NUCLEIC ACID BASED MOLECULAR BIOLOGICAL METHODS FOR QUANTITATION AND CHARACTERIZATION OF HUMAN CYTOMEGALOVIRUS IN THE CLINICAL SPECIMENS FROM IMMUNOCOMPROMISED PATIENTS

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CERTIFICATE

This is to certify that the thesis entitled **"Nucleic acid based molecular biological methods for quantitation and characterization of Human Cytomegalovirus in the clinical specimens from immunocompromised patients"** is a record of research work done by **Ms. P. Sowmya,** during the period of study under my supervision and guidance at L & T Microbiology Research Centre, CU Shah Ophthalmic Post Graduate Training Centre, Medical Research Foundation, Sankara Nethralaya, Chennai. It has not previously formed the basis for the award of any Degree or Diploma of any other university. It is further certified that the work in the thesis represents independent work on the part of the candidate.

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LIST OF ABBREVIATIONS

^{o}C	degree celsius
%	percentage
µg	microgram
µl	microlitre
µM	micromolar
A^{o}	Angstorm
AFLP	Amplified fragment length polymorphism
AIDS	Aquired immunodeficiency syndrome
APAAP	Alkaline phosphatase anti –alkaline phosphatase
BAL	Bronchoalveolar lavage
bDNA	Branched DNA
BLAST	Basic Local alignment search tool
BMT	Bone marrow transplants
bp	Base pair
CDS	Conventional dextran sedimentation
CEC	Cytomegalic endothelial cells
CLTs	Cytomegalovirus latency – specific transcripts
CMC	Christian Medical college
CMV	Cytomegalovirus
CNS	Central nervous system
C_P	Crossing point
CPE	Cytopathic effect
CSF	Cerebrospinal fluid
C_T	Threshold cycle
CV	Coefficient of variation
D+/R-	Donor positive/ Recipient negative
DABCYL	4-(4'-dimethylamino- phenylazo)-benzene
ddNTPs	dideoxynucleotide triphosphates
DEIA	DNA enzyme immunoassay
DEL	Direct erythrocyte lysis
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
E	early
EBV	Epstein Barr virus
EDTA	Ethylene diamine tetrahydrochloric acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immuno sorbent assay
FAM	6-carboxyfluoroscein
fg	femtogram
FITC	Fluorescein iso thiocyanate
FRET	Fluorescence resonance energy transfer
gB	Glycoprotein B
gCI	Glycoprotein complex I
gCII	Glycoprotein complex II

gCIII	Glycoprotein complex III
gH	Glycoprotein H
gL	Glycoprotein L
gM	Glycoprotein M
GM-CSF	Granulocyte-macrophage colony stimulating factor
gN	Glycoprotein N
gO	Glycoprotein O
gp	glycoprotein
gp55	Glycoprotein 55
GVHD	Graft versus host disease
HAART	Highly active anti –retroviral therapy
HCMV	Human Cytomegalovirus
HHV	Human herpesvirus
HIV	Human Immunodeficiency virus
HLA	Human leucocyte antigen
HMA	Heteroduplex mobility assay
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
IE	Immediate-early
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin 10
IRL	Internal long repeat sequence
IRS	Internal short repeat sequence
ISH	In situ hybridization
JOE	2,7-dimethoxy-4,5-dichloro-6-carboxy-fluoroscein
kD	Kilodaltan
L	Late
LA	Late antigen
LCR	Ligase chain reaction
LUX	Light upon extension
M	Molar
MCP	Major capsid protein
mCP	Minor capsid protein
MGB	Minor groove binding
MHC	Major Histocompatibility complex
MIE	Major immediate early
ml	millilitre
mM	milli Molar
mRNA	Messenger ribonucleic acid
mtr II	Morphological transforming region II
N terminus –	Amino terminus
NASBA	Nucleic acid sequence-based amplification
NFQ	Non – fluorescent quencher
NFκB	Nuclear factor kappa B
NK	Natural killer
nm	Nanometre
nmol	nanomoles

ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBLs	Peripheral blood leucocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	picogram
PMNL	Polymorphonuclear leycocytes
pmol	picomoles
PNA	Peptide nucleic acid
pp	Phosphoprotein
pp65	Phosphoprotein 65
PRT	Post renal transplant
PTLD	Post -transplant lymphoproliferative disorders
RFLP	Restriction fragment length polymorphism
RIA	Radio immuno assay
RNA	Ribonucleic acid
rpm	revolutions per minute
RT- PCR	Reverse transcription polymerase chain reaction
SCT	Stem cell transplant
SD	Standard deviation
SDA	Strand displacement assay
SNP	Single nucleotide polymorphism
SOT	Solid organ transplant
SSCP	Single strand conformation polymorphism
ssDNA	Single stranded deoxyribonucleic acid
TAMRA	6-carboxy-tetramethyl-rhodamine
TAP	Transporter of antigenic peptides
TAS	Transcription based amplification system
$TCID_{50}$	Tissue culture infectious dose
T_D	Denaturation temperature
T_M	Melting temperature
TMA	Transcription mediated amplification
TNF α	Tumor necrosis factor alpha
TRL	Terminal long repeat sequence
TRS	Terminal short repeat sequence
UL	Unique long sequence
UNG	Uracil N glycosylase
US	Unique short sequence
UV	Ultraviolet
VZV	Varicella zoster virus

1. INTRODUCTION

Human Cytomegalovirus (HCMV) is the prototype member of Beta Herpesvirinae. As with all members of Herpesviridae, the virus has the ability to persist in the host in a latent state after primary infection (Emery, 2001).

In the immunocompetent individual, the virus and host exist in a symbiotic equilibrium, such that disease manifestations are rarely encountered. However, when the host immune system is compromised, either through immaturity (neonate); infection for example by Human immunodeficiency virus (HIV); or through iatrogenic means following organ transplantation, the virus is able to exert its full pathogenic potential (Emery, 2001).

HCMV is the etiologic agent of the most common congenital virus infection in humans occurring in 0.2%-2.2% of all live births (Revello et al., 1999a). Congenital HCMV infection is silent at birth in 90% of infants yet 5-17% of these neonates may develop neurological impairment (Revello et al., 1999a). In other immunocompromised individuals the symptoms can range from mild disease to disseminated disease involving organs of pulmonary, nervous, gastro intestinal systems including liver. The usual manifestations are severe pneumonitis, encephalitis, chorio retinitis, optic neuritis and mild cholangitis. The reported prevalence of HCMV seropositivity in general population in South India is about 98%. Data from Christian Medical College (CMC), Vellore places the prevalence of clinical HCMV disease post transplant at about 30%, consistent with reinfection and reactivation syndromes (Rao, 2002). It has been documented that at least 25% of

AIDS patients develop serious HCMV disease. Retinitis occurs in 6-15% of patients, gastro duodenal disease in 5-10% and 30% reveal active HCMV infection at autopsy (Mujtaba et al., 2003).

Current scenario involves use of antiviral drugs viz. ganciclovir, foscarnet, and cidofovir as a prophylaxis in these individuals. There has been a considerable reduction in both the morbidity and mortality of HCMV disease in recent years owing to an increase in the antiviral prophylaxis. The toxicity associated with currently available antiviral agents and development of resistance remains a significant problem (Boeckh and Boivin, 1998).

Highly sensitive and quantitative detection methods aid in identifying patients at higher risk for disease prior to the onset of disease, thereby focusing antiviral treatment only to patients at risk for disease without covering the entire immunosuppressed population (Boeckh and Boivin, 1998). Quantitation of the systemic HCMV load may provide a highly sensitive and specific method to predict the development of HCMV disease. The conventional methods of quantitation like the traditional plaque assay, $TCID_{50}$ and other modified tissue culture-based methods like the rapid shell vial based assays are not preferred owing to the time-consuming procedures, poor reproducibility, and a relatively low sensitivity. The major problem faced with the culture techniques includes poor ability of some HCMV strains to form plaques, rapid loss of viability, lack of staining by monoclonal antibodies and nonspecific monolayer toxicity.

The important characteristics required for an optimal diagnostic assay for HCMV monitoring are

(i) A high sensitivity that allows early detection in individuals at high risk for disease.

(ii) Potential to quantify the results to increase the positive predictive value and to measure viral load during antiviral treatment.

(iii) Rapidity to allow early initiation or change of treatment.

(iv) A high degree of reproducibility.

Quantitative pp65 antigenemia assay and quantitative PCR-based methods have one or more of the characteristics of an ideal diagnostic test for HCMV monitoring. The detection of the pp65 antigen in leukocytes is a sensitive method widely used for the early diagnosis of HCMV infection, but it is labor-intensive, requires immediate processing, and relies on a subjective interpretation of the slides (Boeckh and Boivin, 1998; Landry et al., 1995). Qualitative PCR detection of HCMV DNA in leukocytes or plasma is considered the most sensitive method, but it lacks specificity for the diagnosis of HCMV disease (Drouet et al., 1995; Kanj et al., 1996; Weber et al., 1994). Quantification of HCMV DNA has been proposed to be more specifically associated with the disease (Boeckh and Boivin, 1998; Zipeto et al., 1993). Real-time PCR based on the Taqman technology provides an accurate means to quantify viral DNA with the major advantage of avoiding post-PCR handling that can be the source of DNA carryover (Heid et al., 1996; Holland et al., 1991). Several studies report the utility of real-time PCR for the quantification of HCMV DNA (Machida et al., 2000; Nitsche et al., 2000; Nitsche et al., 1999; Tanaka et al., 2000; Yun et al., 2000; Piiparinen et al., 2004; Allice et al., 2006). In

these studies, the primers used for PCR were located in the major immediate-early gene (Nitsche et al., 1999; Tanaka et al., 2000 ; Yun et al., 2000) the US17 gene (Machida et al., 2000), or the glycoprotein B gene (Yun et al., 2000). The sensitivity of quantitative PCR may be dependent on the viral target gene (Yun et al., 2000); however, the most appropriate region for amplification has not been established, as the sequence diversity of clinical HCMV strains remains to be characterized.

The diversity of organs and cell types infected by HCMV in vivo has also led to hypothesis that HCMV disease and tissue tropism may be related to sequence variation among the strains (Chou and Dennison, 1991; Lurain et al., 1999). HCMV clinical isolates have been grouped by immunologic assays, such as neutralization kinetics (Rasmussen et al., 2002). The genetic basis for the biological differences seen among virus isolates is unknown.

Serologically HCMV cannot be classified into any particular subtypes, as significant antigenic differences do not exist (Arens, 1999). Monoclonal antibodies were used to differentiate between two strains of HCMV. They do not have the ability to distinguish individual isolates within a serotype because the major antigenic epitopes that define the serotype are highly resistant to change (Chou, 1989). The need for this capability has provided the impetus for the development of molecular methods for subtyping HCMV. Though complete genome sequences of standard strain AD 169 is known, as many as 22 additional virus genes have been found in low passage clinical isolates and Toledo genome which were absent in AD169 and Towne strains (Rasmussen et al., 2002; Cha et al., 1996).

Restriction endonuclease analyses have shown genetic variation in every region of HCMV genome (Chandler and McDougall, 1986; Chou, 1990a). Many investigations have reported up to 7 subtypes by restriction analysis and up to 22 subtypes by SSCP of the gB gene of HCMV (Chou and Dennison, 1991; Trincado et al., 2000; Meyer-Konig et al., 1998c).

Strain variations related to gB gene coding for gp55 protein and gH gene are well documented. These two genes code for two of the immunologically dominant envelope glycoproteins of HCMV involved in the induction of virus neutralizing antibody. Four major variants and an additional variant have been determined at the region coding for the cleavage site of gB. Two additional sites of variability have been described at restricted regions of the N and C termini of the gB gene product (Rasmussen et al., 2002; Zipeto et al., 1995). gH (UL-75) is one of the component encoding gCIII complex of HCMV which aids in the penetration of HCMV into the host cells. The other components include gL (UL-115) and gO (UL-74). The former is necessary for the transport of the gH glycoprotein to the cell surface and the latter plays a role in fusion of cells. Many studies have confirmed two closely related variants of gH gene at the N terminus (Chou, 1992a; Rasmussen et al., 2002). Four variants each in gL and gO genes (Rasmussen et al., 2002) have been determined. Another HCMV polymorphic surface glycoprotein is gpUL73-gN, encoded by the ORF UL73. It is a component of the envelope gC-II complex, associated with gM and has the ability to induce neutralizing antibodies. UL73 shows four main genomic variants, denoted gN-1, gN-2, gN-3 and gN-4. The gN3 genotype can be further divided into two subgroups (gN3a and gN3b) and gN-4 genotype into three subgroups (gN-4a, gN-4b and gN-4c) (Pignatelli et al., 2003a).

Molecular characterization for the purpose of sub typing though not relevant to treatment, is useful for epidemiologic purposes and for investigations into pathogenesis and disease progression of HCMV. They may also throw light on the most conserved regions of the viral genome and hence aid in defining genomic targets suitable for primers and probes selection for nucleic acid based detections.

1.1 HYPOTHESIS

Nucleic acid based molecular methods aid in rapid detection, quantitation and characterization of HCMV from direct clinical specimens obtained from immunocompromised patients with suspected HCMV infections.

1.2 BROAD AIMS

1.2.1 To develop nucleic acid based molecular methods for detection and quantitation of HCMV genome from direct clinical specimens.

1.2.2 To develop nucleic acid based molecular methods for characterization of HCMV strains in the clinical specimens.

1.3 SPECIFIC OBJECTIVES

1.3.1 Standardization of pp65 antigenemia assay for detection and semi-quantitation of HCMV antigen in peripheral blood specimens

1.3.1.1 To compare the Conventional dextran sedimentation (CDS) and Direct erythrocyte lysis method (DEL) for isolation of leucocytes and standardize immunofluorescence based pp65 antigenemia assay.

1.3.2 Evaluation of the efficiency of primers targeting morphological transforming region II (mtrII), UL -83 and glycoprotein O (gO) regions for the detection of HCMV DNA in clinical specimens

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1.3.2.4 To evaluate the efficiency of the PCRs for mtr II, UL 83, gO and duplex PCR for gO gene against pp65 antigenemia assay as the 'gold standard'.

1.3.3 Development and application of a multiplex PCR for semi-quantitation of HCMV and its evaluation against pp65 antigenemia assay and Taqman – probe based Real time PCR assay for HCMV

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1.3.9. Isolation and characterization of HCMV

1.3.9.1 To isolate HCMV from clinical specimens and to characterize the isolates.

2. REVIEW OF LITERATURE

2.1 THE HUMAN HERPESVIRUSES

The family Herpesviridae consists of eight human herpesviruses HHV1 to HHV 8: herpes simplex virus 1 and 2 (HSV1 and HSV2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), Human cytomegalovirus (HCMV) and human herpesvirus 6, 7 and 8 (HHV 6, HHV7 and HHV 8) (Zhou et al., 2006). The herpesvirus family is divided into three subfamilies, designated alpha, beta, and gamma. HSV-1, HSV-2, and VZV belong to the alphaherpesviruses; HCMV, HHV-6 and HHV-7 to betaherpesviruses and Epstein-Barr (EBV) virus and HHV-8 are gammaherpesviruses (Zhou et al., 2006). HSV-1 and HSV-2 are neurotropic and cause fever blisters, genital sores and infections of the central nervous system. Primary varizella-zoster virus infection causes chickenpox (varicella), usually in children. The recurrent form of VZV is herpes zoster which occurs mostly in immunosuppressed and elderly subjects. In immunosuppressed individuals VZV may also cause infections of the central nervous system or in rare cases even visceral VZV disease without cutaneous involvement (Grant et al., 2002). HCMV primary infections of immunocompetent individuals are usually asymptomatic but might also cause mononucleosis-like illness. Congenital infection caused by HCMV is a major cause of hearing loss and mental retardation. Moreover, reactivation of the virus is important in immunocompromised patients, such as transplant recipients and AIDS patients. HHV-6 is the causative agent of exanthem subitum, an early childhood disease characterized by high fever and a mild skin rash, which are occasionally complicated by seizures or encephalitis (Yamanishi et al., 1988). HHV-6 may also

reactivate during immunosuppression. The clinical manifestations of HHV-7 are less clear, but have been associated with some cases of exanthem subitum (Tanaka et al., 1994; Torigoe et al., 1995; Ueda et al., 1994), pityriasis rosea (Drago et al., 1997), neurological symptoms (Caserta et al., 1998; Torigoe et al., 1996) and hepatitis (Hashida et al., 1995). EBV is the cause of the majority of mononucleosis cases. EBV is also associated with post-transplant lymphoproliferative disorders (PTLD) and other malignancies, such as nasopharyngeal carcinoma and Burkitt`s lymphoma. HHV-8 is etiologically linked to Kaposi's sarcoma and other malignancies. The main characteristics of herpesviruses are their ability to remain in a latent or persistent state in their host after primary infection and reactivate under immunosuppression. Reactivation and infection with human herpesviruses remain one of the most common infectious complications after transplantation (Fishman and Rubin, 1998).

2.2 HUMAN CYTOMEGALOVIRUS (HCMV)

In 1956, Margaret G. Smith recovered the first HCMV isolate from the submaxillary salivary gland tissue of a dead infant and the second isolate from the kidney tissue of a baby dying of cytomegalic inclusion disease (Smith, 1956). The same year, Rowe and coworkers, who recovered adenoviruses by observing cytopathic changes in uninoculated cultures of human adenoids, noted unique focal lesions and intranuclear inclusions primarily in the fibroblast component of cultures of adenoidal tissues from three asymptomatic children (Rowe et al., 1956). The cytopathic effect of the new virus strain (AD169) very closely resembled that of the Davis strain that was observed 1 year later by Weller and colleagues in human

embryonic skin muscle tissue cultures inoculated with a liver biopsy taken from a 3-month-old infant with microcephaly, jaundice, hepatosplenomegaly, chorioretinitis, and cerebral calcifications (Weller et al., 1957). The same group of researchers isolated two additional HCMV strains: the Kerr strain from the urine of a newborn with petechiae, hepatosplenomegaly, and jaundice, and the Esp. strain from the urine of an infant with hepatosplenomegaly, periventricular calcification, and chorioretinitis (Weller et al., 1957).

2.3 MORPHOLOGY

HCMV is the largest member of the herpes virus family (Landolfo et al., 2003). It is spherical, slightly pleomorphic and about 150 – 200 nm in diameter. The HCMV virion consists of an icosahedral capsid surrounded by a trilaminar phospholipid outer envelope about 10 nm thick. The core consists of a large double stranded linear DNA. Four proteins constitute the capsid: pUL 46, pUL 48.5, the minor capsid protein (mCP, UL85) and major capsid protein (MCP, UL86). The outer envelope of the virus contains multiple virally encoded glycoproteins which play multiple critical roles in the viral life cycle, including attachment, penetration, cell-to-cell spread, and envelopment and maturation of nascent viral particles (Huber and Compton, 1998). Three major complexes of glycoproteins can be identified: glycoprotein complex I (gCI), gCII and gCIII. The gCI is composed of glycoprotein B (gB), a glycoprotein expressed early in the replication cycle and existing as a heterodimer of glycoprotein (gp)115 and gp55, that remain complexed in the virion envelope. Up to 50 % of the protein mass of the viral envelope is represented by gB. Prior to infection, gB is involved in both attachment and fusion with the host cell

membrane. The gCII complex includes several different highly glycosylated proteins in the range of 47 kD to 200 kD. One of these is glycoprotein M (gM), a 100 kD protein that may be responsible for the heparin-binding capacity of gCII. The third complex gCIII is composed of a heterotrimeric complex of gH, gL and gO. Although the function of the assembled gCIII complex is unknown, gL is necessary for the transport of gH from the nuclear membrane to the cell surface, and gO has been associated with fusion of CMV-infected cells. The disulfide-bonded, tripartite gCIII complex is displayed on the surface of infected cells and ultimately becomes part of the virion. Aside of gB, gCIII is most abundantly expressed in the viral membrane, and has a role in fusion and penetration into the host cell. The viral tegument (proteinaceous layer between the envelope and the inner capsid) contains 20-25 structural virion proteins that are the major targets of host cell mediated immune response (Baldick and Schenk, 1996). Most of these tegument proteins are phosphorylated and they are also highly immunogenic. The most abundant are ppUL32 (pp150) and ppUL83 (pp65), of which the latter is the target antigen in antigenemia assays for rapid diagnosis of HCMV infections.

2.4 GENOME

HCMV has a double stranded linear DNA of more than 240 kb and codes for more than 200 putative open reading frames (ORFs). The linear HCMV genome is composed of a unique long domain (UL) and a unique short (US) domain. Both ends of these domains are flanked by a repeat sequence, an internal and terminal long repeat sequence and an internal and terminal short repeat sequence, IRL, TRL and IRS, TRS respectively. The UL domain and US domain can be arranged head to tail

or head to head and in the order UL-US or US-UL. Thus, four different isomers of the HCMV genome can be formed. **Table 1** shows the key genes of HCMV with their functions.

2.5 REPLICATION

The cellular receptor of CMV is unknown, but due to the ability of CMV to recognize and productively enter a wide range of cells suggests that the receptor is widely distributed (Mocarski and Courcelle, 2001). The epidermal growth factor receptor (EGFR) and cellular integrins have recently been suggested to function as entry receptors for CMV (Feire et al, 2004; Wang et al., 2003). Viral entry is the result of a cascade of interactions between viral and cellular proteins and it ends in fusion of the virion envelope with the plasma membrane. Binding of the enveloped glycoproteins such as gB and gH to cellular receptors activates cellular transcription factors, such as NFκB and Sp1. The virus then enters the cell, releasing viral DNA, virion proteins and virion mRNA transcripts into the cytoplasm, where virion mRNA are translated. Viral DNA and certain viral proteins are transported to the nucleus. During productive replication the viral genes are expressed in a temporally co-ordinated and regulated cascade of transcriptional events that lead to synthesis of immediate-early (IE), early (E) and late (L) proteins (Mocarski and Courcelle, 2001). In general, the IE proteins are activators of other genes. They have an important role in controlling cellular and viral gene expression, especially of early gene expression. Early genes encode mostly non-structural proteins, which are involved in the replication of viral DNA and in the induction of late gene expression. Late proteins are the virus structural proteins and they have a role in viral assembly

Table 1: Key genes of HCMV and their functions

Genes	Functions
UL 55 (gB)	Envelope glycoprotein; Major target for neutralizing antibodies; Virus entry and cell to cell transmission
UL 83 (pp65)	Major target for CD8 restricted cytotoxic lymphocytes. Major virion structural protein
US27, US28, UL33, UL75	G coupled receptor family members. US 28 acts as a promiscuous chemokine receptor
US2, US3, US6, US11	Involved in downregulation of HLA class I display on infected cells
UL 18	Class I HLA homologue; functions as NK decoy
UL 146	CXC Chemokine homologue; chemoattractant of neutrophils
UL 112/113 (IE1/IE2)	Key transactivators required for CMV replication
UL 80A	Protease; necessary for capsid assembly.
UL 97	Protein Kinase essential for replication. Activates ganciclovir to its monophosphate
UL 54	DNA polymerase. Required for CMV replication. Site of action of antiviral drugs (Ganciclovir, Foscarnet, Cidofovir)
UL 75 (gH)	Viral entry, target for neutralizing antibodies
UL 73 (gN)	Induction of neutralizing antibodies
UL 74 (gO)	Viral entry and fusion of infected cells
UL 115 (gL)	Transport of gH to cell membrane
UL 123	Target of Cytotoxic T lymphocytes
UL 144	Truncated TNF -α- like receptor gene
US28	Functional β chemokine receptor
US 9	Required for replication in polarized epithelial cells of human retina

and morphogenesis of the virion. DNA replication, formation of the viral capsids and DNA packaging occur in the nucleus, whereas the maturation takes place in the cytoplasm before the mature virus particles leave the cell via exocytosis. The whole replication cycle of HCMV takes approximately 48-72 hours.

2.6 TROPISM

During natural infection, it is shown that CMV replicates in many kinds of cells, including epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, mesenchymal cells, hepatocytes, monocytes/ macrophages and lymphocytes (Kahl et al., 2000; Plachter et al., 1996; Sinzger and Jahn, 1996; Sinzger et al., 1996). CMV may also infect several types of tissues, including most of the parenchymal organs, salivary glands, gastrointestinal and genitourinary tract (Pass, 2001).

2.7 EPIDEMIOLOGY AND TRANSMISSION

CMV shares with other herpesviruses the ability to remain latent in tissues after acute infection. Unlike herpes simplex virus and varicella zoster virus, which remain latent in highly restricted areas of the body, latent CMV can be found in multiple body sites, although it causes disease in only some of these that too in certain patient groups (Grundy, 1990). CMV infects humans of all ages, although the peak period of viral acquisition in the general population occurs early in life (Ho, 1990). In the population at large, primary infection occurs by direct close personal contact via exposure to bodily fluids such as saliva, tears, urine, stool, semen, and breast milk. Infants may acquire CMV transplacentally as a result of maternal viremia or perinatally via breast milk.

The term vertical transmission refers to the HCMV transmission from mother to fetus during pregnancy. Due to latency following primary infection and periodic reactivation of HCMV replication causing recurrent infections, in utero transmission of HCMV may follow either primary or recurrent infections (Ahlfors et al., 1984; Fisher et al., 2000; Schopfer et al., 1978). It is commonly recognized that primary HCMV infections are transmitted more frequently to the fetus and are more likely to cause fetal damage than recurrent infections (Fowler et al., 1992). In addition, it seems that primary infection occurring at an earlier gestational age is related to a worse outcome (Demmler, 1991; Stagno et al., 1986). The role of recurrent maternal infections in causing congenital infections is supported by reports and prospective studies describing congenital infections in the infants born to immune mothers (Schopfer et al., 1978; Embil et al., 1970; Krech et al., 1971; Stagno et al., 1973; Stagno et al., 1977). In a study, the incidence of symptomatic congenital HCMV infections in immune mothers was shown to be similar in primary and recurrent maternal infections (Boppanna et al., 1999). Symptomatic congenital infections appear to be mostly caused by reinfection of immune mothers during pregnancy by a new HCMV strain (Boppanna et al., 2001).

In later childhood, close physical contact facilitates transmission. Notably, infection in children is usually asymptomatic. Young children in day care centers transmit the virus to other children as well as to susceptible adults. (Adler, 1989; Adler, 1991; Gurevich and Kunha, 1981). In large day care centers, approximately half of all children experience active CMV infections and 10% to 15% of uninfected children become infected each year (Jones et al., 1996; Hutto et al., 1985). The highest prevalence rates of active viral transmission and excretion are found in

children of 13 to 24 months of age (Dobbins et al., 1994). Depending on the population surveyed, the prevalence of CMV antibody seropositivity in various regions ranges from 40 to 100% (Bale et al., 1999; Ho, 1990; Marshall et al., 1993; Rubin, 1990; Tong, 1997). CMV can be heterosexually and homosexually transmitted; Approximately 50% of transplant patients excrete CMV in body secretions (e.g., saliva and urine) at some stage after organ transplantation which usually begins in the first month following transplant surgery. Viral shedding reaches peak levels during the second and third months following transplantation, at which time it may be associated with disease (Griffiths, 1995; Lam and Khan, 1997). The incidence of symptoms related to CMV infection varies among different types of allograft recipients. In general, liver, pancreas, lung, intestinal, and heart transplant recipients have a greater incidence of CMV disease than do kidney transplant recipients. Symptomatic infections occur in approximately 39 to 41% of heart-lung, 9 to 35% of heart, 22 to 29% of liver and pancreas, and 8 to 32% of renal transplant recipients not receiving antiviral prophylaxis (Grossi et al., 1995 ; Ho, 1994; Patel and Paya, 1997). Recipients of living-related kidney allografts experience less morbidity associated with CMV infection than do those receiving cadaveric renal allografts (Stratta, 1993).

In the transplant population, three patterns of CMV infection are observed, each with a different propensity for causing clinical disease. Primary infection develops in a CMV-seronegative individual who receives blood products and/or an organ from a CMV-seropositive donor. Most primary CMV infections in organ transplant recipients are due to transplantation of an organ carrying latent virus from a seropositive donor. Secondary or reactivation infection occurs when latent CMV

reactivates posttransplantation in a CMV-seropositive recipient. CMV superinfection or reinfection occurs in a CMV seropositive host who receives cells and/or an organ from a seropositive donor, with reactivation of latent virus present in the allograft or reinfection by a new strain of CMV. It is difficult to distinguish superinfection from infection due to reactivation though, there is some indication that reinfection is more frequent than reactivation of endogenous CMV (Chou, 1989).

2.8 LATENCY AND REACTIVATION

Viral latency is defined as the persistence of the viral genome in the absence of production of infectious virions, but with the ability of the viral genome to reactivate under specific stimuli. Sporadic reactivation events in a latently infected immunocompetent host are generally well controlled by cell mediated immunosurveillance (Sinclair and Sissons, 2006). Occasionally HCMV activates from its latent state and infectious virions appear in the saliva and/or urine.

One of the site of carriage of the latent HCMV is considered to be in the peripheral blood compartment. The proof comes from the ability of immunocompetent, HCMV seropositive blood donors to transmit HCMV infection to recipients and the fact that transmission by blood could be reduced by using leucocyte depleted blood products for transfusion (Yeager et al., 1981; Adler, 1983; Tolpin et al., 1985; Graan-Hentzen et al., 1989). It is difficult to isolate the virus from the blood of healthy donors (Jordan, 1983). Highly sensitive PCRs have demonstrated the presence of HCMV DNA in the peripheral blood leukocytes (PBLs) of healthy, HCMV seropositive individuals (Taylor-Wiedeman et al., 1991;

Stanier et al., 1992; Larsson et al., 1998). Study using granulocyte-macrophage colony stimulating factor (GM-CSF) mobilized peripheral blood cells (Slobedman and Mocarski, 1999), suggest that the frequency of cells that carry the HCMV genome is extremely low (probably <1 in 10,000 peripheral blood mononuclear cells), requiring very sensitive PCR conditions. By using sorted peripheral blood – cell populations, for the analyses it has been proved that peripheral blood monocytes are major site of carriage of HCMV DNA in healthy carriers (Taylor – Wiedeman et al., 1991; Larsson et al., 1998). HCMV DNA has also been detected in $CD34^+$ bone-marrow progenitors (Mendelson et al., 1996). Viral genome does not appear to be carried in the polymorphonuclear leucocytes (PMNL), T – cell or B – cell fractions of peripheral blood (Taylor- Wiedeman et al., 1991; Taylor- Wiedeman et al., 1993). Analysis of the conformation of HCMV DNA in peripheral blood mononuclear cells has shown that the viral genome migrates as a circular plasmid (Bolovan-Fritts et al., 1999). The other sites of latency established by detection of viral DNA include early bone marrow haematopoietic progenitors, epithelial cells and endothelial cells (Hendrix et al., 1990; Kondo et al., 1994; Markovic-Lipkovski et al., 1992; Melnick et al., 1983; Taylor-Wiedeman et al., 1991; Taylor-Wiedeman et al., 1993; Sindre et al., 1996).

The cytomegalovirus latency – specific transcripts (CLTs) includes novel spliced and unspliced RNA transcripts mapping to both strands of HCMV major IE region (Kondo et al., 1994). No viral gene products from the CLTs have been shown to play a role in the latency. The roles of UL 82 (pp71), UL 111.5A encoding a homologue of IL-10 in latency are yet to be deciphered. The efforts to detect the viral transcription associated with carriage of HCMV in myeloid cells from naturally

latently infected individuals has been impeded by the low frequency of cells carrying latent HCMV in vivo. Certain experimental models have been able to predict several putative latent viral RNAs to have a role in the latency but their definitive role awaits confirmation.

Reactivation of a latent virus by allogeneic stimulation of T cells has been demonstrated (Soderberg-Naucler et al., 1997). The bone marrow acts as a reservoir of latent CMV, which then seeds latent virus into the peripheral blood via monocytes. Differentiation of monocytes to tissue macrophages seems to lead to virus reactivation and productive infection (Sinclair and Sissons, 2006; Soderberg-Naucler et al., 2001; Taylor-Wiedeman et al., 1994). The reactivation of CMV from latency is the critical first step in the pathogenesis of CMV infection. It is also observed that the load of latent viral genome is one of the factor that can influence reactivation. A higher load of the latent viral genome may make it easier for the virus to be reactivated (Reeves et al., 2005). The key mediators for the reactivation of CMV from latency is Tumor necrosis factor alpha TNF α, which activate protein kinase C and NFκB, a promoter of the immediate early (IE) gene of CMV initiating replication (Prosch et al., 1995; Stein et al., 1993). This observation is important, as it explains why CMV infection and disease are associated with several processes (e.g. sepsis, other viral infections and allograft rejection) (Rubin, 2001).

2.9 IMMUNE EVASION

HCMV encodes for a number of proteins which interfere with antigen processing. These proteins have been designated `immune evasion' molecules (Johnson and Hill, 1998). The US2 and US11 genes in the short unique segment of

the HCMV genome code for proteins which are capable of displacing the class I MHC heavy chain from the endoplasmic reticulum into the cytosol, where they are subsequently degraded. The US6 gene codes for a protein which binds to the endoplasmic reticulum face of the TAP (transporter of antigenic peptides) heterodimer and blocks the transport of peptides produced in the proteosome into the endoplasmic reticulum. The US3 gene product appears to promote the retention of assembled class I MHC molecules in the endoplasmic reticulum. All these proteins thus interfere with antigen processing via the class I pathway and contribute to the downregulation of class I MHC molecules on the surface of HCMV infected cells (Wiertz et al., 1997). Normally, the cells that lack class I MHC molecules are usually identified and lysed by Natural killer (NK) cells. Signal peptide sequences of class I MHC molecules normally bind to the non-classical class I molecule HLA-E and allow its egress to the cell surface where it can be recognized by, and inhibit, NK cells. It has been shown that the UL40 gene product of HCMV contains a sequence homologous to the signal peptides which can facilitate HLA-E expression (Tomasec et al., 2000). HCMV may thus have a mechanism for simultaneously inhibiting Cytotoxic T lymphocyte recognition and, by still allowing HLA-E expression, inhibiting NK cell recognition.

2.10 PATHOGENESIS & CLINICAL MANIFESTATIONS

2.10.1 *POST – ORGAN TRANSPLANT RECIPIENTS*

HCMV infection and disease are important causes of morbidity and even mortality among transplant patients. Three virus associated factors have been attributed to the pathogenesis of HCMV: the cell bound nature of the virus, ability to

disseminate throughout the body via blood stream and monocytotropic character. The latent virus in the monocytes forms a circulating reservoir which on differentiation into macrophage is capable of active replication thus causing the disease (The et al., 1994). Among the host factors, failure to reconstitute CMV – specific cellular immunity after transplantation is considered to be a potential cause for progression to HCMV disease (Quinnan et al., 1982).

The major risk factors for the development of CMV disease in the transplant recipients are serological status of the donor and recipient [sero-positive donor / CMV sero-negative recipient (D+/R-)], primary infections occurring in sero-negative transplant recipients, modification of immunity by the use of anti-lymphocyte antibodies or other agents that has an influence on the cell-mediated immunity (Birkeland et al., 1998; Chou, 1986; Smith et al., 1998). Transplant recipient receiving the allograft from HLA B7 positive donors have also been attributed to have a higher risk of developing CMV disease (Boland et al., 1993). CMV is also known to be an active inducer of other herpes viruses such as EBV and HHV 6 (Aalto et al., 1998). Simultaneous detection of HCMV and HHV 6 DNA in urine or serum of transplant recipient is considered to be a predictor of HCMV disease (Des Jardin et al., 1998; Ratnamohan et al., 1998).

HCMV reactivation following transplantation and other predisposing factors include type and intensity of immunosuppression, receipt of CMV contaminated blood products, cadaveric allograft transplantation and donor-receipient HLA mismatch (Bock et al., 1997; Glenn, 1981; Pillay et al., 1993, Sawyer et al., 1993).

The load of CMV in the graft has been attributed to be one of the factors that determine severity of the CMV disease in transplant recipients.

Direct viral cytopathogenecity and CMV triggered immune reactivity have been described as major factors for CMV induced organ dysfunctions (The et al., 1994; Smith et al., 1998; Grundy et al., 1987).

Irrespective of the pattern and type of CMV transmission, symptomatic disease in most of the transplant recipients occur between 1st and 4th months post transplant, when the immunosuppression is most intense (Paya and Razonable, 2003). The definitions of CMV infection and disease in transplant recipients have recently been updated (Ljungman et al., 2002). "CMV infection" is defined as isolation of the CMV virus or detection of viral proteins or nucleic acid in any body fluid or tissue specimen (Ljungman et al., 2002). The minimum requirements for definition of "CMV disease" are fever (>38ºC, for at least 2 days within a 4-day-period), neutropenia or thrombocytopenia, and the detection of CMV in blood. In an end-organ disease, e.g pneumonia, hepatitis, gastrointestinal disease, retinitis, nephritis, cystitis, myocarditis, pancreatitis or central nervous disease, CMV needs to be detected in the particular organ.

The consequences of HCMV infection in the transplant recipients include: Infectious disease syndrome caused by the virus itself, opportunistic superinfections with other infectious agents due to CMV associated immunosuppression, allograft dysfunctions and reduced graft survivals, and decreased survival rates among the transplant recipients.

CMV pneumonia remains a life-threatening syndrome, which is usually complicated by other pathogens, such as Pneumocystis, Aspergillus and other fungal copathogens (Fishman and Rubin, 1998). In hematopoietic stem cell transplantation (HSCT) patients, the most frequent clinical manifestations of CMV are fever, pneumonitis and gastrointestinal disease (Boeckh and Ljungman, 2003). In solid organ transplant patients, CMV can infect various organs such as lung, liver, intestines, kidney and heart, and may also affect the transplanted organ (Paya and Razonable, 2003). In addition to direct effects of CMV on the host, there are also indirect effects of the virus (Rubin, 2001). These are an association of CMV with acute graft rejection (Pouteil-Noble et al., 1993; Reinke et al., 1994) and chronic graft rejection, including accelerated transplant vasculopathy in heart transplant recipients (Grattan et al., 1989; Koskinen et al., 1993; Valantine et al., 1999), vanishing bile duct syndrome in liver transplant recipients (Arnold et al., 1992; Evans et al., 1999; Lautenschlager et al., 1997), chronic allograft nephropathy in kidney transplant recipients (Helantera et al., 2003; Humar et al., 1999a; Tong et al., 2002) and bronchiolitis obliterans in lung recipients (Kroshus et al., 1997). CMV is also associated with bacterial and fungal infections (Fishman and Rubin, 1998; George et al., 1997). CMV could interact with other viruses and may accelerate hepatitis C virus pathogenesis (Burak et al., 2002, Razonable et al., 2002a). Chorioretinitis is an uncommon manifestation of CMV disease in transplant recipients and usually occurs late (more than 6 months) in the post transplantation period. The usual symptoms include scotomata with or without decreased visual acuity (Rubin, 1990). Recurrence of HCMV disease after treatment is seen in 6 to 31% of kidney transplant recipients (Humar et al.,1999b; Jordan et al., 1992;

Sawyer et al., 1993). Most cases of the recurrent disease occur within 3 months after treatment of the first episode and tend to involve multiple organs. A cadaveric source of the graft and lack of CMV specific T helper responses following infection have been associated with recurrent HCMV disease (Humar et al.,1999b; Zeevi et al., 1998).

2.10.2 CONGENITAL HCMV INFECTION

Intrauterine transmission occurs in 30 to 40% of cases following primary infection and in reactivated maternal infections, the risk of symptomatic congenital infection is markedly lower (Ahlfors et al., 1984; Griffiths and Baboonian, 1984; Stagno et al., 1986). Although existing immunity does not prevent transmission of the virus to the fetus, reactivated infections are less likely to cause damage to the offspring than primary infections (Fowler et al., 1992). Multiple mechanisms of immune evasion for HCMV relates to the pathogenic role of the virus (Greijer et al., 2001).

The mechanisms of HCMV transmission to the fetus remain largely unexplored. It has been reported that about 15% of women undergoing primary infection during the first months of pregnancy abort spontaneously, showing placental but not fetal infection (Griffiths and Baboonian, 1984; Hayes and Gibas, 1971). Subsequently in the course of pregnancy, placental infection has been shown to be consistently associated with fetal infection (Muhlemann et al., 1992).

Guinea pig models have been helpful in establishing placenta as the reservoir in which CMV replicates prior to transmission to the fetus (Leiser and Kaufmann,

1994). In experimental infection of the guinea pig with species-specific CMV, the virus disseminates hematogenously to the placenta, from which it is transmitted to the fetus in about 25% of cases. The guinea pig CMV also persists in placental tissues long after virus has been cleared from blood (Griffith et al., 1985).

Two in vitro models for the study of trophoblast populations lying at the maternal-fetal interface, villous explants and isolated cytotrophoblasts have been described (Fisher et al., 1985; Fisher et al., 1989; Fisher et al., 2000). Hypotheses on the routes of transmission of HCMV to the fetus in primary and reactivated maternal HCMV infection have been framed based on the results of the in vitro models, immunohistochemical studies of in vivo HCMV-infected placentas (Muhlemann et al., 1992, Sinzger et al., 1993) and findings on HCMV latency (Hahn et al., 1998, Soderberg-Naucler et al., 1997). First model: During primary infection of the mother, leukocytes carrying infectious virus may transmit HCMV infection to uterine microvascular endothelial cells. These cells are in direct contact with cytotrophoblasts of anchoring villi invading maternal arterioles and forming hybrids of maternal-fetal cells. Infected cytotrophoblasts may in turn transmit the infection to underlying tissues of villous cores, including fibroblasts and fetal endothelial cells (Sinzger et al., 1993), thus spreading to the fetus. Second model of transmission envisages the spreading of infection to the villous stroma by infected maternal leukocytes after primary maternal infection through breaches of the syncytiotrophoblast layer (Hemmings et al., 1998; Jacques and Qureshi, 1993). A further hypothesis has been raised suggesting possible transportation of the virus as antibody-coated HCMV virions by a process of transcytosis through intact syncytiotrophoblasts similar to that advocated for transport of maternal IgG to the

fetus (Fisher et al., 2000). Finally, syncytiotrophoblasts may be directly infected, but the infection proceeds slowly and remains predominantly cell associated (Hemmings et al., 1998) until infected cells are eliminated during the physiological turnover (Smith et al., 1997). This hypothesis therefore excludes transmission through virus replication in syncytiotrophoblasts.

In the case of congenital HCMV infection following recurrent maternal infection, the placenta is considered as a hemiallograft inducing local immunosuppression in the uterus (Fisher et al., 2000; Roth et al., 1996). This may cause reactivation of latent virus in macrophages of the uterine wall, with HCMV transmission to the invading cytotrophoblasts. Then, virus could spread in a retrograde manner to anchoring villi and subsequently to the fetus (Muhlemann et al., 1992). In this regard, HCMV establishes a true latent infection in $CD14^+$ monocytes, which can be reactivated upon allogeneic stimulation of monocyte-derived macrophages from healthy blood donors (Soderberg-Naucler et al., 1997). Reactivation of latent HCMV is dependent on the production of gamma interferon in the differentiation process (Soderberg-Naucler et al., 2001). As a consequence of placental infection, HCMV impairs cytotrophoblast differentiation and invasiveness (Fisher et al., 2000). This explains the early abortion occurring in women with primary infection. In addition, HCMV infection impairs cytotrophoblast expression of HLA-G, thus activating the maternal immune response against the cytotrophoblast subpopulation expressing this molecule (Fisher et al., 2000).

Less than 5% of pregnant women with primary infection of HCMV are symptomatic (Pass and Boppana, 1999). The major symptoms include

mononucleosis – like syndrome which includes fever, cervical adenopathy, sore throat, splenomegaly, hepatomegaly and rash. The primary infection acquired just before conception is generally assumed to represent a lower risk than primary infection acquired during pregnancy. Primary infection early in pregnancy implies greater likelihood of congenital disease. The ultrasonographic abnormalities found frequently during fetal HCMV infection include intrauterine growth retardation, hydrops or ascites and central nervous system abnormalities.

The most common clinical sequelae reported in infected infants are jaundice, thrombocytopenia, hepatomegaly, petechiae, purpura, pneumonitis, splenomegaly and seizures. Permanent outcome of HCMV infection in the infants include CNS manifestations like microcephaly, chorioretinitis, sensorineural hearing loss, intracranial calcifications, mental retardation, developmental delay or seizures and death (Munro et al., 2005).

2.10.3 HIV INFECTED INDIVIDUALS

Prior to the introduction of highly active anti retroviral therapy (HAART), Cytomegalovirus (CMV) infection was one of the most important opportunistic infections in HIV-infected patients. Approximately 40% of HIV-infected patients with advanced disease suffer from one of several different manifestations of CMV disease during life-time (Bowen et al., 1996; Jabs et al., 1989; Pertel et al., 1992; Drew, 1992). The median survival period after diagnosis of CMV disease in these patients was 4–9 months (Harb et al., 1991; Gerard et al., 1997). After the introduction of HAART therapy in 1995–1996, life expectancy and quality of life had dramatically improved with the persistent suppression of HIV viremia.

Persistent immune reconstitution, decreased CMV replication and incidence of CMV disease particularly CMV retinitis in HIV-infected patients were observed in few studies (O'Sullivan et al., 1999; Palella et al., 1998; Ledergerber et al., 1999; Mocroft et al., 2000).

But, recent studies have documented rapid progression (2.5 times) of CMV – seropositive individuals to AIDS than seronegative individuals even after HAART therapy (Sabin et al., 2000). In the case of primary CMV infection, even patients with relatively high $CD4^+$ cell count (>100/mm^3) were shown to be at a significantly increased risk for progression to AIDS (Robain et al., 2001). A CD4 cell count <100/mm^3 was identified as the major risk factor for CMV disease in CMV-seropositive HIVinfected patients (Deayton et al., 2004; Deayton, 2001; Salmon-Ceron et al., 2000). The initial months of antiretroviral treatment where in cellular immunity is not fully reconstituted is shown to be the phase at which the HIV patients develop CMV disease (Ledergerber et al., 1999; Salmon-Ceron et al., 2000).

CMV retinitis is the single most common manifestation of CMV disease, accounting for 85% of all cases (Gallant et al., 1992; Yust et al., 2004). It is characterized by haemorrhagic retinal necrosis, including white areas with or without hemorrhages and gray-white areas of retinal necrosis. Lesions have an irregular, dry-appearing, granular border, with little or no overlying vitreous inflammation (Ljungman et al., 2002). Ophthalmologic findings are pathognomonic, so that the diagnosis may be established by an experienced opthalmologist within the context of advanced HIV-infection (Ljungman et al., 2002). The prevalence of

immune recovery vitritis varies in patients with previous CMV retinitis from 18% to 63% (Nguyen et al., 2000; Karavellas et al., 1999).

Gastrointestinal disease is the second most common manifestation of CMV infection accounting for 15% of all cases of CMV disease (Gallant et al., 1992; Yust et al., 2004). CMV infection may involve all parts of the gastrointestinal tract. The major symptoms include odynophagia or persistent diarrhea and in severe cases obstruction or perforation of the gastrointestinal tract may occur. Bleeding of the tract rarely occurs.

Clinical CMV disease of the nervous system accounts for less than 1% of all CMV diseases in HIV-infected patients (McCutchan, 1995; Gallant et al., 1992), but mortality rate approximated 100% in untreated patients (McCutchan, 1995) and median survival from the onset of neurological symptoms was less than 2 months (Salazar et al., 1995; Arribas et al., 1996). Signs and symptoms of CMV encephalitis are unspecific, such as drowsiness, fever, disorientation, confusion, apathy, a relatively abrupt onset of mental status changes or focal neurological signs and may be similar to infections with other viruses such as herpesviruses other than HCMV, polyomaviruses; parasite such as *Toxoplasma gondii* and fungus such as C*ryptococcus neoformans* (Morgello et al., 1987).

CMV pneumonia is a very rare cause of lung disease in HIV-infected patients (Gallant et al., 1992). Only very few well documented studies on HIV-infected patients with CMV pneumonia have been described (Rodriguez-Barradas et al., 1996; Salomon et al., 1997; Vasudevan et al., 1990). The clinical presentation of CMV pneumonia is indistinguishable from pneumonia caused by other infectious

agents such as *Pneumocystis jiroveci* and manifests as fever, cough, hypoxemia and diffuse radiographic opacities.

2.11 DIAGNOSIS OF HCMV INFECTIONS

The clinical manifestations of CMV disease may be relatively nonspecific. The signs and symptoms of CMV disease cannot be easily differentiated from illnesses caused by a variety of other opportunistic microbes or drug toxicity. It is also difficult to establish the cause for acute graft rejections in transplant recipients on clinical grounds since, HCMV plays a role in organ dysfunction. Hence, clinical diagnosis of HCMV is unreliable which necessitates the use of rapid and sensitive laboratory diagnostic tests for its diagnosis. Laboratory methods are useful not only in establishing CMV disease in the patients but also to predict the risk of development of HCMV in the patients. Both qualitative and quantitative methods are currently employed in the diagnosis of HCMV disease in various patient populations. Quantitative methods are employed more as prognostic markers in the development of HCMV disease in post organ transplant recipients and HIV infected individuals. Since, bone marrow transplant recipients as well as neonates develop disease even with a low viral load, qualitative methods itself may be beneficial in these groups. Quantitative methods irrespective of the patient groups are useful in determining the response to treatment and as a surrogate marker in development of antiviral resistance. The diagnostic methods include serology, viral culture, antigen detection and detection of nucleic acids. Several specific definitions for CMV in blood are recommended: viremia is defined as the isolation of CMV by culture from blood, antigenemia as the detection of CMV pp65 in blood leukocytes, and

DNAemia as the detection of DNA in samples of plasma, whole blood, isolated peripheral blood leukocytes or buffy-coat specimens (Ljungman et al., 2002).

2.11.1 CONVENTIONAL LABORATORY METHODS OF DIAGNOSIS

2.11.1.1 Serology

Serology is an insensitive marker of active CMV infection in the organ transplant recipients and HIV infected individuals and is therefore of limited diagnostic usefulness (Marsano et al., 1990; van Son and The, 1989; Robain et al., 2001). The main utility of serologic studies in organ transplantation is as an accurate and sensitive means of determining a past history of CMV infection in potential organ/blood donors and allograft recipients. In patient with AIDS, seroconversion may be significantly delayed, and the maturation of humoral and cellular immunity may be clearly prolonged (Robain et al., 2001). Similarly, reactivation from latency may not be detected by serology due to a less effective recall immune response in AIDS patients (Robain et al., 2001). However, serological techniques have been useful in establishing primary infections in pregnant women. Seroconversion i.e. appearance of CMV specific IgG antibodies in a previously seronegative pregnant women is considered to be a definitive diagnosis of primary HCMV infection. In cases where seroconversion cannot be proved, detection of IgM by one of the numerous diagnostic tests such as complement fixation, indirect hemagglutination, anticomplement immunofluorescence, Radio immuno assay (RIA) or Enzyme linked immuno sorbent assay (ELISA) can be helpful in establishing clinically significant primary HCMV infections (Reynolds and Stagno, 1979; Krishna et al., 1980; Schmitz et al., 1980). The detection of IgM antibody seldom occurs during recurrent

infections in a immunocompetent individual such as pregnant women. Hence, the detection of IgM antibody is considered to be a reliable marker for primary infection in pregnant mothers. However, the presence of IgM can reveal different clinical situations such as, acute phase of a primary infection (peak titres of IgM antibody is seen between 1 and 3 months), convalenscent or late phase of primary infection (the titre drops down after 3 months) mere persistence of IgM antibodies (stable levels of IgM for longer than 3 months) which can occur in around 28.5% of pregnant women (Revello and Gerna, 1999). In cases of persistent IgM levels, test like IgG avidity may be useful in establishing the clinical significance of the presence of IgM antibodies. IgG avidity tests can be helpful in distinguishing primary from non primary HCMV infections (Bodeus et al., 1998; Bodeus and Goubau, 1999; Revello and Gerna, 1999). The test principle is based on the difference in the avidity of the IgG antibody produced during the initial and later phase of infection. During the first few months of infection (primary infections) IgG of lower avidity is produced while in subsequent period (recurrent or remote infections) a maturation process occurs giving rise to IgG antibodies of a higher avidity. The result of the test is expressed as avidity index which is the percentage of IgG antibody bound to the antigen following the treatment with denaturing agents such as 6M urea. The avidity index within 3 months of a primary infection is usually 21 % $\pm$ 13% whereas mean avidity index values for serum samples from subjects with remote HCMV infection were 78% $\pm$ 10% (Revello and Gerna, 1999). Thus, the presence of high IgM levels and a low avidity index are highly suggestive of a recent (less than 3 months) primary HCMV infection. In a study, the ability of three different IgG avidity assays to detect a primary HCMV infection was found to be approximately 100%, whereas the

ability to exclude a recent infection was shown to range from 96% to 32%. These data indicate a need for more standardized assay (Bodeus et al., 2001). Also, the gap between the low and higher avidity index is immense and any value between the two may be inconclusive of the phase of primary infection. The determination of neutralizing antibodies in the immunocompetent host may aid in establishing the period of primary infection. The neutralizing antibodies in majority of the cases tend to appear after 15 weeks (range 14- 17 weeks) of primary infection. Hence, an absence of neutralizing antibody during convalescence is indicative of a recent primary infection and presence of the same rules out infection within 15 weeks (Eggers et al., 1998).

2.11.1.2 Histopathology & Immunohistochemistry

Traditionally, the diagnosis of CMV infection was based on the histologic recognition of cytomegalic inclusion bodies that have the characteristic intranuclear owl-eye appearance in haematoxylin and eosin-stained tissue specimens. Histopathological studies have been considered useful in differentiating allograft dysfunction caused by inflammatory response provoked by CMV from cellular rejection (Barkholt et al., 1994; Colina et al., 1995; Ulrich et al., 1986) in liver and renal transplant recipients. The presence of viral inclusion bodies in liver tissue, for instance, correlates with active disease in most cases (Colina et al., 1995). Conversely, CMV may be detected in cultures of biopsy specimens that are negative on histopathology (Paya et al., 1989). Histopathological studies have also been used in establishing or ruling out CMV associated manifestations in AIDS patient (Rovery et al., 2006). The use of immunohistochemistry has increased the

sensitivity for the histologic diagnosis of CMV disease compared to standard hematoxylin and eosin staining (Barkholt et al., 1994; Colina et al., 1995; Paya et al., 1990). Immunostaining techniques use either a monoclonal or a polyclonal antibody against an early CMV antigen. The sensitivity and specificity of the immunohistochemistry was close to 84 and 97% in one study (Paya et al., 1990). A high rate of false-negativity has been shown in a study, owing to the focal distribution of CMV-positive cells (Colina et al., 1995). Histopathological studies or immunohistochemistry are considered to be important for the identification of localized CMV disease, but their usefulness is limited because of the need to use invasive procedures to obtain samples.

2.11.1.3 Detection of Cytomegalic endothelial cells (CEC) in circulation

The term endotheliemia was introduced to indicate HCMV-infected CEC in the peripheral blood of immunocompromised patients. CEC were first described in 1993 by two independent groups (Grefte et al., 1993; Percivalle et al., 1993) and were shown to be endothelial in origin and fully permissive for HCMV replication. The presence of these cells is considered to indicate extensive endothelial damage. These cells are derived from infected endothelial cells of small blood vessels, which progressively enlarge until they detach from the vessel wall and enter the bloodstream. These cells can be identified by cytocentrifugation of the mononuclear fraction of leukocytes onto glass slides followed by endothelial cell-specific staining. A method for quantification of CEC in peripheral blood has also been described (Kasdeelen et al., 1998). In patients with AIDS, the detection of CEC is associated with lack of anti- CMV treatment, emergence of drug-resistant CMV,

insufficient treatment, or transient response to antiviral therapy (Gerna et al., 1998c). CEC have also been studied in hematopoietic stem cell transplant recipients (Salzberger et al., 1997). In recent years, the introduction of HAART for AIDS patients and the adoption of prophylactic and preemptive therapy approaches for transplant recipients have nearly eliminated CEC from blood of these patient groups. However, CEC is shown to occur in the blood of fetuses and newborns with symptomatic congenital HCMV infection.

2.11.1.4 Virus isolation

Conventional detection of CMV in clinical specimens is achieved by direct viral culture. The detection of CMV in tissue cultures from peripheral sites such as urine or saliva is often uninformative because the virus may be present in these sites for prolonged periods after the acute phase of infection and during viral reactivation. Nevertheless, viral isolation from these sites indicates a relative risk of about twofold for future CMV disease (Pillay et al., 1992; Pillay et al., 1993). Furthermore, viral isolation from any site in a CMV-seronegative patient is indicative of primary CMV infection.

2.11.1.4.1 Conventional tube cell culture

For conventional cell culture, clinical specimens are inoculated onto human diploid fibroblast cells and incubated at 37°C**.** CMV exhibits a typical cytopathic effect (CPE) characterized by foci of flat, swollen cells. The time required for the development of CPE is directly related to the titer of the virus in the sample. Typically, the mean time for CPE to be visible is 8 days, but it can range from 2 to

21 days. The long time required for diagnostic confirmation by this method limits its clinical usefulness. There are two methods of quantitating CMV in conventional tube cultures: the plaque assay and determination of $TCID_{50}$.

(i) Traditional Plaque assay

In a plaque assay, serial dilutions of the specimen are inoculated onto fibroblast monolayers and after infection the cells are overlaid with a semisolid medium. The virus spreads from cell to cell, resulting in a localized plaque. After an incubation period, the infectious plaques are enumerated under microscopic examination. The plaque assay and modifications of it have been used frequently for CMV quantitation in a wide variety of clinical specimens (Chou and Scott, 1988; Churchill et al., 1987). Results are obtained by calculating a logarithmic viral titer from the plaque counts in the dilution giving about 200 plaques.

(ii) $TCID_{50}$ assay

A less precise assay for the quantitation of CMV in tissue culture is determination of the $TCID_{50}$. In this method, the virus titer is ascertained by determining the highest dilution of the specimen which produces a cytopathic effect in 50% of the cell cultures or wells inoculated (Schmidt, 1989). The $TCID_{50}$ has been used to quantify CMV in lung tissue and urine. Serial log or half-log dilutions of the specimen are inoculated onto human foreskin fibroblasts and observed for 6 weeks.

2.11.1.4.2 Rapid shell vial assay

Traditional tissue culture methods have been replaced by the more rapid shell vial centrifugation culture assay system. Detection of CMV-specific early-antigen fluorescent foci by using the shell vial assay permits the detection of CMV prior to the development of CPE in conventional tube cell culture. Shell vial assays are performed in vials containing coverslips containing a fibroblast monolayer. Centrifugation of specimen onto the cell monolayers greatly assists absorption of virus, increasing apparent infectivity of the viral inoculum. The shell vial assay utilizes a monoclonal antibody directed at the immediate-early (IE) or early viral antigen to detect CMV by indirect immunofluorescence after 16 – 48 hours of incubation. CMV is quantified in shell vial-based assays either by keeping the viral inoculum constant and counting the number of infectious foci per shell vial (Gerna et al., 1990) or by inoculating serial dilutions of the sample and determining the titer, which is defined as the reciprocal of the final dilution in which CMV can be detected (Slavin et al., 1992). Shell vial centrifugation culture-based assays have been used for the quantitation of viremia and of CMV in bronchoalveolar lavage (BAL) fluid (Gerna et al., 1990; Slavin et al., 1992).

Due to a high rate of false-negative results, virus isolation procedures are useful only in the setting of a positive result and is considered a parameter of risk for the development of CMV disease. The number of positives identified by the shell vial assay is greater than that detected by conventional culture. Development of viremia detected by shell vial culture more than 2 months after liver transplantation

is a strong predictor of CMV disease (Manez et al., 1996). Viral isolation has been shown to be highly useful for antiviral susceptibility testing.

2.11.1.4.3 Limitations of viral isolation procedures

A delay in specimen processing of longer than 24 hours severely compromises the sensitivity of the assay. The other limitations include:

Cytotoxic effects of clinical specimens on the fibroblasts ; Overgrowth of rapidly growing microorganisms (such as herpes simplex virus or bacteria); Lack of staining by monoclonals in case of shell vial assays; and poor ability of certain CMV strains to form plaques.

2.11.1.5 pp65 antigenemia assay

The antigenemia assay detects and quantifies peripheral blood leukocytes, mostly polymorphonuclear leukocytes (PMNL) and, to a much lesser extent, monocytes/macrophages, which are positive for the HCMV lower matrix phosphoprotein pp65 (Grefte et al., 1992; Revello et al., 1992). The HCMV protein is transferred to PMNL from infected permissive cells via transitory microfusion events between two adhering cells. It has been shown that antigenemia becomes positive earlier than viremia but later than DNAemia at the onset of infection, and it becomes negative later than viremia but earlier than DNAemia in the advanced stage of a systemic infection in immunocompromised patients (Gerna et al., 2001). A high antigenemia level is often associated with HCMV disease. The assay is widely used as a prognostic marker for institution of antiviral therapy and monitoring of HCMV load during therapy. (Boeckh et al., 1992; Gerna et al., 1991). Breakpoints of 10

positive cells per slide in kidney transplant recipients and HIV infected individuals and 100 positive cells per slide in liver and heart transplant recipients in the antigenemia assay have been advocated to institute early antiviral treatment (Boeckh and Boivin, 1998). During ganciclovir treatment of primary HCMV infections, antigenemia levels may increase for up to 2 to 3 weeks despite the efficacy of treatment as shown by the disappearance of viremia. (Boeckh et al., 1996; Gerna et al., 1998b). The antigenemia testing consists of a number of steps, including isolation of blood leukocytes preparation of cytospin slides containing a given cell number by centrifugation of leukocyte-rich supernatant, slide fixation and staining with monoclonal antibody directed against the CMV lower matrix phosphoprotein pp65 antigen. Immunodetection of CMV antigen is possible with either indirect immunoperoxidase, indirect immunofluorescence methods or phosphatase/ anti-alkaline phosphatase (APAAP) staining . The results may be reported as the number of positively stained cells relative to the number of cells used to prepare the slide.

2.11.1.5.1 Advantages and limitations of pp65 antigenemia assay

Advantages include a short processing time (less than 6 hours) and the lack of requirement of a highly specialized laboratory**.** Disadvantages include the need for immediate processing, the relatively time-consuming nature of the different assay steps with large specimen numbers, and the subjective component of slide interpretation.

2.11.1.6 Short comings of Conventional methods of detection

Most of the conventional methods of diagnosis of HCMV suffer from one or more of the following disadvantages: time-consuming, poor reproducibility, low sensitivity, cannot be applied on wide variety of clinical specimens, cannot be automated and cannot be adapted for high throughput.

2.11.2 MOLECULAR METHODS OF DIAGNOSIS

Detection and quantification of HCMV DNA in blood has become a major diagnostic tool for HCMV diseases in immunocompromised patients**.** The molecular methods of diagnosis have one of the several characters of an ideal diagnostic tool. This includes rapidity, high sensitivity of detection, better reproducibility, ability to be applied on a wide variety of clinical specimens, scope for automation and high throughput.

2.11.2.1 In situ Hybridization

In situ hybridization (ISH) with CMV-specific complementary DNA probes applied to cellular material has facilitated the histopathological identification of infected cells in tissues (Paya et al., 1990; Mauch et al., 1995). Hybridization has conventionally been performed with probes labeled with radioactive isotopes, which then allows the detection of sequence specific nucleic acid following autoradiographic analysis (Fox and Emery, 1992). The routine use of radioactive isotopes has largely been replaced by biotinylated DNA and electrochemiluminescent labels (Einsele et al., 1989; Musiani et al., 1996). Biotinylated probes may be used for the direct detection of CMV inclusions in

formaldehyde-fixed, paraffin-embedded tissue sections in cases where active infection is present. Estimation and quantification of nucleic acid present in tissue samples or cellular smears may also be possible with ISH. The comparative sensitivity of ISH for diagnosing CMV disease varies in different studies. Most reports do not favor the use of ISH over conventional histopathologic examination for the diagnosis of CMV organ disease (Barkholt et al., 1994; Colina et al., 1995; Paya et al., 1990). Nonetheless, the test specificity for allograft biopsy specimens is as high as 100% and allows rapid detection of HCMV in tissues (Masih et al., 1988; Paya et al., 1990). Applications for this test include the diagnosis of CMV pneumonitis (Einsele et al., 1989), hepatitis (Masih et al., 1988; Paya et al., 1990) and gastroenteritis (Chou, 1990b). PCR-driven in situ hybridization (PCR-ISH), and Reverse transcription PCR –based in situ hybridization for detection of HCMV during experimental latent infection of cultured granulocyte-macrophage progenitors and natural infection have been described (Slobedman and Mocarski, 1999). However, the techniques involved are cumbersome. Specimen preparation consists of securing the sample to a glass slide and denaturing the DNA without detaching or destroying the morphologic identity of the cells.

2.11.2.2 In vitro nucleic acid based amplification techniques

In 1983, Kary Mullis envisioned a process of in vitro amplification which is eventually known as polymerase chain reaction (PCR) (Mullis, 1990). This is the hallmark of nucleic acid amplification method. PCR became a reality within a short period by its first practical application. (Saiki et al., 1985).

The primary objective of the in vitro amplification methods is to improve the sensitivity of tests based on nucleic acids and to eventually simplify them through the use of automation and the incorporation of non-isotopic detection methods. The in vitro amplification techniques have been classified into three categories (Persing and Landry, 1989). They are—

(1) Target amplification systems, which use PCR, self-sustaining sequence replication, or strand displacement amplification.

(2) Probe amplification systems, which include those involving thermostable DNA ligase chain reaction (LCR).

(3) Signal amplification, in which the signal generated from each probe molecule, is increased by using compound probes or branched-probe technology.

2.11.2.2.1 Target amplification methods

(i) Polymerase chain reaction

The PCR technique is based on the reiteration of a three-step process: denaturing dsDNA into single strands, annealing of primers (specific synthetic oligonucleotides) to the ssDNA, and enzymatically extending the primers complementary to the ssDNA templates. After the primers are annealed to the denatured DNA, the ssDNA segment becomes the template for the extension reaction. Nucleotides, present in the solution in excess, are enzymatically joined to form the complementary DNA sequences. During the second and subsequent cycles, the original DNA segment and the newly generated complementary DNA strands

become templates. Each cycle of PCR therefore doubles the amount of specific DNA present. A typical amplification is 20 to 40 cycles and results in a 10^6-fold amplification of the original DNA (Parsons, 1988 ; van Brunt, 1990). The improvement of PCR by the use of a thermostable enzyme, Taq DNA polymerase, has allowed semi-automation and simplification of the process to the point that it has become widely adopted in research laboratories.

Confirmation of PCR product authencity:

PCR product authencity can be determined by a number of simple methods.

- Agreement between observed and expected size of the PCR product
- Confirmation of the position of a single restriction site within the amplified DNA.
- Dot-blot or Southern blot hybridization or sequencing in rare cases.

Short comings of PCR:

The system is susceptible to contamination with extraneous DNA fragments that could be amplified along with the sample. This is important in that extraneous DNA can be carried over from previous amplifications (amplicons) or introduced from other sources. To eliminate this problem, adherence to careful laboratory procedures, such as rigid quality control of enzyme preparations and the use of dedicated pipettes and prealiquoted reagents, is necessary. Various other techniques for reducing extraneous DNA contamination of PCR products have been described

such as treatment with psoralen and exposure to UV or use of dUTP – UNG system for amplification. The system is also susceptible to various kinds of inhibition that may occur due to problems in the cell lysis (extraction step), nucleic acid degradation (during storage) or polymerase related inhibition (Wilson, 1997).

(ii) Modifications of PCR

(a) Nested PCR

In a typical nested amplification protocol, a first round amplification is performed with a single pair of primers for 15 to 30 cycles. This step produces an amplified product, which is transferred to a new reaction tube for a second round of amplification by using a second pair of primers that is specific for the internal sequence amplified by the first pair of primers. The second amplification usually proceeds for an additional 15 to 30 cycles.

Nested amplification offers both advantages and disadvantages. The sensitivity of most nested amplification procedures is extremely high. A single copy of target can be detected without the need for hybridization with labeled probes (Persing, 1993). Re-amplification with the second set of internal primers also serves to verify the specificity of the first round product. In addition, the transfer of reaction products from the first reaction effectively serves to dilute out inhibitors that might be present in the sample initially.

The incipient risk with the nested PCR is contamination due to the aerosolized amplified DNA during the transfer of the reaction product from the first reaction into a new tube for re-amplification. Several methods have been described

for performing nested reactions without open transfer of amplification products between reaction tubes. These single tube nested protocol involve various approaches. A thick layer of oil above the reaction mixture containing the first primer pair has been used (Feray et al., 1992). After the first amplification, the reaction tubes are removed and spun in a microcentrifuge to mix the reaction components and dilute the first round components, and then the contents are reamplified. This single tube protocol has much less risk of contamination and is reported to be as sensitive as the two-tube nested protocol (Yourno, 1992). Other methods of single tube nested amplification are based on differential annealing of outer and inner primer pairs and differential melting temperatures of the large and small amplification products (Erlich et al., 1991).

(b) Semi-nested PCR

In case of the semi-nested PCR, nested PCR reaction is carried out using two rounds of primers. Of the primer sets used, one is common to both the external and the internal amplification. Thus, the internal target amplified includes one end of the initially amplified target. The sensitivity and the specificity is the same as that of the nested PCR.

(c) Multiplex PCR

Multiplex PCR is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. This was first described by Chamberlain et al in 1988 (Chamberlain et al., 1988). Co-amplification of different targets serves several purposes.

(1) Large regions of the DNA sequence can be scanned for alterations.

(2) Unrelated segments of the target genome can be tested.

(3) Internal controls for the amplifiability of the sample can be included.

(4) Cost-effective panel of tests for multiple pathogens from a single specimen can be developed (Persing, 1993).

In viral diagnosis the utility of multiplex PCR finds its place in detection of multiple viruses causing a syndrome, for genotyping and quantitation.

Critical parameters that require optimization for a successful multiplex PCR assay are:

- Primers – their relative concentrations, length, G+C content, amplification target size (which is easily resolvable on the agarose gel)
- Concentration of the PCR buffer
- Cycling temperatures
- Number of cycles for amplification
- Balance between the concentrations of $MgCl_2$ and dNTPs (Henegariu et al., 1997)

In diagnostic laboratories, cost and availability of adequate sample limit the use of PCR. Multiplex PCR has the potential to reduce the time and effort taken within the laboratory without compromising test utility.

Bias in Multiplex PCRs

Preferential amplification of one target sequence over another is a known phenomenon in multiplex PCRs that are designed to amplify more than one target simultaneously. Two major classes that induce bias are: PCR drift and PCR selection. PCR bias is assumed due to stochastic fluctuation in the interactions of PCR reagents particularly in the early cycles which would arise in the presence of very low template concentrations. PCR selection is a mechanism which inherently favors the selective amplification of certain templates due to the inherent nature of the targets. The properties of the target include interregion differences in the GC content, leading to preferential denaturation, higher binding efficiency of GC rich primers, different accessibility of targets within genomes due to secondary structures, and gene copy number within a genome. Amplification bias dependant strongly on the choice of primers have been described (Elnifro et al., 2000).

Points to be considered during Multiplex PCR development:

- A rational approach for inclusion or exclusion of specific pathogens
- Compatability among the primers
- Primer design must take into account all the strains of the target pathogen as possible

Advantages of multiplex PCR :

- One of the major problems faced in PCR include false negatives due to reaction failure. Each amplicon in a multiplex PCR act as an internal control for the other amplified fragments.

- It is shown that the degraded templates give weaker signals for long bands than for short; hence the quality of the template may be determined more effectively in multiplex than in single locus PCR.

- The cost, time and labour are much reduced in multiplex PCR.

- Multiplex PCR helps in conserving the template that may be in short supply in instances of clinical specimens such as intraocular fluids or CSF.

Application of Multiplex PCR in diagnosis of HCMV infections

Multiplex nested PCR for detection and typing of herpesviruses: HSV1, HSV 2, VZV, CMV, HHV6 and EBV has been applied to CSF from patients with meningitis, encephalitis and other neurological syndromes. The multiplex PCR was based on the DNA polymerase gene. The first round amplified universal herpes viruses with second round identifying the specific herpes viruses (Tenorio et al., 1993). A multiplex short tandem amplification of multiple pathogens (STAMP) primers has been employed for the detection of HSV, VZV, CMV and toxoplasma in a single tube (Dabil et al., 2001). Stair primers was employed for the simultaneous

detection of HSV, VZV, CMV, EBV and HHV-6 in a single multiplex reaction in the tear samples (Robert et al., 2002).

Two different genes of HCMV were detected with a single step multiplex PCR in clinical specimens from renal transplant recipients and other seropositive patients. The test was based on the IE and LA genes of HCMV and allowed monitoring of CMV infection by quantitation (Caballero et al., 1997). Another multiplex PCR was used to simultaneously amplify four different regions of HCMV genome. The test had a high diagnostic utility for detection of CMV variants with maximal sensitivity and specificity (Markoulatos et al., 1999). Multiplex PCR for genotyping of HCMV genome is also described (Tarrago et al., 2003).

(d) Quantitative PCRs

Quantitative PCR assays have been useful in establishing relative or absolute amounts of target DNA. Methods developed for DNA quantitation by PCR may be classified into two categories: semiquantitative and competitive assays.

Semiquantitative PCR

Semiquantitative PCR methods provide relative data. These procedures are designed to perform titer determinations of the target template or of an external control by end-point sample dilution prior to PCR (Drouet et al., 1993b; Kulski, 1994) or by co-amplification of target and an endogenous cellular (e.g., β-globin) DNA sequence (Kellogg et al., 1990). The amount of DNA is extrapolated from a standard curve derived from the amplification of known amounts of the external standard.

The relationship between the amount of initial target DNA to the final amplified product is given by the following formula:

$$Y = X (1 + E)_n$$

Where Y is the amount of final amplicon; X is the amount of initial target DNA; E is the efficiency of the reaction and n is the number of amplification cycle. In a normal PCR, the efficiency of the reaction is seldom 100%. The efficiency of the PCRs can be affected due to several factors such as primer-annealing kinetics; concentrations of DNA, deoxynucleoside triphosphates, $MgCl_2$, and primers; number of cycles, incomplete DNA strand separation and PCR product strand reannealing.

Competitive PCR

The competitive PCR method is based on co-amplification of an exogenous template as an internal standard that competes with the target sequence for the same primers so that any variable affecting amplification has the same effect on both. In competitive PCR, the equation of PCR amplification is as follows:

$$C/T = C'/T'$$

where C and T are the product yields of the competitor and the target templates, respectively, and C' and T' are the initial amounts of both templates.

In quantitative competitive PCRs, two main types of competitors have been used. Homologous competitors containing small deletions or insertions with respect to the target sequence (Boivin et al., 1995; Fox et al., 1992), and heterologous

competitors having the target sequence for primers as the target nucleic acid but differing in the intervening sequence (Gerna et al., 1994; Gerna et al., 1998a). In case of the homologous competitors, the amplicons are differentiated by resolving them on agarose gels or by restriction enzyme digestion. In case of the heterologous competitors, the products of the competitor and the targets are differentiated by different capture probes.

The advantage of the homologous competitor is that the amplification efficiency of the competitor and the target is similar, but, heteroduplexes between the two may form during the plateau phase of reaction. However, the formation of heteroduplexes is avoided in case of the heterologous competitors by difference in the intervening sequences while the amplification efficiency may be lost due to differences in the G+C contents and size of the competitor and target.

Commercial Quantitative PCR assays for HCMV

The Sharp Signal System (Digene Corp., Silver Spring, Md.) and AMPLICOR CMV test (Roche Diagnostic Systems, Branchburg, N.J.) are the commercial assay available for quantitation of HCMV in PMNL or plasma. Both systems use non-isotopic hybridization for viral target detection in a convenient microplate assay. AMPLICOR CMV Test (Roche Molecular Systems, Branchburg, N.J.) is a fully automated system that amplifies, CMV DNA, captures the biotinylated amplification product with a specific oligonucleotide probe, and detects the bound products colorimetrically. The results are available within 6 hours (Didomenico et al., 1996).

Limitations of the Quantitative PCRs

- Almost all the quantitative PCRs are end point quantitation i.e. the quantitative data is obtained from the end point of the amplification. There may not be a strict relationship between the initial template and final amplicon concentration since the final amount of amplicon present may have been affected by inhibitors, poorly optimized reaction conditions, saturation of inhibitory PCR – by products and double stranded amplicons.

- Most of the in house quantitative PCRs suffer from substantial intra and inter assay variability and narrow dynamic range which necessitates dilution of the amplicon prior to conventional detection or use of diluted clinical sample before amplification.

(e) Real-Time PCR

Real- time PCR also known as "Kinetic PCR" is an in vitro amplification technique where in amplification of the template and detection of the amplicons occur simultaneously. The real – time PCR has several advantages over the conventional PCRs, which include: speed, high sensitivity, reduced inter and intra assay variability, broad dynamic range and reduced chances of cross contaminations.

Several factors such as reduced amplicon size, reduced cycling profiles, avoidance of post-PCR handling steps, use of fluorogenic labels and sensitive methods for detecting the emissions contribute to the beneficial characteristics of the Real time PCR.

Different chemistries in Real- time PCR

There are five different chemistries available for detection of genome by Real –time PCR. These are broadly classified as amplicon sequence specific and non – specific methods of Real time PCR detection.

Non specific Real time PCR detection

This method utilizes DNA binding fluorophores. Intercalating agents such as ethidium bromide, YO PRO -1, SYBR green fluoresce when associated with ds DNA and exposed to suitable wavelength of light. The advantage of the method include need for less expertise, less expense and eliminates risk of detection failures due to sequence variations in the template. The limitations however include association of the fluorophore to the primer dimers or other non-specific products which may cause confusion in the interpretation and less power to discriminate variants. The limitations are overcome by use of software capable of fluorescent melting curve analysis. This method makes use of difference in the temperature at which the ds DNA amplicon is denatured (T_D). The primer dimer which is shorter than the specific amplicon can be discriminated due to a reduced T_D.

Amplicon specific Real-time PCR chemistries

This method utilizes the use of amplicon specific fluorogenic probes for quantitation of genomes. Most of the fluorogenic probe based assays rely upon Fluorescence resonance energy transfer (FRET) occurring between fluorophores or a fluorophore and non – fluorescent quencher (NFQ). FRET is a spectroscopic process by which energy is passed between molecules separated by 10 – 100 A^o that have

overlapping emission and absorption spectra (Clegg, 1992). The mechanism is a non – radiative induced – dipole interaction as determined by Forster. The efficiency of the energy transfer is proportional to the inverse sixth power of the distance (R) between the donor and acceptor ($1/R^6$) fluorophores (Selvin, 1995).

Linear oligoprobes based assays

The method uses a pair of adjacent, fluorogenic hybridization oligoprobes. This chemistry is used in the LightCycler equipment (Roche Molecular Biochemicals, Mannheim, Germany) (Wittwer et al.,1997). In this method, the upstream oligoprobe is labeled with a 3' donor fluorophore (fluorescein isothiocyanate, FITC), and the downstream probe is commonly labeled with either a LightCycler Red 640 or Red 705 acceptor fluorophore at the 5'-terminus, so that when both oligoprobes are hybridised, the two fluorophores are located within 10 nucleotides of each other.

Different linear class of oligoprobes are available now. They include: Displacement probes, Light up probes and HyBeacons.

Displacement probes:

These are double-stranded oligoprobes that function by displacement hybridization (Li et al., 2002). In this process, a 5' fluorophore labeled oligonucleotide, in its resting state, is hybridised with a complementary, but shorter, quenching DNA strand that is 3' end-labeled with an NFQ. When the full-length complementary sequence in the form of an amplicon is present, the reporter strand will preferentially hybridise to the longer amplicon, disrupting the quenched

oligoprobe duplex and permitting the fluorophore to emit its excitation energy directly.

Light up probes:

The light-up probe is a peptide nucleic acid (PNA) to which the asymmetric cyanine fluorophore thiazole orange is attached (Svanvik et al., 2001). PNA is a DNA analogue that is formed of neutral repeated N-(2-aminoethyl) glycine units instead of negatively charged sugar phosphates (Egholm et al., 1993). PNA retains the same sequence recognition properties as DNA. When hybridised with a nucleic acid target, as either a duplex or triplex, the fluorophore becomes strongly fluorescent. These oligoprobes do not interfere with the PCR or require conformational change, they are sensitive to single nucleotide mismatches and, because a single reporter is used, they allow the direct measurement of fluorescence instead of the measurement of a change in fluorescence between two fluorophores (Svanik et al., 2001). Non specific fluorescence with extended cycling has been observed (Svanvik et al., 2000)

HyBeacon probes:

The HyBeacon is a single linear oligonucleotide internally labelled with a fluorophore that emits an increased signal upon formation of a duplex with the target DNA strand (French et al., 2001). This chemistry does not require destruction, interaction with a second oligoprobe or secondary structure changes to produce a signal, and it is relatively cheap and simple to design.

The other chemistries utilizing the linear oligoprobe include Q-PNA and DzyNA primers.

Dual Labelled oligoprobes based assays

5'Nuclease Oligoprobes:

This assay utilizes Hydrolysis or Taqman probes for quantitation of the targets. The Taqman probes consist of a 5' reporter fluorophore such as 6-carboxyfluoroscein (FAM) and a 3' quencher which can be a fluorophore such as TAMRA or an NFQ that dissipates energy as heat. When in close proximity the fluorescence emitted by the reporter fluorophore is quenched by the quencher. When the probe hybridizes to the specific target molecule, during the extension step, the probe gets cleaved due to the 5' – 3' endonuclease activity of the DNA polymerase. This separates the reporter from the quencher and the fluorescence of the reporter fluorophore is no longer quenched by the quencher molecule. The fluorescence emitted is monitored by the instrument. This is the chemistry used in ABI Prism sequence detection systems. The quantitation of the target is given based on the threshold cycle (C_T) values. The C_T is defined as the PCR cycle in which the gain in fluorescence generated by the accumulating amplicon exceeds ten standard deviations of the mean baseline fluorescence, using data taken from cycles 3–15. In LightCycler, the fractional cycle is called the crossing point (C_P). Another major advance in the probe design for the Taqman assay is the minor groove binding (MGB) probes, in which NFQ is used with a molecule that stabilizes the probe and target duplex (Afonina et al., 2002). This allows the use of shorter probes and makes the assay more convenient for identifying single nucleotide polymorphisms (SNP).

Hairpin oligoprobes - Molecular beacons:

Molecular beacons are the first hairpin oligoprobes used in real-time PCR. The molecular beacon's fluorogenic labels are positioned at the termini of the oligoprobe. The labels are held in close proximity by distal stem regions of homologous base pairing deliberately designed to create a hairpin structure. The closed hairpin is quenched due to FRET. In the presence of a complementary sequence, the oligoprobe will hybridise, shifting into an open configuration. The fluorophore is now spatially removed from the quencher's influence, allowing fluorescent emissions to be monitored (Tyagi and Kramer, 1996). This structural change occurs in each cycle, increasing in cumulative intensity as the amount of specific amplicon increases. The quencher is usually DABCYL (4-(4'-dimethylamino- phenylazo)-benzene), an NFQ.

Self fluorescing amplicon based assays

In this chemistry the labels become irreversibly incorporated into the PCR product. The two methods described include the sunrise primers, and scorpion primers (Nazarenko et al., 1997; Whitcombe et al., 1999). The sunrise primer consists of a 5' fluorophore and 3' DABCYL NFQ. The labels are separated by complementary stretches of sequence that create a stem when the sunrise primer is closed. The 3' end consists of a target specific primer sequence. This enables the sunrise primer to be duplicated by the nascent complementary strand, thus destabilizing the stem and hence the fluorophores is free to emit the excitation energy for monitoring. The scorpion primer is similar in design to the sunrise primer

except for an adjacent hexethylene glycol molecule that blocks duplication of the signaling portion of the scorpion.

A self-quenching hairpin primer has been described which is commercially entitled the light upon extension (LUX) fluorogenic primer (Nazarenko et al., 2002). This chemistry is dark in the absence of specific amplicon, through the natural quenching ability of a carefully placed guanosine nucleotide. The natural quencher is brought into close proximity with the FAM or JOE 5' 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluoroscein fluorophore via a stretch of 5' and 3'complementary sequences. However, in the presence of specific target, the primer hybridises, opening the hairpin and permitting fluorescence from the fluorophore.

Multiplex Realtime PCR assays & Real–time PCR based genotyping

Multiplex real-time PCR more commonly refers to the use of multiple fluorogenic oligoprobes for the discrimination of amplicons that may have been produced by one or several primer pairs. The development of multiplex realtime PCR has proven problematic because of the limited number of fluorophores available and the frequent use of monochromatic energizing light sources. Some real-time PCR designs have made use of conserved single or multiple nucleotide changes among similar templates to allow their differentiation by concurrent changes to the oligoprobe's T_M or the amplicon's T_D (Espy et al., 2000; Nicolas et al., 2002). Combining the use of multiple fluorophores with the discrimination of additional targets by temperature allows the identification of a significantly larger number of amplicon targets.

The SYBR green and Linear oligoprobe chemistries are most commonly used to perform genotyping assays. Genotyping data are obtained as end point analysis after the completion of the PCR. The amplicon is denatured and rapidly cooled to encourage the formation of fluorophore and target strand complexes. The temperature is then gradually raised, and the fluorescence from each vessel is continuously recorded. The detection of sequence variation using fluorescent chemistries rely upon the destabilization incurred as a result of the changes, thus altering the expected T_M in a manner that reflects the particular nucleotide change. The resulting rapid decrease in fluorescence is presented as a 'melt peak' using software capable of calculating the negative derivative of the fluorescence change with temperature. The other chemistries such as the Taqman probe or the hairpin probes need two different probes with different fluorophores to differentiate the genotypes. Between the above two chemistries, the hairpin probes are more suited for genotyping since, the occurrence of a mismatch between a hairpin oligonucleotide and its target has a greater destabilizing effect on the duplex than that with the linear probe.

Designing of oligoprobes for Real – time PCR assay

- Optimal length of the probe is usually 20 – 40 nucleotides, the MGB probes are usually shorter (around 14 nucleotides)
- The G+ C content must be between 40 -60%
- No runs of single nucleotide, particularly G is recommended

- Presence of G at the 5' end is avoided as it is a natural quencher
- Complementarity with the primers or repeated sequence motifs are not recommended
- T_M of the probe is usually 5 – 10^oC higher than that of the primers, enabling hybridization of probe prior to primer extension
- The Taqman (5'nuclease) probes are usually designed closer to the forward primer
- The Taqman probes for identification of SNPs are designed to have the mismatch at the centre of the probe rather than at the 5' end to prevent knock out by the polymerase enzyme.
- The fluorogenic oligoprobes are labeled at the 3' terminus with a phosphate or octanediol molecule to prevent Taq mediated extension. This prevents the oligoprobes from functioning as a primer.

Limitations of Real – time PCR

- The establishment cost of the real – time PCR may be too high for low – throughput laboratories.
- Monitoring of amplicon size is difficult.
- Only limited fluorescent chemistries may be adaptable on a given platform.
- Restricted multiplexing capabilities.

Application of Real time PCR in diagnosis of HCMV infections

Many real-time quantitative tests for CMV DNA using the chemistries listed are available. Several different gene targets viz. gB (Yun et al., 2000; Schaade et al., 2000; Kearns et al., 2001; Guiver et al., 2001; Kearns et al., 2002; Ando et al., 2002 ; Pang et al., 2003; Li et al., 2003; Hong et al., 2004), IE gene(Tanaka et al., 2000; Limaye et al., 2001; Greenlee et al., 2002; Visconti et al., 2004; Kalpoe et al., 2004), US – 17 (Machida et al., 2000; Aberle and Puchhammer-Stockl, 2002; Ikewaki et al., 2003) , UL – 83 (Gault et al.,2001; Gouarin et al., 2004) , DNA polymerase (Sanchez et al., 2001; Sanchez and Storch, 2002), MIE (Nitsche et al., 1999; Nitsche et al., 2003; Satou et al., 2001),pp150 IL 32 gene (Hanfler et al., 2003), UL 65 (pp 67) gene (Persson et al., 2003) have been used for quantitation of HCMV. It has been shown that the gene of interest can affect the sensitivity of the assay (Yun et al., 2000). The real time PCRs have been applied majorly on fractions of blood or whole blood , though , certain studies have shown the utility of quantitation in clinical specimens such as amniotic fluid, urine, intraocular fluids, respiratory fluids and cerebrospinal fluid (Gouarin et al., 2002; Ando et al., 2002; Kearns et al., 2002; Aberle and Puchhammer-Stockl, 2002). Most studies have compared the real-time PCR assays with antigenemia assay (Tanaka et al., 2000; Gault et al., 2001; Yakushiji et al., 2002), conventional culture (Machida et al., 2000; Najioullah et al., 2001) and conventional PCRs including COBAS AMPLICOR PCR (Schaade et al., 2000; Satou et al., 2001; Funato et al., 2001; Pang et al., 2003; Herrmann et al., 2004) and found good correlation with regard to the quantitative data. Machida et al (2000) found a better correlation of pp65 antigenemia assay with viral loads determined in leucocyte component than in the plasma component by real time PCR.

In general, quantitative real-time PCR has several advantages to the antigenemia test including increased sensitivity for early detection of CMV infection or reactivation, utility for patients with neutropenia, stability of target DNA in blood specimens than the antigen, wide detection range (7 to 8 $\log_{10}$) of CMV DNA, ability to process large number of specimens, flexibility of time of transport and processing of specimens, and the potential for increased accuracy of results through precision instrumentation (Funato et al., 2001; Mengelle et al., 2003; Nitsche et al., 2003; Yakushiji et al., 2002). Real-time PCRs have been shown to be more suitable for monitoring CMV reactivation in immunocompromised patients and for guiding clinical management and monitoring of Bone marrow transplants (BMT), Stem cell (SCT) and Solid organ transplant (SOT) patients (Meyer-Koenig et al., 2004; Piiparinen et al., 2004; Ikewaki et al., 2003). A study revealed, that for the SCT patients the optimal cutoff value of CMV DNA load defining relevant viral reactivation was slightly higher than that for the SOT patients (Kalpoe et al., 2004). Recently, using a real-time PCR assay, the viral load of herpes viruses, including CMV, was assessed in the CSF (Aberle and Puchhammer-Stockl, 2002). Although unclear at this time, assessment of viral load in the CNS may have prognostic implications, may predict distinct CNS manifestations, and may be useful for differentiating between real infection and nonspecific presence of virus in the CSF, especially in severly immunocompromised individuals. Real time PCR has also been used to establish primary infections in the pregnant women by detecting the HCMV DNA in the amniotic fluid (Gouarin et al., 2002). Real time PCR coupled with automated extraction systems have been used for monitoring of immunosuppressed patients. Nitsche et al (1999) compared the Light cycler and Taqman probe based

assay and concluded that both are equal when sensitivity and dynamic ranges were considered. Multiplex real time PCR assays have been developed for simultaneous detection of HSV, CMV and VZV using SYBR green chemistry in separate tubes. A multiplex PCR was also developed for simultaneous detection of HCMV and Human DNA (Sanchez and Storch, 2002).

(iii) Nucleic acid sequence-based amplification (NASBA)

Nucleic acid sequence-based amplification (NASBA) is a commercial development of the isothermal Transcription based amplification system (TAS) method and alternative of Transcription mediated amplification (TMA). Unlike, RT-PCR NASBA allows specific amplification of unspliced RNA in a genomic DNA background. Amplification by NASBA is isothermal and involves the coordinated activities of three enzymes, AMV Reverse Transcriptase, RNase H, and T7 RNA Polymerase. Quantitative detection is achieved by way of internal calibrators, added at isolation, which are co-amplified and subsequently identified along with the wild type of RNA using electrochemiluminescence. In this procedure, a strand of RNA is the target for an oligonucleotide primer that contains a polymerase-binding site. After hybridization of the primer to the target, a reverse transcriptase elongates the primer complementary to the target sequence. This is followed by selective degradation of the template RNA by RNAse H followed by annealing of another oligonucleotide primer to the first strand and extension by Reverse transcriptase. This is followed by the activity of T7 RNA polymerase which produces multiple copies of RNA that are detected by electrochemiluminescence. A 10^5-copy amplification occurs within 15 minutes. NASBA based on pp67 mRNA, a late viral

transcript of HCMV appeared to be a promising tool for initiation and termination of preemptive therapy for solid organ transplant recipients with reactivated HCMV infection (Gerna et al., 1999), whereas monitoring of immediate early mRNA expression appeared to be a useful parameter for initiation of preemptive therapy in HSCT recipients (Gerna et al., 2000).

(iv) Strand displacement amplification (SDA)

SDA is an isothermal amplification method based on the displacement of one probe when DNA polymerase is used to extend a second probe. In the original method (Walker et al., 1992b), the target DNA underwent initial cleavage into 47-bp fragments and heat denaturation. A probe containing a HincII recognition site on the 5' end was hybridized to the target fragments. When it is bound to the target site, the probe possessing the HincII recognition site (6 nucleotides) and several more bases that do not hybridize to the target nucleic acid is extended past the end of the target fragments. DNA polymerase then generates a nucleic acid strand complementary to the target and free end of the probe, incorporating a modified dATP (dATPαS). The HincII enzyme then nicks the probe strand at the HincII recognition site (the hemiphosphorothioate HincII recognition site prevents complete cleavage of the dsDNA). DNA polymerase then generates a nucleic acid strand that is complementary to the target nucleic acid strand and that displaces the original probe segment from the target fragment. Because of the orientation of the HincII recognition site, the complementary probe sequence generated by DNA polymerase retains a functional HincII recognition site. This allows continual nicking of the probe by HincII and subsequent displacement through the action of DNA

polymerase. Strand displacement amplification generates upto 10^7 copies of the template DNA in 2 hours (Walker et al., 1992 a). A loop mediated isothermal amplification based on the principles of SDA has been developed for rapid detection of HCMV genome (Suzuki et al., 2006).

2.11.2.2.2 Probe based amplification methods

(i) Ligase chain reaction (LCR)

The ligase chain reaction, also called the ligase amplification reaction or the oligonucleotide ligation assay is a probe amplification technique for detecting the target sequence. Unlike PCR, which creates new DNA molecules from individual nucleotides, the ligase reaction uses a thermostable ligase enzyme to join two oligonucleotides that are immediately adjacent to each other. Like PCR, this reaction is cyclic; denaturation, annealing, and ligation are the basis for the amplification reaction. As in PCR, the ligated oligonucleotide pairs, along with the original sequence, become templates for the next cycle (Wu and Wallace, 1989). Twenty to 30 cycles of this reaction yield a 10^6-fold increase in the original target. The method has an ability to detect as few as 10 nucleic acid targets (Backman, 1992). In the ligase reaction, the entire target sequence must be known, because a 1-bp mismatch at the point of ligation can prevent ligation of the oligonucleotides. Although this could be detrimental in many situations, failure to ligate can be used to detect point mutations in the target sequence. The end-products are visualized by agarose gel electrophoresis or by hybridization using chemiluminescent probes. Another variety of ligase reaction, the gapped -LCR involves the use of both a polymerase and a ligase (Birkenmeyer and Mushahwar, 1991). Two oligonucleotide primers are

annealed to the target but are spaced so that polymerase fills a gap between the two primers; the ligase then connects the filled gap and the second primer. Ligase chain reaction has been used in identification of mutation related to gancicliovir resistance in the UL 97 gene of HCMV (Bourgeois et al., 1997).

(ii) Cycling Probe Reaction

The cycling probe reaction is almost the exact opposite of LCR. In this procedure, a nucleic acid probe that incorporates DNA-RNA-DNA sequences is synthesized. After the probe is allowed to hybridize to the target, RNase H, an enzyme that specifically degrades the middle RNA portion of the probe, is added. The DNA fragments thus remain dissociate from the target. The target is now free to hybridize with another probe molecule; therefore, the reaction is inherently cyclic without external manipulations. The free DNA fragments can then be detected or amplified by a secondary amplification system (Duck et al., 1990).

2.11.2.2.3 Signal amplification methods

Amplification of the detectable signal also improves the sensitivity of nucleic acid probes. A properly constructed probe system that hybridizes even one target can be detected if the signal is amplified sufficiently.

(i) Hybrid capture assay

The hybrid capture assay is a solution based hybridization assay that involves amplified chemiluminescent detection. Specimens containing the target DNA hybridize with a specific CMV RNA probe complementary to approximately 40,000

bp or 17% of the CMV genome. The resultant RNA-DNA hybrids are captured onto the surface of a tube coated with antibodies specific for RNA-DNA hybrids. The immobilized hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for the hybrids and detected with a chemiluminescent substrate. Because each 38-kb RNA-DNA hybrid binds approximately 1,000 antibody conjugate molecules, each of which is bound to about 3 alkaline phosphatase molecules, the resulting signal is amplified at least 3,000-fold. The amount of light emitted, which is measured as relative light units on a luminometer, is proportional to the amount of target DNA in the specimen. Upto 54 specimens along with controls can be tested qualitatively within 6 hours by the assay.

(ii) Branched DNA signal amplification assay

The branched DNA (bDNA) assay (Bayer, Chiron Corp., Emeryville, California) uses bDNA amplifiers to effect signal amplification during hybridization (Chernoff et al., 1997). It measures viral nucleic acids directly from clinical specimens by boosting the reporter signal rather than amplifying target sequences as the means of detection. The bDNA molecule contains multiple binding sites for an enzyme-labeled probe. The target nucleic acid is bound to the bDNA molecule, and the complex is detected with a chemiluminescent substrate. The test allows the direct quantification of CMV DNA in blood. The lack of amplification makes the test less susceptible to contamination. The test is less sensitive than most antigenemia and PCR assays (Ehrnst, 1996). The bDNA signal amplification assay has been used with blood, CSF, and semen samples (Boeckh and Boivin, 1998). The assay requires at least 2 x 10^6 PBL or 1 to 1.5 ml of ultracentrifuged CSF. PMNL should be

separated within 8 hours of collection, at which time they can be frozen for subsequent testing. The assay procedure is as follows: The PMNL pellet or CSF sample is incubated with proteinase K in a lysis buffer, and the target probes are added. After incubation for 16 to 18 hours in a microtiter plate, the bDNA is added; then the enzyme-labeled probe and the chemiluminescent substrate are added. Finally, the light emission is measured. The quantity of DNA is measured using the standard curve generated by known concentrations of HCMV DNA.

2.11.3 Clinical implications of DNAemia in different patient groups

PCR was first used for HCMV DNA detection in the urine of congenitally infected babies at the end of the 1980s (Demmler et al., 1988). The advantages of PCR over conventional techniques are the small amount of sample required; the short time required for test results (24 to 48 hours versus 2 to 28 days) and the ability to use frozen specimens with noninfectious virus. Detection and quantification of CMV, by PCR is performed in different fractions of the blood (i.e. cellular fractions and plasma), organ fluids (cerebrospinal fluid, urine, throat washing or semen) and tissue samples. Although PCR for CMV DNA detection in peripheral blood leukocytes is most sensitive for detecting viral infection in diverse group of patients, when PCR is carried out in a qualitative manner, it is of little clinical significance since the results obtained do not correlate with clinical symptoms, particularly, in CMV - seropositive patients with HIV infection or those who have undergone solid organ transplantation. (Monte et al., 1996). However, qualitative detection of HCMV DNA in peripheral blood and amniotic fluid has been useful in establishing primary infections in sero-negative patients and

intrauterine infections in pregnant women respectively. There have been controversies on the detection of latent HCMV in the peripheral blood leucocytes by qualitative PCRs. However, most studies support the concept that viral DNA is not detected in the peripheral blood leukocytes of HCMV-seropositive immunocompetent individuals (Revello et al., 1998; Zanghellini et al., 1999). Different fractions of blood have been used for quantitation of HCMV viz: peripheral blood leucocytes, plasma, serum or whole blood. Most studies show that the quantity of viral DNA in leukocytes is generally greater than in plasma for both transplant recipients and subjects with AIDS (Boivin et al., 1998; Gerna et al., 1994). The positive predictive value of PCR of plasma for the development of CMV disease has been evaluated in different settings with somewhat contradictory results. Although CMV infection has been shown to precede the development of clinical disease (Hansen et al., 1994; Spector et al., 1992), PCR of plasma reflected the kinetics of CMV infection poorly, especially during therapy compared to PCR of leukocytes in subjects with AIDS (Gerna et al., 1994). In liver transplant recipients, PCR of serum was the best method of predicting the development of symptomatic CMV infection (Patel et al., 1995). PCR of plasma appeared to be an earlier and more sensitive marker of serious CMV infection in marrow transplant recipients than did PCR of leukocytes in one study (Nolte et al., 1995), while others found either similar (Hebart et al., 1996) or lower (Boeckh et al., 1997) sensitivity than PCR of leukocytes. Several longitudinal studies have utilized quantitative molecular assays to ascertain the relationship of systemic viral load to the onset of symptoms in different patient groups and to predict the subsequent development of CMV disease or relapse of CMV disease before the onset of clinical symptoms.

Conventional quantitative assays such as antigenemia tests and blood cultures allow a reliable prediction of CMV disease (Chevret et al., 1999); however, the interval between first positive test result and occurrence of CMV disease is clearly shorter than that obtained with molecular assays, especially PCR (median, 34 days and 1 day, respectively, versus 46 days) (Dodt et al., 1997). Quantitation of CMV DNA in blood leukocytes have also shown practical implications for the diagnosis of visceral organ disease during viremia in transplant recipients and patients with AIDS. The median quantity of DNA in the leukocytes of patients with visceral organ disease is significantly greater than that in patients with viremia alone (Saltzman et al., 1992). Significantly higher levels of HCMV load are detected in infants with congenital symptomatic HCMV infection than asymptomatic infants. Quantitative molecular assays have also established that the virus clearance from blood occurs spontaneously in both symptomatic and subclinically infected newborns, even though the process takes longer in symptomatic newborns (Revello et al., 1999b). A study with the quantitative PCR, reported that levels of viral DNA of 10^3 to 10^5/ml of amniotic fluid was necessary to transmit the infection, whereas levels 10^5 was required to cause HCMV disease in the fetus. Thus, levels less than or equal to 10^3 genome equivalents/ml were unable to transmit the infection and were cleared by the fetus during fetal life (Guerra et al., 2000; Lazzarotto et al., 2000; Maine et al., 2001). Using the viral load to limit the amount of antiviral drug treatment does not appear to be safe in a clinical setting of rapid progression from first detection of CMV to disease in allogeneic stem cell or bone marrow transplant recipients. In contrast, in patients without or with mild GVHD, measurement of viral load appears to be a good strategy to target antiviral drug treatment (Boeckh and Boivin, 1998).

Drouet et al (1993a) showed in a small group of patients that CMV retinitis in AIDS patients was generally associated with high levels of CMV-DNA in leucocytes as detected by semi-quantitative PCR. The findings have been confirmed in larger cohorts and by evaluating different clinical specimens. Detection of CMV-DNA in plasma (Jabs et al., 2002) or whole blood (Bowen et al., 1997) at the time of initial diagnosis of retinitis was associated with a higher risk of mortality than was a high HIV viral load. Erice et al (2003) found an increased risk for CMV disease in patients with CMV-DNA levels to >100,000 copies/ml (quantification limit of the assay used). Still, a substantial number of patients suffer from CMV disease with CMV-DNA levels significantly lower than this value (Bowen et al., 1997; Spector et al., 1999). The CMV load in HIV-infected individuals seems to be generally higher than in transplant recipients.

Amplification assays for viral mRNA in leucocytes have been used mainly to identify active viral replication (Randhawa et al., 1994). The presence of CMV IE mRNA has been demonstrated in monocytes and PMNL during active CMV infection (Lam et al., 1998; The et al., 1995). The absence of circulating mRNA is associated with a lack of CMV-associated symptoms, irrespective of the presence or absence of CMV DNA, while its presence is detected only in the setting of disease (Randhawa et al., 1994). However, detection of HCMV mRNA appears to be less sensitive than the pp65 antigen test and shell vial culture and PCR in diagnosing CMV disease (Meyer-Konig et al., 1995; Patel et al., 1995).

Despite most diagnostic usefulness as predictive markers of CMV disease, the molecular assays suffer from a major limitation. The identification of a universal

DNA level that indicates reliably a high risk for CMV disease and consequently requires preemptive therapy, however, has not yet been formulated. The factors that attribute to this limitation include: Differences in the test method, the method of quantification, the units with which the viral load is expressed, definition of CMV disease, the kind of immunosuppression and also there are significant differences in the DNA levels associated with disease with regard to different patient populations.

2.12 MOLECULAR TYPING METHODS

Today's molecular techniques have been instrumental in viral subtype analysis that has gone well beyond the realm of antigen-antibody interaction. The viral genomes can vary at the nucleotide levels but maintain their essential characteristics at the protein or virion levels. This variability is the basis for molecular characterization and subtype classification of viruses. Molecular characterization for the purpose of subtyping is not relevant to treatment but is useful mainly for epidemiological purposes and for investigations into pathogenesis and disease progression (Arens, 1999). They may also throw light on the most conserved and hypervariable regions in the viral genome which may be useful in designing primers and probes to be used in the molecular assays described. Serologically HCMV cannot be distinguished and thus molecular methods are sort out for genotyping the virus.

2.12.1 *GENOTYPIC METHODS*

Nucleic acid-based subtyping was first applied to study the epidemiology of a nosocomial outbreak of *Klebsiella pneumoniae* infection (Sadowski et al, 1979).

Initial applications involved primarily the detection and characterization of plasmids in bacteria. Developments in nucleic acid isolation, separation, and amplification since 1975 have led to the application of nucleic acid-based subtyping methods to various specific epidemiologic problems. The different genotypic methods that are available for human viruses include: Nucleotide sequencing, Restriction fragment length polymorphism (RFLP), Blotting techniques, Oligonucleotide fingerprint analysis, Reverse hybridization, DNA enzyme immunoassay (DEIA), RNase protection analysis, Single strand conformation polymorphism (SSCP), Heteroduplex mobility assay (HMA), Heteroduplex tracking assay, Genome segment length polymorphism and Amplified fragment length Polymorphism (AFLP) (Arens, 1999). Other than these genotypic methods, Multiplex PCRs and Real-time PCRs have also been used for genotyping of the viruses. The genotyping methods that are available for HCMV include DNA sequencing, RFLP/ PCR-RFLP, SSCP, Southern blotting, DEIA and AFLP.

2.12.1.1 ***Nucleotide sequencing or DNA sequencing***

DNA sequencing refers to the biochemical methods for determining the order of the nucleotide bases viz. adenine, guanine, cytosine and thymine in a DNA oligonucleotide.

(i) Early methods of DNA sequencing

In 1973, Gilbert and Maxam reported the sequence of 24 base pairs using a method called wandering spot analysis. Maxam and Gilbert (1980) developed a DNA sequencing method based on chemical modification of DNA and subsequent

cleavage at specific bases. The advantage of the procedure included the use of purified DNA without the need for cloning which was necessary for plus-minus sequencing. The limitations of the method included complex techniques, use of hazardous chemicals and difficulties with scale up. The chain terminator method introduced by Sanger became the method of choice owing to its efficiency and rapidity. The key principle of this method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. The classical chain termination or Sanger method requires a single stranded DNA template, a DNA primer, a DNA polymerase, radioactively or fluorescently labeled nucleotides and modified nucleotides that terminate DNA strand elongation. The method also required the sample to be divided into four, each containing all the required reaction mixture along with one of the four ddNTPs. The ddNTPs acted as chain –terminators. After the reaction, the newly synthesized and labeled DNA fragments were heat denatured, and separated on a denaturing polyacrylamide – urea gel with resolution of just one nucleotide. This was either followed by reading of bands in the X ray films after autoradiography or on the gel under UV transilluminator (Sanger et al., 1977).

(ii) Variations in chain termination sequencing

The technical variations of chain termination sequencing included labeling of the primers at 5' end with biotin, radioactive labels or fluorescent dye; or the nucleotides with the dyes. Both the methods required four separate reactions for sequencing. Later, development occurred by means of fluorescently labled ddNTPs and primers that set the stage for automated, high – throughput DNA sequencing.

(iii) Dye terminator sequencing and Automated sequencing

This method involves the use of labeling the chain terminators. The major advantage of the method is that the sequencing can be performed in a single reaction. In this method, each of the ddNTPs is labeled with a different fluorescent dye, each fluorescing at a different wavelength. The advantages include greater expediency and speed and is now the mainstay in automated sequencing with DNA sequencers. The limitations of the method however include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peaks and shapes in the trace chromatogram after capillary electrophoresis. This problem has been overcome by introduction of DNA polymerase and dyes that minimize the incorporation variability. Modern automated DNA sequencers can sequence upto 384 fluorescently labeled samples in a single run and perform as many as 24 runs a day.

Current methods of DNA sequencing involves PCR amplification of the part of the genome to be sequenced followed by cycle sequencing, clean up of the products and re- suspension in a buffer solution before loading onto the sequencer. Most of these methods can directly sequence short DNA fragments (300 – 1000 nucleotides) in a single reaction.

(iv) Large – scale sequencing strategies

Large scale sequencing aims at sequencing very long DNA fragments which may involve the whole genome of the pathogen. The brief steps involved are: Fragmenting of the DNA using restriction enzymes or shearing with mechanical

forces, cloning of the different fragments into DNA vector usually a bacterial plasmid followed by its amplification in the *E. coli*. The fragments are purified from individual bacterial colonies, completely sequenced and assembled electronically into one long contiguous sequence by identifying 100% identical overlapping sequences between them. This method is called "shotgun sequencing". The limitation of such a method includes a complex and error prone assembly process in genomic regions with sequence repeats.

(v) Newer high throughput methods of sequencing

The newer method of sequencing involves in vitro clonal amplification of the region of interest to generate many copies of the individual molecule followed by parallelized sequencing. The in vitro clonal amplification methods produce many physically isolated locations containing many copies of single fragments. The known methods of in vitro clonal amplification include emulsion PCR and bridge PCR (Margulies et al., 2005). In the former the individual DNA molecule is isolated along with primer – coated beads in aqueous bubbles within an oil phase. This is followed by a PCR that coats the beads with clonal copies of the isolated library molecule and the beads are subsequently immobilized for later sequencing. In the latter method, the fragments are amplified upon primers attached to a solid surface.

In parallelized sequencing, the physically localized clonal DNA sequences are sequenced by the dye-termination electrophoretic sequencing or reversible terminator methods. This method, use dye terminators, adding one nucleotide at a time, detecting fluorescence corresponding to that position, then removing the blocking group to allow the polymerization of another nucleotide. Pyrosequencing

also uses DNA polymerization to add nucleotides, adding one type of nucleotide at a time, then detecting and quantifying the number of nucleotides added to a given location through the light emitted by the release of attached pyrophosphates (Ronaghi et al., 1996).

Another recent method of sequencing called "Sequencing by ligation" utilizes DNA ligase to identify the target sequence (Shendure et al.,2005). This method uses a pool of random oligonucleotides labeled according to the sequenced position. Oligonucleotides are annealed and ligated. Preferrential ligation by DNA ligase for matching sequences results in a signal corresponding to complementary sequence at that position.

(vi) Advantages and limitations of DNA sequencing

The advantages of DNA sequencing as a genotypic method include its wide applicability, and ability to identify single nucleotide variation. The limitations however, include technical complexity, high cost of the equipment and large amount of the data which may be complex to analyze.

2.12.1.2 Restriction Fragment length polymorphism (RFLP)

This method utilizes the ability of restriction enzymes to recognize and cleave specific genomic sequences. A particular restriction enzyme generates a unique family of fragments from a particular DNA molecule. The genomic variants are differentiated based on the presence or absence of restriction sites and the pattern of fragments generated by the enzyme**.** The technique requires fairly large amounts of purified or partially purified DNA. Currently, the method involves amplification

of specific regions of the genome by PCR followed by treatment with one or more restriction enzymes. After the restriction enzyme reaction, the fragments are resolved by electrophoresis on agarose gel or polyacrylamide gel depending on the resolution required. Significant advantage of PCR-RFLP over genomic DNA restriction is that problems of poor restriction of genomic DNA as a result of DNA base modifications (methylation) are not encountered (Swaminathan and Matar, 1993). The advantage of the RFLP as a method for genotyping includes simplicity and wide applicability. The major limitation of the method is that any mutation occurring outside the recognition site of the restricition enzyme cannot be detected.

2.12.1.3 Southern Blotting

Southern blotting analysis is a modification of RFLP analysis wherein the family of restriction fragments is transferred onto a nitrocellulose or nylon membrane following restriction enzyme treatment and electrophoretic separation of DNA. The DNA fragments are then probed using specific oligonucleotides. The advantages of the method include wide applicability, ability to analyze complex genomes. The limitations of the method however, include technical complexity, requirement of radioisotopes for analysis and restricted analysis of the sequences that are cleaved by the enzyme or the probe sites.

2.12.1.4 Single strand conformation polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) analysis is the term applied to the method developed (Orita et al., 1989) with which it was demonstrated that a single nucleotide substitution was sufficient to cause a mobility shift of a

fragment of single-stranded DNA in a neutral polyacrylamide gel. Initially, the general procedure was to use RFLP fragments from genomic DNA (one from the wild-type genome and one from a possible mutant), denature the fragments by alkali treatment, subject them to electrophoresis in a neutral polyacrylamide gel, and compare their mobilities. Modifications have been made to accommodate PCR amplification of a specific region of wild-type or mutant genomes prior to denaturation and separation on a neutral gel (Hayashi, 1991). In either case, if mutations are present in the segment of the mutant genome being tested, that segment will likely run at a different position in the gel than the same segment from the wild-type genome. The altered electrophoresis pattern is apparently due to the mutation-altered secondary structure of the restriction fragment or the PCR amplicon (Orita et al., 1989). The separation of the wild-type and mutant fragments is dependent on several environmental factors, including the temperature of the gel during electrophoresis, the concentration and composition of the electrophoresis buffer, and the presence of denaturing agents in the gel. Several sets of conditions should be tried empirically to optimize mutation detection. One major advantage of this method is that it can "sample" the genetic makeup of several hundred base pairs of DNA and the major limitation is that the method can identify the presence of the mutation but not the precise location of the mutation.

2.12.1.5 Amplified fragment length polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a polymerase chain reaction (PCR) based genetic fingerprinting technique (Vos et al., 1995). AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of complementary

double stranded adaptors to the ends of the restriction fragments. A subset of the restriction fragments are then amplified using 2 primers complementary to the adaptor and restriction site fragments. The fragments are visualized on denaturing polyacrylamide gels either through autoradiographic or fluorescence methodologies. The advantages of the method include higher reproducibility, resolution and sensitivity. About 50 -100 fragments can be amplified at a given time. In addition, no prior sequence information is needed for amplification.

2.12.1.6 DNA enzyme immuno assay (DEIA)

In this method, a specific region of the viral genome is amplified by PCR, followed by heat – denaturation of the amplicons. The denatured amplicons are then hybridized to probes bound to mutiwell plates. Hybrids are detected by colorimetric or photometric methods similar to ELISA. The advantage includes, simplicity of the procedure and ease of commercialization. The limitation includes high levels of error due to probe target mismatches.

2.12.2 MAJOR APPLICATIONS OF GENOTYPIC METHODS FOR HCMV

The genotypic methods for HCMV have been used primarily to understand the epidemiology and transmission of the virus. CMV was the first virus to be subjected to RFLP analysis of viral genomic DNA pursuant to epidemiologic studies. In a study among infants in a nursery, RFLP analysis of CMV strains isolated from the urine of eight babies demonstrated that one of the babies had transmitted CMV to two other babies, apparently via fomites (Stagno et al., 1989). Southern blot analysis has been used to demonstrate that an AIDS patient can be

infected with multiple strains of CMV. In a study on transmission of CMV in cadaveric renal transplantation, 15 distinct strains (genotypes) of CMV were isolated from 19 organ recipients (i.e. four pairs [8 patients] of the 19 had paired isolates with the same genotype). In all four pairs of patients who had received kidneys from the same cadaver, both had acquired it from the donor and that seropositive recipients can be reinfected by a new strain of CMV after transplantation (Arens, 1999). Nucleotide sequencing and PCR – based RFLP methods are currently employed for identification of variants and or recombinants of several HCMV genes viz. glycoprotein B, glycoprotein H, glycoprotein O, glycoprotein L, glycoprotein N, Major immediate early gene (UL 123), UL 144, US 28,US 9 (Chou and Dennison, 1991; Meyer-Konig et al., 1998a; Meyer-Konig et al., 1998b; Chou, 1992a; Rasmussen et al., 2002; Pignatelli et al., 2001, Pignatelli et al., 2003a; Retiere et al., 1998; Arav-Boger et al., 2002; Rasmussen et al., 2003). These techniques have also been useful in identifying mutations in the UL – 97 gene and UL -54 gene coding for phosphotransferase and DNA polymerase of HCMV conferring resistance to antiviral drugs (Wolf et al., 1995; Boivin et al., 1996). Verbraak et al (1998) used AFLP technique to study the HCMV variants based on the a-sequence and Immediate early genes of HCMV in the ocular specimens and peripheral blood of AIDS patients.

2.12.3 HCMV STRAIN VARIATIONS AND CLINICAL SIGNIFICANCE

The genome of CMV is highly conserved, but diversity is sufficiently high in various regions of the genome to differentiate between several CMV strains or genotypes (Meyer-Konig et al., 1998a; Pignatelli et al., 2001; Steininger et al.,

2005). The relationship of strain variation in HCMV and its clinical manifestation is still a matter of debate. The variations in the genome of HCMV have been associated with the cell tropism, variations in pathogenesis, or ability of the strains to cause severe disease.

It has been shown that different gB subtypes can exhibit different cell tropism and that, in contrast to the gB2 and gB3 subtypes, the gB1 subtype does not infect lymphocytes in vivo (Meyer-Konig et al. 1998a; Meyer-Konig et al. 1998b). In HIV-infected individuals, the aspect of compartmentalization of different CMV strains has also been investigated, and differences in the distribution of various CMV gB subtypes between blood and eye and between blood and CSF, respectively, have been described (Tarrago et al., 2003; Verbraak et al., 1998). Observations on the predominance of a certain CMV genotype, however, were ambiguous in HIV infected individuals with CMV retinitis (Shepp et al., 1996; Rasmussen et al., 1997; Drew et al., 2002; Bongarts et al., 1996). In a clinical investigation of bone marrow transplant recipients it was found that certain gB subtypes are more frequently associated with death due to myelosuppression than others (Torok-Storb et al., 1997). The contradictory results obtained in various investigations are attributed to the difference in the geographic distribution of strains (Zipeto et al., 1998), demographic characteristics of the infected individuals as was seen in a study between the distribution of HCMV strains in AIDS patients and in transplant recipients (Rasmussen et al., 1997) and the fact that most investigations were carried out to characterize the variations at only one polymorphic gene of the virus genome. Several investigations of CMV infections in children have shown concordantly that congenital and perinatal infections may occur with all CMV gB subtypes (Bale et

al., 2000; Barbi et al., 2001; Trincado et al., 2000), and in overall, no particular gB subtype could be found to be associated with severe disease. Later, however, another group showed that the severity of congenital disease may be associated with viral polymorphisms, not in the gB gene, but in the UL144 gene, and that certain subtypes of this gene coding for the tumor necrosis factor (TNF)-α-like receptor may be associated with unfavorable outcome (Arav-Boger et al., 2002). Considering the large variety of virus–host interactions that are mediated by the numerous viral gene products, it has been difficult to predict the sets of CMV genes that would significantly influence a defined clinical situation. One factor that could contribute to variations in pathogenesis or severity of disease is that different CMV strains may interact differently with the host's immune system. Investigations of renal transplant patients have shown that differential presentation of polymorphic gB or IE-1 peptides by HLA molecules as well as differential recognition of these by host CD8+ and CD4+ T lymphocytes modulate the host's immune response with a possible impact on CMV pathogenesis (Retiere et al., 2003). The action of NK cells has been reported to be dependent on the virus strain infecting the target cells (Cerboni et al., 2000). In addition, variations in CMV immune evasion genes might also influence the virus-host interaction (Reddehase, 2002).

HIV-infected patients and transplant recipients are most frequently co-infected with different CMV strains (Lang et al., 1989, Bongarts et al., 1996). The compromised immune system of these patients may not be capable of limiting dissemination of new CMV variants. Consequently, coinfection by several CMV strains might provide the basis for CMV intragenic recombination and emergence of CMV variants with the possibility for altered biological properties. Cell culture

experiments demonstrated that co-infection with two CMV strains may give rise to viable recombinant CMV-gB strains (Haberland et al., 1999). Intragenic recombination was demonstrated in CMV strains detected in the CSF of HIV-infected patients (Steininger et al., 2005). Therefore, intragenic recombination between different CMV strains may be common in patients with advanced HIV-infection and transplant recipients as they are mostly prone to mixed infections with multiple genotypes of HCMV, which could lead to a source of new CMV strains with altered biological properties. It has been recommended that knowledge on the strains of HCMV circulating in a given region may be essential as efforts to develop a CMV vaccine gain momentum (Britt, 1996; Plotkin, 2001).

2.13 TREATMENT

Because CMV causes a significant disease to transplant patients, strategies aimed at CMV prevention have been developed. These strategies include "prophylaxis", "preemptive therapy" and treatment of established CMV disease (Ljungman, 2002). In the prophylaxis, the patients at risk of disease based upon the pre-transplant CMV serological status of recipient and donor are treated after transplantation. In the pre-emptive therapy, antiviral agents are given in situations, where there is laboratory evidence of viral replication by viral culture, pp65 antigenemia, or detection of CMV DNA. Treatment is given following established CMV disease in transplant recipients. The currently available antivirals for the treatment of CMV disease are ganciclovir, foscarnet, and cidofovir (Paya and Razonable, 2003). Ganciclovir is a choice for the treatment of established CMV disease in solid organ transplant patients, given intravenously for at least two weeks.

Ganciclovir is a nucleoside analogue, which in its active triphosphorylate form inhibits the viral DNA polymerase and competes with deoxyguanosine triphosphate (dGTP) to act as a terminator of biosynthesis for the viral DNA. This phosphorylation in CMV infected cells is mediated by the CMV UL97 protein (Littler et al., 1992). This protein is a phosphotransferase that phosphorylates ganciclovir to ganciclovir monophosphate in infected cells (Biron, 2006). Two subsequent rounds of phosphorylation by cellular kinases produce ganciclovir triphosphate. Resistance to ganciclovir arises from mutations in the UL97 gene. Resistance may also arise from mutations in the viral DNA polymerase gene (UL54). The side-effects of ganciclovir include leukopenia and thrombocytopenia. Foscarnet is a pyrophosphonate analogue, which interferes with the binding of the pyrophosphate to its binding site of the viral DNA polymerase and it is used when ganciclovir is contraindicated. The major dose-limiting toxicity of foscarnet is renal impairment. Resistance to foscarnet arises from the point mutations in the UL54 gene, and cross-resistance has been observed between ganciclovir and foscarnet. Cidofovir is a nucleoside analogue, which may also be used but reports on this antiviral for therapy is very limited (Biron, 2006). In addition, CMV immunoglobulin has been used in lung transplant recipients for the treatment of CMV disease together with ganciclovir (Weill et al., 2003). As a prophylaxis in transplant recipients, ganciclovir is administered before active CMV infection occurs, immediately after the transplantation, especially those D+/R- patients. Ganciclovir is a widely used and effective drug for CMV prophylaxis. Previously, ganciclovir was administered intravenously, thereafter with the availability of the oral ganciclovir still favored CMV prophylaxis, although the bioavailability of oral

ganciclovir is lower than intravenous ganciclovir (Winston and Busuttil, 2004). Valganciclovir, a valine ester of ganciclovir, has been developed more recently to overcome the limitations of oral ganciclovir and it gives the same plasma ganciclovir exposures to those achieved with i.v. ganciclovir (Peskovitz et al., 2000). Valganciclovir prophylaxis in solid organ transplant patients has been found clinically as effective as oral ganciclovir with a comparable safety profile and the absence of ganciclovir resistance (Hodson et al., 2005; Paya et al., 2004). Valganciclovir has also recently been used in the treatment of CMV infection and CMV disease in organ transplant recipients (Babel et al., 2004; Humar et al., 2005). In preemptive therapy, antiviral treatment is initiated when CMV is detected in the blood using sensitive quantitative virological methods, such as PCR or tests for viral antigen. Preemptive therapy is based on frequent monitoring of the viral load in the peripheral blood of the recipient. It has been shown that peak viral loads correlate with CMV disease and that the rate of increase in viral load can identify recipients at risk of developing CMV disease (Emery et al., 2000). The advantages of the pre-emptive therapy include reduction in the number of patients exposed to antivirals, lowering the risk of drug resistance and maximizing the cost benefit ratio. The effectiveness of pre-emptive ganciclovir therapy has also been shown in randomized, placebo-controlled trials (Strippoli et al., 2006). Recently, valganciclovir has also been used pre-emptively (Singh et al., 2005). Currently, clinical antiviral trials of the drug, Maribavir is under way. Maribavir is a potential inhibitor of UL – 97 kinase of HCMV. Initial research has shown a good anti HCMV effect of Maribavir. Maribavir had shown to antagonize the effect of ganciclovir though; the antiviral effects of foscarnet and cidofovir remained

unaffected (Chou and Marousek, 2006). Antiviral resistance to Maribavir has been shown to be conferred by mutations in a region upstream to that conferred by ganciclovir in the UL97 gene (Chou et al., 2007).

2.14 MOLECULAR METHODS FOR DETECTION AND CHARACTERIZATION OF HCMV IN INDIA

In India, several studies on HCMV have been based on the use of conventional methods particularly employing serological techniques for the detection of IgG and IgM antibodies of HCMV in different patient populations (Pal et al., 1972; Mathur et al., 1981; Shanmugam et al., 1982; Broor et al., 1991; Kapil and Broor, 1992; Ray and Mahajan, 1997; Chakravarty et al., 2005; Sheevani et al., 2005; Gandhoke et al., 2006; Kothari et al., 2006). Few reports are available on the use of pp65 antigenemia assays to establish CMV disease in renal transplant recipients (Sakhuja et al., 2002; Minz et al., 2004).There are handful reports on the use molecular assays for the detection of HCMV in different patient populations. Sivakumar et al (2001) used Mac ELISA and a nested PCR simultaneously for establishing congenital CMV infection in neonates with clinically proven HCMV. The study suggested that Mac ELISA had parallel sensitivity and specificity to the nested PCR and more preferred since it was less expensive, less cumbersome and more user friendly. A nested PCR targeting mtr II region was used to detect HCMV genome in the urine specimens of congenitally infected and healthy neonates. The study emphasized the value of molecular detection of HCMV to establish an association with symptomatic disease in congenitally infected infants (Shoby et al., 2002). A highly sensitive polymerase chain reaction (PCR) for immediate early gene of CMV and pp65 antigenaemia assay were applied on clinical specimens from

patients with AIDS and healthy HIV seropositives to establish CMV infection. The study suggested that PCR is a powerful tool for detection of CMV in blood and is superior to the antigenaemia assay (Mujtaba et al., 2003). Nucleic acid sequence-based amplification (NASBA) technology was used to detect pp67 mRNA of cytomegalovirus (CMV) in transplant recipients. The study concluded that the pp67 mRNA assay was an accurate, rapid, and effective diagnostic tool to detect active CMV disease in symptomatic transplant cases (Wattal et al., 2004). Kishore et al (2004) employed PCR for detection of CMV disease and graft rejection in renal transplant recipients. The study suggested that PCR may not be sensitive enough in detecting CMV disease or rejection (Kishore et al., 2004). Rao et al., 2000 in a longitudinal study, demonstrated the importance of quantitation of HCMV genome to diagnose HCMV disease in seroendemic transplant population. In this study quantitation of the HCMV DNA was performed by limiting dilution. The study showed that a cutoff of 0.001 μg/ 5 μl input DNA had a good positive predictive value of 92.3% for symptomatic CMV disease (Rao et al., 2000). A similar study was performed on the bone marrow transplant recipients (Finny et al., 2001). Qualitative PCR have also been employed in detection of HCMV in intraocular specimens (Priya, 2001; Biswas et al., 2002). Studies on molecular characterization of HCMV however have been scarce. The only report from India on CMV genotypes showed that gB subtypes 2 and 3 were prevalent in immunocompromised patients with suspected HCMV infections in Chennai region, India. Genotypes 1 and 4 were not found in the study (Madhavan and Priya, 2002b). Therefore, due to the paucity of reports on the use of nucleic acid based molecular assays for detection, quantitation and characterization of HCMV such a study was undertaken.

3. STANDARDIZATION OF pp65 ANTIGENEMIA ASSAY TO DETECT AND QUANTITATE HCMV ANTIGEN IN PERIPHERAL BLOOD SPECIMENS

3.1 BACKGROUND

pp65 antigenemia assay is a rapid, simple, quantitative test for the diagnosis of active HCMV infection. Antigenemia assay is more sensitive than serology and viral isolation by conventional tube culture or shell vial assays. The test has a prognostic value to identify immunocompromised patients at risk of developing HCMV disease (Boeckh and Boivin, 1998). The lead time for the test is usually several days to one week before the onset of symptoms in immunocompromised patients (The et al., 1992; Le Goff et al., 1995). The test is used for monitoring the development of CMV disease and /or antiviral resistance in solid organ transplant recipients and AIDS patients. The pp65 antigenemia assay uses monoclonal antibodies to detect the lower matrix protein pp65 in the leucocytes. Several modifications in the steps involved for the assays have evolved over period of time including automation of the procedure by the use of flow cytometry (Toulemonde et al., 2000).

3.2 OBJECTIVE

3.2.1 To compare conventional dextran sedimentation (CDS) and direct erythrocyte lysis method (DEL) for isolation of leucocytes and standardize immunofluorescence based pp65 antigenemia assay.

3.3 MATERIALS AND METHODS

3.3.1 Clinical specimens

EDTA anticoagulated peripheral blood specimens were collected from 25 healthy blood donors (controls) and 25 post-renal transplant recipients with suspected HCMV infections.

3.3.2 Total leucocyte count by manual method

The total leucocyte count on the peripheral blood was performed manually by using haemocytometer as described in Appendix I**.** The values obtained were used as a reference to calculate the recovery rates of the leucocytes obtained by CDS and DEL methods.

3.3.3 pp65 antigenemia assay

The details of preparation of reagents are provided in Appendix I. The various steps involved in the pp65 antigenemia assay include: isolation of leucocytes followed by counting of cells, preparation and fixation of smears and detection of pp65 antigen by immunofluorescence staining.

3.3.3.1 Isolation of leucocytes

Two different protocols were followed for isolation of leucocytes:

i. Conventional dextran sedimentation method (CDS)

ii. Direct erythrocyte lysis method (DEL)

3.3.3.1.1 Conventional dextran sedimentation method (CDS):

To 5ml of EDTA-anticoagulated blood, 1.5ml of 6% sterile dextran solution was added and incubated at 37^{o}C for 30 minutes to allow aggregation and sedimentation of the erythrocytes. The leukocyte rich supernatant was mixed with 10ml of sterile phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 10 minutes. The cell pellet was suspended in 1ml of 0.8% ammonium chloride for 5 minutes to lyse the remaining erythrocytes. The mixture was centrifuged at 3000 rpm for 5 minutes. The cells were then washed thrice with sterile PBS. The final leukocyte pellet was suspended in 1ml of PBS.

3.3.3.1.2 Direct erythrocyte lysis method (DEL):

Twenty five ml of 0.8% ammonium chloride was added to 2ml of EDTA anticoagulated blood, mixed and incubated at 37^{o} C for 5 minutes. The mixture was centrifuged at 3000 rpm for 5 minutes. The cell pellet was washed with sterile PBS thrice. The final leukocyte pellet was suspended in 1ml of PBS.

3.3.3.2 Cell count & smear preparation

Cell counts were determined using a hemocytometer (Appendix I). Cytospin smears containing 2x 10^{5} leucocytes/ slide were made by centrifugation at 1000 rpm for 5 minutes. Cytospin slides were fixed in methanol for 10 minutes; treated with 0.5% Nonidet -P40 for 5 minutes for improving cell permeablilization; air dried for 15 minutes.

3.3.3.3 Detection of pp65 antigen by immunofluorescence staining

The Cytospin smear was covered with 25µl of 1/5 diluted CMV monoclonal antibody directed against pp65 antigen (DAKO, A/S, Denmark) and incubated in a moist chamber for one hour. After incubation, the smear was washed thrice with PBST and incubated with 25µl of 1/5 diluted FITC conjugated rabbit anti-mouse IgG antibody (DAKO, A/S, Denmark) for one hour in a moist chamber. The smear was washed thrice with PBST, counter stained with 0.5% Evans blue and mounted with glycerol. Number of nuclei with apple green fluorescence was scored under UV microscope at 400 X magnification.

3.3.3.4 Interpretation of the results

Positive result:

Presence of at least 1 positively stained leukocyte on the slide.

Result of semi-quantitation:

Total no. of cells positive for pp65 antigen / $2x10^5$ leucocytes.

3.3.3.5 Analysis of results

The recovery rates (%) of the leucocytes for the CDS and DEL method were calculated individually. Since different volumes of blood were used for the two methods the total number of leucocytes were converted per milliliter of blood and following formula was applied:

Leucocytes isolated by CDS/DEL /ml of blood x 100

Total no. of leucocytes/ ml (as determined by manual method)

3.4 RESULTS

3.4.1 Comparison of CDS & DEL method on recovery rates of leucocytes

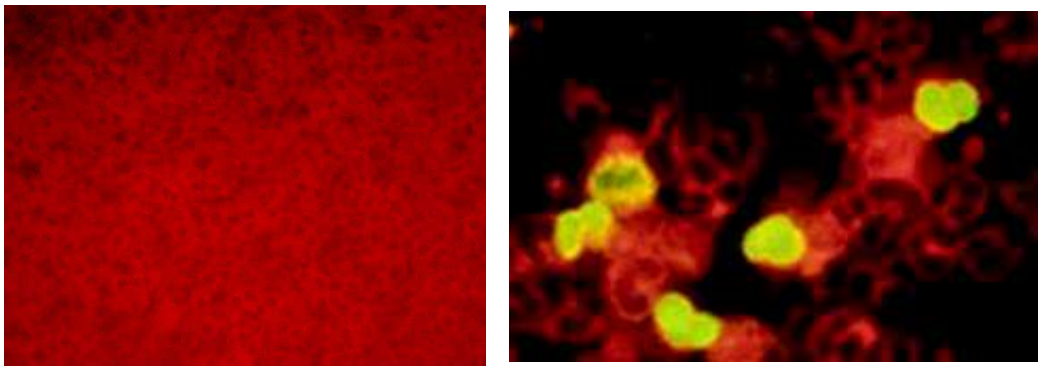
No significant difference in the recovery rates of leucocytes was observed between the two methods. The mean ± SD recovery rate of leucocytes by CDS method was 64 ± 15.2% (range 30.7% to 89%) and by DEL method was 64.8 ± 14.3% (range 33 % to 87.7%).

3.4.2 Results of pp65 antigenemia assay on controls and clinical specimens

Irrespective of the method of leucocyte recovery all the 25 peripheral blood specimens from healthy blood donors were negative for pp65 antigenemia **(Figure 1a)**. Of the 25 clinical specimens from post – renal transplant recipients, 13 were positive by both DEL and CDS methods **(Figure 1b)**. Discrepancy in determining positivity of the sample was not observed between the two methods. **Table 2** shows the pp65 antigenemia values of the 13 positive specimens with both CDS and DEL method. A positive correlation was found with the semi-quantitation results of CDS and DEL method (r_s = 0.8338, Spearman correlation coefficient test; $p < 0.001$) **(Figure 2a)**. A good agreement was found between the two methods by Bland – Altman plots. The bias (difference between the means) was 0.2 and the 95 % limits of agreement were between -19.8 to 19.4 **(Figure 2b)**.

Figure 1: Results of pp65 antigenemia assay

(a) **(b)**



(a) pp65 antigenemia assay performed on PBLs of healthy control negative for HCMV antigenemia (100x magnification).

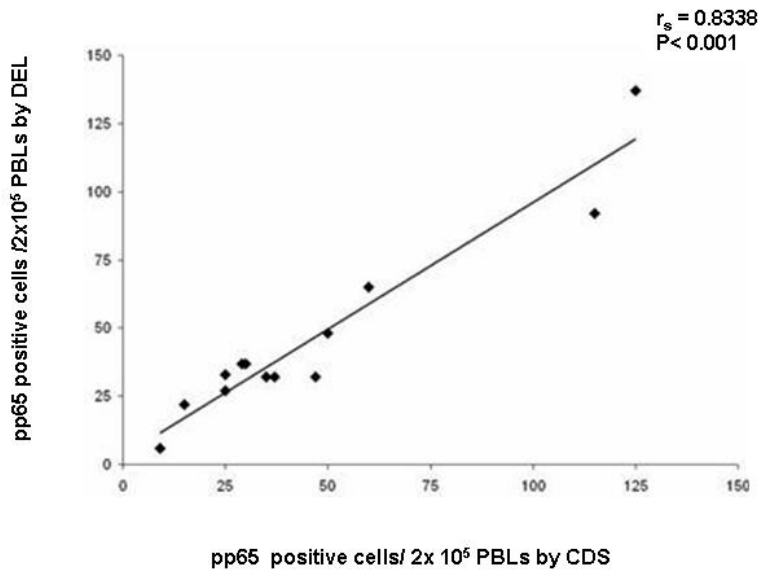
(b) pp65 antigenemia assay performed on PBLs of post renal transplant recipient positive for HCMV antigenemia (400x magnification).

Table 2: Results of pp65 antigenemia values obtained with CDS and DEL method on 13 peripheral blood specimens from post – renal transplant recipients with HCMV antigenemia

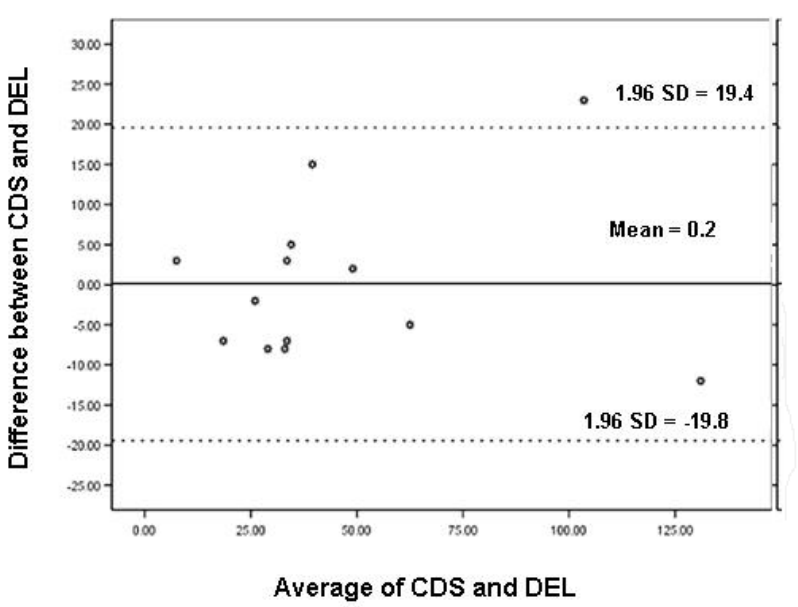
S.No.	pp65 antigenemia values	
	CDS method	DEL method
1	29	37
2	50	48
3	35	32
4	30	37
5	115	92
6	25	27
7	125	137
8	25	33
9	60	65
10	37	32
11	9	6
12	15	22
13	47	32

Figure 2: Correlation and agreement between CDS and DEL methods

(a)



(b)



(a) XY Scatter plot . pp65 positive cells obtained with DEL method plotted against that of CDS method. The correlation between the method was examined by spearman rank test and was found significant with a correlation coefficient (r_s) = 0.8338 (P<0.001).

(b) Bland Altman plot showing good agreement between CDS and DEL methods. The bias was 0.2 and the 95% limits of agreement was between -19.8 to 19.4

3.5 DISCUSSION

The sensitivity, specificity and relative rapidity of pp65 antigenemia assay are well established. The assay is widely used as differential marker for active and latent HCMV infection, prognostic marker for development of HCMV disease, and surrogate marker for the development of antiviral resistance in transplant recipients and AIDS patients (Boeckh and Boivin, 1998). The maiden pp65 antigenemia assay protocol comprised of isolation of leucocytes by dextran sedimentation, fixation and detection of pp65 antigen by indirect immunoperoxidase staining (Van der Bij et al., 1988). Isolation of leucocytes may be a critical step, since; the pp65 antigen is detectable more in the granulocytes particularly polymorphonuclear leucocytes PMNL than other fractions. Ficoll gradient may not be useful for isolation of leucocytes as it provides a monocyte rich fraction (Boeckh and Boivin, 1998). Dextran sedimentation, Polymorphprep method, direct erythrocyte lysis methods have all been used previously for preparation of leucocytes (Ho et al., 1998; Garcia et al., 1996). Significant losses of the leucocytes have been reported irrespective of the methods of leucocyte isolation in different studies. Garcia et al (1996) carried out a prospective, parallel, and blind study comparing both qualitatively and quantitatively, the efficiency of Polymorphprep method and dextran sedimentation method. The study concluded that both methods were equally effective (Garcia et al., 1996). However, Ho et al (1998) compared the dextran sedimentation and direct erythrocyte lysis methods and found that direct erythrocyte lysis method gave higher yields and better results than the dextran sedimentation. Taking these into consideration, simple and inexpensive methods of leucocyte isolation namely the Conventional dextran sedimentation (CDS) and Direct erythrocyte lysis (DEL)

methods were compared as a first step in optimization of pp65 antigenemia assay. The results showed no significant difference in terms of the recovery rates of the leucocytes or qualitative/ quantitative detection of antigenemia in healthy controls as well as the post renal transplant recipient groups. However, considering the time taken, expenditure and volume of specimen required, DEL method might have a marginal advantage over CDS method. On the other hand, in patients with severe leucopenia (neutrophils < 200/mm^3) since a larger volume of blood (around 10 ml) would be required for preparing the slides the CDS may be preferred over DEL method. Also, in patients with lymphocytosis, it may be advisable to use the CDS method as it selectively provides the PMNL fraction, since lymphocytes do not express pp65 antigen of HCMV. No efforts were made to compare different fixation or staining procedures since methanol fixation and immunofluorescence staining techniques were well developed and routinely performed for other infectious agents in the laboratory. The results of the study suggest that CDS and DEL methods can be alternatively used for pp65 antigenemia assay.

4. EVALUATION OF THE EFFICIENCY OF PRIMERS TARGETING MORPHOLOGICAL TRANSFORMING REGION II (mtrII), UL -83 AND GLYCOPROTEIN O (gO) REGIONS FOR DETECTION OF HCMV IN CLINICAL SPECIMENS

4.1 BACKGROUND

Conventional methods of laboratory diagnosis of HCMV infection include serology, virus culture by conventional tube method or rapid shell vial assay and antigen detection. Culture is the "gold standard" but is a relatively insensitive laboratory method. Serology results are difficult to interpret especially in immunocompromised patients (Madhavan and Priya, 2002a; Boeckh and Boivin, 1998). pp65 antigenemia assay is used as a test for monitoring those at higher risk of developing CMV disease and to initiate pre-emptive therapy (Kusne et al., 1999; Amorim et al., 2001). Detection of HCMV DNA in clinical specimens by nucleic acid based amplification methods such as Polymerase chain reaction (PCR) contributes to a rapid and early diagnosis (Yuen et al., 1995; Mansy et al., 1999; Lo et al., 1999). Primer pairs for the detection of the genes coding for the Immediate Early (IE) antigen and Late antigen (LA) were initially used for the detection of HCMV genome in urine and peripheral blood leucocyte specimens (Demmler et al., 1988; Shibata et al., 1988). Since then, a variety of primer pairs are being used for routine diagnosis of HCMV infection in various patient populations. Different factors including sequence variations in the viral genome have been shown to affect the ability of the PCR using different primer sets to detect HCMV DNA (Brytting et

al., 1992; Chou, 1992b; Distefano et al., 2004; Wirgart et al., 1998). Little is known about the regions under the present study viz: morphological transforming region II (mtr II), UL 83 and glycoprotein O (gO) gene as targets for the detection of HCMV genome by PCR. In the present study, pp65 antigenemia assay was considered as "gold standard" to evaluate the efficacy of the Polymerase chain reaction tests to detect HCMV genome in the clinical specimens from immunocompromised patients with suspected HCMV infection. pp65 antigenemia test was considered as a "gold standard" since detection of antigenemia in immunocompromised patients is an indicator of active HCMV disease (Mazzulli et al., 1999; George and Rinaldo, 1999).

4.2 OBJECTIVES

4.2.1. To standardize uniplex PCR for UL-83 gene of HCMV.

4.2.2. To standardize nested PCRs for gO gene of HCMV using two sets of primers.

4.2.3. To standardize duplex PCR by combining the two sets of primers targeting gO gene.

4.2.4. To evaluate the efficiency of the PCRs for mtr II, UL 83, gO and duplex PCR for gO gene against pp65 antigenemia assay as the 'gold standard'.

4.3 MATERIALS AND METHODS

4.3.1 Standardization of Uniplex PCR for UL-83 gene of HCMV

4.3.1.1 Standard strain of HCMV – AD169 (Christian Medical College and Hospital [CMCH], Vellore, India).

4.3.1.2 DNA extraction

DNA extraction from the standard strain of HCMV was carried out by QIAamp DNA mini Kit (QIAGEN, GMbH, Germany) strictly adhering to manufacturer's instructions. The detailed procedure is provided in Appendix II**.**

4.3.1.3 PCR amplification

The uniplex PCR for UL-83 gene of HCMV was performed as described previously (Gouarin et al., 2002) with some modifications. The original PCR in Taqman probe-based real time PCR format was modified to suit conventional uniplex PCR. The primer pairs are provided in **Table 3.** For amplification, a 50µl reaction was set with 160 µM of each dNTPs, 10 picomoles of each primer, 1x buffer (10mM Tris-Cl [pH 8.3], 50mM KCl, 0.01% gelatin, 1.5mM $MgCl_2$) and one unit of *Taq* polymerase. Ten microlitres of extracted DNA was added as the template. The reactions were carried out in a thermal cycler (Perkin Elmer model no. 2400, USA). The thermal profile consisted of an initial denaturation at 94°C for five minutes for one cycle followed by 45 cycles of denaturation at 94°C for 15 seconds, annealing at 65°C for 20 seconds and extension at 72°C for 20 seconds and a single cycle of final extension at 72°C for five minutes. Negative control containing the reaction mixture with milli Q water in the place of extracted DNA; spiking reaction (to rule out inhibitors) and positive control containing HCMV AD-169 DNA were used to validate the PCR reaction in each run. The amplified products were then subjected to gel electrophoresis using two percent agarose gel containing 0.5µg/ml

Table 3: Primer sequences for the detection of mtr II, UL 83 and gO genes of HCMV

Genes	Primer sequences (5' – 3')	Amplicon size
mtr II	**I round** **F:** CTG TCG GTG ATG GTC TCT TC **R:** CCC GAC ACG CGG AAA AGA AA	234 bp
	II round **F:** TCT CTG GTC CTG ATC GTC TT **R:** GTG ACC TAC CAA CGT AGG TT	168 bp
UL 83	**F:** GGG ACA CAA CAC CGT AAA GC **R:** GTC AGC GTT CGT GTT TCC CA	283 bp
gO (AD169)	**I round** **F:** CAG CTT CGA AAA CCG GCC AAA TAC G **R:** AAT ATA CTT GGG GAC GCG AAA TAG A	375 bp
	II round **F:** GCT TCG AAA ACC GGC CAA ATA CG **R:** ATA CTT GGG GAC GCG AAA TAG A	370 bp
gO (Towne)	**I round** **F:** CAA CTC CGT AAA CCG GCC AAA T **R:** ATA TAC TTG GGA ACG CGG	375 bp
	II round **F:** CTC CGT AAA CCG GCC AAA TAT G **R:** TAC TTG GGA ACG CGG AAT	370 bp

F – Forward primer; R – Reverse primer

of ethidium bromide as described in Appendix II. The specificity and sensitivity of the PCR was determined as described in Appendix II.

4.3.2 Standardization of nested PCRs for gO gene of HCMV using two sets of primers

The PCRs for gO gene of HCMV was carried out as described previously (Rasmussen et al., 2002) with certain modifications. In brief, two nested PCRs were standardized individually using two sets of primers designed for AD169 and Towne strains of HCMV. The primer sequences are provided in **Table 3.** Similar reaction mixtures except for the primers were used for both the PCRs. For the first round amplification, a 50µl reaction was set with 160 µM of each dNTPs, 10 picomoles of each primer, 1x buffer (10mM Tris-Cl [pH 8.3], 50mM KCl, 0.01% gelatin, 1.5mM $MgCl_2$) and one unit of *Taq* polymerase. Ten microlitres of extracted DNA was added as the template. The reactions were carried out in a thermal cycler (Perkin Elmer model no. 2400, USA). The thermal profile consisted of an initial denaturation at 94°C for five minutes for one cycle followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds and a single cycle of final extension at 72°C for five minutes. AD169 strain of HCMV and a clinical isolate of HCMV were used as positive controls for standardization of the PCRs for gO (AD169) and gO (Towne) respectively. The second round of amplification consisted of reaction mixture similar to the first round except for the primers. Two microlitres of the first round product was used as template for the second round and subjected to a thermal profile similar to the first round with 20 cycles. The amplified products were then subjected

to gel electrophoresis using two percent agarose gel containing 0.5μg/ml of ethidium bromide as described in Appendix II. The specificity and sensitivity of the PCRs were determined as described in Appendix II.

4.3.3 Standardization of duplex PCR by combining the two sets of primers targeting gO gene

Duplex nested PCR for gO gene was standardized by combining the two sets of primers for AD169 and Towne strains. The reaction mixture similar to that of the individual PCRs (section 4.3.2) was used except for the primer concentration. A concentration of 15 picomoles of gO (AD 169), and 10 picomoles of gO (Towne) primers were used. The thermal profile for the amplification was similar to the individual PCRs (section 4.3.2). Specificity and sensitivity of the duplex PCR was determined as described in Appendix II.

4.3.4 Evaluation of the efficiency of the PCRs for mtr II, UL 83, gO (AD169) and duplex PCR for gO gene against pp65 antigenemia assay as the 'gold standard'

4.3.4.1 Study design and Definition of gold standard

Clinical specimens received at L & T Microbiology Research Centre, Vision Research Foundation, in Sankara Nethralaya, Chennai, India during December 2004 to July 2005 were investigated for the possible association of CMV infection in 74 immunocompromised patients. Clinical specimens were investigated because of clinical suspicion of CMV- related disease in these patients. The patients with pp65 antigenemia positivity were defined to have activation of HCMV disease and this

test was used as the "gold standard" to evaluate the PCRs for mtrII, UL 83, gO (AD169) and duplex PCR for gO gene for their clinical specificity, sensitivity and predictive values. The nested PCR for mtrII, uniplex PCR for UL83 and nested PCR for gO (AD169) were applied prospectively while the duplex PCR for gO was applied retrospectively on the stored DNA as it was standardized at a later stage.

4.3.4.2 Patients, Controls and Clinical specimens

The distribution of the clinical specimens in relation to the clinical status of the patients from whom they were collected is provided in **Table 4.** Ninety two clinical specimens [74 blood, 18 urine] from 74 immunocompromised patients [Solid organ transplant recipients – 59, Bone marrow transplant recipients -3, HIV infected individuals -7 and Congenital/ neonates -5] were analyzed during the course of the study. Among the 74 patients, 47 were males and 27 were females. The age of the patients ranged from 40 hours after birth to 67 years. The patients clinically presented with multiple symptoms such as fever, jaundice and leucopenia. Three of the renal transplant recipients had clinical evidences of a moderate graft rejection. CMV retinitis was predominant in the HIV infected individuals. In addition blood samples from 45 healthy blood donors seropositive for HCMV were used as controls for all the tests.

4.3.4.3 pp65 antigenemia assay, PCR for mtrII, UL-83, gO (AD169) and duplex PCR for gO gene of HCMV

pp65 antigenemia assay was performed as described in section 3.3.3. CDS or DEL methods were alternatively used depending on the volume of blood available.

Table 4: Distribution of 92 clinical specimens in relation to the clinical status of the 74 patients with suspected CMV infections

Clinical Status of the Patients	Total number of patients (n = 74)	Clinical Specimens collected (n= 92)	
		Blood only (n= 56)	Blood and urine (n = 18 x 2 : 36)
Solid organ transplantation	59 (79.7%)	43	16
Bone marrow transplantation	3 (4.0%)	3	-
HIV infected individuals	7(9.5%)	7	-
Congenital / neonates	5(6.8%)	3	2

The extraction of the DNA for the PCRs was performed using two commercially available DNA extraction columns strictly adhering to the manufacturer's instruction. The detailed procedures of the DNA extraction are provided in Appendix II. The PCR for detection of mtrII region was performed as described by Madhavan and Priya (2002a). The primer sequences are provided in **Table 3.** For the amplification a 50µl reaction was set with 80 µM of each dNTPs, 10 picomoles of each primer, 1x buffer (10mM Tris-Cl [pH 8.3], 50mM KCl, 0.01% gelatin, 1.5mM $MgCl_2$) and one unit of *Taq* polymerase. Ten µl of extracted DNA was added as the template. For the nested amplification, one microlitre of the first round product was added as the template DNA for the second round with a reaction mixture similar to the first round. The reactions were carried out in a thermal cycler (Perkin Elmer model no. 2400, USA). The amplification profile for CMV consisted of 30 cycles for the first round and 20 cycles for the second round each consisting of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute with a final extension at 72°C for ten minutes. The amplicons were visualized on a 2% agarose gel incorporated with ethidium bromide as described in Appendix II.

The uniplex PCR for UL83, nested PCR for gO (AD169) and duplex PCR for gO were performed as described in sections 4.3.1; 4.3.2; 4.3.3 respectively.

4.3.4.4 Analysis of Results

Diagnostic data from clinical specimens of 74 patients with suspected HCMV infections were used for the determination of the clinical sensitivity,

specificity, positive and negative predictive values of the different PCRs using pp65 antigenemia results as the "gold standard". The difference in the clinical sensitivity and the specificity of the PCRs were statistically analyzed by Fisher's exact test for two proportions.

4.4 RESULTS

4.4.1 Specificity and Sensitivity of UL-83 PCR

The uniplex PCR for the UL-83 gene of HCMV generated amplified product of 283 bp. The PCR was specific for HCMV and sensitive enough to detect 50fg of HCMV DNA.

4.4.2 Specificity and Sensitivity of nested PCRs for gO gene using two sets of primers

Both the nested PCRs for gO gene of HCMV generated amplified products of 375 bp in the first round of amplification and 370 bp in the second round. The PCRs were specific for HCMV and sensitive enough to detect 500fg of HCMV DNA.

4.4.3 Specificity and Sensitivity of duplex PCR for gO gene

The duplex PCR for gO gene of HCMV generated amplified products of 375 bp in the first round and 370 bp in the second round of amplification. The PCR was specific for HCMV. The duplex PCR detected 500fg of HCMV DNA.

4.4.4 Results of the application of pp65 antigenemia assay, PCRs for mtr II, UL 83, gO (AD169) and duplex PCR for gO gene on 74 immunocompromised patients

Of the 74 patients tested, the pp65 antigenemia assay became positive in 38 patients. Hence, of the total 92 clinical specimens from 74 patients, 48 clinical specimens from 38 patients were considered as positive for the "gold standard". The 48 clinical specimens positive included blood alone from 28 patients and pairs of blood and urine from 10 patients. Of the 48 clinical specimens positive by the "gold standard", when tested by PCR methods all were positive for mtr II region, 27 for UL –83 gene, 21 for gO (AD169) and 35 by duplex PCR for gO gene. The increase in clinical sensitivity by the PCR for mtr II over the UL 83, gO (AD169) PCR and duplex gO PCR were 44 %, 56 % and 27 % respectively. The increase in the clinical sensitivity of the PCR for mtr II was statistically significant ($P < 0.0001$ Fisher's exact test for two proportions). However, the difference in the clinical specificity between the PCRs was not statistically significant. A summary of the results is presented in **Table 5**. All the 45 peripheral blood leucocytes obtained from the controls (seropositive healthy donors) remained negative by pp65 antigenemia assay and all the PCR tests for HCMV. **Figures 3a, 3b**, **3c** and **3d** shows the agarose gel electrophoretogram of amplicons obtained with PCR for mtr II, UL 83, gO (AD-169) and duplex gO respectively.

4.5 DISCUSSION

Human Cytomegalovirus has long been recognized as a major cause of life-threatening complications in immunosuppressed individuals. There is perceived need for the use of a reliable technique that allows an early detection of the viral

Table 5: Results of the mtrII, UL 83, gO(AD169), duplex gO PCR for the diagnosis of suspected HCMV infections in comparison to pp65 antigenemia (gold standard)

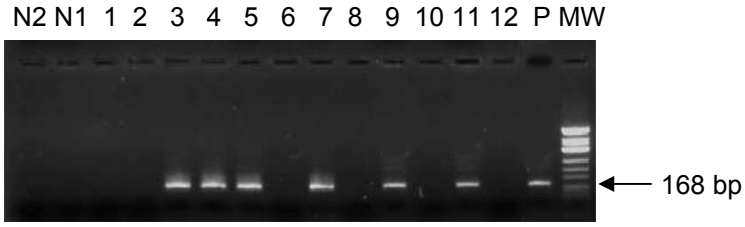
Diagnostic test result	pp65 Positives (n = 48)				pp65 Negatives (n = 44)			
	No. of Clinical specimens	SENS (%)	PPV (%)	FN (%)	No. of Clinical specimens	SPEC (%)	NPV (%)	FP (%)
PCR for mtrII positive	48	100	87	0	7	84	100	16
PCR for mtr II negative	0				37			
PCR for UL 83 positive	27	56	87	44	4	91	66	9
PCR for UL 83 negative	21				40			
PCR for gO (AD169) positive	21	44	91	56	2	95	61	5
PCR for gO(AD169) negative	27				42			
PCR for duplex gO positive	35	73	90	27	4	91	75	9
PCR for duplex gO negative	13				40			

Abbreviations **:** SENS, sensitivity; PPV, Positive predictive value; FN, False negative; SPEC, specificity; NPV, Negative predictive value; FP, False positive

Increase in clinical sensitivity of the PCR for mtr II gene over other PCRs was statistically significant ($P<0.0001$, Fishers exact test)

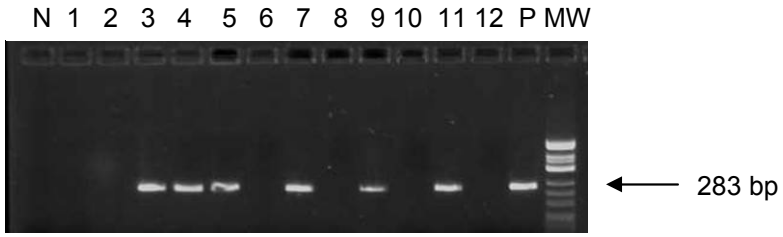
Figure 3: Agarose gel electrophoretogram showing the amplified products of the PCRs applied on peripheral blood and urine specimens

(a)



(a) Nested PCR for mtr II gene. Lanes: N2 – Negative control (II round) ; N1 - Negative control (I round); 1,2,6,8 –Blood negative for mtrII PCR; 3,4,5,7, 9 –Blood positive for mtrII PCR; 10, 12 – Urine negative for mtr II PCR; 11 – Urine positive for mtr II PCR; P- Positive control (AD- 169 DNA); MW – *Hinf I* digest of ϕX – 174 DNA.

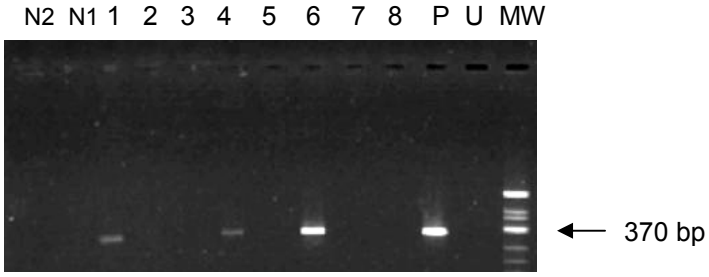
(b)



(b) Uniplex PCR for UL-83 gene. Lanes: N – Negative control; 1,2,6,8 –Blood negative for UL 83 PCR ; 3,4,5,7,9 –Blood positive for UL 83 PCR; 10, 12 – Urine negative for UL 83 PCR ; 11 – Urine positive for UL 83 PCR ; P- Positive control (AD- 169 DNA); MW – *Hinf I* digest of ϕX – 174 DNA.

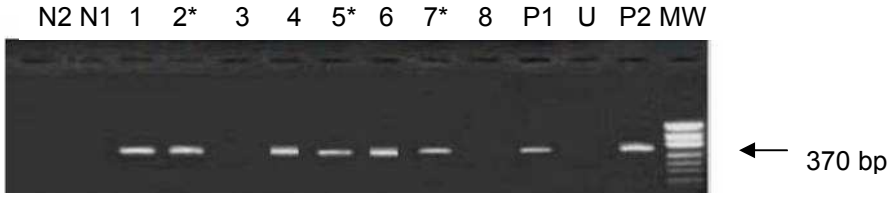
Figure 3 (continued)

(c)



(c) **Nested PCR for gO (AD 169).** Lanes: N2 – Negative control II round ; N1 - Negative control I round; 1 –Urine positive for gO (AD169)PCR; 2,3–Urine negative for gO (AD169) PCR; 4,6 – Blood positive for gO (AD169)PCR; 5,7,8 – Blood negative for gO (AD169)PCR; P- Positive control (AD- 169 DNA); U – Unloaded well; MW – *Hinf* I digest of ϕX – 174 DNA.

(d)



(d) **Duplex PCR for gO gene.** Lanes: N2 – Negative control II round ; N1 - Negative control I round; 1,2 –Urine positive for duplex gO PCR; 3–Urine negative for duplex gO PCR; 4,5,6,7 – Blood positive for duplex gO PCR; 8 – Blood negative for duplex gO PCR;P1 – Positive control (CMV isolate (control for gO (Towne)); U – Unloaded well; P2- Positive control (AD-169 DNA); MW – *Hinf* I digest of ϕX – 174 DNA.

***** denotes specimens negative by PCR for gO (AD169)

activation to help decide on early use of pre-emptive therapy in those at greater risk of the disease. Technique such as virus isolation though most specific cannot be practiced on a regular basis due to lack of its sensitivity and non-availability of human diploid fibroblasts in this part of the world. Quantitative pp65 antigenemia assay, used to monitor and detect HCMV disease, is well established to have a higher positive predictive value for the disease (Boeckh and Boivin, 1998). Since, antigenemia is cell based and a low frequency event, a sufficient number of granulocytes are necessary for a reliable result (Boeckh and Boivin, 1998). This becomes difficult in Bone marrow transplant or other patients with severe leucopenia. Other difficulties include the necessity for immediate processing of the specimen (within 6 hrs, stored specimens may give erroneous results), difficulty in processing a large number of specimens at a time and subjective component in slide reading, which requires expertise (Boeckh and Boivin, 1998). The drawbacks of pp65 antigenemia assay or virus isolation may be overcome by the use of rapid, sensitive and normalized method such as PCR for the detection of HCMV genome in the clinical specimens, which can be applied on a large scale of clinical specimens without any difficulty in a standard laboratory for routine diagnosis of HCMV infection.

Amplification of HCMV genome by PCR is a rapid and sensitive method for detection of HCMV in clinical specimens. The choice of PCR primers for HCMV genome detection in a clinical specimen is crucial, since the genome of HCMV is reported to be highly variable (Brytting et al., 1992; Chou, 1992b; Distefano et al., 2004; Wirgart et al., 1998). The primers targeting regions such as the Major immediate early gene exon 4, regions of gene coding late antigen, glycoprotein B (gB) and glycoprotein H (gH) which are widely used have failed to detect the

HCMV genome in certain clinical specimens due to primer target mismatch owing to the large sequence variations in HCMV genome. Nucleotide substitutions and even deletions of certain ORFs have been found along the genome of HCMV (Cha et al., 1996). Studies by Distefano et al (2004) suggested that PCR for gB gene was more reliable than the Major immediate early gene exon 4 or Late antigen gene in detecting HCMV genome in the clinical specimens from congenital and perinatal infections in Argentina. Studies by Wirgart et al (1998) suggested that the DNA polymerase gene and gB gene were more conserved and can be used for diagnosis of HCMV infections in different patient populations. PCRs for gB and gH genes of HCMV were compared in a Brazilian study on the renal transplant recipients by Aquino and Figueiredo (2001) where in a multiplex format of both the genes was suggested for reliable detection of HCMV genome. PCRs for Major Immediate early (MIE; IE1) gene, glycoprotein B (gp 58) and structural phosphoprotein (pp 150) were compared with pp65 antigenemia in heart and lung transplant recipients by Barber et al (1996) where in all the PCR showed a high sensitivity of 100% though gp 58 was associated more with a positive PCR signal than the other two PCRs.

There are only a few reports available on the primers targeting the regions of HCMV genome under the present study (Madhavan and Priya, 2002a; Rasmussen et al., 2002, Gouarin et al., 2002). Therefore, the efficacy of these three primers was evaluated on different clinical specimens obtained from different high risk immunocompromised patient populations against pp65 antigenemia assay as gold standard. Blood from the healthy seropositive controls did not yield any detectable CMV DNA by any of the three PCRs following amplification. Though PCR is

considered as a highly sensitive method, they did not detect low - level latent HCMV infection present in the healthy immunocompetent individuals in this study.

pp65 antigenemia assay detected active CMV disease in 38 of 74 patients . A high level antigenemia (>50 cells/ 2x 10^5 PBLs) was seen in clinical specimens positive by PCR for all the three regions. We hypothesize that PCRs for all the three regions may become positive only with a high viral load.

The failure of the PCR targeting UL 83 may be due to its lower analytical sensitivity, as it is a uniplex PCR. The PCR for gO gene though a nested PCR, showed already a lower analytical sensitivity than PCR for UL 83 and this may be attributed to the strain variations in gO gene leading to the primer target mismatch and hence loss of an amplification signal. This was confirmed with the results of the duplex PCR for gO gene since, the duplex PCR for gO increased the sensitivity over the individual gO PCR for AD-169. Results of the study showed that the PCR for mtr II had 100% sensitivity, 100 % negative predictive value, 87% positive predictive value and 84% specificity. Therefore, PCR for mtrII is the most suitable test for qualitative detection of HCMV in clinical specimens from immunocompromised patients in Chennai region, India.

5. DEVELOPMENT AND APPLICATION OF A MULTIPLEX PCR FOR SEMI- QUANTITATION OF HCMV AND ITS EVALUATION AGAINST pp65 ANTIGENEMIA ASSAY AND TAQMAN – PROBE BASED REAL TIME PCR ASSAY FOR HCMV

5.1 BACKGROUND

Early diagnosis of HCMV infection in high-risk patients is essential in order to initiate antiviral therapy (Gault et al., 2001; Roberts et al., 1998). Qualitative detection of the HCMV by a highly sensitive PCR may be sufficient in case of infants infected congenitally, establishing intrauterine infections in anti-HCMV IgM positive pregnant women and allogeneic marrow transplant recipients with severe graft versus host disease where in systemic viral load is of limited value. On the other hand, quantitative assays may be required for monitoring HIV infected individuals or solid organ transplant recipients who develop HCMV disease when there is a high viral load (Boeckh and Boivin, 1998). Considering the limited resources available for health care in developing countries like India, simplicity and the cost of the diagnostic assay are important. The detection of pp65 antigen in leucocytes although a sensitive method is laborious, requires immediate processing and relies on subjective interpretation of the slide. In India, quantitation of HCMV DNA by limiting dilution was attempted successfully (Rao et al., 2000). The major constraint in this method includes the use of multiple reactions for each dilution of the specimen. Quantitative assays available commercially do not have the levels of sensitivity of nested PCR and are thus not appropriate where initial qualitative

diagnosis is required (Caballero et al., 1997). Real-time PCR based on 5' nuclease assays are expensive on a routine basis. Hence, there is a need for developing cost effective method that are as sensitive as qualitative PCRs with element of quantitation in it and would not require multiple reactions for its performance. Such an assay would allow the rational use of preemptive therapy for the patients at high risk of developing symptomatic disease.

5.2 OBJECTIVES

5.2.1 To develop a multiplex PCR assay for detection of the HCMV genome viz. morphological transforming region II (mtr II), the UL-83 gene and gO region by combining the individual primer pairs.

5.2.2 To evaluate the multiplex PCR by simultaneously applying it with pp65 antigenemia assay on peripheral blood specimens obtained from post-renal transplant recipients.

5.2.3 To standardize Taqman probe-based real time PCR assay for quantitation of HCMV.

5.2.4 To compare the results obtained with Taqman probe- based real time PCR assay and multiplex PCR on different clinical specimens obtained from immunocompromised patients.

5.2.5 To correlate the results obtained with pp65 antigenemia assay and Taqman probe-based real time PCR assay.

5.3 MATERIALS AND METHODS

5.3.1 Standardization of multiplex PCR for HCMV

A multiplex nested PCR for HCMV was standardized by combining the individual primers for mtr II, UL 83 and gO gene (**Table 3**). The reaction mixture and thermal profiles were restandardized to achieve maximum sensitivity. The extraction of the DNA was carried out using commercially available DNA extraction columns adhering strictly to the manufacturer's instruction (Appendix II). The primer sets of the individual PCRs for mtr II, UL-83 and gO gene (individual primer sets for AD-169 and Towne strains) (**Table 3**) were combined and used in a nested multiplex PCR reaction. For amplification a 50 µl reaction was set with 200µM of each dNTPs, 1× PCR buffer (10mM Tris–HCl (pH 8.3), 50mMKCl, 0.01% gelatin, 1.5mMMgCl_2) and 2 units of Taq DNA polymerase. Twenty picomoles of each primer for gO gene, 10 picomoles of each primer for mtr II region and 15 picomoles of each primer for UL-83 gene were added. Ten microlitre of extracted DNA was added as template. The first round thermal profile consisted of an initial denaturation at 94°C for 5 minutes for 1 cycle followed by 35 cycles each consisting of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds followed by a single cycle of final extension at 72°C for 10 minutes. Only the primers targeting mtr II and gO genes were used for the second round. A 50µl reaction was carried out with 200µM of each dNTPs, 1× PCR buffer (10mM Tris–HCl (pH - 8.3), 50mM KCl, 0.01% gelatin, 1.5mM $MgCl_2$) and 2 units of Taq DNA polymerase. Twenty picomoles of each primer for gO gene and 10 picomoles for mtr II gene were used. From the first round 2 µl of

the amplified product was used as template for the nested round. The profile was similar to the first round of amplification with 25 cycles. For each specimen separate negative controls (containing water with PCR reagents), positive control (DNA extracted from AD 169) and spiking reaction (for validation of true negatives by ruling out inhibitors) were set. The amplified products were analyzed by gel electrophoresis using 4% agarose gel (Sisco Research Laboratories, Mumbai, India) containing 0.5μg/ml of ethidium bromide (Sigma, USA) (Appendix II). The specificity and sensitivity of the PCR was determined as described in Appendix II.

5.3.2 Application of multiplex PCR on retrospective clinical specimens and controls from healthy blood donors

The standardized multiplex PCR was applied on 25 retrospective peripheral blood specimens proven to be positive for mtr II region of HCMV and 25 peripheral blood specimens from healthy blood donors.

5.3.3 Evaluation of the multiplex PCR by simultaneously applying it with pp65 antigenemia assay on peripheral blood specimens obtained from immunocompromised patients

5.3. 3.1 Study design: Prospective study

5.3.3.2 Clinical specimens

Seventy peripheral blood specimens from seventy post-renal transplant recipients were included in the study.

5.3.3.3 pp65 antigenemia assay and multiplex PCR

pp65 antigenemia assay and multiplex PCR for HCMV were performed as described in sections 3.3.3 and 5.3.1 respectively. The 70 peripheral blood specimens were classified arbitrarily into four groups based on the results of pp65 antigenemia assay. Group 1 consisted of the patients who had a low pp65 antigenemia levels (0–10 cells/2×10^5 peripheral blood leucocytes (PBLs). Group 2 consisted of patients with moderate pp65 antigenemia levels (11–50 cells/2×10^5 PBLs). Group 3 consisted of patients with high pp65 antigenemia levels (51–100 cells/2×10^5 PBLs). Group 4 consisted of patients with very high pp65 antigenemia levels (>100 cells/2×10^5 PBLs).

5.3.3.4 Analysis of results

The results of the pp65 antigenemia assay and multiplex PCR applied simultaneously on 70 peripheral blood specimens obtained from 70 post-renal transplant recipients were compared. The various patterns of amplification obtained by application of the multiplex PCR was grouped into one of the four categories of pp65 antigenemia values. Mann–Whitney test was used for the statistical analysis of the results of multiplex PCR with pp65 antigenemia assay. P values < 0.05 were considered significant.

5.3.4 Standardization of Taqman probe based Real-time PCR assay for quantitation of HCMV

The sequences of the PCR primers and that of the probe used to quantify HCMV were selected from the mtr II region of HCMV. The sequences of the forward (CMV PKSF3) and reverse primers (CMV-PKR 11) were

5’ - TTACGCGACCAGATTGCAAGA - 3’ and 5’ - TACCTACGTGACCTACCAACG - 3’, respectively. The Taqman probe used was 5’- (6FAM) - CTCCGCGTCACCTTTCATCGAGTAAA - (TAMRA)- 3’. PCR was performed with 25µl of HCMV primer probe mixture, containing each of the primers at a concentration of 30 pmol, 0.006 nmol Taqman probe, and 20µl of DNA in a total volume of 50µl. The PCR product was detected as an increase in fluorescence with the Rotor-Gene 3000 (Corbett Research, Australia). A plasmid containing the target sequence of 74bp was used as standard. A standard graph of the cycle threshold (C_T) values obtained from serial dilutions (10–10^5 copies) of the plasmid was constructed for HCMV. The C_T values from unknown samples were plotted on the standard curve, and the number of HCMV genome copies/ml was calculated with Rotor-Gene 3000 Sequence detector (Corbett Research, Australia).

5.3.4.1 Sensitivity, Linearity and Reproducibility of Real-time PCR

The sensitivity and linearity of the Real-time PCR was determined using the plasmid standards (1 to 10^5 corresponding to 2.5 x 10^2 to 2.5 x 10^7 copies) in a single run. The intra-assay (within run) and inter-assay (between runs) reproducibility were evaluated using triplicates of plasmid dilutions (10^1, 10^3 and 10^5) corresponding to an input of 2.5×10^3, 2.5×10^5 and 2.5×10^7 copies/ml per reaction in the same and four independent runs, respectively.

5.3.5 Application of Real-time PCR on 50 peripheral blood controls from healthy blood donors

The Taqman-probe based real time PCR was applied on the DNA extracted from 50 peripheral blood controls from healthy blood donors. The pp65 antigenemia

assay, qualitative PCR for mtrII, multiplex PCR as described in sections 3.3.3. 4.3.4.3 and 5.3.1 respectively were also applied on these clinical specimens.

5.3.6 Comparison of the results obtained with Taqman probe-based Real time PCR assay and multiplex PCR on different clinical specimens obtained from immunocompromised patients

5.3.6.1 Study design: Prospective study

5.3.6.2 Clinical specimens

Thirty one clinical specimens (peripheral blood – 23, urine – 3, nasopharyngeal aspirates – 3, bronchoalveolar lavage – 1, aqueous humor – 1) from 29 immunocompromised patients belonging to post-renal transplant recipient, congenitally infected infants or HIV infected individuals were included in the study. The specimens were received at L & T Microbiology Research centre during the period April 2006 to June 2006.

5.3.6.3 Multiplex PCR and Taqman probe-based Real time PCR

The multiplex PCR and Taqman probe-based Real time PCR were carried out as described in the sections 5.3.1 and 5.3.4 respectively.

5.3.6.4 Analysis of the results

The results of the multiplex PCR applied on 31 clinical specimens obtained from 29 immunocompromised patients were compared with those of the real-time PCR independently to derive the cut-off value for the different amplification patterns of the multiplex PCR. Mann–Whitney test was used for the statistical analysis of the

results of multiplex PCR with real-time PCR. The results were expressed as medians, and P values <0.05 were considered significant.

5.3.7 Correlation of the results obtained with pp65 antigenemia assay and Taqman probe-based Real time PCR assay

5.3.7.1 Study design: Prospective study

5.3.7.2 Clinical specimens

Twenty three peripheral blood specimens from 22 post-renal transplant recipients were included in the study. The specimens were received at L & T Microbiology Research centre during the period April 2006 to June 2006.

5.3.7.3 pp65 antigenemia assay and Taqman probe-based real time PCR

pp65 antigenemia assay and the real-time PCR were carried out as described in the sections 3.3.3 and 5.3.4 respectively.

5.3.7.4 Statistical analysis

Spearman rank order correlation was used to correlate antigenemia levels and viral loads determined by Real-time PCR. P values < 0.05 were considered significant.

5.4 RESULTS

5.4.1 Sensitivity and specificity of multiplex PCR

The multiplex PCR was specific for HCMV genome. The multiplex PCR was sensitive enough to detect 20fg of HCMV DNA.

5.4.2 Application of multiplex PCR on 25 retrospective clinical specimens and 25 controls

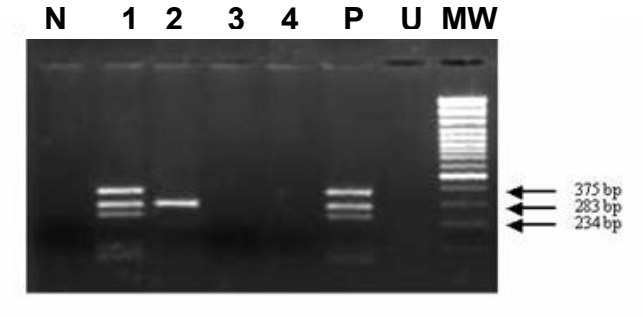
The multiplex PCR generated one of the four patterns when applied on retrospective peripheral blood specimens positive for mtr II region of HCMV. Pattern I generated all the three amplicons in the first round (n = 3); Pattern II generated a single amplicon in the first round (n = 2); Pattern III generated two amplicons in the nested round (n = 14) while Pattern IV generated a single amplicon in the nested round (n = 6). **Figure 4a** and **4b** shows the patterns generated by the multiplex PCR. All the peripheral blood leucocytes obtained from the controls (seropositive healthy donors) remained negative by multiplex PCR.

5.4.3. Results of comparison of the pp65 antigenemia levels and multiplex PCR applied on the 70 peripheral blood samples from 70 post-renal transplant recipients

Of the 70 peripheral blood specimens obtained from 70 post-renal transplant recipients, 60 were positive for pp65 antigenemia assay while an additional 4 were positive by the multiplex PCR. The comparative data showing the patterns of multiplex PCR amplification observed with that of the pp65 antigenemia levels in the patients is shown in **Table 6**. The proportion of the results belonging to the different patterns of the multiplex PCR varied in relation to the pp65 antigenemia values. With the pp65 antigenemia levels 0–10, 21/28 (75%) belonged to Pattern IV, between 11 and 50, 11/16 (68.75 %) belonged to Pattern III, between 51 and 100, 10/14 (71.43%) showed Pattern II and >100, 9/12 (75%) showed Pattern I. As shown in **Figure 5** the pp65 antigenemia levels in the specimens from which all the

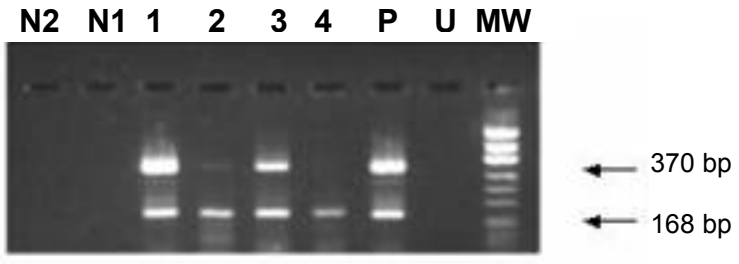
Figure 4: Agarose gel electrophoretogram showing the patterns obtained with the multiplex PCR applied on four different peripheral blood specimens

(a)



(a) **First round amplified products.** Lanes: N – Negative control; 1 – Positive for all three (mtr II, UL-83, gO) regions (Pattern I); 2 – Positive for a single region (UL-83) (Pattern II) ; 3 and 4 – Negative for all three regions; P – Positive control (CMV AD-169 strain), U – Unloaded well; MW – 100 bp ladder.

(b)



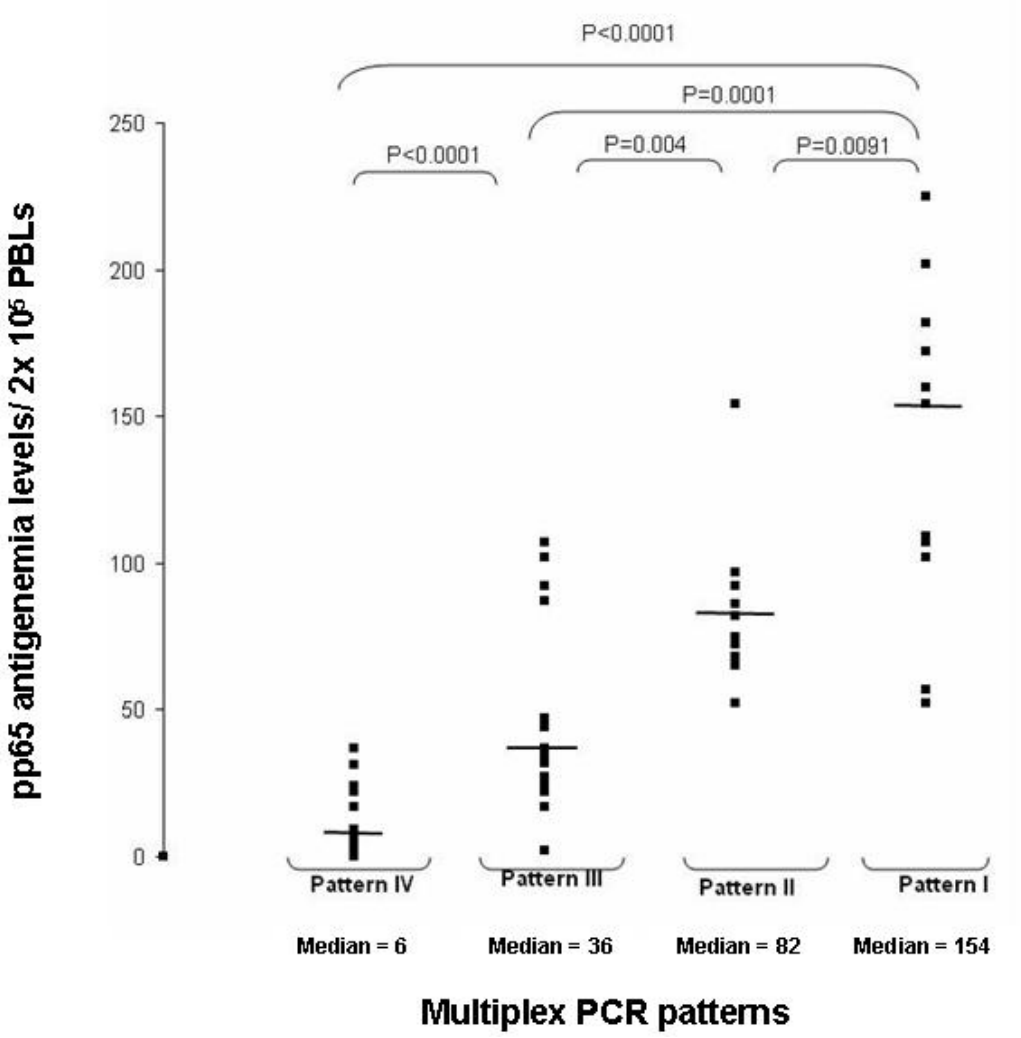
(b) **Second round amplified products.** Lanes: N2- Negative control (II round); N1 – Negative control (I round); 1 – Positive for mtr II and gO in II round; 2 – Positive for mtr II and gO in II round ; 3 – Positive for mtr II and gO regions in II round only (Pattern III); 4- Positive for mtr II region in II round only (Pattern IV); P – Positive control (CMV AD-169 strain); U – Unloaded well; MW –*Hinf I* digest of ϕX – 174 DNA

Table 6: Results of the comparison of pp65 antigenemia levels with the amplification patterns of multiplex PCR applied on 70 peripheral blood specimens obtained from 70 post-renal transplant recipients

pp65 antigenemia range	No. of specimens	Characteristic pattern of amplification in multiplex PCR			
		Pattern I	*Pattern II*	*Pattern III*	*Pattern IV*
0 to 10 cells	28*	-	-	01	21
11-50 cells	16	-	-	11	05
51- 100 cells	14	02	10	02	-
> 100 cells	12	09	01	02	-

* six peripheral blood specimens were negative for both pp65 antigenemia assay and multiplex PCR.

Figure 5: **Comparison of different patterns of multiplex PCR with pp65 antigenemia levels/2×10^5 PBLs in the 70 peripheral blood specimens**



The amplification pattern I in multiplex PCR where in all three regions were amplified showed significantly higher pp65 antigenemia levels than patterns IV and III where in one or two regions were positive ($P\leq0.0001$, Mann–Whitney test). Bars show the median pp65 antigenemia level for each amplification pattern.

three regions were amplified were significantly different from those from which one or two regions were amplified ($P \leq 0.0001$, Mann–Whitney test). There was a statistically significant difference in the median pp65 antigenemia levels observed with different amplification patterns of multiplex PCR.

5.4.4 Sensitivity, Linearity and Reproducibility of Real-time PCR

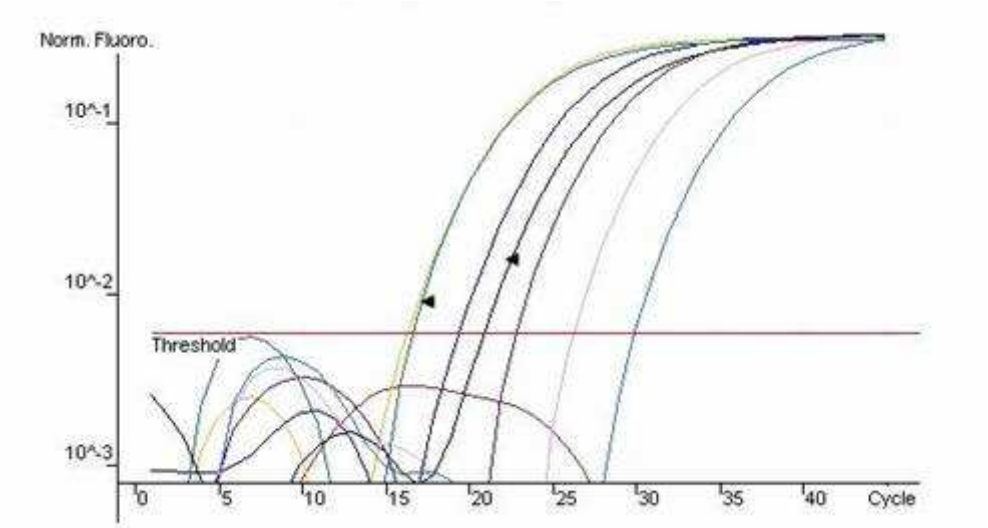
A single copy of the plasmid corresponding to 250 copies/ml of clinical specimen was detected regularly per reaction. The relationship between the C_T and $\log_{10}$ plasmid copy numbers was linear over the range of 10^1–10^5 copies corresponding to 2.5×10^3 to 2.5×10^7 copies/ml (**Figure 6**). The intra-assay variability and inter-assay variability was expressed as coefficient of variation (CV, calculated as the standard deviation divided by the mean). The percentage of CV obtained for 10^1, 10^3, 10^5 copies (corresponding to 2.5×10^3, 2.5×10^5 and 2.5×10^7 copies/ml) of plasmid in intra-assay variation testing were 6.7, 7.5 and 1.3%, respectively, and in inter-assay variation testing, the CV (%) were 8.7, 1.1 and 2.5%, respectively. **Table 7** shows the results of the intra-assay and inter-assay variability.

5.4.5 Results of the application of Real-time PCR on 50 peripheral blood controls from healthy blood donors

Of the 50 peripheral blood controls, 8 (16%) samples gave positive amplification signal. The viral load ascertained by Taqman probe based Real-time PCR for the eight samples were in the range of 3 to 836 copies/ml. Median viral load was 26.5 copies/ml. The pp65 antigenemia assay, qualitative PCR and multiplex PCR were negative in all the 50 controls.

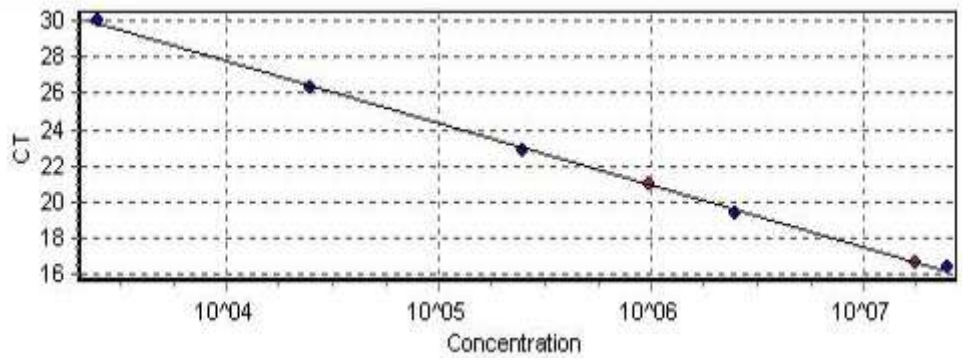
Figure 6: Amplification plot and Standard curve obtained with application of Taqman probe based Real time PCR

(a)



(a) **Amplification plot of five standards and two specimens for CMV real-time PCR.** Specimens are marked with arrow heads (◄). Standard DNA corresponding to 2.5 x 10^7 to 2.5x 10^3 were amplified for 45 cycles.

(b)



(b) **Standard curve for CMV real-time PCR.** C_T values were plotted against various numbers of copies of standard DNA. The blue spots indicate the standards and the red spots indicate clinical specimens. The correlation coefficient was 0.999.

Table 7: Results of intra and inter assay variability of Taqman probe based Real time PCR

(a) Intra-assay variability (within run)

Plasmid Copies*	Copies/ml obtained (Replicates)			Standard Deviation (SD)	Mean	Coefficient of variation (CV)	CV (%)
	I	II	III				
10^1	2550	2627	2895	181.0976	2690.667	0.0673	6.7%
10^3	25570	25720	29139	2018.656	26809.67	0.0753	7.5%
10^5	25073050	25203210	25692710	326733.9	25322990	0.0129	1.3%

* plasmid dilutions 10^1, 10^3 and 10^5corresponds to an input of 2.5×10^3, 2.5×10^5 and 2.5×10^7 copies/ml per reaction

(b) Inter-assay variability (between runs)

Plasmid Copies*	Copies/ml obtained (Runs)				Standard Deviation (SD)	Mean	Coefficient of variation (CV)	CV (%)
	I	II	III	IV				
10^1	2550	2379	2920	2573	226.8252	2605.5	0.0870	8.7%
10^3	25570	25555	24975	25250	283.093	25337.5	0.01117	1.1%
10^5	25073050	24397650	24087943	25429967	613502	24747153	0.0247	2.5%

* plasmid dilutions 10^1, 10^3 and 10^5corresponds to an input of 2.5×10^3, 2.5×10^5 and 2.5×10^7 copies/ml per reaction

5.4.6 Results of the quantitative measure of viral DNA in clinical specimens by real-time PCR and calculation of cut-off value for different patterns observed with the multiplex PCR

Table 8 shows results obtained with the different patterns of multiplex PCR and the median number of viral copies/ml and median $\log_{10}$ copies/ml as determined by the real time PCR applied on 31 clinical specimens obtained from 29 immunocompromised patients. The median $\log_{10}$ number of viral copies/ml differed significantly between each pattern ($P < 0.01$, Mann–Whitney test) (**Figure 7**).

5.4.7. Correlation of the pp65 antigenemia assay and real-time PCR applied to 23 peripheral blood specimens obtained from 22 immunocompromised patients

Table 9 shows the detailed results of the pp65 antigenemia assay and real-time PCR applied on 23 peripheral blood specimens obtained from 22 post renal transplant recipients. **Figure 8** shows a moderate positive correlation (Spearman rank order correlation coefficient (r_s) = 0. 7778; $P < 0.001$) between the increase in the pp65 antigenemia levels with that of the viral copy numbers/ml.

5.5 DISCUSSION

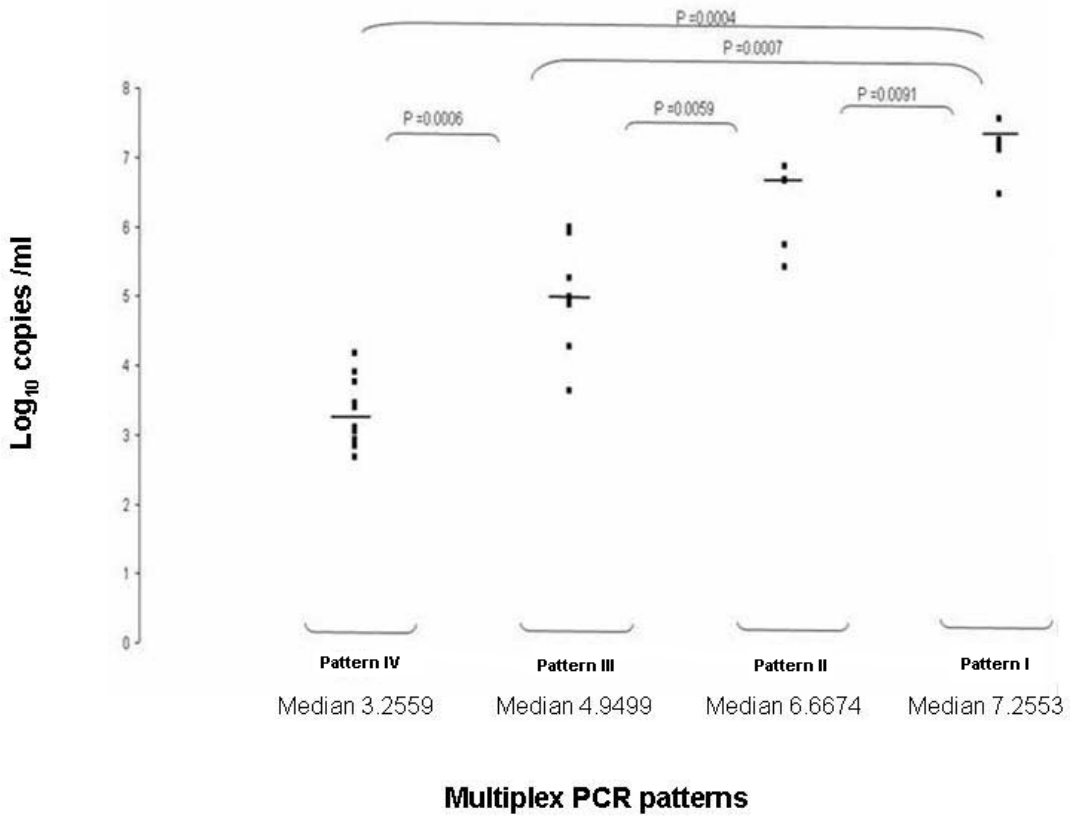
Quantitation of HCMV plays an important role in identifying patients at high risk of developing disease and also for assessing the response to antiviral treatment. Post-organ transplant recipients and HIV-infected individuals are monitored routinely for HCMV load, as increasing levels of systemic HCMV loads precede end-organ disease in majority of these patients. Several quantitative competitive

Table 8: Results of the comparison of the amplification patterns of multiplex PCR and viral load obtained with Real time PCR on 31 clinical specimens from 29 immunocompromised patients

Characteristic pattern of amplification in multiplex PCR	No. of specimens	Median Copy numbers/ml (median $\log_{10}$ copy numbers/ml) by Real-time PCR
Pattern I	7	1.8×10^7 (7.2553)
Pattern II	6	4.65×10^6 (6.6674)
Pattern III	8	8.95×10^4 (4.9499)
Pattern IV	10	1.9×10^3 (3.2559)

The median viral copies/ml present in the samples positive for all three targets (Pattern I and II) were significantly higher than those which were positive for one or two regions (Pattern III and IV)

Figure 7: **Comparison of different patterns of multiplex PCR with HCMV DNA copy numbers obtained by performing real-time PCR on 31 clinical specimens**



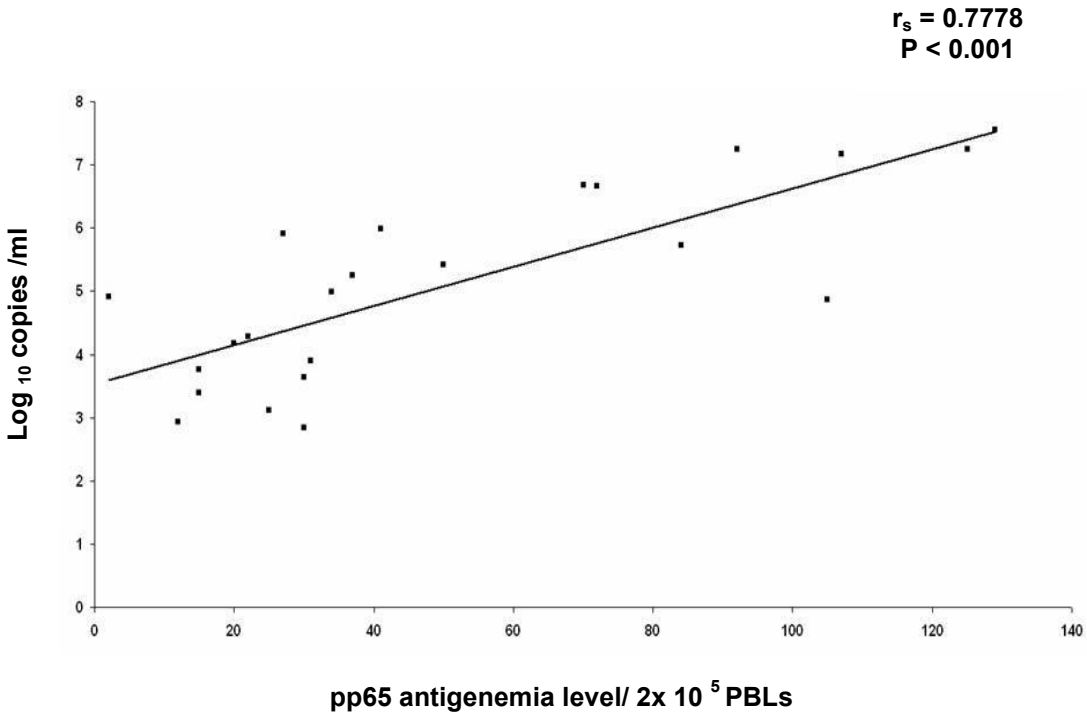
The amplification patterns I and II in multiplex PCR where in all three regions were amplified showed significantly higher HCMV DNA copy numbers than patterns IV and III where in one or two regions were positive ($P < 0.01$, Mann–Whitney test). Bars show the median HCMV DNA copy number for each amplification pattern.

Table 9: Results of pp65 antigenemia assay, and Real-time PCR performed on 23 peripheral blood specimens from 22 post renal transplant recipients

Sample no.	pp65 antigenemia level / 2x 10^5 PBLs	Real time PCR (log_{10} copies/ml)
1	129	7.5563*
2	107	7.1761
3	92	7.2553*
4	72	6.6628
5	34	4.9912
6	41	5.9912
7	37	5.2553
8	15	3.3979
9	20	4.1761
10	27	5.9138
11	12	2.9395
12	2	4.9085
13	84	5.7324
14	31	3.8976
15	50	5.4150
16	30	3.6335
17	125	7.2553
18	25	3.1139
19	70	6.6721
20	105	4.8751
21	30	2.8389
22	15	3.7559
23	22	4.2788

* Peripheral blood sample collected from same patient at an interval of one week.

Figure 8: **Correlation of pp65 antigenemia levels in PBLs with HCMV DNA copy numbers on the basis of the results of 23 clinical specimens positive by both antigenemia assay and real-time PCR**



The HCMV DNA $\log_{10}$ copies/ml was plotted against the number of pp65-positive cells detected by the antigenemia assay. The correlation between the HCMV DNA copy number and the pp65 antigenemia levels was examined by Spearman rank test and was found to be significant, with a correlation coefficient (r_s) = 0. 7778 ($P < 0.001$).

methods are available for quantitation by PCR to accurately and reproducibly determine the systemic and site-specific HCMV load, but they are technically difficult to carry out routinely in clinical laboratories, time consuming and require a good amount of expertise. In contrast, semi-quantitative method as described in the study is applicable more easily to laboratories in which PCR is regularly carried out.

The pp65 antigenemia assay has shown false-negative results due to a low-level expression of the pp65 antigen in white blood cells in a small number of patients with definite HCMV disease (Piiparinen et al., 2002; Razonable et al., 2002b; Seropian et al., 1998). The labour-intensive nature of the procedure, the requirement for immediate sample processing, and the subjective interpretation of slides place limitations on this assay as a routine diagnostic procedure (Boeckh and Boivin, 1998; Razonable et al., 2002b; Landry et al., 1995). In India, it is difficult to procure the monoclonals as they are not indigenous and needs to be imported which also make the assay a little expensive. PCR-based qualitative detection of HCMV DNA in blood samples provides 100% sensitivity for the diagnosis of HCMV disease, but the specificity is generally 50% or less (Nitsche et al., 1999; Shapiro et al., 2000; Weber et al., 1994). The NucliSens assay, which detects the presence of HCMV late mRNA pp67, has a lower sensitivity compared to DNA amplification and antigenemia assays for detection of HCMV (Razonable et al., 2002b). The commercial COBAS Amplicor CMV Monitor assay has been shown to have certain disadvantages, such as cost, narrow dynamic range and the time-consuming nature of the procedure that limits its use as a surveillance tool in high-risk populations (Razonable et al., 2002b; Yun et al., 2000). In India, Rao et al., 2000 successfully quantitated HCMV genome in peripheral blood specimens of post-renal transplant

recipients in a longitudinal study (Rao et al., 2000). However, the method of quantitation employed was limiting dilution which involved the use of several replicates of PCR reaction for every specimen. This makes the procedure laborious.

There have been many studies describing the role of real-time PCR for monitoring HCMV infections in solid organ transplant recipients. The innate advantages of the technique include reliable quantitation of HCMV DNA for moderate and high viral load with precise information on viral load kinetics for evaluation of disease progression and antiviral response, the rapidity in thermal cycling and simultaneous detection of amplified products during amplification yielding test results within 3 hours, a high inter- and intra-assay reproducibility and high-throughput without significant risk of cross-contaminations. (Piiparinen et al., 2004; Allice et al., 2006).

In this study the linearity of the real-time PCR was maintained between 10^1 and 10^5 copies of the plasmid corresponding to 2.5 x 10^3 to 2.5 x 10^7 copies / ml. This characteristic bestowed the technique with accurate quantitation of high HCMV viral load without the need for diluting the clinical specimens. The total technique needed not more than 2 hours to be performed including extraction of DNA. The high degree of reproducibility of the real-time PCR was reflected by the CVs of inter- and intra-assay variations. Hence, the real-time PCR was chosen to provide the cut-off value for the multiplex PCR.

Numerous studies have investigated the correlation of HCMV load and symptomatic HCMV disease in immunocompromised patients by the pp65 antigenemia assay and variety of quantitative PCR assays (Kalpoe et al., 2004;

Ikewaki et al., 2003; Mazulli et al., 1999). Although, there is a consensus between laboratories on the cut-off value for pp65 antigenemia in different patient populations, a comparison of quantitative PCR assay results has been elusive due to marked differences in PCR methods and reporting of results.

The sensitivity of the quantitative PCR may be dependent on the viral target gene. The most appropriate region for amplification has not been established, as the sequence diversity among the clinical isolates needs further characterization. The multiplex PCR was developed with three different regions of HCMV DNA, since detection of a single target has a greater probability of giving a false negative result as proven by previous studies (Distefano et al., 2004; Wirgart et al., 1998). This approach served as an alternative to taking up replicate assay for the same gene fragment. It was hypothesized that the average amount of the HCMV DNA in those samples from which a single gene was amplified was significantly less than in those from which two or more regions were amplified. The hypothesis was made on the basis of observation in the previous study comparing the three different PCRs for mtr II, UL 83 and gO with pp65 antigenemia, that all the three regions became positive whenever the antigenemia value was high. The quantitative element of the multiplex PCR lies in the combination of the three regions with different sensitivities. The region of UL-83 was deliberately included as uniplex PCR for providing an intermediate sensitivity between the first round and second round of the other two nested PCRs. In the present study, the results of the pp65 antigenemia assay and the real time PCR correlated well with the multiplex PCR confirming the hypothesis. In agreement with other published data the pp65 antigenemia levels showed moderate correlations with the real-time PCR viral loads (Gault et al., 2001;

Pang et al., 2003; Piiparinen et al., 2004; Allice et al., 2006). This was an absolute necessity to validate the results of the multiplex PCR as it was compared with both the tests individually. Nevertheless, inconsistencies between the results of antigenemia and Real time PCR was observed in at least two samples. Sample 12 and sample 20 in **Table 9** showed a lower antigenemia with high viral DNA load and higher antigenemia with lower viral DNA load respectively. Findings similar to the former have been documented in several studies (Ikewaki et al., 2003; Gault et al., 2001; Piiparinen et al., 2004). The clinical significance of such a finding is not yet established though such condition is observed more in patients at early stage of CMV disease (Ikewaki et al., 2003). However, the higher antigenemia with lower viral load as in sample 20 **(Table 9)** has not been observed in many studies, but intermittent rise in antigenemia levels during the first three weeks of antiviral treatment have been suggested owing to the phagocytosis of the degrading CMV matrix protein pp65 from lysed, infected leukocytes or from circulating CMV-infected endothelial cells by PMNL (Boeckh and Boivin, 1998). Concurrent measure of viral load in such a condition has not been reported. Hence, it is assumed in the absence of any solid clinical data that the phase at which the clinical specimen is analyzed, may account for such discrepancies.

There was a statistically significant difference in the quantity of the viral DNA between the array of specimens, which was positive for all the three targets, and those that were positive for either one or two regions as analyzed by the real-time PCR. The real-time PCR was based on the mtr II region of HCMV as the previous study showed that this region was most conserved and provided 100% sensitivity for detection of HCMV DNA in immunocompromised patients.

The real – time PCR detected HCMV DNA circulating in healthy controls while the pp65 antigenemia and multiplex PCR were negative in this population. The median load (26.5 copies/ml) however was comparatively less than that observed in immunocompromised patient group. This is in agreement with previous studies (Nitsche et al., 2000; Machida et al., 2000).

The crude way of assessing the viral load by multiplex PCR may be of very little significance when analyzing single samples but may be useful when serial samples are used in conjunction with clinical data. The quantity of HCMV DNA in immunocompromised individuals who develop active infection can approximately be predicted by cohort of samples collected at regular time intervals. Such studies can aid in differentiating the individuals positive for HCMV genome who are at a higher risk of developing an end-organ disease and those who would have a milder form and to know the response of an individual to antiviral therapy. The concept of using the multiplex PCR both for increased sensitivity and for initial assessment of viral load may have a significant effect on the clinical application of PCR for the diagnosis of HCMV.

6. ANALYSIS OF gB GENOTYPES OF HCMV IN DIFFERENT CLINICAL SPECIMENS OBTAINED FROM IMMUNOCOMPROMISED PATIENTS

6.1 BACKGROUND

Glycoprotein B (gB) of the human cytomegalovirus (HCMV) plays an important role in virus infectivity. This protein is a major component of the virion envelope and is transported to the plasma membrane of infected cells. It has been shown that monoclonal antibodies to gB inhibit virus penetration into cells and block transmission of infectious virus from cell to cell (Navarro et al., 1993). Furthermore, anti-gB antibodies constitute up to 70% of the total serum neutralizing activity found in persons with past HCMV infection, indicating that gB is a major target for neutralizing antibodies (Britt et al., 1990; Utz et al., 1989). Common and strain-specific epitopes have been described (Meyer et al., 1992; Basgoz et al., 1992). In addition, it has been shown that gB protein induces cytotoxic T cell responses (Riddell et al., 1991; Liu et al., 1993).

Human Cytomegalovirus (HCMV) strains display nucleotide polymorphisms in the gene encoding glycoprotein B. There are four common genotypes (types 1–4) determined based on the region around the proteolytic cleavage site (Chou, 1990a; Chou and Dennison, 1991). Additional genotypes have also been described (Shepp et al., 1998). In addition, variability have also been identified in regions coding for the amino terminal and carboxy terminal (Meyer-Konig et al., 1998c). Presence of mixed gB genotypes in immunocompromised patients is associated with a higher viral load, higher prevalence of HCMV disease and concomitant infection with other

herpesviruses such as Epstein-Barr virus (EBV) (Coaquette et al., 2004).Genotyping of the gB gene of HCMV is carried out usually by amplification of the proteolytic cleavage site (gp UL 55) of the glycoprotein B gene followed by restriction fragment length polymorphism of the amplified product. Most of the genotyping studies have been carried on isolates of HCMV, using a uniplex PCR for the amplification of the gB gene followed by RFLP. There are studies emerging on the genotyping of HCMV on direct clinical specimens (Chou, 1990a; Barbi et al., 2001; Tarrago et al., 2003; Carraro and Granato, 2003). In India, only one study has been carried out for genotyping of gB, which utilized application of the uniplex PCR on direct clinical specimens followed by RFLP of the amplified product (Madhavan and Priya, 2002b). The study suggested that the uniplex PCR was less sensitive and 50% of the clinical specimens known to be positive for HCMV could not be genotyped. The other methods employed for genotyping of gB gene include single-stranded conformation polymorphism, heteroduplex mobility analysis, direct DNA sequencing, multiplex PCR and DNA Enzyme Immuno assay.

6.2 OBJECTIVES

6.2.1 To standardize PCR-based RFLP for genotyping of gB gene of HCMV.

6.2.2 To standardize multiplex PCR for genotyping of gB gene of HCMV.

6.2.3 To evaluate the efficiency of PCR-based RFLP and multiplex nested PCR by simultaneously applying them on different clinical specimens for gB genotyping of HCMV strains.

6.2.4 To study the distribution of gB genotypes in different clinical specimens by multiplex PCR.

6.3 MATERIALS AND METHODS

6.3.1 Standardization of nested PCR- based RFLP for genotyping of gB gene of HCMV

6.3.1.1 Nested PCR for gB gene

A nested PCR was performed as described by Carraro and Granato, 2003. DNA from HCMV-AD169 strain was used for standardization of the PCR. A 50µl reaction mixture was prepared with 100µM of each dNTPs, 10 picomoles of primers (gB1246 5'CGAAACGTGTCCGTCTT3' and gB1724 5'GAGTAGCAGCGTCCTGGCGA3') 1 x buffer (10mM Tris–HCl 127(pH 8.3), 50mMKCl, 0.01% gelatin, 2.5mMMg$Cl_{2)}$, 1 U of Taq DNA polymerase. Ten µl of DNA was used as the template. The thermal profile consisted of an initial denaturation at 95^{o}C for 3 min followed by 30 cycles of denaturation at 94^{o}C for 45 seconds, annealing at 55^{o}C for 30 seconds, and extension at 60 sec at 72^{o}C for 60 seconds in a thermal cycler. The second round of amplifications was carried out with conditions similar to the first round, with internal primers (gB1319 5'TGGAACTGGAACGTTTGGC3' and gB1604 5'GAAACGCGCGGCAATCGG3'). Two µl of the first round amplicon was used as the template for the second round of amplification. Amplicons were analyzed by electrophoresis on a 2% agarose gel incorporated with ethidium bromide (Appendix II). Sensitivity and specificity of the PCR was determined as described in Appendix II.

6.3.1.2 Restriction fragment length polymorphism (PCR-RFLP) of gB products

Ten µl of unpurified second round amplicons were subjected to digestion with *HinfI* and *RsaI* (Fermentas Inc., USA) in two separate reactions of 25 µl volume. The amount of enzyme, buffer composition and temperature for incubation are provided in Appendix II. A control reaction containing the amplicon and buffer was included during each digestion. Digested fragments were analyzed after electrophoresis on 4% agarose gels incorporated with ethidium bromide (Appendix II).

6.3.1.3 Application of nested PCR based RFLP on clinical specimens

The standardized nested PCR based RFLP was performed on DNA extracted from 46 clinical specimens from 37 patients with HCMV infections proven by nested PCR for mtrII region of HCMV. The 46 clinical specimens included 25 blood, 18 urine, 1 bronchoalveolar lavage and 2 nasopharyngeal aspirate.

6.3.2 Conversion of nested PCR-based RFLP to seminested PCR-based RFLP based on the results obtained with nested PCR- based RFLP of gB gene

(section 6.4.1.3).

6.3.2.1 Analysis of lack of amplification signal in 18 clinical specimens by multiple sequence alignment

The prototype sequences of the gB subtypes and HCMV AD169 (GenBank accession number: X17403) were aligned with the primer sequences used for the amplification to determine the reason for the failure of the amplification signals and total absence of gB3 genotype that existed in Chennai during 2002 (Madhavan and

Priya., 2002b) (section 6.4.1.3). The gB subtypes gB1 (GenBank accession numbers: M60927 and M60929); gB2 (GenBank accession numbers: M60931 and M60932); gB3 (GenBank accession numbers: M60933 and M60934); gB4 (GenBank accession numbers: M60924 and M60926) were aligned with individual primer sequences using the Clustal X program.

6.3.2.2 Protocol for semi-nested PCR-based RFLP for gB gene

The nested PCR was converted to semi-nested PCR by excluding the outer forward primer (gB 1246). The first round of amplification was carried out with 10 picomoles of the forward and reverse primers gB 1319 and gB 1724 maintaining the other reaction mixture components as for the nested PCR for gB gene (section 6.3.1). The thermal profile consisted 30 cycles of 45 seconds at 94^{o}C, 45 seconds at 55^{o}C and 60 seconds at 72^{o}C. The second round of amplification was carried out with 10 picomoles of gB1319 and gB 1604 primers with reaction mixture similar to the first round. Two µl of the first round amplicon was used as the template for the second round. The reaction mixture was subjected to 20 cycles of amplification with thermal profile of first round. Sensitivity and specificity of the PCR was determined as described in Appendix II. The RFLP protocol was not modified (section 6.3.1.2).

6.3.2.3 Application of seminested PCR based RFLP for genotyping of gB gene of HCMV on the 46 clinical specimens

The semi-nested PCR-based RFLP for gB gene of HCMV was applied retrospectively on the 46 clinical specimens from 37 patients. The details of the clinical specimen are provided in section 6.3.1.3.

6.3.3 Standardization of multiplex nested PCR for genotyping of gB gene of HCMV

A multiplex nested PCR was standardized for genotyping of gB gene of HCMV using DNA from HCMV AD-169 strain. A common pair of primers was used to amplify consensus of all the genotypes in the first round followed by a genotype specific amplification in the second round (Tarrago et al., 2003). The primer sequences and the size of the amplicon generated by each of the genotypes are provided in **Table 10**. For amplification, a 50µl reaction mixture was prepared with 100µM of each dNTPs, 10 picomoles each of CMVQ1+ and CMVQ1- primers, 1 x buffer (10mM Tris–HCl 127(pH 8.3), 50mMKCl, 0.01% gelatin, 2.5mM $MgCl_{2)}$, 1U of Taq DNA polymerase. Five µl of DNA was used as the template. The thermal profile consisted of an initial denaturation of 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds and final extension step at 72°C for 5 minutes. The second round of amplifications was carried out with reaction mixture containing 200µM of each dNTPs, 10 picomoles of each inner primer (CMV GT1+, CMV GT2+, CMV GT3+, CMV GT4+, CMV GT5+ and CMV Q2-), 1x buffer (10mM Tris–HCl (pH 8.3), 50mMKCl, 0.01% gelatin, 2.5mM $MgCl_{2)}$, 1U of Taq DNA polymerase in a final volume of 50µl. One µl of the first round amplicon was used as the template for the second round. The second round was carried out under conditions identical to those used in the first round except the annealing temperature was 58°C instead of 60°C. Amplicons were analyzed by electrophoresis on a 3% agarose gel incorporated with ethidium bromide (Appendix II). Sensitivity and specificity of the PCR was determined as described in Appendix II.

Table 10: Primer sequences of multiplex nested PCR for gB gene of HCMV

Primers	Primer sequences	Amplicon size
FIRST ROUND		
Forward CMV Q1+	5' TTT GGA GAA AAC GCC GAC3'	751 bp
Reverse CMV Q1-	5'CGC GCG GCA ATC GGT TTG TTG TA3'	
SECOND ROUND		
Forward primers		
CMV GT1+ (gB1)	5' ATG ACC GCC ACT TTC TTA TC 3'	420 bp
CMV GT2+ (gB2)	5' TTC CGA CTT TGGA AGA CCC AAC 3'	613 bp
CMV GT 3+ (gB3)	5'TAG CTC CGG TGT GAA CTC C 3'	190 bp
CMV GT 4+ (gB4)	5' ACC ATT CGT TCC GAA GCC GAG GAG TCA 3'	465 bp
CMV GT 5+ (gB5)	5' TAC CCT ATC GCT GGA GAA C 3'	139 bp
Common reverse primer CMV Q2-	5' GTT GAT CCA CAC ACC AGG C 3'	

The genotypes are specified within brackets ( ).

6.3.4 Evaluation of the efficiency of semi-nested PCR based RFLP and multiplex nested PCR by simultaneously applying them on different clinical specimens for gB genotyping of HCMV strains

6.3.4.1 Clinical specimens

Seventy clinical specimens [40 blood, 23 urine, 3 nasopharyngeal aspirates, 1 tracheal aspirate, 2 bronchoalveolar lavage and 1 gastric wash] from 51 patients were included in the study. Presence of HCMV was proven in these patients by nested PCR for mtr II region. Single samples were tested from 38 patients while two or more samples of either similar or different types of clinical specimens were tested from 13 patients.

6.3.4.2 Semi-nested PCR-based RFLP and multiplex PCR for gB gene of HCMV

The semi-nested PCR-based RFLP and multiplex PCR for gB gene were performed as described in sections 6.3.2.2 and 6.3.3 respectively. The methods were compared based on the results of genotyping, time taken and expenditure incurred for their performance.

6.3.5 Validation of mixed genotypes by DNA sequencing

Second round amplicons obtained by semi-nested PCR on three blood specimen that gave mixed genotypes each of gB1+gB3; gB1+gB2; gB1+gB2+gB3 by multiplex PCR were subjected to DNA sequencing. The DNA sequencing was performed as described in Appendix II.

6.3.6 A study on the distribution of gB genotypes in different clinical specimens from immunocompromised patients by multiplex PCR

6.3.6.1 Clinical specimens and patients

Two hundred and eighty four clinical specimens from 239 immunocompromised patients were included in the study. Only those clinical specimens which showed positivity for HCMV by nested PCR for mtrII were included. Two hundred single specimens (Peripheral blood – 156; Urine – 22; Respiratory fluids– 18; intraocular fluids - 4) were obtained from 200 patients (Post renal transplant (PRT) – 156; Infants – 28; HIV infected individuals (HIV) – 11 and Bone marrow transplant (BMT) – 5). Pairs of similar or different clinical specimens accounting to 72 clinical specimens were obtained from 36 patients (PRT – 23 (Pairs of blood and urine from 20 patients; a pair of blood samples, blood and gastric wash; and blood and respiratory fluid from one patient each); Infants – 3 (2 pairs of blood and urine and 1 pair of urine and respiratory fluid); HIV infected individuals– 9 (9 pairs of blood and intraocular fluid) and BMT – 1 (a pair of urine and respiratory fluid). Four samples of clinical specimens were obtained from 3 PRTs (Two pairs of blood and urine from two patients, one sample of blood and three samples of urine from one patient).

6.3.6.2 Application of multiplex PCR for gB gene

The multiplex PCR for gB gene was applied on the extracted DNA of clinical specimens as described in section 6.3.3.

6.3.6.3 Analysis of results

The distribution of the genotypes was analyzed for each patient population irrespective of the type of specimens. The results of the patients from whom single specimens were obtained and those patients whose multiple specimens showed concordant results were analyzed together. Those patients who had a discordant result with multiple clinical specimens were analyzed separately. The results were statistically analyzed by Chi-square test and was considered significant when P value <0.05.

6.4 RESULTS

6.4.1 Results obtained with nested PCR-based RFLP of gB gene of HCMV

6.4.1.1 Specificity and sensitivity of nested PCR for gB gene of HCMV

The first round yielded a product of 477 bp with HCMV AD-169. A 302 bp product was obtained in the second round. The nested PCR was specific for gB gene of HCMV and sensitive to detect 0.5 pg of HCMV DNA.

6.4.1.2 RFLP pattern of AD-169

The second round amplified products of AD-169 strain gave restriction fragments of 202bp and 100 bp with *Hinf I* enzyme and 239 and 63 bp with *Rsa I* enzyme.

6.4.1.3 Results of the application of nested PCR-based RFLP on 46 clinical specimens from 37 patients

Of the 46 clinical specimens tested by the nested PCR-based RFLP, the genotyping results were available for 28 clinical specimens from 24 patients. Of

these 18 were genotyped as gB1 and 10 as gB2. Eighteen other clinical specimens from 13 patients could not be genotyped due to lack of amplification signal. **Figure 9** shows amplified products obtained by application of nested PCR for gB gene of HCMV on clinical specimens.

6.4.2 Results of multiple sequence alignment of prototype sequences with gB primers

The outer forward primer showed mismatches at three sites with that of the gB3 prototype sequences (**Figure 10**). It was hypothesized that these mismatches were the reason for the lack of amplification signal in the 18 clinical samples.

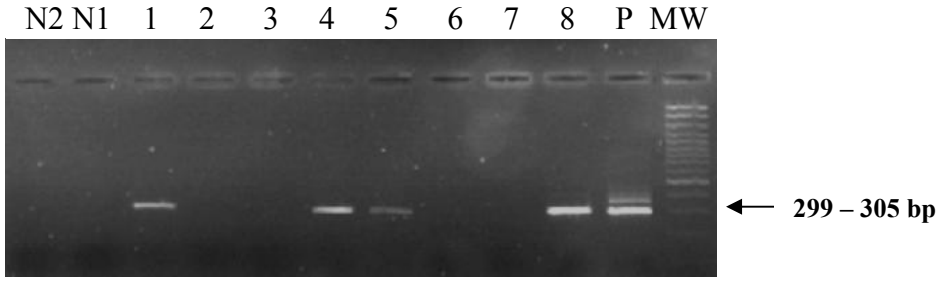
6.4.3 Specificity and sensitivity of the semi-nested PCR for gB gene

The first round yielded a product of 406bp with HCMV AD-169. A 302 bp product was obtained in the second round. The seminested PCR was specific for gB gene of HCMV and sensitive to detect 0.5 pg of HCMV DNA.

6.4.4. Results of the application of seminested PCR-based RFLP on 46 clinical specimens from 37 patients

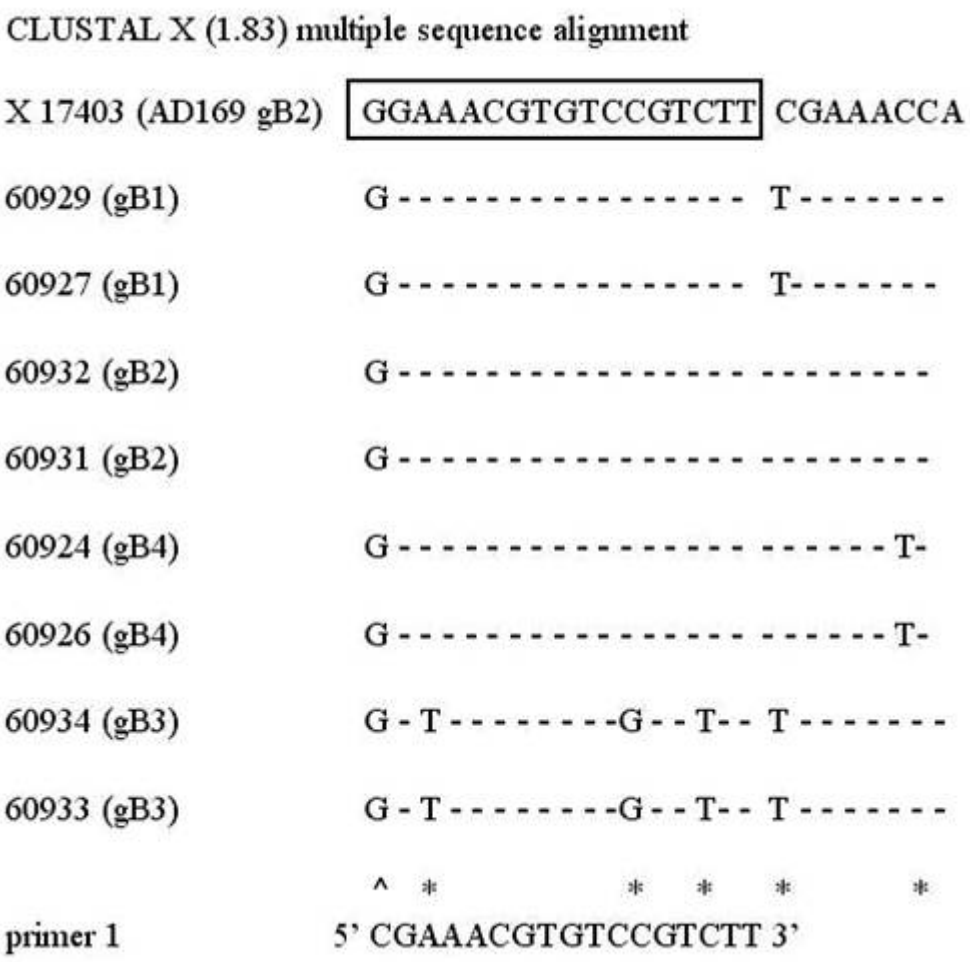
Of the 46 clinical specimens tested by the semi-nested PCR-based RFLP, the genotyping results were available for all the 46 clinical specimens from 37 patients. Of these 18 were genotyped as gB1 and 10 as gB2. Eighteen other clinical specimens from 13 patients which could not be genotyped by the nested PCR for gB gene was genotyped as gB3 by seminested PCR – based RFLP. gB4 genotype was not observed in the study. Similar genotypes were shed by patients in different samples. **Table 11a and 11b** shows the various RFLP fragments (expected pattern) generated by *Hinf I & Rsa I* in different CMV gB genotypes.

Figure 9: **Agarose gel electrophoretogram showing the amplified products of nested PCR for gB gene applied on clinical specimens**



Lanes : N2 – Negative control (II round) ; N1 - Negative control (I round); 1,4 - Blood positive for gB gene; 2, 3 - Blood negative for gB gene; 5, 8 - Urine positive for gB gene; 6,7 - Urine negative for gB gene; P - Positive control (AD 169); MW - 100 bp ladder

Figure 10 : Multiple sequence alignment of eight sequences of different prototype HCMV strains (gB1–gB4) with the HCMV AD 169 strain (GenBank accession number: X17403; gB2 subtype) and the forward outer primer (gB 1246)



Identities at nucleotide positions are shown by dashes. The region of the forward outer primer (gB 1246) is shown in the box. Asterisk (*) indicates mismatches with respect to the HCMV AD 169 strain and symbol (^) indicates a primer–target mismatch seen in all genotypes.

Table 11: Expected sizes of the RFLP fragments generated by (a) *Hinf I* and (b) *Rsa I* on the amplicons of gB gene of HCMV by semi-nested PCR based RFLP

(a) *Hinf I*

gB1	gB2	gB3	gB4
202bp 67 bp 36bp	202bp 100bp	202bp 97 bp	202bp 67 bp 36bp

(b) *Rsa I*

gB1	gB2	gB3	gB4
239bp 66 bp	239bp 63bp	195bp 63 bp 41 bp	195bp 66 bp 44 bp

6.4.5 Specificity and sensitivity of multiplex PCR for genotyping of gB gene of HCMV

The multiplex nested PCR generated a 751 bp product in the first round and 613 bp product corresponding to genotype gB2 for HCMV AD169 in the second round. The multiplex nested PCR for gB genotyping was specific for HCMV and sensitive enough to pick up 50fg of AD169 DNA.

6.4.6 Results of comparison of seminested PCR-based RFLP and multiplex PCR

6.4.6.1 Results of application of semi-nested PCR based RFLP and multiplex nested PCR for gB gene on 70 clinical specimens from 51 patients

Of the 70 clinical specimens included in the study (**Table 12**), all were genotyped by multiplex nested PCR while 5 clinical specimens could not be typed by PCR-based RFLP for gB gene due to lack of amplification signal. Of the 65 clinical specimens typed by both the methods, concordant results were obtained in 41 (15 - gB1,11- gB2 and 15 gB3). Of the 24 clinical specimens in which discordant results were obtained mixed infection with multiple genotypes were detected by multiplex nested PCR (gB 1 + gB3 - 21; gB1 + gB2 – 2 ; gB1 +gB2 + gB3 – 1) where as only single genotypes were identified by PCR- based RFLP (gB1 – 10; gB2 – 2; gB3 - 12). The genotypes of the 5 clinical specimens, which could not be typed by PCR-based RFLP, showed a distribution of gB1 in 4 and gB1+gB2 in one. gB4, gB5 genotypes and other mixed genotypes were not found in any clinical specimen by both the methods. **Figures 11 and 12** shows the results obtained by application of PCR-based RFLP and multiplex PCR for gB gene on clinical specimens respectively.

Table 12: Results of PCR- based RFLP and Multiplex nested PCR for gB genotyping of 70 clinical specimens obtained from 51 HCMV infected patients

Clinical Specimens	*Method Of Genotyping*								
	PCR - RFLP			**Multiplex Nested PCR**					
	gB1	**gB2**	**gB3**	***Single genotypes***			***Multiple genotypes***		
				gB1	**gB2**	**gB3**	**gB1+ gB 3**	**gB1+ gB2**	**gB1+gB2 + gB3**
Blood Leucocytes (40)	14	9	16	8*	8	9	13	1	1
Urine (23)	9	3	8	9**	2	5	5	2***	-
Nasopharyngeal aspirate (03)	-	-	2	1****	-	1	1	-	-
Broncho alveolar lavage (02)	-	1	1	-	1	-	1	-	-
Tracheal aspirate (01)	1	-	-	1	-	-	-	-	-
Gastric wash (01)	1	-	-	-	-	-	1	-	-
TOTAL (70) *****	25	13	27	19	11	15	21	3	1

* One of the blood specimen genotyped as gB1 by multiplex nested PCR could not be genotyped by PCR-based RFLP.

** Two urine specimens genotyped as gB1 by multiplex nested PCR could not be genotyped by PCR-based RFLP.

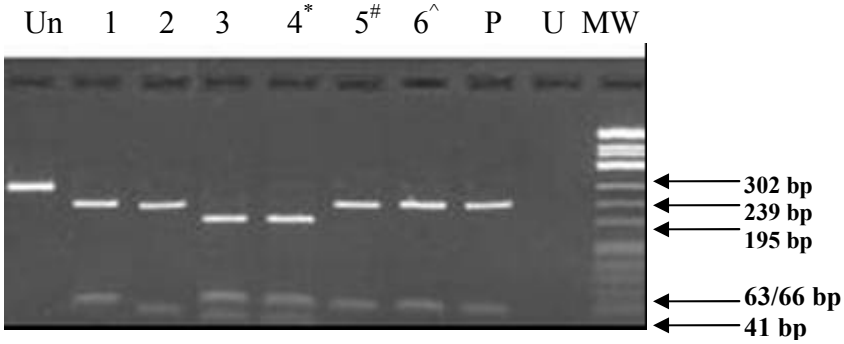
*** One of the urine specimen genotyped as mixed infection of gB1 and gB2 by multiplex nested PCR could not be genotyped by PCR-based RFLP.

**** One of the Nasopharyngeal aspirate genotyped as gB1 by multiplex nested PCR could not be genotyped by PCR-based RFLP.

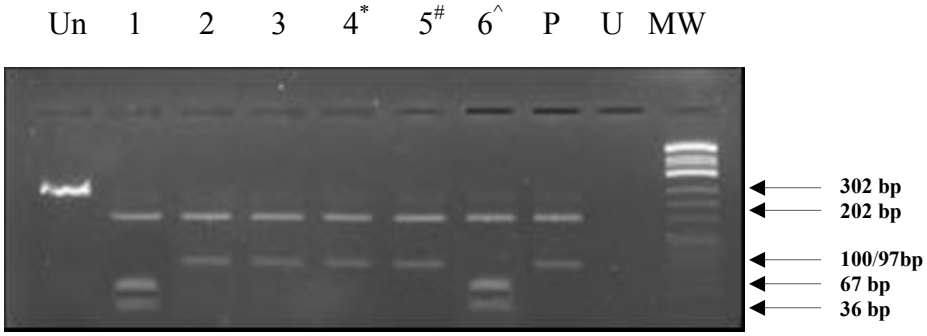
***** Five clinical specimens could not be genotyped by PCR-based RFLP.

Figure 11: **Agarose gel electrophoretogram showing the restriction fragments obtained by semi-nested PCR based RFLP of gB gene of HCMV**

(a)

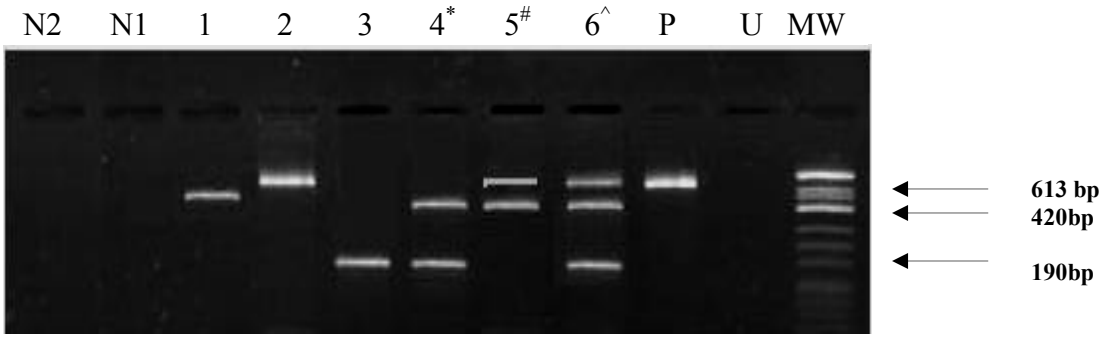


(b)



(a) ***Rsa I* enzyme (b) *Hinf I* enzyme.** Lanes :Un – Undigested product ; 1 – Blood CMV genotype gB1; 2 – Urine CMV genotype gB2; 3 – Nasopharyngeal aspirate CMV genotype gB3 ; 4* - Blood CMV genotype gB3 (determined as gB1 + gB3 by multiplex PCR); 5#- Blood CMV genotype gB2 (determined as gB1 + gB2 by multiplex PCR) ; 6^- Blood CMV genotype gB1 (determined as gB1 + gB2 +gB3 by multiplex PCR); PC – CMV AD-169 (genotype gB 2); U – Unloaded well ; MW – *Hinf I* digest of ϕX 174 DNA

Figure 12: Agarose gel electrophoretogram showing the results of genotyping of gB gene of HCMV by multiplex PCR



Lanes: N2- Negative control (II round); N1- Negative control (I round); 1 – Blood CMV genotype gB1; 2 – Urine CMV genotype gB2; 3 – Nasopharyngeal aspirate CMV genotype gB3; 4* - Blood CMV mixed genotype gB1 + gB3 (determined as gB3 by PCR-RFLP); 5#- Blood CMV mixed genotype gB1 + gB2 (determined as gB2 by PCR-RFLP); 6^- Blood CMV mixed genotype gB1+gB2+gB3 (determined as gB1 by PCR-RFLP) ; PC – CMV AD-169 (genotype gB 2); U – Unloaded well; MW – *Hinf I* digest of ϕX 174 DNA

6.4.6.2 Comparison of the time taken and expenditure incurred in performing genotyping by multiplex PCR and PCR based RFLP for gB gene of HCMV

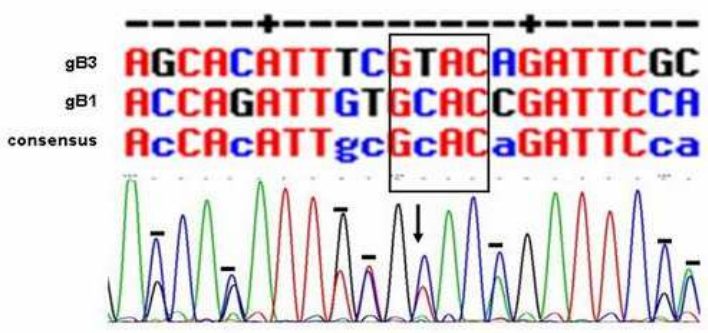
The time taken for performing multiplex PCR and semi-nested PCR-based RFLP were 5 hours and 10 hours respectively. The total cost of the reagents and other consumables incurred for performing the study of 70 samples along with respective controls by multiplex nested PCR was Rs.3400.00 as against Rs. 5990.00 for PCR-based RFLP (US$=About RS 42.0). The cost calculated did not include the expenditure incurred on extraction of DNA from clinical specimens, labour, establishment and maintenance costs. There was a 43.24 % decrease in the expenditure incurred when multiplex nested PCR was performed instead of PCR-based RFLP.

6.4.7 Results of the DNA sequencing of the three amplicons that showed mixed genotype by multiplex PCR for gB gene

Figures 13 a, b, c show the results of DNA sequencing of the amplicons obtained by semi-nested PCR on blood specimens that gave gB 1+3, gB 1+2, gB1+2+3 genotypes by multiplex nested PCR. The DNA sequences revealed mixed bases spanning gB gene. The mixed bases at and around the *Rsa I* recognition site in the gB gene of HCMV is depicted in **Figure 13a** (This differentiates gB1 from gB3 genotype). The sequence around the *Hinf I* recognition site lacked clarity owing to the frame shifts due to deletions before *Hinf I* recognition site in the gB sequence. Hence, gB1 and gB2 in case of amplicons with genotypes gB1+gB2 and gB1+2+3 could not be differentiated by sequences at the *Hinf I* recognition site. The other regions of the gB gene carrying the variations are depicted to identify the gB1+gB2 (**Figure 13b**) and gB1+2+3 (**Figure 13c**). The results confirm the presence of mixed

Figure 13: Results of DNA sequencing of amplicons obtained by semi-nested PCR for gB gene for validation of mixed genotypes obtained with multiplex PCR

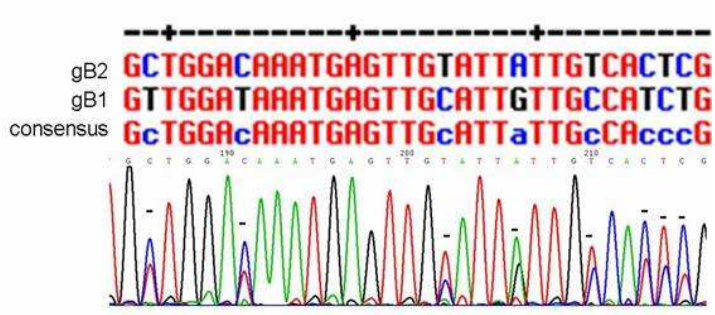
(a) gB 1 + gB 3



(a) The box shows the recognition site of *Rsa I* enzyme that differentiates gB3 from gB1. The arrow shows the mixed bases (C and T) at the recognition site of *Rsa I.*

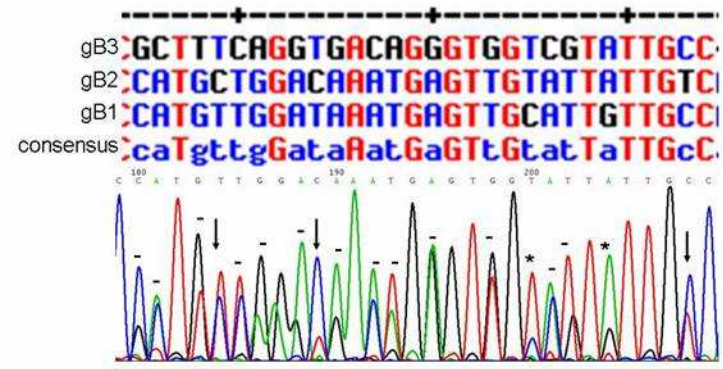
The bars (‒) show other mixed bases distributed near the recognition site of *Rsa I* enzyme in the gB gene. Multiple sequence alignment with consensus is also shown.

(b) gB1+gB2



(b) The bars (–) show mixed bases of gB1 and gB2 genotypes. Multiple sequence alignment with consensus is also shown.

(c) gB1+gB2+gB3



(c) The arrow, bars (‒) and asterisk (*) indicate the bases that differentiates gB2; gB3 and gB1 from rest two genotypes respectively. Multiple sequence alignment with consensus is also shown.

genotypes in the clinical specimens. Multiple sequence alignment of relevant genotypes is also shown (**Figure 13**).

6.4.8 Results of the study on the distribution of gB genotypes in different clinical specimens from immunocompromised patients by multiplex PCR

6.4.8.1 Patientwise distribution of gB genotypes

Genotyping results for the gB gene was available for 220 (92.1%) of 239 patients analyzed. The 220 patients consisted of 164 PRTs, 30 infants, 20 HIV infected individuals and 6 BMT. Discordances between multiple clinical specimens were observed in 11 patients (8 PRTs and 3 HIV infected individuals). The distribution of gB genotypes were analyzed in 209 patients (156 PRTs, 17 HIV infected individuals, 30 infants and 6 BMTs). **Table 13** shows the distribution of gB genotypes in different patient population. gB4 and gB5 genotypes were not observed in the study. Single genotypes were observed in 141 (67.5%) and mixed genotypes were observed in 68 (32.5%) of 209 patients. Among the single genotypes a predominance of gB1 (51.8%) was seen irrespective of the nature of patients. This result was statistically significant ($P<0.0001$, Chi- square test). When the distribution was studied as individual genotypes which comprised of gB1, gB2, gB3 and gB mix, frequency of gB mix in PRT was 38.5% and in BMT was 50%. No mixed gB genotypes was found in the infant group ($P<0.001$). There was a predominance of males in the study population. There was no difference between the distribution of gB genotypes between males (n = 141) and females (n = 68) in the 209 patients.

Table 13: Distribution of gB genotypes in different patient groups

Genotypes	Patient groups				Total
	PRT	***HIV***	***Infants***	***BMT***	
gB1	51	3	16	3	73
gB2	20	6	5	0	31
gB3	25	3	9	0	37
gBmix	60	5	0*	3	68
Total	**156**	**17**	**30**	**6**	**209**

* The absence of mixed genotype in the infant group was statistically significant. ($P<0.001$, Chi square test).

6.4.8.2 Discordance in gB genotypes between multiple clinical specimens

Figure 14 summarizes the results of the discordance observed with gB genotypes in multiple clinical specimens. Discordances between multiple clinical specimens were observed in 11 patients (8 PRTs and 3 HIV infected individuals). Of the 8 PRTs, a pair of blood and urine was collected from 6 patients; a pair of blood specimens from one and three samples of urine and one blood from one patient. Of the six pairs of blood and urine collected, genotyping results were available in the urine component only for 2 (one each of gB1 and gB3) ; Out of the rest 4 PRTs, mixed genotypes were seen in 3 urine with single genotypes in the blood component and vice-versa in 1 patient. Of the two blood samples collected from the PRT, the second sample alone revealed the genotype gB2 and first sample was negative. The genotyping results on 3 urine and one blood sample collected from a single patient revealed mixed genotypes in first three samples (two urine and one blood specimen) while a single genotype in the fourth (urine)sample. Of the three blood and intraocular fluids from 3 HIV patients all the three revealed mixed genotype in the blood while a single genotype in the intraocular fluid. The strain other than gB1 present in the blood (gB2 or gB3) was encountered in the intraocular fluid.

6.4.8.3 Specimenwise distribution of gB genotypes

Results of gB genotypes irrespective of the patient group was available for 261 specimens (Blood- 174, Urine- 52, Respiratory fluids – 21, intraocular fluids – 13 and gastric wash -1). **Table 14** shows the distribution of gB genotypes in different clinical specimens. Compared to other clinical specimens, a higher frequency of mixed genotype and gB2 were observed in blood and intraocular fluids respectively ($P<0.05$, Chi – square test).

Figure 14: Flow chart showing the discordant results observed with gB genotypes in multiple clinical specimens from 11 patients

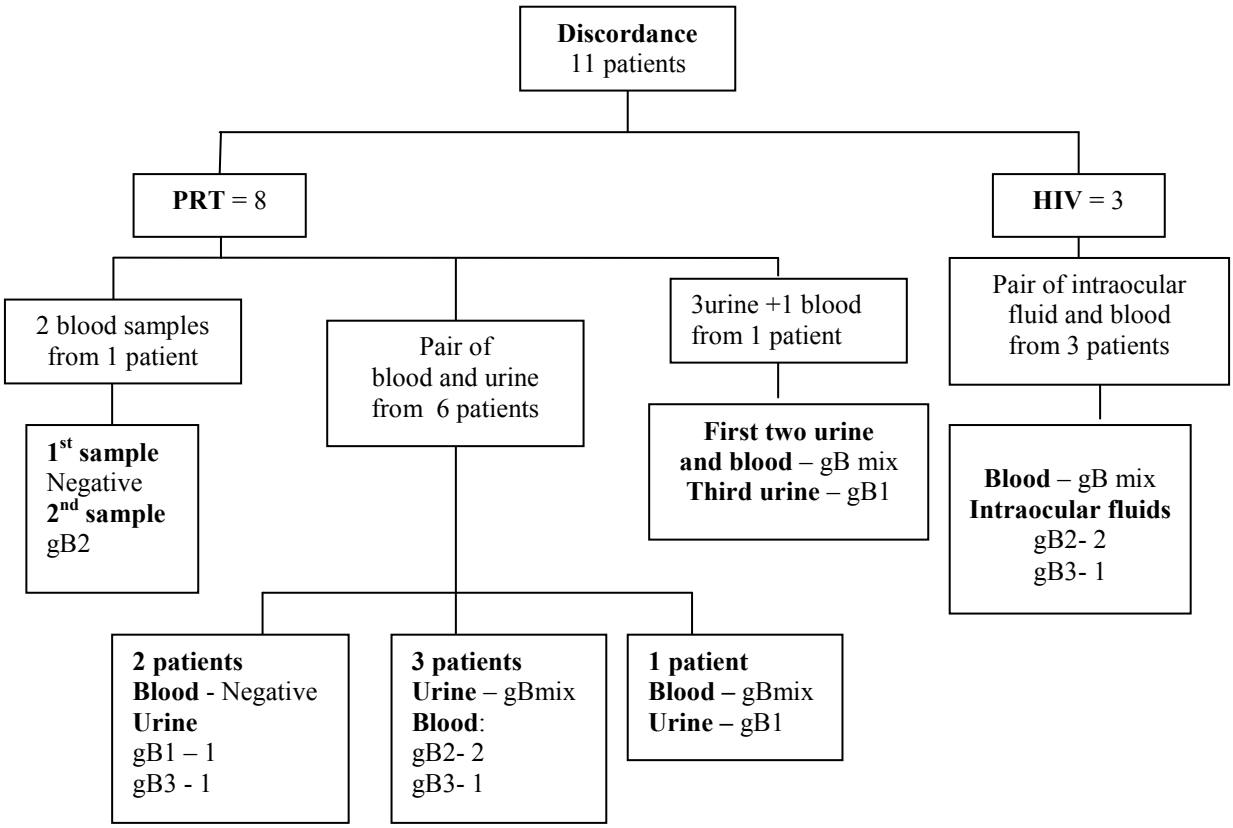


Table 14: Distribution of gB genotypes of HCMV in different clinical specimens

Genotypes	Clinical specimens					Total
	Blood	***Urine***	***Respiratory fluids***	***Intraocular fluid******	***Gastric wash***	
gB1	52	17	11	2	0	82
gB2	26	6	4	6**	0	42
gB3	25	13	3	4	0	45
gBmix	71*	16	3	1	1	92
Total	**174**	**52**	**21**	**13**	**1**	**261**

* Frequency of gB mix higher in peripheral blood

** Frequency of gB2 higher in intraocular fluids
($P<0.05$, Chi – square test).

*** All the intraocular fluids were obtained from HIV infected individuals

6.5 DISCUSSION

Glycoprotein B is considered as a major viral glycoprotein playing a role in initiating strong immune response in humans. A correlation between gB genotype and CMV-associated disease in immunocompromised patients has long been sought for, but no convincing evidence has been found. The present study was carried out to analyze the distribution of gB genotypes in clinical specimens obtained from immunocompromised patients in Chennai region, India. There is only one study available on gB genotypes in India wherein, a uniplex PCR-based RFLP was employed on direct clinical specimens for genotyping (Madhavan and Priya, 2002b). Since, the sensitivity of the technique was low owing to the uniplex nature of amplification, initial attempt was made to perform the genotyping by an already existing nested PCR for gB gene (Carraro and Granato, 2003) followed by RFLP in the present study. The result of the nested PCR for gB gene showed no significant increase in the sensitivity with only 60.87% of clinical specimens being genotyped as opposed to 50% by uniplex PCR. A sudden absence of gB3 genotype which existed during the year 2002 was also observed in the results of genotyping with nested PCR based RFLP. To analyze the controversy, multiple sequence alignment of the primer sequences were performed with the prototype sequences of gB1 to gB4 genotypes procured from Genbank database. The results indicated primer target mismatches in the outer forward primer with respect to gB3 genotype. It was hypothesized that this mismatch was responsible for the lack of amplification from clinical specimens. Hence, the nested PCR was converted to semi-nested PCR by excluding the outer forward primer. The results of application of the semi-nested PCR on the 46 clinical specimens proved the hypothesis since, all the clinical

specimens that did not give an amplification signal with nested PCR for gB gene belonged to gB3 genotype. The study also indicated that the sequence variations in the gB genome of HCMV may complicate genotyping of HCMV. Confirmation of primer specificity by the BLAST program may not suffice for the genotyping studies. Complete alignment of the prototype sequences of all the genotypes that exist for the gene of interest by a multiple sequence alignment program with the primers used for amplification may be necessary for genotyping of HCMV. Errors in the published primer sequences with respect to several genes of HCMV have already been reported (Habbal et al., 2005).

The next study was undertaken to compare the efficiency of the semi-nested PCR based RFLP and multiplex PCR for genotyping of gB gene of HCMV. Though various nucleic acid based molecular methods are available for genotyping of HCMV, PCR-based RFLP and multiplex nested PCR were chosen in the study due to lower technical complexity and cost effectiveness. In this study, the multiplex nested PCR showed a higher efficiency in genotyping HCMV compared to the conventional PCR-based RFLP. There was no significant difference in the positivity rate of the multiplex nested PCR and semi nested PCR for gB gene. The positivity rates were 100 % and 92.8 % for multiplex nested PCR and semi-nested PCR respectively. Mixed infection with multiple genotypes was effectively picked up by the multiplex nested PCR compared to PCR –based RFLP. A similar pattern was observed in a study on serotyping of subgenus C Adenoviruses (Adhikary et al., 2004). The presence of mixed genotype in the clinical specimens was confirmed by DNA sequencing. The inability of the PCR- based RFLP to identify multiple infections by mixed genotypes may be attributed to the selective amplification of the strain predominant in the clinical specimen and hence the generation of the

restriction pattern respective to that strain. The PCR-based RFLP proved to be expensive than the multiplex nested PCR owing to the digestion step, which required two restriction enzymes and use of an additional agarose gel electrophoresis to view the digested products. The cost of the four extra primers used in the second round of the multiplex nested PCR was negligible as they were used in very low concentrations. The study showed that the multiplex nested PCR for gB gene is more advantageous than the commonly employed PCR based RFLP since the former method allowed easier detection of mixed infections with multiple gB genotypes in clinical specimens. It was a single step reaction devoid of the restriction digestion step, simple, easy to perform and less expensive than the PCR based RFLP and required 5 hours for completion of the test as opposed to 10 hours in case of the PCR based RFLP. Hence, the multiplex PCR was employed to determine the distribution of gB genotypes from different patient groups.

In the study determining the distribution of gB genotypes by multiplex PCR, the results of the genotype with disease outcome could not be correlated, since, the clinical details of the patients were not available. There was no difference in the distribution of gB genotypes with regard to gender in this study, though the previous study on gB genotypes suggested that gB3 was predominant in females and gB2 in males (Madhavan and Priya, 2002b). The study in 2002 on genotyping of gB gene of HCMV using the uniplex PCR based RFLP revealed absence of gB1 and gB4 in the study population. In contrary, the result of the present study saw a predominance of gB1 genotype followed by gB3 and gB2 in the study population irrespective of the patient group. The result is in agreement with reports from other countries (Xanthakos and Schleiss, 2003; Yu et al., 2006 ; Wu et al., 2005; Humar et al., 2003; Barbi et al., 2001;Bale et al., 2000). Other findings of the present study included

absence of gB4 and gB5 in the study population and gB mix in the infant group. The absence of gB mix genotype in the infants was in agreement to recent studies (Yamamoto et al., 2007; Jin et al., 2007; Xanthakos and Schleiss, 2003). Yu et al (2006) reported mixed genotypes in congenitally infected infants (10.13%) in China. However, the samples tested in the study were predominantly urine. The present study, however, was carried out on different clinical specimens obtained from infants with 44% being urine. Whether the nature of the clinical specimens could be attributed for the total absence of gB mix in the infant group requires to be established.

Predominance of gB2 in intraocular fluids may be attributed to the fact that all the intraocular fluids belonged to HIV patients and gB2 is generally known to be predominant in HIV patients with CMV retinitis (Drew et al., 2002) though the predominance of gB2 in HIV patient group was not statistically significant in this study group. However, reports on the predominance of a certain CMV genotype in patients with CMV retinitis is ambiguous (Shepp et al., 1996; Rasmussen et al., 1997; Drew et al., 2002; Bongarts et al., 1996).

In the HIV patient group with discordant results between the blood and intraocular fluids, the genotype other than gB1 in the blood, was detected in the intraocular fluid. Whether different levels of neurotropism of gB subtypes as established by previous studies could be attributed to such an observation needs confirmation (Shepp et al., 1996; Tarrago et al., 2003). The frequency of mixed gB genotypes in PRT and HIV groups are little less than the reports available. It has been shown for renal transplant patients (Aquino and Figueiredo, 2000) as well as for lung transplant recipients (Puchhammer- Stockl et al., 2006) that up to 70 and

90% of the patients, respectively, develop mixed CMV-strain infections in the follow-up after transplantation. The results on the mixed gB genotypes support the existing notion that infection with one strain does not protect against reinfection with another CMV strain. The significance of infections with mixed genotypes in the patient groups is attributed to the recombination events that might occur between the CMV strains leading to the generation of recombinant strains (Steininger et al., 2005). Humar et al (2003) showed that infections with mixed CMV gB-subtype strains in solid organ transplantation may be associated with a delayed clearance of the virus from blood during therapy. This finding is of special interest because delayed virus clearance under antiviral therapy may also signify a higher risk for development of drug resistance. Coaquette et al (2004) described that mixed gB-subtype infections were associated with increased graft rejection and disease progression.

The study on the distribution of gB genotypes in Chennai region, India suggests that there was no absolute segregation of gB genotypes with gender, patient groups or clinical specimens though certain genotypes were prevalent in different groups. The major limitations of the study however include, the non-availability of the clinical data of the patients to correlate the gB genotype distribution with respect to the clinical outcome; difference in sample sizes of different patient groups and cross-sectional design of the study.

7. A STUDY ON THE DISTRIBUTION OF gH, gO AND gL GENOTYPES OF THE gCIII COMPLEX OF HCMV

7.1 BACKGROUND

The gCIII viral-envelope complex consists of UL75 (gH), UL74 (gO), and UL115 (gL) (Gretch et al., 1988; Huber and Compton 1997; Huber and Compton 1998; Huber and Compton 1999; Li et al., 1997) glycoproteins. Although the function of the assembled gCIII complex is unknown, the gH component is known to induce virus-neutralizing antibody (Rasmussen et al., 1984) and facilitates penetration of CMV into host cells (Keay and Baldwin 1991; Keay and Baldwin 1996; Keay et al., 1989). gL is necessary for the transport of gH from the nuclear membrane to the cell surface (Kaye et al., 1992; Spaete et al., 1993) and gO has been associated with fusion of CMV-infected cells (Milne et al., 1998; Paterson et al., 2002). A genetic homologue of CMV gO in EBV is gp42 and is indispensable for infecting B lymphocytes. Whether HCMV gO plays any role in cell tropism is yet to be determined. The disulfide-bonded, tripartite gCIII complex is displayed on the surface of infected cells and ultimately becomes part of the virion (Theiler and Compton, 2001; Theiler and Compton, 2002).

In view of the importance of the viral-envelope glycoprotein complexes for both replication and induction of a host immune response, one might predict a high level of genetic conservation. Several reports have confirmed the consistent detection of two closely related variants of the gH gene; however, very limited studies are available on the genetic variability of the individual gL and gO genes. A

study on the distribution of the three genes of gCIII complex showed that there is predictable variations in the genes of the gCIII complex and the clinical strains of HCMV from different patient groups clustered into four groups based on the gO and gL sequences (Rasmussen et al., 2002). No studies have been available on the genotyping of the three genes of gCIII complex from India. Also, most methods developed for identification of the genotypes of these three genes have not been applied directly on the clinical specimens. This study was undertaken to elucidate the genetic configuration of the three genes of gCIII complex and their distribution by applying PCR-based RFLP methods on direct clinical specimens from different immunocompromised patient groups in India.

7.2 OBJECTIVES

7.2.1 To standardize PCR-based RFLP for gH gene of HCMV

7.2.2 To study the distribution of gH genotypes in different clinical specimens from immunocompromised patients

7.2.3 To standardize PCR-based RFLP for gO gene of HCMV

7.2.4 To study the distribution of gO genotypes in different clinical specimens from immunocompromised patients

7.2.5 To standardize PCR-based RFLP for gL gene of HCMV

7.2.6 To study the distribution of gL genotypes in different clinical specimens from immunocompromised patients

7.2.7 To study the distribution of gCIII complex and genetic linkages between the genes of gCIII complex.

7.3 MATERIALS AND METHODS

7.3.1 Standardization of seminested PCR-based RFLP for gH gene of HCMV

7.3.1.1 Semi-nested PCR for gH gene

A semi-nested PCR was standardized for gH gene of HCMV using DNA of AD-169 strain by including an outer forward primer in the first round of amplification to an existing uniplex PCR (Chou, 1992a). A 50µl reaction mixture was prepared with 160µM of each dNTPs, 20 picomoles of primers gH1 and gH3 (primer sequences are provided in **Table 15)**, 1 x buffer (10mM Tris–HCl 127(pH 8.3), 50mMKCl, 0.01% gelatin, 3.0mMMg$Cl_{2)}$, 1 U of Taq DNA polymerase. Ten µl of DNA was used as the template. The thermal profile followed is as shown in **Table 15.** The second round of amplification was carried out with reaction mixture similar to the first round with 10 picomoles each of primers gH1 and gH2. Ten µl from the first round amplicon was used as the template for the second round of amplification. The thermal profile followed for the second round is provided in **Table 15**. Amplicons were analyzed by electrophoresis on a 2% agarose gel incorporated with ethidium bromide (Appendix II). Sensitivity and specificity of the PCR was determined as described in Appendix II.

7.3.1.2 Standardization of Poly Acrylamide Gel Electrophoresis based RFLP (PAGE – RFLP) of gH gene product

Ten µl of unpurified second round amplicon of HCMV AD 169 was subjected to digestion with *Hha I* enzyme in a 25µl reaction. The concentration of

Table 15: Primer sequences, thermal profile and amplicon size for PCR targeting gH and gL genes of HCMV

Gene	Primer sequences	Thermal Profile	Amplicon Size
gH	gH1 : 5' ccacctggatcacgccgctg 3' gH2: 5' tggtgttttcacgcaggaa 3' gH3: 5' aggtattgacagatcaatgg 3'	I round: (35 cycles) 94°C - 2 minutes 94°C - 45 seconds 62°C - 30 seconds 72°C - 45 seconds 72°C - 5 minutes II round: (25 cycles) 94°C - 2 minutes 94°C - 45 seconds 62°C - 30 seconds 64°C - 45 seconds 64°C - 5 minutes	I round 536bp II round 213bp
gL	gL1: 5'ttgatgtgccgccgcccggatt 3' gL2: 5'gcaccagctcgaagcctaac 3' gL3 5'atgtgccgccgcccggatt 3' gL4 5'ccagctcgaagcctaac 3'	I round: (35 cycles) 94°C - 5 minutes 94°C - 45 seconds 65°C - 45 seconds 72°C - 45 seconds 72°C - 10 minutes II round: (25 cycles) Profile Same as I round	I round 556bp II round 550bp

the enzyme, buffer composition and temperature for incubation used are provided in Appendix II. A control reaction containing the amplicon and buffer was included during each digestion. Digested fragments were analyzed after electrophoresis on 20% polyacrylamide gels and visualized by silver staining (Appendix II).

7.3.2 Application of PCR –based RFLP on clinical specimens

The standardized seminested PCR-based RFLP was applied on 34 clinical specimens from thirty immunocompromised patients with CMV infection proven by a positive PCR for mtr II gene. The 34 clinical specimens included in the study were as follows: Peripheral blood – 25, Urine – 6, Respiratory fluids – 3. The clinical specimens which gave a specific amplification signal by the PCR were genotyped by PAGE based RFLP.

7.3.3 Modification of PAGE based RFLP to Agarose gel based RFLP

The restriction digestion was modified to visualize the restriction fragments on an agarose gel. The restriction enzymes were designed using a web based program (http://www.restrictionmapper.org) by providing consensus sequences of gH1 and gH2 genotypes after alignment of sequences available in the Genbank database (Accession numbers M94228 to M94237). Two restriction enzymes *Hpa II* and *Stu I* were selected for differentiation of the genotypes. Ten µl of unpurified second round amplicon of HCMV AD 169 was subjected to digestion with *Hpa II* and *Stu I* enzymes in separate 25µl reactions. The concentration of the enzyme, buffer composition and temperature for incubation used are provided in Appendix II. A control reaction containing the amplicon and buffer was included during each

digestion. Digested fragments were analyzed after electrophoresis on 4% agarose gels incorporated with ethidium bromide (Appendix II).

7.3.3.1 Validation of agarose gel based RFLP for gH

The agarose gel based RFLP for gH gene was validated against the conventional PAGE based RFLP. The stored amplified products of the clinical specimens (section 7.3.2) that was already genotyped by PAGE based RFLP were subjected to restriction enzyme digestion by *Hpa II* and *Stu I* enzymes.

7.3.4 A study on the distribution of gH genotypes in different clinical specimens from immunocompromised patients

7.3.4.1 Clinical specimens and patients

Two hundred and forty one clinical specimens from 201 immunocompromised patients were included in the study. Only those clinical specimens which showed positivity for HCMV by mtr II PCR were included. One hundred and sixty seven single specimens (Peripheral blood –136; Urine – 12; Respiratory fluids– 15; intraocular fluids - 4) obtained from 167 patients (Post renal transplant – 132; Infants – 20; HIV infected individuals – 11 and Bone marrow transplant – 4) were included. Pairs of similar or different clinical specimens accounting to 62 clinical specimens were obtained from 31 patients (Post renal transplant – 19 (Pairs of blood and urine from 18 patients; a pair of blood sample from one patient); Infants – 2 (pairs of blood and urine from 2 patients); HIV infected individuals – 9 (pairs of blood and intraocular fluids from 9 patients) and Bone marrow transplant – 1 (a pair of urine and respiratory fluid). Four samples of

clinical specimens were obtained from 3 post renal transplant patients (Two pairs of blood and urine from two patients, one sample of blood and three samples of urine from one patient).

7.3.4.2 PCR based RFLP for gH

Seminested PCR and agarose gel based RFLP for gH gene was carried out on the extracted DNA of the clinical specimens as described in sections 7.3.1.1 and 7.3.3 respectively.

7.3.4.3 Analysis of results

The distribution of the genotypes for gH gene was analyzed similar to gB gene (section 6.3.6.3).

7.3.5 Validation of mixed genotypes of gH gene by DNA sequencing

Second round amplicon of gH gene from a blood specimen that gave mixed genotype gH1 + gH2 was subjected to DNA sequencing. The DNA sequencing was performed as described in Appendix II.

7.3.6 Standardization of PCR-based RFLP for gO gene of HCMV

The duplex nested PCR for gO gene of HCMV was carried out as described in section 4.3.3. Two restriction enzymes *Hpa II* and *Hae III* were selected for differentiation of four genotypes of the gO gene of HCMV. Ten µl of unpurified second round amplicon of HCMV AD 169 was subjected to digestion with *Hpa II* and *Hae III* enzymes in separate 25µl reactions. The concentration of the enzyme,

buffer composition and temperature for incubation used are provided in Appendix II. A control reaction containing the amplicon and buffer was included during each digestion. The products were visualized on 4% agarose gel incorporated with ethidium bromide (Appendix II).

7.3.7 A study on the distribution of gO genotypes in different clinical specimens from immunocompromised patients

7.3.7.1 Clinical specimens and patients

Two hundred and sixteen clinical specimens from 178 immunocompromised patients were included in the study. Only those clinical specimens which showed positivity for mtr II PCR were included. One hundred and forty six single specimens (Peripheral blood –117; Urine – 11; Respiratory fluids– 14; intraocular fluids - 4) were obtained from 146 patients (Post renal transplant – 111; Infants – 20; HIV infected individuals – 11 and Bone marrow transplant – 4). Pairs of similar or different clinical specimens accounting to 58 clinical specimens were obtained from 29 patients (Post renal transplant – 17 (Pairs of blood and urine from 16 patients; a pair of blood sample from one patient); Infants – 2 (2 pairs of blood and urine); HIV infected individuals – 9 (9 pairs of blood and intraocular fluids) and Bone marrow transplant – 1 (a pair of urine and respiratory fluid). Four samples of clinical specimens were obtained from 3 Post renal transplant patients (Two pairs of blood and urine from two patients, one sample of blood and three samples of urine from one patient).

7.3.7.2 PCR based RFLP for gO gene and Analysis of the results

PCR based-RFLP for gO gene was carried out on the extracted DNA of the clinical specimens as described in section 7.3.6. The distribution of the genotypes for gO gene was analyzed as done for gB gene (section 6.3.6.3).

7.3.8 Validation of mixed gO genotype by DNA sequencing

Two blood specimens that gave mixed genotypes by duplex gO PCR-based RFLP were subjected to amplification by individual gO PCRs for AD169 and Towne strains respectively as in section 4.3.2. The second round amplicons of gO gene obtained by applying individual PCRs were subjected to DNA sequencing. The DNA sequencing was performed as described in Appendix II.

7.3.9 Standardization of PCR-based RFLP for gL gene of HCMV

7.3.9.1 Nested PCR for gL gene

A nested PCR was standardized for gL gene of HCMV using CMV-AD169 DNA (Rasmussen et al., 2002). A 50µl reaction mixture was prepared with 200µM of each dNTPs, 10 picomoles of primers gL1 and gL2 (primer sequences are provided in **Table 15)**, 1 x buffer (10mM Tris–HCl 127(pH 8.3), 50mMKCl, 0.01% gelatin, 1.5mMM$gCl_{2)}$, 1 U of Taq DNA polymerase. Ten µl of DNA was used as the template. The second round of amplification was carried out with reaction mixture similar to the first round with 10 picomoles each of primers gL3 and gL4. Two µl from the first round amplicon was used as the template for the second round of amplification. The thermal profile followed for both the rounds is as shown in

Table 15. Amplicons were analyzed by electrophoresis in a 2% agarose gel incorporated with ethidium bromide (Appendix II). Sensitivity and specificity of the PCR was determined as described in Appendix II.

7.3.9.2 Standardization of RFLP of gL gene product

Ten µl of unpurified second round amplicon of HCMV AD 169 was subjected to digestion with *Rsa I* and *Taq I* enzyme in a 25µl reaction. The concentration of the enzyme, buffer composition and temperature for incubation used are provided in Appendix II. A control reaction containing the amplicon and buffer was included during each digestion. Digested fragments were analyzed after electrophoresis on 4% agarose gel incorporated with ethidium bromide. (Appendix II).

7.3.10 A study on the distribution of gL genotypes in different clinical specimens from immunocompromised patients

7.3.10.1 Clinical specimens and patients

The same group of clinical specimens and patients included for the analysis of gO genotypes (section 7.3.7.1) was subjected to analysis of gL genotypes.

7.3.10.2 PCR based RFLP for gL

PCR based-RFLP was carried out on the extracted DNA of the clinical specimens as described in section 7.3.9. The results were analyzed similar to gB gene (section 6.3.6.3).

7.3.11 Validation of mixed genotype of gL gene by DNA sequencing

A blood specimen that gave mixed genotype (gL3+gL4) by nested PCR-based RFLP for gL gene was subjected to DNA sequencing. The DNA sequencing was performed as described in Appendix II.

7.3.12 A study on the distribution of gCIII complex in immunocompromised patients and a study on linkage of genes of gCIII complex

7.3.12.1 Study design : Retrospective study

7.3.12.2 Study Population

7.3.12.2.1 Inclusion criteria for the analysis:

Patients who had a positive result and harbored a single genotype for all the three genes of gCIII complex (gH, gO and gL).

In case of patients, whose multiple specimens showed concordant results, one of the specimen was chosen for analysis.

In case of patients, whose multiple specimens showed discordant results, the specimen showing single genotype for all the three genes, if available, was considered.

7.3.12.2.2 Exclusion criteria for the analysis:

The patients who had a negative result for at least one gene of the gCIII complex.

The patients who had a mixed genotype for any gene of the gCIII complex.

In case of patients, who had a discordant result between clinical specimen, if the multiple specimens showed the mixed genotype or negative results, they were excluded.

7.3.12.2.3 Details of Patients included in the analysis:

Ninety eight immunocompromised patients (Post renal transplant - 57, HIV infected individuals - 20, infants - 18, Bone marrow transplants - 3) were included in the analysis.

7.3.12.2.4 Analysis of results:

The patientwise distribution of the variants of gCIII complex was analyzed. In case of genetic linkages the distribution of different gL and gO genotypes with respect to gH1 and gH2 genotypes and with respect to each other were analyzed. Chi-square test was performed to find any significant linkage between the variants of different genes. P value $\leq$0.05 was considered significant.

7.4 RESULTS

7.4.1 Results of standardization of seminested PCR and PAGE based RFLP for gH gene of HCMV

The seminested PCR for gH gene generated a first round product of 536 bp and second round product of 213 bp with CMV –AD169 strain. The PCR was specific for HCMV and sensitive enough to pick up 1pg of HCMV DNA. The

RFLP of the PCR product of AD169 revealed the following restriction fragments 81, 75, 29, 20 and 8 bp and belonged to gH1 genotype.

7.4.2 Results of application of semi-nested PCR and PAGE based RFLP for gH gene on 34 clinical specimens:

Of the 34 clinical specimens, 25 clinical specimens were positive for gH gene. **Figure 15** shows the amplified products of gH gene of HCMV applied on clinical specimens. The gH 1 genotype gave digested products of following sizes 81, 75, 29, 20 and 8 bp and gH2 gave 92, 81, 29 and 8 bp (**Figure 16**). Of the 25 clinical specimens, 18 were genotyped as gH1 and 7 as gH2 by PAGE based RFLP.

7.4.3 Results of the standardization and validation of Agarose gel based RFLP for gH gene

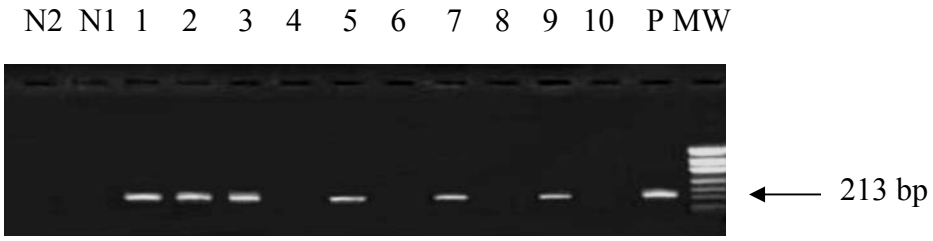
Genotype gH1 gave 162bp and 51bp fragments with *Hpa II* and remained undigested with *Stu I*. Genotype gH2 gave 158bp, 52bp fragments with *Stu I* and remained undigested with *Hpa II*. There was an absolute concordance of results between the conventional PAGE based RFLP and Agarose gel based RFLP. The latter was used in further studies due to simplicity and rapidity of the procedure.

7.4.4 Results of the study on the distribution of gH genotypes in different clinical specimens from immunocompromised patients

7.4.4.1 Patientwise distribution of gH genotypes

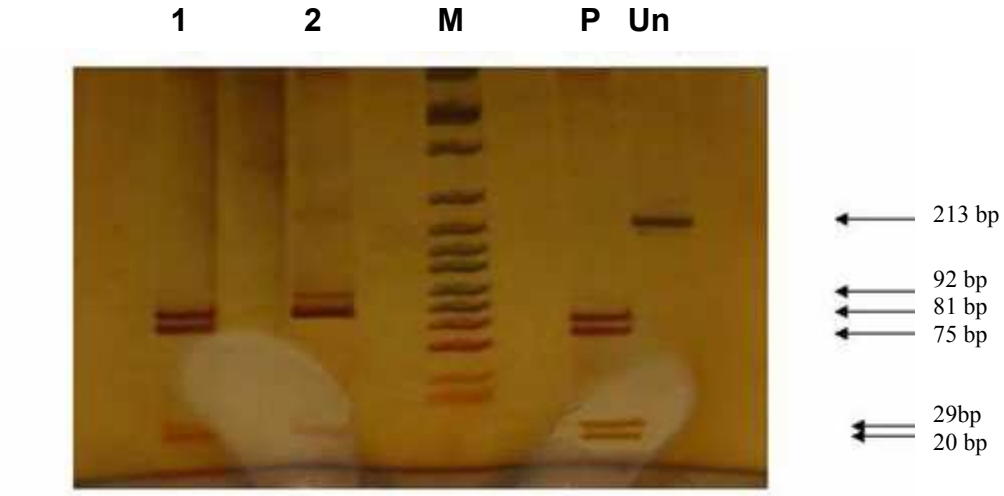
Genotyping results of the gH gene was available for 148 (73.6%) of 201 patients analyzed. The 148 patients consisted of 105 PRTs, 19 infants, 20 HIV infected individuals and 4 BMT. Discordances between multiple clinical specimens

Figure 15: Agarose gel electrophoretogram showing the amplified products of gH gene of HCMV



Lanes: N2- Negative control (II round); N1 - Negative control (I round); 1,2,3,5 - Blood positive for gH; 4,6 - Blood negative for gH ; 7- Urine positive for gH; 8, 10 - Urine negative for gH; 9 – Respiratory fluid positive for gH; P – Positive control (HCMV AD169); MW –*Hinf I* digest of ϕX – 174 DNA

Figure 16: Polyacrylamide gel electrophoretogram showing the results of PCR based RFLP for determination of strain variation in gH gene sequence



Lanes: 1 – Blood specimen (genotype gH 1); 2- Blood specimen (genotype gH 2) MW - ϕX174 DNA /*Hinf I* digest; P – HCMV AD 169 strain (genotype gH 1); Un - Undigested product

were observed in 8 patients (7 PRTs and 1 HIV infected individual). The distribution of gH genotypes were analyzed in 140 patients (98 PRTs, 19 HIV infected individuals, 19 infants and 4 BMTs). **Table 16** shows the distribution of gH genotypes in different patient groups. Single genotypes in 135 (96.4%) and mixed genotypes in 5 (3.6%) of 140 patients were observed. Among the single genotypes, a predominance of gH1 (69.6%) was seen irrespective of the patient groups. When the distribution was studied as individual genotypes which comprised of gH1, gH2, and gH mix, a statistically significant difference in the distribution of gH genotypes was seen among the different patient groups ($P<0.05$). A higher frequency of gH1 genotype in PRTs and HIV patients and gH2 genotype in BMT group was observed. The distribution of gH1 and gH2 was almost equal in the infant group. The presence of mixed gH genotype was observed only in PRT patient group. There was a higher frequency of males among the patients. There was no difference in the distribution of gH genotypes between males (n = 99) and females (n = 41) in the 140 patients. **Figure 17** shows the results of PCR –based RFLP of gH gene on clinical specimens.

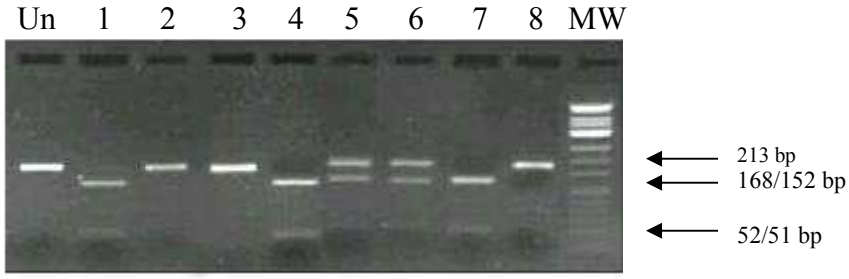
7.4.4.2 Discordance in gH genotypes between multiple clinical specimens

Figure 18 summarizes the results of the discordance observed with gH genotypes in multiple clinical specimens. Discordance between multiple clinical specimens was observed in 8 patients (7 PRTs and 1 HIV infected individual). Of the 7 PRTs, a pair of blood and urine was collected from 4 patients; two pairs of blood and urine from 2 patients and 3 samples of urine and one blood from one patient. Of the four pairs of blood and urine collected, genotyping results were available in the blood component alone for 2 patients (one each of gH1 and gH2) ; The rest 2 patients harbored, mixed genotype (gH1+gH2) in the blood while single

Table 16: Distribution of gH genotypes in different patient groups

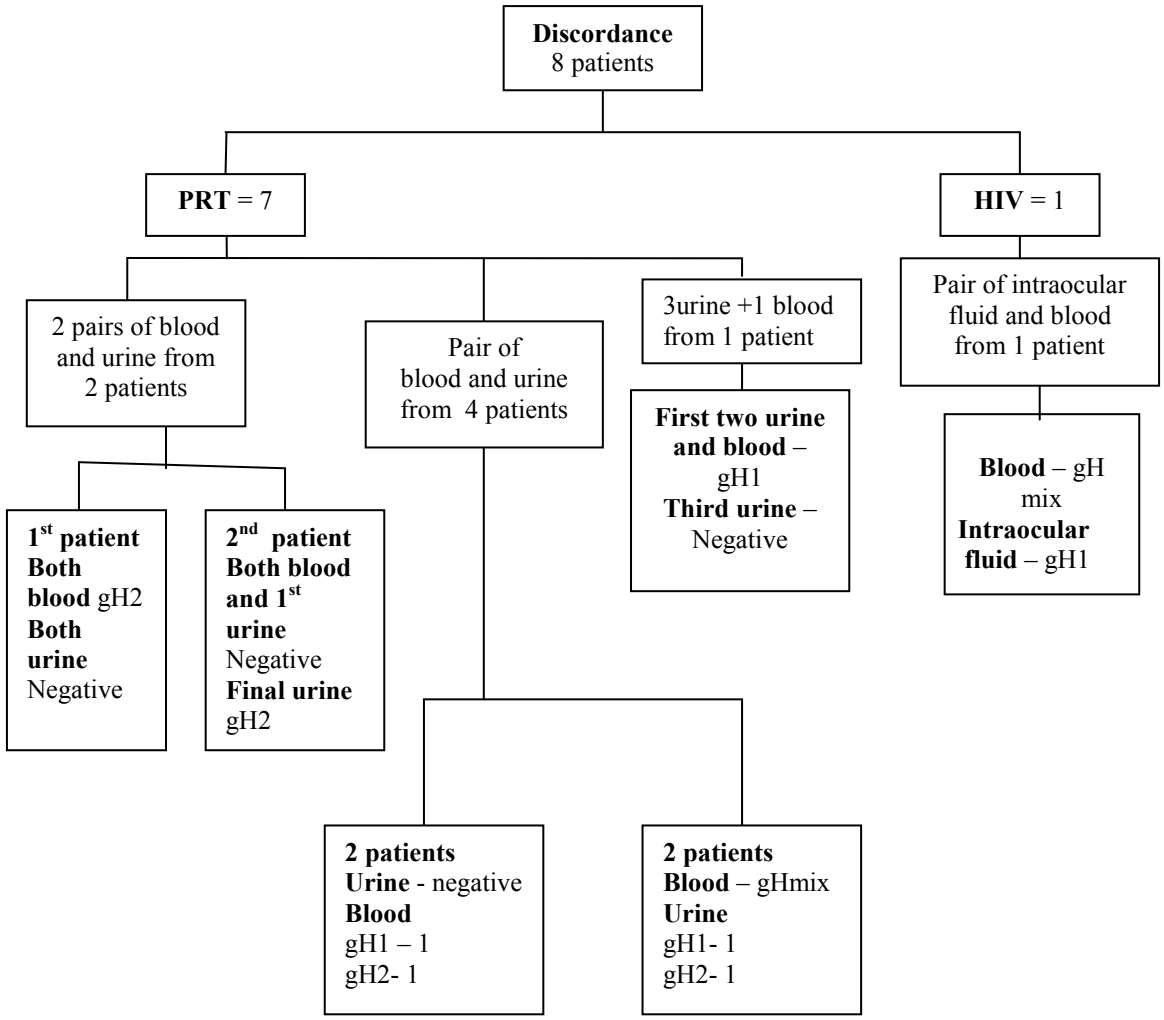
Genotypes	Patient groups				Total
	PRT	***Infant***	***HIV***	***BMT***	
gH 1	71	9	13	1	**94**
gH 2	22	10	6	3	**41**
gH mix	5	0	0	0	**5**
Total	**98**	**19**	**19**	**4**	**140**

Figure 17: Agarose gel electrophoretogram showing the *Hpa II* and *Stu I* products of gH gene



Lanes: Un - Undigested product; 1 – *Hpa II* digest of blood specimen (Genotype gH1); 2 – *Stu I* digest of blood specimen (Genotype gH1); 3 – *Hpa II* digest of blood specimen (Genotype gH2); 4 – *Stu I* digest of blood specimen (Genotype gH2); 5 – *Hpa II* digest of blood specimen (Genotype gHmix); 6 – *Stu I* digest of blood specimen (Genotype gHmix); 7 – *Hpa II* digest of HCMV AD- 169 (Genotype gH1); 8 – *Stu I* digest of HCMV AD- 169 (Genotype gH1); MW – *Hinf I* digest of ϕX – 174 DNA

Figure 18: Flow chart showing the discordant results observed with gH genotypes in multiple clinical specimens from 8 patients



genotypes of gH1 and gH2 were shed in the urine specimen of each patient. The discordant results in case of further 3 PRTs were as follows: Of the two patients from whom 2 pairs of blood and urine were collected, one patient showed presence of gH2 genotype in the pairs of blood component alone, while the pairs of urine were negative for gH gene. In the second patient, the first three samples (2 blood and 1 urine) were negative while the final urine sample revealed the presence of gH2 genotype. The patient from whom 3 samples of urine and a single sample of blood was collected harbored gH1 in all samples except the final urine sample. The blood and intraocular fluid of the HIV patient revealed mixed genotype in the blood while gH1 genotype was shed in the intraocular fluid.

7.4.4.3 Specimenwise distribution of gH genotypes

Results of gH genotypes irrespective of the patient group was available for 176 specimens (Blood- 122, Urine- 26, Respiratory fluids – 15 and intraocular fluids – 13). **Table 17** shows the distribution of gH genotypes in different clinical specimens. A higher occurrence of gH1 genotype irrespective of the nature of specimens was seen. There was no statistically significant difference in the distribution of gH genotypes between the different clinical specimens. gH mix was exclusively observed in blood specimens.

7.4.5 Result of DNA sequencing of mixed gH genotype from blood specimen

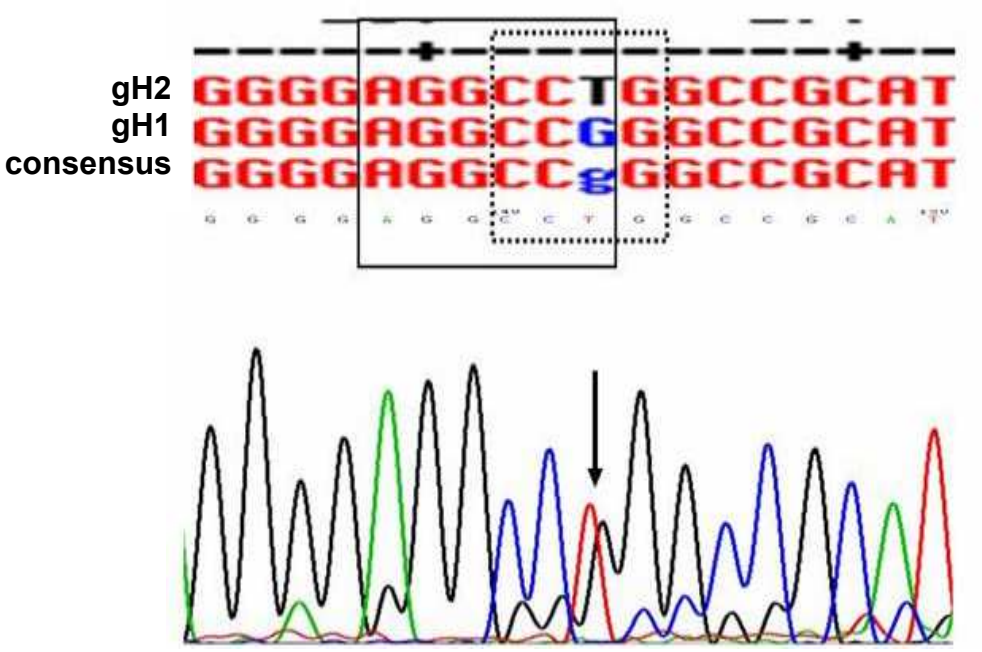
Figure 19 shows the result obtained by DNA sequencing of the amplicon of gH gene of HCMV from blood specimen that gave gH1+ gH2 genotype. The mixed

Table 17: Distribution of gH genotypes in different clinical specimens

Genotypes	Clinical specimens				Total
	Blood	***Urine***	***Respiratory fluids***	***Intraocular fluid****	
gH1	82	16	8	9	115
gH2	32	10	7	4	53
gHmix	8	0	0	0	8
Total	**122**	**26**	**15**	**13**	**176**

* All the intraocular fluids were obtained from HIV infected individuals

Figure 19: DNA sequencing of amplicons obtained by semi-nested PCR for gH gene for validation of mixed genotypes obtained by RFLP



Mixed bases at the recognition sites of *Hpa II* and *Stu I* . The plain box shows the recognition site of *Stu I* enzyme and box with dotted lines show the recognition site of *Hpa II* enzyme. The arrow indicates the mixed bases (T and G) at the recognition site of both the enzymes. Multiple sequence alignment with consensus sequence is also shown.

bases were seen in the recognition sites of Hpa *II* (CC↓GG) and *Stu I* (AGG↓CCT) enzymes. Multiple sequence alignment of gH1 and gH2 genotypes is also shown.

7.4.6 Result of standardization of PCR-based RFLP of gO gene of HCMV

The AD169 strain belonged to gO1 genotype and generated restriction digests of the following sizes for *Hpa II* enzyme: 345 bp, 25 bp and remained undigested with *Hae III* enzyme.

7.4.7 Results of the study on distribution of gO genotypes in immunocompromised patients

7.4.7.1 Patientwise distribution of gO genotypes

The RFLP patterns obtained for different gO genotypes are provided in Appendix II.

Genotyping results for the gO gene was available for 132 (74.2%) of 178 patients analyzed. The 132 patients consisted of 89 PRTs, 20 infants, 20 HIV infected individuals and 3 BMTs. Discordances between multiple clinical specimens were observed in 8 patients (7 PRTs and 1 HIV infected individual). The distribution of gO genotypes was analyzed in 124 patients (82 PRTs, 19 HIV infected individuals, 20 infants and 3 BMTs). **Table 18** shows the distribution of gO genotypes in different patient groups. Single genotypes were observed in 119 (96%) of 124 patients and mixed genotypes were observed in 5 (4%) of 124 patients. Among the single genotypes a predominance of gO1 (41.1%) and gO4 (31.5%) genotypes were seen over gO2 and gO3 irrespective of the patient groups. When the

Table 18: Distribution of gO genotypes in different patient groups

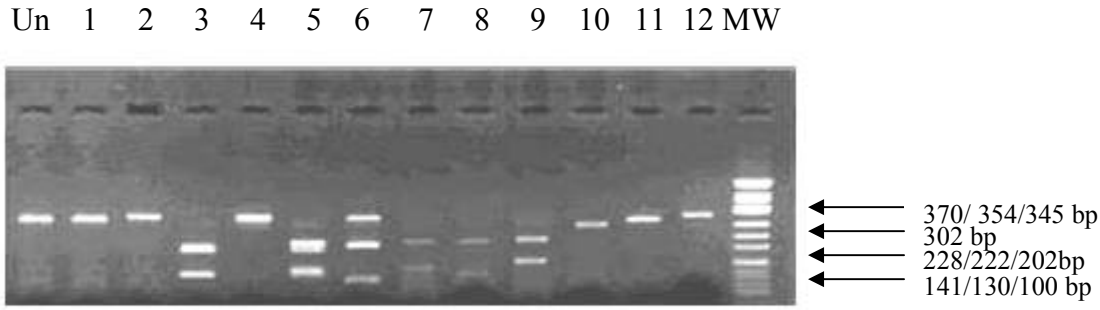
Genotypes	Patient group				Total
	PRT	***HIV***	***BMT***	***Infant***	
gO1	21	14	3	13	**51**
gO2	7	1	0	4	**12**
gO3	14	1	0	2	**17**
gO4	35	3	0	1	**39**
gOmix	5	0	0	0	**5**
Total	**82**	**19**	**3**	**20**	**124**

distribution was studied as individual genotypes which comprised of gO1, gO2, gO3, gO4 and gO mix, a higher frequency of gO4 (42.7%) in PRTs and gO1 in all other patient groups ($P<0.01$) was seen. The presence of mixed gO genotype was observed only in PRT patient group. There was a higher frequency of males in the study population. There was no significant difference between the gO genotypes between males (n = 85) and females (n = 39) in the 124 patients. **Figure 20** shows the RFLP patterns observed with different gO genotypes.

7.4.7.2 Discordance in gO genotypes between multiple clinical specimens

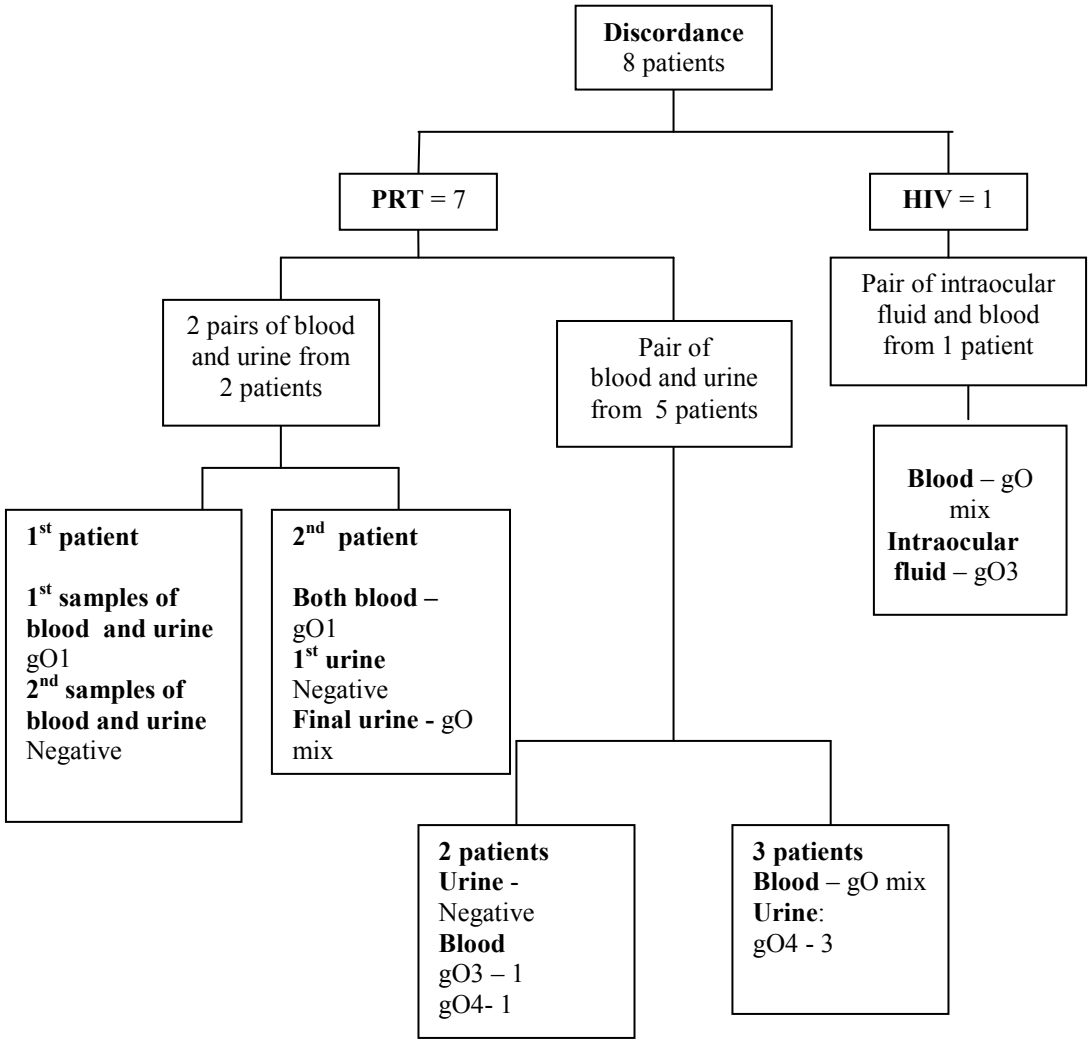
Figure 21 summarizes the results of the discordance observed with gO genotypes in multiple clinical specimens. Discordances between multiple clinical specimens were observed in 8 patients (7 PRTs and 1 HIV infected individual). Of the 7 PRTs, a pair of blood and urine was collected from 5 patients; two pairs of blood and urine from 2 patients. Of the five pairs of blood and urine collected, genotyping results were available in the blood component alone for 2 patients (one each of gO3 and gO4); the rest 3 patients harbored, mixed genotype (gO3+gO4) in the blood while single genotypes of gO4 were shed in the urine specimen of each patient. The discordant results in case of further 2 PRTs were as follows: Of the two patients from whom 2 pairs of blood and urine were collected, one patient showed presence of gO1 genotype in first samples of blood and urine specimens and negative results in the second samples of blood and urine specimens. In the second patient, both the samples of blood showed gO1 genotype, while the first urine specimen was negative and the second sample of urine showed a mixed genotype of gO1+gO3. The blood and intraocular fluid of the HIV patient revealed mixed

Figure 20: Agarose gel electrophoretogram showing the *Hpa II* and *Hae III* products of gO gene



Lanes: Un - Undigested product; 1 – *Hpa II* digest of blood specimen (Genotype gO1); 2 – *Hae III* digest of blood specimen (Genotype gO1); 3 – *Hpa II* digest of blood specimen (Genotype gO4); 4 – *Hae III* digest of blood specimen (Genotype gO4); 5 – *Hpa II* digest of blood specimen (Genotype gOmix); 6 – *Hae III* digest of blood specimen (Genotype gOmix); 7 – *Hpa II* digest of blood specimen (Genotype gO3); 8 – *Hae III* digest of blood specimen (Genotype gO3); 9 – *Hpa II* digest of blood specimen (Genotype gO2); 10 – *Hae III* digest of blood specimen (Genotype gO2); 11 – *Hpa II* digest of HCMV AD- 169 (Genotype gO1); 12 – *Hae III* digest of HCMV AD- 169 (Genotype gO1); MW – *Hinf I* digest of ϕX – 174 DNA

Figure 21: Flow chart showing the discordant results observed with gO genotypes in multiple clinical specimens from 8 patients



genotype (gO3+4) in the blood while gO3 genotype was shed in the intraocular fluid.

7.4.7.3 Specimenwise distribution of gO genotypes

Results of gO genotypes irrespective of the patient group were available for 162 specimens (Blood- 109, Urine- 28, Respiratory fluids –12 and intraocular fluids –13). **Table 19** shows the distribution of gO genotypes in different clinical specimens. There was a statistically significant difference in the distribution of gO1 genotype among the different clinical specimens ($P<0.05$). In the blood specimens, gO1 and gO4 were almost equally distributed. In all other clinical specimens there was a higher frequency of gO1 over the other genotypes.

7.4.8 Result of validation of mixed gO genotype by DNA sequencing

Of the two blood specimens one was identified as gO1+ gO2 and other as gO3+gO4 by DNA sequencing. The specimen carrying gO1+gO2 was amplified by gO (AD169) primer only. In case of the specimen carrying gO3+ gO4 genotype, amplicons were obtained with both gO (AD169) and gO (Towne) primers. On sequencing both the amplicons pure gO3 sequence and gO4 were obtained with gO (AD169) and gO (Towne) primers respectively. **Figure 22** shows the results obtained by DNA sequencing of the amplicon of gO gene of HCMV from blood specimen that gave gO mix (gO1+gO2) genotype. The mixed bases were seen at the recognition sites of Hpa *II* (CC↓GG) and *Hae III* (GG↓CC) enzymes. Multiple sequence alignment of gO1 and gO2 genotypes is also shown.

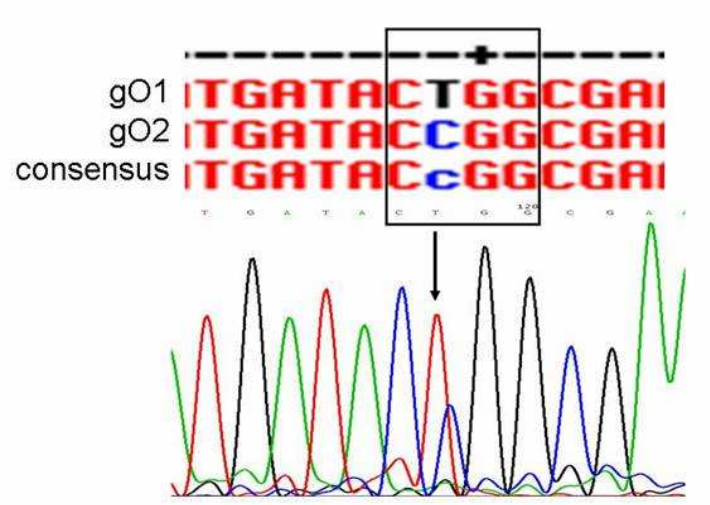
Table 19: Distribution of gO genotypes in different clinical specimens

Genotypes	Clinical specimens				Total
	Blood	***Urine***	***Respiratory fluids***	***Intraocular fluids****	
gO1	37	15	8	7	**67**
gO2	9	3	2	1	**15**
gO3	16	3	1	2	**22**
gO4	38	6	1	3	**48**
gOmix	9	1	0	0	**10**
Total	**109**	**28**	**12**	**13**	**162**

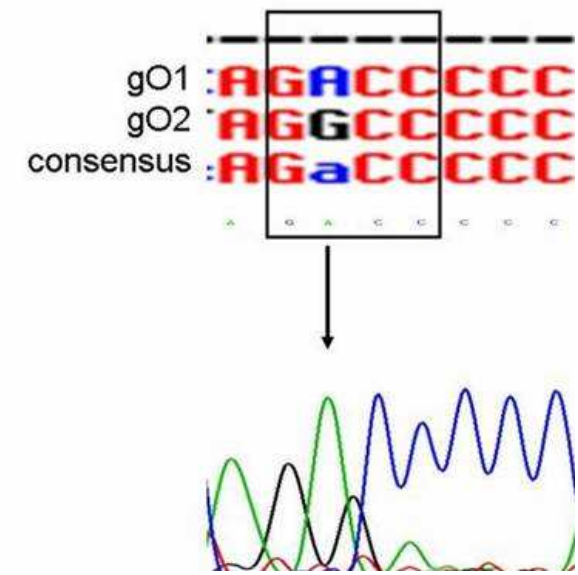
* All the intraocular fluids were obtained from HIV infected individuals

Figure 22: DNA sequencing of amplicons obtained by duplex PCR for gO gene for validation of mixed genotypes obtained by RFLP

(a)



(b)



a) **Mixed bases at the recognition sites of *Hpa II* of gO1+gO2 genotype** The box shows the recognition site of *Hpa II* enzyme. The arrow indicates the mixed bases (T and C) at the recognition site . Multiple sequence alignment with consensus is also shown.

(b) **Mixed bases at the recognition sites of *Hae III* of gO1+gO2 genotype.** The box shows the recognition site of *Hae III* enzyme. The arrow indicates the mixed bases (A and G) at the recognition site. Multiple sequence alignment with consensus is also shown.

7.4.9 Results of standardization of nested PCR based RFLP for gL gene of HCMV

The size of the first round and second round amplicons with AD-169 DNA were 556bp and 550bp respectively. The PCR was specific for HCMV and detected up to 0.5pg of HCMV DNA. The RFLP of the gL PCR product of AD169 revealed the following restriction fragments 287, 117, 96, 50bp with *Rsa I* and 386, 156, 8bp with *Taq I* and belonged to gL1 genotype.

7.4.10 Results of the study on the distribution of gL genotypes in immunocompromised patients

7.4.10.1 Patientwise distribution of gL genotypes

The expected RFLP patterns for gL genotypes are provided in Appendix II. Genotyping results for the gL gene was available for 128 (71.9%) of 178 patients analyzed. The 128 patients consisted of 84 PRTs, 21 infants, 20 HIV infected individuals and 3 BMT. Discordances between multiple clinical specimens were observed in 5 patients (4 PRTs and 1 HIV infected individuals). The distribution of gL genotypes were analyzed in 123 patients (80 PRTs, 19 HIV infected individuals, 21 infants and 3 BMTs). **Table 20** shows the distribution of gL genotypes in different patient groups. Single genotypes were observed in 121 (98.4%) of 123 patients and mixed genotypes were observed in 2 (1.6%) of 123 patients. gL1 and gL2 genotypes were not observed in the study population. Difference in the distribution of genotypes between PRT, HIV and BMT group were not observed. gL4 genotype predominated in all patient groups. There was a higher frequency of males among the patients. There was no considerable difference between the gL

Table 20: Distribution of gL genotypes in different patient groups

Genotypes	**Patient group**				**Total**
	PRT	***Infants***	***HIV***	***BMT***	
gL3	25	9	4	0	**38**
gL4	53	12	15	3	**83**
gLmix	2	0	0	0	**2**
Total	**80**	**21**	**19**	**3**	**123**

genotypes between males (n = 88) and females (n = 35) in the 123 patients. **Figure 23** shows the amplified products of PCR for gL gene. The restriction pattern observed in different clinical specimens is shown in **Figure 24.**

7.4.10.2 Discordance in gL genotypes between multiple clinical specimens

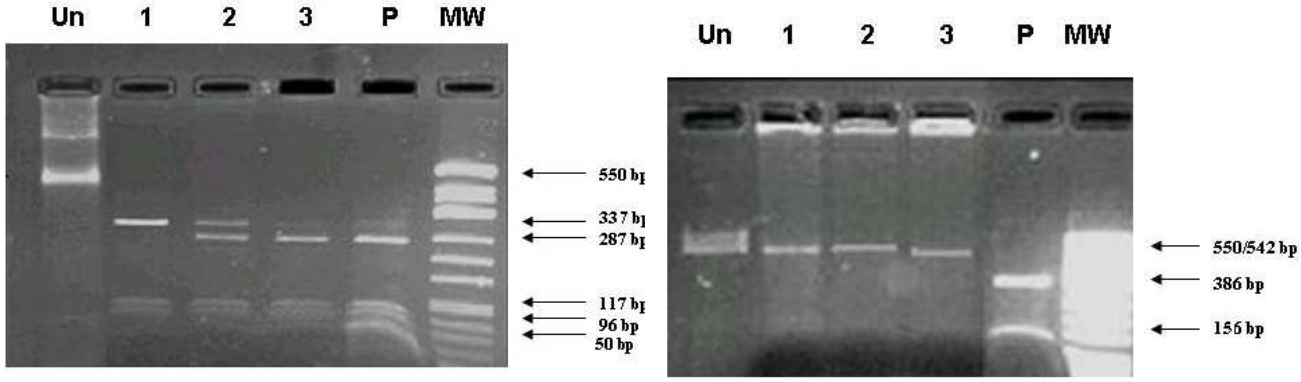
Figure 25 summarizes the results of the discordance observed with gL genotypes in multiple clinical specimens. Discordances between multiple clinical specimens was observed in 5 patients (4 PRTs and 1 HIV infected individuals). Of the 4 PRTs, a pair of blood and urine was collected from 2 patients; two pairs of blood and urine from 1 patient and three samples of urine and one blood from one patient. Of the two pairs of blood and urine collected from 2 PRTs, a mixed genotype (gL3+4) was observed in the urine component with blood negative in one patient, and a mixed genotype (gL3+4) in blood with single genotype of gL4 in the urine of the other patient. In case of the PRT from whom two pairs of blood and urine were collected, gL4 was observed in both samples of urine and second sample of blood, while the first sample of blood was negative for gL gene. The PRT from whom three samples of urine and a sample of blood was collected revealed gL4 genotypes in the first three samples (one blood and two urine) while the final sample of urine was negative for gL gene. The blood and intraocular fluid of the HIV patient revealed mixed genotype (gL3+4) in the blood while gL4 genotype was shed in the intraocular fluid.

Figure 23: Agarose gel electrophoretogram showing the amplified products of gL gene of HCMV

Lanes: N2- Negative control (II round); N1 – Negative control (I round); 1,2,6,8 – Blood specimens negative for gL gene; 3-5, 7,9 – Blood specimens positive for gL gene; 10, 12 – Urine specimens negative for gL gene; 11 – Urine specimen positive for gL gene; PC – Positive control HCMV-AD169; MW – *Hinf I* digest of ϕX-174 DNA

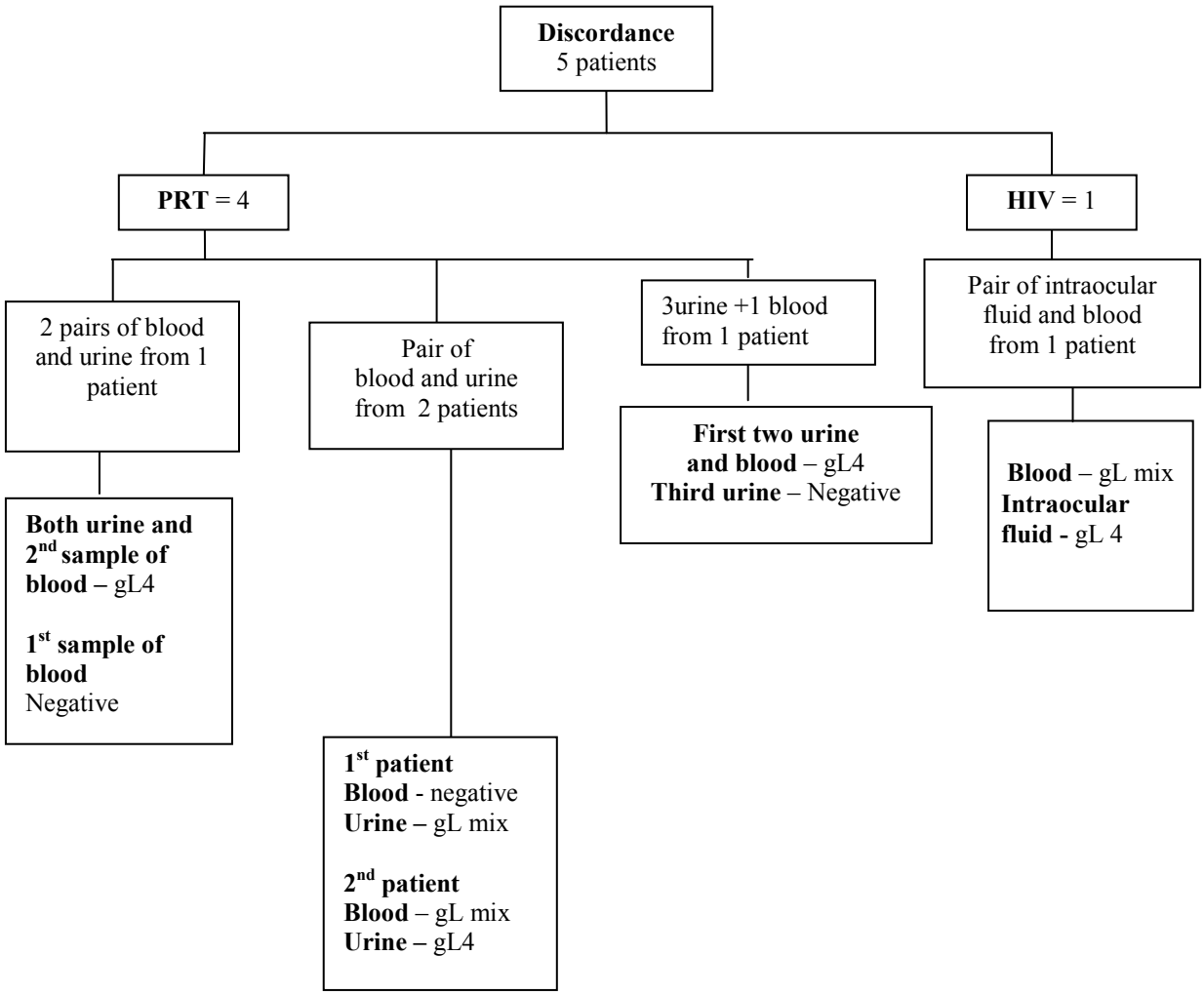
Figure 24: Agarose gel electrophoretogram showing the restriction fragments obtained by nested PCR based RFLP of gL gene of HCMV

(a) **(b)**



(a) ***Rsa I* digests; (b) *Taq I* digests**. Lanes: Un – Undigested product; 1 – Blood specimen (Genotype gL4); 2 – Blood specimen (Genotype gL mix (gL3 + gL4)); 3 – Blood specimen (Genotype gL3); P - Positive control HCMV AD-169 (Genotype gL1); MW – *Hinf I* digest of ϕX-174 DNA

Figure 25: Flow chart showing the discordant results observed with gL genotypes in multiple clinical specimens from 5 patients



7.4.10.3 Specimenwise distribution of gL genotypes

Results of gL genotypes irrespective of the patient group were available for 161 specimens (Blood- 104, Urine- 32, Respiratory fluids –12 and intraocular fluids –13). **Table 21** shows the distribution of gL genotypes in different clinical specimens. A higher frequency of gL4 genotype over gL3 was observed in all groups of specimens. There was no significant difference in the distribution of gL genotypes among the different specimen groups.

7.4.11 Result of validation of mixed gL genotype by DNA sequencing

Figure 26 shows the result obtained by DNA sequencing of the amplicon of gL gene of HCMV from blood specimen that gave gL mix genotype. The mixed bases are seen in the recognition site of Rsa *I* (GT↓AC) enzyme that differentiates gL3 and gL4 genotypes. Multiple sequence alignment of gL3 and gL4 genotypes is also shown.

7.4.12 Results of study on distribution of gCIII complex in 98 immunocompromised patients

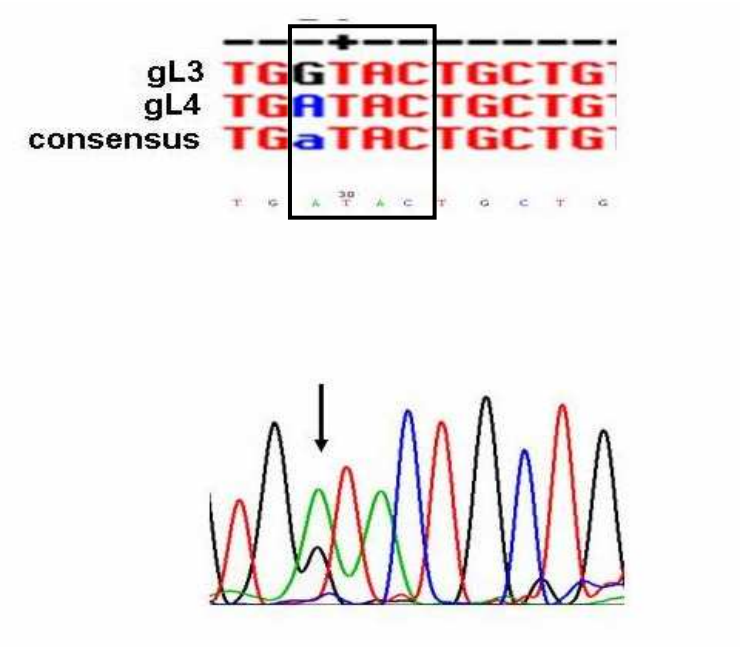
Table 22 shows the distribution of genes of gCIII complex in different patient groups. Sixteen strains of HCMV could be identified with results of the three genes. The number of different strains that existed in each patient population was as follows: PRT – 14 strains; HIV – 8 strains; Infants – 9 strains and BMT – 2 strains. Only 2 strains were represented in all patient groups. A higher frequency of gH1-gO1-gL4 strain was observed irrespective of the patient group. Fifty five percent of the HIV patients carried strains with gH1-gO1-gL4 configuration. There was a

Table 21: Distribution of gL genotypes in different clinical specimens

Genotypes	Clinical specimens				Total
	Blood	***Urine***	***Respiratory fluids***	***Intraocular fluid****	
gL3	31	9	2	4	**46**
gL4	69	22	10	9	**110**
gL mix	4	1	0	0	**5**
Total	**104**	**32**	**12**	**13**	**161**

*All the intraocular fluids were obtained from HIV infected individuals

Figure 26: DNA sequencing of amplicon of nested PCR for gL gene for validation of mixed genotypes obtained by RFLP



Mixed bases at the recognition site of *Rsa I* of gL3+gL4 genotype. The box shows the recognition site of *Rsa I* enzyme The arrow indicates the mixed bases (G andA) at the recognition site. Multiple sequence alignment with consensus sequence is also shown.

Table 22: Distribution of genes of gCIII complex in different patient groups

HCMV strain based on gCIII complex	Patient groups				Total
	PRT	***HIV***	***Infant***	***BMT***	
gH1-gO1-gL4*	10	11	4	1	26
gH1-gO1-gL3	1	0	1	0	2
gH1-gO2-gL4	1	0	1	0	2
gH1-gO2- gL3	1	1	0	0	2
gH1-gO3-gL4	3	0	1	0	4
gH1-gO3-gL3	2	0	0	0	2
gH1-gO4-gL4	7	1	1	0	9
gH1-gO4-gL3	12	1	0	0	13
gH2-gO1-gL4	4	3	4	2	13
gH2-gO1-gL3*	0	0	4	0	4
gH2-gO2-gL3	2	0	0	0	2
gH2-gO2-gL4	1	0	1	0	2
gH2-gO3-gL3	0	1	1	0	2
gH2-gO3-gL4	4	1	0	0	5
gH2-gO4-gL3	2	1	0	0	3
gH2-gO4-gL4	7	0	0	0	7
Total	**57**	**20**	**18**	**3**	**98**

*Distribution of the strains differed significantly between different patient groups

statistically significant difference in the distribution of gH1-gO1-gL4 and gH2-gO1-gL3 strains among the different population (P<0.05). Three strains existed only in PRT group and one strain existed only in infants.

7.4.13 Results of the study on genetic linkages among components of the gCIII complex:

The genotypes of each gene were analyzed with respect to the other genes in the gCIII complex. There was no significant association in the distribution of individual gO, gL genotypes or both together between gH1 or gH2 groups (**Table 23**). When genotypes of gO and gL genes were analyzed irrespective of the gH genotype, a statistically significant association of gO1 with gL4 (P<0.001) and gO4 with gL3 (P<0.05) was observed (**Table 24**).

7.5 DISCUSSION

The relationship between genetic variants of the genes coding the gCIII complex and disease outcome has been the focus of recent studies, because these variants are major targets for virus-neutralizing antibody and are essential for some functions, such as binding to receptors and cell fusion (Rasmussen, 1999).

Limited studies are available showing the different possible genetic configurations of genes associated with gCIII complex in different patient groups (Rasmussen et al., 2002; Rasmussen et al., 2003). Till now there has been no study on the distribution of the genes of gCIII complex in Indian population. Hence, this study was undertaken. The methodology for determining the different variants of the three genes was devised as the first step to study the distribution of the genotypes of

Table 23: Distribution of different gO and gL genotypes irrespective of the patient groups with (a) gH1 and (b) gH2 genotypes

(a)

gL genotypes	gH1				Total
	gO genotypes				
	gO1	gO2	gO3	gO4	
gL3	2	2	2	13	**19**
gL4	26	2	4	9	**41**
Total	**28**	**4**	**6**	**22**	**60**

(b)

gL genotypes	gH2				Total
	gO genotypes				
	gO1	gO2	gO3	gO4	
gL3	4	2	2	3	**11**
gL4	13	2	5	7	**27**
Total	**17**	**4**	**7**	**10**	**38**

Table 24:Distribution of different gO and gL genotypes irrespective of gH genotypes

gO genotypes	gL genotypes		Total
	gL3	gL4	
gO1	6	39*	45
gO2	4	4	8
gO3	4	9	13
gO4	16*	16	32
Total	**30**	**68**	**98**

*A statistically significant association of gO1 with gL4 ($P<0.001$) and gO4 with gL3 ($P<0.05$) was observed.

gCIII complex. The uniplex PCR for gH gene was converted to a semi-nested PCR to achieve optimal analytical sensitivity. The PAGE based RFLP was replaced by an in-house agarose based RFLP for simplifying the process of genotyping. The in-house agarose based RFLP proved to be a better technique than the PAGE based RFLP for genotyping of strain variants of gH gene by being less time consuming, easy to perform and cost effective as the latter requires visualization of products on PAGE gel followed by silver staining. Also, the mixed genotypes identified by the in house agarose based RFLP, might have been missed by the PAGE based RFLP as the family of restriction fragments generated in gH1 and gH2 by the use of *Hha I* enzyme did not differ significantly. In case of the gO and gL genes, since the nested PCRs were available they were adapted for genotyping of the direct clinical specimens. However, the restriction analysis was modified to suit the detection of the fragments on agarose gel.

After standardization of the methods for genotyping, the distribution of the genes of the gCIII complex in various clinical specimens of different patient groups were analyzed individually as well as cumulatively. The analysis revealed no significant differences in the distribution of the gCIII variants based on gender. A predominance of gH1 genotype in PRT and HIV, gO4 in PRT, absence of gL1 and gL2 genotypes and predominance of gL4 genotype irrespective of the patient groups were in disagreement with the limited number of studies that were available**.** (Chou, 1992a; Rasmussen et al., 2002; Rasmussen et al., 2003; Vogelberg et al., 1996). The frequency of the mixed genotypes of the genes of gCIII complex was considerably low compared to the mixed genotypes encountered in the genotyping of gB gene. The presence of mixed genotypes was validated by subjecting the

amplicons to DNA sequencing. In contrary to gB, gO and gL genes, mixed gH genotype was found only in the blood specimens of immuncompromised patients. In case of the former genes, though less frequent mixed genotypes were detected at least in the urine of the patients. Whether the observations have any clinical significance or limited to the difference in the sample size needs to be elucidated.

Considering the two variants of gH, four each of gO and gL the number of theoretical strains with different gCIII configuration would be 32 strains. Taking into account the absence of gL1 and gL2 genotypes in the study, theoretically, 16 strains is expected to be circulating. In reality, all the sixteen strains were represented in the study population irrespective of the patient groups although, certain strains were more represented in a particular patient group than others. Around 55% of the HIV patients harboured the strains with gH1-gO1-gL4 configuration, the significance of the same needs to be deciphered.

In a study by Rasmussen et al (2002) a strong genetic linkage between the gH1 and the gO1; lower frequency of gL2 in gH1 than other gL variants; higher frequency of gO2-gL4 in gH2 than gH1 group were found. The study by Rasmussen et al (2002) also showed a lack of genetic linkage between most of the gL variants with either gH or gO variant. In contrary, in this study the frequency of occurrence of gL3 with gO4 and gL4 with gO1 were statistically significant but no significant difference in the distribution of either gL, gO or both in gH1/gH2 groups were found.

The potential for the gCIII complex to exist in different genetic configurations in clinical strains may have implications for cellular tropism and

provide some insight into the mechanism of the pantropism of CMV in vivo. It is being hypothesized that the different combinations of the gCIII complex may vary in their immunological potential with some combinations favouring immune evasion, while others promoting a vigorous immune response that favours virus eradication.

More studies particularly on cross neutralization of virus strains depending on the variability in the gH, gO, and gL components of the gCIII complex may be required to prove its significance. Report on the genotype specific immunity have been available for gH genotype (Urban et al., 1992). Should the gCIII complex prove to be more important than its single components in the induction of virus-neutralizing antibody, then it will be important to know whether all genetic variants are similarly susceptible to gCIII antibody to a single virus strain. This issue becomes relevant when considering the data showing that preexisting immunity to CMV does not ensure protection from reinfection as evidenced by the presence of mixed genotypes in the study.

8. A STUDY ON THE DISTRIBUTION OF gN GENOTYPES IN DIFFERENT CLINICAL SPECIMENS FROM IMMUNOCOMPROMISED PATIENTS

8.1 BACKGROUND

Glycoprotein N (gpUL73) encoded by the ORF UL73, is a component of the envelope gC-II complex in association with gM (Mach et al., 2000; Dal Monte et al., 2001; Pignatelli et al., 2002) and has the ability to induce neutralizing antibodies (Britt and Auger, 1985). UL73 shows four main genomic variants, denoted gN1, gN2, gN3 and gN4. gN3 and gN4 genotype can be further divided into subgroups gN3a, gN3b and gN4a, gN4b and gN4c respectively (Pignatelli et al., 2001). The UL73 gN-encoding gene is more polymorphic than gB (Pignatelli et al., 2001) and is located just upstream of the UL74 gO gene, a hypervariable locus in HCMV (Paterson et al., 2002). Pignatelli et al (2003a) studied the geographical distribution of gN genotypes of HCMV clinical isolates obtained from regions of Europe, Australia, Northern America and China. Recent studies have shown that gN genomic variants to be related to CMV-induced immunopathogenesis in the immunocompromised host and in congenital infected infants (Pignatelli et al.,2003b; Pignatelli et al., 2003c). No reports have been available on the distribution of gN genotype from India, hence the study was undertaken.

8.2 Objectives

8.2.1 To standardize PCR-based RFLP for gN gene of HCMV

8.2.2 To study the distribution of gN genotypes in clinical specimens by PCR-based RFLP.

8.3 MATERIALS AND METHODS

8.3.1 Standardization of PCR-based RFLP for gN gene of HCMV

8.3.1.1 Semi-nested PCR for gN gene

A semi-nested PCR was standardized for gN gene of HCMV using CMV-AD169 DNA by including an outer forward primer U -73 (Pignatelli et al., 2001) to an already existing uniplex PCR (Pignatelli et al., 2003a). A 50µl reaction mixture was prepared with 160µM of each dNTPs, 10 picomoles of primers U-73 and gN2 (primer sequences are provided in **Table 25)**, 1 x buffer (10mM Tris–HCl 127(pH 8.3), 50mMKCl, 0.01% gelatin, 2.0mMMg$Cl_{2)}$, 1 U of Taq DNA polymerase. Ten µl of DNA was used as the template. The thermal profile followed is as shown in **Table 25.** The second round of amplification was carried out with reaction mixture similar to the first round with 10 picomoles each of primers gN1 and gN2. Five µl from the first round amplicon was used as the template for the second round of amplification. The thermal profile followed for the second round is provided in **Table 25**. Amplicons were analyzed by electrophoresis on a 2% agarose gel incorporated with ethidium bromide (Appendix II). Sensitivity and specificity of the PCR was determined as described in Appendix II.

8.3.1.2 Standardization of RFLP of gN gene product

Ten µl of unpurified second round amplicon of HCMV AD 169 was subjected to digestion with *Sal I*, *Sac I* and *Sca I* in separate reactions (25 µl). The concentration of the enzyme, buffer composition and temperature for incubation used are provided in Appendix II. A control reaction containing the amplicon and

Table 25: Primer sequences, thermal profile and amplicon size for PCR targeting gN genes of HCMV

GENE	PRIMER SEQUENCES	THERMAL PROFILE	AMPLICON SIZE
gN	U73: 5'ttcggtcggtcaacatcgtaag 3' gN1: 5'tggtgtgatggagtggaac3' gN2:5'-tagcctttggtggtggttgc3'	I round: (30 cycles) 94°C - 5 minutes 94°C - 45 seconds 55°C - 45 seconds 72°C - 45 seconds 72°C - 10 minutes II round: (20 cycles) Profile Same as I round	I round 453bp II round 411-420bp

buffer was included during each digestion. The products were visualized on a 4% agarose gel incorporated with ethidium bromide (Appendix II).

8.3.2 Distribution of gN genotypes in different clinical specimens from immunocompromised patients

8.3.2.1 Clinical specimens and patients

The same group of clinical specimens and patients included for the analysis of gO genotypes (section 7.3.7.1) was subjected to analysis of gN genotypes.

8.3.2.2 PCR based RFLP for gN gene

PCR based-RFLP was carried out on the extracted DNA of the clinical specimens as described in section 8.3.1. The results were analyzed similar to gB gene (section 6.3.6.3).

8.3.3 Validation of mixed genotypes of gN gene by DNA sequencing

A blood specimen that gave mixed genotypes by PCR-based RFLP for gN gene was subjected to DNA sequencing. The DNA sequencing was performed as described in Appendix II.

8.4 RESULTS

8.4.1 Results of standardization of seminested PCR-based RFLP of gN gene of HCMV

The size of the first round and second round amplicons with AD-169 DNA were 453 bp and 420bp respectively. The PCR was specific for HCMV and

sensitive enough to pick up 50fg of HCMV DNA. The RFLP of the gN PCR product of AD169 revealed the following restriction fragments: 297, 123bp with *Sac I* and remained undigested with *Sal I* and *Sca I* and belonged to gN1 genotype.

8.4.2 Results of the study on distribution of gN genotypes in immunocompromised patients

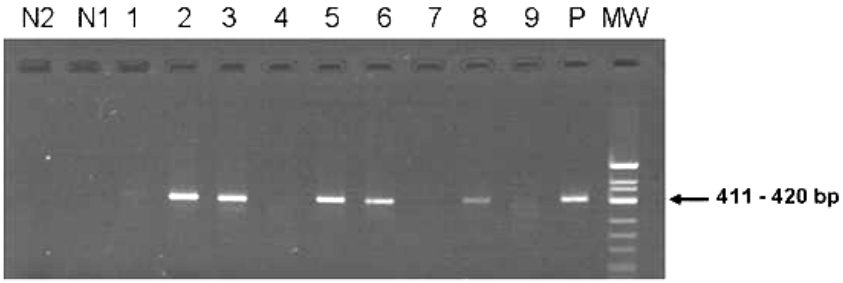
8.4.2.1 Patientwise distribution of gN genotypes

The different RFLP patterns observed with the different gN genotypes is provided in Appendix II. Genotyping results for the gN gene was available for 157 (88.2 %) of 178 patients analyzed. The 157 patients consisted of 112 PRTs, 21 infants, 20 HIV infected individuals and 4 BMT. Discordances between multiple clinical specimens were observed in 2 patients (one each of PRT and HIV infected individuals). The distribution of gN genotypes were analyzed in 155 patients (111PRTs, 19 HIV infected individuals, 21 infants and 4 BMTs). **Table 26** shows the distribution of gN genotypes in different patient groups. Single genotypes were observed in 152 (98.1 %) of 155 patients and mixed genotypes were observed in 3 (1.9 %) of 155 patients. Among the single genotypes a predominance of gN4c genotype was seen irrespective of the patient groups. There was a statistically significant difference in the frequency of gN4c among the different patient groups ($P<0.05$). gN2, gN3b and gN mix were found only in PRT group. There was a higher frequency of males among the patients. There was no difference between the distribution of gN genotypes between males (n = 112) and females (n = 43) in the 155 patients. **Figure 27** shows the amplified products of PCR for gN gene. The restriction pattern observed in different clinical specimens is shown in **Figure 28.**

Table 26: Distribution of gN genotypes in different patient groups

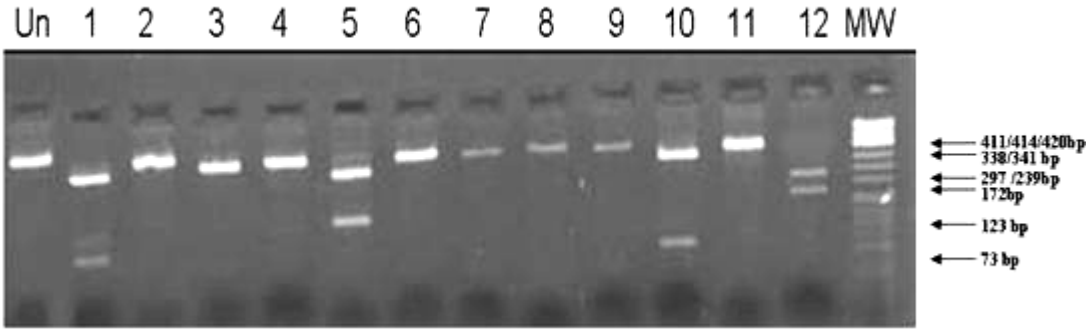
gN genotypes	Patient groups				Total
	PRT	***HIV***	***Infants***	***BMT***	
gN1	21	2	6	1	**30**
gN2	2	0	0	0	**2**
gN3a	12	2	3	0	**17**
gN3b	8	0	0	0	**8**
gN4a	9	3	2	0	**14**
gN4b	19	0	1	0	**20**
gN4c	37	12	9	3	**61**
gNmix	3	0	0	0	**3**
Total	**111**	**19**	**21**	**4**	**155**

Figure 27 : Agarose gel electrophoretogram showing the amplified products of PCR for gN gene of HCMV



Lanes: N2- Negative controls (II round); N1 – Negative control (I round); 1,4,7,9 – Blood specimens negative for gN gene; 2,3,5,6 – Blood specimens positive for gN gene ; 8- Urine positive for gN gene; P – Positive control HCMV – AD169; MW – *Hinf I* digest of ϕX-174 DNA

Figure 28: Agarose gel electrophoretogram showing RFLP products of gN gene of HCMV



Lanes: Un - Undigested product; 1 – *Sal I* digest (Genotype gN4b); 2 – *Sac I* digest (Genotype gN 4b); 3 - *Sca I* digest (Genotype gN 4b); 4 – *Sal I* digest (Genotype gN1) 5 – *Sac I* digest (Genotype gN 1); 6- *Sca I* digest (Genotype gN 1); 7 – *Sal I* digest (Genotype gN3a); 8 – *Sac I* digest (Genotype gN3a); 9- *Sca I* digest (Genotype gN3a) 10 – *Sal I* digest (Genotype gN4c); 11 – *Sac I* digest (Genotype gN4c) ; 12- *Sca I* digest (Genotype gN4c); MW – *Hinf I* digest of ϕX – 174 DNA

8.4.2.2 Discordance in gN genotypes between multiple clinical specimens

Figure 29 summarizes the results of the discordance observed with gN genotypes in multiple clinical specimens. Discordance between multiple clinical specimens was observed in 2 patients (one each of PRT and HIV infected individual). In case of a pair of blood and urine from PRT, gN4c genotype was observed in blood while the urine was negative. The blood and intraocular fluid of the HIV patient revealed mixed genotype in the blood while gN4b genotype was shed in the intraocular fluid.

8.4.2.3 Specimenwise distribution of gN genotypes

Results of gN genotypes irrespective of the patient group was available for 194 specimens (Blood- 134, Urine- 34, Respiratory fluids –13 and intraocular fluids –13). **Table 27** summarizes the distribution of gN genotypes in different clinical specimens. A higher frequency of gN4c genotype over other genotypes was observed irrespective of the clinical specimens. There was no significant difference in the distribution of gN genotypes between the different specimen groups.

8.4.3 Results of DNA sequencing of mixed genotype of gN gene of HCMV

Figure 30 shows the result obtained by DNA sequencing of the amplicon of gN gene of HCMV from blood specimen that gave gN mix genotype. The mixed bases are seen in the recognition sites of Sca *I* (AGT↓ACT). The *Sal I* and *Sac I* also showed mixed bases which were not clear due to the frame shifts that occurred due to the deletion of bases before the restriction sites. The mixed genotype was found to be gN1 + gN4c.

Figure 29: Flow chart showing the discordant results observed with gN genotypes in multiple clinical specimens from 2 patients

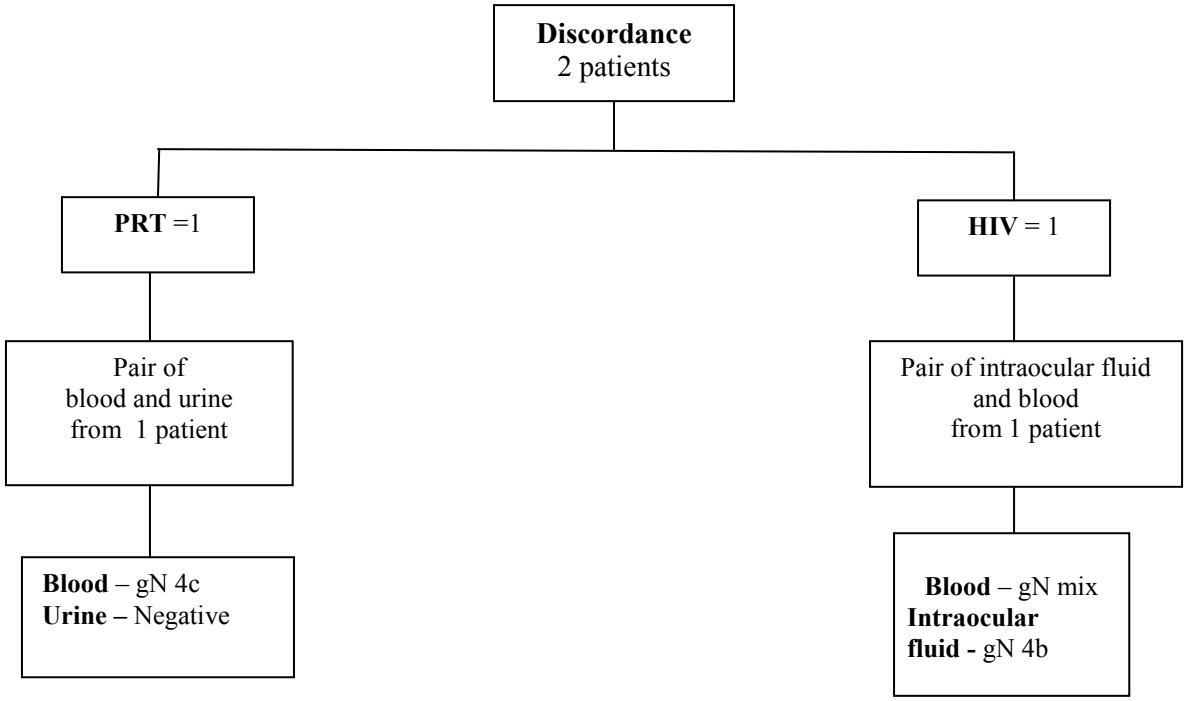
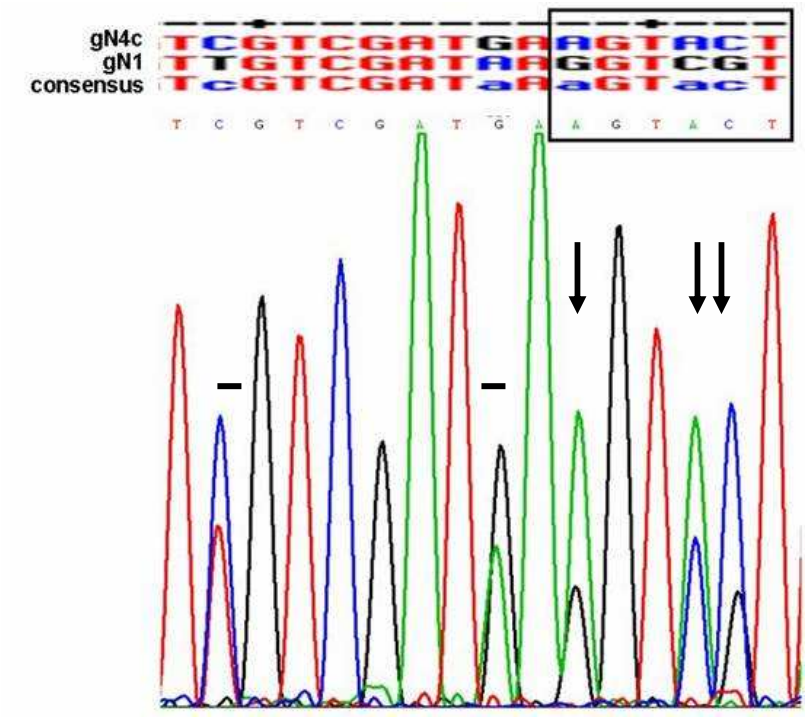


Table 27: Distribution of gN genotypes in different clinical specimens

gN genotypes	Patient groups				Total
	Blood	*Urine*	*Respiratory fluid*	*Intraocular fluids**	
gN1	24	8	4	2	**38**
gN2	2	0	0	0	**2**
gN3a	15	4	0	1	**20**
gN3b	8	0	0	0	**8**
gN4a	12	3	1	3	**19**
gN4b	19	2	0	1	**22**
gN4c	50	17	8	6	**81**
gNmix	4	0	0	0	**4**
Total	**134**	**34**	**13**	**13**	**194**

* All the intraocular fluids were obtained from HIV infected individuals.

Figure 30: DNA sequencing of amplicon of nested PCR for gN gene for validation of mixed genotypes obtained by RFLP



Mixed bases at the recognition site of *Sca I*. The box shows the recognition site of *Sca I*. The arrows represent mixed bases at the recognition site and bars (-) represent mixed bases outside the recognition site. Multiple sequence alignment with the consensus sequence is also shown.

8.5 DISCUSSION

The polymorphic locus ORF -73 has been the next best studied loci after gB and gH. Previous reports (Mach et al., 2000) have demonstrated that gN associates with its glycosylated counterpart, gM, forming a highly immunogenic envelope complex, gCII (Kari et al., 1986). It has been shown that gM is highly conserved (Lehner et al., 1991) and that the polymorphic gpUL73 (gN) could be the main protein responsible for the immunogenicity of gCII. UL73 also seems to be more variable than gB in the HCMV genome (Pignatelli et al., 2001), and hence proven to be an efficient marker for epidemiological studies. Studies are emerging from all regions of the world on the association of gN genotypes with clinical outcome of HCMV. In this study, an existing uniplex PCR-based RFLP was converted to a semi-nested PCR based RFLP for anlaysing the distribution of gN genotypes of HCMV from direct clinical specimens. All the gN genotypes known till now was represented in the study population. When the distribution was considered as gN1, gN2, gN3 and gN4 the distribution of the genotypes irrespective of the patient groups were 19.4%, 1.3%, 16.1% and 61.3% respectively. The frequencies of the genotypes of this study is in accordance to the previous studies suggesting that in immunocompromised patients with active HCMV infections, the gN4 genotype was the most common and gN 2 was the rarest (Pignatelli et al., 2003a; Pignatelli et al., 2003 b). Considering the geographical distribution of the gN genotypes, the distribution of the gN genotypes in this study is in accordance with that of Europe (Pignatelli et al., 2003a). The difference in the frequency of occurrence of gN2 genotype was observed with other regions of the world. In this study gN2 occurred in a low frequency of 1.3%. A total absence of gN2 was seen in the clinical strains

from Australia and China while the same was well represented in the Northern America in the study by Pignatelli et al (2003a). The study showed a higher frequency of gN4c subtype irrespective of the specimens analyzed. There was no statistically significant difference in the distribution of gN genotypes in different clinical specimens though a statistically significant difference in the distribution of gN4c among the patient groups was seen. The genotypes gN2 and gN3b occurred only in the PRT group in this study. Whether the finding has any significance or limited to the difference in the sample sizes of different patient groups needs to be further explored though these genotypes were encountered in intrauterine infections by Pignatelli et al (2003b). There has been reports on the prevalence of gN1 genotype in HIV patients as these strains seem to replicate favourably in these immunocompromised hosts (Pignatelli et al., 2003c), however this study did not confirm the finding since gN1 genotype occurred at a frequency of 10.3% in HIV patients with 63% of HIV patients infected with gN4c genotype. There has been a consensus in the association of gN4 genotype with severe clinical outcome of HCMV disease in immunocompromised patients and gN1 with more favourable outcome (Pignatelli et al., 2003b). Such conclusions could not be arrived at due to unavailability of well documented clinical details of the patients. Studies have also shown that gN1 was detected at a higher rate in the monocytes of immunocompetent patients with latent HCMV infections (Pignatelli et al., 2006) and gN4b was more associated with a peak pp65 antigenemia in patients (Rossini et al., 2005). Such analysis was out of scope of the present study. The association of a specific gN genotype with levels of pp65 could not be performed due to short supply of the monoclonals during the study, also, the cross sectional nature of the study was a

major limitation in establishing such conclusions. In this study, the discordant results were limited to two patients, of which the HIV patient showed a mixed genotype in blood, shedding a single genotype in the intraocular fluid. Two different strains of gN genotypes have been detected in saliva and urine in a patient by Pignatelli et al (2001). Hence, it can be concluded that in accordance with the recent studies, gN is more polymorphic than the other loci of HCMV genome studied till now and all the genotypes of gN known till now are represented in the Indian population as well.

9. A STUDY ON THE DISTRIBUTION OF gB-gCIII-gN VARIANTS IN IMMUNOCOMPROMISED PATIENTS AND A STUDY ON THE LINKAGE OF THE GENE COMPONENTS

9.1 BACKGROUND

Several investigators have associated polymorphisms in single loci of HCMV genomes to cell tropism and pathogenesis of HCMV in different patient groups. (Bale et al., 2000; Barbi et al., 2001; Trincado et al., 2000). However, other investigators have disapproved such associations and related it to some other loci of HCMV genome. Considering the large variety of virus–host interactions that are mediated by the numerous viral gene products, it seems difficult to predict most significant CMV gene influencing a defined clinical situation. It is being hypothesized that CMV pathogenesis may involve interactions of multiple genes. Use of multiple genetic markers have been suggested to be more useful in predicting the outcome of CMV infection (Rasmussen et al., 2003). Also, since certain studies show genetic linkages between the genes of the three glycoprotein complexes a study was taken up to analyze the distribution and presence of linkage in the genetic markers such as gB, gH, gO, gL and gN in different immunocompromised patient groups.

9.2 OBJECTIVE

9.2.1 To study the distribution of HCMV variants with respect to gB, gCIII and gN genes.

9.2.2 To study the possible genetic linkages with respect to gB, gCIII and gN variants.

9.3 MATERIALS AND METHODS

9.3.1 Study design: Retrospective study.

9.3.2 Study Population

9.3.2.1 Inclusion criteria for the analysis

Patients who had a positive result and harbored a single genotype for all the five genes viz. gB, gH, gO, gL and gN. In case of patients whose multiple specimens showed concordant results, one of the specimens was chosen for analysis. In case of patients, whose multiple specimens showed discordant results, the specimen showing single genotype for all the five genes, if available, was considered.

9.3.2.2 Exclusion criteria for the analysis

The patients who had a negative result for at least one gene.

The patients who had a mixed genotype for any of the five genes.

In case of patients, who had a discordant result between clinical specimen, if the multiple clinical specimens showed the mixed genotype or negative results, they were excluded.

9.3.2.3 Details of Patients included in the analysis

Seventy one immunocompromised patients (Post renal transplant - 36, HIV infected individuals - 15, infants - 18, Bone marrow transplants - 2) were included in the analysis. These patients were selected from a group of 178 patients (section 7.3.7.1) based on the criteria for analysis.

9.3.2.4 Analysis of results

The patientwise distribution of HCMV variants was analyzed. In case of genetic linkages the results obtained were subjected to pairwise analysis. The results were cross tabulated. Chi-square test was performed to find any significant linkage between the variants of different genes. P value ≤ 0.05 was considered significant.

9.4 RESULTS

9.4.1 Patientwise distribution of HCMV variants with respect to the five genes

Forty eight strains of HCMV could be identified with results of the five genes. The number of different strains that existed in each patient population was as follows: PRT – 29 strains; HIV – 11 strains; Infants – 14 strains and BMT – 2 strains. There was not a single strain that was represented in all the patient groups. Of the 48 strains, 25 strains were exclusively detected in PRT, 9 in infants and 7 in HIV patient group. Only 7 strains were represented in more than one patient group. There was no significant association of HCMV strains with any patient population. **Table 28** shows the different strains encountered in different patient groups.

9.4.2 Genetic linkages among gB, gN and gCIII complex

For simplification, a pairwise analysis was carried out. Statistically significant associations were seen in the distribution of gB3 with gL3; gN3b with gH2; gN 4b with gH1; gO1 with gL4 and gO4 with gL3; gO2 with gN1 and gO3 with gN4c ($P<0.05$). The results are shown in the cross tabulations. (**Table 29 (a to j)**.

Table 28: Distribution of 48 different HCMV strains in different patient groups

HCMV strains based on five genes of HCMV	Patient groups				Total
	PRT	HIV	Infant	BMT	
gH1-gO1-gL4-gN4c-gB1*	Nil	2	2	1	5
gH1-gO1-gL4-gN3a-gB1	Nil	Nil	2	Nil	2
gH1-gO1-gL4-gN4c-gB2*	1	2	Nil	Nil	3
gH1-gO1-gL4-gN4a-gB1	1	Nil	Nil	Nil	1
gH1-gO1-gL4-gN4a-gB3	1	Nil	Nil	Nil	1
gH1-gO1-gL4-gN4b-gB2	2	Nil	Nil	Nil	2
gH1-gO1-gL4-gN4a-gB2	Nil	2	Nil	Nil	2
gH1-gO1-gL4-gN3a-gB2	1	Nil	Nil	Nil	1
gH1-gO1-gL3-gN1-gB3	Nil	Nil	1	Nil	1
gH1-gO1-gL3-gN1-gB1	1	Nil	Nil	Nil	1
gH1-gO2-gL4-gN1-gB2	Nil	Nil	1	Nil	1
gH1-gO2-gL3-gN1-gB1	Nil	1	Nil	Nil	1
gH1-gO3-gL4-gN4a-gB1	1	Nil	Nil	Nil	1
gH1-gO3-gL4-gN4c-gB2	Nil	Nil	1	Nil	1
gH1-gO3-gL3-gN4c-gB1	1	Nil	Nil	Nil	1
gH1-gO4-gL4-gN4c-gB1*	1	Nil	1	Nil	2
gH1-gO4-gL4-gN1-gB2	Nil	1	Nil	Nil	1
gH1-gO4-gL4-gN3a-gB1	2	Nil	Nil	Nil	2
gH1-gO4-gL4-gN4b-gB3	1	Nil	Nil	Nil	1
gH1-gO4-gL4-gN2-gB3	1	Nil	Nil	Nil	1
gH1-gO4-gL3-gN4a-gB3	Nil	1	Nil	Nil	1
gH1-gO4-gL3-gN4b-gB1	2	Nil	Nil	Nil	2
gH1-gO4-gL3-gN4a-gB1	2	Nil	Nil	Nil	2
gH1-gO4-gL3-gN3a-gB1	1	Nil	Nil	Nil	1
gH1-gO4-gL3-gN4c-gB1	1	Nil	Nil	Nil	1
gH1-gO4-gL3-gN4a-gB2	1	Nil	Nil	Nil	1
gH1-gO4-gL3-gN4c-gB2	1	Nil	Nil	Nil	1
gH1-gO4-gL3-gN4b-gB3	2	Nil	Nil	Nil	2
gH2-gO1-gL4-gN4c-gB1*	Nil	Nil	2	1	3
gH2-gO1-gL4-gN4c-gB2*	Nil	2	1	Nil	3
gH2-gO1-gL4-gN4c-gB3	Nil	Nil	1	Nil	1
gH2-gO1-gL4-gN3a-gB2*	1	1	Nil	Nil	2
gH2-gO1-gL4-gN1-gB3	1	Nil	Nil	Nil	1
gH2-gO1-gL4-gN4a-gB2	2	Nil	Nil	Nil	2
gH2-gO1-gL3-gN4a-gB3	Nil	Nil	1	Nil	1
gH2-gO1-gL3-gN1-gB1	Nil	Nil	1	Nil	1
gH2-gO1-gL3-gN1-gB3	Nil	Nil	2	Nil	2
gH2-gO2-gL4-gN1-gB1*	1	Nil	1	Nil	2
gH2-gO2-gL3-gN3b-gB2	1	Nil	Nil	Nil	1
gH2-gO3-gL4-gN4c-gB1	1	Nil	Nil	Nil	1
gH2-gO3-gL4-gN4c-gB3	Nil	1	Nil	Nil	1
gH2-gO3-gL3-gN4c-gB3	Nil	1	Nil	Nil	1
gH2-gO3-gL3-gN4a-gB2	Nil	Nil	1	Nil	1
gH2-gO4-gL4-gN3b-gB1	1	Nil	Nil	Nil	1
gH2-gO4-gL4-gN4a-gB2	1	Nil	Nil	Nil	1
gH2-gO4-gL4-gN4c-gB1	1	Nil	Nil	Nil	1
gH2-gO4-gL3-gN3b-gB1	2	Nil	Nil	Nil	2
gH2-gO4-gL3-gN3a-gB3	Nil	1	Nil	Nil	1

* Strains represented in more than one patient group

Table 29: Results of pairwise analysis of different glycoprotein genes to ascertain genetic linkage

(a) gH vs gO

gH genotype	gO genotype				Total
	gO1	gO2	gO3	gO4	
gH1	19	2	3	18	**42**
gH2	15	3	5	6	**29**
Total	**34**	**5**	**8**	**24**	**71**

(b) gH vs gL

gH genotype	gL genotype		Total
	gL3	gL4	
gH1	15	27	**42**
gH2	9	20	**29**
Total	**24**	**47**	**71**

(c)gH vs gB

gH genotype	gB genotype			Total
	gB1	gB2	gB3	
gH1	22	13	7	**42**
gH2	12	9	8	**29**
Total	**34**	**22**	**15**	**71**

(d) gL vs gO

gL genotype	gO genotype				Total
	gO1	gO2	gO3	gO4	
gL3	6	2	3	13*	**24**
gL4	28*	3	5	11	**47**
Total	**34**	**5**	**8**	**24**	**71**

* Association of the genotypes were statistically significant ($P < 0.05$, Chi-square test).

(e) gL vs gB

gL genotype	gB genotype			Total
	gB1	gB2	gB3	
gL3	11	4	9*	**24**
gL4	23	18	6	**47**
Total	**34**	**22**	**15**	**71**

(f) gB vs gO

gB genotype	gO genotype				Total
	gO1	gO2	gO3	gO4	
gB1	13	3	4	14	**34**
gB2	14	2	2	4	**22**
gB3	7	0	2	6	**15**
Total	**34**	**5**	**8**	**24**	**71**

(g) gN vs gL

gN genotype	gL genotype		Total
	gL3	gL4	
gN1	6	5	**11**
gN2	0	1	**1**
gN3a	2	7	**9**
gN3b	2	2	**4**
gN4a	6	7	**13**
gN4b	4	3	**7**
gN4c	4	22	**26**
Total	**24**	**47**	**71**

(h) gN vs gH

gN genotype	gH genotype		Total
	gH1	gH2	
gN1	5	6	**11**
gN2	1	0	**1**
gN3a	6	3	**9**
gN3b	0	4*	**4**
gN4a	9	4	**13**
gN4b	7*	0	**7**
gN4c	14	12	**26**
Total	**42**	**29**	**71**

* Association of the genotypes were statistically significant

(P< 0.05,Chi-square test).

(i) gN vs gB

gN genotype	**gB genotype**			**Total**
	gB1	**gB2**	**gB3**	
gN1	5	2	4	**11**
gN2	0	0	1	**1**
gN3a	5	3	1	**9**
gN3b	3	1	0	**4**
gN4a	4	6	3	**13**
gN4b	2	2	3	**7**
gN4c	15	8	3	**26**
Total	**34**	**22**	**15**	**71**

(j) gN vs gO

gN genotype	**gO genotype**				**Total**
	gO1	**gO2**	**gO3**	**gO4**	
gN1	6	4*	0	1	**11**
gN2	0	0	0	1	**1**
gN3a	5	0	0	4	**9**
gN3b	0	1	0	3	**4**
gN4a	6	0	2	5	**13**
gN4b	2	0	0	5	**7**
gN4c	15	0	6*	5	**26**
Total	**34**	**5**	**8**	**24**	**71**

* Association of the genotypes were statistically significant ($P < 0.05$, Chi-square test).

9.5 DISCUSSION

Several epidemiologic studies have focused on the importance of variation in the glycoprotein genes as a factor in the ability of HCMV to infect various cells in vivo and to cause multiple organ manifestations. However, the conclusions of studies seeking an association of either gB, gN or variants of gCIII complex with either the outcome of CMV infection or to cellular tropism are inconsistent. Demographic characteristics of infected individuals have been attributed as one of the factor that may explain some of the discrepancies. For example, the association of gB genotype 2 with retinitis was related to a high prevalence of gB2 among patients with AIDS (Zipeto et al., 1998).

Taking into consideration the existing numbers of variants for each gene, theoretically 1120 strains of HCMV must exist. Considering the absence of certain genotypes such as gB4, gB5, gL1 and gL2 in the study population as shown in preceding sections; there was a possibility of 336 strains in the study population. Forty eight (14.3%) of these 336 strains were found in the analysis of all the five genes. The lower representation of the number of different strain is attributable to the inadequate sample size and the study design. In the beginning of the analysis there were 178 patients (study population provided in section 7.3.7.1) for whom genotyping results for all the genes were available. Of which, 107 had to be excluded due to non – compliance to the inclusion criteria. Little more than half of these 107 harboured mixture of genotypes in the specimens analyzed. One of the most important finding was that mixed genotypes in any loci was not observed in the

infants. It may be hypothesized from the finding that increase in age may be directly proportional to a higher re-infection episodes.

The study also revealed the presence of certain strains exclusively in a particular patient group. Whether the observation would hold good with an increasing sample size is a question in itself. But, the result suggests that infinite number of strains may circulate at a given period and any conclusion drawn based on single loci of the genome with regard to its biological properties may not be of much utility considering the huge genomic background of HCMV. At the start of the study it was hypothesized that any variation in a single loci of HCMV genome may not reflect changes occurring in other loci of the genome and genetic linkages were rare. Earlier studies in this ground have shown that genetic linkages are seen among the clinical isolates with respect to the glycoprotein genes. Genotypes gB 2 and gB4 have been shown to occur frequently in isolates with gH-1 (Fries et al., 1994), and the gO-1 genotype appears to be linked to the gH-1 genotype in the genes encoding the gCIII complex. Recently, discrete association have been shown between the gO and gN variants in isolates collected from different parts of the world (Mattick et al., 2004). In the present study, significant association were seen between the gO and gL genes of gCIII complex; gO and gN genes; gN with gH and gB with gL. The association between gO, gN and gH may be attributable to the vicinity of the ORFs (gN- UL73; gO – UL74 and gH – UL 75). The associations of gO and gL may be attributable to their function since both their products constitute the gCIII complex. The importance of such associations requires confirmation.

The data in the present study lead to the conclusion that HCMV are highly heterogenous group of viruses and exhibit several genetic combinations, study of a single gene is inadequate for investigating the molecular epidemiology of HCMV as the changes at one genetic locus do not necessarily reflect changes at other sites since genetic linkages are limited. Also, preexisting immunity to a HCMV strain may not protect from reinfection with a new strain as evident from the detection of mixed genotypes in the patient groups. At present, the standard strains AD-169 and Towne have been recommended as vaccine candidates, this means their genetic configuration with respect to the five genes under study would be gB2-gH1-gO1-gL1-gN1 and gB1-gH2-gO4-gL2-gN4b. The genetic configuration of strains similar to AD-169 or Towne was not encountered in the study. However, the biological or functional impact of the variability of these genes requires direct analysis before arriving at any conclusion. One approach is to construct experimentally modified recombinant viruses and to develop *in vitro* systems that can functionally differentiate the genetic variants. Based on the functional differences the genetic variants related to morbid clinical outcomes may be identified and chosen to design vaccine to achieve maximum protection against important HCMV strains.

10. A STUDY ON THE RELATIONSHIP OF HCMV GENOTYPES WITH VIRAL LOADS

10.1 BACKGROUND

Several studies have proved an association of high viral load and HCMV diseases in different immunocompromised patient groups. The CMV load is also a predictor for the development of CMV disease and response to treatment, and a high CMV load is an independent predictor of poor survival in most studies (Boeckh and Boivin, 1998). Independent studies on the association of different HCMV genotypes with clinical outcome of the patients with HCMV disease have also been available. It is still unclear, whether presence of a single HCMV or mixed infections with multiple HCMV strains is associated with an increased frequency of disease. Previous study on mixed gB genotypes with viral load as determined by a semi-quantitative PCR and Real –time PCR has shown that presence of mixed genotypes are associated with a high viral load (Coaquette et al., 2004). Since, the analysis on the loci other than gB can also show the presence of mixed genotype, the present study was undertaken to find if there is any association of mixed genotypes in any loci of HCMV genome with the viral loads in clinical specimens from different patient groups.

10.2 OBJECTIVES

10.2.1 To study the possible relationship of HCMV genotypes with the viral load.

10.2.2 To analyze the distribution of genotypes with respect to the five glycoprotein genes of HCMV in paired clinical specimens obtained from different patient groups.

10.3 MATERIALS AND METHODS

10.3.1 Study on the relationship of genotypes with viral load

10.3.1.1 Clinical specimens and Patients

Seventy three clinical specimens (Peripheral blood -58, Urine - 10, Respiratory fluids - 3, intraocular fluids - 2) from 63 immunocompromised patients (PRT - 56, HIV -2, Infants - 5) were included in the study. Single specimens were obtained from 53 patients (PRT – 50, infants – 3). Multiple specimens were obtained from 10 patients. PRT – 6 (Pair of blood and urine from 6 patients), HIV infected patients – 2 (pair of blood and intraocular fluid from 2 patients) and infants – 2 (a pair of blood and urine from 2 patients). In case of blood specimens the leucocyte fraction and plasma fraction were analyzed separately.

10.3.1.2 Genotyping and Taqman probe based real – time PCR

Multiplex PCR for gB, PCR based RFLP for gH, gO, gL and gN genes were carried out on all the 73 clinical specimens as described in sections 6.3.3; 7.3.1.1; 7.3.3; 7.3.6; 7.3.9 and 8.3.1 respectively. The leucocyte and plasma fractions of the peripheral blood were analyzed separately to determine the genotypes. Taqman probe based Real-time PCR was performed on the 73 clinical specimen as described in the section 5.3.4. In case of the peripheral blood specimens, the Taqman probe based Real-time PCR was applied only on the leucocyte fractions.

10.3.1.3Analysis of results

Specimen wise genotyping results of 73 clinical specimens obtained from 63 immunocompromised patients were compared to the viral load as established by

Taqman probe based Real time PCR. The clinical specimens were grouped into three for simplification of the analysis. Group I consisted of clinical specimens showing positivity for all the five regions with mixed genotypes in at least one region, Group II consisted of clinical specimens with single genotypes for all five regions; Group III consisted of clinical specimens with negativity in one or more regions. The analysis of the genotyping results of leucocyte and plasma fractions of the 58 peripheral blood were performed separately. The distribution of genotypes were categorized as described earlier as Group I, Group II and Group III for both leucocyte and plasma fractions individually. The median viral loads obtained between the groups with respect to leucocyte and plasma fractions were also compared. Mann–Whitney test was used for the statistical analysis of results of genotyping with viral load. The results were expressed as medians and P values <0.05 were considered significant.

10.3.2 Distribution of genotypes in the five glycoprotein genes of HCMV strain in paired clinical specimens obtained from different patient groups

10.3.2.1 Study design and Study population

A retrospective analysis was carried out on paired clinical specimens obtained from different patient groups. Fifty four paired clinical specimens from 27 patients out of the 58 clinical specimens from 29 patients as described in section 7.3.7.1. was included in the analysis. A pair of blood specimens from a PRT and a pair of urine and respiratory fluid from BMT (section 7.3.7.1) were excluded from the analysis. The details of clinical specimens and patients included for the analysis is as follows: Fifty four clinical specimens (Peripheral blood – 27, Urine – 18,

Intraocular fluids – 9) from 27 patients (PRT- 16, Infants – 2 and HIV -9) was carried out. The specimens included pairs of blood and urine from 16 PRTs and 2 infants, pairs of blood and intraocular fluids from 9 HIV infected individuals.

10.3.2.2 Analysis of results

The results of genotyping obtained with blood and paired clinical specimens such as urine or intraocular fluids was analyzed for discordances in the distribution of genotypes with respect to the number (whether discrepancy in single or multiple loci) and nature of genetic loci (whether gB/gCIII/gN) and variety of discrepancy (mixed genotype in one with single genotype in other etc.).

10.4 RESULTS

10.4.1 Comparison of results of specimenwise genotyping with viral load on the 73 clinical specimens from 63 patients

The results obtained with the three groups of clinical specimens based on the genotypes in five genes of HCMV and the median number of viral copies/ml and median $\log_{10}$ copies/ml as determined by the real-time PCR are shown in **Table 30.** The median number of viral copies/ml differed significantly between the groups ($P < 0.0001$, Mann–Whitney test) (**Figure 31**).

10.4.2 Comparison of genotyping results of leucocyte and plasma fraction of peripheral blood for genotyping of the five genetic loci of HCMV

Table 31 shows the distribution of genotypes with respect to the groups described in section 10.3.1.3 between plasma and leucocyte fraction of peripheral

Table 30: Results of 73 clinical specimens from 63 immunocompromised patients showing the comparison of three groups of clinical specimens with viral load as ascertained by Taqman probe based real-time PCR

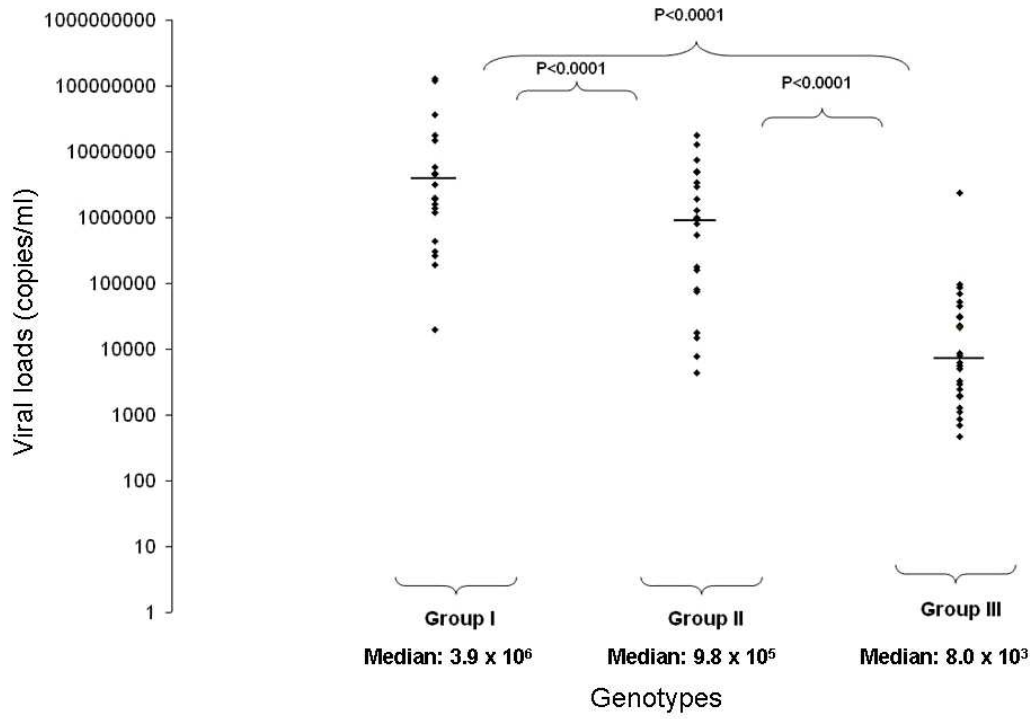
Group of clinical specimen	No. of clinical specimens	Median copy number/ml (Median $\log_{10}$ copy number/ml) by Real-time PCR
Group I	22	3.9×10^6 (6.5911)
Group II	23	9.8×10^5 (5.9912)
Group III	28	8.0×10^3 (3.9031)

The difference between the median viral copies/ml present in different groups of clinical specimens is statistically significant ($P<0.0001$). Group I consists of clinical specimens positive in all genes with mixed genotype in at least one gene, Group II consists of clinical specimens with single genotypes in all genes, Group III consists of clinical specimens with negativity at least in one gene

Table 31: Distribution of genotypes between leucocyte and plasma fractions of 58 peripheral blood

Genotypes determined from blood plasma	Genotypes determined from blood leucocytes			Total
	Group I	***Group II***	***Group III***	
Group I	5	0	0	**5**
Group II	12	3	0	**15**
Group III	3	13	22	**38**
Total	**20**	**16**	**22**	**58**

Figure 31: Comparison of viral load with distribution of genotypes in 73 clinical specimens from 63 immunocompromised patients



Bars indicate the median values. The difference between the median viral loads between the groups was statistically significant. (P<0.0001, Mann Whitney test).

blood specimens from 58 patients. Discrepancy with respect to the detection of mixed genotypes (Group I) and single genotypes (Group II) were observed between the leucocytes and plasma fraction of the peripheral blood samples from the patients. Seventy five percent of mixed genotype (Group I) as evident from the leucocyte fractions were characterized as single genotype (Group II) or negative (Group III) when the plasma fraction was analyzed. Sixteen (42.1%) of 38 clinical specimens that had a mixed or single genotype as per the analysis of leucocyte fraction was classified as negative when plasma fraction was analysed. The results of genotyping obtained with the three groups between the plasma and leucocyte fractions and the median number of viral copies/ml and median $\log_{10}$ copies/ml as determined by the real-time PCR applied on leucocyte fractions of the 58 peripheral blood is provided in **Table 32.** The median number of viral copies/ml was compared between each of the three groups for plasma and leucocyte fractions of peripheral blood. The median values differed significantly between each group of plasma and leucocyte fractions ($P < 0.0001$ for group I and group II; $P<0.01$ for group III, Mann–Whitney test) (**Figure 32**).

10.4.3 Results of genotyping between blood and paired clinical specimens

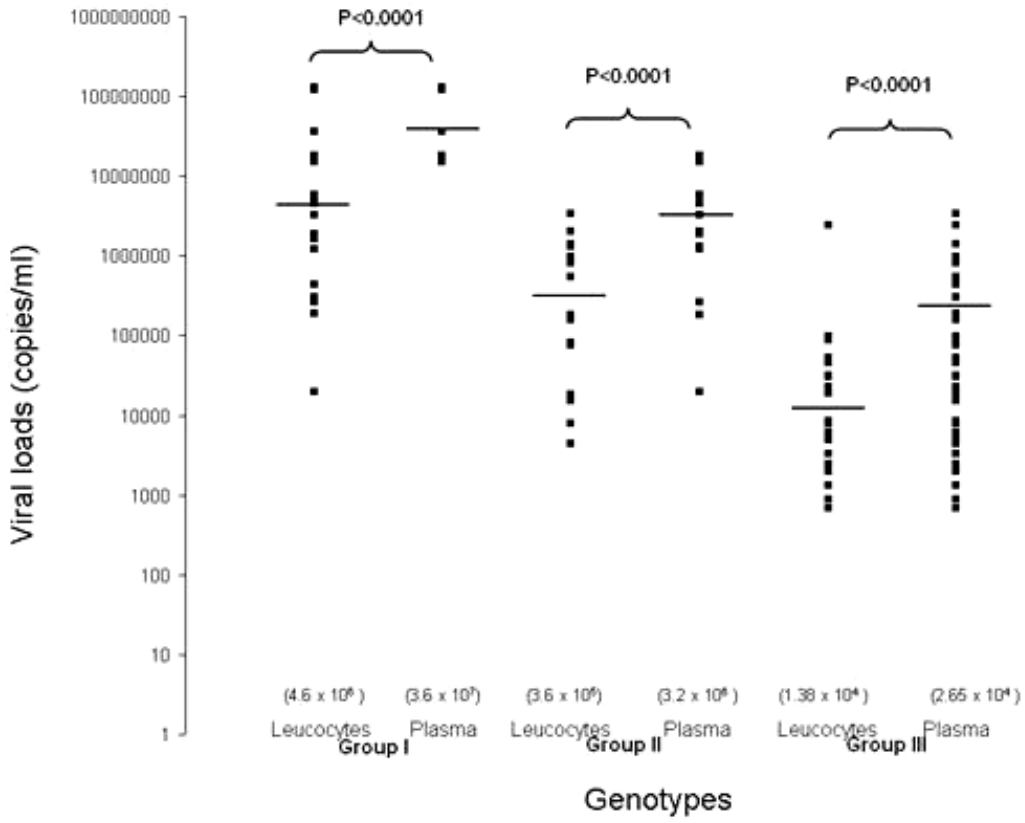
Concordant results between all the loci analyzed were obtained in case of 10 (37.0%) of 27 patients (PRT- 4; HIV- 4 and Infants – 2). **Table 33** shows the discordance in the distribution of genotypes with respect to the number and nature of genetic loci in PRT (n = 12) and HIV (n = 5) group. A variety of discrepancies were observed that included detection of mixed genotypes in one specimen with detection

Table 32: Results of comparison of the viral load with genotypes between plasma and leucocytes fractions of peripheral blood of 58 patients

Group of clinical specimen	No. of clinical specimens		Median copy number/ml (Median log_{10} copy number/ml) by Real-time PCR	
	Leucocytes	*Plasma*	*Leucocytes*	*Plasma*
Group I	20	5	$4.6x10^6$ (6.6628)	$3.6x10^7$ (7.5563)
Group II	16	15	$3.6x10^5$ (5.5563)	$3.2x10^6$ (6.5052)
Group III	22	38	$1.38x10^4$ (4.1399)	$2.65x10^4$ (4.4232)

The difference between the median viral copies/ml present within the groups between the plasma and leucocyte fractions was significant (Group I and Group II $P<0.0001$; Group III $P<0.01$). Group I consists of clinical specimens positive in all genes with mixed genotype in atleast one gene, Group II consists of clinical specimens with single genotypes in all genes, Group III consists of clinical specimens with negativity at least in one gene.

Figure 32: Comparison of viral loads with distribution of genotypes in leucocyte and plasma fractions of 58 peripheral blood specimens



Bars indicate the median values. The difference between the median viral loads within groups between plasma and leucocyte fraction was statistically significant (P<0.0001, Mann Whitney test). The actual median for each group is shown within brackets ( ).

Table 33: Discordances in the distribution of genotypes with respect to the number and nature of genetic loci in 17 patients (PRT-12; HIV-5)

Number and nature of loci		Patient group	
		PRT	HIV
One	gB	3 (2)	2 (2)
	gH	1 (0)	0
	gO	2 (2)	0
	gL	0	1(1)
Two	gB & gH	1*	0
	gB& gO	1(1)	1(1)
	gB & gN	1(0)	0
	gH & gL	1(1)	0
	gH & gO	1**	0
	gO & gL	1**	0
	gH & gN	0	1(1)***
Total		12(9***)	5 (5)

The value in the brackets () represent the number of discrepancy with regard to mixed genotypes in a clinical specimen with single genotype or negativity in the paired clinical specimen.

* Discrepancy with regard to mixed genotypes seen in gB loci alone

** Discrepancy with regard to mixed genotypes seen in loci other than gO

*** Mixed genotype with respect to gB was found in both blood and intraocular fluid

**** Includes the patients who showed discrepancy with regard to mixed genotype in at least one loci

of single genotypes in other; negativity in one specimen with detection of mixed or single genotypes in the other. The distribution of nature of discrepancies in different genetic loci irrespective of patient group is shown in **Figure 33.** All the discrepancies in case of the HIV patient were of a single kind viz. detection of mixed genotypes in blood with detection of single genotypes in the intraocular fluids. Discrepancy with the detection of mixed genotypes was observed in 18 loci of 14 patients (**Table 34**). The results show that mixed genotypes irrespective of the loci was found more in the blood than the paired clinical specimens in PRT and HIV patients.

10.5 DISCUSSION

Prospective studies of immunocompromised patients have shown that CMV viral detection and quantification are useful for identifying those individuals at highest risk for disease but do not consistently predict clinical outcome. Some individuals develop symptomatic CMV disease with a relatively low viral load, while others maintain chronic high-level viremia yet remain free of symptomatic disease. Thus, factors in association with, or in addition to, viral load will affect the development of symptomatic CMV disease. Reports are emerging on relationship of strain differences in HCMV associated with virulence (Torok – Storb et al., 1997; Fries et al., 1994; Shepp et al., 1996). Earlier studies have thrown light on the fact that reinfections with different HCMV strains are common in different patient groups. CMV reinfections have been shown to lead to an accumulation of more than one latent virus in a host (Bale et al., 1996; Chandler et al., 1987). Multiple CMV gB-genotypes have been detected even in the tissues of immunocompetent patients

Figure 33: Distribution of discrepancies in different genetic loci irrespective of patient group

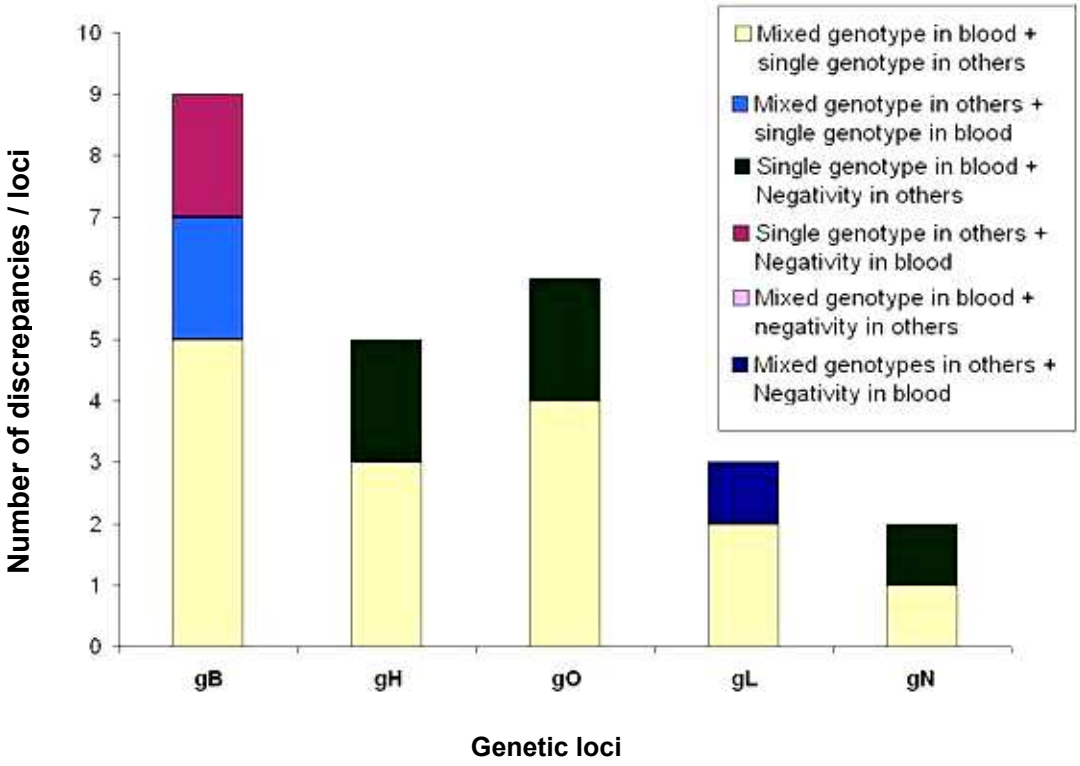


Table 34: Discrepancies in detecting mixed infections at different genetic loci in paired clinical specimens from 9 PRT and 5 HIV patients

Patient group	Number of patients showing discrepancies with respect to detection of mixed genotypes	Number of genetic loci showing discrepancy	Clinical specimens in which mixed genotype was detected	
			Blood	Urine/ Intraocular fluids*
HIV	5	7	7	0
PRT	9	11	8	3
Total	14	18	15	3

*Urine and intraocular fluids were analyzed in case of PRT and HIV respectively.

(Meyer-Konig et al., 1998a, Meyer-Konig et al., 1998b). It is known that HIV patients may harbor a number of different CMV strains, which reactivate especially during stages of significant immunosuppression (Spector et al., 1984). In post – organ transplant recipients a variety of CMV strains may be involved, including strains of the recipient acquired during the course of pre-transplantation life as well as ones that were acquired, possibly in substantial amounts, by receiving an organ from a CMV-seropositive donor. Most of these studies were based on genotyping of single genetic loci, particularly gB gene of HCMV. In this study the results have shown that the mixed infections with multiple genotypes of HCMV are found though at a low frequency in the genetic loci other than gB gene of HCMV. The identification of such mixed genotypes would have been missed if only one gene was studied at a time. In this study, detection of the mixed genotypes in minimum of 6 and maximum of 16 patients would have been missed if only one genetic loci was studied. The order of genetic loci suitable for detection of mixed genotype as confirmed by the study is gB>gO>gH=gL>gN. The higher rate of detection of mixed genotypes in the gB gene may be partly attributed to use of multiplex PCR for detection of genotypes. PCR- RFLP may be less sensitive in detection of the mixed genotypes although, the method was devised with great care for particularly identifying the mixed genotypes by choosing the restriction enzymes that would provide maximum difference between the existing genotypes for a particular genetic locus.

A statistically significant difference was observed in the viral loads as determined by Taqman probe-based real time PCR between the specimens carrying single genotype and multiple genotypes. This finding is in accordance with a

previous study where in identification of mixed infections with multiple gB genotypes of HCMV was associated with an increase in viral load, graft rejection and disease progression (Coaquette et al., 2004). Hence, detection of mixed genotypes in the hypervariable regions of HCMV can be a surrogate marker to an increase in viral load and disease progression.

It becomes increasingly important to identify the right clinical specimen for detection of the mixed genotypes. This study shows that peripheral blood may be more useful in the detection of mixed genotypes than other clinical specimens in case of PRT and HIV. Different fractions of the peripheral blood such as leucocyte, plasma or whole blood have been used by several investigators for the purpose of genotyping. The study shows that the results of genotyping may depend on the fraction of the blood analyzed. The leucocyte fraction of the peripheral blood may be more suitable for identification of the mixed genotypes than the plasma fraction. The analysis of the viral load in the samples revealed that the mixed genotypes were detected in the plasma when the median viral load was greater than that required for their detection in the leucocytes. The utility of whole blood in this regard needs to be deciphered.

The discrepancies observed between the distribution of genotypes in the intraocular fluids and peripheral blood of HIV patients in this study was quite different from that reported in other studies. In studies by Peek et al (1998) and Verbraak et al (1998), total compartmentalization of different CMV genotypes in blood and eye components, for eg. gB4 in eye and gB3 in blood of a patient was observed. The studies hypothesized that the intraocular cytomegalovirus strain was a

persistent CMV genotype of a previous viremia that has already been cleared from the blood attributed to limited ocular penetration of systemically administered ganciclovir in these patients. Both the studies were from developed countries where the treatment modality is different from that in India. Since, intravitreous ganciclovir without systemic administration is the standard therapy for treating CMV retinitis in developing countries, selective elimination of HCMV strain in the eye without being eliminated from the blood could partially answer the presence of mixed genotype in the blood with single genotypes in the intraocular fluids. But, in case of all the five HIV patients in this study, the clinical specimens were collected prior to any intravitreal treatment, hence ruling out the hypothesis. Alternatively, after a first infection with CMV, infection with a new CMV strain can occur, which, although present in blood, might not have reached the eye. If the patient is followed up for a brief period, it might be possible to identify the mixed genotypes in the intraocular fluids also, as seen with one of the HIV patient in this study.

Another interesting observation with regard to the HIV patients showing mixed genotype in blood with single genotypes in intraocular fluid is with respect to pattern of gB genotypes. In all the 3 HIV patients with discrepancies at gB loci, gB1 genotype was associated with blood and gB2 or gB3 were shed in the intraocular fluid. The study on the distribution of the gB genotypes in the intraocular fluids from HIV patients (**Table 14**) revealed 10/13 with gB2 and gB3 genotypes. Also, the plasma fraction of the three blood analyzed from these HIV patients showed the genotypes that were shed in the intraocular fluid. Hence, whether the observation is to be related to tropism or spread of a genotype predominant in the blood to the intraocular fluid needs to be ascertained. However, reports on the association of a

certain CMV genotype in patients with CMV retinitis is ambiguous (Shepp et al., 1996; Rasmussen et al., 1997; Drew et al., 2002; Bongarts et al., 1996).

The discrepancies in finding mixed genotypes in blood and urine components of PRT can also be addressed similarly. A longitudintal study with proper clinical data is advocated to establish whether therapy, or load or tropism has any role to play in such pattern of shedding. Due to the cross sectional nature of the study, lack of clinical data and lower sample size no solid conclusions could be made with such observations.

With respect to the biological aspects of the presence of more than one strain, the presence of mixed strains may allow transcomplementation between the strains with defects or disadvantageous polymorphisms of one virus strain being overcome by the co infecting strains thus greatly enchancing the virus tropism, replication, or immune evasion (Cicin –Sain et al., 2005). It was also observed that recombination events between CMV strains can occur, leading to the generation of recombinant strains (Steininger et al., 2005) that could further influence the evolutionary development of CMV strains.

11. ISOLATION AND CHARACTERIZATION OF VIRAL ISOLATES

11.1 BACKGROUND

Most of the studies in literature on the characterization of HCMV strains utilize nucleotide sequencing of specific genes from HCMV isolates (Rasmussen et al., 2002; Pignatelli et al., 2001; Tanaka et al., 2005). Isolation of the virus from clinical specimen indicates the active replication of the virus *in vitro*. In India, there has been paucity on the isolation of HCMV from clinical specimens due to non-availability of good quality Human diploid fibroblast cell lines. Hence, in this study an attempt was made to isolate HCMV from clinical specimens on Tenon's capsule fibroblast cell line. Nucleotide sequencing of HCMV isolates have been attempted to establish emergence of recombinant strains of HCMV in HIV patients, to screen for novel mutations with respect to antiviral resistance and to establish the evolutionary patterns of clinical strain with respect to prototype strains (Steininger et al., 2005; Lurain et al., 2001; Rasmussen et al., 2002; Pignatelli et al., 2001). In this study, nucleotide sequencing and phylogenetic analysis of clinical isolates and other clinical strains from direct clinical specimens was attempted to validate the results of other genotyping methods (multiplex PCR and PCR-based RFLP) and to establish presence of novel strains if encountered in the study population.

11.2 OBJECTIVE

(i) To isolate HCMV from clinical specimens and to characterize the isolates.

11.3 MATERIALS AND METHODS

11.3.1 Clinical specimens and Patients

Twelve clinical specimens (pairs of peripheral blood leucocytes and urine specimens) from 6 patients were included in the study. All the twelve clinical specimens were subjected to virus isolation. Fifteen other clinical specimens carrying different HCMV strains as revealed by genotyping of the five glycoprotein genes were also included. The details of the fifteen different HCMV strains included in the study are provided in **Table 35.**

11.3.2 Virus isolation

Virus isolation was performed using rapid shell vial technique. Human Tenon's capsule fibroblasts (Dr. Michelle C Madiagan, Save Sight Institute, Sydney) grown on cover slips were inoculated with the clinical specimens. For inoculation, 100µl of peripheral blood leukocyte suspension of blood specimens and 100µl of decontaminated centrifuged deposits of the urine specimens were used. The details of processing of clinical specimens are provided in Appendix I. The cover slips were stained at 48 hours with mouse monoclonal antibody (DAKO, A/S, Denmark) raised against the early antigen of HCMV and rabbit anti-mouse fluorescein iso thiocyanate conjugate (DAKO, A/S, Denmark). One or more positive fluorescent nuclei indicated positive result. The test was carried out in duplicates. One of the shell vial was used for confirming the presence of the virus. The contents of the second shell vial was harvested and used for subsequent passages. The specimens were considered negative after three passages. Those that became positive, the culture

Table 35: Details of the patient group, clinical specimens of the fifteen HCMV strains

Strain number	Strain	Patient group	Clinical specimen
1	gH2-gO1-gL4-gN4c-gB2	HIV	Blood
2	gH1-gO1-gL4-gN4c-gB1	HIV	Blood
3	gH1-gO4-gL4-gN4c-gB1	PRT	Urine
4	gH2-gO1-gL4-gN4c-gB1	Infant	Respiratory fluid
5	gH2-gO2-gL4-gN1-gB1	PRT	Blood
6	gH2-gO3-gL4-gN4c-gB1	PRT	Blood
7	gH1-gO1-gL4-gN4a-gB1	PRT	Blood
8	gH2-gO4-gL4-gN3b-gB1	PRT	Blood
9	gH2-gO1-gL3-gN1-gB1	Infant	Respiratory fluid
10	gH1-gO1-gL4-gN4c-gB2	HIV	Intraocular fluid
11	gH2-gO1-gL4-gN4a-gB2	PRT	Blood
12	gH1-gO4-gL3-gN4b-gB1	PRT	Blood
13	gH2-gO1-gL4-gN3a-gB2	HIV	Blood
14	gH2-gO3-gL4-gN4c-gB3	HIV	Intraocular fluid
15	gH2-gO4-gL3-gN3a-gB3	HIV	Blood

harvests of the first and subsequent passages were pooled and utilized for genotyping. The culture harvests were stored at -80^{o}C till further processing.

11.3.3 Genotyping of HCMV isolates

Multiplex PCR for gB gene and PCR based-RFLP for gB gene, genes of gCIII complex and gN gene of HCMV were performed as described in the sections 6.3.3; 6.3.2.2; 7.3.1.1,7.3.3, 7.3.6,7.3.9 and 8.3.1 respectively.

11.3.4 DNA sequencing

The viral isolates and the fifteen other HCMV strains were subjected to DNA sequencing. DNA sequencing on the second round amplicons of semi-nested PCR for gH, gL and gN gene were carried out as described in Appendix II. In case of the gB gene, the amplicon generated by semi-nested PCR was sequenced. With respect to the gO gene, the PCRs for gO (AD-169) and gO (Towne) were performed individually and not as duplex. The amplicon generated in the respective PCRs were sequenced.

11.3.5 Phylogenetic analysis

DNA sequences of the five glycoprotein genes (gB, gH, gO, gL and gN) were aligned and analyzed using MEGA version 4.0 software for analysis of relationships by the generation of phylogenetic trees using Neighbour joining methods. The evolutionary distances were computed using the Kimura 2-parameter method. For each gene, a single tree with 1000 bootstrap values was generated.

11.3.6 Nucleotide sequence accession numbers included in the analysis

gB gene : AD169 - X17403, Towne - AY315197

Prototypes : gB 1 - M60929, gB2 - M60932, gB3 - M60934, gB4 - M60924

gH gene: AD 169 - AC146999, Towne - AY315197 , Toledo - AC146905

Prototypes : gH1 - M94236, gH2 - M94229

gL gene: AD169 - AF530173, Towne - AY315197, Toledo -AF530182

gL1 -AF530172, gL2- AF530169, gL3 - AF530175, gL4 - AF530181

gO gene: AD169 - AF531331, Towne - AF531356, Toledo - AF531355

Protoypes : gO1 - AF531340, gO2 - AF531353, indeterminate type - AF531321, gO3 -AF531352, gO4 - AF531323

gN gene: AD169 - X17403, Towne - AF224677, Toledo - AY486475

Prototypes: gN1 - AF309974, gN2 - AF309976, gN3a - AF309980, gN3b – AF390802 gN4a - AF309993, gN4b - AF309996, gN4c - AF310006

11.4 RESULTS

11.4.1 Virus isolation

HCMV was isolated from 4 (33.3%) of 12 specimens. One peripheral blood and urine from one patient and urine specimens from two other patients were positive for isolation.

11.4.2 Results of genotyping by multiplex PCR, PCR based RFLP of the isolates

The four isolates were characterized for all the five genes. The genotyping results of the four isolates were as follows:

Isolate 1 from urine gB1-gH1-gO4- gL4- gN4c

Isolate 2 from blood gB1-gH1-gO4- gL4- gN4c

Isolate 3 from urine gB1-gH1-gO1-gL4 -gN1

Isolate 4 from urine gB3-gH2-gO1- gL4- gN4c

The result of genotyping on the direct clinical specimens from which the HCMV was isolated was available only for the gB gene. The direct clinical specimens of isolate 3 and 4 were genotyped as mixed genotypes gB1+gB3 by multiplex PCR while isolates 1 and 2 were genotyped as gB1.

11.4.3 Phylogenetic analysis of the four HCMV isolates and 15 strains from direct clinical specimens

The analysis of the phylogenetic relationships for intragenic variants of the five glycoprotein genes of the four HCMV isolates and 15 other HCMV strains are depicted in **Figure 34 a to e**. Bootstrap values of greater than or equal to 50% are shown above the branches of the phylogenetic trees. With respect to gB, gH, gL and gN genes, there were no discrepancy in determination of the genotypes between DNA sequencing and other methods of genotyping. Chromatographic evidence of mixed genotypes was not seen in any of the strains sequenced.

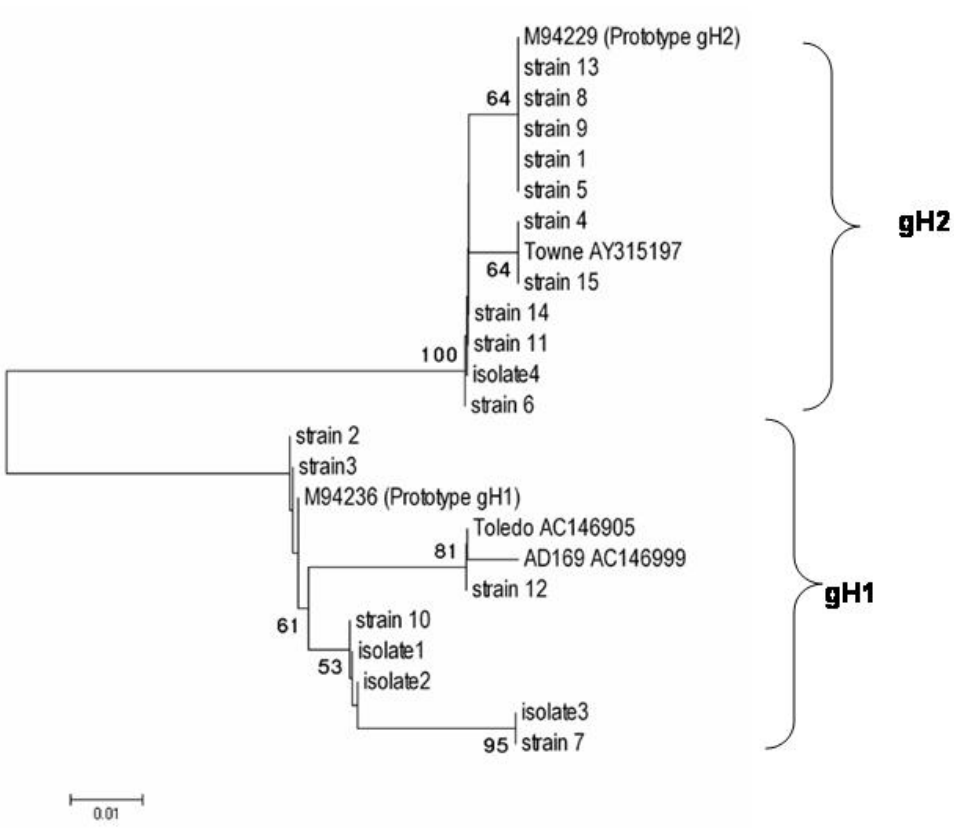
In case of the gO gene, the strains 6 and 14 that were genotyped as gO3 by PCR- based RFLP did not cluster with any of the four prototype strains. To characterize these two sequences further, they were compared with sequences of HCMV strains available in the Genbank database by a BLAST search. In this

Figure 34: Phylogenetic analysis of the four isolates and 15 CMV strains from direct clinical specimens

(a)

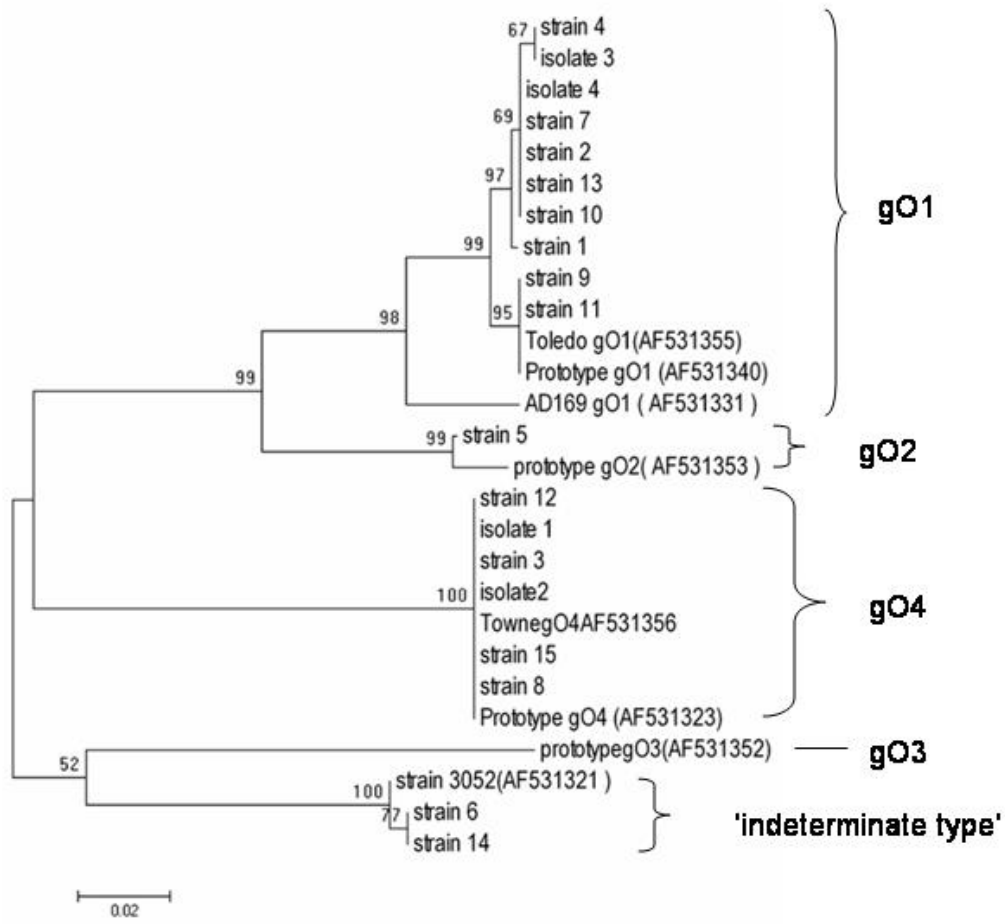
(a) **Results of phylogenetic analysis of the gB DNA sequences.** The tree was generated using the MEGA version 4.0 software. Scale measures distance among sequences. The reliability of the branching orders was estimated by bootstrapping (1000 re-iterations). Bootstrap values (≥50 % after 1000 iterations) for branches are shown.

(b)



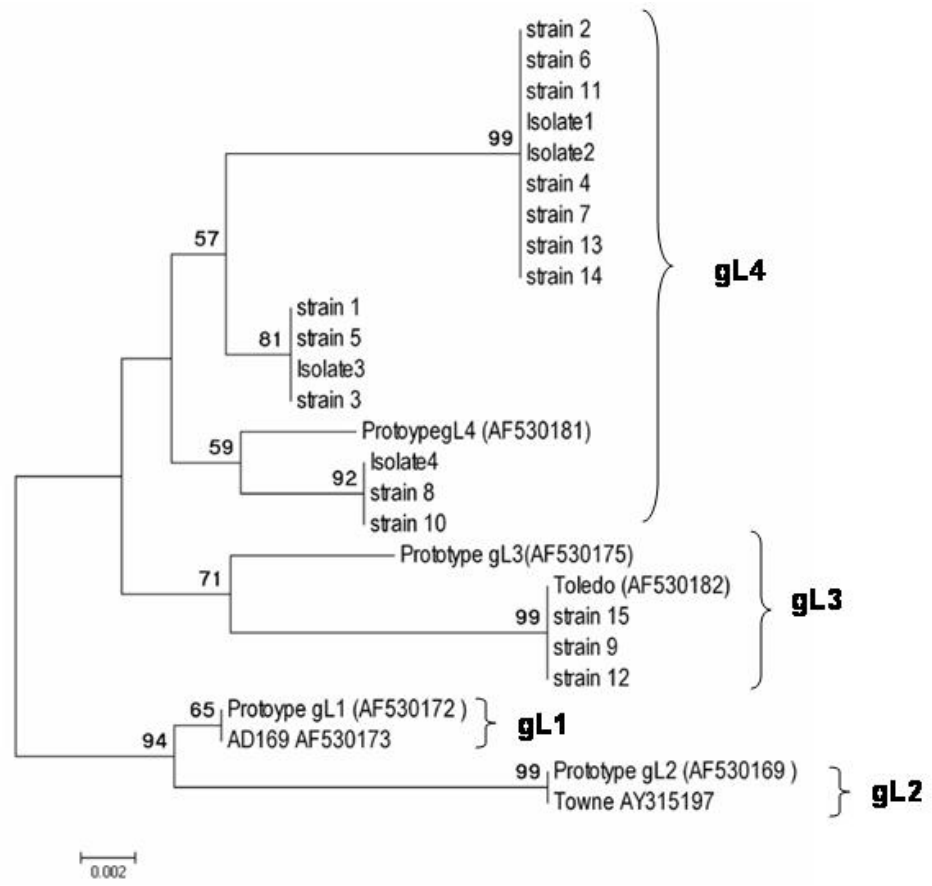
(b) **Results of phylogenetic analysis of the gH DNA sequences.** The tree was generated using the MEGA version 4.0 software. Scale measures distance among sequences. The reliability of the branching orders was estimated by bootstrapping (1000 re-iterations). Bootstrap values (≥50 % after 1000 iterations) for major branches are shown.

(c)



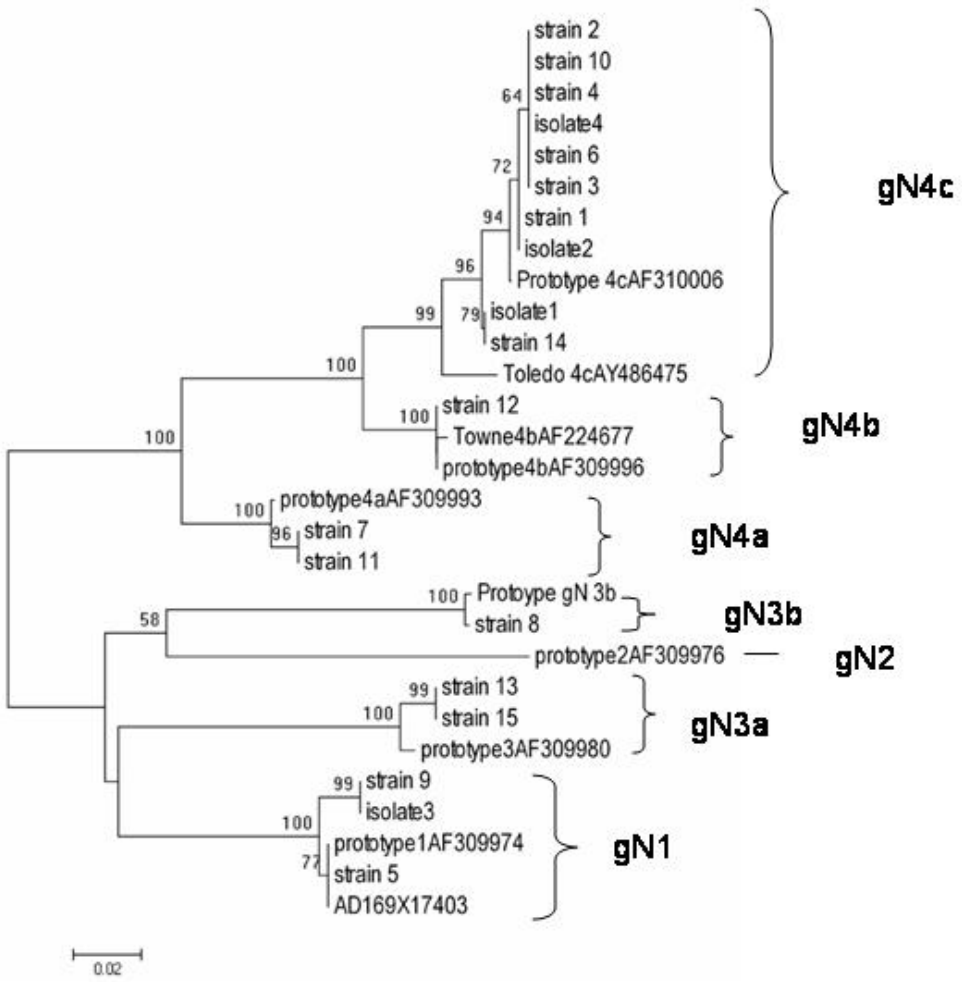
(c) **Results of phylogenetic analysis of the gO DNA sequences.** The tree was generated using the MEGA version 4.0 software. Scale measures distance among sequences. The reliability of the branching orders was estimated by bootstrapping (1000 re-iterations). Bootstrap values (≥50 % after 1000 iterations) for branches are shown.

(d)



d) **Results of phylogenetic analysis of the gL DNA sequences.** The tree was generated using the MEGA version 4.0 software. Scale measures distance among sequences. The reliability of the branching orders was estimated by bootstrapping (1000 re-iterations). Bootstrap values (≥50 % after 1000 iterations) for branches are shown.

(e)



(e) **Results of phylogenetic analysis of the gN DNA sequences.** The tree was generated using the MEGA version 4.0 software. Scale measures distance among sequences. The reliability of the branching orders was estimated by bootstrapping (1000 re-iterations). Bootstrap values (≥50 % after 1000 iterations) for branches are shown.

analysis, CMV strain 3052 (GenBank accession no. AF 531321) (Rasmussen et al., 2002) and Merlin strain (AY446894) were closest to strains 6 and 14. Strains 6 and 14 showed a 99% homology to the strains Merlin and strain 3052. The sequences at the *Hpa II* and *Hae III* recognition sites of the strain 3052 was similar to that of genotype gO3. The homology of the strains 6 and 14 with prototype gO3 however was 85%. The difference with regard to gO genotype of the two strains was not associated with any particular patient group or clinical specimen.

11.5 DISCUSSION

To our knowledge, this is the first study in which isolation of HCMV was attempted on Tenon's capsule fibroblast cell line. About 33.33% of the clinical specimens were positive for isolation. Urine was a better specimen than peripheral blood for the purpose of isolation in Tenon's capsule fibroblast. All the three patients from whom HCMV was isolated had a pp65 antigenemia > 100 cells and showed Pattern I of multiplex PCR suggestive of a high viral load.

More specimens could not be subjected to isolation due to lack of quality fibroblast cell lines in this part of the world. The genotyping results of three of the four isolates by PCR-based RFLP, multiplex PCR and DNA sequencing revealed one of the 48 strains encountered in the previous study (**Table 28**). Isolate 3 showed a pattern of gB1-gH1-gO1-gL4 -gN1 which was not encountered in the study (**Table 28**). This may be attributed to the non-inclusion of mixed genotypes in the analysis. It is assumed that the above mentioned strain could have occurred in combination with another strain.

There was a difference in the genotyping results of gB gene of the isolates 3 and 4 and the corresponding direct clinical specimens. It is assumed that there might be a selection of the strain based on their predominance in the clinical specimen. It is hypothesized that the strain that is in a higher proportion has a higher chance of getting isolated, since; the fibroblast cell line is known to support growth of all the CMV strains.

It was hypothesized in the beginning of the study that the use of RFLP or multiplex PCR in the study to identify genotypes may lead to underestimation of the true number of sequence variants or genotypes, since these methods detect only a limited number of variant nucleotides specific to the recognition site of the restriction enzyme or primer binding site. Strain variation may be even more extensive than that indicated by these genotyping methods.

Hence, DNA sequencing was considered for characterizing the isolates and other clinical strains. Due to the high cost of DNA sequencing fifteen different HCMV strains were selected for sequencing of the five glycoprotein genes. The selection of the strains was made on the basis of including almost all the genotypes for a given gene from different patient groups. The result of nucleotide sequencing however was in accordance with the other methods of genotyping in case of all the genes except gO.

The phylogenetic analysis of the gO gene of strains 6 and 14 revealed that these strains did not cluster with the known genotypes and on BLAST analysis showed similarity to strain 3052 described by Rasmussen et al (2002). The strain 3052 as described by Rasmussen et al (2002) was indeterminate type but the study

ruled out it from being a recombinant. The RFLP pattern of strain 3052 as determined by web based program (http://www.restrictionmapper.org) showed a gO3 pattern as seen with strains 6 and 14 in this study. This study confirms the earlier finding that intragenic variations in gO gene may be very high compared to other genes of gCIII complex.

Thus the DNA sequencing of the HCMV strains in this study, does favour the already established genotyping system for all the five genes. No new genotype was detected with respect to any of the five glycoprotein genes, though strains diverging from prototype strains were encountered in the phylogenetic analysis pertaining to gO gene of HCMV. However, in lieu of a moderate frequency of mixed genotypes encountered in the HIV and PRT patients in the study, intragenic recombination between the different CMV strains may be common in these patients and may act as a source of new CMV strains with altered biological properties.

12. SUMMARY AND CONCLUSIONS

The main objective of the study was to develop and establish the usefulness of nucleic acid based molecular methods for detection and quantitation of HCMV in various clinical specimens from patients with clinically suspected HCMV infections. In addition the HCMV strains encountered in these patients were characterized based on five different glycoprotein genes viz. glycoprotein B belonging to gCI complex; glycoprotein H, glycoprotein O and glycoprotein L belonging to gCIII complex and glycoprotein N belonging to gCII complex.

- pp65 antigenemia assay, a rapid, sensitive and semi-quantitative assay based on the detection and quantitation of lower matrix protein pp65 of HCMV for detection of active HCMV infection was standardized. Initially, two different methods namely the Conventional Dextran sedimentation (CDS) and Direct erythrocyte lysis (DEL) were compared for isolation of leucocyte from peripheral blood. Since, a good agreement in the quantitation of pp65 positive cells was found between the two methods, they were alternatively used in the rest of the study. pp65 antigen was not detectable in the peripheral blood of the controls (healthy blood donors seropositive for HCMV) confirming the earlier view that detection of the antigen in a patient is an evidence for active HCMV infection.

- Since, nucleic acid based molecular methods showed great promise in detection of several viruses and also reports suggesting that the region of interest can affect the sensitivity of the Polymerase chain reaction, three

different targets viz. mtrII, UL-83 and gO gene of HCMV were evaluated against pp65 antigenemia assay as 'gold standard'. PCR for mtr II was found to be more suitable for qualitative detection of HCMV in Chennai region, India. The other findings from the study include the inability of the qualitative PCRs to detect latent viruses circulating in the seropositive controls and all the three regions becoming positive with high level of antigenemia (>50 cells/ 2x 10^5 PBLs).

- Since, Post renal transplant recipients and HIV patients with HCMV infections remain asymptomatic with lower viral load, quantitation of HCMV in these patient groups become increasingly important. Also, since the results of the previous study suggested a high viral load when all the three regions became positive, multiplexing of the three primers were attempted. The multiplex PCR for the three regions of HCMV proved to be useful in quantitation of HCMV since it generated four different patterns that segregated with different pp65 antigenemia levels.

- Taqman probe based Real-time PCR was standardized for the mtrII region of HCMV. The mtr II region was chosen as it provided 100% sensitivity for detection of HCMV DNA in immunocompromised patients. The real-time PCR was sensitive, rapid (results available in 2 hours) and had a high reproducibility. It had an ability to detect the HCMV genome circulating in peripheral blood specimens of healthy controls. The median viral load in the healthy controls was 26.5 copies/ml.

- The multiplex PCR was evaluated against pp65 antigenemia assay and Taqman-probe based real time PCR for its efficacy in quantitation of HCMV. Each pattern of the multiplex PCR was assigned a viral load based on the results of Taqman-probe based real time PCR. The median antigenemia levels and viral loads in clinical specimens showing Pattern I and Pattern II of the multiplex PCR was significantly higher than Pattern III and Pattern IV. A moderate positive correlation ((r_s) = 0. 7778) was seen between the results of pp65 antigenemia assay and Taqman – probe based real time PCR in the study. The multiplex PCR was less expensive and had the ability to distinguish clinical specimens with high and low viral load which may be effective in predicting patients who might develop HCMV disease and to monitor effectiveness of antiviral therapy.

- Since, the genotypic difference of HCMV affects pathogenesis and tropism of HCMV, the genotypic methods for determining the distribution of HCMV genotypes in the study population was developed and evaluated.

- Initially, a nested PCR based RFLP for gB gene was attempted however the method was unable to identify genotype gB3 in the study population. The study indicated that the sequence variations in the gB genome of HCMV may complicate genotyping of HCMV. Confirmation of primer specificity by the BLAST program may not suffice for the genotyping studies. Complete alignment of the prototype sequences of all the genotypes that exist for the gene of interest by a multiple alignment program with the primers used for amplification may be necessary for genotyping of HCMV.

- Later, seminested PCR-based RFLP and multiplex PCR were developed for genotyping of gB gene. Multiplex nested PCR for gB gene was more advantageous than the commonly employed PCR based RFLP for gB genotyping since it was rapid and allowed easier detection of mixed infections with multiple gB genotypes in clinical specimens.

- In the study determining the distribution of gB genotypes by multiplex PCR a predominance of gB1 genotype followed by gB3 and gB2 irrespective of the patient group or specimen type was found. Absence of gB4 and gB5 in the study population and gB mix in the infant group was observed. Presence of gB2 in the intraocular fluid was statistically significant. No absolute segregation of gB genotype based on gender or patient group was observed. In the HIV patient group with discordant results between the blood and intraocular fluids, the genotype other than gB1 in the blood, was detected in the intraocular fluid.

- PCR-based RFLPs were standardized to determine the distribution of genotypes pertaining to the genes of gCIII complex viz. gH, gL and gO. In all the three cases, the existing protocols were modified to achieve the following: direct application on clinical specimen without requirement for isolation, detection of mixed genotypes and visualization of restriction products on agarose gel.

- The analysis revealed no significant differences in the distribution of the gCIII variants based on gender. Statistically significant difference in the distribution of gH genotypes and gO genotypes were found in different

patient groups. gL1 and gL2 genotypes were not found in the study population and predominance of gL4 genotype irrespective of the patient groups and specimen types were also observed. Fifty percent of HIV patients in the study was shown to harbour HCMV strains with gH1-gO1-gL4 configuration.

- An existing uniplex PCR based RFLP was converted to seminested PCR based RFLP to attain optimal sensitivity for genotyping gN gene of HCMV strains from direct clinical specimens without the need for viral isolation. The distribution of gN genotypes in the study population was in accordance to the distribution seen in other geographical areas. There was no statistically significant difference in the distribution of gN genotypes with respect to gender or clinical specimens. The genotype gN4c predominated among the genotypes irrespective of the patient types or specimen though its frequency was little less in PRT than other groups of patients.

- Following conclusions were drawn from the studies on cumulative genotyping of the five regions of HCMV:

  - The study confirms that HCMV are highly heterogenous group of viruses and exhibit several genetic combinations. Theoretically 1120 strains may exist based on the assumption of number of genotypes existing for the five glycoprotein genes and the present study showed 48 different strains circulating in the study population.

- Genetic linkages are limited though some associations of genotypes occur more frequently than others. Hence, variation in one part of the gene does not reflect variations in other parts of HCMV genome. Analysis of single genes may not aid in understanding the tissue tropism or pathogenesis of HCMV.

- Infection with one HCMV strain does not provide protection against reinfection with a new strain, since a higher number of mixed infections with multiple genotypes were encountered in Post-renal transplant patients and HIV infected individuals.

- The order of genetic loci suitable for detection of mixed genotype as confirmed by the study is gB>gO>gH=gL>gN. The leucocyte fraction of peripheral blood is recommended for detection of mixed genotypes in PRT and HIV patients.

- A statistically significant difference was observed in the viral loads as determined by Taqman probe-based real time PCR between the specimens carrying single genotype and multiple genotypes. Hence, detection of mixed genotypes in the hypervariable regions of HCMV genome can be a surrogate marker for increase in viral load.

- The mixed genotype in any loci was not observed in the infant group in this study.

♦ Phylogenetic analysis of the four isolates and fifteen other HCMV strains revealed that no novel variant existed with respect to the regions of the gene

analyzed and all the strains could be accommodated into one or the other genotypes already established.

12.1 LIMITATIONS OF THE PRESENT STUDY

- The study was carried out on the clinical specimens submitted to the Clinical laboratory of L&T Microbiology Research Centre for the investigation of suspected HCMV infections.

- The clinical details of the patients except for their age, gender and status (eg. HIV infected individual, organ transplant recipient etc.) were not available for many and hence, clinical correlations with the results were not possible.

- The study was cross-sectional and the discrepancies in the sample sizes of the patient groups and clinical specimens hindered useful conclusions that could be drawn from the observations.

12.2 FUTURE RECOMMENDATIONS

- The pp65 antigenemia assay, multiplex PCR can be applied prospectively on serial clinical specimens collected from PRTs and HIV infected individuals in conjunction with clinical data. Such a longitudinal study if planned can help in establishing the cut-off value for antigenemia assay and multiplex PCR that can aid in initiating, monitoring and terminating the antiviral treatment. The antigenemia levels > 10 cells is advised for the renal transplant patients based on reports from outside India. Whether the same holds good for the patients in this region can then be established.

- The patients showing an increase in the antigenemia load or the viral load by multiplex PCR can be further screened for antiviral resistance by sequencing DNA polymerase gene (UL-54) and/or phosphotransferase gene (UL-97).

- The DNA sequencing of the genes of glycoproteins included in the study on serial samples of patients carrying mixed genotypes can be performed to establish any recombinant or novel strain that may be emerging.

- Follow-up of the patients whose paired clinical specimens show discrepant results with respect to mixed genotypes in conjunction with clinical data and treatment modality can throw light on the hypothesis of whether therapy or viral load or tropism have role to play in such pattern of shedding.

- Monocyte subfractions from seropositive healthy blood donors may be concentrated; DNA extraction and methods of genotyping can be restandardized to ascertain the genotypes of latent CMV strains circulating in them. The standardized methodology can then be applied on Donor/Recipients in case of transplant patients, mother/foetus in case of pregnant women to identify the prevalence of reactivations and reinfections of HCMV.

- Experimentally modified recombinant viruses identical in all genetic aspects except for the altered region of the hypervariable gene can be constructed. These viruses can then be studied in either in vivo or in vitro model systems to pinpoint genes that are crucial for virulence.

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APPENDIX I

COLLECTION AND TRANSPORT OF SPECIMENS

Non-ocular specimens

All non-ocular specimens were transported in a sterile container as soon as they were collected to Clinical Microbiology Laboratory, L&T Microbiology Research Centre, Vision Research Foundation, Sankara Nethralaya, Chennai. In case of any delay the clinical specimens were transported in ice.

Intraocular specimens

Vitreous fluids were collected aseptically and transported to the laboratory immediately in syringes in which they were collected after expelling the air inside the syringe and capping the needle with a sterile rubber bung. Aqueous humors were collected aseptically in a tuberculin syringe with a 30G needle and sent to laboratory immediately for processing.

PROCESSING OF CLINICAL SPECIMENS

Peripheral blood

The processing of specimens for pp65 antigenemia assay is provided in section 3.3.3**.** The leucocytes remaining after the pp65 antigenemia assay was centrifuged and the deposits were reconstituted in 1 ml of Dulbeccos modified eagles' medium (DMEM) (HiMedia, India), containing 3% foetal bovine serum (FBS) (HiMedia, India). For isolation, 100 µl of the leucocytes was utilized. For PCR 200 µl of buffy coat fraction was used unless and otherwise specified.

Intraocular fluids

Hundred microlitres of the undiluted clinical specimen was taken for polymerase chain reaction (PCR). The rest of the specimen was stored at -80°C for future use. If the volume available was less than 100µl, the whole specimen was taken as such for extraction of DNA.

Urine

The specimen was centrifuged at 3000rpm for 15 minutes. For PCR, 100µl of the deposit was used. For virus isolation, Urine deposit was decontaminated before inoculation with 100µg/ml of gentamycin and 10µg/ml of amphotericin B. 100µl of the deposit was used for inoculation. Only the deposits were stored at -80^{o}C and supernatants were discarded.

Respiratory specimens

In case of purulent respiratory specimens 100 µl of the specimen was used as such for DNA extraction. In case of washes, the specimens were was centrifuged at 3000rpm for 15 minutes. 100 µl of deposits were used for DNA extraction. Both the supernatants and deposits were stored at -80^{o}C.

pp65 ANTIGENEMIA ASSAY

Reagents

1. Dextran solution (6%):
   Dextran - 6 gms
   Sterile water - 100 ml
   The solution was autoclaved.

2. Ammonium Chloride solution (0. 8%):
   Ammoniuum Chloride - 0. 8 gms
   Sterile water - 100 ml
   Required volumes of the solution was prepared fresh before every use and sterilized by filtration.

3. Phosphate Buffered Saline (PBS) :

NaCl	-	8 gms
Na_2HPO_4	-	1.21 gms
KH_2PO_4	-	0.34 gms
Distilled water	-	1000 ml
pH	-	7.3

The solution was autoclaved and stored.

4. Phosphate Buffered Saline – Tween (PBST) :

NaCl	-	8 gms
Na_2HPO_4	-	1.21 gms
KH_2PO_4	-	0.34 gms
Distilled water	-	1000 ml
Tween	-	0.5 ml
pH	-	7.3

5. Non-idet P-40 (0. 5%) :

0.5 ml of Non-idet P-40 made upto 100ml with sterile PBS.

6. Evans Blue (0. 5%) :

0.5 g of evans blue stain dissolved in 100ml of water.

TOTAL LEUCOCYTE COUNTS BY MANUAL METHODS

Twenty microlitres of blood and 380 µl of Turk's fluid (2% glacial acetic acid with two drops of 1 % methyl violet) was mixed and incubated at room temperature for five minutes. Ten microlitre of the sample was loaded in Neubauer chamber and the cells were allowed to settle for five minutes. The four corner (WBC) squares were counted. The total count was given by the following formula:

No. of cells counted in 4 squares x 50 cells/ mm^3. The number of cells was converted to cells/ ml for further calculation.

DETERMINATION OF CELL COUNTS BY HAEMOCYTOMETER

The surface of the chamber and the cover slip was cleaned with 70% alcohol. The cell suspension was mixed well with pasteur pipette and the suspension was dispensed on the chamber. The suspension was drawn under the cover slip into the counting chamber by capillary action. Similarly, the second counting chamber was filled. The cells were allowed to settle for a few minutes before counting .The excess of fluid was blotted off. The slide was then viewed under an inverted phase contrast microscope with 200x magnification. The slide was positioned to view the large central area of the grid; Subdivisions within the large central area were bordered by three parallel lines and each subdivision was divided into sixteen smaller squares by single lines. Care was taken for even distribution of the cells within the area. If cells were not evenly distributed, the slide was washed and reloaded after vortexing the cell suspension. The cell suspensions were diluted suitably to ensure proper distribution and ease of counting. The dilution factor was taken into account during the final calculation. The number of cells in each of the four corner squares was counted in each counting chamber. The number of cells/ ml was obtained by the following calculations:

Cells/ml = average count per square x dilution factor x 10^4

NOTE: 10^4 is the volume correction factor for the chamber. Each square is 1x 1 mm and depth is 0.1 mm.

APPENDIX – II

EXTRACTION OF DNA USING COMMERCIAL KITS

DNA from all the clinical specimens were extracted using one of the three commercial kits

a) QIAamp DNA mini kit (QIAGEN, Germany),
b) Biogene DNA extraction kit (BIOGENE Inc., CA, USA) or
c) AccupPrep Genomic DNA extraction kit (Bioneer corporation, Rockville, USA)

Predominantly QIAamp DNA mini kit was used for peripheral blood specimens and standard strain and Biogene DNA extraction kit was use for specimens other than blood till January 2005. AccuPrep Genomic DNA extraction kit was used for all the specimens and standard strain after January 2005. In case of blood, the buffy coat fraction was used for extraction of DNA unless otherwise specified.

Procedure for the extraction of DNA using QIAamp DNA mini Kit (QIAGEN, Germany)

Two hundred microlitres of AL buffer (Lysis buffer) and 20 µl of proteinase K were added to 200 µl of clinical specimen, cyclomixed and incubated at 56°C for 10 minutes. Two hundred µl of absolute ethanol was added to the lysate and cyclomixed well. The contents were transferred into the spin column provided in the kit. The spin column was microfuged at 8,000 rpm for 1 minute. The collection tube of the spin column was discarded and the spin column transferred onto a fresh collection tube. To this 500 µl of AW1 buffer (Washing buffer 1) was added and

microfuged at 8,000 rpm for 1 minute. The collection tube was discarded and the washing step was repeated with AW2 wash buffer (Wash buffer 2) at 14,000 rpm for 3 minutes. The spin column was transferred to a fresh collection tube and microfuged at 14,000 rpm for 1 minute to remove the residual washing solution. The collection tube was discarded and the spin column was transferred onto a fresh 1.5 ml microfuge tube. 200 µl of AE buffer (elution buffer) was added at the center of the collection tube and incubated at room temperature for 1 minute. The spin column was centrifuged at 10,000 rpm for 1 minute and the eluted DNA was stored at -20°C till use.

Procedure for the extraction of DNA using* BIOGENE *DNA extraction kit (BIOGENE Incorp., CA, USA)

Two hundred microlitres of digestion buffer and 3 µl of proteinase K were added to 100 µl of clinical specimen, cyclomixed and incubated at 56°C for 30 minutes. Two hundred µl of absolute ethanol was added to the lysate and cyclomixed well. The contents were transferred into the spin column provided in the kit. The spin column was microfuged at 8,000 rpm for 1 minute. The collection tube of the spin column was discarded and the spin column transferred onto a fresh collection tube. To this 500 µl of working wash solution was added (100 µl concentrated wash solution and 400 µl of absolute ethanol) and microfuged at 8,000 rpm for 1 minute. The collection tube was discarded and the washing step was repeated once. The spin column was transferred to a fresh collection tube and microfuged at 12,000 rpm for 1 minute to remove the residual washing solution. The collection tube was discarded and the spin column was transferred onto a fresh 1.5

ml microfuge tube. 100 µl of elution buffer was added at the center of the collection tube and incubated at 56°C for 2 minutes. The spin column was centrifuged at 10,000 rpm for 1 minute and the eluted DNA was stored at -20°C till use.

Procedure for the extraction of DNA using AccupPrep Genomic DNA extraction kit (Bioneer corporation, Rockville, USA)

Two hundred microlitres of Binding buffer (Lysis buffer) and 20 µl of proteinase K were added to 200 µl of clinical specimen, cyclomixed and incubated at 60°C for 10 minutes. Hundred µl of isopropanol was added to the lysate and mixed well with micropipette, vortexing was avoided. The contents were transferred into the spin column provided in the kit. The spin column was microfuged at 8,000 rpm for 2 minute. The collection tube of the spin column was discarded and the spin column transferred onto a fresh collection tube. To this 500 µl of Wash buffer 1 was added and microfuged at 8,000 rpm for 3 minutes. The collection tube was discarded and the washing step was repeated with Wash buffer 2 at 8,000 rpm for 3 minutes. The spin column was transferred to a fresh collection tube and microfuged at 12,000 rpm for 5 minutes to remove the residual washing solution. The collection tube was discarded and the spin column was transferred onto a fresh 1.5 ml microfuge tube. 200 µl of elution buffer was added at the center of the collection tube and incubated at room temperature for 1 minute. The spin column was centrifuged at 10,000 rpm for 3 minutes and the eluted DNA was stored at -20°C till use.

Quantitation of DNA by spectrophotometry

One microlitre of the extracted DNA was diluted to one millilitre in Milli Q water and was spectrophotometrically (Beckman DU 640, USA) read at 260nm. One

OD corresponds to 50μg/ml of DNA. The concentration of DNA was found from the corresponding OD value.

POLYMERASE CHAIN REACTION (PCR)

All the PCR reagents were obtained from Bangalore genei Pvt Ltd., Bangalore, India.

Primer dilution

The lyophilized primer was reconstituted in 100μl of sterile Milli Q water. One microlitre of this primer was diluted to one millilitre in Milli Q water and was spectrophotometrically read at 260nm. One optical density (OD) corresponds to 33μg/ml of primer. The concentration of primer was found from the corresponding OD value. Based on the molecular weight and concentration of the primer it was diluted to the required concentration as mentioned in the detailed procedure for each of the PCRs in the methodology section.

Specificity of PCR

The specificity of the PCR was determined with the DNA extracted from HSV-1 (ATCC 733-VR), HSV-2 (SP753167), VZV (Oka vaccine strain), Epstein-Barr virus (EBV) culture filtrate of B 958 marmoset cell line (obtained from National Eye Institute, Bethesda, USA), Adenovirus serotypes 7a (ATCC 848-VR), and 10 human leucocyte DNA. Briefly, the DNA extracted from the above organisms was submitted to amplification along with the controls. Absence of amplification with the DNA of above mentioned organisms with amplification in the positive control alone indicated absolute specificity of the PCR.

Sensitivity of PCR

The sensitivity of the PCR was determined by subjecting serial log dilutions of neat DNA to amplification with specific PCR. The concentration of the neat DNA was ascertained spectrophotometrically (Beckman DU 640, USA) at 260nm. The highest dilution that gave a visible band was used to calculate the sensitivity of the PCR.

For eg. Neat DNA = 1 μg

Final dilution that gave a visible band = 10^{-6}

Sensitivity of the PCR = 1 μg x 10^{-6} = 1 pg of HCMV DNA.

Titration of $MgCl_2$

The magnesium chloride was titrated using the following concentrations for all the PCRs: 1.5mM, 2.0mM, 2.5mM, 3mM, 3.5mM and 4mM concentrations. The optimal magnesium ion concentration was decided based on the intensity of the specific band.

Precautions for PCR

The DNA extraction and PCR preparation, PCR amplification, and detection of amplified products were physically separated into different rooms to prevent contamination. To prevent amplicon carry over, dedicated pipettes, filter guarded tips for adding DNA and single use aliquots of all the reagents were used. Appropriate negative buffer and water controls and positive controls were included along with every PCR reaction.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP):

Details of concentration of restriction enzymes, composition of buffer, temperature of incubation for RFLP of amplified products of PCR for gB, gH, gO, gL and gN gene of HCMV

In all the cases 10 µl of unpurified amplicon was subjected to RFLP in 25µl reaction mixture and enzyme inactivation was carried out by snap cooling. All the restriction enzymes were obtained from Fermentas Inc., USA.

Genetic loci	**Restriction enzyme**	**Concentration (units)**	**Temperature & Time of incubation**	**Buffer composition (used in 1X concentration)**
gB	*Hinf I*	10u	37°C for 2 hrs	10mM Tris-HCl (pH 8.5) 10mM $MgCl_2$, 100mM KCl and 0.1mg/ml BSA
	Rsa I	10u	37°C for 2 hrs	33mM Tris-acetate (pH 7.9) 10mM Mg-acetate, 66mM K-acetate and 0.1mg/ml BSA
gH	*Hha I*	15u	37°C for 2 hrs	33mM Tris-acetate (pH 7.9) 10mM Mg-acetate, 66mM K-acetate and 0.1mg/ml BSA
	Hpa II	10u	37°C for 2 hrs	33mM Tris-acetate (pH 7.9) 10mM Mg-acetate, 66mM K-acetate and 0.1mg/ml BSA
	Stu I	10u	37°C for 2 hrs	10mM Tris-HCl (pH 7.5) 10mM $MgCl_2$ and 0.1mg/ml BSA
gO	*Hpa II*	20u	37°C for 2 hrs	33mM Tris-acetate (pH 7.9) 10mM Mg-acetate, 66mM K-acetate and 0.1mg/ml BSA
	Hae III	20u	37°C for 2 hrs	10mM Tris-HCl (pH 8.5) 10mM $MgCl_2$, 100mM KCl and 0.1mg/ml BSA
gL	*Rsa I*	20u	37°C for 3 hrs	33mM Tris-acetate (pH 7.9) 10mM Mg-acetate, 66mM K-acetate and 0.1mg/ml BSA
	Taq I	20u	65°C for 3 hrs	10mM Tris-HCl (pH 8.0) 5mM $MgCl_2$, 100mM NaCl and 0.1mg/ml BSA
gN	*Sal I*	10u	37°C for 3 hrs	50mM Tris- HCl(pH 7.5), 100mM KCl, 10mM 2-mercaptoethanol, 0.1mM EDTA, 0.2mg/ml BSA and 50% (v/v) glycerol
	Sac I	10u	37°C for 3 hrs	10mM Bis-Tris Propane-HCl (pH 6.5) 10mM $MgCl_2$ and 0.1 mg/ml BSA
	Sca I	10u	37°C for 3 hrs	10mM Bis-Tris Propane-HCl (pH 6.5) 10mM $MgCl_2$, 100mM KCl and 0.1 mg/ml BSA

Expected RFLP patterns of different genotypes for the five glycoprotein genes of HCMV

gB

gB genotype	***Hinf I***	***Rsa I***
gB1	202,67,36 bp,	239, 66 bp
gB2	202, 100 bp	239, 63 bp
gB3	202, 97 bp	195,63,41 bp
gB4	202,67, 36 bp	195,66,44 bp

gH

gH genotype	***HpaII***	***StuI***	***Hha I***
gH1	162, 51 bp	Undigested (213 bp)	81, 75, 29, 20, 8 bp
gH2	Undigested (210bp)	158, 52 bp	92, 81, 29, 8 bp

gL

gL genotype	***RsaI***	***TaqI***
gL 1	287, 117, 96, 50 bp	386, 156, 8 bp
gL 2	337, 117, 96 bp	386, 156, 8 bp
gL 3	287, 117, 96, 50 bp	542, 8 bp
gL 4	337, 117, 95 bp	542, 8 bp

gO

gO genotype	***HpaII***	***HaeIII***
gO1	345, 25 bp	Undigested (370 bp)/ 270,100 bp (for Toledo)
gO2	202, 141, 26 bp	302, 68 bp
gO3	229, 130, 11 bp	222, 100, 34, 14 bp
gO4	202, 116, 26, 14, 12 bp	354, 16 bp

gN

gN genotypes	***Sac I* (bp)**	***ScaI* (bp)**	***Sal I* (bp)**
gN1	297,123	420	420
gN2	229,123,65	417	296,121
gN3a	420	420	420
gN3b	420	221,172,27	420
gN4a	291,123	221,166,27	341,73
gN4b	414	414/387,27	341,73
gN4c	411	239,172/239, 145,27	338,73

VISUALISATION OF PCR/ RFLP PRODUCTS

Agarose gel electrophoresis

Reagents

1. *Tris Boric acid EDTA buffer (TBE) 10x:* The buffer was prepared by dissolving 54.1gm of Tris base, 3.65gm of EDTA and 27.8gm of Boric acid in Milli Q water in the respective order. The pH was checked to be 8.0 and finally the volume was made upto 500ml. Working concentration (1x) was made by diluting the above prepared 10x buffer ten times in Milli Q water.

2. *Bromophenol blue:* Equal volumes of 0.1% bromophenol blue dissolved in 1x TBE buffer and 40% sucrose solution prepared in Milli Q water were mixed.

3. *Ethidium Bromide:* Two milligram of ethidium Bromide was dissolved in one millilitre of Milli Q water. This stock dye was added to the agarose gel at a final concentration of 0.5μg/ml.

Procedure

The gel trough was cleaned with ethanol and the ends were sealed with cellophane tape with the combs placed in the respective positions to form wells. Agarose gels were prepared in 2% to 4% concentrations in 1x TBE buffer as required depending on the molecular weight of the amplified products. The agarose gel was incorporated with 0.5μg/ml of Ethidium Bromide (Sambrook et al., 1989). The amplified products were loaded onto the gel after mixing them with a tracking dye, bromophenol blue. First the negative controls, samples, positive control and finally the molecular weight marker (ϕX 174 DNA/*Hinf* I digest or 100bp ladder) were loaded. The molecular weight marker loaded contains one microlitre of the

molecular weight marker, 9µl of the 1x TBE buffer and 1µl of the dye. Electrophoresis was carried out at 100 volts. The gel was visualized and documented in CN-2000W Vilber Lourmat photo documentation and imaging systems (Cedex, France).

Polyacrylamide gel electrophoresis (PAGE) **(**Sambrook et al., 1989)

Reagents

1. *30% Acrylamide*

Acrylamide	-	29.2 gm
Bis acrylamide	-	0.8 gm

These were dissolved in 30ml of Milli Q water and the volume was finally made upto 100ml with Milli Q water. This was filtered and stored in refrigerator.

2. *Tris – Cl (pH-8.8)*

Eighteen grams of Tris was dissolved in 40ml of Milli Q water. The pH was adjusted to 8.8 with 1N HCl. The volume was finally made upto 100ml with Milli Q water.

3. *Ammonium persulphate (APS)*

Ammonium persulphate	-	0.1 gm
Milli Q water	-	1.0 ml

This was freshly prepared and can be used for one day at 25ºC.

4. N,N,N',N'tetramethylethylenediamine (TEMED)

Procedure

The glass plates (16x19 cms) were assembled with spacers. This was then transferred to casting slots of the casting stand. 2 % agarose was poured onto the casting slot to make it leak proof. A 20% polyacrylamide gel consisting of 26.5 ml of 30% acrylamide, 10 ml of Tris [pH 8.8], 200 µl of 10% APS and 20µl of N_1 N_1 N^1 N^1 - tetramethylene diamine was prepared and poured in between the plates and the comb was inserted. The gel was allowed to polymerize for 30 minutes. After complete polymerization the wells were washed with milli Q water to remove unpolymerized acrylamide. The plate was removed from the casting slot and attached firmly to the electrophoresis tank. Working buffer (1X TBE) was added to the upper and lower tanks. The wells were loaded with digested products along with the molecular weight marker and electrophoresis was carried out at 100 volts. After 3/4 of the run was over the plates were removed and the gel was transferred into the fixative for silver staining.

Silver nitrate staining technique

The polyacrylamide gel was placed in 100ml of the following reagents and rocked for the stipulated time mentioned. Fixation was done in two changes of 10% methanol and 0.5% acetic acid for five minutes each. The gel was stained with 0.1% silver nitrate for 20 minutes and then washed in three changes of water. The gel was differentiated in 1.5% sodium hydroxide containing 0.1% formalin till bands of satisfactory intensity were visualized. The differentiated bands were fixed in 0.75% sodium carbonate for ten minutes. The gel was finally stored in water till it was documented in the CN- 2000W Vilber Lourmat photo documentation and imaging systems (Cedex, France). The gel picture was also captured by a digital camera.

NOTE: For the silver staining technique, all the reagents were freshly prepared only in Milli Q water. Care was taken to avoid contact of the gel with bare hands till the completion of the staining technique.

DNA SEQUENCING

The DNA sequences were either determined by custom sequencing at Bangalore Genei Pvt Ltd or carried out by an "in- house" DNA sequencing procedure. In case of the custom sequencing amplicons purified by QIAquick Gel extraction kit was provided along with 10 picomoles of primers for every sequencing. The in-house procedure for DNA sequencing is as follows:

'In-house' DNA sequencing

Different procedures were followed depending on the type of template. In case of the mixed templates the amplicons were subjected to exonuclease-Shrimp Alkaline phosphatase (Exo-SAP) treatment. In case of sequencing of pure sequences crude amplicons were as such subjected to DNA sequencing. In case of background in sequencing of pure sequences, the products were treated with Exo-SAP and subjected to DNA sequencing.

Exo-SAP treatment of amplicons

Reaction mixture containing 10u of Exonuclease I (Fermentas Inc., USA), 1u of Shrimp alkaline phosphatase (SAP), 1X buffer (67mM glycine-KOH (pH 9.5), 6.7mM $MgCl_2$, 1mM DTT) was prepared. Five ml of the crude PCR product was added to the mixture and incubated at 37^{o}C for 15 minutes followed by heat inactivation at 85^{o}C for 15 minutes.

Cycle Sequencing

Both the sequences of the amplified products were sequenced in cases of mixed templates and isolates. For pure sequences of the amplified products from other clinical strains any one of the sequence was determined. As a rule upstream primer was used for DNA sequencing. Downstream primers were used if the DNA sequencing with upstream primers did not give a proper result.

For cycle sequencing a 10µl reaction was set with 2 picomoles of respective primer, 5x sequencing buffer (400mM Tris-Cl, 10mM $MgCl_2$ [pH 9.0]) and Ready Reaction mix (RR mix) containing dye labeled terminators (ddNTPs), dNTPs, and Ampli*Taq* DNA polymerase FS. The concentration of the template subjected to sequencing differed based on the quality and size. In cases of crude amplicons of less than 500bp, 1 µl were used, in case of amplicons more than 500bp and Exo-SAP treated amplicons 2µl and 3µl of templates were used respectively. The reactions were carried out in a thermal cycler (Perkin Elmer model no. 2700, USA). The thermal profile for cycle sequencing consisted of a single cycle of initial denaturation at 96°C for one minute followed by 25 cycles of denaturation at 96°C for ten seconds, annealing at 50°C for five seconds and extension at 60°C for four minutes.

Purification of Extension Products & Analysis of sequences:

The products were purified to remove the unincorporated dye terminators before subjecting the samples to capillary electrophoresis. Two µl of 125mM EDTA and 10 µl of 3M sodium acetate (pH4.8) were mixed to the cycle sequenced

prodcuts followed by the addition of 50µl of absolute ethanol and incubated at room temperture for 15 minutes follwed by centrifugation at 8000rpm for 20 minutes to precipitate the amplified product and remove the unutilized ddNTPs, primer (short length molecules) etc. The pellet was washed twice with 75% ethanol followed by air drying. The purified samples were suspended in 20µl of template suppressor reagent (TSR), denatured at 90^{o}C for 3 minutes and subjected for capillary electrophoresis in ABI PRISM 310/3100 genetic analyser. The sequences were then analyzed in Sequence Navigator software (version 1.0.1; ABI Prism 310) or Seq scape manager (version 2.1; ABI Prism 3100 AVANT).