secondary infection after activation of latent virus is also an important source (Ho, 1977).

The severity of end-organ disease caused by CMV is related to the degree of immune suppression and the type of transplant. With immune suppression the most severe in bone marrow transplantation, CMV disease is also the greatest in BMT recipients. Immunosuppressive therapy, and in particular, the use of very high doses of corticosteroids plus azathioprine, also play a major role in reactivation of CMV (Ho, 1977; Rubin, 1981). Preemptive therapy with mycophenolate mofetil, first introduced in 1994, has been associated with an increased risk of development of CMV diseases compared with azathioprine in cadaveric renal transplantation (Basic-Jukic et al, 2000) but the disease course is less severe, and less

frequently accompanied with deterioration of renal function in comparison to the azathioprine group. In a retrospective study of pediatric renal transplant recipients in Singapore, use of mycophenolic acid was significantly associated with CMV disease in the early post-transplant period (Sim et al, 1997).

The main risk factors for CMV disease in recipients of allogeneic stem cell transplants are recipient CMV seropositivity and acute graft-versus-host disease (Meijer et al, 2003). In the era before introduction of ganciclovir (GCV), CMV infection and pneumonia developed in 38 and 17%, respectively, of recipients of allogeneic stem cell transplants, while mortality due to CMV interstitial pneumonia was 85% (Myers et al, 1986). This very serious complication occurred mainly in CMV-seropositive patients, with acute graft-versus-host disease being the most important risk factor. Treatment of CMV pneumonia with GCV and immunoglobulin decreased mortality to 30 to 50% (Emmanuel et al, 1988; Reed et al, 1988). Currently, two antiviral strategies, prophylactic or preemptive antiviral treatment, are used for prevention of CMV disease. Nevertheless, CMV infection remains a life-threatening disease.

CMV remains the most common pathogen isolated after solid organ transplantation, including liver transplantation (Patel et al, 1996;

Stratta et al, 1989). CMV hepatitis occurs frequently, and the incidence is greatest after transplantation from a CMV-seropostive donor. In managing liver transplant patients the signs of severe CMV hepatitis can be difficult to distinguish from graft rejection. Liver biopsy is the only reliable way to distinguish rejection from CMV hepatitis (Demetris et al, 1985). However, prophylaxis and antiviral treatment have been shown to result in a mild or moderate intensity of CMV infection, and among the immunosuppressive drugs, only anti-IL-2Rab was proved to significantly reduce the incidence of CMV (Oldakowska-Jedynak et al, 2003).

The morbidity due to CMV is lowest in kidney transplant recipients, and the most common manifestation of CMV-associated illness is termed CMV syndrome (Sia and Patel, 2000). This usually presents insidiously with fever, anorexia, and malaise without additional signs or symptoms. This viral syndrome may be self-limited or may progress to clinically evident organ involvement (Sia and Patel, 2000).

The association of CMV with allograft dysfunction is controversial. Infection with CMV has been associated with early-onset allograft rejection in renal transplant recipients (Fox et al, 1988). In a cohort of 242 renal transplant patients, 65% developed CMV infection

(Pouteil-Noble et al, 1993). The incidence of rejection was significantly higher in those with antecedent CMV infection: 45% among infected patients versus 11% among noninfected patients. In another study, CMV-seropositive donor status was associated with lower graft survival rates than CMV-seronegative donor status (Hirata et al, 1996).

1.4 Diagnosis

Virological and molecular detection of CMV and serological demonstration of a specific immune response are used for diagnosis, although acute infection is usually diagnosed through detection of the virus in body fluids. There is no single best assay method for monitoring infection but molecular assays have a shorter turnaround time and can be automated, promise earlier treatment, and have reliable results.

Table 1.2 summarizes the different laboratory diagnostic strategies. The diagnosis of invasive disease is not straightforward, since the presence of CMV in a diseased tissue does not necessarily imply a causal relation. Diagnosis should be based on appropriate clinical signs and symptoms of disease in conjunction with detection of virus

from the appropriate involved tissue. For the purpose of consistent reporting of CMV in clinical trials, definitions of infection and disease have been developed (Ljungman et al, 2002).

The first laboratory test that was developed was viremia (detection of infectious virus in peripheral blood leukocytes) after 16 – 24 hours cocultivation with human fibroblasts in shell vials (Gregory and Mengus, 1983). Quantification of CMV in blood was also made possible by inoculating a predetermined number of leukocytes onto shell vial monolayers (Gerna et al, 1995). This approach was found to be the most reliable in evaluating the efficacy of antiviral treatment, because the test, as a rule, became negative 24 – 48 hours after initiation of therapy unless a drug-resistant strain emerged.

Developed at the end of the 1980s, assay for antigenemia entails identification of peripheral blood leukocytes carrying a viral protein (pp65) in the nucleus within a few hours after blood collection (van der Bij et al, 1988). Although representing only an indirect marker of virus replication, antigenemia has been very useful in monitoring CMV infection in immunocompromised patients for several years. However, problems arose in interpretation when rising levels of

antigenemia were observed in association with decreasing or stable levels of viremia and viral DNAemia after initiation of ganciclovir treatment in primary infections of both solid organ transplant recipients and hematopoietic stem cell transplant recipients. This often prompted clinicians to change antiviral treatment with the erroneous assumption that a ganciclovir-resistant strain had been selected, though the efficacy of treatment was documented by the negative results of the viremia assay (Gerna et al, 2004).

Polymerase chain reaction (PCR) methods were developed in the early 1990s for the qualitative and quantitative detection of viral DNA in blood (DNAemia) by either PCR or hybridization techniques, and has been reviewed extensively by Hodinka (1998) and Boeckh and Boivin (1998). Aw et al (2000) have described the usefulness of a quantitative CMV PCR technique for monitoring peripheral blood CMV DNA in pediatric recipients of kidney and liver allografts who had recurrent CMV retinitis, particularly for the surveillance of disease resolution and recurrence as this group of patients may remain asymptomatic for a period of time, despite recurrences. Gerna et al (1998; 2003) have reported that while viremia lacks sensitivity, quantitative DNAemia has been found to correlate with virus replication and clinical symptoms much more closely than

antigenemia, both during the natural history of HCMV infection and antiviral treatment. Weinberg et al (2000) evaluated serial CMV blood cultures, antigenemia, and qualitative and quantitative plasma PCR tests for their value in predicting CMV disease and for guiding preemptive therapy after lung transplantation. They found that PCR and antigenemia tests were the most effective predictors of symptomatic CMV infections and the response to therapy.

Viral DNAemia, however, is possibly a result of virus latency. To this end, viral messenger RNAs are directly related to virus replication. Either IE or late mRNAs may be detected by using either reverse transcriptase PCR (RT-PCR) or the nucleic acid sequence-based amplification (NASBA) technique. A randomized trial comparing antigenemia with NASBA for determination of CMV IE messenger RNA (IE mRNA) as the guiding assay for initiation of pre-emptive antiviral treatment showed that the NASBA technique applied to the detection of IE mRNA was found to be more sensitive than antigenemia in detecting earlier a greater number of infected patients (Gerna et al, 2003).

Table 1.2. Diagnosis of cytomegalovirus infection and disease (adapted from Gandhi and Khanna, 2004)

Diagnostic strategy	Comments
History/examination	Presence of CMV in tissue might simply be a bystander effect and does not necessarily imply causality. CMV-associated disease should be diagnosed on clinical grounds in combination with detection of virus.
Viral cultures	Requires at least 21 days to be reported negative. Not routinely done, but can be useful in antiviral resistance testing.
Detection of early antigen fluorescent foci	Twenty-four hour turnaround. Infected fibroblasts stained with fluorescent antibody specific for the antigen MIEp72 (Gregory and Menegus, 1983). Insufficiently sensitive to be routinely recommended after allogeneic stem cell transplantation (Einsele et al, 1995).
Antigenemia assay	Quantitates leucocytes positive for pp65. Since uninfected cells can harbor CMV protein, this provides an indirect assessment of infection. Reliable, rapid and sensitive. In common use (Boeckh et al, 1996).

Qualitative PCR	Can be used on whole blood, leucocytes and plasma. Rapid and sensitive, easily automated. Threshold needs careful calibrating to avoid over detection of CMV infection.
Quantitative PCR	Allows response to treatment to be monitored. Useful as a surrogate marker of clinical or viral resistance. Correlation between high degrees of viral DNA and presence of clinical symptoms in transplant recipients and patients with AIDS and the congenital infection of pregnancy has been shown ((Emery et al, 2000; Limaye et al, 2001, Trincado and Rawlinson.2001).
Hybrid capture assay	RNA probes used to detect viral DNA in an ELISA-type format. Can be used on whole blood stored for up to 48 hours. Limited experience but results to date promising (Ho et al, 2000).
Nucleic assay sequence-based amplification	Allows the specific nucleic assay sequence-based amplification of unspliced viral mRNAs in a background of DNA. Experience in transplant and AIDS patients encouraging (Blank et al, 2002; Gerna et al, 2003).
Serology	Highly specific and sensitive in immunocompetent individuals. IgM titers rise 2-6 weeks after infection and can persist for 2 years and be detectable

during episodes of reactivation. IgG seropositivity is usually lifelong although antibody concentrations can decline with age. Unreliable and not used in monitoring of CMV reactivation in immunocompromised hosts. IgG antibody avidity assays (low avidity suggests infection within 3 months) can be useful in identifying pregnant women at risk of transmitting intrauterine infection (Bodeus et al, 2002).

1.5 Objectives of the thesis

Despite remarkable progress made in the fields of antiviral therapy and laboratory diagnosis, infections with the human CMV continues to pose serious problems and challenges in certain patient groups, in particular, those with suppressed or impaired immune systems.

Data collection, statistics and publications with respect to CMV infections and routine or specialized laboratory testing (such as detection of antiviral resistance) are almost exclusively from non-Asian sources. There is also a paucity of information in Singapore regarding CMV infections in the general population, or in the immunocompromised groups of patients. A PubMed index search (accessed June 2006) revealed only six such publications, excluding the recent publication of Yeo et al (2005) on molecular detection of ganciclovir resistant CMV. There were two on CMV epidemiology in women and children (Sng and Tobin, 1977; Wong et al, 2000); two case reports on AIDS-related CMV retinitis and pneumonitis (Law et al, 1990; Eng et al, 1992, respectively); a survey of infections (including CMV disease) in paediatric renal transplantation (Sim et al, 1997), and a publication on the utility of quantitative PCR for

monitoring CMV retinitis in pediatric solid organ transplant recipients (Aw et al, 2000).

The scope of this thesis is as follows:

1 Elucidate the molecular epidemiology of CMV in Singapore:

a. Molecular epidemiology studies of CMV infections in Asia have been limited to those carried out in Japan (Wada et al, 1997; Kawasaki et al, 1999; Numazaki et al, 2000; Kashiwagi et al, 2002), Taiwan (Shen et al, 1996) and Hong Kong (Woo et al, 1997). We analyzed the genomes of CMV strains recovered from immunocompromised patients (specifically, the solid organ transplant cohorts admitted to the National University Hospital (NUH), the designated pediatric liver and renal transplant center in Singapore). To the best of our knowledge, this analysis of CMV interstrain variations to determine the types circulating in the country and their association, if any, to the different types of transplantation, represents the first such study in Singapore.

b. CMV serostatus is one of the traditional risk factors for early-onset CMV disease and now appears to also contribute to the occurrence of late-onset CMV disease during the first year after transplantation for both solid organ and hematopoietic stem cell recipients (Razonable, 2005). As an adjunct to the molecular epidemiology study, we also determine the seroprevalence of antibodies to CMV in the apparently healthy, young adult male and female populations. No previous study has also been reported for this age-related cohort in Singapore who is potentially at risk as CMV seronegative organ transplant recipients.

2 **Screen for strains of CMV resistant to ganciclovir in Singapore.** Antiviral drug resistance in CMV is a major concern in transplantation. While the majority of infections by antiviral resistant CMV have been described to occur in patients with AIDS receiving prolonged antiviral therapy for CMV end-organ disease, other immunocompromised individuals on antiviral prophylaxis and or therapy, including bone marrow and solid-organ transplant recipients, are also affected. The frequency of active CMV disease seen in children from the Paediatric Renal and

Liver Transplant Programs at NUH is 30.8%, including one death, in renal allograft recipients (Sim et al, 1997) and 25% in liver allograft recipients. This is despite the routine use of ganciclovir prophylaxis.

Our hypothesis is that the high incidence of active CMV disease, which includes pneumonitis, retinitis and colitis, is due to ganciclovir-resistant CMV mutant strains. We sought to determine the frequency of CMV mutant strains in (i) CMV isolates from the non-transplant population sent to the Virology Laboratories of NUH and the Singapore General Hospital (SGH), where the culture-based detection of CMV areperformed and (ii) CMV DNA isolated directly from clinical samples such as blood, saliva and urine from a cohort of pediatric solid organ transplant patients from NUH. Different laboratory methods currently available, including conventional drug susceptibility assay (plaque reduction assay) and previously published genotypic assays (PCR-RFLP, DNA sequencing) would be employed.

3 **Develop new PCR-based assays for rapid and improved detection of specific mutations in the CMV genome that are known to confer ganciclovir resistance and to**

discover novel mutations. Resistance of CMV to ganciclovir is related to mutations in the CMV UL97 gene and or mutations in the viral DNA polymerase (UL54 gene). Limitations of currently available laboratory tests for resistant strains of CMV include long turnaround time and wide interlaboratory variability owing to the slow and often variable growth patterns of CMV in the plaque reduction assay (Harmenberg and Brytting, 1999) and the fact that PCR-RFLP can detect CMV mutants only they reach approximately 10% of the viral population in a clinical isolate. Nonetheless, genotypic tests that are PCR-based and target specific change(s) in the genome have the advantage of being amenable to producing rapid results and automation. We attempted to develop laboratory tests for detection of antiviral resistant CMV strains by employing new molecular-based strategies that could produced a short turnaround time for results and with improved detection limits.

4 **Define the role of CMV infection in allograft rejection.** While infection has been shown to have a high association with chronic rejection in extra-renal transplants such as heart/lung transplants, there is conflicting evidence on the

role of CMV infection in chronic allograft rejection in renal transplantation. In Singapore the seropositivity rate of CMV in kidney donors is approximately 90%. Assessment of CMV infection of the renal allograft as well as other contributory factors to the process of chronic rejection is therefore important. In this thesis, we attempted to answer the following questions:

a. Does CMV infection contribute significantly to chronic allograft rejection in renal transplant recipients? Upon recruitment of renal allograft recipients transplanted at two hospitals (i.e. NUH and SGH), clinical and laboratory parameters would be recorded to allow for statistical analysis of risk factors for development of chronic allograft rejection at one year post-transplant

b. Is the mechanism of late allograft loss related to possible CMV-induced changes in the antigenic structure of the endothelial cells, namely via anti-endothelial cell antibodies (AECA)?

2 METHODS AND MATERIALS

2.1 Seroprevalence of cytomegalovirus infection in healthy young adults and paediatric transplant subjects in Singapore

Randomly selected and apparently healthy volunteer subjects were approached in 2003 to 2004. There were 151 subjects (aged 17 – 24 years, mean age was 18.6 years), who were students studying in Singapore Polytechnic. Whole blood was drawn by venipuncture; upon informed consent, and serum collected was stored at -20°C prior to batch analysis by immunoassay for serological evidence of CMV infection. Commercially available enzyme-linked immunosorbent assay (ELISA) test kits (DRG Diagnostics Co, Germany) were used to test for the presence or absence of CMV IgG. Microtiter well washing and absorbance readings utilized the Model 1575 Immunowash and Model 680 Microplate Reader (both from Bio-Rad Laboratories, Hercules, CA, USA), respectively. Determination of a positive or negative result was as per the manufacturer's instructions. Data was statistically analyzed using the test for difference between two population proportions to see the relation between gender and CMV seropositivity. $P < 0.05$ was considered statistically significant. Serostatus of the cohort of paediatric transplant subjects were obtained from the clinical database.

2.2 Molecular epidemiology of cytomegalovirus infection in Singapore

This study examined a total of 200 clinical samples, comprising (a) 96 clinical isolates of CMV propagated in human foreskin fibroblasts (MRC5) and saved in frozen storage and (b) CMV DNA isolated directly from 104 samples such as blood, saliva and bronchoalveolar lavage using a modified phenol-chloroform method (Ratnamohan et al, 1992). DNA from the primary isolates in (a) were extracted using the DNeasy Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's specifications. All samples were from patients admitted to Singapore General Hospital (SGH) and the National University Hospital (NUH), Singapore, from the period July 1998 to 2001. Two laboratory strains, AD169 and Towne (both from American Type Culture Collection, Manassas, VA, USA), were used as reference viruses.

Genotyping of CMV for interstrain variation was performed using a previously described PCR-RFLP method (Chou and Dennison, 1991). PCR amplification of part of the CMV envelope glycoprotein B (gB) gene that encodes a portion of gp55 using primers gB1319 (5' TGGAACTGGAACGTTTGGC 3') and gB1604 (5'

GAAACGCGCGGCAATCGG 3') yielded a 293 to 296 bp target (depending on the gB group).

All the PCR primers were synthesized commercially (either by QIAGEN Operon, Alameda, CA, USA or Research Biolabs Pte Ltd, Singapore). PCR reactions were performed in a final volume of 25µL with 2.0mM Mg^{2+}, 200µM of each dNTP (Finnzymes Oy, Espoo, Finland), 1.5 units of *Taq* polymerase (QIAGEN GmbH, Germany) , standard buffer (QIAGEN GmbH) and 0.2µM of each primer. Amplification involved 1 cycle of denaturation for 5 minutes at 95°C and 37 programmed cycles (denaturation at 95°C for 1.5 minutes, annealing at 55°C for 2 minutes, and extension at 72°C for 1 minute) on the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). CMV AD169 and Towne strains were used as positive controls, and nuclease-free water (Promega Corporation, Madison, WI, USA) as the negative control.

To identify the genotype to which a strain belonged, the amplified products were subjected to restriction analysis in separate reactions using *Hinf*I and *Rsa*I (both from New England Biolabs Inc, Ipswich, MA, USA). Digested DNAs were subjected to electrophoresis on 6% polyacrylamide gels and ethidium bromide staining, and the images digitally captured on the Gel Doc 2000 (Bio-Rad Laboratories,

Hercules, CA, USA). Product sizes were measured against the AmpliSize and EZ load 20 bp molecular rulers (all from Bio-Rad Laboratories). RFLP patterns obtained with the two restriction enzymes were used to classify the CMV isolates or DNAs according to one of four gB types (1 – 4).

Data was statistically analyzed using pairwise comparisons of proportions, and statistical significance for all analyses was assumed at $P < 0.05$ level.

2.3 Cytomegalovirus antiviral resistance

Samples for this study comprised the same two cohorts as described in Section 2.2, namely (a) 96 clinical isolates of CMV propagated in human foreskin fibroblasts (MRC-5) and saved in frozen (-70°C) storage and (b) CMV DNA isolated directly from 104 clinical samples using a modified phenol-chloroform method (Ratnamohan et al, 1992). DNA from the primary isolates in (a) were extracted using the DNeasy Tissue Kit. All the clinical samples were initially screened, and were shown positive for presence for CMV by quantitative conventional PCR of the immediate early gene (Jiwa et al, 1990; Aw et al, 2000). The two cohorts were made up of patients admitted to

SGH and NUH hospitals from the period July 1998 to 2002. Included in the study, as a wild-type positive control, was the laboratory CMV strain AD169.

The clinical isolates ("live" cultures) of CMV were screened for ganciclovir resistance by a plaque reduction assay as described by Wentworth and French (1970). In brief, each well in 24-well plates were inoculated with virus suspension when MRC-5 cells (from the human embryonic fibroblast cell line (American Type Culture Collection, Manassas, VA, USA)) were just confluent. The virus suspensions were prepared from stocks consisting extracellular virus from infected monolayers (harvested when extensive cytopathology occurred in at least 90% of the cell layer). After adsorption for 90 minutes at 37°C, the wells were overlaid with 1.5mL of 0.4% agarose (FMC Bioproducts, Rockland, ME, USA) containing the appropriate concentration (0, 1.5, 3, 6, 12 and 24μM) of ganciclovir (Cymevene, F Hoffman-La Roche, Basel, Switzerland) and growth medium (Minimum Essential Medium, Gibco Invitrogen Corporation, Carlsbad, CA, USA) in triplicates. The cultures are then observed for the presence of viral plaques over two weeks, with a second overlay of agarose after the first week of incubation. Two mL of 10% formalin was added to each well to fix the monolayers and the monolayers then removed and discarded. After staining with 0.03%

aqueous methylene blue for two minutes, the plaques were counted visually.

The IC_{50} (50% inhibitory concentration) of the agent for the isolate is defined as the concentration of agent causing a 50% reduction in the number of plaques produced. Sensitive, indeterminate and resistant ranges were as follows: $IC_{50} \leq 6\mu M$ of ganciclovir, sensitive; $IC_{50} >$ 6μM to 12μM, indeterminate; >12μM, resistant

In addition, the clinical isolates were screened using genotypic methods for detection of mutations known to confer ganciclovir resistance in the CMV UL97 phosphotransferase and UL54 DNA polymerase genes.

UL97 mutations were screened using PCR-RFLP, with primers and types of restriction enzymes as previously described (Chou et al 1995a; Chou et al, 1995b; Hanson et al, 1995). This nested PCR protocol enabled improved, specific amplification, and hence, direct detection of the virus in clinical samples.

In brief, extracted CMV DNA was subjected to an initial round of PCR amplification using universal primers as previously described (Chou et al, 1997) to amplify the entire UL97.

Subsequently, regions within the UL97 gene flanking frequently encountered UL97 resistance mutations at codons 460 (Met to Val), 594 (Ala to Val), 595 (Leu to Ser, or Leu to Phe) and 520 (His to Glu) were further amplified by PCR with the relevant primers.

The sequences of the nested PCR primers for amplification of UL97 are as follows: for the first round of PCR, universal primers CPT0 (5' GTCGACGACGCCGTCTAA 3') and CPT2096 (5' CGTCAAGGTCCTCCTCGCA 3') for the entire UL97 gene; for the second round, primer set CPT1088 (5' ACGGTGCTCACGGTCTGG AT 3') and CPT1619 (5' AAACGCGCGTGCGGGTCGCAGA 3') for region surrounding codon 460; primer set CPT1713 (5' CGGTCT GGACGAGGTGCGCAT 3') and CPT1830M (5' AATG AGCAGACAGGCGTCGAAGCAGTGCGTGAGCTTGCCGTTCTT 3') for region surrounding codon 594 and 595; primer set CPT1088 and CPT1587M (5' CTGCAGCGGCATGGGTCGGAAA GCAAG 3') for region surrounding codon 520 (Chou et al 1995a; Chou et al, 1995b; Hanson et al, 1995).

All the PCR primers were synthesized commercially (QIAGEN Operon or Research Biolabs). PCR reactions were performed in a final volume of 25μL with 2.0mM Mg^{2+}, 200μM of each dNTP

(Finnzymes Oy, Espoo, Finland), 1.5 units of *Taq* polymerase (QIAGEN GmbH, Germany) , standard buffer (QIAGEN GmbH) and 0.2µM of each primer. 1×Q-solution (QIAGEN GmbH), a proprietary PCR additive, was included in amplification reactions for templates that had low copy numbers of CMV DNA.

PCR amplification involved 1 cycle of denaturation for 15 minutes at 95°C and 37 programmed cycles (denaturation at 95°C for 1.5 minutes, annealing at 55°C for 1 minute, and extension at 7°2C for 1 minute) on the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). CMV AD169 and Towne strains (both from American Type Culture Collection) were used as wild type positive controls, and nuclease-free water (Promega Corporation) as the negative control.

PCR products obtained were then digested separately with restriction enzymes (*Nla*III, *Hha*I, *Taq*I, *Mse*I and *Alu*I (all from New England Biolabs Inc, Ipswich, MA, USA)) in order to identify mutations in codons 460, 594, 595 (two different mutations) and 520, respectively. Digest conditions were according to the manufacturer's instructions. The digest patterns were visualized upon electrophoresis on 8% or 15% polyacrylamide gels and ethidium bromide staining, the images digitally captured on the Gel Doc 2000 (Bio-Rad Laboratories), and

compared against that for wild type laboratory CMV strain AD169 DNA (Advanced Biotechnologies Inc, Columbia, MD, USA) and various molecular rulers (Table 2.1). Mutations at codons 460 and 594 result in the loss of naturally occurring restriction enzyme sites but mutations at codons 520 and 595 result in additional restriction sites (Table 2.1).

Specific identification of mutations was by DNA sequence analyses of the PCR products using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 310 genetic analyzer (Applied Biosystems), and by comparison with reference strain AD169 sequence (EMBL accession no X17403)..

CMV UL54 mutations were detected by sequencing the relevant portions of the polymerase-encoding gene, in particular, regions of the gene that have been shown to confer ganciclovir resistance (Chou et al, 2003). PCR reactions were done under the following conditions: 37 cycles at 95°C for 1 minute, 55°C for 2minutes and 72°C for 1 minute on the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). Sequencing reactions were carried out using the BigDye Terminator Cycle Sequencing kit and the ABI Prism 310 genetic analyzer. PCR primers specific for codons 300 to 1000 (as previously described by Erice et al, 1997) were as follows: Z4957F

(5' GCTGCACTTCGGAGGGTGTGA 3'), Z4958R (5' TGCC GCTTAAAACCCACGGCG 3'), Z3583F (5' GGGCTCGAAACATCG TCAGG 3'), Z3584R (5' CGTGGCACGCCGTACTTCTT 3'), Pol1474F (5' GTGATATCGAGGTAGACTGCGATG 3'), Pol2536R (5' GCGGCGGTAGAGATGATAGC 3'), Pol2401F (5' GTATCT ACACCTCGCTGCTG 3') and Pol3895 (5' ACGGCAATGT GCGGCAGGTTAG 3'). Comparison of UL54 sequences obtained was undertaken with reference strain AD169 sequence, based on the published strain AD169 reference sequence (EMBL Accession number X17403).

2.4 CMV UL97 mutation analysis by discriminatory PCR

This study involved the development of discriminatory PCR assays for mutation analysis of CMV UL97 codons 594 and 595. Wild type laboratory CMV strain AD169 DNA was obtained commercially (Advanced Biotechnologies Inc).

(The newly developed discriminatory PCR assays were subsequently used to prospectively screen samples received in the Dept of Paediatrics, NUS for anti-ganciclovir resistance testing, in parallel

with conventional PCR-RFLP methods for mutation analysis in the UL97 gene)

A total of 40 clinical samples (phenol-chloroform extracted and purified DNAs) from a cohort of pediatric solid organ transplant patients admitted to the National University Hospital, Singapore or the Dept of Paediatrics, National University of Singapore (NUS) was studied. Samples from these renal (n = 12) and liver (n = 5) transplant recipients were previously analyzed for the presence of CMV by quantitative conventional PCR and the wild type and or mutant genotype determined using PCR-RFLP, as previously described (Section 2.3). Mutations at the two codons of interest (namely CMV UL97 codons 594 and 595) were confirmed by DNA sequence analyses of the PCR products.

New PCR primers were designed using Primer3 software program (Rozen and Skaletsky, 2000), again based on the published strain AD169 reference sequence (EMBL Accession number X17403).

All the PCR primers were synthesized commercially (QIAGEN Operon or Research Biolabs). Optimized PCR reactions were performed in a final volume of 25µL with 2.0mM Mg^{2+}, 200µM of each dNTP (Finnzymes Oy, Espoo, Finland), 1.5 units of HotStarTaq

DNA polymerase (QIAGEN GmbH, Germany) , standard PCR buffer (QIAGEN GmbH), 1×Q-solution, 0.2μM of each primer and 1μg of total DNA. Samples with low copy number of CMV were subjected to a nested PCR protocol and 2uL of PCR product from the first round of amplification using universal primers (as previously described (Chou et al, 1997) to amplify the entire UL97)) were used for the second round (discriminatory PCR) amplification.

Controls included the CMV AD169 strain as the wild type positive control, and nuclease-free water (Promega Corporation) and DNAs from culture isolates of herpes simplex virus type 1 and adenovirus (American Type Culture Collection) as the negative controls.

CMV UL97 mutation analysis of codon 594 involved a two-step PCR amplification profile as follows: 1 cycle of denaturation for 15 minutes at 95°C and 37 programmed cycles (denaturation at 95°C for 1.5 minutes, and annealing/extension at 58°C for 0.5 minute). For mutation analysis of codon 595, the profile was similar except that the annealing/extension were at 60°C for 0.5 minute. The iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) was utilized.

PCR products obtained were resolved by electrophoresis on 8% polyacrylamide gels and ethidium bromide staining, the images digitally captured on the Gel Doc 2000 (Bio-Rad Laboratories), and compared against that for the wild type CMV strain and various molecular rulers (Bio-Rad Laboratories).

2.5 CMV UL97 mutation analysis by real time PCR using molecular beacons

2.5.1 Real time PCR using molecular beacons

The aim of this study was to develop a PCR-based protocol using the real time platform for mutation analysis. As proof of concept, the mutation in the CMV UL97 codon 460 gene served as the focus of the study. An A to G change at position 1378 of the gene leads to amino acid changes (Met to Val) which have previously been shown to confer ganciclovir resistance (Chou et al, 1995a).

DNA extraction was performed directly from blood samples using a modified phenol-chloroform method, and spectrophotometrically quantified as previously described. The 40 samples were randomly selected from a cohort of pediatric solid organ transplant patients

(renal (n = 12) and liver (n = 5)) admitted to NUH. Prior to coding for unbiased reexamination, all samples were analyzed for the presence of CMV by quantitative conventional PCR, as previously described (Aw et al, 2000). This was followed by codon 460 mutation analysis via PCR-RFLP (that is, conventional nested PCR, *Nla*III restriction enzyme digestion and polyacrylamide gel electrophoresis), also as previously described (Chou et al, 1995a). Included in the study, as a calibrated wild-type positive control, is the laboratory CMV strain AD169 DNA, obtained commercially (Advanced Biotechnologies Inc) in the form of quantitated viral DNA (10^4 DNA copies/μL).

The PCR primers and molecular beacons were also synthesized commercially (Proligo SAS, France or QIAGEN Operon), and both primers and beacons were designed using the Beacon Designer 2 version 2.06 software (Premier Biosoft International, Palo Alto, CA, USA). PCR primers were designed to amplify a portion of the UL97 CMV gene region flanking codon 460, based on the published strain AD169 reference sequence (EMBL Accession number X17403). The product generated by these primers is 321 bp long. The molecular beacons were constructed following criteria first described by Tyagi and Kramer (1996). They consisted of probe sequences 16 nucleotides long that anneal either to the wild-type or to mutant

sequences, 5-nucleotide arm sequences, the fluorophores FAM (carboxy-fluorescein) and HEX (hexachlorofluorescein) covalently linked to the 5' ends of the wild-type and mutant beacons, respectively, and the quencher DABCYL (4-[4'-dimethylaminophenylazo]benzoic acid) linked to the 3' ends of both beacons. The sequences of the primers and molecular beacons are summarized in Table 2.4.

Using the iCycler iQ detection system (Bio-Rad Laboratories, Hercules, CA, USA), PCR conditions were optimized by varying molecular beacon (100 – 500 nM), primer (200 – 1000 nM), and magnesium (4 – 6 mM) concentrations and the annealing temperature (50 - 60°C).

Optimal PCR reactions contained 25µL of 2× QuantiTect Probe PCR Master Mix (QIAGEN GmbH, Germany), 200nM of primers, 200nM of molecular beacon for the wild-type or mutant sequence, and 1µg of total DNA in a 50µL final volume. The thermal cycling program consisted of 15 minutes at 95°C to activate the HotStarTaq DNA polymerase, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. Fluorescence was monitored during the 60°C annealing phases.

2.5.2 Generation of CMV UL97 M460V mutants by PCR mutagenesis

Whilst wild type CMV DNA are readily available through commercial sources (for instance, American Type Culture Collection, or Advanced Biotechnologies, Inc), DNA from ganciclovir-resistant strains of CMV has to be sourced by screening clinical samples or isolates from patients. Using an *in vitro* PCR mutagenesis protocol, we aimed to generate CMV DNA containing mutant sequence arising from ATG to GTG change (methionine to valine amino acid substitution) at codon 460 of the UL97 gene. These mutants would serve either as controls for PCR assays or for use in generating mixed populations of known proportions of wild type and mutant sequences.

By means of mismatched primer mutagenesis first described by Higuchi (1990), new primers were designed (using the Beacon Designer 2 version 2.06 software (Premier Biosoft International)) to be only partially complementary to the target site but in such a way that it will still bind specifically to the target (Table 2.3). Two mutagenic reactions were carried out, generating two separate PCR products which have partially overlapping sequences containing the M460V mutation. These reactions were carried out as asymmetric PCR assays, in order to preferentially amplify the designed strand

containing the mutant sequence. A symmetric PCR results in exponentially grown double stranded DNA whereas an asymmetric PCR generates one of the strands by linear amplification and a fraction of its total product as double-stranded DNA limited by the concentration ratio of the primers used (Poddar, 2000). The denatured products were then combined in a final PCR reaction (using outer primers previously described by Chou et al, 1995a) to generate a larger product with the mutation in a more central location.

All PCR reactions were carried out in 50µL volumes consisting of 2.0mM Mg^{2+}, 200µM of each dNTP (Finnzymes Oy), 1.5 units of HotStarTaq DNA polymerase (QIAGEN GmbH), standard PCR buffer (QIAGEN GmbH), 1×Q-solution, 0.2µM of outer primers CPT1088 and CPT1619 or 0.3µM of inner primers 460_F and 460_R and 1µg of CMV AD169 DNA (for first round of PCR) or 2µL of PCR product. Cycling conditions were 1 cycle of 95°C for 15 minutes; 37 cycles with denaturation at 94°C for30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. Amplification was carried out on the iCycler thermal cycler (Bio-Rad Laboratories).

Electrophoresis on 1% agarose gel and ethidium bromide staining were used to size the desired PCR products. The M460V mutation was confirmed by DNA sequence analysis of the PCR products.

2.6 CMV UL54 mutation analysis

PCR-based assays - either involving restriction analysis (PCR-RFLP) or discriminatory PCR primers - were developed for detection of a mutation at codon 412 of the CMV UL54 gene, occurring at a conserved region in the codons 300 to 1000 of the polymerase genes where the majority of mutations leading to ganciclovir resistance are known to occur (Chou, 1999).

Primers for PCR were designed using the Jellyfish version 1.4 for Windows software (Biowire.com, Inc.). A nested PCR strategy was adopted to increase the sensitivity and specificity of amplification. For the first round of PCR, universal primers were selected to amplify the entire CMV UL54 gene sequence (NCBI, Human CMV strain AD169 complete genome Accession and Version No: 59591, Sequence feature view of the region: gi|59591:c80631-76903).

2.6.1 Analysis of CMV UL54 F412C mutation by PCR-RFLP

For the second round of PCR, primers were designed to amplify the gene region surrounding codon 412 within UL54. Also taken into account during primer design and selection was the number of restriction site (and thus the number and size of fragment generated) as well as the commercial availability of the restriction enzymes. These newly designed primers and corresponding restriction enzymes are listed in Table 2.5. All primers were synthesized commercially by QIAGEN Operon or Research Biolabs Pte Ltd.

Optimized conditions for the first (universal) round of amplification of the nested PCR protocol were as follows: 2.0mM Mg^{2+}, 200µM of each dNTP (Finnzymes Oy), 1.5 units of HotStarTaq DNA polymerase (QIAGEN GmbH), standard PCR buffer (QIAGEN GmbH), 1×Q-solution, 0.2µM of each universal primer (TJ54N_F1 and TJ54N_B1) and 1µg of total DNA to a total volume of 25µL. Cycling conditions were 1 cycle of 95°C for 15 minutes; 37 cycles with denaturation at 94°C for 2 minutes, annealing at 50°C for 2 minutes and extension at 72°C for 2 minutes; to ensure the complete synthesis of all strands, the samples were further extended at 72°C for

5 minutes. Amplification was carried out on the iCycler thermal cycler (Bio-Rad Laboratories).

PCR-RFLP involved a second round of PCR amplification as above except that the universal primers were replaced by specific primers (RJ54_412F and RJ54_412R) designed to amplify the region surrounding codon 412 and 2 – 3µL of the first round PCR product were used as test template.

Validation of the assay involved the use of control templates, with CMV AD169 strain as the wild type positive control, DNAs from culture isolates of herpes simplex virus type 1 and adenovirus (American Type Culture Collection) as the negative template controls as well as nuclease-free water (Promega Corporation) as the negative no template control.

Sensitivity of assay was determined by using two-fold serial dilutions of calibrated wild type laboratory CMV strain AD169 DNA, obtained commercially (Advanced Biotechnologies Inc) in the form of quantitated viral DNA (stock concentration = 10^4 DNA copies/µL).

Cycling conditions were similar to the initial round of amplification above and also carried out on the iCycler thermal cycler (Bio-Rad Laboratories).

Restriction enzyme digestion using *Mbo*II (New England Biolabs Inc) was under conditions as specified by the manufacturer. Electrophoresis on 8% polyacrylamide gels and ethidium bromide staining were followed by fragment analysis against the wild type laboratory CMV strain AD169 and the AmpliSize molecular ruler (Bio-Rad Laboratories).

Upon optimization, the PCR-RFLP assay was used for a retrospective analysis of 52 randomly selected clinical samples (phenol-chloroform extracted and purified DNAs) from the same cohort of pediatric solid organ transplant patients admitted to NUH. These patients were also determined to be positive for CMV and also genotyped for presence or absence of CMV UL97 mutations (see Section 2.3).

2.6.2 Analysis of CMV UL54 F412C mutation by discriminatory PCR

This discriminatory PCR strategy was based on that for our previous study on mutation analysis of UL97 codons 594 and 595. Primer

pairs were designed with changes in the 3' terminal sequence to anneal either to wild type or mutant sequences but not to both (Table 2.5). All primers were synthesized commercially by QIAGEN Operon or Research Biolabs Pte Ltd.

Reagent mixture for PCR was also as previously described for UL97 mutation analysis by this method, except that primer set CMV_412FW1 and CMV_412RW1 was used for the detection of wild type sequence and primer set CMV-412FW1 and CMV_412RM2 for the mutant. Cycling conditions –with the optimal annealing temperature again determined by performing gradient PCR – were as follows: 1 cycle of 95°C for 15 minutes; 37 cycles with denaturation at 94°C for 1 minute, annealing at 64.7°C for 15 seconds minutes and extension at 72°C for 30 seconds. Amplification was carried out on the iCycler thermal cycler (Bio-Rad Laboratories).

Electrophoresis on 8% polyacrylamide gels and ethidium bromide staining enabled fragment sizing against that for various template controls and molecular ruler (Bio-Rad Laboratories).

Upon optimization, a retrospective analysis of the 52 clinical samples from the same cohort of pediatric solid organ transplant patients for the PCR-RFLP study was made. F412C mutations were confirmed by

DNA sequence analyses of the PCR products using BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and the ABI Prism 310 genetic analyzer (Applied Biosystems), and by alignment with the reference strain AD169 sequence (EMBL accession no X17403).

2.7 Role of cytomegalovirus infection in the development of chronic allograft dysfunction

2.7.1 Patient population

A prospective study was done involving a total of 119 consecutive renal allograft recipients transplanted at the two major transplant centers in Singapore, i.e. NUH or SGH hospitals, recruited over a two year period. Informed consent was obtained from all the patients enrolled in the study, which was approved by the Institutional Review Boards of both hospitals.

Clinical data on all renal allograft recipients were obtained by chart review, case notes and the clinical database. The standard induction immunosuppression regime used included methylprednisolone, azathioprine and cyclosporin A. Patients who were at high

immunological risk received induction with anti-thymocyte globulin (ATG) and/or anti-CD3 monoclonal antibodies (OKT3). Patients who were seronegative prior to the transplantation and who received a kidney from a seropositive donor were treated prophylactically with intravenous ganciclovir as were patients with proven CMV infection. Chronic allograft dysfunction at one year post-transplant was defined as serum creatinine greater than 150 μmol/L. Clinical and laboratory parameters recorded in the study included transplantation type (cadaveric or living-related or living-unrelated), the number of acute rejections, the degree of immunosuppression and the administration of ganciclovir. Whole blood was obtained from each subject serially at the following time intervals: pre-transplant, day one, one month, three months and five months post-transplant.

2.7.2 Determination of CMV DNAemia in the pre- and post-transplant period

To study the relationship between the presence of CMV DNA at various time intervals in the post-transplant period and subsequent allograft function at one year, nucleic acid was extracted from peripheral blood leucocytes by a modified phenol-chloroform procedure using protocols previously described (Jiwa et al, 1990;

Aw et al, 2000). PCR amplification and quantitation of CMV were performed using primers specific for the CMV immediate early gene, namely 5'-AGC TGC ATG ATG TGA GCA AG and 3'-GAA GGC TGA GTT CTT GGT AA (Jiwa et al, 1990). The 146bp PCR products were visualized upon electrophoresis with 2% agarose gel and staining with 0.5µg/mL ethidium bromide; laser densitometric analysis carried out using a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA) at 260 nm. Quantitation of the test CMV copy number was accomplished by comparison with a standard curve constructed from the densitometric analysis of the PCR amplicons obtained by running known copy numbers (10, 50, 100, 500 and 1000) of an external control plasmid containing CMV DNA. The results were expressed as CMV copy number/µg of total DNA tested.

2.7.3 Anti-endothelial cell antibodies activity (AECA) in sera

Enzyme immunoassay (EIA) for AECA in sera was performed as previously described by Yap et al (1988), Jordan et al (1988) and Rappaport et al (1990),with minor modifications.

In brief, patient sera, diluted 1:10 for IgM specific and 1:50 for IgG specific AECA, were tested against human umbilical venous endothelial cells (American Type Culture Collection, Manassas, VA, USA) that were cultured in Clonetics endothelial cells basal medium (Cambrex Corp, Baltimore, MD, USA) supplemented with antibiotic and antimycotic and endothelial cell growth supplement (Sigma-Aldrich Co, St Louis, MO, USA). The cells were grown on 96-well microtiter well plates (Nunc A/S, Roskilde, Denmark) until they formed at least a 90% confluent monolayer. After fixation with 0.2% gluteraldehyde and quenching with PBS-Tween 0.1%, the plates were ready for testing. Sera were tested in quadruplicates. .

To standardize the AECA EIA, the relevant antibody levels were measured in 30 normal controls, and four controls were used to statistically represent the distribution of the normal controls, and were subsequently tested on every microtiter well plate. A standardized score was derived based on the mean OD of the four standard controls (M) on each plate, expressed as follows:

$$\text{AECA score} = \text{OD of test serum} - M$$

The upper 95th percentile confidence limit (one-tailed test) of the control population was calculated to determine the upper limit of

normality; scores of greater than 0.06 for IgG specific AECA, and greater than 0.08 for IgG specific AECA were considered abnormal.

2.7.4 Statistical analysis of risk factors for development of chronic allograft dysfunction at one year post-transplant

Statistical analysis of risk factors for development of chronic allograft rejection at one year post-transplant were performed using SPSS for Windows, Rel. 13.0 2004 (SPSS Inc., Chicago, USA). Initially, univariate analyses employing a chi-square test for interdependence were performed. $P < 0.05$ was considered statistically significant. This was followed by logistic regression analyses considering chronic allograft rejection as the dependent variable. Potentially significant variables identified in the univariate analysis were tested in this model. The odds ratio (OR) for each variable, and their 95^{th} percentile confidence intervals were calculated, and their significance were then determined.

Table 2.1. Screening for CMV UL97 mutations related to ganciclovir resistance based on distinctive restriction digestion patterns from PCR products (adapted from Erice, 1999).

Restriction enzyme	UL97 Codon	Product size (bp)*	
		Wild-type sequence	Mutant sequence
*Nla*III	460	198, 168, 126, 9	324, 168, 9
*Alu*I	520	295, 189, 17	268, 189, 27, 17
*Hha*I	594	50, 38, 18, 12	62, 38, 18
*Mse*I	595	76, 42	46, 42, 30
*Taq*I	595	99, 19	71, 28, 19

*Underlined fragments are normally seen in polyacrylamide gels after ethidium bromide staining.

Table 2.2. CMV UL97 mutation analysis by discriminatory PCR: primers and sequences targeting codons 594 and 595.

Target	Primer	Sequence(5′-3′)*	Product size (bp)
UL97 codon 594			
	CMV_594R	CGAATGTTACCACCCTGCTT	
	CMV_594LW	CTTACCGTTCTCCAACG	269
	CMV_594LM	CTTACCGTTCTCCAACA	
UL97 codon 595			
	CMV_595R	TATTGCATGTCGGAGCTGTC	
	CMV_595LW	GAGCTTACCGTTCTCCA	159
	CMV_595LM	GAGCTTACCGTTCTCCG	

** underlined nucleotide indicates point mutation*

Table 2.3. Generation of CMV UL97 mutants by PCR mutagenesis: sequences of primers targeting codon 460.

Primer	Sequence(5′-3′)
Outer primers	
CP1088[a]	ACGGTGCTCACGGTCTGGAT
CP1619[a]	AAACGCGCGTGCGGGTCGCAGA
Inner primers	
460_F	GCACGTTCAGGGGTGTAATG
460_R	CATTACACCCCTGAACGTGC

[a] *previously described by Chou et al, 1995a*
** new primers*
***underlined nucleotide indicates site of mutation (A to G change)*

Table 2.4. CMV UL97 mutation analysis by real time PCR using molecular beacons: sequences of primers and molecular beacons targeting codon 460.

Name	Sequence[a]
Primer	
Forward	5′-TGCATCGACAGCTACC-3′
Reverse	3′-GCAGATGAGCAGCTTC-5′
Molecular beacon	
MB-Wild-type 460	5′-HEX GCGAGAGCACGTTCATGGGTGCTCGC DABCYL-3′
MB-Mutant 460	5′-FAM GCGAGAGCACGTTCAC*GGGTGCTCGC DABCYL-3′

[a] *underlined sequences indicate stem sequences of the molecular beacon*
** indicates site of mutation*

Table 2.5. CMV UL54 mutation analysis by PCR-RFLP and discriminatory PCR: sequences of primers for the entire UL54 gene, and primers and restriction enzyme targeting codon 412.

Primer	Sequence(5′-3′)	Product size (bp)
Complete UL54 gene		
TJ54N_F1	TTTTTCAACCCGTATCTGAGC	
TJ54N_ B1	CAGCACCATGTGCAAAAACT	3663
PCR-RFLP		
RJ54_412F	TCTCTTTACGATCGGCACCT	
RJ54_412R	GCCGGATTGTTGTGAGAAG	318*
**Restriction enzyme MboII digest patterns for wild type (318 bp, i.e. no digestion) and for mutant genotype (134 bp, 184 bp)*		
Discriminatory PCR		
CMV_412FW1	TGTGCTACGAGACGGGGGG	
CMV_412RW1	CGCGTGAGGATGTACTTCAAGTCAA	280
CMV_412RM2	CGCGTGAGGATGTACTTCAAGTCTG**	
** underlined nucleotide indicates point mutation and deliberate mismatch*		

3 EPIDEMIOLOGY OF CYTOMEGALOVIRUS IN SINGAPORE

3.1 Introduction

CMV is endemic rather than epidemic in all parts of the world. It is present throughout the year, with no seasonal variation seen in infection rates.

Every mammal appears to be infected with its own species-specific CMV, and no evidence exists for infections across species in nature. Hence, humans are the only natural host for CMV infection. Although most adults eventually become infected with CMV, the epidemiology of this infection is complex, and the age at which an individual acquires CMV depends greatly on geographic location, socioeconomic status, cultural factors, and child-rearing practices.

Although CMV is not noted for being highly communicable, any person with a CMV infection can pass it to others throughout life, chiefly via contact with infected secretions. In developed countries congenital CMV infection is the leading cause of congenital viral infection, with a stable incidence in 0.4 to 2.2% of all live births

(Alford et al, 1990). Intrauterine infection occurs in up to 50% of pregnancies following primary maternal infection, and 10% of the infected infants are symptomatic at birth (Stagno et al, 1986). The virus may be transmitted readily to susceptible children via saliva, urine, and fomites; these children, in turn, may transmit infection to their parents.

In adulthood, sexual activity is probably the most important route of acquisition of CMV, though the observation that virus is present in saliva, cervicovaginal secretions, and semen makes it unclear which route or routes of transmission are primarily responsible for establishment of infection. Other important routes of transmission include blood transfusion and solid organ transplantation.

The immune response to CMV in immunocompetent individuals involves synthesis of anti-CMV IgM antibodies several weeks after primary infection and which persist for 3-4 months; this is followed, a few weeks later, by the appearance of anti-CMV IgG antibodies that persist for life (Landolfo et al, 2003).

In Singapore, very few studies have been conducted to estimate the seroprevalence rate for CMV infection. To date, only two seroprevalence studies were conducted locally (Sng and Tobin, 1971;

Wong et al, 2000); these studies focused on and women and children in the former, and on pregnant women in the latter.

Advances in the field of molecular biology have greatly augmented not only the laboratory's capability to detect nonculturable or slow-growing microorganisms and detect antimicrobial resistance but also to monitor a variety of disease processes by quantitation of nucleic acid sequences, as well as evaluate the relatedness of organisms involved in epidemic and nosocomial infections. Specifically, molecular strain characterization aids in the detection of infectious disease outbreaks and helps guide control efforts to protect public health, identify pseudo-outbreaks due to contamination events, and, in the case of a number of infectious diseases, distinguish between reactivation disease and reinfection (Arens, 1999 and 2001; Clarke and Bloor, 2002; Hungnes et al, 2000).

A variety of typing methods have been used, including nucleotide sequencing, Southern blotting, oligonucleotide fingerprint analysis, single-strand conformation polymorphism (SSCP) analysis, heteroduplex mobility assay, or restriction fragment length polymorphisms (RFLP) (Arens, 1999).

PCR-based RFLP methods, in which amplification of selected segments of the viral genome is followed by restriction enzyme digestion of the amplicons, are most widely used in the elucidation of CMV epidemiology.

The importance of CMV strain variability, recognized since 1976, is now receiving renewed interest (Rasmussen, 1999), in particular, as part of investigations into why in hosts with comparable levels of immunosuppression, some CMV infections result in symptomatic CMV disease while others are limited to asymptomatic virus shedding with no discernible clinical consequences.

There has been no previous study on the molecular epidemiology of CMV infection in Singapore. Therefore, in this chapter, we sought to determine the frequency distribution of the genotypes of clinical isolates of CMV directly from blood in Singapore on the basis of envelope glycoprotein gene sequences by PCR-RFLP (Chou and Dennison, 1991).

Additionally, the seroprevalence rate for CMV infection was determined from a cohort of randomly selected and apparently healthy young adults (aged 17 to 24 years) by screening for serological evidence of CMV infection (presence of IgG antibodies) by

immunoassay (ELISA). CMV serostatus is one of the traditional risk factors for early-onset CMV disease and now appears to also contribute to the occurrence of late-onset CMV disease during the first year after transplantation for both solid organ and hematopoietic stem cell recipients (Razonable, 2005). No previous study has also been reported for this age-related cohort in Singapore.

3.2 ***Results***

3.2.1 Cytomegalovirus seroprevalence in healthy young adults and pediatric solid organ transplant subjects

Of the total number of 151 subjects (Table 3.1) tested for CMV antibodies by ELISA, 46.36% (70/151) were CMV IgG seropositive. With respect to gender, 42.47% (31/73) of males and 50.0% (39/78) of females were positive for CMV IgG antibody ($P = 0.18$) (Table 3.2).

Pre-transplant CMV seropositivity for the cohort of 17 pediatric solid organ transplant recipients was 23.52%; all 5 liver transplant subjects were CMV seronegative (Table 3.2).

3.2.2 Molecular epidemiology of cytomegalovirus infection in Singapore

A total of 200 clinical samples (104 from peripheral blood, saliva and bronchoalveolar lavage of 17 pediatric solid organ transplant subjects, and 96 clinical isolates of CMV, Table 3.3) were subjected to PCR amplification of the CMV gB gene.

Seven samples (from renal transplant subjects) did not yield any PCR product (possibly owing to DNA degradation from prolonged storage or amplification inhibition) and were therefore not typed. Restriction enzyme digestion by *Hinf*I and *Rsa*I of the PCR products of the remaining 193 samples showed distinct patterns for gB types 1 – 3 (Figure 3.1). The correct gB type 1 and gB type 2 were obtained for the reference strains Towne and AD169, respectively.

For the 193 clinical samples assayed, the overall frequency distribution of CMV strains on the basis of gB types 1, 2, 3, and 4 was 58.5%, 19.1%, 14.1% and 0%, respectively, with 8.3% of samples containing mixed genotypes (Table 3.3). Pairwise comparisons of the different genotypes were carried out; the percentage of gB type 1 was found to be statistically significant from the other types ($P = 0.03$) for the two cohorts (transplant and non-transplant subjects) surveyed independently as well as when both are combined

Table 3.1. Summary of cohort characteristics for CMV epidemiological study

Cohort	Characteristics
Healthy volunteer subjects (n = 151)	Sera from 73 males and 78 females; mean age = 18.6 ± 1.5 years, median = 19.0 years
Pediatric solid organ transplant subjects with CMV disease (n = 17)	
Liver transplant (n = 5)	CMV DNAs isolated from peripheral whole blood, saliva and bronchoalveolar lavage of 4 males and 1 female
Renal transplant (n = 12)	CMV DNAs isolated from peripheral whole blood, saliva and bronchoalveolar lavage of 12 males and 0 female
Clinical isolates of CMV (n = 96)	Viruses propagated in human foreskin fibroblasts (MRC-5) and saved in frozen (-70°C) storage

Table 3.2. CMV serostatus of healthy young adults and pediatric solid organ transplant subjects

Category	Number tested	Presence of CMV IgG antibody
Healthy young adults		
Male	73	42.47% (31/73)*
Female	78	50.0% (39/78)
Total	151	46.36% (70/151)
Pediatric solid organ transplant subjects (pre-transplant)		
Liver transplant	5	0% (0/5)
Renal transplant	12	33.33% (4/12)
Total	17	23.52% (4/17)

* $P = 0.18$

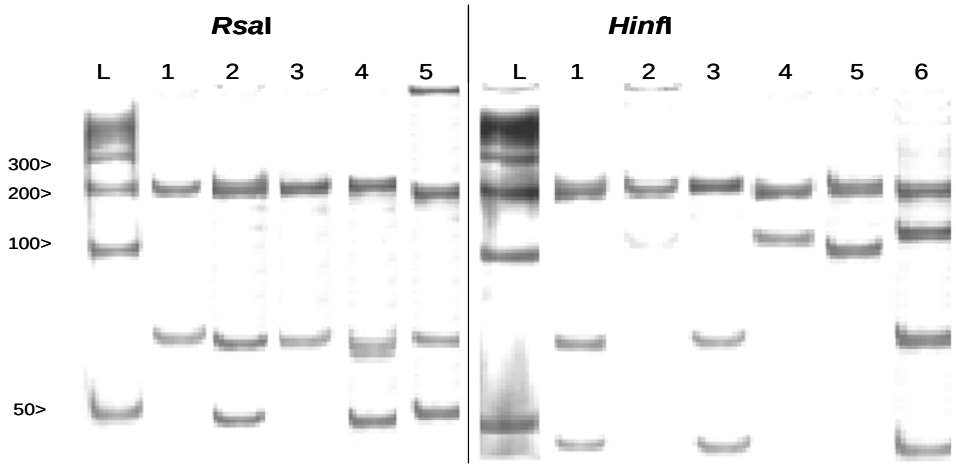

Figure 3.1. Restriction digest profiles of PCR amplified part of CMV envelope glycoprotein B (gB) using (**A**) *Rsa*I. Lanes 1-2: Towne (gB type 1), AD169 (gB type 2); Lanes 3 – 5: CMV isolates types 1 – 3, respectively; Lane L, AmpliSize molecular ruler (Bio-Rad Laboratories, Hercules, CA, USA), and (**B**) *Hinf*I. Lanes 1-2: Towne (gB type 1), AD169 (gB type 2); Lanes 3 – 5: CMV isolates types 1 – 3, respectively, Lane 6: mixed infection (types 2 and 3); Lane L, AmpliSize molecular ruler.

Table 3.3. CMV envelope glycoprotein B (gB) genotype distribution from clinical samples

	gB type				
	1	2	3	4	Mixed types
Cohort A: Pediatric solid organ transplant subjects (n=17)					
Liver transplant (n = 5); 15 clinical samples	5 (33.3)	5 (33.3)	4 (26.7)	0 (0	1 (6.7)
Renal transplant (n = 12); 89 clinical samples received but 82 samples typed (see text)	46 (56.0)	18 (21.9)	12 (14.6)	0 (0)	6 (6.7)
Total: 97 (100%)	51 (52.5)*	23 (23.7)	16 (16.5)	0 (0)	7 (7.2)
Cohort B: Clinical isolates of CMV (n = 96, non-transplant subjects)					
Total: 96 (100%)	62 (64.5)*	14 (14.5)	11 (11.5)	0 (0)	9 (9.4)
Overall Total: 193(100%)	113 (58.5)*	37 (19.1)	27 (14.1)	0 (0)	16 (8.3)

* $P = 0.03$

3.3 Discussion

3.3.1 Cytomegalovirus seroprevalence in healthy young adults and pediatric solid organ transplant subjects

In populations with good socioeconomic status, the seroprevalence of CMV is approximately 40% in adolescents, increasing thereafter by about 1% annually (Griffiths and Baboonian, 1984). Approximately 70% of adults in good socioeconomic conditions eventually become infected with CMV. In contrast, most children in developing countries acquire CMV infection early in life, with adult seroprevalence approaching 100% by early adulthood.

The overall seroprevalence of CMV infection for young adults in Singapore (mean age = 18.6 ± 1.5 years), on the basis of CMV IgG antibody status, is 46.36%. This is not significantly different from the results of a recent study of 12 to 19 year-olds (Staras et al, 2006) in the United States (41.7%, $P = 0.23$).

A recent survey by Wong et al (2000) of Singapore's antenatal population (mean age = 29.2 years) revealed that 87% of 200 patients tested positive for CMV IgG, suggesting that CMV is endemic in Singapore. The percentage obtained is, however, a significant (P = 0.03) drop from the 99% of 100 expectant mothers who tested positive in Sng and Tobin's 1971 survey, reflecting the improvement in socioeconomic conditions and in standards of public health in Singapore. But Singapore's CMV seroprevalence is still high compared to that for Europe (51.5% in France (P = 0.03), 54.4% in London, UK (P = 0.03)), reflecting the high rates seen in the tropics, as also seen in countries like Thailand (100%).

Of the 73 male and 78 females in the study cohort, 42.47% (31/73) of males and 50.0% (39/78) of females were positive for CMV IgG antibody. However, gender and CMV IgG seropositivity were found to be independent of each other (P = 0.18), as was also seen in a study of the blood donor population in Taiwan by Lu et al (1999).

In contrast to the 46.36% seropositive rate in young adults obtained in this study, the pre-transplant CMV seropositive rate of the 17 pediatric solid organ transplant subjects was only 23.52% (Table 3.3).

The trend is similar to that seen in the West, where CMV IgG seropositivity increases with age (Griffths and Baboonian, 1984, Staras et al, 2006).

CMV seronegative patients who receive kidneys from seropositive donors are at highest risk of developing CMV-associated disease (Ho, 1982). Symptomatic disease occurs in 60-80% of these patients with a mortality of 10%. Seropositive patients are not immune from the disease, as up to 30% of them can develop clinical disease, either from reactivation or reinfection.

McLaughlin et al (2005) showed recently that CMV seronegative recipients of renal allografts from CMV seropositive donors (D+/R-) have an increased risk of early allograft loss due to acute rejection even if the D+/R- group does not appear to have poorer renal allograft function 3 years post-transplant. In another study of the incidence and risk factors for CMV disease during the first post-transplant year in a cohort of liver transplant recipients who received antiviral prophylaxis with oral ganciclovir, late CMV disease developed in a substantial proportion of D+/R- recipients after prophylaxis was discontinued and was associated with increased mortality (Limaye et al, 2004). With the majority of the transplant recipients likely to be CMV seropositive (R+), Singh et al (2005) has shown CMV infection

developed in 32.5% of 177 R+ liver transplant recipients, and, in the same study, donor CMV seropositivity is identified as the major risk factor in R- patients.

The increasing trend of CMV seropositivity with age has implications for the management of both seropositive and seronegative, immunocompromised recipients in Singapore. Our data suggests that pediatric transplant recipients, majority of whom is CMV seronegative, are likely to receive organs from adults, who are CMV seropositive donors. In fact, the seropositivity rate of CMV in kidney donors in Singapore is 89.1% (Table 6.2).

3.3.2 Molecular epidemiology of cytomegalovirus infection in Singapore

The CMV envelope glycoprotein B, encoded by UL55, is a major target of neutralizing antibodies as well as cell-mediated immune responses (Britt, 1984, Hopkins et al, 1996). In addition, it has been shown to be involved in the viral entry into cells, cell-to-cell viral transmission and fusion of infected cells (Navarro et al, 1993).

Several studies have been conducted to find a correlation between the gB genotype and the occurrence of CMV-associated disease in immunocompromised patients because it has been observed that in hosts with comparable levels of immunosuppression, some CMV infections result in symptomatic CMV disease while others are limited to asymptomatic virus shedding with no discernible clinical consequences. But it remains unclear whether certain gB genotypes are associated with an increased frequency of disease. Although an earlier study had suggested an association between gB type 2 and development of CMV retinitis in patients with AIDS (Shepp et al, 1996), data from recent studies have not supported this (Gilbert et al, 1999; Drew et al, 2002). Also, Humar et al (2003) have shown that gB genotype did not affect the rate of disease recurrence or occurrence of tissue-invasive disease in solid-organ transplant recipients with CMV disease. Candidate genes other than UL55 and their biological activities should be studied for their contribution to virulence.

On the basis of CMV strains obtained in Singapore (from a cohort of pediatric solid organ transplant patients, and from the two clinical microbiology laboratories in Singapore performing isolation and culture of CMV from non-solid organ transplant patients), the overall frequency distribution (Table 3.3) for gB types 1, 2, 3 and 4 are

58.5%, 19.1%, 14.1% and 0%, respectively; 8.3% of the isolates studied contained mixed types. The percentage of gB type 1 strains isolated is found to be statistically significant from the other types using pairwise comparisons of proportions ($P < 0.05$) for the overall frequency distribution.

This study showed that CMV gB type 1 is also the predominant CMV strain for the pediatric solid organ (both cohorts of liver and renal) transplant subjects in Singapore ($P = 0.03$). This observation is in agreement with other Asian reports of renal transplant recipients in Hong Kong (Woo et al, 1997) and also in immunocompetent patients with CMV infection in Japan (Kashiwagi et al, 2002), whereas gB type 2 CMV strains were more frequently recovered in Brazilian renal transplant recipients (Carraro and Granato, 2003).

A predominance of gB type 1 genotype has also been observed in the majority of American studies of solid organ transplant population (Fries et al, 1994; Rosen et al, 1998; Humar et al, 2003; Carraro and Granato, 2003) although gB type 3 was the mostly frequently observed in liver transplant patients in a Canadian study (Sarcinella et al, 2002). Another exception is the predominance of type 2 among bone marrow transplant recipients (Wada et al, 1997; Woo et al, 1997). A recent study of CMV strains isolated in the pediatric

population, equal numbers of gB types 1 and 3 were obtained (Xanthankos, 2003). Therefore, it remains unclear whether or not gB genotype is a specific CMV-virulence factor with respect to association with a particular type of organ transplantation.

In the Singapore context, our finding was likely a reflection of gB type 1 strain as the predominant CMV genotype in circulation in the population as gB type 1 strains were isolated in 64.5% (versus 14.5%, 11.5% and 9.4% of gB types 2, 3 and mixed types, respectively ($P < 0.05$)) in the non-transplant population (Table 4.3),
Different mechanisms of the response to CMV infection, rather than different degrees of virulence associated with certain gB types might be at work, as shown by the dominance of different gB types in different types of transplant patients (Woo et al, 1997). The pathological damage caused by CMV in renal transplant recipients is believed to be a result of the viral lysis following replication associated with gB type 1. The induction of a strong cell-mediated or cytotoxic immune response may be more strongly associated with gB type 2 CMV strains in bone marrow transplant patients (in whom disease is immunopathological) rather than in renal transplant recipients in whom the immune response is likely to be protective.

The fact that gB is the principal target of neutralizing antibodies towards CMV in humans and is the most highly conserved envelope glycoprotein of human herpesviruses (Marshall et al, 2000) has made it the target of CMV vaccine development. CMV gB vaccines using recombinant gB protein expressed from plasmid DNA and gB expressed in several different viral vectors have been investigated with animal models (Endresz et al, 2001; Wang et al, 2004). Evaluations in humans have been based on a live attenuated gB type 1 (Towne) strain of CMV (Adler et al, 1998; Pass et al, 1999), more specifically its envelope glycoprotein B. The initial safety data from a Phase 1 clinical trial of a CMV immunotherapeutic vaccine by Vical Inc, USA were reported at the Interscience Conference on Antimicrobial Agents and Chemotherapy in November 2004.

More recent clinical trails have shown that the Towne vaccine induced neutralizing antibodies and cell-mediated immunity (both antigen-specific CD4+ and CD8+ T cell responses) mitigated CMV disease in seronegative renal transplant recipients and protected against a low-dose virulent CMV challenge in normal volunteers although it did not prevent infection in mothers of children excreting CMV (Gonczol and Plotkin, 2001; Jacobson et al, 2006).

While CMV gB type 4 isolates were not found in this study (they were also relatively rare in previously published reports), the overall frequency distribution of gB types 2 and 3 in Singapore adds up to 33%. Whether or not the gB type 1 (Towne) vaccine is sufficient to generate cross protection against gB types 2 and 3 strains of CMV remains unknown and warrants investigation.

In 8.3% of all the clinical samples typed, mixtures of at least two different CMV strains were found. This has also been also reported elsewhere for renal transplant recipients (Aquino and Figueiredo, 2000; Coaquette et al, 2004) although there was no association of gB genotypes with the development of symptomatic CMV infection in these two reports. As only select clinical samples for each patient in the cohort of pediatric transplant recipients were available for gB genotyping we were thus unable to determine if there is indeed an association of viral genotype with the development of CMV disease in our local solid organ transplant population.

4 CYTOMEGALOVIRUS ANTIVIRAL RESISTANCE

4.1 Introduction

CMV infections are a major cause of morbidity and mortality among immunocompromised patients, especially recipients of bone marrow and solid-organ transplants and patients with AIDS. In severely immunocompromised patients who develop CMV disease, prolonged antiviral therapy is often necessary. To prevent CMV disease in the transplant population, typically one of two approaches are taken: prophylactic regimens (initiated in all transplant patients, regardless of risk) and pre-emptive regimens (administered only to high risk individuals). Studies have shown that both strategies are effective in decreasing the incidence of CMV disease in solid organ transplants and hematopoietic stem cell transplants (Razonable and Paya, 2003) though there is debate as to which strategy is optimal (Emery, 2001; Hart and Paya, 2001). Antiviral prophylaxis has also been shown to reduce other clinically relevant and indirect CMV-related outcomes such as graft rejection (Lowance et al, 1999).

Preventive strategies via the use of antiviral prophylaxis and pre-emptive therapy have, however, resulted in changing the natural course of CMV infection ('late-onset CMV disease') (Razonable and

Paya, 2003). Also, widespread and prolonged use of antiviral drugs have lead to the emergence of ganciclovir- (and other drug)-resistant CMV infections.

Risk factors for ganciclovir-resistant CMV infection include lack of CMV-specific immunity (i.e. donor CMV-seropositive and recipient CMV-seronegative), high viral replication and a high viral load, multiple episodes of CMV disease, receipt of potent immunosuppression, prolonged antiviral drug administration, and suboptimal tissue-plasma antiviral drug concentration (Limaye et al, 2000).

The majority of infections caused by drug-resistant CMV have been found in patients with AIDS (Erice, 1999). The frequency of these infections has varied among different studies. In one study that analyzed the prevalence of infections caused by ganciclovir-resistant CMV strains in 31 patients with AIDS and CMV retinitis, 5 (38%) of 13 patients who were treated with intravenous ganciclovir for more than 3 months were excreting ganciclovir-resistant CMV strains in their urine (Drew et al, 1991). This represented an overall incidence of ganciclovir resistance of 7.8% in this patient population. A 1997 randomized trial conducted by Studies of Ocular Complications of

AIDS (SOCA), in collaboration with the AIDS Clinical Trials Group, comparing intravenous ganciclovir and foscarnet found ganciclovir-resistant CMV in 5.3% of 207 patients who remained viremic after being treated with ganciclovir for a minimum of 2.5 months. Ganciclovir-resistant CMV infections in AIDS patients who had received prophylaxis or preemptive therapy with oral ganciclovir was 4.8% (Smith et al, 1998). In that study, the detection of these UL97 mutations in plasma was associated with clinical progression.

CMV drug resistance represented a major problem in patients with AIDS until the recent introduction of highly active antiretroviral combination therapy, which dramatically decreased the incidence in this clinical setting (Baldanti et al, 2004). However, CMV resistance to antiviral drugs remains a major problem in the transplantation setting.

The incidence of ganciclovir-resistant CMV infections varies according to the type of organ transplant, with the highest incidence observed in lung and combined kidney-pancreas transplant recipients (9% and 13%, respectively) (Bhorade et al, 2002; Limaye, 2002; Limaye et al, 2002). Studies in the United States indicate that CMV

ganciclovir resistance occurs in 0%, 0.3% and 1% of liver, heart and kidney transplant recipients, respectively (Limaye et al, 2000; Limaye et al, 2002; Bhorade et al, 2002). A Western Australian retrospective study of orthotopic liver transplant patients on universal ganciclovir prophylaxis did not find antiviral resistance in this type of organ transplant (Leong et al, 2004).

Until recently, there were only sporadic cases of ganciclovir-resistant CMV infection among hematopoietic stem cell transplant recipients but it may be an emerging problem in pediatric transplantation. In a case report by Springer et al (2005), two such recipients died with persistent CMV infection complicated by resistance to antivirals, with resistance detected after only 6 weeks of foscarnet treatment in the first patient and after 11 weeks of ganciclovir treatment in the second. Other reports have also suggested that resistance may develop more rapidly in severely immunocompromised children (Wolf et al, 1998).

4.2 Characterization of mutations conferring cytomegalovirus resistance to ganciclovir

Antiviral agents for the treatment of CMV infections include ganciclovir, foscarnet, and cidofovir. All three compounds inhibit CMV DNA synthesis by inhibiting the viral DNA polymerase. Valganciclovir, an orally administered prodrug of the standard drug ganciclovir, is also used for the treatment of AIDS-related CMV retinitis and as prophylaxis of CMV infection and disease in high-risk solid organ transplant recipients (Baliga et al, 2004; Cvetkovic, 2005).

Ganciclovir (9-[1,3-dyhydroxy-2-propoxymethyl]guanine) is a deoxyguanosine analogue that must be phosphorylated to ganciclovir *triphosphate* to exert its antiviral activity (Crumpacker, 1996). The CMV UL97 gene of CMV encodes a viral protein kinase that phosphorylates ganciclovir to ganciclovir monophosphate, with cellular kinases carrying out further phosphorylations to ganciclovir di- and triphosphate. Ganciclovir triphosphate is a competitive inhibitor of the natural substrate (deoxyguanosine triphosphate) for the CMV DNA polymerase. Also, incorporation of ganciclovir

triphosphate into viral DNA inhibits CMV DNA chain elongation (Crumpacker, 1996; Field et al, 1983).

The evidence that CMV could become resistant to antiviral compounds was initially obtained in the laboratory by selecting a ganciclovir-resistant CMV strain after passaging a susceptible reference virus (laboratory CMV strain AD169) in the presence of increasing concentrations of ganciclovir (Biron et al, 1986). Initial studies of resistant laboratory and clinical CMV strains related ganciclovir resistance to inadequate intracellular phosphorylation of the compound in cells infected with ganciclovir-resistant viruses. Although experimental evidence at that time suggested that ganciclovir phosphorylation was controlled by a CMV-encoded function, the mechanism by which ganciclovir is phosphorylated in CMV-infected cells was eventually confirmed by two independent reports providing biochemical, immunological, and genetic evidence that the phosphorylation of ganciclovir in CMV-infected cells is controlled by a protein kinase homologue encoded by the UL97 open reading frame of the virus (Littler et al, 1992; Sullivan et al, 1992).

Analyses of UL97 sequences in clinical CMV isolates resistant to ganciclovir have also demonstrated the presence of point mutations or deletions in this region of the viral genome. To date, the majority of

the ganciclovir-resistant isolates of CMV contained mutations at codon 460, 520, 594 or 595 (Erice, 1999). In fact, resistance mutations are all within a region of about 150 codons or 450 base pairs making genotyping a viable method for antiviral testing. Sequencing of the UL54 gene is much more cumbersome since resistance-conferring mutations occur over a region of about 600 codons or 1800 base pairs.

The aforementioned isolates contained mainly single UL97 mutations, with few isolates containing two mutations in this region (Table 5.1). Marker transfer experiments have shown that the mutations confer ganciclovir resistance when introduced into the genome of recombinant viruses. Similar to the studies done with ganciclovir-resistant laboratory CMV strains, anabolism studies with ganciclovir-resistant clinical CMV isolates have demonstrated that the functional consequence of these UL97 mutations is an impaired intracellular phosphorylation of ganciclovir into ganciclovir monophosphate, with the subsequent lack of synthesis of ganciclovir di- and triphosphate.

A study by Lurain et al (2001) has also demonstrated that, in the complete sequences of the UL97 phosphotransferase genes in 28

phenotypically ganciclovir-sensitive CMV clinical isolates, the gene was highly conserved, with nucleotide sequence identity among strains ranging from 98.6 to 100% and amino acid sequence identity of >99%. Mutations in the UL97 gene eventually lead to decreased levels of the active (tri-phosphorylated) form of ganciclovir so that the CMV polymerase is no longer inhibited.

The CMV DNA polymerase is also the target of the antiviral agents such as ganciclovir. Fully activated ganciclovir is a competitive inhibitor of the DNA polymerase (UL54 gene) and also causes a slowing and then cessation of chain elongation (Arens, 2001). Mutations in the UL54 gene can also directly result in resistance of the virus to these drugs (Table 5.1). Sequence analysis of the DNA polymerase of ganciclovir-resistant CMV strains selected in the laboratory has shown the presence of mutations in this region of the viral genome (Lurain et al, 1992; Sullivan et al, 1993). Recent marker transfer studies by Chou et al (2003) have confirmed CMV DNA polymerase resistance mutations at both conserved and nonconserved loci. It has been postulated that these DNA polymerase mutations could be involved in substrate recognition and could cause drug resistance by decreasing the affinity of the viral polymerase for antiviral compounds.

4.3 Laboratory methods for the diagnosis of ganciclovir-resistant cytomegalovirus

Diagnosis of ganciclovir resistance requires the demonstration of reduced susceptibility of a clinical CMV isolate to ganciclovir *in vitro*. Methods used in the laboratory to determine susceptibilities of CMV isolates to antiviral compounds may be classified as phenotypic or genotypic.

Phenotypic methods are designed to determine the concentration of an antiviral agent that would inhibit the virus in culture. In general, these are culture-based methods in which a known amount of infectious virus is grown in the presence of different concentrations of the antiviral agent.

The plaque reduction assay (PRA) is considered the gold standard for antiviral susceptibility testing of CMV and other viruses (Biron et al, 1986). In this assay, a standardized amount of a stock virus (previously subjected to titer determination in cell culture) is

inoculated into cultures and incubated in the presence of the antiviral agent. The cultures are then observed for the presence of viral plaques. On the basis of the results of phenotypic assays, viruses are classified as susceptible or resistant to a given antiviral compound. Phenotypic assays require a viral isolate, thus prolonging the time necessary for their completion and are not therapeutically useful (Lurain et al, 2001).

Genotypic assays are designed to determine whether mutations known to confer antiviral resistance are present in the genome of the viruses being studied. Generally, genotypic methods involve the use of molecular technologies to obtain sequence information concerning target regions of the viral genome. The clustering of mutations at specific UL97 codons in ganciclovir-resistant CMV isolates has led to the development of diagnostic screening assays based on restriction digest analysis of selected PCR products amplified from CMV-infected cell cultures or directly from clinical specimens (Wolf et al, 1995). These assays are useful when the genetic basis for antiviral resistance has been identified and when there is a predictable relationship between the presence and absence of a genetic variant and measurable antiviral resistance. The significance of mutations found in genotypic studies must be further evaluated by assessing the

effect of each mutation on the phenotype of recombinant viruses and on the structure and function of mutated viral proteins (Chou et al, 2002).

Genotypic assays for resistance to anti-CMV drugs examine two CMV gene products which are implicated in resistance: the CMV UL97 and the UL54 gene products. Point mutations or deletions of portions of UL97 can lead to ganciclovir resistance. To date, the most common mutations conferring ganciclovir resistance are at codons 460, 520, 594, 595 and deletions 595 to 607 (Chou et al, 1995a; Chou et al, 1995b; Erice et al, 1989; Handson et al, 1995; Spector et al, 1995; Wolf et al, 2001 and Wolf et al, 1995).

Ganciclovir-resistant strains can be classified into two groups: those with low resistance to ganciclovir with mutations on the UL97 gene, but wild-type for the UL54 gene; and those with high resistance to the drug, with mutations on both viral genes (Uwe-Vogel et al., 1997).

UL54 mutations, depending on their locations, can confer resistance to one or more antivirals (Chou et al, 2003). The majority of

genotypic assays are PCR-based methods involving restriction digest analysis and/or DNA sequencing analysis. They may be more sensitive than phenotypic assays but may be limited by incomplete characterization of all the mutations that confer resistance either by lack of marker transfer experiments or recombinant virus data.

The main aim of this chapter is to determine the frequency of CMV mutant strains in (i) CMV isolates ("live" cultures) sent to the Virology Laboratories of NUH and the Singapore General Hospital (SGH), where the active programs for solid organ transplantations in Singapore are located and (ii) CMV DNA isolated directly from clinical samples such as blood, saliva and urine for a cohort of pediatric solid organ transplant patients from NUH. Different laboratory methods currently available, including conventional drug susceptibility assay (plaque reduction assay) and previously published genotypic assays (PCR-RFLP, DNA sequencing) would be employed.

Table 4.1. CMV UL97 and UL54 gene mutations and phenotype analysis (adapted from Erice, 1999)

Gene region (codon) and mutation	CMV phenotype*
UL97 (confirmed by marker transfer experiments)	
M460V, M460I, H520Q, 591-594 deleted, A594V, L595S, L595F, 595 deleted, C603W, C607Y	R
Additional UL97 mutations (found in resistant clinical isolates)	
N510S, A590T, A591D, A591V, C592G, A594T, L595T, L595W, E596G, E596D, N597I, G598V, K599M, 600 deleted, C503Y, C606D	R
UL54 (confirmed by marker transfer studies/recombinant virus data)	
F412C, D413E, L501I, A809V	R
L802M	S or R
Additional UL54 mutations (found in resistant clinical isolates)	
N408D, F412V, L501F, T503I, P522S, L545S, G678S, I722V, Y751H, V781I, T821I, V812L, A987G, K513R	R

***** S, Sensitive; R, Resistant

4.4 Results

4.4.1 Drug susceptibility testing by plaque reduction assay (PRA)

Laboratory strains of clinical isolates originally propagated in human foreskin fibroblasts (MRC-5) and saved in frozen (-70°C) storage were subjected to plaque reduction assay (Figure 4.1). Of the total of 96 clinical isolates of CMV re-cultured to prepare stock virus concentrations for the assay, seven (7.3%) either did not show CPE characteristic of CMV (confirmed negative by direct immunofluorescence staining using FITC-conjugated monoclonal anti-CMV (Dako A/S, Denmark)) or failed to produce the requisite 90% cytopathology of the infected cell layer.

IC_{50} values obtained for PRA for the wild type control (laboratory CMV strain AD169) ranged from 1.6 to 2.2µM (mean IC_{50} = 1.9µM, CV% = 72.4), which were within the expected sensitive range (≤ 6µM of ganciclovir). The 89 laboratory strains of clinical isolates of CMV that were tested by PRA had also IC_{50} values below the 6.0uM value for sensitivity, and ranged from 0.95 to 2.4µM (Table 4.2).

4.4.2 Genotypic analysis of the CMV UL97 and UL54 genes

Examination of the extracted DNAs from the 96 clinical isolates of CMV by (a) PCR-RFLP did not reveal any of the UL97 mutations at codons 460, 594, 595 and 520 that were known to confer ganciclovir resistance, and by (b) DNA sequencing of the codons 300 to 1000 region of the CMV polymerase (UL54) gene also did not reveal any mutation, when compared against results obtained for the wild type laboratory CMV strain AD169 (Table 4.2).

As shown in Table 4.2, of the 12 renal transplant patients (comprising a total of 89 clinical samples) screened by PCR followed by restriction analysis, three patients (Patients R1, R2 and R3) harbored CMV strains with mutations in the UL97 gene that were known to confer ganciclovir resistance by clinical studies as well as marker transfer experiments (Chou et al, 1995a; Chou et al, 1995b). These mutations (either as single mutant strain or a mixture of wild type and mutants) were at codons 594 and 595, and were confirmed by DNA sequencing (Table 4.2, Figures 4.2 and 4.5). CMV DNAs from the rest of the patients were wild type genotypes with respect to both UL97 and UL54 genes.

Using the same screening strategies to examine a cohort of five liver transplant patients (with a total of 15 clinical samples comprising blood and liver, colon and sigmoid biopsies), mutant CMV genotypes were obtained for the UL97 gene (in codons 460 and 594) in two patients, Patients L1 and L2 (Table 4.2, Figures 4.2, 4.4 and 4.5). The remaining patients' CMV DNAs exhibited only wild type genotypes for both the UL97 and UL54 genes.

While CMV disease such as retinitis and pneumonitis was found to occur in all transplant patients in this study cohort irregardless of presence or absence of mutant CMV genotypes, all five patients with CMV resistant genotypes also had peripheral blood or bronchoalveolar lavage cellular CMV DNA copy numbies of > 1000 µg DNA, compared to one kidney and one liver transplant patient with > 1000 µg DNA but with wild type CMV genotype. Viral copy number of > 1000 µg DNA has been shown to associate with recrudescence of CMV retinitis in pediatric solid organ transplant recipients (Aw et al, 2000).

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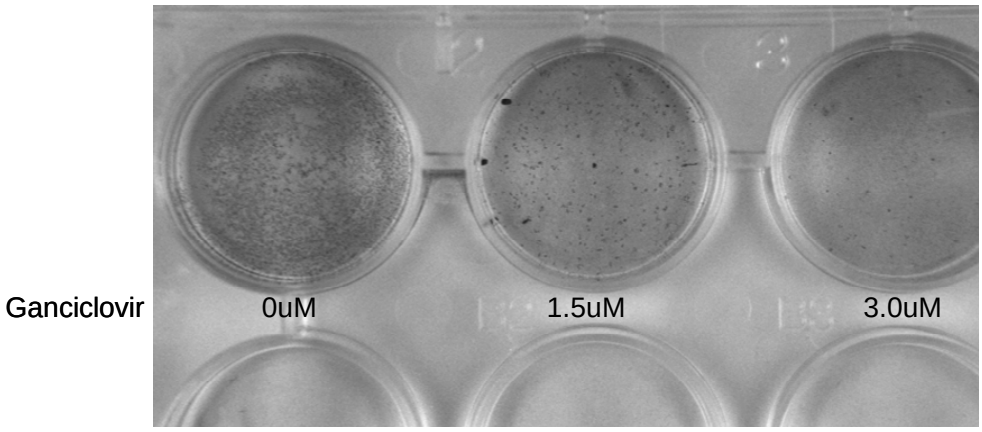

Figure 4.1. Cytomegalovirus susceptibility to ganciclovir by plaque reduction assay. Plaques (cells stained with methylene blue) produced by a laboratory strain from a clinical isolate grown in monolayers of human embryonic fibroblasts under agarose overlay medium with increasing concentrations of ganciclovir.

Table 4.2. Screening results for CMV mutations conferring ganciclovir resistance in (a) pediatric renal and liver transplant patients, and (b) clinical isolates of CMV in Singapore

Cohort A: Pediatric solid organ transplant subjects (n = 17)			
Renal transplant subjects (n = 12; 89 clinical samples assayed)	*Ganciclovir resistant CMV detected in 3/12 subjects by PCR-RFLP*		
	UL97 changes*		
	Position(s); Amino acid(s) changes	Nucleotide change(s)	Remarks
Patient R1	L595S	T to C	mutants or mixture of wild type and mutants
Patient R2	A594V	C to T	mixture of wild type and mutants
Patient R3	A594V, L595S	C to T (codon 594), T to C (codon 595)	mixture of wild type and mutants
Liver transplant subjects (n = 5, 15 clinical samples assayed)	*Ganciclovir resistant CMV detected in 2/5 subjects by PCR-RFLP*		
	UL97 changes*		
	Position(s); Amino acid(s) changes	Nucleotide change(s)	Remarks
Patient L1	M460V	A to G	mutants or a mixture of wild type and mutants
Patient L2	A594V	C to T	mixture of wild type and mutants

*No simultaneous UL54 mutations noted in the clinical samples received for all patients with UL97 mutants at time of screening; UL54 mutation was detected in additional samples received for Patient R1 (see Chapter 6)

Table 5.2. (continued)

Cohort B: Clinical isolates of CMV (n = 96)

Laboratory strains of clinical isolates ("live" cultures) originally propagated in human foreskin fibroblasts (MRC-5) and saved in frozen (-70°C) storage	7/96 of isolates did not show CPE or produce 90% cytopathology of infected cell layer; 89/96 of isolates tested by plaque reduction assay (PRA). No ganciclovir resistance demonstrated. IC_{50} values = 0.95 – 2.4µM (mean IC_{50} = 1.9µM, CV% = 72.4). IC_{50} values for laboratory CMV strain AD169 = 1.6 – 2.2µM. Extracted DNAs from 96/96 clinical isolates tested by PCR-RFLP and DNA sequencing for ganciclovir resistance. No UL97 or UL54 mutations conferring ganciclovir resistance detected.

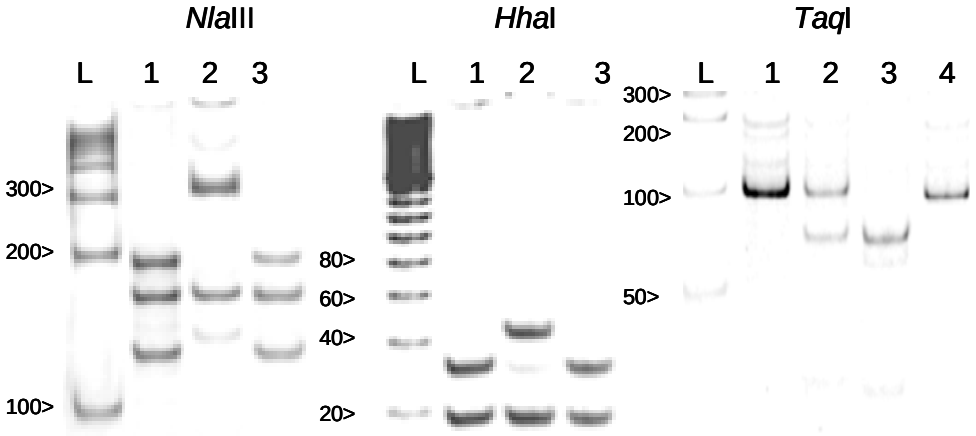

Figure 4.2. Genotypic analyses of CMV UL97 gene by PCR-RFLP for M460V, A594V and L595S mutations that confer ganciclovir resistance. (a) *Nla*III restriction digests of PCR amplified CMV UL97. Lanes 1, 2 and 3 contain CMV strains from a patient who harboured wild type CMV, patient L1 (M460V mutant) and wild type laboratory control CMV AD169, respectively. Lane L, AmpliSize molecular ruler (Bio-Rad Laboratories, Hercules, CA, USA); (b) *Hha*I restriction digests of PCR amplified CMV UL97. Lanes 1, 2 and 3 contain CMV strains from a patient has wild type CMV, patient R3 (both A594V mutant and wild type CMV) and wild type laboratory control CMV AD169, respectively. Lane L, 20bp EZ Load molecular ruler (Bio-Rad Laboratories, Hercules, CA, USA); (c) *Taq*I restriction digests of PCR amplified CMV UL97. Wild type CMV strains isolated from a patient with wild type CMV and the laboratory control (AD169, wild type) are shown in lanes 1 and 4, respectively. CMV strains present in patient R1 are either a mixture of wild type and L595S mutant strains (lane 2) or mutant strains alone (lane 3). Lane L, AmpliSize molecular ruler (Bio-Rad Laboratories).

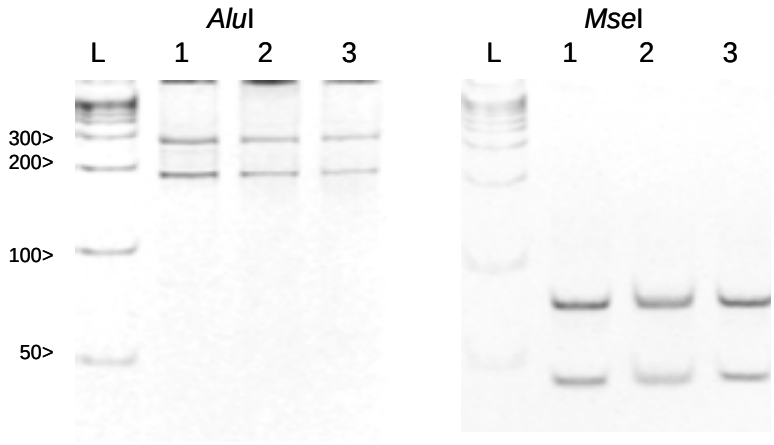

Figure 4.3. Genotypic analyses of CMV UL97 gene codons by PCR-RFLP for H520Q and L595F mutations. (a) *Alu*I restriction digests of PCR amplified CMV UL97. Lanes 1, 2 and 3 contain CMV strains from two patients with the wild type and the laboratory control CMV AD169 strain, respectively. Lane L, AmpliSize molecular ruler; (b) *Mse*I restriction digests of PCR amplified CMV UL97. Lanes 1, 2 and 3 contain CMV strains from two patients with the wild type and the laboratory control CMV AD169, respectively. . Lane L, AmpliSize molecular ruler (Bio-Rad Laboratories).

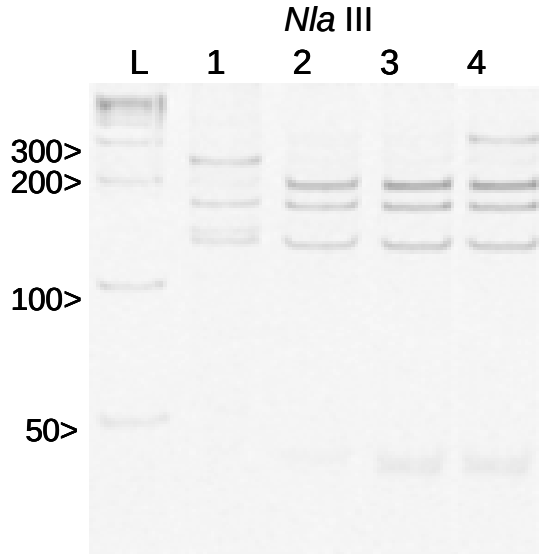

Figure 4.4. Genotypic analyses of CMV UL97 gene codons by *Nla*III restriction digests of PCR amplified CMV UL97 for M460V mutations. Lanes 1, 2, 3 and 4 contain CMV strains from a patient with wild type variant, a patient with the wild type, laboratory control CMV AD169, and patient L1 with the mutant CMV, respectively. Lane L, AmpliSize molecular ruler (Bio-Rad Laboratories).

	460		594 595
AD169	ACACCCATGAACGTG	……	TGCCGCGCGTTGGAGAAC
	Met		Cys Arg Ala Leu Glu Asn
M460V	GTG	……	
	Val		
A594V		……	GTG
			Val
L595S		……	TCG
			Ser

Figure 4.5. CMV UL 97 gene in the vicinity of codons 460, 594 and 595. Sequence of reference (laboratory control CMV strain AD169) is shown, aligned with sequence of mutant strains M460V, A594V and L595S.

4.5 Discussion

Plaque reduction assay has been considered the gold standard for antiviral susceptibility testing (Wentworth and French, 1970) and is used successfully for rapidly growing, cell-free clinical isolates of herpes simplex virus. CMV presents a more challenging problem because clinical isolates are often slow growing and cell associated. This study illustrates several of its limitations. Time required for viral isolation (in particular in CMV, noted for its slow growth) and subsequent performance of the assay typically required several weeks at a minimum to perform, itself a disadvantage in guiding clinical decisions.

In the preparation of viral stocks for the PRA assay in this study, 7.3% (7/96) of the clinical isolates of CMV either did not show CPE characteristic of CMV or failed to produce the requisite 90% cytopathology of the infected cell layer in spite of multiple passages in culture. As adequate amounts of virus were not achieved, the PRA assays were therefore not carried out for these clinical isolates. This inherent difficulty in CMV drug susceptibility testing has been noted elsewhere by Landry et al (2000) and Jabs et al (1998a; 1998b).

Nevertheless, the 89/96 laboratory strains of clinical isolates of CMV examined by this in vitro method were all phenotypically non-resistant to ganciclovir. The IC_{50} values obtained were in the range of 0.95 to 2.4μM of ganciclovir, against the non-ganciclovir resistant laboratory control CMV strain AD169's average value of 1.9μM. The expected sensitive range is ≤ 6μM of ganciclovir.

However, it has been demonstrated that certain CMV viruses that were susceptible in phenotypic assays actually contained UL97 or UL54 mutations conferring antiviral resistance. One study reported a clinical isolate of the CMV virus with a A594V mutation in the UL97 gene that was susceptible to ganciclovir by PRA (Wolf et al, 1995), and, in another study, a CMV isolate with a similar UL97 codon 594 mutation, obtained from a bone marrow transplant recipient, was also susceptible to ganciclovir using a genotypic assay for ganciclovir resistance (Erice et al, 1998). Resistance mutations affect the growth rate of the CMV virus and growth attenuation has been reported elsewhere for mutant strains (Baldanti et al, 1996). Thus, slow growing mutant viruses may affect phenotypic assays such as the PRA, whereby the delayed appearance of plaques may be misinterpreted as drug susceptibility.

The two genotypic methods (namely PCR-RFLP and DNA sequencing) adopted in this study were also utilized for ganciclovir resistance

screening of CMV DNAs extracted from these clinical isolates. However, no mutations known to confer resistance were detected by these methods.

In contrast, screening CMV DNAs extracted directly from blood of a cohort of pediatric solid organ transplant subjects revealed the presence of UL97 gene mutations conferring ganciclovir resistance in three of 12 (25%) renal and two of five (40%) liver transplant patients (Table 4.2). Owing to the small sample numbers per transplant type, it is not possible to draw comparisons between the percentages obtained and data from previously studies. Nonetheless, the mutations detected all occurred in the two strongly clustered sites (at codons 460 and 594 and 595, corresponding to subdomains VI and IX, respectively) in the UL97 phosphotransferase gene where all known UL97 mutations occur (Table 4.1).

This study confirms the presence of ganciclovir resistance in CMV strains in Singapore. It also demonstrates – in the mutations at UL97 codons 460, 594 and 595 that were detected in CMV strains obtained locally - that the majority of the types of mutations in ganciclovir-resistant isolates of CMV were genotypically similar to those occurring in the West, particularly from the immunocompromised patients (Erice, 1999).

It was also noted that either mutant or mutant and wild type strains of the CMV virus were isolated from the cohort screened. As was also observed by Erice et al (1998), immunocompromised patients such as transplant patients can harbor multiple isolates, including the mixed populations of mutant and wild type strains.

The fact that mutant CMV strains were not detected by the PRA assay but by the genotypic screening method could be attributed to the improved sensitivity of the PCR based method. In a previous study, mutants could be detected only when they reach 10% of the viral population in a clinical isolate of CMV (Chou et al, 1995b).

The discrepancy between a resistant genotype (with a mutant UL97 or UL54 sequence) as determined by a method such as PCR-RFLP and a susceptible phenotype, as demonstrated by the PRA assay, could also be explained by the fact that *in vitro* anti-viral susceptibility assays have a lower sensitivity than that for PCR-based amplification methods for specific amplification and detection of antiviral resistance mutations.

Additionally, the co-existence of multiple isolates of the virus in a patient may make it possible that culture-based susceptibility assays

will select a different virus population from that selected by PCR, thereby producing different sets of results. .

A disadvantage of this PCR-RFLP method is that specific mutations involving base changes that do not result in a change in the restriction enzyme pattern may not be detected. Also, strain variation at *Nla*III sites other than codon 460 occasionally resulted in a variant restriction pattern but this was easily distinguished from that of the M460V mutation (Figure 4.4). In this study, the wild type variant CMV strain was observed in patients who harbored CMV that were either ganciclovir resistant or sensitive or both, and thus, there did not appear to be any association between their occurrence and susceptibility or resistance to ganciclovir.

Resistance of CMV to antiviral agents is a well-documented complication of long-term antiviral therapy. This problem has been observed mostly in patients with AIDS and CMV retinitis, in whom drug-resistant CMV infections have been associated with clinical progression and therapeutic failure. Currently available data suggest that the incidence of infections caused by drug-resistant CMV in bone marrow and solid organ transplants is low. In a study by Boivin et al (1993), none of 33 blood or tissue CMV isolates recovered from solid-organ transplant recipients who developed CMV viremia after receiving

prophylaxis and or treatment with intravenous ganciclovir were resistant to this drug. Gilbert et al (2001) showed that following preemptive ganciclovir therapy in allogeneic stem cell transplant recipients, there was no emergence of CMV UL97 mutations conferring ganciclovir resistance

Nevertheless, with the extensive use of ganciclovir as prophylaxis and or prolonged preemptive therapy and the use of oral ganciclovir, reports of emergence of drug-resistant mutants in the various transplant populations have accumulated in recent years (Chou, 1999; Singh, 2001; Limaye, 2002).

In a 2000 retrospective study by Limaye et al of solid organ transplant recipients who had received oral ganciclovir and highly potent immunosuppression, ganciclovir-resistant CMV disease developed in five (7%) of 67 seronegative recipients of CMV-seropositive organs (D+/R-) compared with none of 173 seropositive recipients (P = 0.002). Among the 25 (10.4%) patients who developed CMV disease within one year after transplantation, five had ganciclovir-resistant CMV disease.

We have also demonstrated the presence of CMV ganciclovir resistance in Singapore in three of 12 (25%) renal transplant recipients.

A retrospective study of all the clinical samples received from the first such case (Patient R1, Appendix A) showed emergence of the ganciclovir resistant CMV approximately seven months post-transplantation (Figure 7.1). Wild type strains of CMV were eventually replaced by a mixture of both resistant and wild type strains in a renal transplant recipient on long-term anti-CMV prophylaxis and treatment in immunocompromised patients. The patient experienced several episodes of severe CMV disease, leading to the administration of repetitive courses of antiviral therapy. Selective pressure allowed for strains of CMV with a point mutation (TTG-to-TCG; Leu-to-Ser at codon 595 of the UL97 gene) to co-circulate with wild type CMV.

Our data also showed that ganciclovir resistance is also extended to CMV strains associated with liver transplantation with clinical samples from two of five (40%) of liver transplant patients containing CMV strains with UL97 gene mutations.

To date, on the basis of a PubMed index search, no molecular studies on CMV resistance to ganciclovir have been conducted in Singapore or in the region. There is a single Japanese report (Harada et al, 1997) of a clinical isolate of CMV exhibiting ganciclovir resistance, in the UL54 polymerase gene.

Our findings confirm the presence of ganciclovir-resistant CMV strains in Singapore. That they are found in a cohort of transplant patients who are on ganciclovir prophylaxis (and not in the non-transplant population) warrants the adoption of antiviral screening assays for these patients as the assays have the potential to guide therapy and assist in the confirmatory diagnosis of clinical antiviral resistance.

Based on our own experience, a culture-based susceptibility assay such as the PRA was laborious compared to the relative ease of use and shorter turnaround time of the PCR-RFLP test. Of particular usefulness will be molecular assays that can be performed on DNA extracted directly from clinical samples and that also are amenable to automation.

5 DEVELOPMENT OF RAPID NUCLEIC ACID TESTS FOR DETECTION OF GANCICLOVIR RESISTANCE IN CYTOMEGALOVIRUS

5.1 Introduction

Traditional methods for detection of microbial drug resistance follow the basic paradigm of isolation of a microorganism in pure culture followed by detection of phenotypic drug resistance by one or more methods, for example, plaque reduction assay for resistance to antiviral agents. These methods are invariably labor intensive and time-consuming. Delays in providing timely results for effective patient management and treatment can be substantial.

CMV resistance studies have been limited by the difficulty and lack of standardization of antiviral susceptibility assays although efforts are being made towards the development of a standardized plaque reduction assay for CMV cell-associated clinical isolates (Landry et al, 2000). The recognition that specific mutations in the UL97 and UL54 genes of CMV are associated with different antiviral susceptibility patterns has prompted the development of molecular laboratory methods for detection of mutant viral sequences in viral isolates and directly in clinical specimens. The incorporation of these methods in

the routine of diagnostic virology laboratories have contributed to a more rapid and sensitive detection of drug-resistant CMV strains.

5.2 Current and new strategies for the genotypic detection of mutations conferring ganciclovir resistance in cytomegalovirus

While phenotypic assays have the advantage of measuring the growth characteristics of a virus even in the context of a complex assortment of mutations in which it may be very difficult to sort out and identify their individual actions and collective interactions. Genotyping, on the other hand, can be a fairly simple, straightforward procedure that yields clinically useful information at a reasonable cost and in a short amount of time and is routinely performed on the nucleic acid extracted from patient plasma, thus precluding the necessity of an *in vitro* culture. The major requirement for interpretation of genotypes for resistance testing is that the location of mutations that confer resistance must be known and there should be some concept of which mutations confer what level of resistance.

For all practical purposes, there are only four viruses for which a genotypic analysis is clinically useful, HBV, HCV, CMV and HIV. This is mostly a function of the fact that there are not many viral

infections that can be treated with antiviral drugs and thus there are few opportunities for viruses to become resistant to antiviral drugs (Arens, 2001).

Currently the majority of genotypic screening tests for ganciclovir resistance are based on PCR, entailing amplification of the region of UL97 gene surrounding specific codons where mutations are known to occur, followed by restriction digest analysis of the PCR products. Mutations at specific UL97 codons in conserved regions of the gene in ganciclovir-resistant CMV isolates are in the form of amino acid deletions and or substitutions, and result either in the loss or acquisition of naturally occurring restriction enzyme sites. Distinctive restriction patterns can be visualized by gel electrophoresis and ethidium bromide staining (Table 2.1). This screening method can detect mutants when they reach 10% of the viral population in a clinical isolate; in one study, it identified 78% of the isolates resistant to ganciclovir in phenotypic assays (Chou et al, 1995b). A major advantage of this restriction digestion analysis is that preliminary results can be obtained in less than 48 hours (compared to a minimum of six to seven weeks for the plaque reduction assay). This method (using a nested PCR format) can be applied directly to clinical samples such as leukocytes, plasma and cerebrospinal fluid. In addition, direct genotypic detection of a mutant strain of the virus indicates that the

mutation is not due to *in vitro* phenotypic selection. A limitation of phenotypic methods for sensitivity testing of CMV strains is that the selection process during growth in cell culture may lead to changes in the features of the virus present in the original clinical sample. Also, the virus in blood or urine may be different from the virus causing the disease (for example, in the case of CMV retinitis) and it is thus questionable how representative a virus isolated in cell culture is for the disease causing virus in the retina.

However, a drawback of this PCR-RFLP method is that specific mutations involving base changes that do not result in a change in the restriction enzyme pattern may not be detected. Also, it does not detect ganciclovir-resistant CMV isolates containing DNA polymerase mutations but wild-type UL97 sequences (Erice et al, 1997). In other words, while a positive result is virtually diagnostic of ganciclovir resistance, a negative result does not necessarily indicate that a virus is susceptible to ganciclovir.

Confirmed ganciclovir resistance mutations on the CMV DNA polymerase gene occur at both conserved and nonconserved loci (Chou et al, 2004). These are mainly point mutations with the exception of a two-codon (codons 981 and 982) deletion (Chou et al, 2000). At

present, the method of choice for detection of these mutations is by sequencing the relevant portions of the UL54 gene.

The aims of this chapter were to (a) improve on existent PCR-RFLP methods for mutation analysis in the CMV UL97 gene and (b) develop assays for analysis of mutations in the CMV UL54 gene that obviate the need for DNA sequencing. A secondary aim of this chapter was to discover novel mutations that may also confer ganciclovir resistance.

For mutation analysis in the CMV UL97 phosphotransferase gene, we sought to develop rapid PCR-based methods using the current conventional PCR assay as well as the more recently available real time PCR platforms. PCR-RFLP as a screening method can detect mutants when they reach 10% of the viral population in a clinical isolate (Chou et al, 1995b); we sought to improve on this rate of detection as well as the turnaround time for reporting of results by simultaneous and direct detection of mutant and wild type genotypes in the same clinical samples.

Although DNA sequencing is now less costly than in the past, there is still the need to invest in and maintain an automated DNA sequencer. In Singapore and neighboring countries, this facility is usually only available in research laboratories. Thus, routine analysis of CMV

UL97 DNA polymerase to detect ganciclovir resistance is not often possible. To date, there is a limited number of publications on the use of non-DNA sequencing methods such as restriction enzyme analysis of PCR fragments for mutation analysis in the CMV UL54 polymerase gene (Harada et al, 1997; Chou et al, 1998). We therefore also aimed to develop PCR-based screening assays that did not necessitate the use of DNA sequencing.

5.3 Results

5.3.1 CMV UL97 mutation analysis by discriminatory PCR

We developed rapid molecular assays for the detection of these point mutations by a novel discriminatory PCR assay strategy involving two sets of amplifications, one each for detection of wild type and of mutant sequences. The first set of primers, designed using the Primer 3 software program, was a pair (CMV_594R and CMV_594LW for analysis of codon 594, CMV_595R and CMV_595LW for codon 595, Table 2.2), corresponding to the sequence of the reference strain AD169. This allowed for amplification and therefore detection of wild type sequences (amplification product size 269 bp and 161 bp, respectively) but not A594V or L595S mutants that contain a single nucleotide substitution (C to T or T to C) at positions 1781 and 1784, respectively. The second set of primers (CMV_594R and CMV_594LM for codon 594, CMV_595R and CMV_595LM for codon 595) included either a 3' substitution of A for G nucleotide on primer CMV_594LM or a 3' substitution of G for A to enable detection of the L595S mutant sequence but not the wild type for the respective codons (Figures 6.1 and 6.2). Therefore, by simultaneously performing two PCR reactions for each unknown clinical sample, this assay can

differentiate between wild type and mutant genotypes, and, if amplification products are detected in both reactions, the detection of mixed populations of wild type and mutant CMV strains.

In order to reduce the turnaround time, a two-step protocol (versus the standard three-step) was devised, combing the annealing and elongation step of the PCR cycle. Selection of the optimal annealing temperature is often the most critical component in the optimization phase of PCR assay development to achieve increased specificity and minimize mispriming and amplification of non-target sequences (Harris and Jones, 1997).

During the assay development, the thermal gradient functionality of the iCycler thermal cycler – which allowed programming of a temperature gradient in increments of 0.5°C across the thermal cycling block - was exploited to determine the best annealing/extension temperature for amplification of the desired sequence (for example, the wild type) but not the non-specific product (for example, the mutant genotype) or artefacts and secondary products such as primer-dimers.

These newly developed PCR assays were performed on 40 clinical samples randomly selected from our study cohort of renal and liver transplant recipients. There was complete concordance with the results obtained previously by PCR-RFLP methods for ganciclovir resistance with respect to detection of wild type, mutant or a mixture of wild type and mutant genotypes (Table 6.1).

5.3.2 CMV UL97 mutation analysis by real time PCR using molecular beacons

Nucleic acids isolated from 40 blood samples (from the same study cohort of solid organ transplant recipients) were subject to real-time PCR molecular beacon analysis for the presence of the A to G mutation in codon 460 of the UL97 CMV gene. Each sample was simultaneously analyzed in two separate reaction wells, each well containing PCR reaction mixture with either the wild-type beacon or the mutant beacon. Fluorescence was measured during every cycle of the PCR using the 490 and 530 filters for FAM and HEX, respectively. Comparison of the multiple amplification curves was made following normalization of the fluorescence for each molecular beacon as previously described (Smit et al, 2001). In brief, the formula $(F - F_{min})/(F_{max} - F_{min})$ is used, where F is the measured fluorescence, and

F_{min} and F_{max} are the minimum and maximum fluorescence, respectively.

Figure 5.3 shows the amplification curves obtained, after normalization of fluorescence, with PCR amplifications using both molecular beacons of three different patient samples that harbor wild-type strains of CMV (sample a), both wild type and mutant strains (sample b) or mutant strains alone (sample c) with respect to codon 460 of the UL97 gene.

The results indicate that PCR amplification of CMV in each sample produced an increase in the fluorescence of only the molecular beacon that was complementary to the intended target DNA. For instance, there was increase in fluorescence when wild-type beacon was tested against samples a and b (containing wild-type CMV and a mixed population, respectively) but not against sample c (which contained no wild-type CMV). When the mutant beacon was tested against the same samples, only samples b and c (which contained the mixed population and mutant CMV, respectively), displayed increase in fluorescence. Simultaneous fluorescence of both molecular beacons in sample b indicated the presence in sample c of both wild-type and mutant strains of the virus. Thus the genotypes of different combinations of CMV genomes could be correctly predicted by this strategy. It was also observed that samples negative for CMV DNA as well as negative

(nuclease-free water) controls did not generate any fluorescence for either beacon.

Analysis of the scatter plot of fluorescence values received in the last PCR cycle for each clinical sample tested against both wild type and mutant molecular beacons (Figure 5.4) show three distinct clusters, reflecting the three possible combinations of wild type and mutant CMV strains in any given sample with CMV DNA. .

Table 5.2 summarizes the results obtained by both PCR-RFLP and real-time PCR analysis by molecular beacons for the detection of wild-type and mutant strains in clinical samples. With one exception, the results obtained by molecular beacon analysis show general concordance with the other molecular approach for the clinical samples tested with the exception of one sample which was genotyped as mixture of wild-type and mutant strains by molecular beacons but as wild type only by PCR-RFLP.

To further evaluate the ability of the molecular beacon assay to discriminate genotype artificial mixtures of different percentages of M460V mutant and wild-type (AD169) strains of the virus of known copy numbers were prepared and assayed in triplicates. The M460V

mutants were generated by mismatched primer mutagenesis using wild type CMV AD169 as template and newly designed primers.

The results are comparable to PCR-RFLP and indicate that a sample with approximately 10% or more of the mutant strains against a background population of wild type virus can also be correctly clustered in the wild type/mutant group (Figure 5.4). However, molecular beacon assay was able to identify artificial mixtures at a ratio of 95:5 wild-type to mutant strains in two out of the triplicate samples whereas all samples were genotyped as wild type using PCR-RFLP.

5.3.3 Analysis of CMV UL54 F412C mutation by PCR-RFLP

We designed primers for PCR amplification of the CMVL UL54 gene region surrounding codon 412 yielding a 318 bp amplicons, to be followed by its digestion by *Mbo*II to produce a single fragment (318 bp) for wild type CMV and two fragments (134 and 184 bp) for mutant strains (Table 2.5).

Screening 52 clinical samples using this PCR-RFLP assay, two of three samples containing a mixture of wild type and F412C mutant CMV

strains were correctly genotyped when compared with the results obtained by DNA sequencing (Table 5.3, Figure 5.7). Arrow (bottom figure) indicates the 1235 position (T) of the UL54 gene and shows a T to C substitution (resulting in an amino acid change from phenylalanine to cysteine in codon 412.

5.3.4 Analysis of CMV UL54 F412C mutation by discriminatory PCR

Applying a strategy similar to that of the discriminatory PCR-based assay for detection of CMV UL97 gene mutations, we designed primers for and developed a discriminatory PCR assay for direct, rapid detection of the F412C mutation in the UL54 gene from clinical samples. Primer set CMV_412FW1 and CMV_412RW1 were designed to only anneal to and amply wild type sequences whereas primer set CMV_412FW1 and CMV_412RM2 contain, in the3' end of latter primer, a 'wobble' sequence (TG) which allowed for annealing to complementary mutant sequence but not to the wild type owing to the mismatch sequence (Table 2.5). As shown in Figure 5.6, this assay allowed for discrimination between wild type and mutant templates as well as for identification of templates with mixed populations of viruses.

Compared with results obtained by DNA sequencing of 52 samples from the same cohort of transplant patients that was also screened using the PCR-RFLP method, there was complete agreement between DNA sequencing and this newly developed PCR method. And unlike the PCR-RFLP method, where the restriction patterns were ambiguous and the genotype for 13 samples could not be determined, this discriminatory PCR assay was able to correctly identify wild type strains and to distinguish them from samples with mixed populations of wild type viruses and viruses with the F412C mutation, previously confirmed by DNA sequencing (Table 5.3, Figure 5.7).

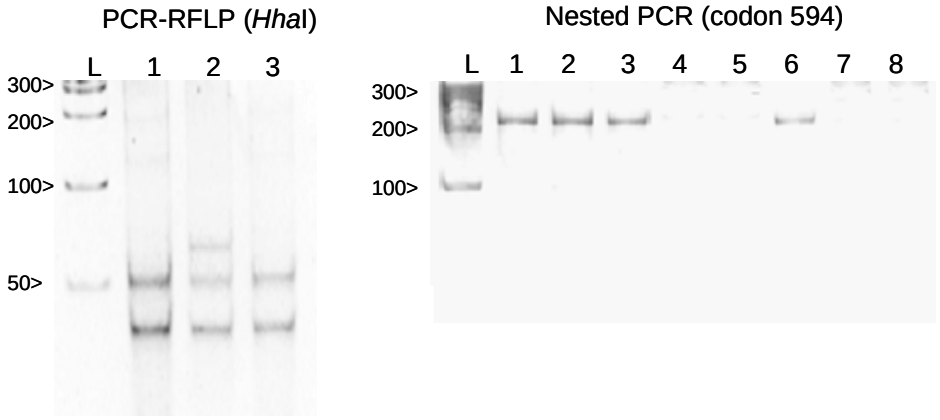

Figure 5.1. (a) *Hha*I restriction digests of PCR amplified CMV UL97. Lanes 1, 2 and 3 contain CMV strains from patient X (wild type), patient L2 (both A594V mutant and wild types) and control (AD169, wild type) strain, respectively. Lane L, AmpliSize molecular ruler. (b) Products of discriminatory PCR assay for wild type and A594V mutant CMV UL97. CMV strains from patient X, patient L2, positive control (AD169) plus a negative control (water) are present in lanes 1, 2, 3 and 4 and lanes 5, 6, 7, 8, respectively. Lane L, EZ Load 100 bp molecular ruler (Bio-Rad Laboratories, Hercules, CA, USA)

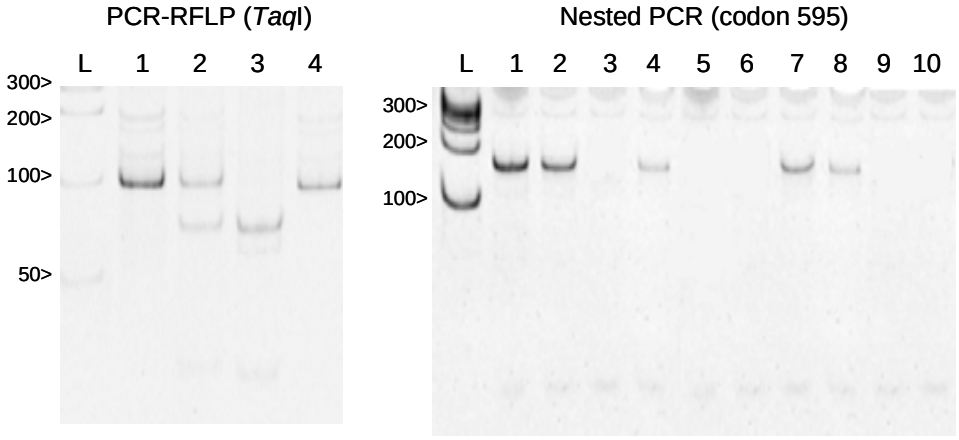

Figure 5.2. (a) *Taq*I restriction digests of PCR amplified CMV UL97. Wild type CMV strains isolated from patient X and the control (AD169) are shown in lanes 1 and 4, respectively. CMV strains present in patient A are either a mixture of wild type and L595S mutant strains (lane 2) or mutant strains alone (lane 3). Lane L, AmpliSize molecular ruler. (b) Products of discriminatory PCR assay for wild type and L595S mutant CMV UL97. CMV strains from patient X (wild type), patient R1 (mixed population), patient R1 (mutant alone) and positive control (AD169) plus a negative control (water) are present in lanes 1, 2, 3, 4 and 5 and lanes 6, 7, 8, 9 and 10, respectively. Lane L, EZ Load 100 bp molecular ruler.

Table 5.1. Comparison of PCR-RFLP and novel two-step discriminatory PCR (D-PCR) assays for codons 594 and 596 UL97 CMV gene mutation analysis.

	UL97 gene region (codon 594)		UL97 gene region (codon 595)	
	PCR-RFLP	D-PCR	PCR-RFLP	D-PCR
Clinical samples				
Wild-type strain only	37	37	38	38
Mixed (both mutant & wild type strains)	3	3	1	1
Mutant strain only	1	1	1	1
Total number	40	40	40	40

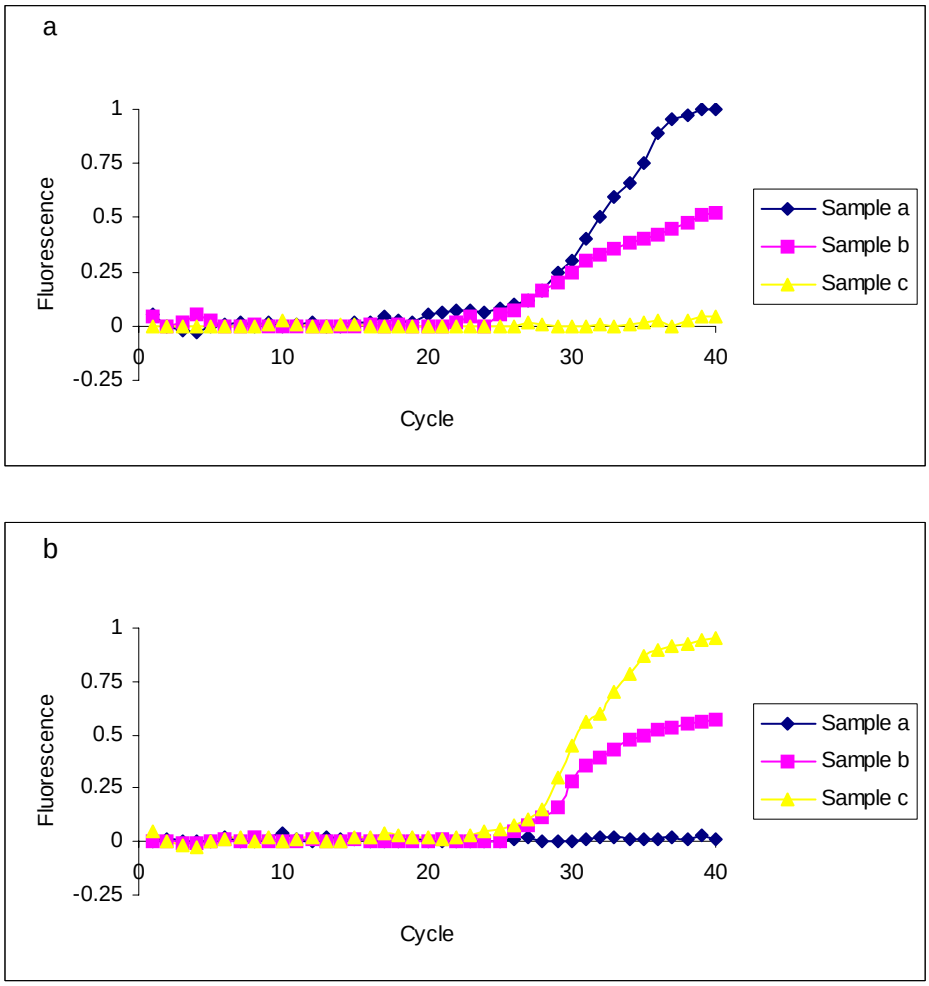

Figure5.3. Real-time PCR amplification plots of three different viral genotypes with respect to codon 460 of the UL9 CMV gene: sample a (wild-type CMV only), sample b (both wild-type and mutant CMV, sample c (mutant CMV only). All samples were probed with both wild-type and mutant molecular beacons (top and bottom graphs, respectively). Normalized fluorescence of both the wild-type and mutant beacons is plotted on the y-axis and the number of PCR cycles on the x-axis.

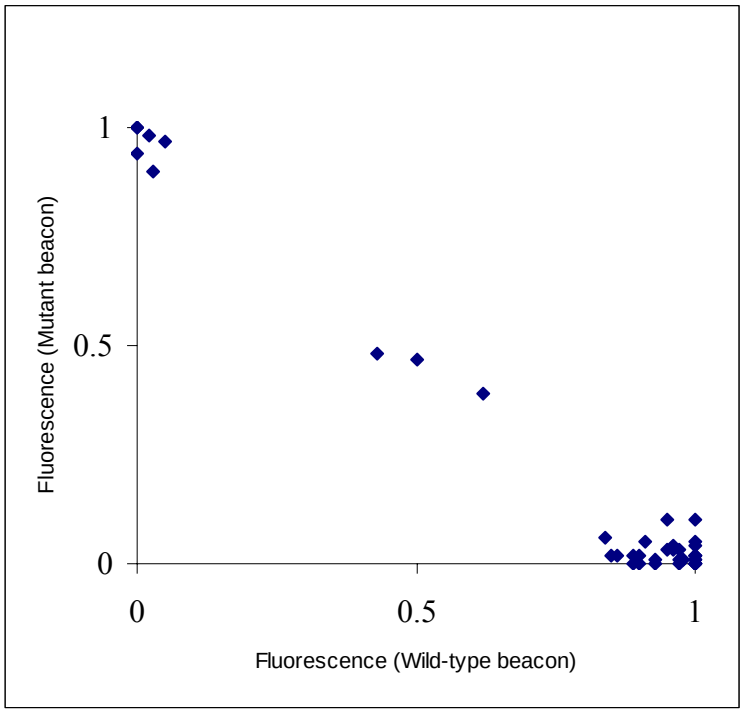

Figure 5.4. Scatter plot of 40 clinical samples based on fluorescence values obtained at the last PCR cycle when analyzed by real-time PCR using both wild-type and mutant molecular beacons. The three clusters obtained correspond to the three possible genotypes.

Table 5.2. Comparison of two DNA-based methods (PCR-RFLP and real-time PCR using molecular beacons) for codon 460 UL97 CMV gene mutation analysis.

	PCR-RFLP	Molecular beacons PCR
A. Clinical samples		
Wild-type strain only	31	30
Mixed (both mutant & wild type strains)	3	4
Mutant strain only	6	6
Total number	40	40
B. Artificial mixtures (% CMV AD169 wild type to mutant strains, each combination assayed in triplicates)	Genotype detected	Genotype detected
100:0	Wild-type (3/3)	Wild-type (3/3)
95:5	Wild-type (3/3)	Mixed (2/3) Wild-type (1/3)
90:10	Mixed (3/3)	Mixed (3/3)
80:20	Mixed (3/3)	Mixed (3/3)
70:30	Mixed (3/3)	Mixed (3/3)
0:100	Mutant (3/3)	Mutant (3/3)

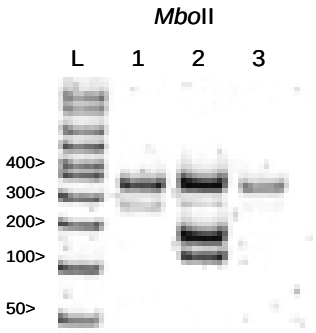

Figure 5.5. *Mbo*II restriction digests of PCR amplified CMV UL54 for codon 412 mutation analysis. Lanes 1, 2 and 3 contain CMV strains from patient X (wild type), patient R4 (both F412C mutant and wild types) and control (AD169, wild type) strain, respectively. Lane L, AmpliSize molecular ruler.

Table 5.3. Comparison of DNA sequencing analysis with newly developed PCR-RFLP and discriminatory PCR assays for F412C mutation in the CMV UL54 gene.

	DNA sequencing	PCR-RFLP	Discriminatory PCR
Clinical samples			
Wild-type strain only	49	37	49
Mixed (both mutant & wild type strains)	3	2	3
Mutant strain only	0	0	0
'Indeterminate'	-	13	-
Total number	52	52	52

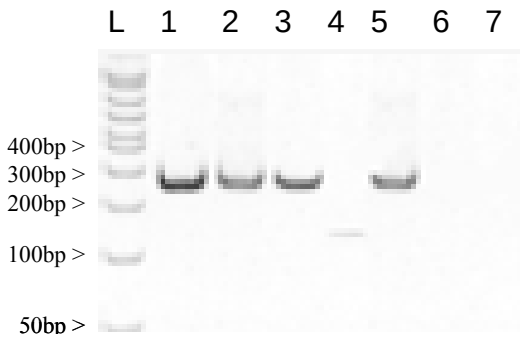

Figure 5.6. Products of discriminatory PCR assays for CMV UL54 codon 412 mutation analysis. CMV strains from patient 1 (wild type), patient 2 (mixed of wild type and mutant populations) and wild type positive control (AD169) are present in lanes 1, 2, 3, respectively for PCR using wild type primer set (CMV_UL54FW1 and CMV_UL54RW1), and also in lanes 4, 5 and 6, respectively, for PCR using primer set (CMV_UL54FW1 and CMV_UL54RM2) for amplification of mutants. Lane 7 is a negative (water) control. Lane L, EZ Load 100 bp molecular ruler.

Figure 5.7. DNA sequence alignment using Jellyfish software version 1.4 for CMV UL54 codon 412. ul54 refers to published UL54 sequence for wild type CMV AD169; A is wild type patient sample. Arrow (top figure) indicates the 1235 position (T) of the UL54 gene. E is patient sample with mutant F412C genotype. Black color arrow (bottom figure) indicates the 1235 position (T) of the UL54 gene and shows a T to C substitution. Red color arrow (bottom figure) points to G to A change at position 1237.

5.4 Discussion

5.4.1 CMV UL97 mutation analysis by discriminatory PCR

The most common CMV UL97 codons 594 and 595 mutations conferring ganciclovir resistance involve single nucleotide substations at positions 1781 and 1784 (Erice, 2000) which result in amino acid changes from Ala to Val and from Leu to Ser, respectively.

A novel discriminatory PCR assay strategy was devised for detection of these point mutations. This involves two sets of newly designed primers, the first set of which allowed for amplification and therefore detection of wild type sequences (amplification product size 269 bp and 161 bp, respectively) but not A594V or L595S mutants that contain a single nucleotide substitution (C to T or T to C) at positions 1781 and 1784, respectively. The second set of primers enables detection of the L595S mutant sequence but not the wild type for the respective codons (Figures 5.1 and 5.2). Furthermore, a two-step protocol (versus the standard three-step) was devised, combing the annealing and elongation step of the PCR cycle. Selection of the optimal annealing temperature via use of the thermal gradient functionality of the iCycler

thermal cycler was an important component in the optimization phase of PCR assay development to achieve increased specificity and minimize mispriming and amplification of non-target sequences. Complete concordance was observed with the results obtained previously by PCR-RFLP methods for ganciclovir resistance with respect to detection of wild type, mutant or a mixture of wild type and mutant genotypes (Table 5.1).

Mismatches resulting in non-authentic priming events in these assays were controlled in our assay by optimising the annealing/extension temperature for mutation analysis of each UL97 codon by gradient PCR selection to allow for reliable analysis of CMV strains of unknown status. This carefully selected temperature allowed for maximal stringency in the pairing between, for instance, a reverse primer (CMV_594LW) designed for wild type codon 594 sequences but a strongly destabilized 3' terminal guanosine when this primer is paired with a mutant sequence containing thymidine instead of cytosine in position 1781 of the UL97 gene.

Alain et al (1997) described the use of a conventional three-step discriminatory PCR protocol for detection of the M460V mutation in

the CMV UL97 gene without the necessity of restriction analysis. With the two-step discriminatory PCR protocol for detection of A594V and L595S mutations, we have expanded on the repertoire of PCR-based assays available for rapid detection of ganciclovir resistance directly on clinical samples.

5.4.2 CMV UL97 mutation analysis by real time PCR using molecular beacons

A rapid real-time PCR assay using molecular beacons for the simultaneous detection of wild-type and mutant CMV viruses with respect to codon 460 of the UL97 gene has been developed. The molecular beacons were designed to complement the wild-type codon 460 or the mutant sequence arising from ATG to GTG change (methionine to valine amino acid substitution). We elected to perform the detection of wild type and mutant CMV strains in each sample simultaneously but in separate reaction tubes in order to avoid competition and other problems inherent in multiplex PCR assays (Elnifro et al, 2000).

Discrimination between wild-type and mutant templates was demonstrated as the beacons did not generate fluorescence with their

respective mismatch targets but only with those that they were designed to perfectly anneal with (Figure 5.3). In fact, samples that harbor mixed populations of wild type and mutant viruses could also be recognized by examining the amplification curve (Figure 5.3) or by a scatter plot (Figure 5.4). The mixed genotypes are indicated by a separate (central) cluster hat is distinguishable from the clusters of wild type and mutant genotypes when the fluorescence values of the last PCR cycle obtained using either wild type or mutant molecular beacon were plotted.

Applied to a small number of clinical samples, the retrospective mutation screening results generated by the molecular beacons real-time PCR assays are in general concordance with that obtained by PCR-RFLP. Using molecular beacons strategy, codon 460 mutation was detected in ten out of the total number of 40 samples, whereas the latter method identified nine samples as containing the mutation.

The discrepant result arose from the genotyping of one of the clinical sample as mixed (containing both wild-type and mutant CMV Strains) by molecular beacons but as wild-type by PCR-RFLP. It has been reported that PCR-RFLP is able to detect mutant CMV templates only when the percentage of mutants in the viral population reaches 10% (Chou et al, 1995a). Hence, genotyping by use of a more sensitive

probe-based PCR amplification method such as molecular beacons is probably more accurate.

In a separate experiment using artificial mixtures of wild-type and mutant CMV templates of varying percentages (Table 5.2), we demonstrated that the molecular beacons used in this study were able to correctly and reproducibly genotype samples that contained 10% or more mutant templates against a wild-type background. However, we observed that two out of three samples in the triplicates containing mixtures of 5% mutant CMV DNA templates could also be unambiguously genotyped by this method.

The improved sensitivity of real-time over conventional PCR for the detection of viruses in routine and research laboratories has been documented and attributed mainly to improved detection by fluorogenic chemistries such as molecular beacons as well as advances in instrumentation (Mackay et al, 2002; Abravaya et al, 2003; Poddar, 2000). Our data is also in support of this observation, and indicate that the molecular beacons assay is possibly more sensitive for mutation analysis of codon 460 mutations than PCR-RFLP.

As real-time PCR also allows for quantitation of the copy number or amount of starting template, an area for further investigation with this

new technology is the determination of the exact percentage of mutants in the viral population in an isolate that is correspondent to the appearance of clinical resistance to ganciclovir.

5.4.3 Analysis of CMV UL54 F412C mutation by PCR-RFLP and discriminatory PCR

Published data (reviewed by Erice, 1999) indicate that susceptibility patterns of CMV to antiviral agents are dependent upon whether the CMV strain contains mutations only in UL97, only in UL54, or in both genes. The general observation is that CMV strains with only UL97 mutations are resistant to ganciclovir but susceptible to foscarnet and cidofovir, whereas CMV strains with certain UL54 mutations are cross-resistant to ganciclovir and cidofovir, and CMV strains containing UL97 and UL54 mutations (double-mutant strains) are highly resistant to ganciclovir on the basis of IC_{50} values obtained for the corresponding clinical isolates (Smith et al, 1997).

The mutation in codon 412 consists of a single-base change at position 1893(G to T) of the UL54 gene, which results in a change from phenylalanine to valine at residue 412 (F412C mutation). This mutation is located in the conserved region IV, and it has been

suggested this DNA polymerase mutation could be involved in substrate recognition and could cause drug resistance by decreasing the affinity of the viral polymerase for antiviral compounds (Lurain et al, 1992; Sullivan et al, 1993). Antiviral susceptibility studies have revealed that these CMV strains were resistant to ganciclovir, susceptible to foscarnet, and resistant to cidofovir (Chou et al, 1997; Smith et al, 1997).

A survey of published data revealed that DNA sequencing is extensively and almost exclusively used for detection of mutations in codons within the CMV UL54 gene. Whilst automated DNA sequencers are now available and the cost per run is reduced, its utility remains limited to research institutes or clinical laboratories with adjunct research and development facilities.

Chou et al (1999) have recommended that genotypic assays for UL54 resistance should focus on the codon ranges 379 to 421, 492 to 545, 696 to 845, and 978 to 988, based on confirmed marker transfer experiments. However, to date, there have only been two publications citing a PCR-based method (Harada et al, 1997; Chou et al, 1998) for mutation analysis in the UL54 gene at codons 501 and 809, respectively. It is with these reasons in mind that the study aimed to develop PCR-based methods which are rapid and relatively less

complex to perform for the detection of mutations in other regions of the UL54 genes.

PCR-RFLP entails PCR amplification followed by restriction analysis of the amplified product at specific recognition sites that may or may not be affected by changes such as point mutation, inversion and deletion. The presence and absence of fragments resulting from changes in recognition sites are used identifying wildtype and mutant strains.

Analysis of the published sequence of the UL54 gene at codon 412 where a known mutation (F412C) exists showed a potential *Mbo*II recognition site ($^{v}(N)_{7}$ TCTTC) for the mutant sequence (TCT) but not in the wildtype (TTT). The T to C substitution results in amino acid change from phenylalanine to cysteine. We therefore designed primers for PCR amplification of the gene region surrounding codon 412 followed by *Mbo*II digestion to produce distinguishable electrophoretic patterns, namely a single fragment (for wild type CMV and two fragments for mutant strains, distinguishable u (Figure 5.5).

The mutant F412C genotype was detected in two samples (sputum) belonging to patient R1, a renal transplant recipient whom we have previously also shown, in screening of his blood samples, to harbor

mixed populations of wild type CMV strains and a L595S mutation in the UL97 gene by PCR-RFLP and DNA sequencing (Chapter 3) as well as the discriminatory PCR assay we had developed. UL54 polymerase mutations usually appear after prolonged therapy and may increase the drug resistance beyond that conferred by a preexisting UL97 mutation (Smith et al, 1997). The detection of the F412C in the sputum of patient R1 approximately a year after ganciclovir treatment had been initiated and the earlier appearance of a UL97 mutation in codon 595 confirms this observation.

DNA sequencing of the region surrounding codon 412 of the UL54 gene also revealed nucleotide change (G to A) at position 1237 (Figure 5.7), one codon downstream of codon 412, leading to a change in amino acid from aspartic acid (GAC, for wild type genotype) to asparagine (AAC).

This appears to be a novel mutation. A D413E mutation (aspartic acid to glutamic acid) had previously been reported (Erice et al, 1997) in a blood isolate for an AIDS patient with CMV colitis and retinitis and shown by marker transfer studies to confer ganciclovir resistance (Chou et al, 2004). There have been no other reports of any codon 413 changes in this conserved region IV of the viral DNA polymerase.

This mutation was seen in the sample of Patient R1 who demonstrated clinical resistance to ganciclovir, in addition to CMV disease markers such as pneumonitis and retinitis (Appendix A). But whether or not this particular mutation confers ganciclovir resistance can not be ascertained at this juncture as it appeared in clinical samples that also contained CMV strains with other, known ganciclovir-resistant markers (namely L595S in the UL97 gene and F412C in the UL54 gene). Multiple mutations may occur in a viral genomes as large as that of CMV (230 kb) so new mutations suspected of conferring drug resistance requires validation experimentally by a process of marker transfer whereby the mutation is transferred to a drug-susceptible CMV laboratory strain (Chou et al, 2003). Marker transfer study of this new mutation is thus warranted.

In the screening of 52 clinical samples, randomly selected from our study cohort of renal and liver transplant recipients, by this PCR-RFLP method, non-specific restriction of PCR products yielding DNA fragment sizes other than the expected 134 bp and 184 bp was encountered for 13 of the clinical samples, rendered as 'indeterminate' (Table 5.3). For these samples, it was difficult to distinguish between a true mutation, a mixed population (both mutant and wild type strains present) and non-specificity owing to restriction sites resulting from polymorphism in this particular region of the UL54 gene.

Harada et al (1997) have also reported unusual restriction digest patterns in the assay they had developed for genotyping codon 501. Indeed it has been shown that while no amino acid variation exists for codon 412 other than the F412C mutation, interstrain variations exist for the polymerase gene, with a total of 282 variant nucleotides, of which greater than 80% produce silent mutations (Chou et al, 1999). Changes within the region amplified by PCR which produce additional recognition sites for *Mbo*II will therefore result in variant restriction digest patterns.

Difficulties encountered by this PCR-RFLP approach to genotyping prompted us to seek an alternative PCR-based method.

We had previously developed rapid, discriminatory PCR assays for detection UL97 mutations conferring ganciclovir resistance (see Section 6.4.1). Screening samples obtained from a cohort of paediatric solid organ transplant patients for A594V and L595S mutants, we have also shown complete concordance with results obtained by published PCR-RFLP methods (Tables 5.1 and 5.2).

Applying a strategy similar to that of the discriminatory PCR-based assay for detection of CMV UL97 gene mutations, we designed primers

for and developed a discriminatory PCR assay for direct, rapid detection of the F412C mutation in the UL54 gene from clinical samples. As shown in Figure 5.6, this assay allowed for discrimination between wild type and mutant templates as well as for identification of templates with mixed populations of viruses.

We compared results obtained by DNA sequencing of 52 samples from the same cohort of transplant patients and by the PCR-RFLP method, there was complete agreement between DNA sequencing and this newly developed PCR method (Table 5.3). Restriction patterns were ambiguous for the PCR-RFLP method and the genotype for 13 samples could not be determined whereas this newly developed discriminatory PCR assay was able to correctly identify wild type strains and to distinguish them from samples with mixed populations of wild type viruses and viruses with the F412C mutation, previously confirmed by DNA sequencing (Table 5.3, Figure 5.7).

A major problem with PCR, however, is that all polymerases invariably generate errors during amplification (Fujimura et al, 1990). Polymerase misincorporations invariably become disguised as mutations and result in false positives (Reiss et al, 1990). To this end, we have attempted to minimize amplification errors by developing PCR-based assays that allow for direct detection from clinical samples

in addition to confirmation of positive results by RFLP (restriction digests) and DNA sequencing. RFLP-based methods used for detection of known mutations examine few sites for mutations relative to mutation scanning methods such as denaturation gradient gel electrophoresis (DGGE). Therefore, in principle they are less affected by PCR errors since at the particular DNA position the polymerase is likely to cause less misincorporation (Makrigiorgos, 2004). This is also valid for real-time PCR-based detection of known mutations (Neoh et al, 1999).

To this end, we propose a strategy for laboratory detection of CMV ganciclovir resistance using a variety of molecular assays. Following detection of total CMV by PCR in whole blood (and other sample types) screening of the common mutations in the UL97 gene using PCR-RFLP, discriminatory PCR or real-time PCR strategies, or a combination of strategies to detect select mutations rather than all possible mutations simultaneously will – with its short, same-day turnaround time – enable correlation with clinical suspicion of antiviral resistance. Laboratory confirmation of the mutation detected can subsequently be made by DNA sequencing, utilizing Sanger or dye terminator sequencing or even pyrosequencing.

6 ROLE OF CMV INFECTION IN CHRONIC ALLOGRAFT DYSFUNCTION

6.1 Introduction

Renal transplantation has become the preferred treatment modality for patients with end-stage renal disease. Over the past two decades, the success rates of renal transplantation have improved with the introduction of a number of new and effective immunosuppressive agents into clinical use, particularly in the 1990s. Short-term outcomes have improved with decline in the incidence of early graft failures and the number of acute rejection episodes but, despite these advances, evidence exists that long-term outcomes have not improved in a similar manner (Hariharan et al, 2000). Recent studies by Meier-Kriesche et al (2003; 2004) have shown that there is little or no impact in the improvement in long term outcomes.

Chronic rejection is currently the most prevalent cause of renal transplant failure (Joosten et al, 2005). While studies have shown that episodes of acute rejection are major risk factors for chronic rejection, various nonimmunologic factors have been found to be important as well (Pascual et al, 2002). As multiple factors are now known to play a part in chronic rejection, the term "chronic allograft nephropathy

(CAN)" has been introduced (Halloran et al, 1999). In fact, CAN remains the most common cause of late graft loss in renal transplantation (Joosten et al, 2005; Mekeel et al, 2006, Pascual et al, 2002).

Clinically, chronic rejection is characterized by a slow loss of function (demonstrated by a reduction in the glomerular filtration rate or, more commonly, rising serum creatinine levels), often accompanied by low-grade proteinuria and hypertension, usually occurring months or years after transplantation (Halloran et al, 1999; Morris, 1999). A biopsy is often obtained to help determine the etiology of graft dysfunction. The histopathology is not specific in most cases but transplant arteriopathy, chronic transplant glomerulopathy, tubular atrophy, and interstitial fibrosis are characteristic (Colvin, 1996).

The pathogenesis of CAN involves both immunologic (antigen-dependent) and nonimmunologic (antigen-independent) mechanisms (Table 7.2). CAN develops in grafts that undergo intermittent or persistent damage from cellular and humoral responses resulting from indirect recognition of alloantigens. Poggio et al (2004) examined alloreactivity in renal transplant recipients with and without CAN and concluded that persistent cell-mediated and humoral alloimmunity

contribute to the development of CAN, and that direct, donor-specific cellular immunity predominated in patients with CAN.

Nonimmunologic factors also have a role in the development of CAN. At the tissue level, acute peri-transplantational injuries causing delayed graft function and the role of senescence conditioned by ischemia/reperfusion may contribute to the development of chronic allograft nephropathy (Halloran, 1999; Joosten et al, 2005). The age of the donor, the quality of the graft, and the number of nephrons in the donor organ have been implicated as important predictors of the long-term survival of the graft (Halloran, 1999). Histopathologic findings from 2-year protocol biopsies from a U.S. multicenter kidney transplant trial comparing tacrolimus versus cyclosporine suggest that drug-induced toxicity resulting from long-term treatment with calcineurin inhibitors – in addition to other nonimmunologic factors such as CMV infection in the first year – may also play a part in CAN (Solez, 1998).

Infection by CMV in the allograft recipient continues to be a serious complication of transplantation owing to long term use of immunosuppressive regimes to prevent rejection of the transplanted kidney from the host's immune system. While the newer immunosuppressive agents have been able to induce a greater degree of

tolerance for the allograft, their use has also resulted in the undermining of the body's ability to contain opportunistic infections such as CMV. Polyomavirus-associated nephropathy is an emerging disease in renal transplant patients with variable prevalence of 1-10% and graft loss up to 80%; BK virus is the primary etiologic agent, but JC virus and possibly simian virus SV40 may account for some cases (Hirsch et al, 2006).

While CMV infection has been shown to have a high association with chronic rejection in extra renal transplants such as heart/lung transplants, there is conflicting evidence on the role of CMV infection in chronic allograft rejection in renal transplantation.

The proposed mechanisms by which CMV induces an alteration of the cellular and humoral responses to the allograft include upregulation of HLA class I and II-like antigens, and production of anti-endothelial cell antibodies (AECA), shown previously to be deleterious to the allograft (Jordan et al, 1988; Yap et al, 1988). Assessment of CMV infection of the renal allograft as well as identification of other contributory factors to the process of chronic rejection is therefore important.

In this chapter, we sought to determine if CMV infection in the renal allograft contributes significantly to chronic allograft dysfunction in renal transplant recipients by:

1. Examining the potential factors that may be associated with chronic allograft dysfunction in a cohort of renal transplant recipients at one year post transplant, including the presence of CMV DNA in the blood of transplant recipients within the first five month post transplant using logistic regression analysis, and;
2. Determining the relationship between anti- endothelial cell antibodies (AECA) and CMV infection in the same post transplant period.

Table 6.1. Putative risk factors for chronic allograft nephropathy (adapted from Sahadevan and Kasiske, 2005)

Immunologic risk factors
Acute rejection
Histocompatability mismatch
Prior sensitization
Suboptimal immunosuppression
Medication noncompliance
Ongoing humoral injury
Non-immunologic risk factors
Ischemic injury and delayed graft function
Older donor age
Donor and recipient size mismatching
Calcineurin-inhibitor nephrotoxicity
Hyperlipidemia
Hypertension
Cigarette smoking
Hyperhomocysteinemia
Oxygen free radicals
Infection
Proteinuria

6.2 Results

6.2.1 Clinical characteristics of the study group

A total of 119 consecutive renal allograft recipients were enrolled in the study. The population characteristics of the patients in the study are shown in Table 6.2. The mean age (± SD) at the time of renal transplantation was 41.5 years (± 9.4 years). In terms of transplant type, 79.4% (94/119) had a cadaveric donor transplant while 20.6% (25/119) underwent live (related and unrelated) donor transplant. Of the study population 20.7% (25/119) received either ATG or OKT3 in addition to the standard induction immunosuppression regimen of methyl prednisolone, azathioprine or mycophenolate mofetil and cyclosporin A. At least one acute rejection episode was observed in 35.7% (42/119) of the patients. Twenty-seven percent (32/119) of the study population also received intravenous ganciclovir either prophylactically or as a form of treatment in CMV infection. Donor and recipient CMV seropositivity were 89.1% (106/119) and 95.8% (114/119), respectively. Mean serum creatinine of the study group at one year post-transplant was 136 μmol/L (± 45 μmol/L). Defining chronic allograft dysfunction at one-year post transplant as serum creatinine greater than 150 μmol/L, 38.6% (46/119) of the study

population had chronic allograft dysfunction at one year post-transplant.

6.2.2 CMV DNAemia in the pre- and post-transplant periods

The detection of CMV DNA in blood by quantitative PCR was monitored for 119 renal allograft transplant recipients who had whole blood drawn consecutively during pre-transplant and at day one, months one, three and five post-transplant. Negative CMV DNAemia was interpreted as CMV levels at less than 100 copies, and positive CMV DNAemia as CMV levels at 100 or more copies of CMV DNA/μg of total DNA in whole blood. Percentages of recipients with or without CMV DNAemia were plotted against time (in the pre- and post-transplant periods) for recipients grouped according to status of renal allograft function at one year post-transplant (as defined by (a) serum creatinine <150 μmol/L and (b) serum creatinine ≥150 μmol/L) (Table 6.3 and Figures 6.1). As shown in Figure 7.1 (a), patients classified as having a normal functioning allograft at one year post-transplant were able to clear CMV DNAemia by five months post-transplant; on the other hand, there was an increasing trend in the percentages of patients with allograft dysfunction who were positive for CMV DNA in blood, with 61.9% having positive CMV DNAemia

at five months post-transplant, compared to 38.1% in the subgroup of patients without allograft dysfunction ($P < 0.01$) (Figure 6.1 (b)).

6.2.3 Statistical analysis of risk factors for development of chronic allograft rejection at one year post-transplant

Univariate analysis employing chi-square test showed a significant correlation between chronic allograft dysfunction at one year post-transplant and presence of CMV DNAemia at five months post-transplant ($P < 0.01$) but not the other periods pre or post-transplant (Table 6.4). There was no significant association between renal allograft dysfunction and transplantation type, previous acute rejection episodes, or the administration of anti-CD3 monoclonal antibody, anti-thymoglobulin, mycophenolate mofetil and ganciclovir (Table 6.3).

With chronic allograft dysfunction as the dependent variable in a multivariate logistic regression analysis, positive CMV DNAemia was again a significant risk factor ($P = 0.04$; OR 2.864, 95% CI 1.046-7.839).

6.2.4 Anti-endothelial cell antibodies (AECA) activity in sera

Fifty one of the renal allograft recipients had sera available at five months post-transplant for the EIA assay for AECA. Twenty-four percent (12/51) of these patients' sera were positive for IgG specific AECA activity as compared to 6% of controls ($P < 0.01$), and 29% (15/51) of sera were also positive for IgM specific AECA activity, compared to 9% of controls ($P < 0.01$). The levels of AECA activity in the transplant recipient and control groups, expressed as the standardized score, are shown in Figure 6.2.

Chi square analysis of these patients, re-grouped as recipients who were positive or negative for CMV DNAemia at five months post-transplant, versus AECA activities revealed a significant association between the presence of CMV DNAemia and presence of IgG specific activity ($P < 0.01$) (Table 6.5). There was, however, no statistically significant association with IgM specific AECA activity ($P = 0.31$).

Table 6.2. Characteristics of the study population (n = 119)

	No. (%)
Gender	
i. Male	70 (58.8)
ii. Female	49 (41.2)
Race	
i. Chinese	95 (79.8)
ii. Non-Chinese	24 (20.1)
Mean age at transplantation (years) (± SD)	41.5 (± 9.4)
Transplant type	
i. Cadaveric	94 (79.4)
ii. Live donor	25 (20.6)
Acute rejection episodes	
i. No rejection episodes	77 (64.3)
ii ≥1 episode	42 (35.7)
Ganciclovir prophylaxis and/ or treatment	32 (27)
Mean serum creatinine at one year post-transplant (150 µmol/L) (± SD)	136 (± 45)
CMV serostatus	
i. Donor seropositive	106 (89.1)
ii. Recipient seropositive	114 (95.8)

Table 6.3 Potential risk factors that may be associated with chronic allograft dysfunction in a cohort of renal transplant recipients at one year post transplant.

Factor	Renal allograft function at one year post-transplant, measured by		*P* value*
	Serum creatinine <150 µmol/L	Serum creatinine ≥150 µmol/L	
Transplant type			
i. Cadaveric	62.7%	37.3%	0.38
ii. Live donor	72.7%	27.3%	
Acute rejection episodes			
i. No rejection episodes	71.8%	28.2%	0.09
ii. ≥1 episode	56.5%	43.5%	
Immunosuppression type			
i. Anti-CD3 monoclonal antibodies (OKT3)	50.0%	50.0%	0.30
ii. Anti-thymocyte globulin (ATG)	58.3%	41.7%	0.62
iii. OKT3 and/ or ATG	52.2%	47.8%	0.17
iv. Mycophenolate mofetil	73.2%	26.8%	0.16
Ganciclovir	56.7%	43.3%	0.27

*Univariate analysis using chi square test

Table 6.4 CMV DNAemia in the pre- and post-transplant periods for renal allograft recipients, grouped according to graft function at one year post-transplant, as measured by (a) serum creatinine <150 µmol/L and (b) serum creatinine ≥150 µmol/L.

Factor	Renal allograft function at one year post-transplant, measured by		*P* value*
	Serum creatinine <150 µmol/L	Serum creatinine ≥150 µmol/L	
CMV DNAemia			
Pre-transplant			
i) Negative	60.0%	40.0%	0.25
ii)Positive	83.3%	16.7%	
Post-transplant Day 1			
i) Negative	60.8%	39.2%	0.72
ii) Positive	66.7%	33.3%	
Post-transplant Month 1			
i) Negative	61.0%	39.0%	0.82
ii) Positive	63.3%	36.7%	
Post-transplant Month 3			
i) Negative	62.3%	37.7%	0.73
ii) Positive	58.6%	41.4%	
Post-transplant Month 5			
i) Negative	70.1%	29.9%	<0.01
ii) Positive	38.1%	61.9%	

*Univariate analysis using chi square test

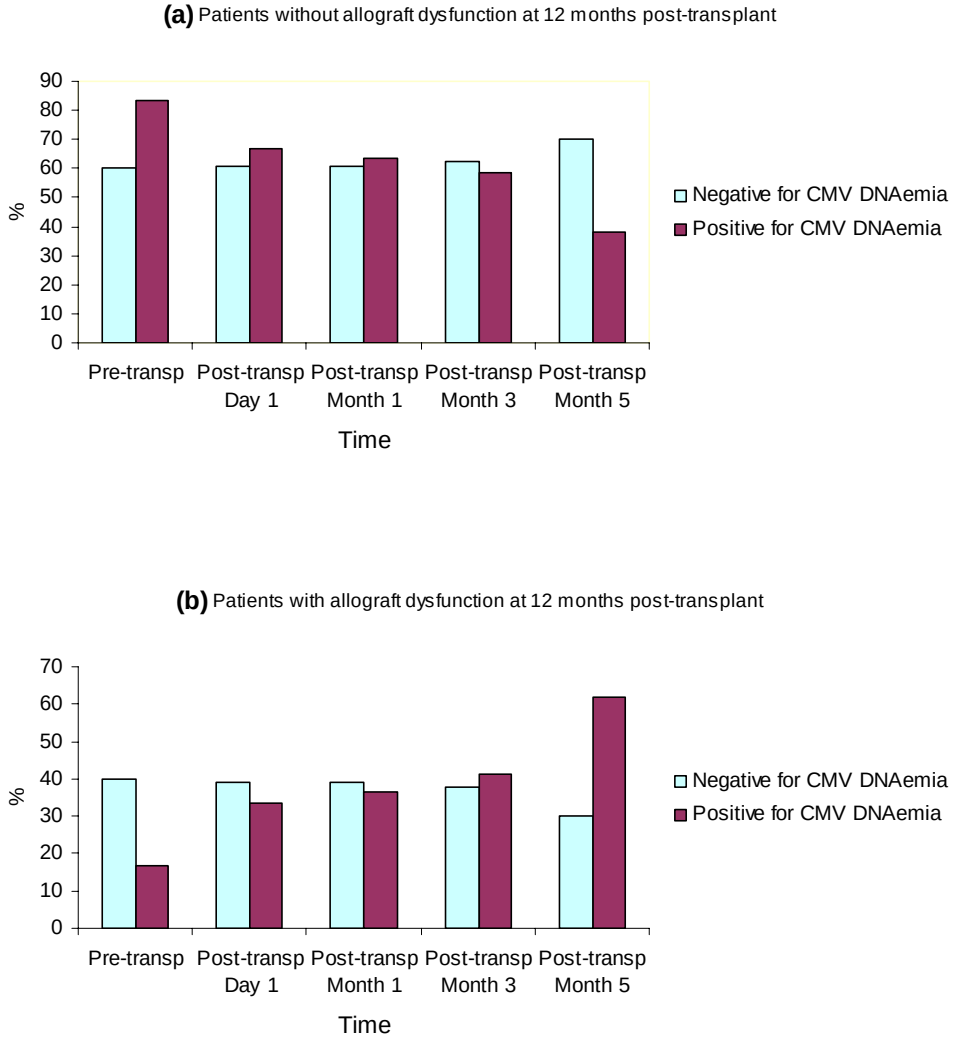

Figure 6.1. Serial CMV DNAemia in the pre- and post-transplant periods for renal allograft recipients, grouped according to graft function at one year post-transplant: (a) Patients without allograft dysfunction (serum creatinine <150 μmol/L), and (b) Patients with allograft dysfunction (serum creatinine ≥150 μmol/L). Negative CMV DNAemia is interpreted as CMV levels at <100 copies, and positive CMV DNAemia as CMV levels > 100 copies of CMV DNA/μg of total DNA in whole blood.

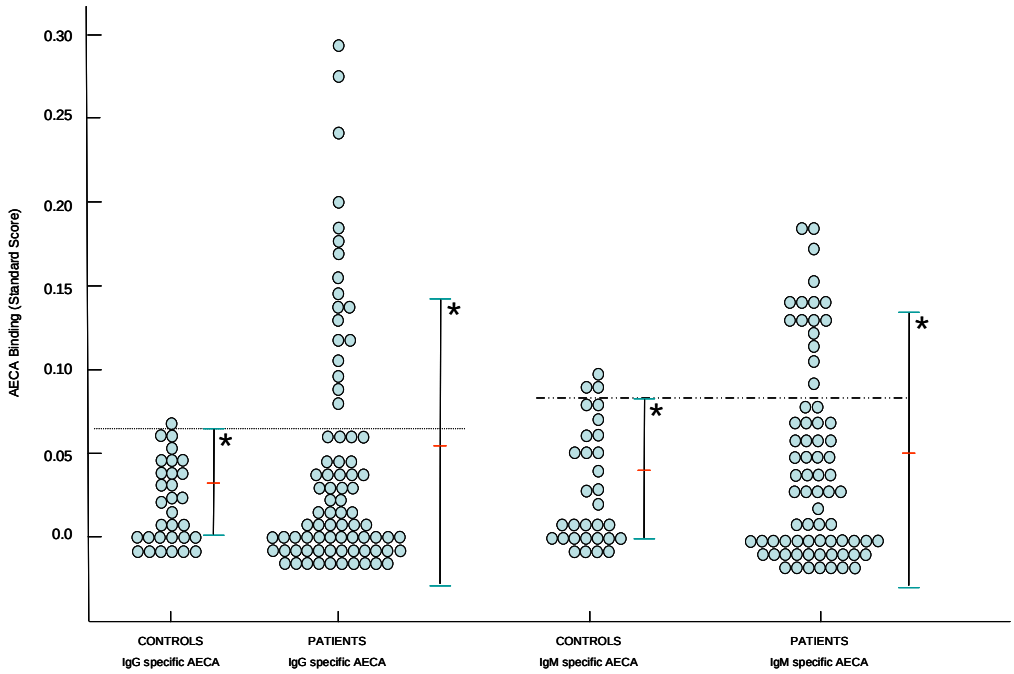

Figure 6.2. Distribution of IgG and IgM specific anti-endothelial cell antibodies (AECA) activity in renal allograft transplant recipients and controls. AECA activity is expressed as a standard score. Dashed lines represent the upper 95th percentile confidence limit of the normal population. (*$P < 0.01$).

Table 6.5. Anti-endothelial cell antibodies (AECA) activity in sera versus CMV DNAemia of renal allograft recipients (n = 51) at five months post-transplant.

		CMV DNAemia at five months post-transplant		
		Negative	Positive	% of Total
IgG specific AECA activity	Negative	61.5%	38.5%	75%
	Positive	16.7%	83.3%*	24%
IgM specific AECA activity	Negative	55.6%	44.4%	71%
	Positive	40%	60%**	29%

*$P < 0.01$; **$P = 0.31$

6.3 Discussion

6.3.1 Cytomegalovirus infection and chronic allograft nephropathy

CAN is said to represent the cumulative and incremental damage to the kidney from time-dependent immunologic and non-immunologic causes (Nankivell et al, 2004; Table 6.1) but its pathophysiology remains poorly understood. Despite the implementation of new antiviral agents and practice guidelines addressing CMV infection in organ transplantation, CMV remains an important cause of morbidity in renal transplantation. Becker et al (2002) showed that while ganciclovir was associated with trends toward lower rates of infection and disease, 9% of renal transplant and 13.4% of simultaneous pancreas-kidney transplant recipients still experienced symptomatic CMV infection or CMV disease. CMV seronegative recipients of an organ from a seropositive donor are at the highest risk, with a 25.2% rate of CMV infection or CMV disease in that study, although a 60% to 80% incidence of subsequent CMV infection has been reported elsewhere (EBPG Expert Group on Renal Transplantation, 2000). In Singapore the incidence of CMV disease in pediatric transplant recipients is 28.6% (Aw et al, 2000).

Several studies have shown a definite link between CMV-related disease and risk of acute rejection in renal transplant recipients. In a historical cohort study of 192 consecutive renal transplant recipients by Toupance et al (2001), transplant patients with CMV disease had a significant likelihood of developing acute rejection after CMV infection or reactivation ($P < 0.01$, OR 5.98, CI 1.21-29.40). In a multiple time-dependent Cox analysis of data from 477 consecutive renal allograft recipients CMV infection (RR = 1.6 (1.1-2.5, $P = 0.02$) and CMV disease (RR = 2.5 (1.2-5.1, $P = 0.01$) were independent significant predictors for clinical acute rejections (Sageda et al, 2002). The impact of CMV infection and disease on long-term outcome of kidney transplantation is less clear.

Clinical evidence for the association between CMV infection and disease in CAN and graft loss have come from studies by Humar et al (1999), Sola et al (2003), Nett et al (2004) and Sagedal at al (2004). However, contradictory data have also been reported (Dickenmann et al, 2001; Ricart et al, 2005). CMV has additionally been associated, after kidney transplantation, with increased cardiac complications (Humar et al, 2000), increased gastrointestinal complications (Sarkio et al, 2005) and new onset diabetes mellitus (Hartmann A et al, 2006).

In this chapter, we prospectively examined the potential factors that may be associated with chronic allograft dysfunction (on the basis of serum creatinine) at one year post-transplant in a cohort of adult renal transplant recipients from the local population. Serum creatinine measured at varying stages post-transplant (usually at six months and one year) as a predictive marker of long-term outcome and graft survival (Prommool et al, 2000; Sahadevan and Kasiske, 2005; Boratynska et al, 2006).

Univariate analysis employing a chi-square test for interdependence showed no significant association between allograft dysfunction at one year post-transplant and transplantation type, previous acute rejection episodes, or the administration of anti-CD3 monoclonal antibody, anti-thymoglobulin, mycophenolate mofetil and ganciclovir (Table 6.2). There was, however, a significant correlation between allograft dysfunction and presence of CMV DNAemia at five months post-transplant ($P < 0.01$) but not the other periods pre or post-transplant (Table 6.4). Similarly using a logistic regression method, with chronic allograft dysfunction at one year post-transplant as the dependent variable, post-transplant CMV DNAemia was also a significant risk factor ($P< 0.01$; OR 3.578, 95% CI 1.417-0.031).

CMV infection in the organ transplant recipient is frequently identified by serological CMV IgM status or CMV IgG seroconversion, viral culture, CMV early antigen detection (pp65 antigenaemia) or PCR to detect CMV DNA. While quantitative PCR methods for CMV DNA have emerged as the most clinically useful method to (a) detect CMV infection in kidney transplant recipients (Sola et al, 2005), (b) monitor viral load during antiviral treatment (Piiparinen et al, 2006), and (c) predict risk for development of CMV diseases such as retinitis in patients with AIDS (Rasmussen et al, 1997) or in paediatric solid organ transplant patients (Aw et al, 2000), there are few studies employing molecular detection of genomic CMV as an index to define active CMV infection in allograft dysfunction.

Sebakova et al (2005) recently showed, in biopsies that were performed as protocol biopsies or when renal function deteriorated, that tissue viral DNA is associated with chronic allograft nephropathy. In this study we have demonstrated, by means of quantitative PCR detection of CMV DNA in blood, that CMV infection is a significant independent prognosticator for renal allograft dysfunction (Table 6.4). This finding agrees with a study by Geddes et al (2003) who, in monitoring CMV PCR positivity for the first three months in consecutive renal transplants and analyzing renal function at one year post-transplant, found a mean estimated creatinine clearance at one

year of 70.0 mL/minute in patients who experience no CMV infection, 47.7 mL/minute in patients who experienced infection but no disease and 39.6 mL/minute in patients who experienced CMV disease (*P* <0.01). Our data also supports similar findings in studies which utilized pp65 antigenaemia as a marker for CMV infection (Sola et al, 2003; Sagedal et al, 2004).

Our data also revealed a time-dependent relationship between presence of CMV infection and development of renal allograft dysfunction. By monitoring CMV DNAemia in the transplant recipients in the pre- and post-transplant periods and subdividing the study cohort into those with or without allograft dysfunction, we showed that inability to clear CMV DNAemia by five months post-transplant was a characteristic of recipients who eventually developed allograft dysfunction one year post-transplant (Figure 6.1(b)). Conversely, there was a decline in the percentage of recipients with positive CMV DNAemia in the subgroup without allograft dysfunction to 38.1% at five months post-transplant (compared to 61.9% in the subgroup with allograft dysfunction)(Figure 6.1(a), Table 6.3). This is in spite of the 83.3% positive for CMV DNAemia pre-transplant (compared to 16.7% in the subgroup with allograft dysfunction), indicating that these patients were adequately immunocompetent to clear the virus.

6.3.2 Correlation between anti-endothelial cell antibodies and cytomegalovirus infection related chronic allograft nephropathy

Although acute rejection and immunosuppression, putative risk factors known to affect long term kidney transplant outcome, were not significantly associated with allograft dysfunction in this study, it is possible that other causal factors, whether in addition to, or in association with or consequential to CMV infection, are also involved in the development of chronic allograft dysfunction.

Also, as histological data were not available in this study (protocol biopsies were not performed during this time period), we were not able to show whether CMV infection as defined by renal allograft histopathologic changes were associated with CAN. It has become increasingly apparent that examination of graft histology can help to identify some of the specific factors involved in damaging the allograft in an individual recipient (Nankivell et al, 2001; Nickerson et al, 2001)

Recent studies examining the impact of CMV infection on histopathologic changes in protocol biopsy specimens of kidney allografts have revealed that the presence of CMV proteins or genome in the kidney allograft, together with a previous history of acute

rejection episodes, was associated with increased vascular changes (Helantera et al, 2003). Persistent CMV infection has also been associated with the increased expression of fibrogenic molecules in the development of CAN (namely transforming growth factor-β (TGF- β) and platelet-derived growth factor (PDGF)) and the increased expression of intercellular adhesion molecule-1 (ICAM-1) (Helantera et al, 2005). In a model of rat kidney allograft, prolonged, increased expression of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) in the CMV-infected vascular endothelium was associated with accelerated chronic allograft nephropathy (Kloover et al, 2000).

Jeannet et al (1970) first observed that post-transplant, *de novo*, donor-specific antibodies are associated with a poor outcome in human renal transplantation. More recently, deposition of C4d, the cleavage product of the complement component C4 in the peritubular capillaries of the graft has been demonstrated in patients, including paediatric transplant recipients, with chronic renal allograft rejection and CAN, permitting the recognition of a form of ongoing antibody-mediated or humoral mechanism in the pathology (Mauiyyedi et al, 2001; Aiello et al, 2004, Herman et al, 2005).

CMV infection after transplantation has been associated with the occurrence of various autoantibodies and monoclonal gammopathies

(Hebart et al, 1996; Mengarelli et al, 2000). Among these, high levels of AECA have been demonstrated in 80% of renal and heart and in 43% of liver transplant recipients with CMV infections and to play a role in the development of antibody-mediated rejections (Jordan et al, 1988; Dunn et al, 1992; Toyoda et al, 1997; Fredrich et al, 1999; Toyoda et al, 1999; Varani et al, 2002**.** Toyoda et al (1999) have shown that AECA induced by CMV infection are primarily autoantibodies that were also able to react with multiple cell types such as human fibroblasts, keratinocytes, peripheral blood mononuclear cells and platelets. They suggested that the elevated AECA levels seen in CMV-infected transplant recipients represent a polyclonal activation of humoral immune responses by CMV.

The exact role of AECA in the pathogenesis of CAN has not been fully elucidated. Poggio et al (2004) have shown that direct and indirect cellular immunity as well as humoral immunity (by detection of post-transplant anti-HLA antibody by flow cytometry) are significantly associated with renal allograft transplant recipients with CAN, suggesting that persistent cell-mediated and humoral alloimmunity contribute to its development.

In our study IgG specific AECA activity was observed in 24% of renal transplant recipients (n = 51) versus 6% of the non-transplant control

population ($P < 0.01$); IgM specific AECA activity was also present in 29% of the recipients compared to 9%, of controls ($P < 0.01$). In our study we also sought to evaluate the correlation between the presence of AECA and CMV DNAemia monitored at various periods pre- and post-transplant periods in the cohort of renal allograft recipients with CAN or normal renal function. At five months post-transplant, presence of IgG specific ($P < 0.01$) but not IgM specific ($P = 0.31$) AECA activity was significantly associated with CMV DNAemia. We have shown, in the first part of the study, that CMV DNAemia monitored five months post-transplant was significantly associated with abnormal renal function at one year post-transplant. However, we did not find a statistically significant direct correlation between AECA activity and abnormal renal function at one year post-transplant ($P = 0.85$). We noted that Fault et al (1999) found a significant association of anti-endothelial antibodies with transplant-induced coronary artery disease, a primary cause of graft failure in cardiac allograft recipients after the first year of transplantation. In that study, serum antibodies to endothelial cells were detectable by flow cytometry, enzyme-linked immunosorbent assay and immunoblotting techniques but only significant associations were found for the amount of antibody identified by Western immunoblotting.

The association between AECA and CMV DNAemia post-transplant suggests that CMV-induced endothelial damage leads to the production of AECA. While these alloantibodies may represent a marker of cell injury, their detection in sera – unlike positive CMV DNAemia – does not correlate directly with the development of chronic allograft dysfunction. Perhaps it is the actual binding of AECA to the renal vascular endothelium and the bystander effect of subsequent complement activation via the classical pathway that contributes to chronic allograft injury. Further studies are required to examine the correlation between CMV infection, presence of AECA in sera and renal histological evidence of CAN and the presence or absence of chronic allograft dysfunction.

6.3.3 Conclusion

Our data suggests that current strategies to improve long term outcomes after renal transplantation should also include serial post-transplant assessment of CMV infection by means of PCR amplification of viral DNA in sera. Both immunologic and nonimmunologic factors contribute to long term graft outcome. The genesis and progression of chronic allograft dysfunction and CAN involves more than a single pathogenic mechanism.

We speculate that humoral mediated insult to the allograft may involve AECA via CMV induction in association with some other immunological process such as complement activation. Although protocol biopsies were not routinely performed during the period of this study, histological assessment of donor kidneys – at various post transplant intervals – to correlate viral tissue DNA, presence of AECA, evidence of antibody-mediated injury and renal function may serve to elucidate the role of virus-induced immune injury to the allograft.

7. CONCLUDING REMARKS

This thesis has addressed several issues relating to the clinical challenges that CMV infections pose to immunocompromised patients from a Singapore perspective. In a previously reported series of children with renal transplantation, 30.8% had complications of CMV disease (Sim et al, 1997).

We had hypothesized that the high incidence of active CMV disease was due to ganciclovir-resistant CMV mutant strains. By determining the frequency of CMV mutant strains in CMV isolates from the non-transplant population and CMV DNA isolated directly from clinical samples from a small cohort of pediatric solid organ transplant patients from NUH, and employing different laboratory methods currently available, we have demonstrated the presence of CMV ganciclovir resistance in Singapore in three of 12 (25%) renal transplant recipients two (40%) of the five patients who underwent liver transplantation.

There was no genotypic evidence of ganciclovir resistance in our screen of CMV isolates from the non-transplant population. Clinical information from the latter was not made available in our study so it was not possible to establish a clear relationship between laboratory

resistance and clinical unresponsiveness. Nevertheless, our laboratory analyses confirm the presence of ganciclovir-resistant CMV strains in Singapore. That they are found in a cohort of transplant patients who were on ganciclovir prophylaxis and who had active CMV disease which were relatively resistant to treatment is of significance.

As antiviral screening assays have the potential to guide therapy and assist in the confirmatory diagnosis of clinical antiviral resistance – and the fact that resistant CMV strains are found in the local population - we advocate that these tests be made available for testing of patients who are positive for CMV and, in particular, those undergoing kidney and lung transplantation.

We have developed molecular assays that can be directly performed on clinical samples, that are with greater sensitivities than currently available methods in their ability to detect low copy number of mutant CMV strains (against a high background of wild type CMV, mixed viral populations being a feature of the cohort in our study) and with short turnaround time. These new assays have enabled us to discover a novel codon 413 mutation in one of the renal transplant patients with CMV disease.

A potential area for further research is the quantitative determination of mutant strains. This has application in ascertaining the exact percentage of mutants in the viral population in an isolate that is correspondent to the appearance of clinical resistance to ganciclovir. Modifications of existing PCR techniques or novel methods are necessary in order to ensure that mutant and wild type sequences amplify with near equal efficiency, so that they are eventually present in proportions representative of the starting material.

We have begun to prospectively evaluate the clinical utility of these novel assays – in tandem with conventional PCR testing - for the laboratory detection of ganciclovir resistance and correlating presence or absence of clinical features suggestive of ganciclovir resistance, such as the need for an alternative antiviral agent or the development of late CMV disease

Although ganciclovir-resistance in CMV strains exist in Singapore and this has now to be considered a risk factor in patients with CMV infections – at least in the paediatric transplant population, as we have demonstrated – follow up studies are necessary to better define whether this particular immunologically impaired group has an increased risk of CMV infection or disease. One option is to employing recently available immunological assays to determine the number and

functionality of CMV specific lymphocytes (by flow cytometry or enzyme-linked immunospot technique) in subsets of adult, paediatric and non-transplant subjects. An investigation into the kinetics of cellular immune responses (early versus late lymphocyte proliferation) for these subsets may shed additional light.

In an attempt to correlate CMV infection and other risk factors with allograft outcome, a prospective study of a cohort of renal transplant recipients pre- and post-transplantation was carried out in this study.

In renal transplantation, there is conflicting evidence on the role of CMV infection in chronic allograft rejection. This could be because previous retrospective and prospective studies have only looked at the relationship between CMV antibody positivity and allograft function. CMV antibody positivity reflects not only possible active CMV infection, but also host immunity and latent infection. Therefore, CMV antibody status may not be the best index to use to define the role of active CMV infection in allograft rejection. Detection of CMV genome by PCR has been shown to correlate well with CMV antigenemia which occurs in acute CMV disease. Both univariate and multivariate analyses of clinical data from a prospective study of 119

consecutive renal transplant patients at the two major transplant centers in Singapore revealed a significant correlation between allograft dysfunction and presence of CMV DNAemia at five months post-transplant.

Our data also revealed a time-dependent relationship between presence of CMV infection and development of renal allograft dysfunction. Our results indicated that inability to clear CMV DNAemia by five months post-transplant was a characteristic of recipients who eventually developed allograft dysfunction one year post-transplant, a feature not observed in the group of patients with normal renal function. Our data provides argument that current strategies to improve long term outcomes after renal transplantation should also include serial post-transplant assessment of CMV infection by means of PCR amplification of viral DNA in sera.

CMV infection after transplantation has been associated with the occurrence of various autoantibodies and monoclonal gammopathies, including high levels of AECA in solid organ transplant recipients who had CMV infections. We noted a relationship between AECA and CMV infection in the same post transplant period by demonstrating that at five months post-transplant, presence of IgG specific AECA activity was significantly associated with CMV DNAemia. We

propose the histological assessment of donor kidneys to elucidate immunopathogenic mechanisms involving CVM mediated AECA injury.

With the emergence of ganciclovir resistance CMV infections remain an important infectious complication after solid organ transplantation. Rapid genotypic analysis of clinical isolates of CMV for characterization of mutations known to confer antiviral resistance as well as the monitoring of novel mutations is also warranted in order to effectively guide therapy and patient management. CMV DNAemia has also been identified as a significant risk factor in the development of long term renal allograft dysfunction. The laboratory detection and quantitation of CMV should be part of pre- and post-transplant strategies to prevent the recipient from returning to end stage renal disease and dialysis.

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APPENDIX A **CASE REPORT: GANCICLOVIR – RESISTANT CYTOMEGALOVIRUS INFECTION IN A PAEDIATRIC RENAL TRANSPLANT RECIPIENT**

Introduction

This report describes the first documented laboratory-confirmed case of ganciclovir resistant CMV infection in Singapore.

A 16-year-old boy first presented (in 1998) at two years old with renal impairment and was diagnosed to have congenital dysplastic kidneys. He developed end stage renal failure at 12 years old, requiring peritoneal dialysis and later, at 15 years old, haemodialysis due to recurrent peritonitis. The patient, who was CMV seronegative, underwent a renal transplant from a cadaveric CMV seropositive donor.

The immunosuppressants given post transplant included anti thymocyte globulin and methylprednisolone, followed by prednisolone, cyclosporin and mycophenolate mofetil.

The patient was given prophylactic ganciclovir at a dose of 5mg/kg twice daily, with adjustment for renal function and total white cell count. Ganciclovir, as an intravenous preparation, was given for a period of 21 days post transplant followed by oral ganciclovir for three months.

At three months post transplant, the patient developed CMV retinitis (diagnosed by indirect ophthalmoscopy through dilated pupils) which showed some clinical resolution after two months of treatment with ganciclovir. However, the retinitis flared up again at seven months post transplant while the patient was on ganciclovir prophylaxis. He also developed CMV pneumonitis (demonstrated by instititial infiltrate and CMV DNA by PCR of bronchoalveolar lavage cells) at three months post transplant which improved clinically initially but developed recurrences at six months, 17 months and 20 months post renal transplant despite ganciclovir prophylaxis.

During each infectious episode, he was treated with a four-month course of intravenous ganciclovir, as well as intravenous hyperimmune CMV immunoglobulin for four weeks followed by intravenous gamma globulin 0.5g/kg twice weekly for a further three months.

He developed chronic allograft dysfunction at 18 months post transplant and finally allograft rejection when he stopped his

medication at 33 months post transplant. Allograft nephrectomy was performed, and this did not show any evidence of CMV renal disease. All immunosuppressants were stopped post nephrectomy, and two weeks after that his blood was cleared of CMV DNA (Figure 7.1).

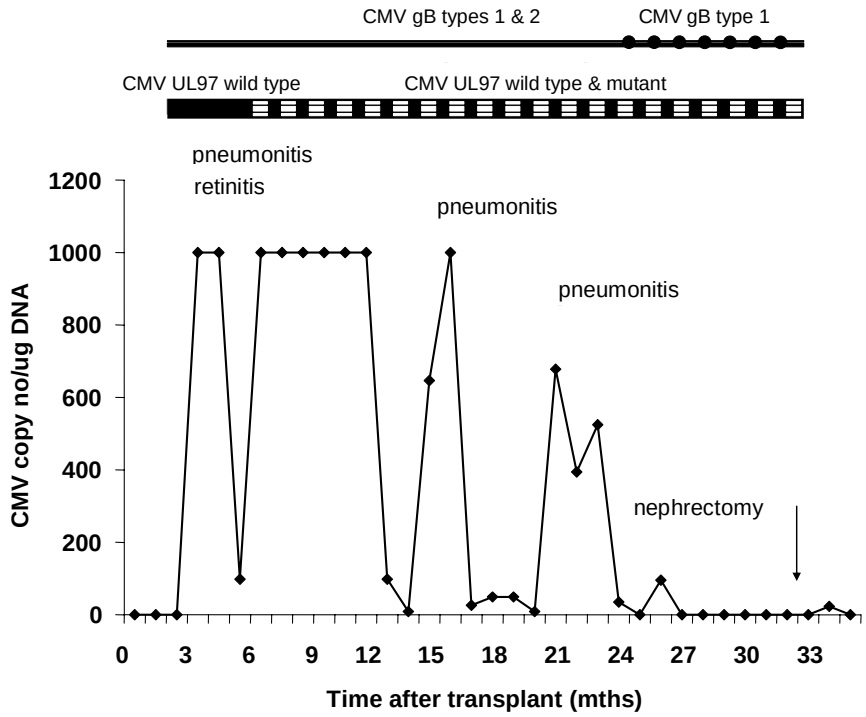

Figure 7.1. Summary of the paediatric renal transplant patient's post transplant history and clinical course of CMV disease.

Virus isolation and genetic analysis

Serial samples (whole blood) obtained from the patient were subjected to PCR for detection and quantitation of CMV DNA as previously described (Aw et al, 2000).

Analyses of CMV UL97 phosphotransferase coding sequences for mutations conferring ganciclovir resistance were carried out by nested PCR- restriction enzyme digest protocols as previously described (Chou et al 1995; Hanson et al, 1995). CMV DNA was subjected to an initial round of PCR to amplify the entire UL97. Subsequently, regions within the UL97 gene flanking frequently encountered UL97 resistance mutations at codons 460 (Met to Val), 594 (Ala to Val), 595 (Leu to Ser, or Leu to Phe) and 520 (His to Glu) were further amplified by PCR with relevant primers. PCR products obtained were then

digested separately with restriction enzymes (*Nla*III, *Hha*I, *Taq*I, *Mse*I and *Alu*I) in order to identify mutations in codons 460, 594, 595 (two different mutations) and 520, respectively. Restriction digest patterns were visualized upon electrophoresis on 8% or 15% polyacrylamide gels and ethidium bromide stained, and compared against wild type CMV reference strain AD169 (American Type Culture Collection, Manassas, VA, USA).

The specific identification of a mutation was confirmed by DNA sequence analysis of the PCR products using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 310 genetic analyzer (Applied Biosystems).

To assess the genetic relatedness of serial CMV DNAs recovered from the patient, genotyping of part of the CMV envelope glycoprotein B was also performed as previously described (Chou and Dennison, 1991).

As shown in Figure 7.1, in the patient's initial blood samples, only wild type strains of CMV were recovered. The presence of a Leu-to Ser mutation at codon 595 of the CMV UL97 gene was first detected – approximately seven months post-transplantation - as part of a mixture of wild type and mutant CMV strains. DNA sequence analysis confirmed the TTG-to-TCG base change. The L595S mutation was noted in all subsequent blood samples. Other known UL97 gene mutations were not detected in the patient.

Genotyping of the gB gene revealed that CMV strains (whether wildtype or mutant) in blood samples collected serially were either a mixture of gB types 1 and 2 in each sample, or gB type 1 alone.

Discussion

The first three cases of clinical resistance to ganciclovir were described in 1989 although a resistant strain of CMV was isolated in the laboratory in 1986 (Erice et al, 1989). More recently, mutations in clinical isolates of CMV which confer ganciclovir-resistance have been elucidated. Ganciclovir-resistant CMV strains selected in the laboratory have amino acid deletions or substitutions in conserved regions of the UL97 gene region and/or point mutations in the DNA polymerase gene of the virus (Erice, 1999).

It has been observed that the majority (94%) of ganciclovir-resistant CMV strains contain specific UL97 mutations (Chou et al, 1995; Smith et al, 1997). By combining PCR and restriction enzyme digestion

methods, it has been shown to be possible to detect 78% of ganciclovir-resistant CMV isolates containing the most common UL97 mutations (Chou et al, 1995).

There is a high incidence of CMV disease (28.6%) in the National University Hospital's paediatric solid organ transplant recipients, with a mortality rate of 3.5 % (Sim et al, 1997; Aw et al, 2000). This is the first documented case of ganciclovir resistant CMV infection due to UL97 mutation but not necessarily the first occurrence as mutation analysis was not performed in Singapore prior to inception of our study.

Wild type strains of CMV were first observed in the blood of the patient, to be replaced by a mixture of both resistant and wild type strains. This supports the observation by Drew (2000) and Erice (1999) of emergence of antiviral drug-resistant CMV in patients on prolonged and or long-term anti-CMV prophylaxis and treatment in immunocompromised patients. The patient experienced several episodes of severe CMV disease, leading to the administration of repetitive courses of antiviral therapy. Selective pressure allowed for strains of CMV with a point mutation (TTG-to-TCG; Leu-to-Ser at codon 595 of the UL97 gene) to co-circulate with wild type CMV.

It was observed that the appearance of CMV strains resistant to ganciclovir coincided with high levels of CMV DNA in the patient's blood as well as with CMV pneumonitis and retinitis.

CMV gB genotypes 1 and 2 or gB genotype 1 alone was detected in blood samples collected serially – the occurrence of mixed genotypes agrees with the findings in Coaquette et al (2004)'s recent study of a group of 64 transplant patients (22 patients received kidney transplant), in whom 46.9% of the patients showed multiple strains of the virus. Similar to that study, infection with a mixture of gB genotypes for this patient appeared to be associated with a higher virus load (> 100 copies of CMV DNA/μg of total DNA), while infection with a single genotype (gB type 1) was seen when copy numbers were less than 100.

Coaquette et al have hypothesized that patients with mixed infection are more heavily immunosuppressed, compared with patients with single gB genotype infection. Post-transplantation immunosuppressants given this patient included anti thymocyte globulin, methylprednisolone, oral prednisolone, cyclosporin and mycophenolate mofetil. The patient had chronic allograft dysfunction, resulting in slow tapering of the immunosuppressive doses, thus predisposing him to CMV infection. In addition, specific treatment of CMV disease

probably contributed to the mixed infection seen in the patient, probably by preventing virus-specific cellular immune responses to infection with diverse CMV gB genotypes.

Based on genotyping of CMV DNAs recovered serially from the patient, there were two different strains of CMV from the onset of infection. Mixed infection and the subsequent detection of ganciclovir-resistant mutant CMV strains meant that the patient harboured multiple gB genotypes in the heterogeneous population of wild type viruses and anti-viral resistant strains. Given the limitation of current molecular methods such as PCR-RFLP (where detection of ganciclovir-resistant mutant CMV strains is possible only when the mutant population reaches 10% or more (Chou et al, 1995; Erice et al, 1999), it was not possible to ascertain whether resistance arose from mutation of wild type strains as a result of ganciclovir therapy or from a mutant strain already present when the patient was infected.

Also, it was fortuitous that the patient's CMV DNAemia was eliminated two weeks post nephrectomy –probably a result of cessation of immunosuppressants and recovery of immune responses.

APPENDIX B Clinical data for the study population (n = 119) of renal allograft recipients (see Chapter 7)

Patient Code	*Serum creatinine at 1 yr post-transplant*	*CMV DNAemia pre- and post-transplant*					*AECA activity*			*Acute rejections*	*Transplant type*							
		Pre	*At transp*	*Month 1*	*Month 3*	*Month 5*	*IgG specific*	*IgM specific*	*IgG and/or IgM specific*			*OKT3*	*ATG*	*OKT3 and/or ATG*	*OKT3, ATG and/or MFF*	*MFF*	*MPL*	*GAN*
CV 01	0	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0
CV 02	1	1	0	2	1	-	1	0	1	1	1	0	0	0	0	0	1	0
CV 03	0	0	0	0	2	3	0	0	0	0	2	0	0	0	1	1	0	1
CV 04	0	2	1	1	1	1	0	0	0	0	1	0	0	0	0	0	1	0
CV 05	0	1	1	2	3	1	0	0	0	0	1	0	0	0	0	0	0	0
CV 06	0	0	0	1	3	1	0	0	0	1	1	0	1	1	1	0	1	1
CV 07	0	0	0	2	2	0	1	0	1	0	1	0	0	0	0	0	0	0
CV 08	0	1	1	3	2	2	1	1	1	0	1	0	0	0	0	0	0	0
CV 09	0	0	2	1	5	1	1	0	1	0	3	0	1	1	1	0	0	0
CV 10	0	0	1	0	0	0	1	0	1	0	2	0	0	0	0	0	0	0
CV 11	0	0	0	0	3	0	1	0	1	0	1	0	0	0	0	0	0	0
CV 12	0	0	3	1	1	3	1	0	1	0	1	0	0	0	0	0	0	0
CV 13	1	1	1	2	3	3	1	1	1	1	2	1	0	1	1	1	1	1
CV 14	0	0	-	3	1	1				0	1	0	0	0	1	1	0	0
CV 15	0	1	1	5	1	3	0	0	0	1	1	0	0	0	0	0	1	0
CV 16	1	1	1	2	2	3	1	0	1	0	1	0	0	0	0	0	0	0
CV 17	1	5	1	2	1	5	0	1	1	1	1	0	0	0	0	0	1	0
CV 18	1	0	1	0	0	5				0	1	1	0	1	1	0	1	1
CV 19	1	0	0	0	0	4				1	1	0	0	0	0	0	0	0
CV 20	1	0	0	0	2	3	0	0	0	0	1	1	0	1	1	1	1	1
CV 21	0	1	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0
CV 22	0	0	0	0	3	3				1	1	0	0	0	0	0	1	0
CV 23	0	1	1	3	3	2	0	0	0	1	1	0	0	0	0	0	1	0
CV 24	0	1	1	1	-	-	0	0	0	1	1	0	0	0	1	1	1	1
CV 25	0	0	0	5	-	-	1	1	1	1	1	1	0	1	1	1	1	1
CV 26	0	0	0	0	-	-	0	1	0	0	2	0	1	1	1	0	0	1
CV 27	1	0	0	0	-	-	1	1	1	0	1	0	0	0	0	0	0	0
CV 28	0	0	0	3	1	1	0	0	0	1	2	1	0	1	1	0	1	1
CV 29	1	0	0	0	1	0				1	1	1	0	1	1	0	1	1
CV 30	1	0	0	0	5	5				0	1	0	1	1	1	0	0	1
CV 31	0	1	0	0	0	-	1	0	1	0	1	0	0	0	0	0	0	0

CV 32	1	0	1	3	0	-	1	1	1	1	1	0	1	1	1	0	1	1
CV 33	1	0	3	0	2	2				0	1	0	1	1	1	0	0	1
CV 34	0	-	0	0	1	0	1	1	1	1	1	0	1	1	1	0	1	0
CV 35	0	5	1	3	-	-	1	1	1	1	1	0	0	0	0	0	0	0
CV 36	0	0	0	0	0	0				1	2	1	0	1	1	1	1	1
CV 37	0	2	1	0	-	-	0	0	0	1	1	0	0	0	0	0	1	0
CV 38	1	1	1	3	5	5				1	1	0	0	0	0	0	1	0
CV 39	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0
CV 40	0	0	0	0	4	0				0	1	0	0	0	0	0	0	0
CV 41	1	1	1	3	3	1				1	1	1	0	1	1	0	1	1
CV 42	0	1	1	3	4	1				0	1	0	0	0	0	0	0	1
CV 43	0	0	0	0	3	0	0	1	1	0	1	0	0	0	0	0	0	0
CV 44	1	2	2	2	5	0	0	0	0	0	1	0	0	0	0	0	0	0
CV 45	0	2	0	5	-	-	1	1	1	0	1	0	0	0	1	1	0	0
CV 46	1	2	3	2	2	0	0	1	1	0	1	0	0	0	0	0	0	0
CV 47	0	0	0	0	0	0				0	2	0	0	0	0	0	1	0
CV 48	0	0	0	0	0	0				0	1	0	0	0	0	0	0	0
CV 49	0	0	0	3	2	2	0	0	0	0	1	0	0	0	0	0	0	0
CV 50	0	0	0	5	0	-	1	0	1	0	1	0	0	0	0	0	0	0
CV 52	1	0	0	1	0	1				0	1	0	0	0	0	0	0	0
CV 55	0	1	2	2	2	4				0	1	1	0	1	1	1	1	1
CV 59	0	1	0	2	3	0				0	1	0	0	0	1	1	0	1
CV 60	0	2	0	1	5	5				0	3	0	0	0	0	0	1	0
CV 61	0	1	0	3	5	2				0	2	0	1	1	1	0	0	1
CV 62	1	0	0	3	3	0				0	1	0	0	0	1	1	0	0
CV 63	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0
CV 65	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0
CV 66	0	0	0	0	0	-	0	0	0	1	1	0	0	0	1	1	0	0
CV 67	0	0	1	0	1	2				0	1	0	0	0	1	1	0	1
CV 68	0	0	1	0	3	4				0	1	0	0	0	0	0	0	0
CV 69	0									0	2	0	1	1	1	0	0	1
CV 71	0	0	0	2	3	0				0	1	0	0	0	0	0	0	0
CV 72	0	1	3	0	1	0				0	1	1	0	1	1	1	1	1
CV 73	0	3	4	0	0	3				0	1	0	0	0	0	0	0	0
CV 75	1	0	0	0	0	2				0	2	0	0	0	1	1	0	0
CV 76	1	0	1	5	3	1				0	1	0	0	0	1	1	0	0
CV 77	1	0	1	2	-	-	1	1	1									
CV 79	1	0	0	0	3	2				0	1	0						
CV 80	1	1	0	0	2	0	0	0	0	0								
CV 81	1	0	1	3	1	3				0	1	0	0	0	0	0	0	0
CV 82	0	0	0	3	0	0				1	1	0	0	0	1	1	0	0

CV 83	0	3	0	3	0	0	0	0	0	0	2	0	0	0	0	0	0	0
CV 84	0	0	0	0	5	0	0	0	0	1	3	1	1	1	1	0	1	1
CV 85	1	0	0	0	0	3				0	1	0	0	0	1	1	0	0
CV 87	1	0	0	0	5	5	0	0	0		1	0		0	0			
CV 88	1	0	1	1	0	1				0	1	0	0	0	0	0	0	0
CV 89	1	0	5	0	0	0				0	2	0	0	0	0	0	0	0
CV 90	1	0	1	1	1	1	1	0	1	0	2	0	1	1	1	0	0	1
CV 92	1	1	1	0	1	3	0	0	0	0	2	0	0	0	0	0	0	0
CV 95	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	1	0	0
CV 96	1	2	2	3	5	3				1	1	0	0	0	1	1	0	0
CV 97	1	0	0	3	3	2	0	0	0	1	3	0	0	0	0	0	1	0
CV 98	0	0	0	1	3	0	0	1	1	1	3	1	0	1	1	0	1	1
CV 100	0	0	0	2	1	0	0	1	1	0	3	0	0	0	0	0	0	0
CV 107	0	0	4	5	0	0				0								
CV 109	0	3	3	3	3	0				0	2	0	1	1	1	0	0	1
CV 112	0	1	0	5	1	0				1	1	0	0	0	1	1	0	0
CV 115	0	0	0	1	1	0				0	1	0	0	0	0	0	0	0
CV 119	0	0	0	0	0	0				0								
CV 120	0	0	0	0	0	0				1	1	0	0	0	1	1	1	0
CV 126	1	1	1	1	1	0				1	1	0	0	0	1	1	1	0
CV 127	1	0	0	0	0	0				1	1	0	0	0	1	1	0	0
CV 128	0	0	0	0	0	0				0	1	0	0	0	1	1	0	0
CV 129	0	0	0	1	0	0				0	1	0	0	0	1	1	0	0
CV 130	1	1	2	1	-	-	1	1	1	0								
CV 136	1	1	1	3	4	-	0	0	0	1	1	0	0	0	0	0	1	1
CV 147	1	0	0	4	1	0	1	1	1	1	1	0	0	0	1	1	1	1
CV 154	0	0	0	1	0	1	0	0	0	0								
CV 168	1	0	0	0	0		0	0	0	0	1	0	0	0	1	1	0	0
CV 170	0	0	2	1	0	2	0	0	0	1	1	0	0	0	0	0	0	0
	0 : <150 µmol/L 1: ≥150 µmol/L	CMV DNAemia measured at levels 0 - 5; levels 0 - 2: < 100 copies of CMV DNA/µg of total DNA in whole blood; levels 3 - 5: ≥ 100 copies					0: no AECA activity; 1: activity present			0 : no rejection 1: ≥1 episode	0: cadaveric 1: living (related or unrelated)	0: not administered; 1: administered						

PUBLICATIONS

1 **Yeo AC,** Yeo WS, Liang AW, Seah CC, Vathsala A, Lee EJC, Yap HK (2006). Post-transplant CMV DNAemia but not CMV-induced anti-endothelial cell antibodies predisposes to chronic renal allograft dysfunction. *Submitted*

With the use of newer immunosuppressive protocols, allograft survival rates have improved tremendously due to the marked reduction in incidence of graft loss to acute rejection. However, chronic rejection still remains a problem. This study aimed to assess the impact of cytomegalovirus (CMV) infection on chronic renal allograft dysfunction. A total of 126 new renal allograft recipients transplanted either at National University Hospital or the Singapore General Hospital were recruited over a two year period, with 4 withdrawals and 3 deaths before completion of the study. Post-transplant parameters recorded included transplantation type, rejection episodes, type of immunosuppression and ganciclovir adminstration. Blood samples were obtained pre-transplantation and subsequently at monthly intervals for five months post-transplant, and the presence of CMV DNA was determined by quantitative polymerase chain reaction. Statistical analysis of risk factors for development of chronic allograft rejection at one year post-transplant, defined as serum creatinine >150 μmol/L was performed using SPSS for Windows 13.0. Univariate analysis employing chi-square test showed a correlation between chronic allograft dysfunction at one year post-transplant and cytomegalovirus DNAemia at five months post-transplant ($P <$

0.01) but not previous acute rejection episodes, type of transplantation or type of immunosuppression or ganciclovir administration. With chronic allograft dysfunction as the dependent variable in a multivariate logistic regression analysis, only cytomegalovirus DNAemia was significant ($P< 0.01$; OR 3.578, 95% CI 1.417-0.031). CMV infection in the post-transplant period was a significant risk factor for long-term renal allograft dysfunction. Further studies will address possible mechanisms by which the virus affects immune responses.

2 **Yeo AC**, Chan KP, Kumarasinghe G, Yap HK. (2005) Rapid detection of codon 460 mutations in the UL97 gene of ganciclovir-resistant cytomegalovirus clinical isolates by real-time PCR using molecular beacons. *Mol Cell Probes* 19(6):389-93.

A rapid real-time polymerase chain reaction (PCR) assay using molecular beacons has been developed for the simultaneous detection of wild-type and mutant strains of cytomegaloviruses (CMV) with respect to codon 460 of the UL97 gene has been developed. The molecular beacons were designed to complement the wild-type codon 460 or the mutant sequence arising from a single base-pair difference (point mutation). Discrimination between wild-type and mutant templates was demonstrated as the beacons did not generate fluorescence with their respective mismatch targets but only with those that they were designed to perfectly anneal with. Samples that harbor mixed populations of CMV could also be readily recognized. Applied to a small number of clinical samples, results from the retrospective

screening by this assay are in general concordance with that obtained by PCR-RFLP. Using molecular beacons strategy, codon 460 mutation was detected in ten out o the total number of 40 samples, whereas the latter method identified nine samples as containing the mutation. The discrepant result arose from the genotyping of one clinical sample as mixed (containing both wild-type and mutant CMV strains) by molecular beacons but as wild-type by PCR-RFLP, suggesting that this real-time strategy is possibly more sensitive for mutation analysis.

3 **Yeo A,** Aw M, Seah CC, Liang AW, Chan KP, Kumarasinghe G, Yap HK. PCR-based detection of gene mutations conferring ganciclovir resistance in cytomegalovirus. *In* Abstracts of 10th International Congress of Infectious Diseases (11-14 March 2002), Singapore.

Use of ganciclovir to treat cytomegalovirus (CMV) infections and diseases or as prophylaxis has led to the emergence of resistant strains. Mutations in several regions of the CMV UL97 phosphotransferase as well as the UL54 DNA polymerase genes have been shown to confer resistance to ganciclovir both clinically and *in vitro* by conventional plaque reduction assay. We describe PCR-based strategies for the detection of CMV strains with mutant UL97 and UL54 sequences that are rapid, non-culture dependent and directly from blood samples. PCR-Restriction Fragment Length Polymorphism (RFLP) assays using newly developed primers were used to screen for mutations in the UL54 gene. To discriminate between CMV strains with and without mutations in the UL97 gene, primers

sets were designed to specifically amplify either one but not both. Thirty-six blood samples from a cohort of solid-organ transplant recipients, which were PCR positive for CMV and which contain either wild type, mixed population or mutant strains (with UL97 mutations in codons 594 and 595), were correctly identified, and confirmed by the standard PCR-RFLP assays. PCR-based detection methods are an alternative to culture for detection of CMV ganciclovir resistance, and their short turnaround time will allow for prompt therapeutic modification.

4 Lim DL, Yeo AW, Liang AW, Seah CC, **Yeo AC**, Koay E, Yap HK. Improved prediction of active cytomegalovirus (CMV) infection in high risk patients. *In* Abstracts of 10th International Congress of Infectious Diseases (11-14 March 2002), Singapore.

Background: CMV infection is a major cause of morbidity and mortality in allograft recipients, bone marrow transplant recipients and in patients with immunodeficiencies. Preemptive therapy is a promising strategy in the prevention of serious CMV infection. We have shown in our previous study that >1000 copies of CMV DNA/mg DNA tested gives a positive predictive value (PPV) of 70%, with a sensitivity and specificity of 63% and 87% respectively. The presence of CMV late mRNA as pp67 is thought to reflect active viral replication. The aim of this study was to determine if the addition of pp67 to the semi-quantitative polymerase chain (PCR) would improve the predictive value of detecting active disease.

Methods: From May 2001 to July 2001, blood was taken for at-risk patients with suspected CMV disease. CMV DNA was detected using semi-quantitative PCR amplification and pp67 mRNA was detected via nucleic-acid sequence-based amplification (NASBA). Active CMV disease was defined as either (i) proven organ involvement, eg. involvement of the lung as proven by CMV detection in bronchoalveolar lavage specimen, or (ii) a presumptive viral syndrome, eg. unexplained fever for at least 72 hours with associated leucopenia.

Results: From May 2001 to July 2001, 117 samples were received from 79 patients. Sixty percent were transplant recipients. Of these samples, 20 were positive for both CMV >1000 copies and pp67. Nineteen had active CMV disease, giving a significantly increased positive predictive value of 95.0% ($P = 0.031$) as compared to CMV PCR alone (PPV = 70%). CMV pp67 alone gave a positive predictive value of 72.9% and this was also significantly lower ($P = 0.042$) than the predictive value of the combined tests. The sensitivity of the combined test was 63.0% and the specificity was 98.9.

Conclusion: The combination of CMV PCR and pp67 provides a clinician with a highly predictive tool that is also very specific for active CMV disease. Anti-viral therapy has its inherent risks and hence this combination is extremely useful when deciding on whether to start preemptive therapy in already immunocompromised patients.

5 **Yeo A,** Yeap SY, Yap HK. PCR-based detection of UL97 gene mutations conferring ganciclovir resistance in cytomegalovirus *In* Abstracts of The Institute of Biomedical Science Congress 2001 (25-27 September 2001), Birmingham, England, UK

Use of ganciclovir to treat cytomegalovirus (CMV) infections or as prophylaxis has led to the emergence of resistant strains. Mutations in several regions of the CMV UL97 phosphotransferase as well as the UL54 DNA polymerase genes have been shown to confer resistance to ganciclovir both clinically and in vitro by conventional plaque reduction assay. We describe a PCR-based strategy for the detection of CMV strains with mutant UL97 sequences that is rapid, non-culture dependent and directly from blood samples. To discriminate between sensitive and resistant CMV strains, primer sets were designed to specifically amplify either one but not both. In a preliminary study, which was carried out prospectively, 32 blood samples, which were PCR positive for CMV and which contain either wild type, mixed population or mutant strains (with UL97 mutations in codons 594 and 595), were correctly identified, and confirmed by the standard PCR-Restriction Fragment Length Polymorphism (RFLP) assays.

6 **Yeo A,** Lee CY, Aw M, Seah CC, Liang AW, Chan KP, Kumarasinghe G, Yap HK. Detection of cytomegalovirus UL97 gene mutations conferring ganciclovir resistance in local allograft recipients. *In* Abstracts of 7th Asian Congress of Pediatric Nephrology (4-6 November 2000), Singapore.

Use of ganciclovir to treat cytomegalovirus (CMV) infections or as prophylaxis has led to the emergence of resistant strains. Mutations in several regions of the CMV UL97 phosphotransferase as well as the UL54 DNA polymerase genes have been shown to confer resistance to ganciclovir both clinically and *in vitro* by conventional plaque reduction assay. The aim of this study was to devise a nested PCR-RFLP strategy for the rapid, direct detection in blood of CMV strains with mutant UL97 sequences. Analysis of UL97 phosphotransferase coding sequences was carried out by restriction enzyme digestion using *Nla*III, *Hha*I, *Taq*I, *Mse*I and *Alu*I. A total of 50 blood specimens from 11 transplant recipients were studied. In two local paediatric transplant recipients with CMV infections, ganciclovir-resistant CMV strains were detected. The mutations were present at either codon 594 or codon 595 of the UL97 gene. In both cases, a mixed population of CMV mutant and wild type strains was observed although genotyping of part of the CMV envelope glycoprotein B (gB) revealed the presence of only one (gB1) of four defined gB types. In conclusion, diagnosis of ganciclovir-resistant CMV infection can be performed rapidly using a nested PCR-RFLP strategy on patients' blood specimens.

7 Elnifro EM, Cooper RJ, Klapper PE, **Yeo AC**, Tullo AB. (2000) Multiplex polymerase chain reaction for diagnosis of viral and chlamydial keratoconjunctivitis. *Invest Ophthalmol Vis Sci.* 41(7):1818-22.

PURPOSE: To develop a multiplex polymerase chain reaction (PCR) for the detection of adenovirus, herpes simplex virus, and Chlamydia trachomatis in conjunctival swabs. METHODS: Oligonucleotide primers for detection of the 3 agents were combined in one reaction and evaluated for optimal performance using control DNAs of adenovirus type 2, herpes simplex virus, and C. trachomatis plasmid. The multiplex PCR was evaluated prospectively against its corresponding uniplex PCRs, virus isolation, Chlamydia Amplicor PCR, and an immunoassay technique (immune dot blot test) in a total of 805 conjunctival swabs from patients with suspected viral and chlamydial keratoconjunctivitis. RESULTS: The multiplex PCR was as sensitive as uniplex PCRs for the detection of the agents in clinical specimens. In the prospective study, 48 of 49 (98%) clinical specimens were positive for adenovirus by the multiplex PCR compared with 26 of 49 (53%) by adenovirus isolation. For herpes simplex virus detection, the multiplex PCR had a sensitivity of 92% (34/37) compared with 94.5% (35/37) by cell culture. The multiplex PCR produced identical results to the Amplicor PCR (21/21; 100%) compared with 71% (15/21) by the immune dot blot test. CONCLUSIONS: With clinical specimens the multiplex PCR was as sensitive as its respective uniplex PCRs but more sensitive than adenovirus isolation and as sensitive as herpes simplex virus isolation or C. trachomatis Amplicor PCR. It has the potential to replace several diagnostic tests with consequent savings in cost. The test also reduces the risk of misdiagnosis by the clinicians.