Detection of Novel Mutations in UL23 Gene of Herpes Simplex Virus and UL97 Gene of Human Cytomegalovirus Conferring Drug Resistance And Evaluation of pp65 Antigenemia Assay

THESIS

Submitted in partial fulfilment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

Y. SAMSON MOSES

Under the Supervision of

Dr. J. MALATHI

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled ***"Detection of Novel Mutations in UL23 Gene of Herpes Simplex Virus and UL97 Gene of Human Cytomegalovirus Conferring Drug Resistance And Evaluation of pp65 Antigenemia Assay"*** and submitted by **MR. Y. SAMSON MOSES** ID No **2006PHXF433** for award of Ph. D. Degree of the Institute embodies original work done by him/her under my supervision.

Signature in full of the Supervisor: _____

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Date:

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"My God Shall supply all my needs according to His Riches in Glory " -Philip 4 :18

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ABSTRACT

Herpesviruses infect members of all groups of vertebrates, and, indeed, the same host can be infected with multiple distinct and unique types of viruses. In India the seroprevalence of HSV among HIV positive has been reported to be 34.3% and 28.6% for HSV1 and HSV 2 respectively and among HIV negative it is 34.3% and 17.1% for HSV 1 and HSV 2 respectively. It has been reported that HCMV is prevelant in about 95% of normal population.

Immune Response to infections are generally triggered by Toll like Receptors localised at multiple cellular locations. The viral protein sensors include the TLRs, which have been shown to recognize viral lipopeptides. Hence the TLR's responsible for the innate immune response to HSV was studied, whereby TLR4 expression which has not been described by other workers on HSV have been identified on infection of the Retinal Pigment epithelial cells.

Drug resistance to the commonly used drugs (Acyclovir(ACV), Ganciclovir(GCV)) has been posing a serious problem in these viral infections. Hence we proposed to study the phenotypic and genotypic characterization of the mutations occurring in the Thymidine kinase gene drug resistance to ACV. Two novel mutations have been identified and reported in two ocular isolates. Molecular modeling and docking studies were carried out with the help of the Bioinformatics tools to fully characterize the resistance due these mutations in two of the ocular isolates.

Genotyping of the HSV1 isolates was done based on the DNA sequencing of the glycoproteins gG, gI, gC and also based on the Thymidine kinase(TK) gene, most isolates have been found to be genotype A and only two isolates have been found to be genotype B based on analysis of gC and intragenic recombinants have been identified which are type A in gG and type B in gI and TK.

The usefulness of molecular methods(Real Time PCR) and the gold standard test pp65 antigenemia assay for monitoring the viral load in case of HCMV infection among renal transplant patients and to monitor the efficacy of drug therapy was evaluated in our study, where it has been proven that Real time PCR has been found to be more sensitive in the detection of HCMV disease. The same has been proven in the recipient groups who were followed up for monitoring the HCMV disease treatment may be restricted to symptomatic patients alone as the presence of HCMV DNA was detected by this assay.

The ganciclovir resistance among HCMV strains has reported with increasing frequency in these transplant patient groups, and it is becoming an emerging clinical problem. Mutations in UL97 gene may induce resistance to ganciclovir. It has been reported that UL97 gene contain specific mutations which prompted the development of screening assays to detect mutant viruses. Novel mutations were observed in our study, which have been thought to confer resistance to the drug. Further analysis based on docking studies for conformation of ganciclovir resistance needs to be performed.

The detection of HCMV pp65 antigenemia has been widely used for monitoring CMV infection and for guidance of preemptive therapy in patients at risk of developing HCMV disease, as a semi-quantitative analysis of HCMV viral

replication. Hence a study was undertaken to determine the significant pp65 antigenemia count among renal transplant patients, by evaluating the test against viral isolation which in turn may be useful for the treatment of the disease. In this study we have concluded that pp65 count of 23 was determined to be the value of clinical significance as it gives culture positive results among renal transplant patients which indicates the value of the test to be helpful in the therapy of these patients.

In conclusion TLR4 expression in HSV infections has been reported for the first time ever. Novel mutations have been reported in the Thymidine kinase gene, which are responsible for the resistance to Acyclovir in two of the ocular isolates. These mutations have been characterized by molecular modeling and docking studies which has been reported for the first time in our study. Two sub groups of A1 and A2 which has been reported for the first time based on our study. There is no association of between genotypes and the site of infection as well no association has been found between genotypes and resistant strains. Molecular methods like Real time PCR has been reported to be more sensitive in the detection of HCMV infections as well monitoring drug therapy in renal transplant patients. Novel mutations responsible for resistance to ganciclovir, has been reported in our study. Significant pp65 count has been reported to be 23 for therapeutic purposes in Indian scenario for the first time in our study.

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LIST OF ABBREVATIONS

ABBREVIATION	DESCRIPTION
Å	Armstrong
Ab	Antibody
ACV	Aciclovir
AIDS	Acquired Immuno Deficiency Syndrome
ARN	Acute Retinal Necrosis
ARPE-19	Retinal Pigment Epithelium
ATP	Adenosine Tri-phosphate
bp	Base Pair
cDNA	Complementary DNA
CFT	Complement fixation Test
CMI	Cell mediated immunity
CO_2	Carbondioxide
CPE	Cytopathic Effect
CSF	Cerebrospinal fluid
°C	Degree Celsius
DAI	DNA-dependent activator of IRF
DNA	Deoxy Ribonucleic Acid
DNA Pol	DNA Polymerase
DNAHA	DNA hybridization assay
dsRNA	Double Stranded Ribonucleic Acid
DU	Dye uptake assay
eIF2-α	eukaryotic initiation factor 2-α
ELISA	Enzyme Linked Immno Sorbent Assay
FITC	Flurescin Iso-thio cynate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPI	Glycosylphosphatidyl Inositol
HAART	Highly Active Anti-Retroviral Therapy
HBD	Human beta defensin
HCF	Human Corneal Fibroblast
HCMV	Human Cytomegalovirus
Hr	Hour

ABBREVIATION	DESCRIPTION
HSCT	Hematopoietic Stem Cell Transplantation
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
IC	Inhibitory Concentration
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate Early Protein
IFN	Interferon
IgM	Immuno globulin M
IRAK	Interleukin 1 Receptor associated Kinase
IRE	Interferon Response Elements
JNK	c-jun N-terminal kinase
LAT	Latency-Associated Transcripts
LPS	Lipopolysaccharide
LRR	Leucinerich Repeats
Mab	Monoclonal Antibodies
MAPK	Mitogen-Activated Proteins Kinase
MCMV	Murine Cytomegalovirus
MDA	Melanoma Differentiation Associated gene
MEM	Minimum Essential Medium
MHC	Major Histocompatibility complex
Mins	Minutes
MKK	Mitogen activated kinase kinase
ml	Millilitre
mM	Milli mole
mRNA	Mitochondrial Ribonucleic Acid
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa B
NLR	Nod-like receptors
nt	Nucleotide
ºC	Degree Celsius
PAMP	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PKR	dsRNA-dependent protein kinase R

ABBREVIATION	DESCRIPTION
PME	Phosphonylmethoxyalkyl
PMN	Peripheral blood Mononuclear cells
pp65	Phosphoprotein 65
PRR	Pattern Recognition Receptors
RFLP	Restriction Fragment Length Polymorphism
RIA	Radio Immuno assay
RIG 1	Retinoid-Inducible Gene 1
RLR	Retinoid-inducible gene 1Like Receptors
RT PCR	Real Time Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SARM	Sterile alpha and HEAT/Armadillo motif
Sec	Seconds
SNP	Single Nucleotide Polymorphism
TBK-1	Tank Binding Kinase 1
$TCID_{50}$	Tissue culture Infective Dose $_{50}$
TFT	Trifluorothymidine
TGF	Transforming growth factor
TH	T-helper
TIR	Toll-interleukin 1 receptor
TIRAP	TIR domain-containing molecule
TK	Thymidine Kinase
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TRAF	Tumor Necrosis Factor Receptor (TNFR)-Associated Factor
UL	Unique Long Sequence
US	Unique Short sequence
VNTR	Variable Number Of Tandem Repeat
VZV	Varicella zoster virus
µg	Microgram
µl	Microlitre

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CHAPTER 1

INTRODUCTION

The Herpes simplex virus (HSV) serotypes 1, 2 and Human Cytomegalovirus (HCMV) belong to the Herpesviridae family. One of the characteristic features of the herpesviridae family is the establishment of latent infection in the host and may get reactivated at any stage of his/ her life[1,2,3]. The most common human ocular infections namely keratitis, conjunctivitis are caused by HSV 1 and 2 and HCMV[3,4,5]. Herpes viruses (Herpes simplex, Cytomegalovirus) are the causative agents of a wide variety of infections in humans[6,7]. Worldwide rates of HSV infection are between 65% and 90%. In India 33.3% of individuals are seropositive for HSV-1 and 16.6% are seropositive for HSV-2. Those with both HSV-1 and HSV-2 antibodies are estimated at 13.3% of the population. Indian men are more likely to be infected with HSV-2 than women, and increasing seroprevalence of this virus is associated with an increasing age[8]. In a study by Shubha priya *et al.*, the sero-prevelance among HIV positive patients has been reported to be 34.3% and 28.6% for HSV1 and HSV 2 respectively. In HIV negative patients it has been reported as 34.3% and 17.1% for HSV 1 and HSV 2 respectively[9].

The prevalance of HCMV screened from blood donors was reported to be 95% in one of the studies conducted by Atul Kothari *et al..*, at Delhi[10]. Many studies have been performed around the world to estimate the number of individuals infected with HSV-1, HSV-2 and CMV by determining if they have developed antibodies against either viral species. This information provides

population prevalence of HSV and CMV viral infections in individuals with or without active disease[7].

Herpes is a very common viral infection throughout the world; it can be caused by either strain of Herpes simplex Virus (HSV), designated HSV-1 and HSV-2. The HSV's, although commonly associated with Oral and Genital Herpes, are responsible for various clinical manifestations, including; cutaneous infections (whitlow, herpes gladatorlum), ocular infections, neonatal herpes, herpes encephalitis, disseminated infection and erythema multiform[1-7]. From 40% to 50% of adolescents and 60% to 90% of adults are infected with HSV1[11,12]. Recurrent lesions occur in approximately 20 to 30% of HSV infected individuals[13]. Human Cytomegalovirus (HCMV) is the cause of lifelong latent infection in immune competent host. HCMV reactivation is a frequent event and represents a significant cause of morbidity and mortality in immunocompromised patients. Early treatment prevents morbidity caused by these viral infections[14,15].

Human cells are equipped with a range of pattern recognition receptors (PRRs) which recognize microbial pathogen-associated molecular patterns (PAMPs), and mount innate immune responses following infection. Anti-viral sensors can be broadly classified into two groups; toll-like receptor family members and retinoid-inducible gene 1 (RIG-I) like receptors (RLRs)[16]. Herpes simplex virus 1 (HSV-1) is the most commonly diagnosed cause of sporadic (non-epidemic) encephalitis in humans. Without early treatment, HSV-1 encephalitis is a devastating disease that is typically fatal. Among survivors, serious residual neurological defects are commonly seen. Whereas HSV causes a variety of

illnesses in immunocompromised hosts, including disseminated infection, such as pneumonia and hepatitis; encephalitis is commonly seen in patients with normal immune responses[17,18,19].

The viral protein sensors include the TLRs, which have been shown to recognize viral lipopeptides. The major trigger for cellular recognition of a viral infection appears to be viral nucleic acid, which can be recognized at multiple cellular locations[20,21]. Extracellular dsRNA is detected at the cell surface by the class A scavenger receptors. Recent data suggest that, these membrane-bound receptors recognize extracellular dsRNA and serve as chaperones to bring viral dsRNA into the cell for cytosolic recognition. In the endosomal compartment, TLR3 serves as a receptor for viral dsRNA and TLR9 recognizes CpG-rich DNA. In the cytoplasmic compartment, RIG-I and melanoma differentiation associated gene 5 (MDA5) recognize viral dsRNA, with redundancy and specificity with respect to both nucleic acid length and structure[22]. The most frequent cause of acute retinal necrosis is HSV 1 and 2, associated with a history of encephalitis and meningitis in patients older and younger than 25 years, respectively. Triggering events such as periocular trauma, neurosurgery, and high-dose corticosteroids have been reported. T lymphocyte infiltration of the brain and cytokine production cannot be detected until 1–2 days after virus infection. The necrotic process seems to be driven by CD4+ cells, macrophages, polymorphonuclear cells, B cells, and the inflammatory cytokines TNF-α and IFN-γ. HSV-1 tegument proteins have been characterized as major targets for T cells within the vitreous fluid[23,24]. Hence a preliminary *in-vitro* study on the expression of TLR in ARPE-19 cells on challenging with HSV was undertaken in the current research.

To determine the host immune response to these HSV infections, especially ocular (acute retinal necrosis) which causes devastating effects such as blindness, an *in-vitro* study was designed using retinal pigment epithelial cells. In this study we have determined the immune response through toll like receptors on ARPE-19 cells.

Once the disease is established acyclovir is given for treatment of the disease. Prolonged antiviral treatment is often required for the clinical management of these patients, which could lead to the emergence of drug-resistant viruses. Due to prolonged use of the drug, strains resistant to ACV have been isolated, with a prevalence of less than 1% in immunocompetent patients but 5% in immunocompromised patients and 25% in allogenic bone marrow transplant patients[25,26,27]. In general, HSV resistance to ACV is most commonly due to mutations (substitutions, deletions, or additions) in the viral Thymidine Kinase (TK) gene[28,29]. In western literature, some of the mutations have been shown to be associated with ACV resistance[27-32]. In India, hardly any data is available on the same. Therefore, we have studied the phenotypic and genotypic resistance exhibited by HSV to acyclovir due to the mutations occurring in TK gene among HSV isolates recovered from patients in our geographical location especially Tamilnadu by standardization and application of molecular techniques. The mutations were further characterized by *in-silico* analysis of the TK gene using molecular modeling and docking studies to confirm the structural conformational changes of the gene.

Three genogroups (namely Genotype A, B and C) of HSV 1 have been detected and reported in western literature, which have been used for molecular epidemiology studies of different populations[33,34]. Genotyping of HSV has

been done by many methods. Phylogenetic analysis is considered superior as it can reveal clonality. The basic concepts of phylogenetic analysis are quite easy to understand, but understanding what the results of the analysis mean, and avoiding errors of analysis can be quite difficult. Functionally related proteins often share sequence similarity as conserved sequence motifs. Genetic diversity among clinical strains of herpes simplex virus type 1 (HSV 1), is of potential importance for diagnostics and vaccine development[33,34]. Therefore, in this study, we have analyzed the genotyping by phylogenetic analysis, to detect if there is any genotypic relationship of the virus with the site of infection as well as the relationship of the resistant strains with respect to others.

Human cytomegalovirus infections after transplantation account at least for half the deaths that occur among renal transplant patients in India. The spectrum of infections, their chronological occurrence, and the risk factors are different from that of developed regions[35]. The diagnostic and therapeutic protocols are adapted to the different medical and socioeconomic environment[36,37,38]. Cytomegalovirus infection was found in 20% of renal transplant patients in western India and produced acute renal failure in 60%. More than 90% of patients and donors tested are infected with cytomegalovirus (CMV). The availability of diagnostic assays for the early identification of reactivated HCMV replication has considerably improved the clinical management of immunocompromised patients (by monitoring the viral load), thus reducing the risk of HCMV disease and allowing pre-emptive treatment as an alternative to universal prophylaxis[39]. The determination of pp65 antigenemia has long been the reference test for monitoring HCMV reactivation and therapeutic monitoring. One of the most rapid approaches to the diagnosis and monitoring of systemic

human cytomegalovirus (HCMV) infections especially after initiating the treatment is the detection of HCMV antigens in nuclei of peripheral blood leukocytes, and in particular of PMN. This assay is known as the pp65 antigenemia assay. The major HCMV antigen detected in this assay is the 65-kD lower matrix phosphoprotein or pp65 (ppUL83; 2)[38,40]. In addition to pp65, PMN can carry infectious HCMV particles that can be detected by immunoflourescence technique but it requires highly skilled interpretation and processing of fresh samples[38,40]. Furthermore, the evaluation of results may be particularly difficult in leukopenic patients. More recently, the quantitation of viral DNA by real-time PCR methods has been proposed as a convenient alternative approach, in order to overcome the drawbacks of antigenemia assays[39]. Therefore, we attempted to evaluate the real time technique in detecting HCMV. Moreover the exact relationship between HCMV DNA and antigenemia levels, is still under investigation. In the current study the relationship was investigated to determine sensitive methods for monitoring HCMV infected patients on treatment of the disease by evaluating both the Real Time PCR against pp65 antigenemia assay.

Cytomegalovirus (CMV) retinitis is the most common cause of visual loss in patients with AIDS[41]. Current treatment strategies rely on the use of short-term induction therapy with anti-CMV drugs followed by long-term maintenance therapy. As experience with therapy for CMV retinitis has accumulated, it has become evident that long-term maintenance therapy is associated with increased virological resistance and diminished clinical responsiveness to systemically administered anti-CMV drugs. It has been suggested that sub-therapeutic drug

delivery to the vitreous humor, as achieved by oral or intravenous administration, may be one cause of this problem.

Jabs *et al.*, have revealed 2% prevalence of resistance to ganciclovir and a 4% prevalence of resistance to foscarnet in their study population. However, different strains of HCMV may be present at different sites in the body, and these strains may have different genotypic resistance patterns[42]. Assessing the prevalence of ganciclovir drug-resistant virus in the eyes of patients with CMV retinitis would therefore be a more direct and, perhaps, more accurate approach to this problem. Unfortunately, it has been proven that propagation of HCMV in cell culture from ocular samples is a cumbersome and insensitive method. Recent studies on the molecular mechanisms of HCMV antiviral resistance have demonstrated that single-point mutations in the HCMV UL97 gene may confer resistance to ganciclovir. We screened our study population to detect the presence of HCMV UL97 mutations that may confer resistance to antivirals in the DNA of HCMV detected in the peripheral blood samples of renal transplant recipients.

Therefore in the current study the laboratory tests commonly used for monitoring patients with HCMV(i.e. pp65 antigenemia and Real Time PCR) was evaluated and the drug resistance conferred by the virus was detected by DNA sequencing method. The genotypic prevalence of HSV and its drug resistance exhibited was also evaluated.

1.2 Hypothesis:

Application of nucleic acid based molecular methods are more sensitive to detect drug resistance in Herpes simplex virus and Human cytomegalovirus compared to phenotypic method, and real time polymerase chain reaction (RT-PCR) method is superior to the commonly used pp65 antigenemia assay to monitor HCMV viral load.

1.3. Objectives

Objective 1: Determination of expression of Toll like receptors 3, 4 and 9 in HSV infection using ARPE cells

- To Standardize the Reverse Transcriptase polymerase chain reaction for the amplification of Toll Like Receptors(TLR) 3, 4 and 9 and Housekeeping gene(GAPDH) of ARPE-19 on challenging with Herpes simplex virus 1 and 2

- To Apply the Reverse Transcriptase polymerase chain reaction for the amplification of Toll Like Receptors (TLR) 3, 4 and 9 and GAPDH of ARPE 19 on challenging with Herpes simplex virus 1 and 2 clinical isolates

- To Analyse of the results of amplification of Toll like receptors 3, 4 and 9 in ARPE-19 cells in HSV infection

Objective 2: Genotypic and phenotypic characterization of acyclovir resistance in Herpes simplex virus

- To Characterize of antiviral Drug resistance of Herpes simplex virus isolates against acyclovir by plaque reduction assay.
- To Standardize the polymerase chain reaction for the amplification of thymidine kinase gene of Herpes simplex virus 1 and 2.

- To apply of the standardized Polymerase chain reaction onto clinical isolates for the detection of mutations responsible for resistance to acyclovir.
- To perform PCR based DNA sequencing of the thymidine kinase gene, of Herpes simplex virus, for the detection of mutations responsible for resistance to acyclovir.
- To analyze the sequences for mutations responsible for the resistance to acyclovir by Herpes simplex viral isolates and molecular modeling of the wild type and mutant thymidine kinase gene.

Objective 3: Genotyping of Herpes simplex virus 1 isolates helps in identifying new recombinants

- Genotyping of Herpes simplex virus 1 using PCR based RFLP method
  - To standardize and apply the Polymerase chain reaction for the amplification of gG gene of HSV 1 isolates
  - To standardize and apply the Polymerase chain reaction for the amplification of gI gene of HSV 1 isolates
  - To perform Restriction fragment length polymorphism analysis of gG using *PflMI* and *DdeI* and gI genes using *PleI* and *Sac1*
  - To Analyze of the RFLP pattern to identify the genotypes
- Phylogenetic comparison of exonic US4 and US7 and UL44 regions of clinical Herpes simplex virus type 1 isolates for the establishment of association between the clinical specimen and its genotype
  - To Standardize and apply the Polymerase chain reaction for the amplification of US4 gene of Herpes simplex virus 1 isolates

- ❖ To Standardize and apply the Polymerase chain reaction for the amplification of US7 gene of Herpes simplex virus 1 isolates

- ❖ To Standardize and apply the Polymerase chain reaction for the amplification of UL44 gene of Herpes simplex virus 1 isolates

- ❖ To perform DNA Sequencing of the amplified products of US4, US7 and UL44

- ❖ To Perform Phylogenetic analysis of the PCR based DNA sequences of US4, US7, UL44 and UL23 (Thymidine kinase).

Objective 4: Real time PCR assay is a sensitive and reliable technique in the diagnosis of HCMV infections among renal transplant patients

- ➢ To standardize the real time Polymerase reaction(RTPCR) for the quantification of HCMV DNA in peripheral blood samples collected from renal transplant patients
- ➢ To perform pp65 antigenemia assay by immunoflourescence method for the semi-quantitative detection of HCMV from leucocytes prepared from the peripheral blood samples of renal transplant patients.
- ➢ To analyze the sensitivity of the RTPCR and pp65 antigenemia assay for the quantification of HCMV infection

Objective 5: Determination of mutations in UL97 gene responsible for the drug resistance in HCMV

- ➢ To standardize Polymerase chain reaction for the amplification of UL 97 gene
- ➢ To apply the PCR on blood samples from Renal transplant patients and to sequence the amplified products

- To analyze the sequenced products for mutations responsible for the ganciclovir resistance

Objective 6: Determination of significance of pp65 antigenemia against viral isolation of Human Cytomegalovirus.

- To determine the pp65 count of the peripheral blood leucocytes collected from renal transplant patients.
- To isolate the HCMV from corneal fibroblast cells
- Analysis- Comparison of pp65 antigenemia assay and viral isolation

1.4 Review of Literature:

Herpes simplex virus:

History:

Herpes disease has been known for at least 2,000 years. *Herpesviridae* are a large family of DNA viruses that cause diseases in animals, including humans. The members of this family are also known as herpesviruses. The family name is derived from the Greek word *herpein* ("to creep"), referring to the latent, recurring infections typical of this group of viruses. *Herpesviridae* can cause latent or lytic infections[1-6].

Taxonomy

The genus *Herpesvirus* was established in 1971 in the first report of the International Committee on Taxonomy of Viruses (ICTV). This genus consisted of 23 viruses and 4 groups of viruses. In the second ICTV report in 1976 this genus was elevated to family level - the *Herpetoviridae*. Because of possible confusion with viruses derived from reptiles this name was changed in the third report in 1979 to *Herpesviridae*. In this report the family *Herpesviridae* was divided into 3 subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*). In 2009 the family *Herpesviridae* was elevated to the order *Herpesvirales*. This elevation was necessitated by the discovery that the herpes viruses of fish and molluscs were only distantly related to those of birds and mammals. Two new families were created - the family *Alloherpesviridae* which incorporates bony fish and frog viruses and the family *Malacoherpesviridae* which contains those of molluscs[1-4].

The Herpesviridae family is classified into three major groups based on certain biological properties namely *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae.*

The *Alphaherpesvirinae*, consisits of Herpes simplex virus 1 and 2 and Varicella zoster virus. The *Betaherpesvirinae*, consisits of Human cytomegalovirus, Human herpes virus 6A, 6B and 7. The *gammaherpesvirinae,* consisits of Epstein- Barr virus and Human herpes virus 8[1-4].

The *Alphaherpesvirinae,* has a short replicative cycle, causes latent infection in ganglia and has a variable host range. The *Betaherpesvirinae,* has a slow reproduction cycle, causes cytomegalia and latent infection in lympho-reticular cells and they have a narrow host range. The *Gammaherpesvirinae* replicate in lympho-blastoid cells (B or T cells), exhibit latent infection in lymphoid tissues and has a very narrow host range[1-4]. Table 1 describes the properties of the common members of herpesvirinae family.

TABLE 1.1 - Properties of Herpes viruses

Human herpes type	Name	Sub Family	Target cell type	Latency	Transmission
1	Herpes simplex-1 (HSV-1)	Alphaherpesvirinae	Mucoepithelia	Neuron	Close contact
2	Herpes simplex-2 (HSV-2)	Alphaherpesvirinae	Mucoepithelia	Neuron	Close contact usually sexual
3	Varicella zoster virus (VSV)	Alphaherpesvirinae	Mucoepithelia	Neuron	Contact or respiratory route
4	Epstein-Barr Virus (EBV)	Gammaherpesvirinae	B lymphocyte, epithelia	B lymphocytes	Saliva
5	Cytomegalovirus (CMV)	Betaherpesvirinae	Epithelia, monocytes, lymphocytes	Monocytes, lymphocytes and possibly others	Contact, blood transfusions, transplantation, congenital
6	Herpes lymphotropic virus	Betaherpesvirinae	T lymphocytes and others	T lymphocytes and others	Contact, respiratory route
7	Human herpes virus-7 (HHV-7)	Betaherpesvirinae	T lymphocytes and others	T lymphocytes and others	Unknown
8	Human herpes virus-8 (HHV-8) Kaposi's sarcoma- associated herpes virus (KSHV)	Gammaherpesvirinae	Endothelial cells	Unknown	

Discovery of Herpes simplex virus:

In 1736 Jean Astruc classified the condition as a sexually transmitted disease and noted its frequency among homosexuals. In the early 19th century, 6 clinical entities were delineated including facial and genital herpes, but the disease was not considered communicable, probably because of the idiosyncratic appearance of symptoms in conjunction with disparate, well defined febrile illness[1-4].

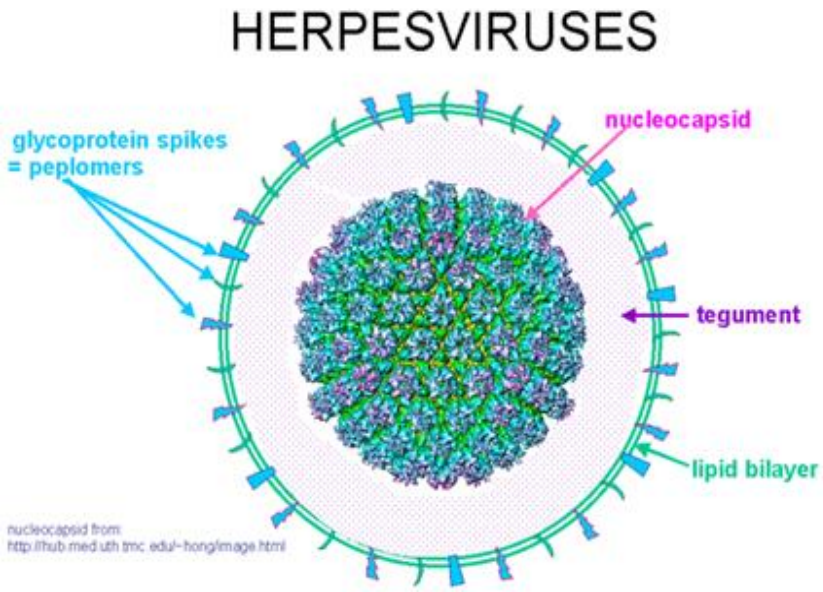
In 1921, Luger and Lauda demonstrated that the material derived from human herpetic lesions that was passed through a filter with pores small enough to retain bacteria, produces a serially transmissible keratoconjunctivitis in rabbits. In 1929, Goodpasture postulated the existence of the virus in latent state and Cushing speculated that HSV exists in the neural ganglion in latent state[1-4].

In early 1930's it became evident that the manifestation is gingivostomatitis in seronegative subjects and recurrent "fever blisters" in seropositive subjects. In early 1960's Schneweis, Dowdle and Nahmias reported the existence of two antigenic strains of HSV based on Neutralization test. Now it is widely known that herpesvirus causes recurrent facial lesions commonly identified as "fever blisters" and sexually transmitted recurrent genital lesions [1-4].

Herpes simplex virus (HSV) is endemic in virtually every human society throughout the world, from urban to remote native tribes. Humans are the only natural reservoirs for HSV[1-4].

Herpes Virus Structure – General

Fig 1.1: Structure of Herpesviruses



Viral structure

Herpesviruses all share a common structure. All herpesviruses are composed of relatively large double-stranded, linear DNA genomes encoding 100-200 genes encased within an icosahedral protein cage called the capsid which is itself wrapped in a protein layer called the tegument containing both viral proteins and viral mRNAs and a lipid bilayer membrane called the envelope. This whole particle is known as a virion[1-4].

Envelope

Herpes viruses are enveloped viruses. They bud from the inner nuclear membrane which has been modified by the insertion of herpes glycoproteins (in the mature virus, these glycoproteins determine the cell to be infected because of the availability of the appropriate receptors). The viral membrane is quite fragile and a virus with a damaged envelope is not infectious (This means that the virus readily falls apart and so the virus can only be obtained by direct contact with

mucosal surfaces or secretions of an infected person - it cannot be caught from toilet seats). Besides drying, the virus is also sensitive to acids, detergents and organic solvents as might be expected for a virus with a lipid envelope[1-4].

Tegument

The space between the envelope and the capsid is the tegument. This contains virally-encoded proteins and enzymes involved in the initiation of replication[1-4].

Capsid

These viruses have a doughnut shaped capsomere of about 100-200 nm in diameter with an icosahedral nucleocapsid. The latter contains 162 capsomeres [1-4].

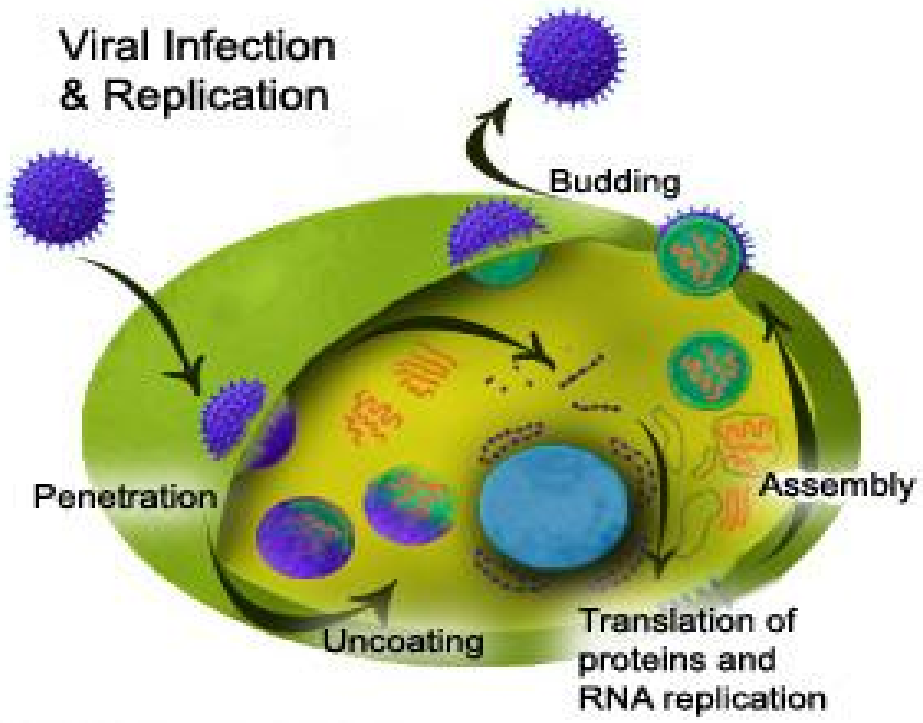
Genome

These viruses have double stranded DNA. The size of the genomes differ with cytomegalovirus having the largest genome[1-4].

HSV replication

Almost any human cell type can be infected by HSV. In many cells, such as endothelial cells and fibroblasts, infection is lytic but neurons normally support a latent infection[1-4].

Fig 1.2: Viral replication



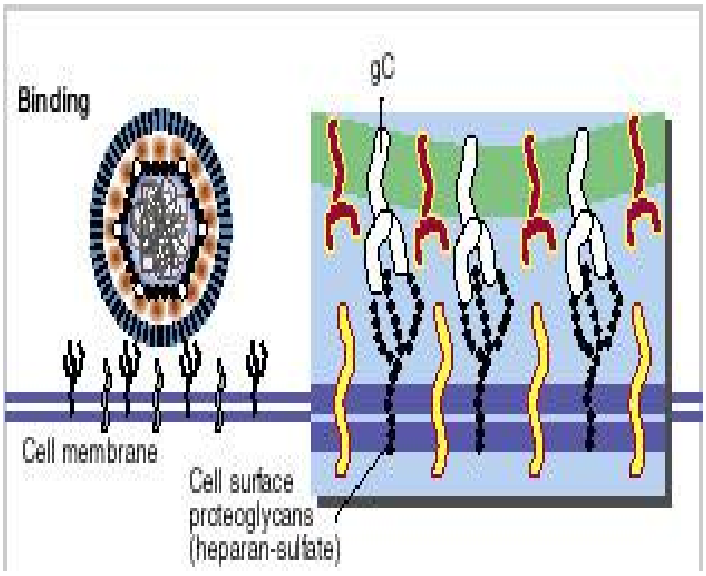
Binding:

The initial step of the interaction of virus with the cell is binding to the proteoglycan, heparan sulfate. This molecule is found on the surfaces of many human epithelial cells and other cells[1-4].

Fusion:

After binding, the virus fuses directly with the plasma membrane (no entry into low pH endosomes/lysosomes is necessary). After fusion occurs, the virus releases some proteins into the cytoplasm. These include some toxins, a protein kinase and a gene transcription initiator[1-4].

Fig: 1.3 Viral Binding



Protein synthesis:

Immediate early genes are first transcribed which promote transcription of early genes. If the infection is to be latent, the only mRNAs that are made are the latency-associated transcripts (LAT). The early gene products include the DNA polymerase plus enzymes that degrade cellular mRNA and proteins. If early and late proteins are made, the cell is set on a route to lysis[1-4,41].

As noted above, only a few DNA polymerase proteins need to be made for replication of viral DNA. At first, circular concatomers are made but then synthesis switches to linear chains of individual molecules that are cleaved into monomers. This occurs by a rolling circle mechanism. Late genes are now transcribed in large amounts, probably triggered by the synthesis of DNA. They are translated in the cytoplasm and transported back into the nucleus where they are assembled into the procapsid. The latter is filled with viral DNA[1-4,41].

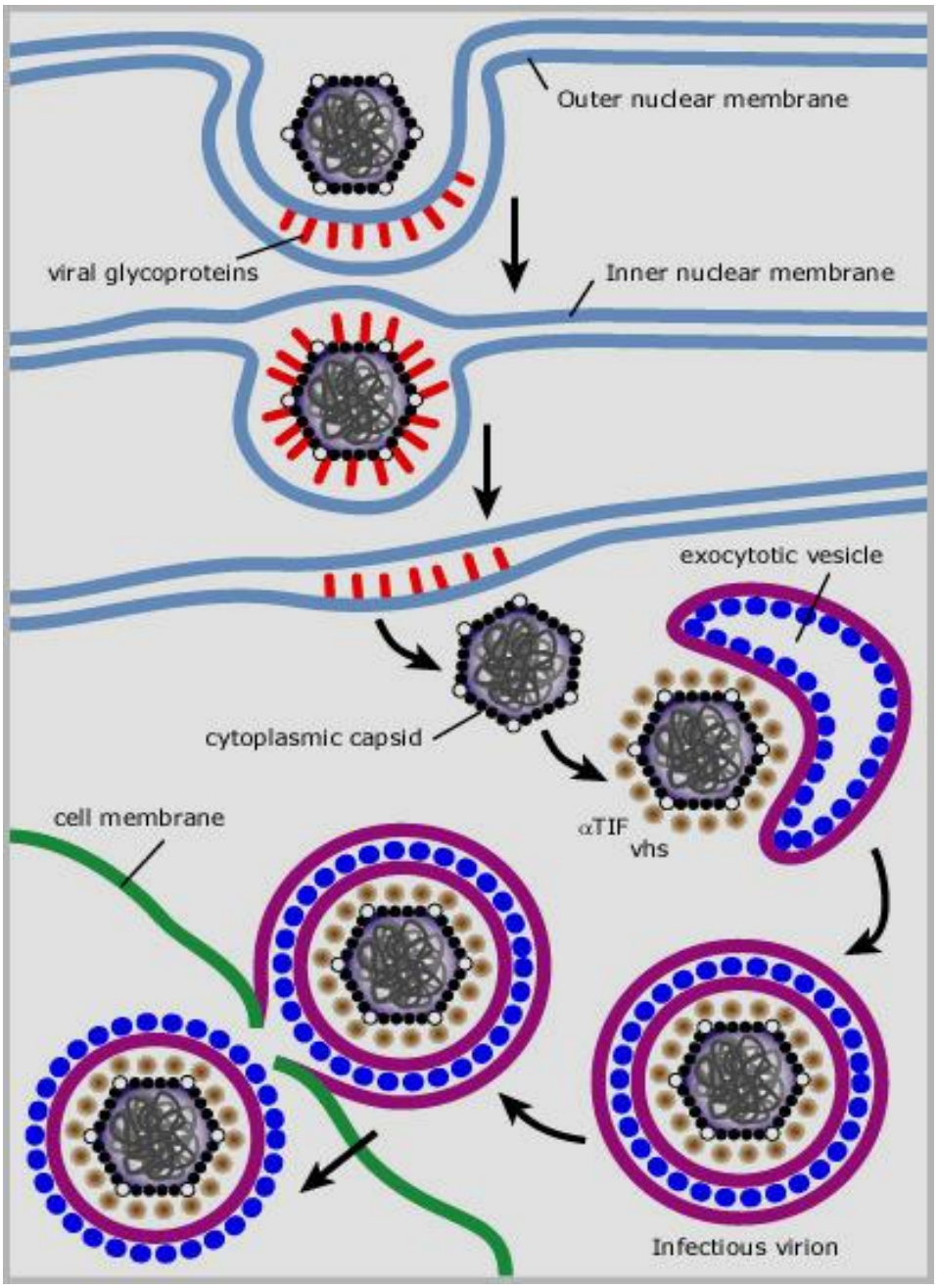
Glycoprotein synthesis:

All glycoproteins are made in the rough endoplasmic reticulum where they receive high mannose sugar chains. The glycoproteins are moved to the nuclear membrane, probably by a process of diffusion since the membrane of the endoplasmic reticulum is continuous with the outer nuclear membrane. How the proteins get around the nuclear pore is unknown. The nucleocapsids now bud through the nuclear membrane via areas in which the viral proteins are concentrated. In some way, the virus enters the exocytotic pathway since it is modified in the Golgi body where it receives sugar chains that are characteristic of Golgi-processed proteins (that is, they contain galactose and sialic acid)[1-4,41].

Release of virus:

Several pathways seem to occur. The virus can proceed along the exocytotic pathway or it can enter the cytoplasm and be released by cell lysis. It also appears to be able to pass through intercellular junctions and thereby spread from cell to cell[1-4, 41].

Fig.1.4: Viral Penetration and Replication- Overview



Herpes virus life-cycle

All herpesviruses are *nuclear-replicating;* the viral DNA is transcribed to RNA within the infected cell's nucleus**.** Infection is initiated when a viral particle contacts a cell with specific types of receptor molecules on the cell surface. Following binding of viral envelope glycoproteins to cell membrane receptors, the

virion is internalized and dismantled, allowing viral DNA to migrate to the cell nucleus. Within the nucleus, replication of viral DNA and transcription of viral genes occur[1-4,41].

During symptomatic infection, infected cells transcribe *lytic* viral genes. In some host cells, a small number of viral genes termed *latency associated transcript* (LAT) accumulate instead. In this fashion the virus can persist in the cell (and thus the host) indefinitely. While primary infection is often accompanied by a self-limited period of clinical illness, long-term latency is symptom-free[1-4,41,42].

Reactivation of latent viruses has been implicated in a number of diseases (e.g. *Shingles*, Pityriasis Rosea). Following activation, transcription of viral genes transitions from LAT to multiple *lytic* genes; these lead to enhanced replication and virus production. Often, lytic activation leads to cell death. Clinically, lytic activation is often accompanied by emergence of non-specific symptoms such as low grade fever, headache, sore throat, malaise, and rash as well as clinical signs such as swollen or tender lymph nodes and immunological findings such as reduced levels of natural killer cells[1-4,40,42].

Infection with HSV is almost universal. The virus is shed from the infected area. It spreads by direct contact. The virus may be shed in saliva and genital secretions. There are 2 clinical patterns of the disease[1-4,40,42].

a) Primary Infection

b) Recurrent disease

Primary Infection:-

Primary infection is usually trivial or subclinical in most individuals. It is a disease mainly of very young children i.e. those below 5 years. There are 2 peaks of incidence, the first at 0 - 5 years and the second in the late teens, when sexual activity commences. About 10% of the population acquires HSV infection through the genital route and the risk is concentrated in young adulthood. Primary infections of all kinds are rare after age 30[1-4].

Most primary infections are silent, they are not even realized by the patient. The vesicles develop at between 1 – 3 days post exposure and the virus remains localized at the site of inoculation. In immunocompromised patients, virus may spread or disseminate. The nature of the disease is determined by the site of inoculation. Some of the clinical manifestations of the primary infection are Gingivostomatitis, Kaposi's Varicelliform eruption, Herpetic Whitlow, Conjunctivitis, Keratitis, Genital Herpes, Neonatal Infection. In neonatal infection the most common infection is encephalitis[1-4].

Recurrent Infection:-

Following primary infection, 45% of orally infected individuals and 60% of patients with genital herpes will experience recurrences. The actual frequency of recurrences varies widely between individuals. The mean number of episodes per year is about 1.6. The actual frequency decreases with age, increases with socioeconomic status and is related to race. Many individuals never experience any clinically apparent reactivation although more than half would be

intermittently shedding virus in saliva, tears, semen or genital (cervical, urethral, prostatic) secretions[1-4,42].

After the primary infection the herpes simplex virus may lie dormant. The viral genome persists in an episomal form (plasmid) in the nucleus of the neuron. HSV 1and HSV 2 can establish a latent infection in the ganglia of the nerves that supply the site of the primary infection. The ganglions involved in HSV infections are sacral ganglia for genital infections, trigeminal ganglion for Oro-facial infections leading to a tingling and numbness around the mouth, then a blister that breaks and forms a crust[1-4,42]

In a percentage of people, the virus may reactivate. Reactivation may be provoked by a number of stimuli which includes sunlight, stress, febrile illnesses, menstruation or immunosuppression. Some of the clinical manifestations exhibited during recurrent infections are cold sores, recurrent genital herpes, keratitis[1-4,42].

HSV infection occurs worldwide. Studies have shown 90% of the population studied, have antibodies to HSV. Antibodies to HSV 2 are not routinely detected until puberty. The antibody prevalence rates for HSV 1 appears to be falling, while those for HSV 2 appears to be rising in many populations which is due to abnormal sexual practices. Many people having detectable antibodies to HSV in their body are asymptomatic and do not exhibit any clinical symptoms. Genital HSV in older adolescents and adults is a major public health problem. It has been increasing in the last 3 decades and has had a marked increase[1,2].

Immunocompromised patients:-

Cell mediated immunity (CMI) is crucial in the control of HSV infection. People with deficiencies in humoral immunity have no problems in controlling HSV infection whereas those with deficiencies in CMI do. Three groups of immunocompromised patients are particularly at risk from serious HSV infection[1-4].

a. Patients receiving cytotoxic drug therapy

b. Organ graft recipients

c. HIV/AIDS patients

Generally, in these patients, the infection can often be controlled on the easing of the immunosuppressive therapy. HSV is a common cause of severe morbidity in bone marrow graft recipients, whereas it causes much fewer problems in renal transplant recipients. AIDS carriers suffer greatly from HSV disease[1-4].

Clinical Features

HSV is involved in a variety of clinical manifestations which includes Acute gingivostomatitis, Herpes labialis (cold sore), Ocular herpes, Herpes genitalis, Other forms of cutaneous herpes, Meningitis, Encephalitis, Neonatal herpes[1-4]

Ocular Herpes

The eye is the site of infection in 3% of all primary ocular infections and occurs in tender childhood or early adulthood. An initial eye infection in an already immune individual can take place at any time. The recurrence rate is 40% and most affected individuals will suffer reactivation at least once within 2 years of the primary episode[1-4].

Two forms of herpetic ulcers are recognized. One form is infectious with active virus replication, the other post-infectious and trophic being secondary to mechanical damage. The gravest form of ocular herpetic disease, occur when the virus spreads to the anterior chamber. The mechanism of pathogenesis is unknown but there appears to be an intense immunological reaction[1-4].

HSV causes a broad spectrum of ocular disease, ranging from mild superficial lesions involving the external eye, to severe sight-threatening diseases of the inner eye[1-4].

a. Primary HSV keratitis:-

In this condition, the patient complains of foreign body sensation, pain, photophobia and lacrimation. In the early stages, small predendritic ulcers are seen on the cornea, neck lymphadenopathy and mild constitutional symptoms may be present. The disease may heal at this stage or progress with the development of a large dendritic ulcer which has a serpentine branching appearance. Corneal sensation can be impaired. The disease normally lasts 3 weeks, during which the ulcer heals. Larger ulcers may take a longer time to resolve[1-4,43].

b. Recurrent HSV keratitis:-

This is a severe ophthalmic problem. There are two forms; (a) Dendritic, which resembles the lesions seen during the primary infection but are often smaller. They do not normally cause much scaring but people subjected to frequent recurrences may suffer a slow deterioration in visual acuity.

(b) Stromal form, which is a severe protracted disease of the eye and causes opacification of the cornea and may lead to necrosis of corneal fibres and rupture of the cornea[1-4,43].

c. HSV conjunctivitis:-

Conjunctivitis, blepharitis, and circumocular dermatitis are commonly present during an attack of primary keratitis. The diagnosis is particularly difficult in the absence of corneal ulceration[1-4].

d. Iridocyclitis, chorioretinitis and neonatal cataract:-

Mild reflex iritis is frequently seen as a complication of HSV kerititis. A more severe form of iridocyclitis may be seen with stromal keratitis. Chorioretinitis and cataract are manifestations of neonatal herpes and can lead to damage or permanent loss of vision[1-4].

Other forms of cutaneous herpes:-

Herpetic dermatitis is a normal complication of the primary mucocutaneous infection. Large areas of the face, perineum and thigh may be involved. Under normal circumstances, HSV does not readily penetrate normal skin. Persons with puncture injuries on their skin may become infected with HSV and develop herpetic whitlows, especially health personnel who constantly manipulate in the oral cavity. (dentists, dental nurses, anaesthetists). HSV may also be spread by contaminated needles, razors, or broken glassware. Herpes gladiatorum is a rare condition seen in wrestlers. All forms of cutaneous disease can recur[1-4].

In herpetic whitlows, the patient complains of pain and tenderness at the site of implantation. A single vesicle develops, but satellite vesicles soon appear and rapidly coalesce into a single necrotic oozing "abscess". Healing takes around

15 days. The normal resistance of the skin to HSV infection may be compromised in various dermatoses. There are 2 forms of Kaposi's varicelliform eruption, and since the withdrawal of smallpox vaccine, the more common form is eczema herpeticum which is seen in patients with atopic eczema. Eczema herpeticum as a primary infection carries a small mortality. A distinct type of cutaneous herpes is called "zosteriform herpes simplex". This is a rare presentation of herpes simplex where HSV lesions appear in a dermatomal distribution similar to herpes zoster. The disease cannot be diagnosed without laboratory help[1-4].

Acute Retinal Necrosis:

Acute retinal necrosis (ARN), first described in 1971, is a rare inflammatory necrotic process affecting one or, in some cases, both retinas in immunocompetent as well as immunocompromised patients[44]. The most frequent cause associated with a history of encephalitis and meningitis in patients older and younger than 25 years, respectively is HSV 1 and 2[44]. Triggering events such as periocular trauma, neurosurgery, and high-dose corticoids have been reported. A pioneering insight into the pathogenesis of ARN was provided by an early animal model in which HSV inoculation into the anterior chamber of rabbits was followed by retinal necrosis of the uninoculated eye[45], later confirmed in mice[46]. The virus spreads through synaptically connected nuclei and neurons to the contralateral, but not ipsilateral, optical nerve and retina. Hence, retinal pigment epithelial cells were used to study the triggering of TLR's 3, 4 and 9 in this study.

Overview of Productive Infection:

A thorough understanding of the HSV lytic infection is required to understand the concepts regarding latent infections and reactivation. The virus causes the initial infection, multiplies in the epithelium, invades the local nerve endings and travels by retrograde axonal transport to neuronal cell bodies in the regional sensory ganglia. After infection with HSV, replication of virus and subsequent death of the host cell usually occur. The viral entry involves the binding of the viral glycoproteins to the cell surface membrane receptors, primarily the heparin sulfate moieties, followed by penetration of the virion by fusion of the viral envelope with the cell plasma membrane, viral protein release, and capsid transport to the nuclear pores where the viral DNA is released into the nucleus of the host cell. Although HSV encodes atleast 74 unique genes, only 5 genes classified as the immediate early (IE) genes are transcribed initially along with the gene encoding the large subunit of the ribonucleotide reductase enzyme. Transcription of the IE genes is activated by VP16, a major component of the tegument. VP 16 acts though the target sequence TAATGARAT, which is present in atleast one copy in all HSV IE promoters. VP16 complexes with an ancillary cellular protein, HCF (Host cellular factor) and OCT- 1. The multiprotein-complex permits the binding of VP16 to the octamer consensus (TAATGARAT). The α genes encode 5 virion polypeptides, ICP0, ICP4, ICP22, ICP27 and ICP47. ICP0, 4, 22 and 27 have regulatory function of viral gene expression whereas, ICP47 prevents host response to infection[41].

The products of the α genes, initiate the production of the early (β) genes, whose role is the increase of nucleic acid metabolism. Replication of the viral DNA occurs in a rolling circle mechanism, in the replication compartment in the

cell nucleus. HSV DNA replication involves three viral origins. They are $Ori_{s1,}$ Ori_{s2} and $Ori_{L,}$ one or more host cell proteins are required to initiate origin dependent replication of HSV DNA[41].

The expression of the late (γ) genes is mediated by the β proteins. The γ genes encode the structural components of the virion and the viron glycoproteins. The assembled viral capsids which bud through the nuclear membrane are surrounded by the tegument proteins and are enveloped as they pass through the inner lamella of the nuclear membrane. Progeny virus spread from cell to cell via virus mediated fusion and potentially enters the dendritic termini.

Epidemiology

HSV is spread by contact, as the virus is shed in saliva, tears, genital and other secretions. By far the most common form of infection results from a kiss given to a child or adult from a person shedding the virus. The risk of infection to a non-immune individual in contact with contaminated secretions can be as high as 80%[1-4]. There are 3 types of herpetic episodes:-

1. Primary episode - the incubation period ranges from 5 - 15 days. The infection may be subclinical.

2. Reactivation - this refers to the reawakening of latent virus

3. Initial or first episode - this refers to infection (clinically apparent or not) on an anatomical site which has never before been the site for endogenous or exogenous virus.

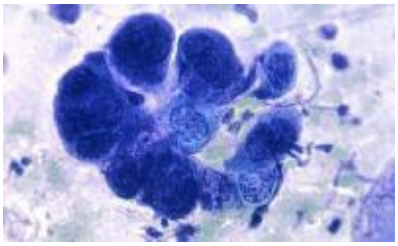
Auto-inoculation for one anatomical site to another is common, especially by scratching. It was said that HSV-1 causes infection above the belt and HSV-2 below the belt. In fact, 40% of clinical isolates from genital sores are HSV-1, and 5% of strains isolated from the facial area are HSV-2. This data is complicated by oral sexual practices[1-4].

Laboratory Diagnosis

Light Microscopy:–

Cells from the base of the lesion, or wiped from a mucous surface, or biopsy material, may reveal intranuclear inclusions (Lipschutz inclusion bodies). Infected cells may show ballooning and fusion[1-4].

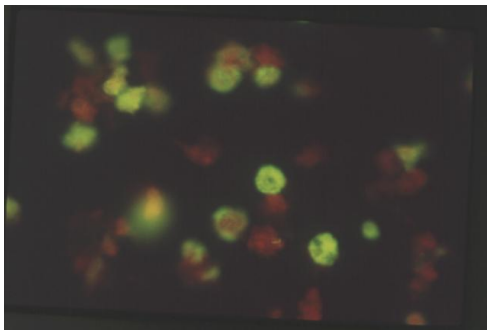
Fig 1.5: Tzanck smear



Electron Microscopy:–

Electron microscopy is not a sensitive tool for the detection of HSV, except in the case of vesicle fluids which often contain 10^8 or more particles per millilitre. However, like light microscopy, electron microscopy cannot distinguish between the different viruses[1-4].

Fig 1.6: Immunoflorescence assay for direct antigen detection of HSV



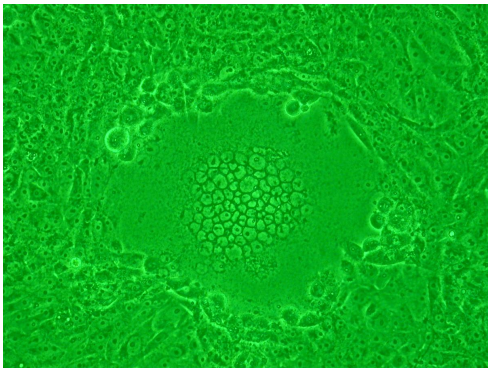
Direct examination by antigen detection:–

Cells from specimens are treated in ice-cold acetone. FITC is generally used for staining of fixed material. It is more sensitive and specific than light and electron microscopy (90% sensitive, 90% specific), but cannot match virus culture. In terms of cost and technical expertise, it is much more demanding[1-4].

Virus Isolation: –

Infectious HSV-1 and HSV-2 are amongst the easiest viruses to cultivate. The fresher the lesion, the better the chance for recovery, ulcers and mucous membranes should be swabbed as vigorously as possible. Virus transport medium should be used but this is not critical provided that desiccation is avoided. Saliva, urine, CSF and biopsy material do not need transport medium. Inoculation should be carried out as soon as possible. A wide range of cell lines are available for isolation. At least 2 cell lines should be chosen. A typical CPE may appear from day 1 onwards. Other viruses can occasionally mimic the CPE of HSV. Formal identification of the isolate can be carried out by immunofluorescence, complement fixation, neutralization or electron microscopy[1-4].

Fig 1.7: Early Cytopathic effect(CPE) of HSV-2 on Vero cells



Serology:–

CFTs are commonly used. But indirect haemagglutination (IFT) assays are more sensitive than CFTs. Weak antigenic cross reaction with VZV may occasionally cause problems in these tests. ELISAs and RIAs are becoming available and may gradually replace CFTs and IFTs. Seroconversion from a zero baseline is usually diagnostic of a primary infection. In the case of recurrent infection, an immune response from a non-zero baseline may be detected. For immune status screen, sensitive tests such as ELISAs and RIAs should be used as insensitive tests such as CFTs may fail to detect very low levels of HSV specific antibodies which are present years after a primary infection[1-4].

In contrast to many other viruses, HSV specific IgM had proved to be unhelpful in the diagnosis of primary infection as HSV IgM may be present in cases of reactivation as well as primary infection. In certain circumstances, HSV IgM may be useful in the case of neonates when the presence of HSV IgM is a highly significant finding, as can be its detection in the CSF of a patient with suspected encephalitis[1-4].

Herpes simplex encephalitis:-

The diagnosis of HSE is difficult, therapy should be commenced as soon as possible where there is suspicion of HSE on clinical grounds before the results of laboratory tests are known. HSE cannot be diagnosed reliably by virus isolation from the CSF or by IF using CSF cells. HSE is diagnosed by the following methods[1-3]:-

(a) HSV antigen by FITC conjugated anti-HSV antibodies from brain biopsy cells.
(b) Virus culture from brain biopsy cells
(c) The presence of HSV virions or Lipschutz inclusion bodies in brain tissue by electron or light microscopy.
(d) The demonstration of intrathecal synthesis of HSV antibody.

Brain biopsy provides the best avenue for diagnosis but is rarely carried out nowadays with the advent of acyclovir. For determination of intrathecal HSV Ab synthesis, the integrity of the blood-brain barrier should be assessed to ensue that any HSV Ab present in the CSF is not a result of transudation from the serum across a compromised blood-brain barrier to the CSF, as is the case during the early stages of acute necrotizing encephalitis. Herpes simplex viruses are the first among the herpes group of viruses to be discovered. Herpes simplex virus (HSV) is responsible for diseases in humans ranging in severity from sub-clinical infection to lethal encephalitis[1-4]. HSV is a large DNA virus, capable of causing lytic and latent infection in humans. The most common human illnesses caused by HSV are the recurrent common cold sore and genital herpes. Lesions occur following recrudescence of latent virus harbored in the sensory neurons

innervating the involved epithelium[1-4,]. Lesions caused in immunocompetent patients may be benign while those in immunocompromised patients can be life threatening with high mortality and morbidity.

Innate Immune Response:

A wide variety of viral products serve as triggers for the cellular recognition of HSV infection, including nucleic acids and lipoproteins. The nucleic acid sensors can be categorized based on both localization and nucleic acid structure, and include the scavenger receptors, the retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), the Toll-like receptors (TLRs), the Nod-like receptors (NLRs) and DNA-dependent activator of IRFs (DAI) [47-52]. The viral protein sensors include the TLRs, which have been shown to recognize viral lipopeptides [53,54].

The major trigger for cellular recognition of a viral infection appears to be the viral nucleic acid, which can be recognized at multiple cellular locations. Extracellular dsRNA is detected at the cell surface by the class A scavenger receptors [51,53]. Recent data suggest that these membrane-bound receptors recognize extracellular dsRNA and serve as chaperones to bring viral dsRNA into the cell for cytosolic recognition. In the endosomal compartment, TLR3 serves as a receptor for viral dsRNA and TLR9 recognizes CpG-rich DNA [47,55,56]. In the present study the response of Toll-like receptors to HSV was studied using Human retinal pigment epithelial cells.

TLRs have been shown to recognize both nucleic acid and protein derived from HSV [52,53,54,56,57]. In mice, four TLRs have been found to play a role in the resistance to HSV-1: TLR3, TLR9, and the TLR2/6 heterodimer, which

recognize dsRNA, CpG-rich DNA and lipopeptides, respectively [52, 53, 58, 59, 60]. HSV-1 infection via intranasal delivery causes 100% mortality in mice deficient in the TLR adaptor protein MyD88, suggesting that innate control via TLRs is important in limiting HSV-1 infection [61]. Indeed, it has been shown *in vivo* that HSV-1-mediated production of IFN requires TLR9 and MyD88 [59,62].

TLRs signalling pathway

MYD88:

MyD88 was the first adaptor protein identified and it is used by all TLRs except TLR3. The main evidence for the role of MyD88 in TLR signalling came from the study of MyD88-deficient mice. These mice were unresponsive to PAMPs detected by TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 [47,48,49]. They were hyporesponsive to LPS and were susceptible to infection by a number of bacteria including *S. aureus* [47], *T. gondii* [48], *Listeria monocytogenes* [51] and *M. tuberculosis* [52,53]. There were no reported developmental defects in the MyD88 knockout mice. Despite the fact that MyD88 knockout mice are susceptible to a number of bacterial infections, they have been shown to still be *Toll-like receptors,* as pathogen sensors are capable of mounting an adaptive immune response [52]. Therefore, the role of MyD88 in initiating an adaptive immune response is dependent on the infection. MyD88 has a TIR domain like TLRs, and it seems that it interacts with TLRs through a TIR–TIR interaction. MyD88 recruits interleukin 1 receptor associated kinase 4 (IRAK-4) which triggers phosphorylation of IRAK1 [54,55]. IRAK1 can activate TRAF6. Both proteins leave the receptor complex and interact with TGF-b-activated kinase 1 (TAK1) and two TAK1 binding proteins TAB1, TAB2 [56]. TAK1 becomes

phosphorylated and activates the IkB kinase (IKK) complex [56, 57]. IkB is then phosphorylated allowing NF-κB to translocate to the nucleus and induce expression of proinflammatory cytokines. TAK1 is also capable for phosphorylating MKK6 and 7 which leads to the activation of p38 and JNK.

The family of TLRs share great similarities with the IL1 receptor especially in its intracytoplasmatic domain with a highly conserved Toll-interleukin 1 receptor (TIR) motif [58,59]. For the TIR domain five different adaptor molecules have been described, namely MyD88, TIRAP, TRIF, TRAM, and SARM of which the first four have activating functions and SARM has inhibitory capacities [60]. The extracellular part is composed of repetitive leucine residues (leucine rich repeat). The intracytoplasmatic signaling structure is grossly divided in a MyD88-dependent and independent pathway both of which show recruitment of crucial adaptor molecules to the Toll-IL-1 receptor motif [62]. The majority of TLRs (namely TLR1,2,4,5,6,7,8, and 9) use the myeloid differentiation factor 88 (MyD88) to initiate a signaling cascade which leads to the downstream activation of kinases and which results in the translocation of the central transcription factors NF-κB and IRF-3 [63]. To this end, MyD88 associates with TIRAP to a complex which then recruits IRAK and subsequently TRAF-6 [64] finally resulting in an activation of the IKK complex (Figure 4). In MyD88-independent signaling which is used by TLR3 and to a certain degree also by TLR4, the adaptor molecule TRIF is recruited to the intracellular part of TLR3 directly [65] or to TLR4 via TRAM [66] which in consequence leads to activation of both, TBK-1 and TRAF-6, a crucial checkpoint for the induction of a NF-κB-dominated immune response or a IRF-3-dominated immune response with an type I interferon activation pattern [66]. Interestingly, it has become clear in the last

years that the recruitment of TRAF-6 and TBK-1 is mutually exclusive, most probably due to steric hindrances, which leads to the exclusive activation of the NF-κB or IRF-3 pathway depending on the accessibility of the binding sites [67].

The divergent activation of these two transcription factors then lead to preferential state of pro-inflammatory immune responses (as for NF-κB) or antiviral immune responses (as for IRF-3). This activation has been shown to be under the negative regulatory control of SARM, an alternative adaptor molecule for the TIR domain [61].

Consequences of TLR activation:

As mentioned above, activation of NF-κB is one of the central activation pathways after recognition of TLR-ligands. This activation not only provides the production of various chemo- and cytokines but also enhances the capacity of phagocytotic cells to ingest microbial compounds. As some of the TLRs, namely TLR2, and 4 are able to co-localize to phagosomes [68,69], a very early contact of the immune system with potentially hazardous microbial antigens are provided. Within the phagosome, two types of innate receptors co-operate to determine the type and magnitude of the immune response. These are phagocytic receptors such as the mannose-receptors and PAMP-receptors such as the TLRs [70]. This combination not only provides a maturation signal for the phagosomes, it also seems to be a crucial help to distinguish self-antigens (which should not be loaded on MHC-II molecules) and foreign antigens (which should be efficiently loaded on MHC-II molecules [70]. In addition to trigger phagocytotic and maturation signals, the engagement of TLRs also enhances co-stimulatory molecule expression such as CD80 and CD86 providing a second signal for the full immune

response [71,72]. Thus, the engagement of TLRs in the phagosome enhances killing of captured microorganisms and the efficient degradation of the ingested proteins for presentation on the cell surface.

The situation is different for the group of intracellular TLRs (TLR 3, 7, 8, and 9) which are located on the endosomal membrane where they sense virus derived pattern molecules and respond with the induction of antiviral genes such as type I interferons [73]. Besides the induction of soluble factors, the engagement of intracellular TLRs also enhances the presentation of antigens by the MHC-I pathway leading to the activation of CD8 cells which are the central weapons against virally infected cells [74,75]. Besides their central role in triggering and shaping the cellular immune response, TLR activation also results in the production of antimicrobial defenses. Thus, several epithelial cell types including airway epithelial cells and keratinocytes react to the stimulation through TLR2 or TLR4 by the up-regulation of human β-defensin 2 [76,77]. Interestingly, after induction of HBD-2, this antimicrobial peptide itself can act as a ligand for the TLR4 and thereby enhance the immune response. In addition to defensin production [78], TLR-engagement also induces reactive oxygen and nitrogen species which are crucial for the killing of intracellular pathogens such as *Mycobacterium tuberculosis*. Of note, in contrast to murine cells the induction of mycobacterial killing in human macrophages and dendritic cells require the parallel engagement of the vitamin D receptor [79]. The most important feature of TLR activation however, is the production of pro-inflammatory milieu which is provided by certain cyto- and chemokines. These are predominately TNFα and IL-12 for NF-κB signaling, TLR-ligands and IFNα/β for IRF-3 signaling. In fact, the combination of the induction of a robust cellular immune response together with a

rapid skewing of the immune response towards a TH-1 dominated profile have rendered TLR-agonists interesting adjuvants for allergy treatment and the design of tumor vaccines [80].

Finally, the engagement of TLRs in some tissues leads to the induction of programmed cell death, using caspase 8 and the FAS-associated death domain protein. This pro-apoptotic effect has been shown for mycobacterial, bacterial, and mycoplasmal lipoproteins and to signal through TLR2 and TLR4 [81,82]. In summary, the sensing of microbial patterns through TLRs leads to the induction of a robust immune response of the TH-1 type including the induction of potent self-defense molecules, and the efficient conversion of immature antigen presenting cells in fully equipped amplifier of a cellular immune response. In tissues not capable of specific antigen presentation, TLR signaling might also induce apoptosis to minimize the spreading of infection.

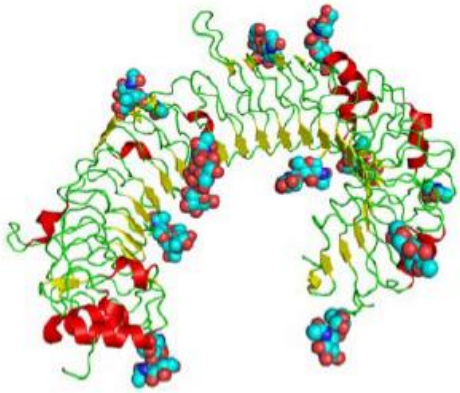
Toll Like Receptor 3:

Double-stranded RNA (ds RNA) is naturally a viral product which is produced by viruses during their life cycle and is recognized by TLR3. Recently the crystal structure of the human TLR3 ectodomain has been obtained [71]. This structure reveals that TLR3 is mostly masked by carbohydrates but there is one face which is glycosylation free and it is predicted that this is the region to which dsRNA binds. TLR3-deficient cells show impaired responses to the synthetic dsRNA ligand polyinosine-polycytidylic acid (Poly (I:C)) [72]. Upon activation, TLR3 signals in a MyD88- independent manner, through Toll-IL-1R domain-containing adaptor-inducing IFN- (TRIF) to up-regulate type I IFN [27]. TLR3 signals also lead to the activation of NF-κB and type I interferons (IFNs) [73].

Structure:

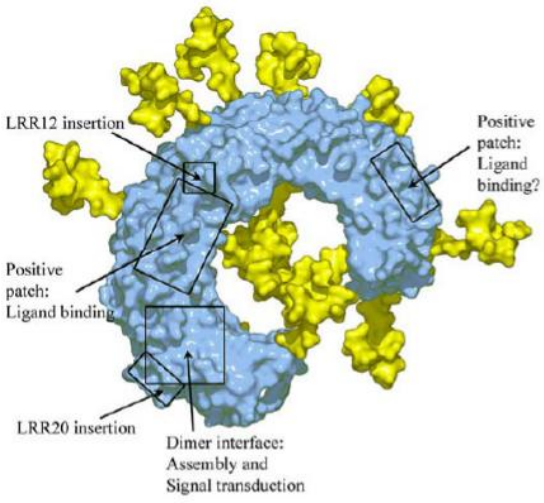
The structure of the human TLR3 ectodomain (ECD) was determined to 2.1 Å resolution using MAD data collected at SSRL Beam Line 11-1 and at the ALS, and represented the first structural look at any TLR ligand binding domain. The overall structure revealed a large horseshoe-shaped, right-handed solenoid structure comprising of 23 leucine rich repeats (LRRs) (Figure 1.8). The inner concave surface is formed from 25 parallel β-strands, 23 from LRRs and one each from the N- and C terminal cap region that makes a highly curved, continuous β-sheet that spans 270º of arc. The outer convex surface contains an assortment of diverse secondary structural elements. The LRRs of TLR3 (ECD) follow the typical consensus motif of a 24-residue repeat consisting of xL2xxL5xL7xxN10xL12xxL15xxxxF20xxL23x, where L represents hydrophobic residues including leucine (most prevalent), isoleucine, valine, methionine and phenylalanine, F is a conserved phenylalanine, and N a conserved asparagine[75]. Seven conserved hydrophobic residues in this motif form a tight hydrophobic core of the solenoid structure and conserved asparagine at position 10 makes extensive hydrogen bonding networks with its own and previous LRR motifs. TLR3 ECD has 15 potential glycosylation sites and electron density for carbohydrate is observed for 8 of these sites. When oligomannans are modeled into all 15 sites, it was revealed that most of the ECD surfaces, with the exception of one side face, are covered with carbohydrates. Contrary to the common belief that the inner concave space contains a ligand binding site, the inner concave surface of TLR3 has two glycosylation sites and many negatively charged residues that make it an unlikely binding site for dsRNA[76].

Fig 1.8: Schematic 3D structure of TLR3



The glycosylation-free face contains two surface patches with a dense cluster of positively charged residues and a TLR3- specific insertion in LRR12 that could play a role in dsRNA binding. This face also contains a highly-conserved surface patch that coincides with a putative homodimer interface observed in the crystal and another TLR3- specific insertion (LRR20) that participates in the dimer interaction (Figure 2). Based on the location of glycosylation sites, the electrostatic surface potential, the TLR3-specific insertion and the dimer formation, we have proposed a model for the dsRNA binding site and mode of signal transduction[76].

Figure 1.9. Functional sites on the glycosylation-free face of TLR3. The locations of the positive patches and one of the two large insertions in LRR12 that are implicated in ligand binding are indicated. The conserved surface and the other large TLR3-specific insertion (LRR20) maps to the dimerization interface. Oligomannose type sugars are drawn in yellow. (From Choe *et al*. 2005).



Although viral dsRNA is known to be sensed by TLR3, DCs or fibroblasts that lack TLR3 still produce type I IFNs after intracellular introduction of dsRNA molecules. This TLR3-independent induction does not require TRIF, but depends on the kinase TBK1 (TANK-binding kinase-1) and the transcription factor IRF-3 (IFN regulatory factor-3) [77]. Therefore, TLR3 is the only TLR which does not use the crucial adaptor molecule, myeloid differentiation primary response gene-88 (MyD88), for intracellular signal transmission [77]. In addition to direct NF-κB activation, TLR3 can also use an alternative signalling pathway leading to the activation of TANK-binding kinase-1 (TBK-1), which results in the phosphorylation and nuclear translocation of the transcription factors interferon regulatory factor-3 (IRF3) and IRF7 [78] followed by the production of type I interferons, namely IFN-β [79].

Although viral dsRNA is known to be sensed by TLR3, dendritic cells or fibroblasts that lack TLR3 still produce type I interferons after intracellular introduction of dsRNA molecules. This TLR3-independent induction does not require TIR domain-containing adapter including IFN-β (TRIF), but depends on the kinase TBK-1 and the transcription factor IRF3 [77]. In this pathway, the dsRNA is recognized by the cytoplasmic helicase domain of the RNA helicase protein retinoic acid–inducible gene-I (RIG-I). The downstream signaling events that result from this recognition require the NH2-terminal caspase recruitment domain (CARD) of RIG-I, which binds to the adaptor molecule Cardif (also known as IPS-1) [80,81]. Another candidate for the sensing of cytoplasmic dsRNA is MDA5 (melanoma differentiation-associated gene 5 or Helicard) which is also interferon-inducible and belongs, like RIG-I, to the DExD/H-box-containing RNA helicases [81]. The third TLR3-independent mechanism by

which mammalian cells recognise dsRNA is the dsRNA-dependent protein kinase R (PKR). PKR is activated upon binding of dsRNA and then undergoes dimerization and autophosphorylation. This 68-kDa, cytoplasmic serine/threonine kinase phosphorylates its physiological substrate eukaryotic initiation factor 2-α (eIF2-α) and inhibits translation and perhaps other substrates which results in activation of a panel of genes that ultimately leads to cessation of virus replication in infected cells[82-84]. It has also been shown that PKR regulates other pathways, including p53, p38, IRF1, and NF-κB [84-86]. Induction of NF-κB has a relevant role in mediating PKR functions, and NF-κB activation by PKR is involved in IFN-β induction in response to dsRNA [87]. Little, if anything, however, is known on the relative contribution of the various dsRNA sensing and signalling pathways in human keratinocytes. As viral infections such as human herpes virus or papilloma virus infections are common in human skin with keratinocytes as the targets for viral attack, the ability to induce a sufficient antiviral response appears to be crucial during the earliest phases of the response when innate defense mechanisms dominate [88].

Distribution and Expression Of TLR3

TLR3 is expressed in a wide variety of cells including epithelial cells [89], fibroblasts [90], microglia [91], astrocytes [92], mast cells [93,94], eosinophils [95], endothelial cells [96] and dendritic cells [97]. In contrast, TLR3 expression is minimal in T cells [98,99] and entirely absent in neutrophils which use alternative sensors to detect dsRNA [100,101]. TLR3 expression is barely detectable [101] to absent [102] in monocytes but expression was markedly increased following cytokine-induced maturation of monocytes to immature dendritic cells (iDC). Muzio and colleagues detected TLR3 in mature DCs but not

iDC [102]. In contrast, other studies found that expression of TLR3 is lost during progression of DCs to the mature form [97, 103]. TLR3 induced DC maturation is believed to bridge the innate and adaptive immune systems [104]. TLR3 mRNA was not detected in T-lymphocytes, B cells or NK cells [102]. Sha *et al.* reported TLR3 to be the most abundant TLR, expressed in Beas-2b airway epithelial cells. In agreement with this, poly I:C was found to be the most effective TLR agonist at upregulating gene expression in micro-arrays, with 7-fold upregulation of IL-8 and IL-6 expression observed [105]. TLR3 expression is upregulated by poly I:C [105-107] or viral infection e.g. RSV [108] and *H. influenza* [89, 109]. TLR3 expression in human monocytes and monocyte-derived macrophages is inducible by treatment with IFN-β. The human promoter for TLR3 contains interferon response elements (IREs) and a STAT element; the cognate transcription factors are suggested to be IRF1 and STAT1 [103]. Indeed, dominant-negative expression of STAT1 can abolish poly I:C induced TLR3 expression in murine cells [110]. LPS also upregulates TLR3 expression *via* the autocrine action of LPS-induced IFN-β secretion. LPS does not however upregulate TLR3 expression in human cells [103].

Intracellular Distribution Of TLR3

TLR3 is mostly thought of as an intracellular receptor, resident on the plasma membranes of endosomal vesicles. Indeed in various subsets of dendritic cells and macrophages TLR3 is exclusively intracellular [89-91]. Other cell types however can display a proportion or all of their TLR3 on the cell membrane. Cell surface TLR3 has been demonstrated in fibroblasts [52], astrocytes [69] and epithelial cells [86, 92, 93]. A neutralizing monocloncal antibody for TLR3 prevents dsRNA signalling in fibroblasts where TLR3 is on the cell surface [6]

but not in dendritic cells where TLR3 is intracellular [91]. Studies on the localization of TLR3 in epithelial cells have been conflicting. Several studies have concluded that TLR3 is located intracellularly in airway [67, 87] and endometrial epithelial cells [94]. As well as upregulating TLR3 expression, viral infection of epithelial cells appears to induce localization of TLR3 to the cell surface [86, 92] where it serves to sensitize or "prime" the cells to better recognize and respond to subsequent viral challenge [86]. A short 26 amino acid "linker" domain between the cytoplasmic TIR domain and the transmembrane portion of TLR3 is necessary for intracellular vesicular localization of TLR3. Truncated versions of TLR3 lacking this region localize to the cell surface [95]. UNC93B is a membrane protein normally resident in the endoplasmic reticulum (ER), which is reported to be involved in trafficking of TLR7 and 9 from the ER to the endolysosome. Although this wasn't demonstrated for TLR3 [96], Brinkmann and colleagues demonstrated that UNC93B interacts with TLR3 *via* its transmembrane domain and that point mutation of UNC93b in mice abrogates TLR3, 7 and 9 signalling, leaving the mice extremely susceptible to viral infection [97]. TLR3 and TLR7 localize in the same intracellular compartments often found adjacent to phagosomes containing apoptotic bodies, suggesting that TLR3 and 7 can be triggered by nucleic acids from apoptotic cells [98].

TLR9:

Studies carried out on TLR9-deficient mice have shown that unmethylated CpG is the natural ligand for TLR9 [121]. TLR9-deficient mice did not produce any inflammatory cytokines in response to CpG, their splenocytes failed to proliferate and there was no maturation of dendritic cells [74]. TLR9, like TLR7 and TLR8, is localized to endosomal compartments which may allow them to

discriminate between self and non-self, as host DNA and RNA tend not to enter into endosomal compartments [111]. TLR9 has been shown to be able to respond to MCMV and herpes simplex virus (HSV), presumably through the presence of unmethylated CpG within these viral genomes [58].

TLR4:

TLR4 is the first described human homologue of *Drosophila* Toll[112]. Lipopolysaccharide (LPS) is the major component of the cell wall of Gram-negative bacteria. It is a potent immunostimulant and can cause the endotoxic shock. LPS consists of a lipid a portion which is the endotoxic component, a core oliogosaccharide and an O-antigen. To respond to LPS, TLR4 requires other co-receptors include CD14 which is a glycosylphosphatidyl inositol (GPI)-anchored glycoprotein[113] and MD2. LPS binds to LPS binding protein in serum which transfers LPS monomers to CD14 which in turn concentrates the LPS to allow binding to TLR4/MD2 complex[114]. This triggers a pathway which leads to the activation of the transcription factor NF-κB which regulates the transcription of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, IL-1 and IL-8. It can also activate other transcription factors include members of the mitogen-activated proteins kinase family (MAPK) notably p38 and JUN N-terminal kinase (JNK)[74]. TLR4 is also capable of responding to components of fungal pathogens such as mannans from *Saccharomyces cerevisiae* and *Candica albicans* and glucuronoxylomannan from *Cryptococcus neoformans* [115-118]. Other TLR4 ligands include taxol [119,120] and the fusion protein from respiratory syncytial virus[121].

The importance and downstream sequelae of TLR recognition of HSV is complex and context-dependent. TLR9 appears to play only a minor role in the defense against HSV despite its requirement for IFN production by pDCs. In the absence of TLR9, there was no change in either viral load in the nervous system or overall survival following infection with HSV-1 [59,62]. Furthermore, Kurt-Jones *et al.* have demonstrated that TLR2 mediates the production of inflammatory cytokines in response to HSV-1 infection, leading to the development of lethal viral encephalitis [54]. Taken together, this evidence suggests that while the precise role of TLRs in the innate response to HSV *in vivo* are not fully understood, they appear to have a role in mediating both immune protection as well as immune pathology. Not surprisingly, there has been a great deal of recent interest in using TLR ligands to treat or prevent HSV infection. A number of groups have revealed that mucosal delivery of ligands for TLR3 and TLR9, but not TLR2 or TLR4, are protective against genital infection with HSV-2 [63-69]. Similarly, intranasal application of a ligand for TLR3, but not for TLR4 or TLR9, protected against HSV encephalitis in mice [70]. The therapeutic approach of using TLR ligands to treat HSV is an area that requires further investigation, since the downstream consequences of TLR engagement *in vivo* remain to be clarified.

Hence this study was planned to demonstrate the expression of TLR's in human retinal pigment epithelial cells in HSV infection using ARPE-19 cells. In this study we have demonstrated the expression of TLR3, 9 and 4 based on reverse transcriptase polymerase chain reaction.

Antiviral treatment:

The development of successful antiviral agents against these infections had been slow until last decade. However progress in organ transplant, cancer therapy and the high incidence of human immunodeficiency virus(HIV) have stimulated efforts towards the development of newer compounds for the treatment of individuals with herpes infection[122].

At present, there are only a few indications of antiviral chemotherapy, with the high cost of antiviral drugs being a main consideration. Generally, antiviral chemotherapy is indicated where the primary infection is especially severe, where there is dissemination and when sight is threatened. Urgent treatment is required in the face of a life-threatening HSV infection in the eczematous or immunocompromised patient, in the neonate, in HSE, and in patients with eczema herpeticum. It is not necessary to treat HSV disease if it is not sufficiently severe to warrant hospital admission. Exceptions to the rule include painful herpetic whitlows and all forms of ocular herpes. Recurrent disease other than ocular disease in normal individuals rarely merits specific chemotherapy[122].

Herpes antiviral therapy began in the early 1960s with the experimental use of medication that interfered with viral replication called deoxyribonucleic acid (DNA) inhibitors. The original use was against normally fatal or debilitating illness such as adult encephalitis, keratitis, in immunocompromised (transplant) patients, or disseminated herpes zoster. The original compounds used were 5-iodo-2'-deoxyuridine, AKA idoxuridine, IUdR, or(IDU) and 1-β-D-arabinofuranosylcytosine or ara-C, later marketed under the name cytosar or cytorabine. The usage expanded to include topical treatment of herpes simplex,

zoster, and varicella. Some trials combined different antivirals with differing results.[1-3,123] The introduction of 9-β-D-arabinofuranosyladenine, AKA ara-A or vidarabine, considerably less toxic than Ara-C, in the mid 1970s, heralded the way for the beginning of regular neonatal antiviral treatment. Vidarabine was the first systemically administered antiviral medication with activity against HSV for which therapeutic efficacy outweighed toxicity for the management of life-threatening HSV disease. Intravenous vidarabine was licensed for use by the U.S. Food and Drug Administration (FDA) in 1977[1-3,123]. Other experimental antivirals of that period included: Heparin, trifluorothymidine (TFT), Ribivarin, interferon, Virazole, and 5-methoxymethyl-2'-deoxyuridine (MMUdR). The introduction of 9-(2-hydroxyethoxymethyl)guanine, AKA acyclovir, in the late 1970s raised antiviral treatment another notch and led to vidarabine vs. acyclovir trials in the late 1980s. The lower toxicity and ease of administration over vidarabine has led to acyclovir becoming the drug of choice for herpes treatment after it was licensed by the FDA in 1998. Another advantage in the treatment of neonatal herpes included greater reductions in mortality and morbidity with increased dosages, something that did not occur when compared with increased dosages of vidarabine[1-3,123]. On the other side of the equation, acyclovir seems to inhibit antibody response and newborns on acyclovir antiviral treatment experienced a slower rise in antibody titer than those on vidarabine[1-3,123].

Antiviral treatment of herpes simplex virus (HSV) infections with nucleoside analogues has been well established for over two decades, but isolation of drug-resistant HSV from immunocompetent patients remains infrequent (0.1 to 0.7% with a mean of 0.3%. [124]) and, with very rare exceptions, resistant HSV is cleared normally with no adverse clinical outcome. The isolation of resistant HSV

from immunocompromised patients is more common (4 to 7% [125]) and more likely to be clinically significant. Nonetheless, even in this population there is no evidence of any increase in resistance despite the progressive increase in antiviral usage.

The use of susceptibility testing could help improve the management of patients with herpesvirus disease who are unresponsive to standard regimens of ACV. Patients whose lesions persist or worsen while they are receiving ACV therapy could benefit from susceptibility studies. In such cases, knowledge of a strain's in vitro susceptibility to ACV would be useful for the selection of alternative antiviral therapy, which might involve increasing the dose of oral ACV or changing to high-dose continuous infusion of ACV (for those patients who do not respond to standard regimens of ACV). It is important to identify a method of susceptibility testing that will diagnose antiviral resistance both rapidly and reproducibly. This will be increasingly important as new candidate antiviral agents become available for the treatment of patients with resistant HSV infection. To determine the susceptibility of HSV to ACV, various tests have been designed. This underlines the clinical importance of HSV drug susceptibility determinations for this patient group.

Phenotypic assays:

Phenotypic susceptibility testing has been an essential tool for identification and monitoring of resistant viruses, elucidation of the mechanisms of antiviral resistance, determination of cross-resistance and for discovery of new antiviral agents[126,127]. So far, phenotypic tests remain the standard for susceptibility testing of HSV.

Since the replication cycle of HSV is rapid(18 hours), the results of susceptibility assays could be theoretically available in time for use in patient management. However, the clinical role of these assays is at present only marginal, mainly due to their laborious and time-consuming character and the lack of the standardization. There are indeed many variables that can influence the susceptibility result. These include: the cell line, the viral inoculum size, the incubation time, the range of drug concentrations, the reference strains, the assay type and the calculation and the interpretation of the endpoint.

Susceptibility assays can be grouped by the type of endpoint that is determined. This can be: i) the number of virus plaques (plaque reduction assay)[127], ii) the amount of infectious virus produced (yield reduction assay)[128], iii) the level of cytopathic effect (CPE) determined either microscopically (CPE reduction assay)[129-131] or colorimetrically (dye uptake assay)[130-132], iv) the production of virus antigen (ELISA, microplate in-situ ELISA) [134,135], v) the number of infected cells (flow cytometric analysis)[136], vi) the viral DNA production determined either by DNA hybridization or real-time PCR[137,138].

The plaque reduction assay (PRA), used in most laboratories for antiviral susceptibility testing of HSV, entails determination of the degree of inhibition of virus growth by counting of the number of plaques present at different concentrations of a given antiviral drug. The dye uptake assay (DU), a semi-automated method based on the uptake of neutral red dye by viable cells, measures a quantitative reduction in cell death and has a colorimetric endpoint measured by a spectrophotometer. The DNA hybridization assay (DNAHA)

quantifies viral DNA after exposure to an antiviral agent by a radioiodinated DNA probe which is specific for HSV-1 and HSV-2.

There is no consensus regarding the level of in vitro susceptibility that indicates a drug-resistant virus, but many different types of assays have shown that ACV-resistant isolates are usually susceptible to an ACV concentration of 12–3 mg/ml. Nevertheless, correlation between clinical findings and in vitro susceptibility to ACV has not been definitely established, and further studies are needed.

Antiviral Chemotherapy

Specific anti-HSV drugs are more effective in the treatment of primary infection and should be given within 3 days of the onset of symptoms. They are rarely effective if given more than 5 days after the onset of symptoms[123]. With recurrent disease, treatment must be initiated in the prodromal phase before the appearance of symptoms or treatment will prove to be valueless. The following are anti-HSV drugs currently used worldwide[123].

Acyclovir:–

This is the drug of choice for most situations at present. Acyclovir {9-[(2-hydroxyethoxy) methyl] guanine, zovirax} is a guanosine analogue with an acyclic side chain at the 9 position. It is a prototype of the group of viral agents that are activated by viral thymidine kinases (Tk) to become inhibitors of viral DNA polymerases and block viral DNA synthesis. Acyclovir uptake and intracellular phosphorylation to monophosphate is mediated by viral thymidine. Acyclovir requires the presence of a HSV-encoded thymidine kinase in order to be converted into its active acyclovir triphosphate form. Acyclovir triphosphate is

a potent selective inhibitor of HSV DNA polymerase and causes premature chain termination when it competes with guanine triphosphate for incorporation into newly synthesized viral DNA[122,123]. Acyclovir is available in 4 formulations:

1. IV (HSV infection in normal and immunocompromised patients)
2. Oral (treatment and long term suppression of mucocutaneous herpes and prophylaxis of HSV in immunocompromised patients)
3. Cream (HSV infection of the skin and mucous membranes)
4. Ophthalmic ointment

Acyclovir has few mild side effects and is generally very safe to use. After prolonged high doses, acyclovir can cause bone marrow depression but this is not likely to be encountered in normal clinical practice. Acyclovir resistant strains of HSV have been reported. Three basic mechanism have been identified-

- ❖ Altered Tk substrate specificity,
- ❖ Absent or partial production of viral Tk and
- ❖ Altered viral DNA polymerase.

The most common mechanism found in clinical isolates is deficient TK activity. Only Tk- variants have emerged in nature following the therapeutic use of acyclovir. However, both types of mutants are associated with reduced pathogenicity. Forscarnet and ara-A are the drugs of choice for the treatment of acyclovir resistant HSV[122, 123]. Acyclovir resistant isolates of HSV have been observed in immunocompromised individuals, especially AIDS patients. Acyclovir resistance in AIDS patients usually includes progressive cutaneous disease with chronic ulcerative mucocutaneous lesions, prolonged viral shedding, poor healing and local spread, despite acyclovir therapy[122].

Famciclovir: -

Famciclovir {[9-(4 hydroxy- 3-hydroxymethylbut l-yl) guanine Famvir]} is the inactive diacetyl 6-deoxy prodrug ester of penciclovir [9-(4 hydroxy-3-hydroxy-3-hydroxymethylbut 1-yl) gaunine], an acyclic nucleoside analog of guanosine. Famciclovir is the prodrug of penciclovir which is the active form and a guanosine analog. It has a very high bioavailabiity of 77%[122,123]. It is converted into penciclovir by a two step process. The first step occurs in the gut and the second step in the liver. It has a long half life in the gut. It has a higher affinity for HSV thymidine kinase than acyclovir but a lower affinity for HSV DNA polymerase than acyclovir. It acts as an inhibitor of viral DNA polymerase and also as a chain terminator. It is used for the treatment of primary and recurrent genital herpes, and for long term suppression of recurrent genital herpes[123].

Valaciclovir:–

Valacyclovir [2-(2 amino-1,6-dihydro- 6-oxo-9H purine 9yl-methothy) ethyl L-valinate hydrochloride, Valtrex] is the L-valylester of acyclovir. It was designed to enhance oral bioavailability of the parent compound. Valiciclovir is the prodrug of acyclovir but is much more readily absorbed by the gut than acyclovir. It is used for the treatment of primary and recurrent genital herpes, and for long term suppression of recurrent genital herpes [122,123].

Idoxuridine and trifluorothymidine:–

Idoxuridine (5' iodo 2' deoxyuridine, stoxili, Herplex) is a pyrimidine nucleoside analogue. It is phosphorylated to its active form by cellular kinase. It acts as a competitive inhibitor of HSV DNA.polymerase and also acts as chain

terminator. Idoxuridine is highly toxic when given systemically and is now only used in the eye. Trifluorothymidine is an excellent drug in ophthalmic practice but it does not have a product license[122,123].

Vidarabine (ara-A):–

Vidarabine (9-B-D-ribofuranosyladenine, ara A, vira A) is a nucleoside purine analogue with activity against all members of human herpes virus group. It is phosphorylated to its active triphosphate form, ara ATP by cellular kinases rather than by virus induced thymidine kinase so it is capable of inhibiting TK - (thymidine kinase) deficient mutants of HSV, which are resistant to acyclovir. Intravenous ara-A can be used for the treatment of neonatal herpes and HSE. It is not available as an oral preparation but can be used topically in the treatment of ocular infections. Ara-A does not have a product license for use in the UK for treatment of HSV infections[122,123].

Acyclovir has all but replaced ara-A for systemic therapy. This shift is not necessarily soundly based as there is little evidence that acyclovir is more effective than ara-A when used systemically. However, there is no doubt that ara-A is a much more toxic compound than acyclovir as it can cause bone marrow depression and its use should be monitored by regular full blood counts. Topical therapy for mucocutaneous herpes is probably of minimal benefit outside ophthalmic practice. Idoxuridine and acyclovir cream are available for the treatment of oral or genital herpes but are of doubtful value[123].

Prophylactic Antiviral Chemotherapy

Prophylactic anti-HSV chemotherapy may be indicated in the following situations:-

Bone marrow graft recipients:–

BM graft recipients become intensely immunodeficient and HSV is an important cause of post- transplant morbidity and death. Generally, acyclovir is given as maintenance prophylaxis for 6 to 9 months, beginning 3 days before transplantation[123].

Patients who suffer frequent recurrences of oral or genital herpes:–

A daily dose of acyclovir 800mg (1000 mg during immunosuppression) will have a favorable effect on recurrences. The response is better for herpes genitalis than labialis lesions[123].

Herpes Simplex Encephalitis

It is still unclear whether the prognosis of HSE is affected to a great extent by specific chemotherapy. The outcome in HSE is dependent to a great extent on the quality of the general medical care. Patients die from this disease because of the unremitting cerebral oedema and damage to critical neurological systems. The cerebral oedema should be aggressively controlled by dexamethasone, osmotherapy (urea, mannitol, or glycerol), hyperventilation, elevation of the head of the bed and if indicated, by surgical decompression. Acyclovir is now the drug of choice for the treatment of HSE. With the best treatment, the mortality of HSE can be reduced from 70% to 30%. Ideally, brain biopsy should be performed before specific antiviral chemotherapy is given but there is at present, a great reluctance to perform brain biopsy[123].

Ocular Herpes

The management of ocular herpes is a highly specialized subject. In general, topical steroids must never be prescribed in any condition of the eye which carries the slightest suspicion of being ocular herpes. Surgical cleansing of the cornea (debridement) makes a significant contribution to healing and complements local antiviral chemotherapy. Secondary bacterial infection should be treated by antibiotics. There is a wide choice of anti- HSV preparations available and selection is difficult and mostly reflects personal preferences. A liberal amount of ointment should be used 5 to 6 times a day. Steroids are harmful and are strongly contraindicated in many forms of ocular herpes. When inadvertently used, dendritic and conjunctival ulcers fail to heal and spread to form large corneal ulcers which may perforate. The only situation when steroids are indicated are in the treatment of ocular herpes of the internal eye[123].

Resistance to Acyclovir:

Chronic, severe and sometimes fatal mucocutaneous herpes simplex virus (HSV) infections may occur upon immunosuppressive conditioning regimens used for patients undergoing hematopoietic stem cell transplantation (HSCT) and patients with AIDS[139,140]. Current prophylactic strategies with oral valacyclovir (ValACV) or intravenous acyclovir (ACV) are quite effective in preventing recurrent HSV disease. The prevalence of HSV infections with reduced susceptibility to ACV in immunocompromised patients generally varies from 4.0 to 7.1%[141-145]. The prevalence of resistant HSV ranges from 3.5 to 7% in HIV-positive individuals[143,146,147], and from 2.8 to 10% in solid organ transplant recipients[142,143].

HSV Thymidine Kinase:

The HSV thymidine kinase is a deoxypyrimidine kinase with a broader range of substrate specificities compared to cellular kinases. Apart from catalyzing the transfer of a γ-phosphate group from ATP to thymidine, it also phosphorylates other pyrimidine and purine nucleoside analogues including those carrying acyclic sugar moieties, such as ACV. This property of lacking specificity in HSV TK already suggests the mutability of this enzyme, which is greater than that of the cellular TK[148].

Chemistry:

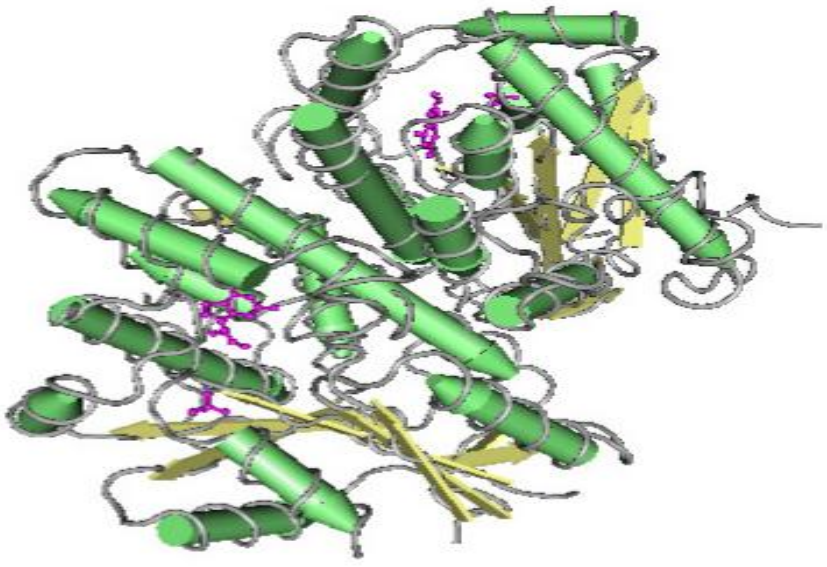
HSV TK is a 376 amino acid (aa) protein functioning as a homodimer in complex with 4 water molecules. Its three dimensional structure is homologous to nucleoside monophosphate (NMP) kinases including a core region with 5 β-sheets and 12 α-helices, a NMP binding domain and a LID domain enclosing ATP-binding site (Fig.10)[148].The active site of TK has been generally described based on the preliminary model proposed by Darby *et al.*[150], and based on regions of high sequence homologies between herpesviral thymidine kinases identified by Balasubramaniam[151].

These studies have defined the location of 6 conserved regions (aa 56-63, 83-88, 162-164, 171-173, 216-222 and 284-289) comprising the ATP-binding site (aa 51-63) and the nucleoside-binding site (aa 168-176). In addition, based on the recently elucidated crystal structure of TK[152,-154] and studies on the functional role of TK amino acid residues[155] it was demonstrated, that not only residues in the conserved regions play an important role in TK function, but also residues outside these regions can have major effect on TK activity. Thus, distant point

mutations that alter relative orientations of the helices, which surround the nucleoside, can result in substantial variances in substrate specificity[148].

The nucleoside-binding domain is hidden in protein interior of TK and is composed of amino acid side chains of 3 parallel helices that interact with substrate through hydrogen bonds. The substrate binding itself is established by specific amino acids in close contact with nucleoside as well as subsidiary stabilizing factors (amino acids from several separate distant locations)[148, 156-159].The ATP binding site is composed of a conserved glycine-rich (G*XX*G*X*GKT) motif (aa 56-63), the so-called P-loop, which is common to all nucleotide kinases. This loop forms a large anion hole which accommodates the β-phosphate of ATP[159]. Together with the arginine-rich LID domain (aa 215-226) it holds ATP in the binding site[160]. The N-terminal 45 residues of TK, does not seem to be essential for TK enzymatic activity[161].

Fig: 1.10. HSV-1 TK dimer with bound ACV and phosphate (both in purple). Backbone of the protein is shown with five central β-sheets (yellow) surrounded by 12 α-helices (green). PDB number 2KI5A.



Thymidine kinase: resistance-associated mutations

There are three types of TK mutations that confer ACV-resistance. The simplest type, which confers the highest level of resistance, totally abrogates the TK activity (TK-negative, TK^-). The second type of mutations leads to reduced levels of TK activity (TK-partial, TK_p). The third type of mutations alters TK so that it can still phosphorylate natural substrates but is impaired for ACV phosphorylation (TK-altered, TK_a). The pathogenicity of these three types of mutants differs, depending on the level of the TK activity[162].

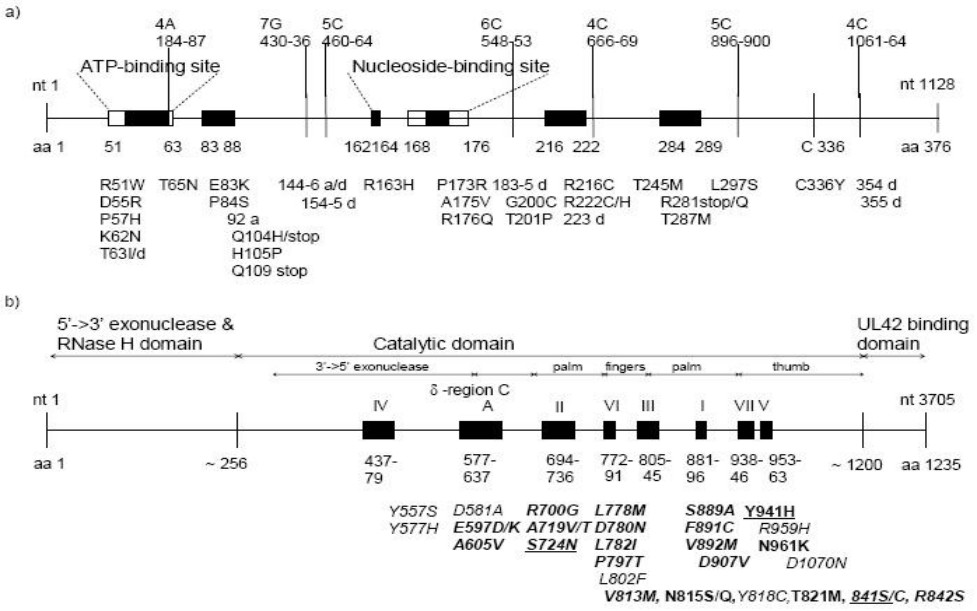
Single mutations in the TK gene may be associated with reduced susceptibility to ACV and/or other nucleoside analogues such as GCV and PCV. Two main types of mutations that occur at approximately equal frequencies have been identified. i) Frameshift (FS) mutations are caused by mostly single nucleotide additions/deletions in one of the homopolymeric repeats of cytosines (C) or guanines (G) that are frequently present throughout the TK gene[162] (Fig. 11a). These frameshifts result in a premature stop codon leading to a nonfunctional TK ii) The second type of mutations comprises single amino acid substitutions. These are usually located in the substrate binding sites, in conserved regions or at highly conserved individual codons (as described above, Fig. 10). A small number of resistance-associated substitutions has been reported outside the above mentioned regions. These, however, have to be carefully differentiated from the relatively frequent mutations due to natural polymorphism[133]. Other type of mutations have been rarely reported; those include deletions of multiple to several hundred nucleotides which ultimately lead to a nonfunctional TK[29,163].

Mutations resulting in a nonfunctional TK confer a highest degree of resistance to ACV (>100-fold increase in the IC50). The degree of resistance conferred by other mutations can range from marginal (2-fold increase in IC50) to high resistance (100-fold increase)[164].

DNA polymerase: structure and resistance-associated mutations:

The HSV-1 DNA polymerase is a 1235 amino acid multifunctional enzyme with DNA-dependent DNA polymerase activity, 5'→3' exonuclease/RNaseH and 3'→5' exonuclease activity. It binds to the UL42 processivity factor that is essential for viral replication[165,166](Fig 11b). Due to the tight linkage of catalytic functions in the DNA pol, single mutations can confer resistance to multiple antiviral drugs. However, compared to TK, these mutations are restricted only to specific regions or residues, since they cannot interfere with the polymerase function. It seems that no specific region is solely involved in recognition of a single class of anti-HSV drugs. Rather, the specific substrate recognition sites are formed through interactions of several non-sequential regions and amino acid residues upon folding[168,169].

Fig. 1.11:Schematic representation of drug resistance-associated mutations in a) HSV-1 TK found in ACV-resistant clinical isolates. Conserved regions, substrate binding sites (conserved regions forming part of ATP and nucleoside-binding sites: aa 56-63, 171-173) and mutational homopolymeric runs are indicated; b) HSV-1 DNA pol gene found in laboratory mutants and clinical isolates (underlined). Mutations indicated in bold, italics and combination of both confer resistance to ACV, PFA and both drugs, respectively. Black boxes indicate conserved regions. a, addition; d, deletion; nt nucleotide.



The presumed catalytic domain of the DNA pol enzyme includes eight conserved regions, designated I to VII and the δ-C region. These regions share sequence homologies with other herpesvirus DNA polymerases. The structure of the HSV DNA polymerase domain resembles a hand, like that of other polymerases, with a thumb, finger subdomains and a palm subdomain. Catalytic center of HSV-1 DNA pol is located in conserved regions I and II of the palm subdomain and is formed by three aspartate residues at positions 717, 886 and 888[154].

Because DNA polymerase mutants are quite rare *in vivo*, most mutations affecting susceptibility to antiviral drugs have been identified in engineered virus mutants[162] and have been broadly located between residues 500 and 1028[170]. Mutations conferring resistance to ACV and PFA cluster mainly in conserved regions II and III. Therefore, these regions seem to be most likely to interact with drugs and natural ligands. Residue Ser724 in region II is highly conserved among herpesvirus polymerases and seems to play a central role in the interaction with multiple classes of antiviral drugs. Mutation Ser724Asn confers resistance to ACV, pyrophosphate analogues and also to phosphonylmethoxyalkyl (PME) derivatives[171-173]. Recently, region VI has also been identified as a multidrug recognition site[174]. Region I is the most highly conserved region in the HSV DNA pol as well as in all α-like DNA polymerases, and is critical for catalytic activity. Consequently, no natural polymorphisms have been identified in this region and engineered mutations in this region are lethal or severely impair virus replication[175]. Region δ-C is a part of the 3'→5' exonuclease domain[176]. Mutations conferring resistance to pyrophosphate analogues and less frequently to nucleoside analogues has been mapped in this region[177]. Some mutations in this

region, especially in the ExoIII motif, significantly increase the mutation rate[178]. Only a few mutations associated with resistance to ACV have been described in regions V and VII. Mutations outside conserved regions, although less frequent, may also confer reduced drug susceptibility[172,173,180]. Most of these mutations were reported to confer unique resistance to cidofovir and not to other drugs[172]. However, some recent findings have undermined the exclusive nature of interaction of cidofovir with the enzyme, through identification of ACV and PFA resistance-associated mutations conferring cross-resistance to cidofovir[174]. However, as yet, resistance to cidofovir has only been reported in vitro[172]. Similarly to TK, natural polymorphisms in the DNA pol gene are frequent.

To date only a small number of clinical isolates with DNA pol mutations has been genotypically characterized[162]. Although evidence for cross-resistance to ACV and PFA, or even primary PFA resistance has been increasingly reported in Haematopoeitic stem cell transplant(HSCT) recipient, no genotypic data on these mutants are available.

Genotyping of Herpes simplex virus:

Phylogenetic methods can be used for many purposes, including analysis of morphological and several kinds of molecular data. The basic concepts of phylogenetic analysis are quite easy to understand, but understanding what the results of the analysis mean, and avoiding errors of analysis can be quite difficult.

Molecular phylogenetic analysis reconstructs the evolutionary history of different organisms. By analyzing DNA-sequences of different isolates or species, conclusions about evolutionary relationships can be drawn.

Genetic variation among strains of human herpes viruses has been used to distinguish viral genotypes and provides useful information for defining molecular epidemiology of infection. Human viruses of the alphaherpesvirinae subfamily have been reported to display a comparatively low degree of genetic inter-strain variability. The existence of intratypic polymorphism in HSV genomes led to the establishment of a new field in terms of molecular epidemiology[181-184].

Recently three genotypes, clearly separated in phylogenetic trees, were described for clinical HSV-1 isolates. The genotypes were arbitrarily designated A, B and C and the classification was based on DNA sequencing of the US4, US7 and US8 genes coding for the glycoproteins G (gG), I (gI) and E (gE) all localized in the unique short region of the genome[180].

Table 1.2: Functions of glycoproteins

Glycoprotein	Functions
gB	**Required for infectivity**
gD	**Required for infectivity, potent inducer of neutralising antibodies**
gC	**Binds to C3b component of complement**
gE	**Binds to Fc of IgG**
gG	**Candidate for type- specific serologic assays**

Genotyping may be a useful screening tool in searching for associations between genotype identity and a wide range of manifestations of HSV infection such as clinical symptoms, immune responses of the host, the transmission of virus, virulence and tropism. Furthermore, the method might also be of interest for epidemiological studies from different geographical regions to determine the genotype identity for a large number of clinical isolates[180,185]. The gE and gI proteins have been shown to form a complex that is involved in cell-to-cell spread and the binding of immunoglobulin G via the Fc receptor. The gG protein has been described as important for virus entry through the apical surfaces of polarized cells. The method can be used to study possible associations between genotype identity and the functions of the gG and gI proteins[186,187].

Based on phylogenetic and recombination analysis of both unique short and long sequences, and additional targets it is possible that recombinants can be detected in between the genotyping targets.

Phylogenetic analysis of Herpes viruses of mammals, birds and reptiles is now a well developed field. Utilizing primarily concatenated alignments of amino acid sequences from a set of up to eight core genes, these studies have yielded a tree that is overall well resolved and robust, but with demarcated localities where branching patterns remain incompletely revealed[188-191]. The tree's fundamental structure has been stable to addition of data for more species as they became available and consistent trees can also be obtained based on fewer genes.

The determination of sequence homology in genes from different organisms is key in identifying conserved functions or pathways[192,193]. Functionally related proteins often share sequence similarity as conserved

sequence motifs. Such information has been used to construct phylogenetic trees based on the number of shared genes between different completely sequenced cellular genomes[194,195] and recently to build a gene-content herpesvirus phylogeny using 13 herpesvirus genomes[196].

The discrimination of HSV-1 from HSV-2 was originally performed using virus culture followed by antibody binding to type-specific determinants (virus neutralization)[197,198]. The application of molecular methods, such as restriction fragment length polymorphism (RFLP) analysis of HSV PCR amplicons is thought to provide a reliable method of typing the virus[197,198]. Whilst serology based typing methods target surface exposed epitopes such as those on glycoprotein C or G. Molecular typing has largely exploited differences between HSV-1 and -2 DNA polymerase I genes. Archetypal HSV-1 and -2 DNA polymerase I genes share 93% sequence identity and 82% amino acid homology. The selection of strain typing polymorphisms for molecular methods is based on the sequence information deposited in public databases coupled with the availability of a convenient restriction endonuclease site. This can identify variation at a selected single nucleotide polymorphism (SNP) site. A rapid SNP typing method is useful because it can yield information about the virus population in the affected host population. This is of value in classification and in epidemiological studies aimed to investigate host-pathogen interplay. HSV SNP sub-typing by 'allele' specific fluorogenic probes offers many advantages over RFLP methods or viral culture. Amplification of the target DNA, and hybridization to a fluorogenic probe are conducted in a single PCR and therefore the chances of possible contamination are minimized.

The HSV-1 genome consists of a 152-kb linear duplex DNA molecule, and characterization of genetic variability of clinical HSV-1 isolates has become an area of growing interest. Restriction fragment length polymorphism analyses have been used earlier in an attempt to classify HSV-1 into different genotypes[180,184,185]. However, these methods are not based on HSV-1 DNA sequencing data and phylogenetic relationships. Recently, three genotypes, clearly separated in phylogenetic trees, were described for clinical HSV-1 isolates[180].

Herpesvirus glycoproteins:

All α-herpesviruses possess several glycoprotein-encoding genes. HSV- 1 and HSV-2 encode at least eleven glycoproteins (g) B, C, D, E, G, H, I, J, K, L and M. All encoded glycoproteins except gK[199] are attached on the virus envelope as well as on virus-infected cell membranes. The herpes viral glycoproteins are involved in several functions, such as in the virus entrance into the host cell through fusion with the lipid envelope or in cell-to-cell fusion, cell-to-cell spread and in the escape of the host's immune system[200]. There are various degrees of sequence homology between HSV-1 and HSV-2, where the most conserved glycoproteins, gD and gB, differ by only 15%. Another example is the gG-gene, of which a large portion is deleted in the HSV-1 (714 nt) in comparison with the HSV-2 gG gene (2097 nt). Although the fundamental functions of HSV-1, and HSV-2 glycoproteins are similar, several functional differences have been demonstrated; HSV-1 and HSV-2 require the combination of the four glycoproteins gH, gL, gB and gD for cell-to-cell fusion[200,201]. HSV-1 gE can be dispensable for replication in cell-cultures[203,204]. The genotypes were arbitrarily designated A, B, and C, and the classification was based on DNA sequencing of the US4, US7, and US8 genes coding for the

glycoproteins G (gG), I (gI), and E (gE), all localized in the unique short region of the genome. Two genotypes of HSV-2 (A and E) has also been described based DNA sequencing. In light of this newly described genetic diversity of HSV-1, the search for associations between specific viral genetic markers and clinical symptoms is of interest. Although DNA sequencing technique is time-consuming in the high-scale format it is still the most accurate method for genotyping,. Comparisons of the gene DNA sequences in the US-segment have demonstrated that US4 and US7 are similar, and have probably evolved by duplication and divergence of the gD gene[203]. Although US8 is distinct from US4 and US7, a more distant relationship has been suggested based on conservation of two clusters of cysteine residues.

HSV-1 contains several glycoproteins, each with varied functions, concerning the overall pathogenesis and immune evasion by the virus. Glycoprotein C plays a significant role in the efficient attachment to the cell surface,

Glycoprotein G-1 (gG-1), a viral envelope glycoprotein[206,207] that was suggested to contribute to viral entry through apical surfaces of polarized cells[208], has been utilized as a prototype antigen for HSV-1 type-specific diagnosis due to lack of cross-reactivity with its counterpart in HSV-2, i.e., gG-2[209,210]. Several commercial gG-based enzyme immunoassays have been evaluated in clinical settings[211]. Furthermore, gG-1 may be a suitable target for typing of HSV isolates by monoclonal antibodies (MAb) that identify type-specific epitopes. Conservation of the gene coding for gG-1 among clinical HSV-1 isolates might therefore be a prerequisite for a reliable assessment of a type-

specific antibody response as well as of correct typing of isolates in individuals infected with HSV-1[212].

It has been demonstrated that gI binds to and forms a complex with gE, and that the gE/gI complex is involved in cell-to-cell spread in epithelial and neuronal tissue[213] as well as in the virus escape from the host immune system by Fc-receptor binding of IgG-antibodies[214-216]. The functions of gG, gE and gI in HSV-2 have not been fully described.

A detailed study of the molecular evolution of glycoprotein genes G and I, in European strains, threw up existence of genotypes arbitrarily labeled A, B, C and intragenic recombinants in a hitherto considered stable genetic makeup.

A detailed analysis of human corneal isolates pointed to the predominance of genotype B, in the European population, though they were not found to have enhanced virulence[180].

Shigeru Ozawa *et al* used RFLP for the epidemiological study of HSV-1 and have identified a major BglII RFLP variant of HSV-1, designated BgK_L, found in 27.0% of 636 HSV-1 clinical isolates and also its dispersion in and around Shikoku islands in Japan[217].

Thirty patients with Recrudescent herpetic keratitis (RHK), sequential corneal HSV-1 isolates were genotyped by PCR amplification of the hypervariable regions located within the HSV-1 genes *US1*, *US10/11*, and *US12* by Lies Remeijer *et al*. Whereas the sequential corneal HSV-1 isolates of 19 (63%) of 30 patients had the same genotype (designated as group 1), the sequential isolates of 11 patients (37%) were genetically different (designated as group 2)[218].

Norberg *et al.,* determined the sequence diversity of the complete genes coding for glycoproteins G (gG), I (gI), and E (gE), comprising 2.3% of the HSV-1 genome and located within the unique short (US) region, for 28 clinical HSV-1 isolates inducing oral lesions, genital lesions, or encephalitis. Laboratory strains F and KOS321 were sequenced in parallel. Phylogenetic analysis, including analysis of laboratory strain 17, revealed that the sequences were separated into, three genetic groups[180].

The identification of different genogroups facilitated the detection of recombinant viruses by using specific nucleotide substitutions as recombination markers. Seven of the isolates and strain 17 displayed sequences consistent with intergenic recombination, and at least four isolates were intragenic recombinants.

The observed frequency of recombination based on an analysis of a short stretch of the US region suggests that most full-length HSV-1 genomes consist of a mosaic of segments from different genetic groups. Polymorphic tandem repeat regions, consisting of two to eight blocks of 21 nucleotides in the gI gene and seven to eight repeats of 3 nucleotides in the gG gene, were also detected. Laboratory strain KOS321 displayed a frameshift mutation in the gI gene with a subsequent alteration of the deduced intracellular portion of the protein[180].

Norberg, *et al.,* further classified HSV-1 into three genotypes using RFLP, clearly separated in phylogenetic trees were described for clinical HSV-1 isolates. The genotypes were arbitrarily designated A, B, and C, and the classification was based on DNA sequencing of the US4, US7and US8 genes coding for the glycoproteins G (gG), I (gI), and E (gE), all localized in the unique short region of

the genome. It was determined that RFLP is rapid and accurate method for genotyping of clinical HSV-1 isolates[185].

Kasubi *et al.,* sequenced gene segment coding for gG, gI and gE as well as the non-coding region between gI and gE from 11 Tanzanian isolates and 10 Norwegian isolates of HSV -2. The sequences were compared to the reference HSV-2 strain HG52. Overall, sequence variation was low, with an overall similarity between the two most distant isolates of 99.6%[186].

Phylogenetic analysis revealed that HSV-2 strains are classified into two genogroups, designated Africa (A) and European (E). All strains classified in A genogroup were isolated in Tanzania, while the E genogroup contained both Tanzanian and Norwegian strains for gI/gE as well as for gG trees. This findings suggests that three clinical HSV-2 isolates collected from patients in Tanzania presenting a genetic pattern consistent with homologous recombination between the A and E genotypes[186] .

Umene *et al.,* investigated Genomic profiles of 66 strains of herpes simplex virus type 1 (HSV-1) isolated in Japan with regard to RFLP and length variation of fragments containing reiterations. There were two predominant genotypes of F1 and F35, and the genomic characteristics of each were studied. The nucleotide change between F1 and F35 was estimated to be 1.5%. An RFLP marker (VR23) peculiar to genotype F35 was identified as the first case of genomic marker specific to a predominant genotype of HSV-1, and is the diagnostic marker of F35[219].

The *a* sequences (repeating in an HSV-1 genome and containing reiterations) of F35 were cleaved by *Sac II* on the DR4 (direct repeat 4) stretch,

while *a* sequences of F1 had a rearranged DR4 and were resistant to *Sac II* digestion. Thus, it was analyzed that fragments containing reiterations, such as *a* sequences, can serve to classify HSV-1 strains as well as for purpose of differentiation. The proportion of strains derived from primary infection to those from recurrent infection was higher in strains of F35 than in those of F1, and this genotypic difference within HSV-1 may possibly influence clinical manifestations. In this study, the intratypic variability of a tandem repeat locus within the DNA polymerase (pol) gene of human herpes simplex virus type 2 (HSV2) was uncovered. The locus contained variable numbers of tandem dodeca nucleotide (5′-GAC GAG GAC GGG-3′) repetitive units. The result showed that approximately 95% of analyzed HSV2 clinical isolates and the current GenBank HSV2 strains contained two copies of the repetitive units[219].

From genital herpes specimens, three new HSV2 strains, which respectively contained 1, 3, and 4 copies of the repetitive units, were identified. This variable number of tandem repeat (VNTR) locus is absent in HSV1, and thus it also contributes to the intertypic variability of HSV 1 and HSV 2[219].

The current study was undertaken to determine the possibility of veritable association between the clinical sites of infection and genotype, not only based on the information provided by the glycoprotein genes G and I, but also deduce to the phylogenetic pattern of these isolates in their gC region, and compare the same with the previously classified I and G regions. As gC region is essential in the initial binding to the HS moiety, any variations detected in these regions classifying itself as a separate genotype, could differentially influence the binding to variegated tissues.

Prevention

On the whole, preventive measures against HSV infection have been disappointing. The virus is ubiquitous and little or nothing can be done to prevent the transmission of infection in environmental terms. HNIG has not been effective in the prevention of HSV infections. Prophylactic chemotherapy may be given to those suffering from frequent and severe recurrent herpes but the cost factor must be taken into account. In clinical trials, γ-interferon eye drops have proved effective for the prevention of recurrent dendritic ulcers[1-4].

The only area where there has been some success is the prevention of transmission of infection to newborn babies by the use of caesarean section. The actual decision of whether to carry out a caesarean section or not is very difficult as maternal genital herpes is common whereas neonatal herpes is rare. Furthermore, asymptomatic shedding of HSV is common. Generally, caesarean sections are not carried out for cases of recurrent herpes (except where florid lesions are present and with the mother's informed consent) but for cases where there is florid primary infection. The neonate should be closely monitored and acyclovir should be given on the merest suspicion of genital herpes. If the mother develops primary HSV infection during the first or second trimester of pregnancy, antiviral therapy may be considered. If the mother contracts primary herpes during weeks 30-34 of pregnancy, she may be treated by acyclovir followed by Caesarean section or normal delivery. The mother with primary genital herpes between week 34 and term should be delivered by Caesarean section. A woman who presents with primary genital herpes while in labour may be treated with IV acyclovir, although it is uncertain whether this will reduce the rate of transmission.[1-4]

Vaccines:–

Several recombinant subunit vaccines are being evaluated at present. There is evidence to suggest that such vaccines may be effective in reducing the frequency and severity of recurrent disease in an already immune individual, but their efficacy in preventing primary infection is uncertain. Primary infection per se is not a condition worth preventing except in immunosuppressed patients. However, such vaccines may be useful in preventing or attenuating recurrent disease[1-4].

Human Cytomegalovirus:

History:

In 1904, Ribbert first identified histopathological evidence of CMV, in tissues from a congenitally infected infant. Ribbert mistakenly assumed that the large inclusion-bearing cells he observed at autopsy were from protozoa (incorrectly named *Entamoeba mortinatalium*). In 1920, Goodpasture correctly postulated the viral etiology of these inclusions[220]. Goodpasture used the term cytomegalia to refer to the enlarged, swollen nature of the infected cells. Human CMV (HCMV) was first isolated in tissue culture in 1956, and the propensity of this organism to infect the salivary gland led to its initial designation as a salivary gland virus.

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. The cytopathic effect of the new virus strain (AD169) very closely resembled that of the Davis

strain that was observed one 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. 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. In the following years, HCMV also showed its pathogenic properties in organ transplant recipients, patients with AIDS, and cancer patients, while it gained the leading position among infectious agents responsible for mental retardation, intellectual impairment, and deafness[221].

Presently, HCMV infection is mostly controlled in immunocompromised patients by available antiviral drugs, yet it continues to maintain its role as the most dangerous infectious agent for the unborn infant. Thus, HCMV infection is still a major health problem, warranting strong preventive measures.

In 1960, Weller designated the virus cytomegalovirus; and during the 1970s and 1980s, knowledge of the role of HCMV as an important pathogen with diverse clinical manifestations increased steadily. Considerable work remains in devising strategies for prevention of HCMV infection and in understanding the role of specific viral genes in pathogenesis although enormous progress has recently been made in defining and characterizing molecular biology, immunology, and antiviral therapeutic targets for HCMV[222].

Human cytomegalovirus(HCMV) poses an important public health problem as it may cause significant morbidity and mortality in congenitally

infected newborn and immunocompromised patients, such as solid organ or bone marrow transplant recipients. It is a self limiting infection in healthy individuals. It is the most frequent cause of infection complication in the early period following kidney transplantation[221,223-225]. HCMV is considered an AIDS-defining infection, indicating that the T-cell count has dropped to low levels. Primary CMV infection in patients with weakened immune systems can lead to serious disease. However, more common problem is reactivation of the latent virus in patients with a depressed immune system. CMV-related disease may be much more aggressive. CMV hepatitis may cause fulminant liver failure. Effective preemptive antiviral therapy that includes ganciclovir and foscarnet depends on detecting HCMV at an early stage of infection. 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. However, because the primary HCMV infection results in a latent lifelong infection and because many transplant patients secrete HCMV without any clinical disease, the mere detection of HCMV does not always indicate the need for treatment[221].

Quantitation of the systemic HCMV load may provide a highly sensitive and specific method to predict whether the patient is responding to therapy. Hence, there is a need to develop highly sensitive and quantitative detection methods to quantify HCMV. Although pp65 antigenemia assay is the most widely used methods for monitoring HCMV infections and reactivations this methods has a lot of limitations. Multiple studies have shown that the real-time quantitative PCR for HCMV DNA in plasma and other biological samples is useful in the rapid diagnosis of HCMV infection and for effective monitoring of clinical course as well as response to therapy[226,227]. Recently reports suggest Real Time PCR

has known to be a rapid and sensitive method in the diagnosis HCMV infections. In our study both these methods were employed for the detection of HCMV infection. The suitability of the assay was compared with pp65 antigenemia assa (gold standard) for detection of HCMV. The assays were standardized to be applied on clinical specimen. Peripheral blood samples of both the donors and recipients (both pre and post transplant) were used in the study to know the reliability of the test and estimate the copy numbers of HCMV.

Cytomegalovirus (from the Greek *cyto*, "cell", and *megalo*, "large") is a viral genus of the viral group known as *Herpesviridae* or herpesviruses. It is typically abbreviated as CMV. The species that infects humans is commonly known as human CMV (HCMV) or human herpesvirus-5 (HHV-5), and is the most studied of all Cytomegaloviruses. Within *Herpesviridae*, CMV belongs to the *Betaherpesvirinae* subfamily. CMV is a lytic virus that causes a cytopathic effect *in vitro* and *in vivo*. The pathologic hallmark of CMV infection is an enlarged cell with viral inclusion bodies. Cells that exhibit cytomegaly are also seen in infections caused by other Betaherpesvirinae. The microscopic description given to these cells is most commonly an "owl's eye". Although considered diagnostic, such histological findings may be minimal or absent in infected organs[1-4].

Infections:

Human cytomegalovirus(HCMV) is a vernacular name of Human herpes virus 5(HHV-5) . Within *Herpesviridae*, HCMV belongs to the *Betaherpesvirinae* subfamily, which also includes cytomegaloviruses from other mammals. It has been reported in studies that CMV IgG antibodies in women of childbearing age.

Risk of seroconversion is observed during pregnancy averages to 2.0- 2.5%. Due to latency and periodic reactivation, *in-utero* transmission may follow either primary or recurrent infection[1-4].

Although they may be found throughout the body, HCMV infections are frequently associated with the salivary glands. HCMV infection is typically unnoticed in healthy people, but can be life-threatening for the immunocompromised, such as HIV-infected persons, organ transplant recipients, or new born infants[221]. After infection, HCMV has an ability to remain latent within the body over long periods. Prevention by hygienic measures is included in information given to pregnant women[228]. Cytomegalovirus, also a herpesvirus, causes retinitis that leads progressively to complete blindness without treatment. CMV retinitis is one of the major problems in HIV infected patients[229]. The clinical presentation of CMV retinitis is considered "gold standard" for the diagnosis of CMV retinitis. It has been shown that the clinical picture of CMV-retinitis is atypical in individuals on HAART therapy probably due to residual amount of immunity against CMV in them. Early detection and institution of appropriate therapy is important to preserve the eye sight of the patient. French guidelines recommend both ophthalmoscopy and CMV quantitative viremia in patients whose CD 4 count is less than 50- 100/mm^3 for the early detection of CMV retinitis. The pp65 antigenemia count among those who have CMV retinitis is comparatively very less than who develop CMV diseases of other organs[229].

Hence a study on the diagnosis of CMV retinitis was carried out to determine the value of real time PCR against qualitative PCR in detecting HCMV in intraocular and peripheral blood of patients who developed HCMV retinitis in spite being on HAART.

HCMV is found throughout all geographic locations and socioeconomic groups, and infects 40% of adults in the worldwide) as indicated by the presence of antibodies in much of the general population. Seroprevalence is age-dependent: 58.9% of individuals aged 6 and older are infected with CMV while 90.8% of individuals aged 80 and older are positive for HCMV. HCMV is also the virus most frequently transmitted to a developing fetus. HCMV infection is more widespread in developing countries and in communities with lower socioeconomic status and represents the most significant viral cause of birth defects in industrialized countries. CMV seems to have a large impact on immune parameters in later life and may contribute to increased morbidity and eventual mortality[221,225].

Graft versus host disease: CMV infection has been associated with acute graft versus host disease in bone marrow transplant recipients. Multiple genotypes (gB 1-4) of CMV exist, each with variations in the gene encoding envelope glycoprotein gB. The association of gB types with acute graft versus host disease and death related to myelosuppression has been examined. Taking into account disease type, donor-recipient HLA matching, donor CMV serostatus, and age, Torok-Storb *et al*., (1997) found that gB3 and gB4 were linked to a higher degree of myelosuppression and death. Interestingly, no specific CMV genotypes were linked to worse outcome in solid organ transplant recipients, although mixed gB genotype infections were associated with higher viral loads and delayed viral clearance[230].

CMV disease among transplant recipients:

Cytomegalovirus (CMV) disease continues to be an important cause of morbidity and mortality in hematopoietic stem cell transplantation (HSCT) and renal transplant recipients[221,223-225].

It is known that CMV remains latent in leucocytes and gets reactivated as age advances. Multiple new strains also arise through recombination of existing strains. Both mechanisms could cause CMV disease in Indian transplant patients where seroprevelance is high. Immunosuppression therapy following transplantation, can also enhance viral replication[221,225].

Primary infection refers to reactivation of donor virus in CMV – seronegative recipients. Secondary infection occurs when endogenous latent virus is reactivated in a sero-positive recipient and re-infection refers to reactivation of the virus of donor origin in seropositive recipient. 60-90% of all renal transplant patients have latent CMV infection but symptomatic infection occurs only in 20 – 60% of them. CMV is the single most frequent cause of transplantation with an overall incidence of CMV infection and disease during the first 100 days post-transplantation are 60% and 25% respectively when no CMV prophylaxis is given[221,225].

Infection is defined as isolation of CMV, or detection of CMV proteins or nucleic acid in any body fluid or tissue specimens. Disease is defined as detection of CMV in a clinical specimen accompanied by either CMV syndrome with fever, muscle pain, leucopoenia and/or thrombocytopoenia or by organ involvement such as hepatitis, gastrointestinal ulceration, pneumonitis, retinitis, central nervous system disease, nephritis, myocarditis, cystitis or pancreatitis. The incidence of

CMV infection is 50 – 75% when the recipient is CMV antibodies negative and the donor tests positive for CMV antibodies. If both are negative the incidence is less than 5%[231].

Human Cytomegalovirus (HCMV) is the cause of lifelong latent infection in immune competent host. HCMV reactivation is a frequent event and represents a significant cause of morbidity and mortality in immunocompromised patients. The availability of diagnostic assays for the early identification of reactivated HCMV replication has considerably improved the clinical management of immunocompromised patients, thus reducing the risk of HCMV disease and allowing pre-emptive treatment as an alternative to universal prophylaxis. The determination of pp65 antigenemia has long been the reference test for monitoring HCMV reactivation, but it requires highly skilled interpretation and processing of fresh samples. Furthermore, the evaluation of its results may be particularly difficult in leukopenic patients. More recently, the quantitation of HCMV DNA by real-time PCR methods has been proposed as a convenient alternative approach, in order to overcome the drawbacks of antigenemia assays. The exact relationship between HCMV DNA and antigenemia levels, however, is still under investigation. Studies addressing this issue would be instrumental in finding suitable threshold levels for the clinical interpretation of HCMV DNA results[221,225,227].

In severely immunocompromised patients who develop HCMV disease, prolonged antiviral therapy is often necessary. A potential risk associated with prolonged use of antiviral compounds is the emergence of resistant strains viruses. HCMV isolates resistant to antiviral agents have been selected in the laboratory and have also been recovered from immunocompromised patients treated with

antiviral agents. Ganciclovir-resistant HCMV strains selected in the laboratory have been found to have amino acid deletions or substitutions in conserved regions of the UL97 region and/or point mutations in the DNA polymerase gene of the virus. In clinical HCMV strains, resistance to ganciclovir has also been associated with mutations in UL97, DNA polymerase, or both viral genes [232-235]. The functional consequence of the UL97 mutations is an impaired phosphorylation of ganciclovir in virus-infected cells, with the consequent lack of synthesis of ganciclovir triphosphate, the active form of the drug[233,234]. The recognition that mutations in the UL97 and/or DNA polymerase region of HCMV are associated with antiviral resistance has led to the development of methods for detection of mutant viruses. The UL97 and DNA polymerase sequences (the viral genotype) are generally characterized by combining PCR and sequencing methods. The observation that the majority (94%) of ganciclovir-resistant HCMV strains contain specific UL97 mutations prompted the development of screening assays to detect mutant viruses[236,237]. These screening methods have been successfully applied to the direct detection of mutant HCMV UL97 sequences in clinical specimens (i.e., plasma, blood leukocytes, and cerebrospinal fluid).

HCMV UL97 was originally identified as a protein kinase homologue [238]. UL97 was then shown to be the HCMV gene product responsible for the phosphorylation of the antiviral drug ganciclovir, a nucleoside analog, thus contributing to the susceptibility of HCMV to this compound[234,239]. Nonetheless, UL97 is a protein serine-threonine kinase[240]. Maribavir, a specific inhibitor of UL97 protein kinase activity[241,242], greatly inhibits viral replication and has shown to have promising results as an anti- HCMV therapeutic

agent in clinical trials[243,244]. Therefore, UL97 is an important antiviral drug target.

Ganciclovir is the antiviral drug most widely used in the management of severe and life-threatening human cytomegalovirus (HCMV) systemic infections in patients with terminal AIDS. The isolation of ganciclovir-resistant HCMV strains is reported with increasing frequency in these patient groups, and it is becoming an emerging clinical problem. Prolonged antiviral maintenance therapy with suboptimal dosages and frequent discontinuation because of the toxic effects of the drug favours the emergence of ganciclovir-resistant HCMV strains[245,246].

Ganciclovir is an analog of guanosine that interferes with viral replication, blocking the DNA polymerase enzyme. Drug activity requires triphosphorylation, and the first phosphate addition is dependent, as previously reported, upon the HCMV protein kinase encoded by the UL97 open reading frame. Mutations in both UL97 and DNA polymerase genes may induce resistance to ganciclovir. However, lack of phosphorylation seems to be the mechanism of resistance most frequently occurring *in vivo*[247].

Primary and Secondary CMV Infection

Approximately 20% to 60% of all transplant recipients develop symptomatic CMV infection[248]. The patient at highest risk for symptomatic disease is the CMV-seropositive donor/CMV-seronegative recipient (D+/R-) who develops a primary infection after transplantation. Such patients are at particular risk for severe manifestations of CMV infection, including tissue-invasive CMV and CMV recurrence[221].

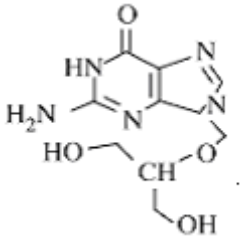
Reactivation infection develops in the patients who becomes CMV-seropositive before transplantation via the traditional routes of transmission and exposure during hemodialysis and blood transfusion, and is more frequent than primary CMV infection. CMV-seropositive recipients are also at risk for superinfection by CMV from a CMV-seropositive donor, especially in the setting of intense immunosuppression (ie, OKT3 and antithymocyte globulin) [221, 224, 225].

Anti-HCMV Drugs

Ganciclovir

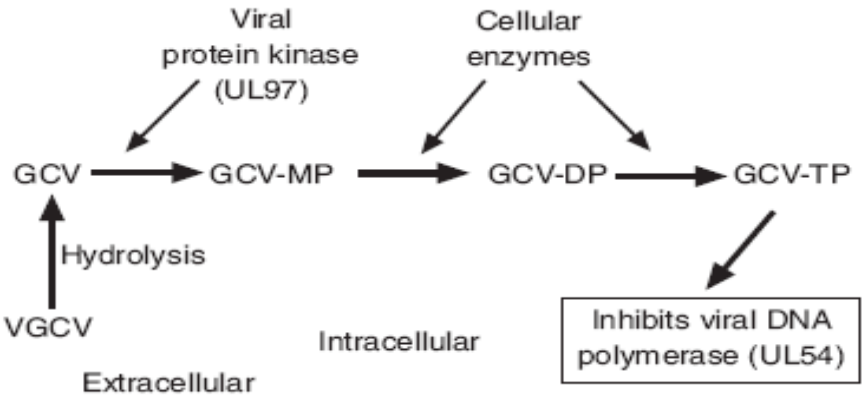
Ganciclovir is the deoxyguanosine analogue and in 1988 was the first drug to be approved for the treatment of HCMV infection. Since then, it has remained the first-line treatment for HCMV infections in immunocompromised patients. It is widely used in patients with impaired cell-mediated immunity, particularly persons with the acquired immunodeficiency syndrome (AIDS) and recipients of solid organ and bone marrow transplantation. It was first synthesized by Julien Verheyden and John Martin at Syntex Research in California in 1980.Ganciclovir sodium is marketed under the trade names Cytovene and Cymevene (Roche)[249]. The chemical name for ganciclovir is (9-[(1,3, -dihydroxy-2-propoxy) methyl] guanine, or DHPG).

Figure 1.12: Structure of Ganciclovir (GCV)



Upon entry into HCMV-infected cells, (Figure 7) GCV is selectively phosphorylated by a viral protein kinase homologue (the product of the UL97 gene, pUL97). Subsequently, cellular kinases convert GCV monophosphate into GCV triphosphate, which acts as a potent inhibitor of the HCMV DNA polymerase (UL 54) by competing with dGTP on the enzyme-binding site. GCV is also incorporated into the viral DNA, where it slows down and eventually stops chain elongation. GCV formulations are available for intravenous (IV) or oral administration and as ocular implants for the local treatment of HCMV retinitis. Due to its poor bioavailability (~6%), efforts were made to develop prodrug of GCV. Valganciclovir (VGCV; Valcyte; Hoffmann-La Roche) is a new valyl ester formulation of GCV that exhibits about 10 times the bioavailability of GCV following oral administration[249,250].

Figure 1.13: Mechanism of action of Ganciclovir

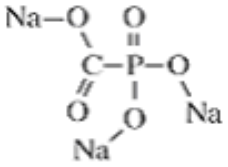


Ganciclovir has been evaluated for and is used primarily in treating HCMV infections, but it also inhibits the replication of other herpesviruses, including herpes simplex virus types 1 and 2 (HSV-1, HSV-2), Epstein-Barr virus (EBV), and human herpes virus 6 (HHV-6), at clinically achievable concentrations. *In-vitro* synergy with Foscarnet has been observed against HCMV[221].

Foscarnet

Foscarnet(PFA) is a DNA polymerase inhibitor acting as a competitor of pyrophosphate. Foscarnet does not require intracellular phosphorylation to inhibit DNA polymerase and, therefore, retains activity against most GCV-resistant strains of HCMV. Intravenous PFA may be used under conditions of failure of GCV treatment, GCV resistance or excessive side effects such as leucopoenia. However, Foscarnet is nephrotoxic, which limits its use in kidney transplantation[221].

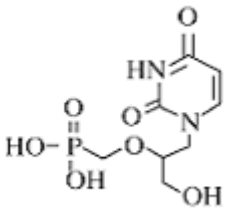
Figure 1.14: Structure of Foscarnet (PFA)



Cidofovir

Cidofovir is a nucleotide analogue inhibiting the viral DNA polymerase. Being already phosphorylated, it does not require activation by UL97. A peculiar characteristic of cidofovir is its extended half-life *in vivo* allowing weekly or bi-weekly administration. The drug is administered intravenously at a dosage of 5 mg/kg once a week during induction and 5 mg/kg once every other week during maintenance treatment.

Figure 1.15: Structure of Cidofovir (CDV)



Clinical Significance, Incidence and Risk Factors For Drug-Resistant HCMV Infections

Drug-resistant HCMV strains first emerged as a significant problem in patients with AIDS. Numerous studies have documented the emergence of drug-resistant HCMV strains (detected by phenotypic or genotypic methods) and their correlation with progressive or recurrent HCMV disease (mainly retinitis) during therapy. The first study to evaluate the prevalence of GCV resistance in AIDS patients was conducted by evaluating the excretion of GCV-resistant strains in the urine of 31 patients with AIDS treated with IV GCV for HCMV retinitis. In that study, no resistant isolates were recovered from patients treated for <3 months, whereas 38% of those excreting the virus in their urine after >3 months of GCV treatment, which represented 8% of the entire cohort of patients, were infected with a resistant isolate. Since then, larger studies have evaluated the temporal emergence of GCV-resistant strains by either phenotypic or genotypic assays. In all studies, GCV resistance at the initiation of treatment was a rare event. Phenotypic evaluation of isolates from the blood or urine of patients treated with GCV (mostly IV) for HCMV retinitis revealed that the percentage of patients excreting a GCV-resistant strain is increasing with duration of drug exposure. A more recent study of AIDS patients treated with VGCV for HCMV retinitis has found similar result, but with the percentage of resistance being lesser than that of HCMV retinitis. The lower incidence of GCV resistance in the latter study, despite the use of more sensitive genotypic methods, might be explained by differences in the study population, notably, improvement in anti-HIV therapy. Due to their less frequent use in the clinic, fewer data on the temporal emergence of PFA- and CDV-resistant HCMV strains in HIV-infected individuals have been reported.

Proposed risk factors for the development of HCMV resistance in this patient population include inadequate drug concentrations due to poor penetration into tissue (e.g., the eyes) or poor bioavailability (e.g., oral GCV), a sustained and profound immunosuppression status (CD4 counts <50 cells/µl), frequent discontinuation of treatment due to toxicity, and a high pretherapy HCMV load.

Linear absorption kinetics has been observed in case of intravenous ganciclovir, which has been described in an approximate to a 2-compartment open model. Accumulation of ganciclovir is unlikely in patients with normal renal function during repeated administration. The drug is eliminated virtually unchanged by the kidneys, with a terminal phase half-life of up to 4.5 hours after oral or intravenous administration. Renal dysfunction reduces elimination of ganciclovir and dosage reductions are therefore required in these patients. Ganciclovir shows minimal binding to plasma proteins. Studies have revealed that Ganciclovir is only 1% to 2% bound to plasma proteins. The volume of distribution after initial and steady state dosing is 15.3 L/1.73 m^2, and 30 to 70 L, respectively. Ganciclovir concentrations in the kidneys are three- to sevenfold higher than in heart blood. Concentrations observed in the liver, testes and lung are similar to heart blood concentrations. Cerebrospinal fluid concentrations are estimated to be 24% to 67% of serum concentrations. Following intravenous ganciclovir, vitreous fluid concentrations are often lower than corresponding plasma concentrations. The volume of distribution in the vitreous is approximately 11.7 mL after a 200 µg intravitreal injection.

The physiochemical properties of acyclovir and ganciclovir are similar, and this explains the similarity in the ocular tissue distribution. The tissue

concentrations are of the same order of magnitude when comparing ganciclovir ophthalmic gel 0.15% with 3% acyclovir ointment despite the difference in dosage strength. In a study by Tabbara et al., the plasma concentration four hours after the administration of ganciclovir gel was very low, this illustrated the limited plasma diffusion of the drug and its lack of systemic toxicity. Low penetration of antiviral drugs to the CNS, along with impaired local immune surveillance, may favor persistent replication with possible emergence of resistant strains.

HCMV resistance to GCV appears to be an emerging problem in Solid Organ Transplant(SOT) recipients and has been associated with an increased number of asymptomatic and symptomatic viremic episodes, the earlier onset of HCMV disease, graft loss, and an increased risk of death[249]. With the widespread use of ganciclovir prophylaxis among transplant recipients, ganciclovir resistance is increasingly reported in the transplant setting, especially among SOT recipients who have had prolonged exposure to ganciclovir. Recently, the early emergence of ganciclovir-resistant virus in children with primary combined immunodeficiency had been described. It was reported that the unusual isolation of multiple drug-resistant variants, containing point mutations and different deletions of the UL97 gene, from one of these patients after 3 weeks of ganciclovir therapy.

Molecular Mechanisms of HCMV Resistance to Current Antiviral Agents

As anticipated from its mode of action, HCMV resistance to GCV can be the result of alterations in two different viral gene products, namely, pUL97 and the viral DNA *pol*(pUL54). The UL97 protein is responsible for GCV monophosphorylation in HCMV-infected cells and is thus involved in HCMV

resistance only to GCV. In fact, this mechanism of resistance (i.e., decreased phosphorylation of GCV) was recognized in laboratory-derived mutants and clinical resistant HCMV strains even before pUL97 was identified as the protein responsible for such activation. Since then, studies of recombinant HCMV UL97 mutants and recombinant vaccinia viruses expressing wild type or mutated forms of UL97 have confirmed this mechanism of resistance. Several genotypic studies have identified UL97 mutations in over 90% of GCV-resistant HCMV clinical isolates, implying that impaired drug phosphorylation is the most important mechanism of GCV resistance in HCMV[242].

Even though the precise role of pUL97 in the HCMV replication cycle has still not been clearly elucidated, major advances in of its biological function have been recently made. The assumption that pUL97 has an important role in the viral replication cycle was confirmed by showing that the replication capacities of recombinant viruses with a UL97 deletion were severely impaired. Whether or not mutations associated with GCV resistance have an influence on viral fitness in the absence of selective pressure remains controversial. Nevertheless, clinical strains with UL97 mutations appear to be fully pathogenic. Even though the protein is able to phosphorylate GCV, acyclovir, and pencyclovir (two other nucleoside analogues mainly used to treat herpes simplex virus [HSV] infections) the natural nucleosides (dA, dC, dT, and dG) are not phosphorylated by pUL97. Indeed, pUL97 shows no homology with known nucleoside kinases but, rather shares sequence homology with protein tyrosine kinases and bacterial phosphotransferases. Those homologies have allowed the recognition of conserved regions within the gene. Finally, pUL97 has been shown to be a structural component of the virion with autophosphorylation properties, and

independent studies have suggested a role for the protein in DNA replication, probably through phosphorylation of the DNA *pol* processivity factor (pUL44), as well as in DNA encapsidation and/or nuclear egress[249].

To date, UL97 mutations associated with GCV resistance that result in either amino acid substitutions or short (1- to 17-amino-acid) in-frame deletions have been found at codons 460 and 520 or in a region defined by codons 590 to 607, which are thought to be involved in ATP binding and substrate recognition, respectively. The cumulative results obtained from three recent studies that have documented the emergence of UL97 mutations in clinical isolates or in blood samples from AIDS and SOT patients are in, general agreement with the proposed frequency of UL97 mutations, based on characterization of 76 independent UL97 mutants gathered in a single laboratory over years. Those data suggest that mutations A594V (30 to 34.5%), L595S (20 to 24%), M460V (11.5 to 14.5%), and H520Q (5 to 11.5%) represent the most frequent UL97 mutations present in GCV-resistant mutants. Other frequent UL97 mutations associated with resistance include C592G and C603W. On the basis of marker transfer experiments, mutation M460V (7-fold), mutation C603W (8-fold), deletion of codons 595 to 603 (8.4-fold), mutation H520Q (10-fold), mutation L595S (4.9- to 11.5-fold), mutation A594V (10.7-fold), mutation C607Y (12.5-fold), and deletion of codon 595 (13.3-fold) appear to be associated with the highest rate of increase in GCV resistance over that of the parental strain, whereas mutations C592G, A594T, and E596G and deletion of codon 600 seem to confer only a modest decrease in susceptibility. Interestingly, analysis of the GCV-phosphorylating activity of mutated UL97 genes expressed in a recombinant vaccinia virus expression system

would have predicted that mutations H520Q and M460V confer the highest decrease in GCV susceptibility.

The second viral protein involved in HCMV resistance to all currently approved systemic antivirals is the viral DNA *pol* (pUL54). However, mutations in UL54 are less common and have been reported only in patients harbouring ganciclovir-resistant UL97-mutant strains maintained on ganciclovir treatment. The catalytic domain of the HCMV DNA *pol*, like the polymerases of other herpesviruses, is composed of eight highly conserved regions (regions I to VII and δ region C) that partially overlap with three conserved Exo motifs (motifs I to III). On the basis of homology with other α-like DNA polymerases, it has been proposed that the Exo I to III motifs constitute the 3' to 5' exonuclease site of the enzyme. However, enzymatic analyses of purified mutated HCMV DNA *pol* proteins (L501F and K513N) suggest that the N-terminal portion of δ region C would also participate in this specific enzyme activity. On the other hand, conserved regions II to V were proposed to be involved in the polymerization activity of DNA *pol* in general, as in that of HCMV. However, more recent enzymatic studies of a purified HCMV DNA *pol* mutated in the N-terminal portion of δ region C (K513N) and of purified HSV DNA *pol* proteins mutated in the Exo II and III motifs and in δ region C show that the two enzymatic functions (i.e., polymerization and 3' to 5' exonuclease activities) may not behave independently in herpesviruses and may indeed overlap. By considering those two enzymatic activities while keeping in mind that GCV triphosphate and CDV diphosphate both act as alternate substrates for the DNA *pol* enzyme, three possible mechanisms of resistance have been postulated for those two antivirals: (i) a decreased affinity of the enzyme for the inhibitor, (ii) a decreased selective

incorporation of the inhibitor into the elongating DNA chain, or (iii) an enhanced selective excision from the DNA chain of the incorporated inhibitors. In the case of PFA, which is not incorporated into the elongating DNA, only the first mechanism of resistance (i.e., decreased affinity of the enzyme) would apply.

More than 80% of the adult population worldwide is infected with Human cytomegalovirus (HCMV). Primary infections occur early in child-hood and may cause exanthema subitum. All herpesviruses especially HCMV, have been implicated as a primary cause of morbidity and mortality in immunosuppressed organ transplant recipients [251-253].

Because the diagnosis of HCMV infections cannot be made reliably on clinical grounds alone, laboratory confirmation is required. The most specific laboratory method for diagnosis of HCMV infection is isolation of the virus in culture. Isolation of HCMV from blood leukocytes (CMV viremia) is considered the most reliable marker of disseminated CMV infection and predicts invasive CMV disease [254-258].

In vivo, Human cytomegalovirus (HCMV) can infect a number of cell types of different origins namely, fibroblasts, epithelial and endothelial cells and smooth muscle cells [259]. In particular, HCMV has been shown to infect and fully replicate in endothelial cells of the vascular tree in immunocompromised patients. Human cytomegalovirus (HCMV) is a ubiquitous human herpes virus present in peripheral myeloid cells of 50% to 90% of normal individuals. In disseminated infection, cytomegalic endothelial cells may also circulate in peripheral blood, and virus dissemination is mediated by peripheral blood leucocytes (PBL) carrying infectious virus acquired from infected endothelium

and transmitting the infection to uninfected endothelial cells [260-262]. The isolation of Human cytomegalovirus (HCMV) from human peripheral blood leukocytes has been documented by workers from various laboratories over more than 10 years. Successful isolation was reported for various leukocyte subpopulations as granulocytes, T lymphocytes, or monocytes[263,264]. pp65 antigen of HCMV is recognized by more than 70% of HCMV-specific circulating T Lymphocytes (CTLs). Because pp65 is processed and presented before endogenous viral replication, pp65-specific CTLs may initiate the HCMV spread [265-270].

The detection of HCMV pp65 antigenemia is widely used for monitoring CMV infection and guide preemptive therapy in patients at risk of developing HCMV disease, as a semi-quantitative analysis of HCMV viral replication [271,272]. As a source of antigen we used the HCMV pp65 matrix protein peptide as a marker for the isolation of the virus in this study. The CMV pp65 antigenemia assay, which quantitates the number of HCMV-infected leukocytes in peripheral blood, has proven efficacy in the detection and monitoring of this virus infection in immunocompromised patients [273-276]. Though sensitive techniques like PCR are available, still laboratories rely on pp65 antigenemia assay to determine the initiation of therapy to renal transplant patients[274]. Therefore, this study was undertaken to determine the significant pp65 antigenemia count among renal transplant patients, by evaluating the test against viral isolation which in turn may be useful for the treatment of the disease.

Chapter 2

2.1. Hypothesis:

Toll like receptors 3, 4 and 9 are triggered when challenged with HSV in retinal infections which could be demonstrated in retinal pigment epithelial cells in the laboratory.

2.2. Objectives:

- To Standardize the Reverse Transcriptase polymerase chain reaction for the amplification of Toll Like Receptors(TLR) 3, 4 and 9 and Housekeeping gene(GAPDH) of ARPE-19 on challenging with Herpes simplex virus 1 and 2
- To apply the Reverse Transcriptase polymerase chain reaction for the amplification of Toll Like Receptors (TLR) 3, 4, 9 and GAPDH of ARPE 19 on challenging with Herpes simplex virus 1 and 2 clinical isolates
- To analyze the results of amplification of Toll like receptors 3, 4 and 9 in ARPE-19 cells in HSV infection

2.2.1 Objective:

To Standardize the Reverse Transcriptase polymerase chain reaction for the amplification of Toll Like Receptors (TLR) 3,4, 9 and GAPDH of ARPE-19 on challenging with Herpes simplex virus 1 and 2.

2.2.1.1 Materials and Methods:

2.2.1.1.1. Cells

ARPE-19(ATCC) cells maintained in Advanced DMEM with F12 (Gibco, USA) with 20% FCS (Hi-media, Australia) were obtained at a passage of 23. The cells were trypsinised and cultured on 24- well plates. The plates were incubated at 37° C with 10% CO_2. Once the cells formed a monolayer the plates were used for inoculation of HSV.

2.2.1.1.2. Standard strains:

HSV – 1: ATCC 733 –VR (Chemicon, California,USA)

HSV 2 Species 753167 (National Institute of Virology, Pune)

2.2.1.1.3. Virus isolation:

Twenty two (22) isolates along with two standard strains were used for inoculation of these, 15 were HSV1 and 7 were HSV2. Ten microlitres (10 µl) of the stock was inoculated onto monolayer of ARPE 19 cell- line after having discarding the growth medium in the plate and the cell lines were kept for rocking for 30 minutes and sterile Advanced DMEM with F12(GIBCO, USA) with 2% FBS was added onto the wells including the uninoculated cell controls. The initial

morphological changes were observed after 24 hours of incubation. The plates were then incubated at 37° C till 48 hours and harvested. The cells were harvested as the CPE was observed and after several times of freezing and thawing.

2.2.1.1.4. RNA extraction:

The RNA was extracted from the culture isolates harvested from ARPE cells and uninoculated cell controls by using the Viral RNA mini kit (Qiagen, Germany), as per the protocol given by the supplier. The RNA was used for the amplification. The protocol followed is described in Appendix 1.

2.2.1.1.5. RT-PCR amplification:

The RT- PCR reagents were procured from USB Corporation, USA.

2.2.1.1.5.1. Preparation of cocktail:

a. RT -PCR Master mix (2X)	25 µl
b. Forward primer (1 in 10 dilution)	1 µl
c. Reverse primer (1 in 10 dilution)	1 µl
d. RNAse free water	21 µl
e. Template RNA	2 µl

2.2.1.1.6. Primer Sequence:

The primer sequences used for the amplification of TLR's 3, 4 and 9 and GAPDH genes and the amplified product size are described in the table below[277,278].

Table 2.2.1 Primer sequences for GAPDH and TLR's

Gene	Primer Orientation	Primer sequence	Amplified product size
TLR3	Forward	5' GATCTGTCTCATAATGGCTTG 3'	304 bp
	Reverse	5' GACAGATTCCGAATGCTTGTG 3'	
TLR4	Forward	5' TCCCTCCAGGTTCTTGATTACAGTC 3'	652 bp
	Reverse	5' TGCTCAGAAACTGCCAGGTCTG 3'	
TLR9	Forward	5''GTGCCCCACTTCTCCATG 3'	259 bp
	Reverse	5' GGCACAGTCAGATGTTGTTG 3'	
GAPDH	Forward	5' CCACCCATGGCAAATTCCATGGCA 3'	600 bp
	Reverse	5' TCTAGACGGCAGGTCAGGTCCACC 3'	

2.2.1.1.6.2. Thermal profile for TLR 3 and 9 Amplification:

The Thermal profile was standardized in house to our laboratory conditions. The thermal profile standardized is as follows:

Table 2.2.2: Thermal profile for TLR3 and 9:

Temperature	Time	Cycles
cDNA conversion **50 ºC**	**30 mins**	**1 cycle**
Denaturation **95 ºC**	**60 secs**	**45 cycles**
Annealing **55 ºC**	**60 secs**	
Extension **72 ºC**	**60 secs**	
Final Extension **72 ºC**	**7 mins**	**1 cycle**

Table 2.2.3: Thermal Profile for TLR4 and GAPDH

Temperature	Time	Cycles
cDNA conversion **50 ºC**	**30 mins**	**1 cycle**
Denaturation **95 ºC**	**60 secs**	**35 cycles**
Annealing **58 ºC**	**60 secs**	
Extension **72 ºC**	**60 secos**	
Final Extension **72 ºC**	**7 mins**	**1 cycle**

2.2.1.1.7. Sensitivity of RTPCR for TLR 3, 4 and 9:

The sensitivity of the RT-PCR was tested by serial tenfold dilutions of the RNA extracted from the positive culture harvest. RT-PCR was performed to determine the sensitivity.

2.2.1.1.8. Detection of amplified product

The amplified product was subjected to electrophoresis on 2% agarose gel incorporated with 0.5 µg/ml ethidium bromide (Appendix 2) for visualization by UV transilluminator (Vilber Lourmet, France).

2.2.1.2. RESULTS:

2.2.1.2.1. Results of viral isolation:

All 22 isolates showed cytopathic effect in the respective inoculated wells. Uninoculated cell controls were also maintained along with these isolates. The pictures of viral isolation are shown in Fig: 2.2.1.1.

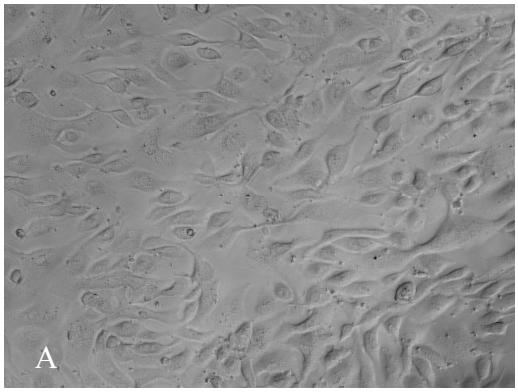
2.2.1.2.2. Results of Standardization of Reverse Transcriptase Polymerase Chain reaction for the amplification of TLR 3, 4 and 9 and GAPDH with the Standard strains:

The Standard strains showed a single bright band corresponding to 304 bp of the 100bp molecular weight marker, thus confirming the specific amplification of the TLR3 genome (Figure 2.2.1.2). The Standard strains showed a single bright band corresponding to 652 bp of the 100bp molecular weight marker, thus confirming the specific amplification of the TLR4 genome (Figure 2.2.1.3). The Standard strains showed a single bright band corresponding to 259 bp of the 100bp molecular weight marker, thus confirming the specific amplification of the TLR9 genome (Figure 2.2.1.4.). The Standard strains showed a single bright band corresponding to 600 bp of the 100bp molecular weight marker, thus confirming the specific amplification of the GAPDH genome (Figure 2.2.1.5.).

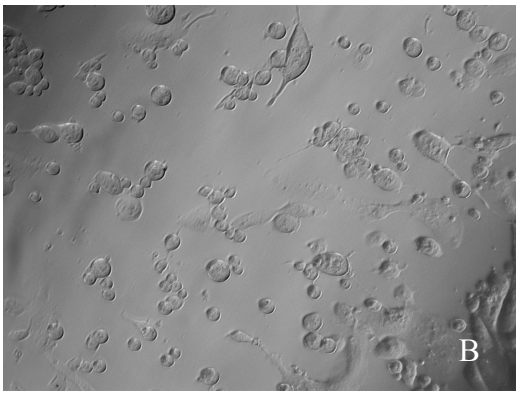
2.2.1.2.3. Sensitivity of the RTPCR for GAPDH and TLR3,4 and 9:

The primers were tested for its sensitivity using serial tenfold dilutions. The sensitivity of the primers was found to be 10 nanograms of RNA of TLR's 3, 4 and 9.

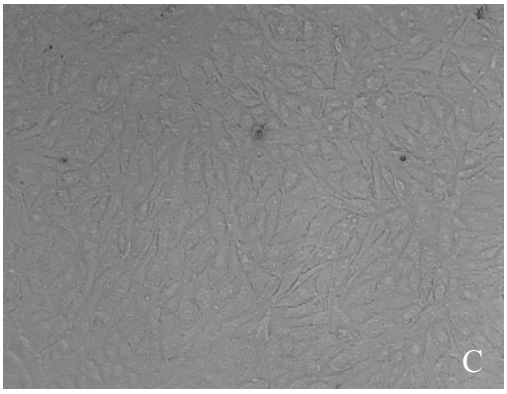
Fig 2.2.1.1: Images of HSV infected and uninfected ARPE-19 cells



A: CPE of HSV1 std strain

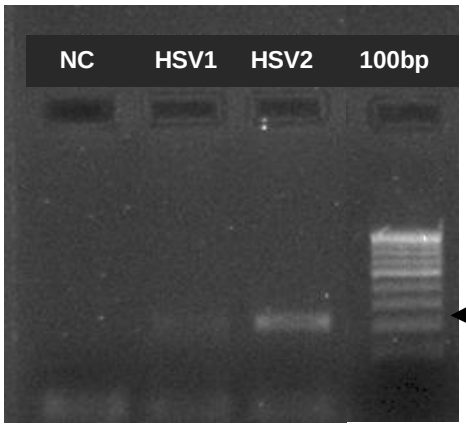


B: CPE of HSV 2 std strain



C: ARPE uninoculated cell control

Fig 2.2.1.2: Electrophoretogram of PCR amplification of TLR3 gene:



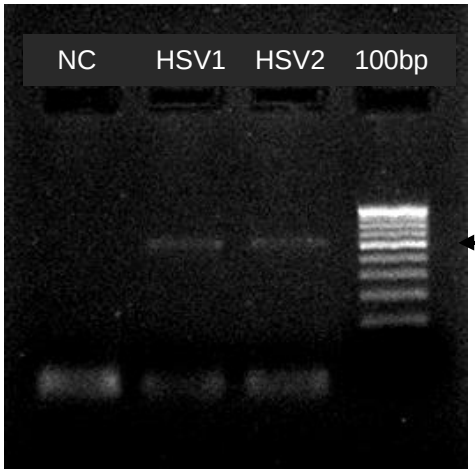
Lane 1: Negative control

Lane 2: HSV1 std strain (ATCC-733 VR)

Lane 3: HSV2 std strain (sp753167)

Lane 4: DNA ladder (100bp)

Fig 2.2.1.3: Electrophotogram of PCR amplification of TLR4 gene:



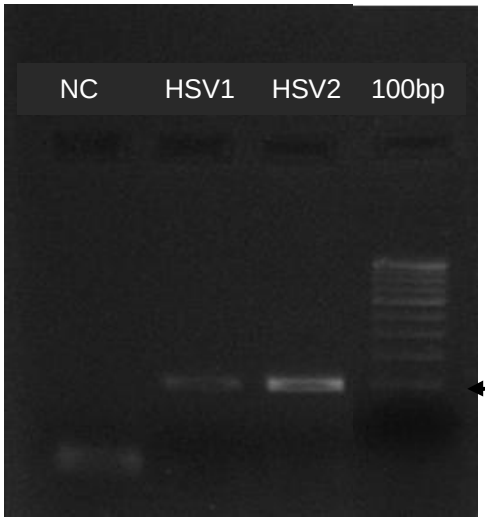
Lane 1: Negative control

Lane 2: HSV1 std strain (ATCC-733 VR)

Lane 3: HSV2 std strain (sp753167)

Lane 4: DNA ladder (100bp)

Fig 2.2.1.4: Electrophoretogram of PCR amplification of TLR 9 gene:



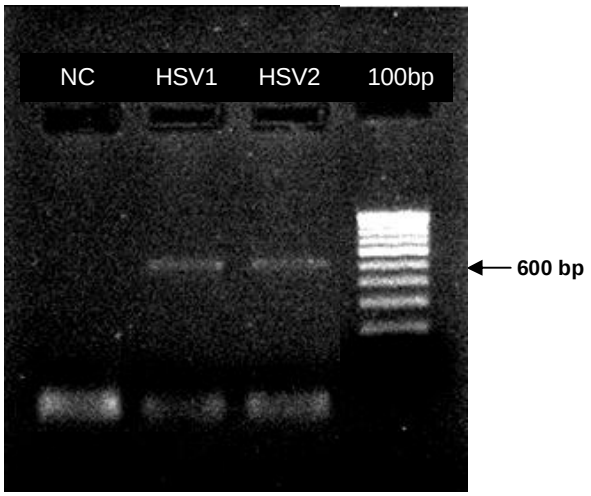
Lane 1: Negative control

Lane 2: HSV1 std strain (ATCC-733 VR)

Lane 3: HSV2 std strain (sp753167)

Lane 4: DNA ladder (100bp)

Fig 2.2.1.5: Electrophoretogram of PCR amplification of GAPDH gene:



Lane 1: Negative control

Lane 2: HSV1 std strain (ATCC-733 VR)

Lane 3: HSV2 std strain (sp753167)

Lane 4: DNA ladder (100bp)

2.2.2. Objective:

Application of Reverse Transcriptase polymerase chain reaction for the amplification of Toll like Receptors (TLR) 3, 4 and 9 and GAPDH of ARPE 19 on challenging with Herpes simplex virus 1 and 2 clinical isolates

2.2.2.1. Materials and Methods:

2.2.2.1.1. Standard strains:

HSV – 1: ATCC 733 –VR (Chemicon, California,USA)

HSV 2 Species 753167 (National Institute of Virology, Pune)

2.2.2.1.2. RNA extraction:

The RNA was extracted from the culture harvests of the viral isolates using the Viral RNA mini kit, (Qiagen, Germany) as per the protocol given by the supplier (Described in Appendix 1). The RNA was used for the amplification.

2.2.2.1.3. Reverse Transcriptase Polymerase Chain Reaction for the amplification of Toll like receptors (TLR) 3, 4 & 9 and GAPDH of ARPE challenged with of Herpes simplex virus clinical isolates:

The TLR's and GAPDH were amplified as standardized using the set of Oligonucleotides already described in section 2.2.1.1.5.

2.2.2.1.3.2. RT-PCR amplification:

The RT-PCR reagents were procured from USB Corporation, USA as described earlier in section 2.2.1.1.6.

2.2.2.1.3.2.1. Preparation of cocktail:

As described in section 2.2.1.1.6.1

2.2.2.1.3.2 Thermal profile:

The details of the thermal profile followed is as described in section 2.2.1.1.6.2.

2.2.2.2. RESULTS:

Results of Application of Reverse Transcriptase Polymerase Chain reaction with the HSV1 and 2 Clinical isolates:

2.2.2.2.1. For GAPDH:

All clinical isolates showed a single bright band corresponding to 600 bp of the 100bp molecular weight marker, thus confirming the expression of the GAPDH genome (Figure 2.2.2.1).

2.2.2.2.2. For TLR3:

All the Clinical isolates showed a single bright band corresponding to 304 bp of the 100bp molecular weight marker, thus confirming the expression of the TLR3 genome (Figure 2.2.2.2).

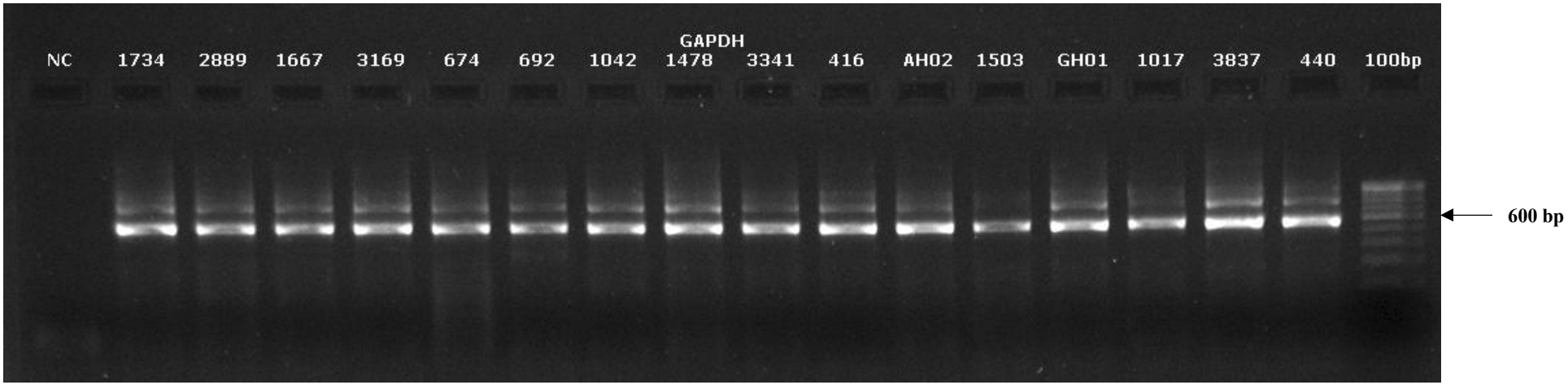
2.2.2.2.3. For TLR9:

Of the 22 clinical isolates 16 showed a single bright band corresponding to 259 bp of the 100bp molecular weight marker, thus confirming the expression of the TLR9 genome (Figure 2.2.2.3). The other 6 isolates did not show the expression of TLR9.

2.2.2.2.4. For TLR4:

All the Clinical isolates showed a single bright band corresponding to 652 bp of the 100bp molecular weight marker, thus confirming the expression of the TLR4 genome (Figure 2.2.2.4.).

Figure 2.2.2.1: Electrophoretogram of the PCR amplification of GAPDH gene on challenging with HSV clinical isolates

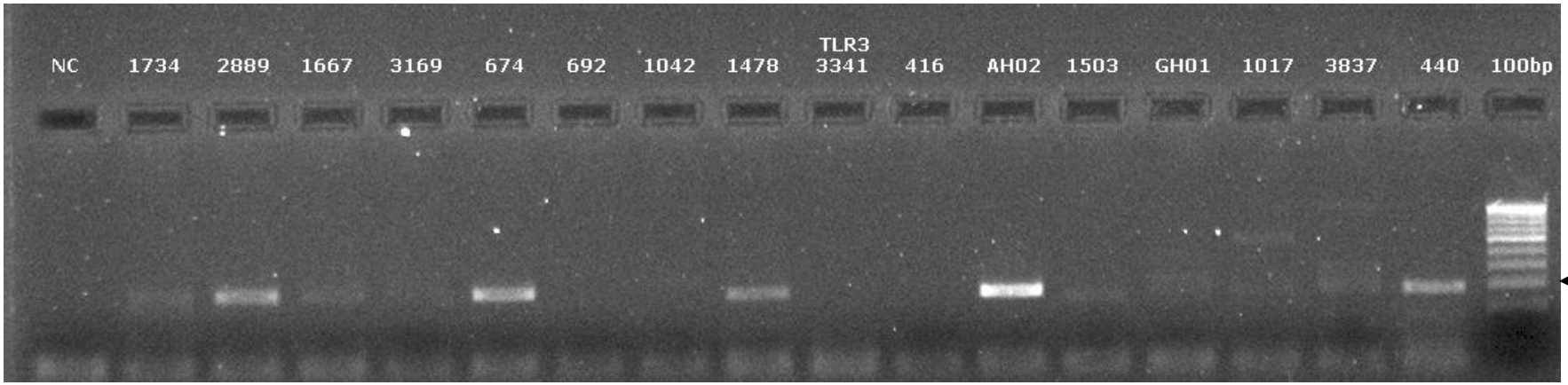


Lane 1 : **Negative control**

Lanes 2-17 : **HSV clinical isolates**

Lane 18 : **DNA Ladder (100 bp)**

Figure 2.2.2.2: Electrophoretogram of the PCR amplification of TLR 3 gene of ARPE cells on challenging with HSV clinical isolates

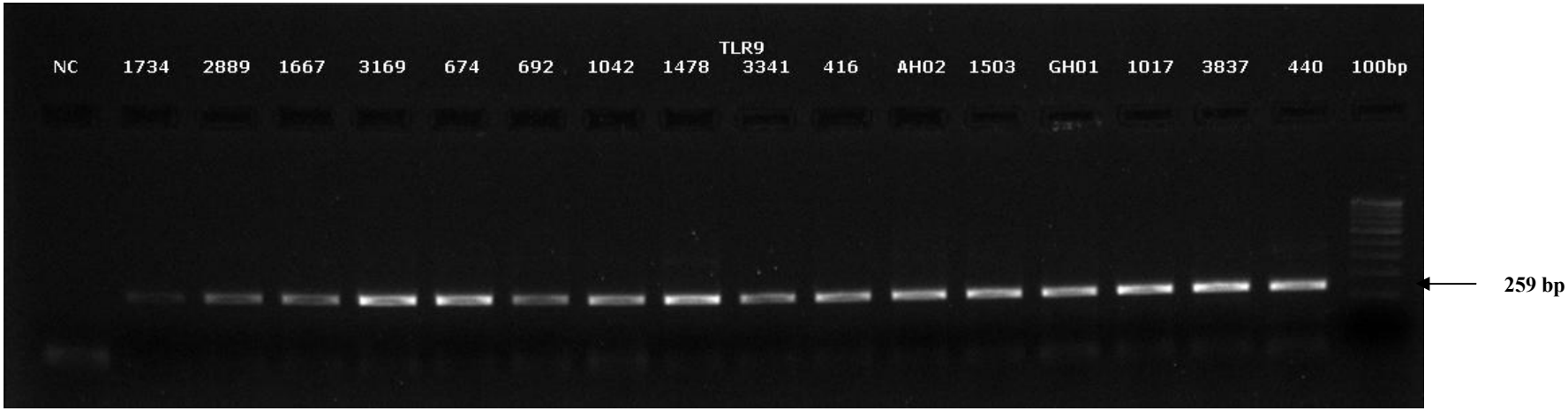


Lane 1 : **Negative control**

Lanes 2-17 : **HSV clinical isolates**

Lane 18 : **DNA Ladder (100 bp)**

Figure 2.2.2.3: Electrophoretogram of the PCR amplification of TLR 9 gene of ARPE cells on challenging with HSV clinical isolates

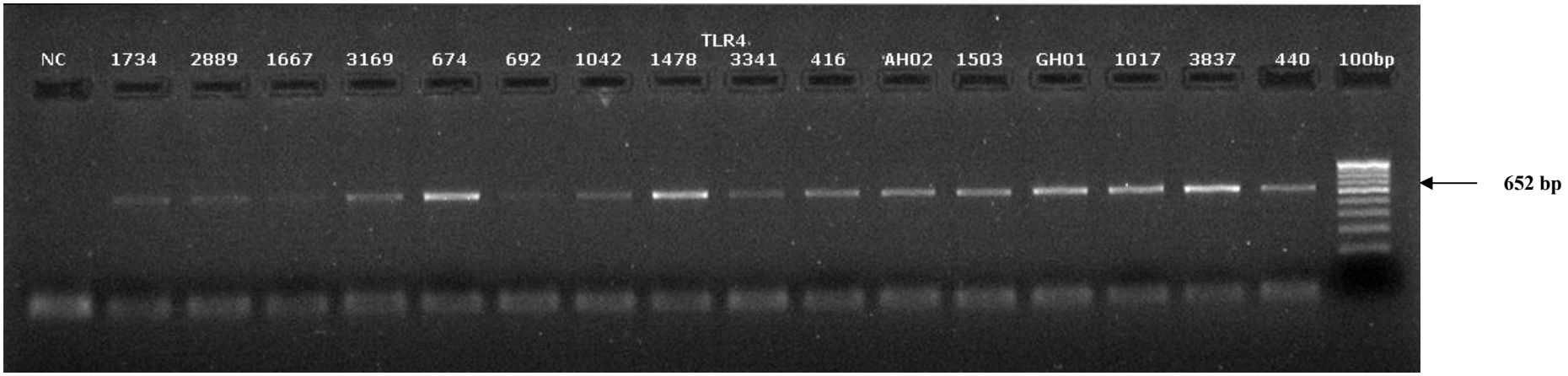


Lane 1 : Negative control

Lanes 2-17 : HSV clinical isolates

Lane 18 : DNA Ladder (100 bp)

Figure 2.2.2.4: Electrophoretogram of the PCR amplification of TLR 4 gene of ARPE cells on challenging with HSV Clinical isolates



Lane 1 : **Negative control**

Lanes 2-17 : **HSV Clinical isolates**

Lane 18 : **DNA Ladder (100 bp)**

2.2.3: DISCUSSION:

Analysis of the results of amplification of Toll like receptors 3, 4 and 9 in ARPE-19 cells in HSV infection.

Host resistance to HSV infections includes nonspecific mechanisms such as IFN's, complement, macrophages, humoral (antibody) immunity, T cell-mediated immunity (such as cytotoxic T cells(CTL's) and T helper cell activity, and cytokine release. The relative importance of these various mechanisms is different in initial or recurrent HSV disease. Animal studies have suggested that activated macrophages, IFN's and to a lesser extent, natural killer cells are important in limiting initial HSV infection, whereas humoral immunity and cell-mediated immunity are important in controlling both initial and recurrent infections[279].

The pathogenesis of Acute Retinal Necrosis (ARN) was provided by an early animal model in which HSV inoculation into the anterior chamber of rabbits was followed by retinal necrosis of the uninoculated eye[44], which was later confirmed in mice[45]. The virus spreads through synaptically connected nuclei and neurons to the contralateral, but not ipsilateral, optical nerve and retina[280-282]. Hence retinal pigment epithelial cells(ARPE-19) serve as the best model to study the expression of TLR's which has been used in this study.

TLRs have been shown to recognize both nucleic acid and protein derived from HSV. In mice, four TLRs have been found to play a role in the resistance to HSV-1: TLR3, TLR9, and the TLR2/6 heterodimer, which recognize dsRNA, CpG-rich DNA and lipopeptides, respectively. HSV-1 infection via intranasal delivery causes 100% mortality in mice deficient in the TLR adaptor protein

MyD88, suggesting that innate control via TLRs is important in limiting HSV-1 infection. Indeed, it has been shown *in-vivo* that HSV-1-mediated production of IFN requires TLR9 and MyD88[283].

A number of groups have revealed that mucosal delivery of ligands for TLR3 and TLR9, but not TLR2 or TLR4, are protective against genital infection with HSV-2. Similarly, intranasal application of a ligand for TLR3, but not for TLR4 or TLR9, protected against HSV encephalitis in mice[46,54,121,284,-286]. The therapeutic approach of using TLR ligands to treat HSV is an area that requires further investigation, since the downstream consequences of TLR engagement *in-vivo* remains to be clarified.

In our study the expression of TLR9 and 4 was seen in all 21 isolates and TLR3 was expressed in 16 of 22 isolates. The non expression of the TLR3 in the rest of the 6 isolates might probably be due to low expression of TLR3 in these isolates. The unusual finding in our study is that TLR4 has been expressed in response to HSV infection in these ARPE 19 cells.

TLR4 expression has usually been reported in response to LPS (in other words in response to bacterial infection). Different human intestinal epithelial cell lines have been shown to have all three types of LPS responsiveness and TLR4 expression: (1) relative hyporesponsiveness to LPS with low level of TLR4, (2) hyporesponsiveness to LPS with intracellular TLR4 localization, and (3) highly LPS-responsive with surface expression of TLR4, suggesting that these cells may comprise different subpopulations with distinct roles in innate immune responses[279]. TLR4 has also been shown to be involved in sensing various viruses including respiratory syncytial virus, mouse mammary tumor virus,

murine leukemia virus, and Coxsackievirus B4, via different viral proteins[46,54,121,284,-286].

The bacterial contamination of the culture harvest was ruled out by performing PCR for the detection of Eubacterial genome targeting the 16s ribosomal RNA subunit and inoculation onto liquid media (Brain Heart Infusion Broth) and checked for sterility for 12 days. The Eubacterial PCR was negative and the bacterial cultures did not show any bacterial growth. The non expression of TLR4 in the uninoculated cell control has also strongly proven the fact that TLR4 has been expressed only because of challenging the cells with HSV.

Hence in conclusion in this study we have observed that TLR4 has been expressed which has not been reported in other studies to be associated with HSV infection. Therefore, we conclude that TLR3, 4 and 9 have been expressed in HSV infections in ARPE cells which have a protective role to play in the immunity of intra-ocular HSV infection. Further studies are required to study the production of IFN's and cytokines.

Chapter 3

3.1 Hypothesis:

Mutations in thymidine kinase gene are majorly responsible for the phenotypic resistance to acyclovir among Herpes simplex virus isolates

3.2. Objective:

- To perform Phenotypic Characterization of antiviral Drug resistance of Herpes simplex virus isolates against acyclovir by plaque reduction assay.
- To Standardize and apply the polymerase chain reaction for the amplification of thymidine kinase gene of herpes simplex virus and application onto clinical isolates of HSV 1 and 2.
- To perform PCR based DNA sequencing of the thymidine kinase gene, of Herpes simplex virus, for the detection of mutations responsible for resistance to acyclovir.
- To analyze the sequences for mutations responsible for the resistance to acyclovir by Herpes simplex viral isolates and molecular modeling of the wild type and mutant thymidine kinase gene.

3.2.1. Objective:

To perform Phenotypic Characterization of antiviral Drug resistance of Herpes simplex virus isolates against acyclovir by plaque reduction assay.

3.2.1.1. Materials and Methods:

3.2.1.1.1. Standard strains:

HSV – 1: ATCC 733 –VR (Chemicon, California,USA)

HSV 2 Species 753167 (National Institute of Virology, Pune)

3.2.1.1.2. Specimens:

Clinical specimens collected from Y.R. Gaitonde Centre for AIDS Research (Y.R.G CARE), Chennai for HIV patients and specimens received at the L & T microbiology laboratory for the isolation of HSV were cultured for virus isolation and stored at -80ºC. The isolates obtained from Christian Medical College & Hospital, Vellore and Aravind Eye Hospital, Madurai were also used for the study.

3.2.1.1.3 Virus isolation:

Hundred (100) µl of the clinical specimen was inoculated onto monolayer of Vero cell-line after having discarded the growth medium in the tubes and the cell lines were kept for rocking for 30 minutes and sterile 2% MEM was added onto the tubes including the controls. The tubes were then incubated at 37° C for 8 days and observed daily for cytopathic effect(CPE). The cells were harvested as the CPE of multinucleated giant cells was observed and after freezing and

thawing. The DNA was extracted from the primary culture harvest and used for PCR amplifications.

3.2.1.1.4. DNA extraction:

The DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Germany) as per the protocol described by the manufacturer.

3.2.1.1.5. Polymerase Chain Reaction for the amplification of Glycoprotein D Gene of Herpes simplex virus:

The PCR targeting glycoprotein D gene was performed using the primer sequences described by *Shyamala et al.,* to characterize the isolates to be HSV 1 / HSV2. DNA was extracted from archival isolates from Christian Medical College Hospital (CMC&H) and those isolated from clinical specimens received at L & T microbiology research centre and those received from YRG care and subjected to PCR[287].

3.2.1.1.6. Determination of $TCID_{50}$[4,5]:

Various dilutions of the viral inoculum were made ranging from 1:10 till 1:150. Ten microlitres of the undiluted viral inoculum and various dilutions were inoculated onto respective wells of tissue culture plate. The plate was rocked for 1 hour at room temperature. 1% MEM was added to the wells and the plate was incubated at 37 ºC in a CO_2 incubator. The test was performed in duplicates. The cultures were interpreted after 24 hours for $TCID_{50}$.

3.2.1.1.7. Plaque Reduction Assay[4,5]:

The standard strain and the isolates of the HSV1 and 2 were subjected to plaque reduction assay against acyclovir. The protocol followed is as follows.

- Medium was aspirated from the wells except the Cell control.
- Ten µl of the diluted stock virus which corresponds to $TCID_{50}$ dose of the virus was added to each well.
- Plate was rocked for 30 mins at room temperature.
- Maintenance medium (1%MEM-500 µl) added to the cell control and virus control.
- Five hundred microlitres of varying concentrations of acyclovir dissolved in 1% MEM was added to the wells (0.5µg to 400.0µg).
- Five hundred microlitres of the highest concentration of acyclovir used for the test was used as drug controls without the virus.
- The plate was incubated at 37ºC in a CO_2 incubator.
- The results were interpreted after 24 hrs.

3.2.1.2 RESULTS:

3.2.1.2.1. Results of Viral culture:

Four samples were collected form HIV patients from YRG care, of which two samples showed cytopathic effect resembling that of HSV. The other two samples were negative. Seven isolates were obtained from corneal scraping

specimens collected from patients suspected to have Herpes keratitis. One isolate was obtained from lip lesion, one isolate from conjunctival swab, one isolate from conjunctival discharge, one isolate form skin scraping, one isolate from throat swab, others were 20 of the archival isolates already preserved at our centre and fourteen isolates from the Department of Virology, Christian Medical College, Vellore and six isolates from the Department of Microbiology, Aravind Eye Hospital, Madurai which were generously given were used in the study.

3.2.1.2.2. Results of PCR for glycoprotein D gene of HSV:

The isolates were typed to be HSV 1 and 2 by PCR. Thirty three isolates were typed to be HSV-1 and twenty three were typed to be HSV-2 which also includes the standard strains.

3.2.1.2.3. Results of $TCID_{50}$:

$TCID_{50}$ was performed for all the isolates and the standard strain. The dilutions which showed atleast 50% infectivity on the cells was considered the $TCID_{50}$ dose of the virus. The dilutions of the HSV 1 and 2 standard strains were 1:50 each respectively. Three of the isolates showed 50% infectivity in undiluted viral stock, twenty one isolates in 1:50 dilution, twenty three isolates in 1:10 dilution, and three isolates in 1:100 dilution. The results of the $TCID_{50}$ have been tabulated as follows (Table 3.1)

Table 3.1: Results of $TCID_{50}$ of HSV isolates

S. No	HSV1 Isolates/Standard strain	Dilution ($TCID_{50}$)	HSV2 Isolates/Standard strain	Dilution ($TCID_{50}$)
1.	Standard strain (HSV 1 733- VR)	1:50	Standard strain (HSV 2 753167)	1:50
2.	Isolate 54	1:50	Isolate 23	Neat (undiluted)
3	Isolate 320	1:10	Isolate 389	1: 50
4	Isolate 342	1:50	Isolate 836	1:10
5	Isolate 416	1:10	Isolate 1204	1:10
6	Isolate 666	1:10	Isolate 1194	1:50
7	Isolate 689	1:50	Isolate 1195	Neat (undiluted)
8	Isolate 922	1:10	Isolate 824	1:10
9	Isolate 1105	1:100	Isolate 674	1:10
10	Isolate 1217	1:10	Isolate 692	1:10
11	Isolate 1362	1:50	Isolate 1089	1:50
12	Isolate 1464	1:50	Isolate 1155	1:10
13	Isolate 1486	1:100	Isolate 1402	1:10
14	Isolate 2857	1:10	Isolate 1503	1:10
15	Isolate 2889	1:50	Isolate 1667	1:10
16	Isolate 3341	1:100	Isolate 1878	1:10
17	Isolate 2307	1:50	Isolate 459	1:10

S. No	HSV1 Isolates/Standard strain	Dilution ($TCID_{50}$)	HSV2 Isolates/Standard strain	Dilution ($TCID_{50}$)
18	Isolate 4184	1:50	Isolate 708	1:50
19	Isolate SN01	1:50	Isolate 1017	1:10
20	Isolate SN02	1:50	Isolate 1042	1:10
21	Isolate AH01	1:50	Isolate 576	1:10
22	Isolate AH02	1:50	YRG 2	1:10
23	Isolate AH03	Neat(Undiluted)	YRG 3	1:10
24	Isolate AH06	1:10	-	-
25	Isolate AH08	1:50		
26	Isolate AH12	1:10		
27	Isolate GH01	1:10		
28	Isolate GH03	1:10		
29	Isolate 1734	1:10		
30	Isolate 1362	1:10		

3.2.1.2.4. Results of Plaque Reduction assay:

The results of the plaque reduction assay were interpreted as sensitive those of which were inhibited to a concentration >2µg of acyclovir and those inhibited between a concentration of (<2 µg - >4 µg) intermittently sensitive and those resistant to <4 µg were Interpreted as Resistant. The standard strains were sensitive to 0.5µg/ml of acyclovir. Twenty six isolates were sensitive to 0.5µg/ml of the drug, two isolates were sensitive to 1.0 µg/ml, two isolates were sensitive to 2.0 µg/ml of the drug, three isolates were resistant upto 6.0 µg/ml of the drug and

15 isolates were resistant upto 160 µg/ml of the drug and two isolates were resistant upto 400 µg/ml. The sensitivity of the isolates with concentration of the drug has been described in the table below (Table 3.2).

Table 3.2.: Results of antiviral assay performed on HSV isolates

S. No	**HSV1 Isolates/Standard strain**	**Results**	**HSV2 Isolates/Standard strain**	**Results**
1.	Standard strain (HSV 1 733- VR)	0.5µg/ml-Sensitive	Standard strain (HSV 2 753167)	0.5µg/ml-Sensitive
2.	**Isolate 54**	**160µg/ml-Resistant**	**Isolate 23**	**160µg/ml-Resistant**
3	**Isolate 320**	**160µg/ml-Resistant**	**Isolate 389**	**160µg/ml-Resistant**
4	**Isolate 342**	**160µg/ml-Resistant**	**Isolate 836**	**160µg/ml-Resistant**
5	Isolate 416	0.5µg/ml-Sensitive	Isolate 1204	1.0µg/ml-Sensitive
6	Isolate 666	0.5µg/ml-Sensitive	**Isolate 1194**	**160µg/ml-Resistant**
7	Isolate 689	2.0µg/ml-Sensitive	**Isolate 1195**	**6.0µg/ml-Resistant**
8	**Isolate 922**	**400µg/ml-Sensitive**	Isolate 824	0.5µg/ml-Sensitive
9	**Isolate 1105**	**400µg/ml-Sensitive**	**Isolate 674**	**160µg/ml-Resistant**
10	**Isolate 1217**	**160µg/ml-Resistant**	**Isolate 692**	**160µg/ml-Resistant**

S. No	HSV1 Isolates/Standard strain	Results	HSV2 Isolates/Standard strain	Results
11	**Isolate 1362**	**160µg/ml-Resistant**	Isolate 1089	0.5µg/ml-Sensitive
12	Isolate 1464	0.5µg/ml-Sensitive	Isolate 1155	0.5µg/ml-Sensitive
13	**Isolate 1486**	**160µg/ml-Resistant**	**Isolate 1402**	**160µg/ml-Resistant**
14	**Isolate 2857**	**160µg/ml-Resistant**	Isolate 1503	0.5µg/ml-Sensitive
15	Isolate 2889	0.5µg/ml-Sensitive	Isolate 1667	0.5µg/ml-Sensitive
16	Isolate 3341	0.5µg/ml-Sensitive	Isolate 1878	1.0µg/ml-Sensitive
17	Isolate 2307	2.0µg/ml-Sensitive	**Isolate 459**	**160µg/ml-Resistant**
18	Isolate 4184	0.5µg/ml-Sensitive	**Isolate 708**	**6.0µg/ml-Resistant**
19	Isolate SN01	0.5µg/ml-Sensitive	Isolate 1017	0.5µg/ml-Sensitive
20	Isolate SN02	0.5µg/ml-Sensitive	Isolate 1042	0.5µg/ml-Sensitive
21	**Isolate AH01**	**6.0µg/ml-Resistant**	Isolate 576	0.5µg/ml-Sensitive
22	Isolate AH02	0.5µg/ml-Sensitive	YRG 2	0.5µg/ml-Sensitive
23	Isolate AH03	0.5µg/ml-Sensitive	YRG 3	0.5µg/ml-Sensitive
24	Isolate AH06	0.5µg/ml-Sensitive	-	-
25	Isolate AH08	0.5µg/ml-Sensitive		

S. No	HSV1 Isolates/Standard strain	Results	HSV2 Isolates/Standard strain	Results
26	Isolate AH12	0.5µg/ml-Sensitive	-	-
27	Isolate GH01	0.5µg/ml-Sensitive		
28	Isolate GH03	0.5µg/ml-Sensitive		
29	Isolate 1734	0.5µg/ml-Sensitive		

3.2.2. Objective:

To Standardize and apply the polymerase chain reaction for the amplification of thymidine kinase gene of herpes simplex virus onto clinical isolates of HSV 1 and 2.

3.2.2.1. Material and Methods:

3.2.2.1.1. DNA Extraction:

The DNA was extracted using the QiAamp DNA mini kit (QIAGEN, Germany). The protocol followed was according to the manufacturer's manual (Appendix 2).

3.2.2.1.2. Standardization and application of Polymerase Chain Reaction for the amplification of thymidine kinase Gene of Herpes simplex virus 1 and 2:

The thymidine kinase gene was amplified as two fragments using two sets of Oligonucleotides are located within the thymidine kinase gene of the HSV genome. The primer sequences are described in Table 3.3[288,289].

Table 3.3: Primers for amplification of HSV thymidine kinase gene

Primer Orientation	Primer sequence	Amplified Product Size
HSV1		
Fragment I		
Forward	5’ CTAGCTACGTCCAGCACCCT 3’	610 bp
Reverse	5’ ACTGGTAGCACCCGGTCACA 3’	
Fragment II		
Forward	5’ CACAAGACCGTCCGCCTCAA 3’	610 bp
Reverse	5’ ACCCGGTCACACGCACATTG 3’	
HSV2		
Forward	5’ AGGGAGTGGCGCAGCTGCTTC 3’	1315bp
Reverse	5’ CCGCGCTTATGGACACAC 3’	

3.2.2.1.3. PCR amplification:

The PCR reagents were procured from Bangalore Genei Pvt. Ltd.

3.2.2.1.3.1. Preparation of cocktail:

a. Dinitro triphosphates 8 µl

b. 10 X assay buffer 5 µl

c. Primer F1/ F2 (1 in 30 dilution) 1 µl

d. Primer R1/R2 (1 in 30 dilution) 1 µl

e. *Taq* DNA polymerase enzyme 0.3 µl

f. Milli Q water 30 µl

3.2.2.1.3.2. Thermal profile:

The thermal profile for the amplification of UL23 gene of HSV1 and 2 are described in tables 3.4 and 3.5.

Table 3.4: Thermal Profile For the amplification of TK gene of HSV- 1:

Initial Denaturation 95ºC	**10 mins**	**1 cycle**
Denaturation 95ºC	**20 secs**	**5 cycles**
Annealing 50 ºC	**30 secs**	
Extension 75 ºC	**10 mins**	
Denaturation 95ºC	**20 secs**	**35 cycles**
Annealing 50 ºC	**30 secs**	
Extension 75 ºC	**1 min**	
Final Extension 75 ºC	**10 mins**	**1 cycle**
Hold 22 ºC	∞	

Table 3.5: Thermal Profile For the amplification of HSV-2 TK gene:

Temperature	**Time**	**Cycles**
Initial denaturation 95 ºC	**10 mins**	**1 cycle**
Denaturation 95 ºC	**1 min**	**40 cycles**
Annealing 50 ºC	**45 secs**	
Extension 72 ºC	**2 mins**	
Final Extension 72 ºC	**10 mins**	**1 cycle**

3.2.2.1.4. Specificity of primers for the amplification of the thymidine kinase gene of HSV-1:

The specificity of the primers was determined with the DNA extracted from, Cytomegalovirus virus, HSV-1 (ATCC 733-VR), HSV -2, VZV (Oka vaccine strain), Adenovirus serotypes 7a (ATCC 848-VR), *Staphylococcus aureus* (ATCC 25293), *Toxoplasma gondii*, and *Candida albicans*, and 2 human leucocyte DNA.

3.2.2.1.5. Sensitivity of primers for amplification of the thymidine kinase gene of HSV-1:

The sensitivity of the primers was tested by serial tenfold dilutions of the DNA extracted from the positive culture harvest. PCR was performed to determine the sensitivity.

3.2.2.1.6. Specificity of primers for the amplification of the thymidine kinase gene of HSV-2:

The specificity of the primers was determined with the DNA extracted from, Cytomegalovirus virus, HSV-1 (ATCC 733-VR), HSV -2, VZV (Oka vaccine strain), Adenovirus serotypes 7a (ATCC 848-VR), *Staphylococcus aureus* (ATCC 25293), *Toxoplasma gondii*, and *Candida albicans*, and 2 human leucocyte DNA.

3.2.2.1.7. Sensitivity of primers for amplification of the thymidine kinase gene of HSV-2:

The sensitivity of the primers was tested by serial tenfold dilutions of the DNA extracted from the positive culture harvest. PCR was performed to determine the sensitivity.

3.2.2.1.8. Detection of amplified product

The amplified product was subjected to electrophoresis on 2% agarose gel incorporated with 0.5 µg/ml ethidium bromide (Appendix 2) for visualization by UV transilluminator (Vilber Lourmet, France).

3.2.2.2. RESULTS:

3.2.2.2.1. Results of Standardization and application of Polymerase Chain reaction for the Amplification of thymidine kinase gene of Herpes simplex virus 1:

The positive control and the isolates showed a single bright band corresponding to 610 bp of the 100 DNA ladder of the molecular weight marker, thus confirming the specific amplification of the HSV 1 genome.

Figures 3.1,3.2 shows the amplification of Fragments 1 and 2 thymidine kinase gene of HSV 1.

3.2.2.2.2. Specificity of the primers for the amplification of thymidine kinase Gene of HSV-1:

The primers for the thymidine kinase gene of HSV was positive for HSV 1 and was negative for HSV 2, Adenovirus, HSV 1, Cytomegalovirus, VZV, *Toxoplasma gondii*, *Staphylococcus aureus*, *Candida albicans, Chlamydiae pneumoniae, Chlamydiae trachomatis* and Human leucocytes thus showing that the primers are very specific for HSV 1.

3.2.2.2.3. Sensitivity of the primers for the amplification of the thymidine kinase Gene of HSV-1:

The primers were tested for its sensitivity using serial tenfold dilutions. The sensitivity of the primers was found to be 100 nanograms of Herpes simplex virus 1 DNA.

3.2.2.2.4. Results of Standardization and application of Polymerase Chain reaction for the amplification of thymidine kinase gene of Herpes simplex virus 2 with the Positive control:

The positive control and the isolates showed a single bright band corresponding to 1315bp of the 100bp molecular weight marker, thus confirming the specific amplification of the HSV 2 genome. **Figure 3.3** the amplification of thymidine kinase gene of HSV 2.

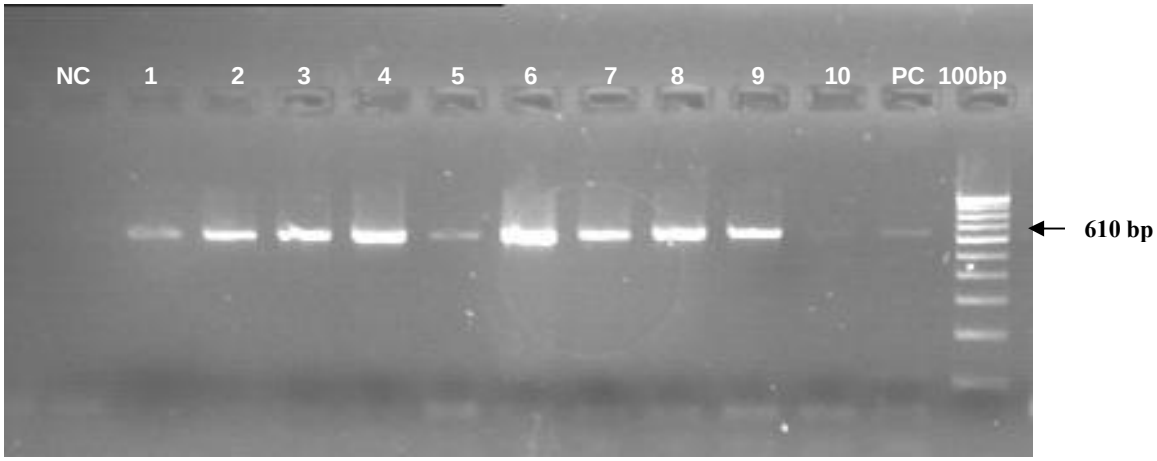
3.2.2.2.5. Specificity of the PCR for thymidine kinase Gene of HSV 2:

The PCR for the thymidine kinase gene of HSV was positive for HSV 2 and was negative for HSV 1, Adenovirus, Cytomegalovirus, VZV, *Toxoplasma gondii*, *Staphylococcus aureus*, *Candida albicans*, *Chlamydiae pneumoniae*, *Chlamydiae trachomatis* and Human leucocytes thus showing that the primers are very specific for HSV 2.

3.2.2.2.6. Sensitivity of the PCR for thymidine kinase Gene of HSV 2:

The primers were tested for its sensitivity using serial tenfold dilutions. The sensitivity of the primers was found to be 50 nanograms of Herpes simplex virus 2 DNA.

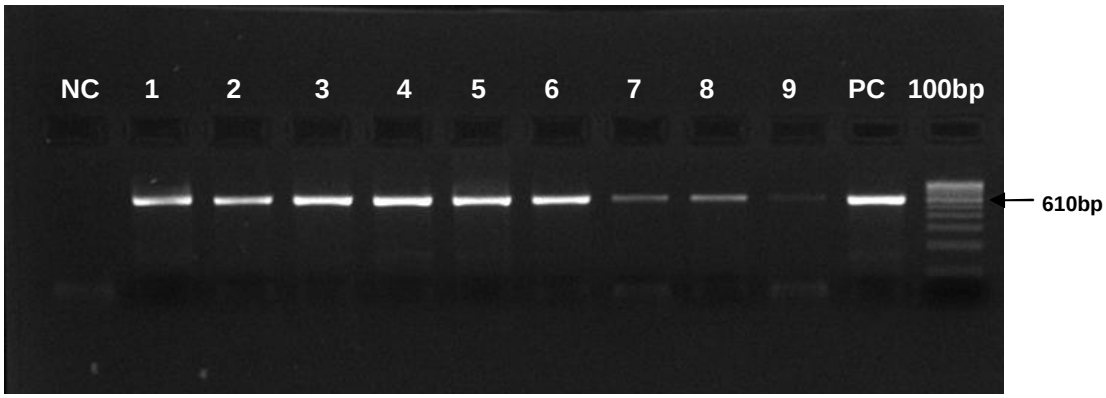
Fig 3.1: Electrophoretogram of PCR amplification of UL23 gene of HSV1 Fragment 1



Lane 1 : Negative control **Lane 12 : Positive control**

Lanes 2- 11 : HSV 1 Clinical isolates **Lane 13 : DNA ladder (100bp)**

Fig 3.2: Electrophoretogram of PCR amplification of UL23 gene of HSV1 Fragment 2

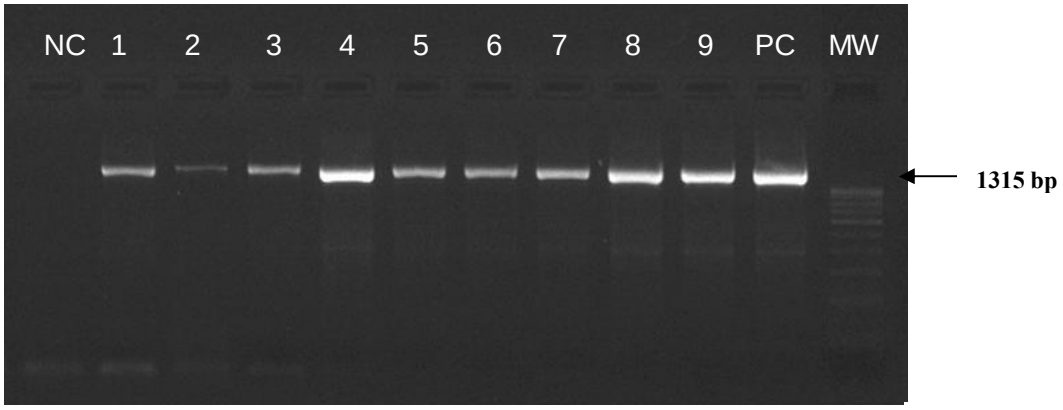


Lane 1 : Negative control

Lanes 2- 10 : HSV 1 Clinical isolates

Lane 11 : Positive control

Fig 3.3: Electrophoretogram of PCR amplification of UL23 gene of HSV 2



Lane 1: Negative control **Lane 11: Positive control**

Lane 2- Lane 10: HSV 2 clinical isolates **Lane 12: 100bp DNA ladder**

3.2.3. Objective:

Sequencing of the thymidine kinase gene, of Herpes simplex virus, for the detection of mutations responsible for resistance to acyclovir.

3.2.3.1. Materials and Methods:

3.2.3.1.1. Sequencing of Thymidine Kinase gene:

Cycle Sequencing:

Cycle sequencing combines amplification and enzymatic DNA sequencing using 5' dye labeled terminators.

Requirements for Cycle Sequencing:

- Forward primer or Reverse primer at the concentration of 1picomole/µl.
- Big Dye Terminator cycle sequencing Ready reaction kit (ABI prism, USA)

Reaction Protocol:

- Ready reaction mix (RR mix) - 1.5 µl
- 5x sequencing buffer - 2.5 µl
- Forward primer (1pmol/µl) - 2 µl
- Milli Q water - 2 µl
- Amplified PCR product (without primer band) - 3 µl

Table 3.6: Thermal profile for Cycle Sequencing:

Initial Denaturation 96˚C	**1 min**	**1 cycle**
Denaturation 96˚C	**10 secs**	**25 cycles**
Annealing 50˚C	**5 Secs**	
Extension 60˚C	**4 mins**	

3.2.3.1.2. Purification of Extension Product (Appendix 3):

The extension products were purified to remove the unincorporated dye terminators before the samples were analyzed.

3.2.3.1.3. Loading of samples:

The sequence of the PCR amplified DNA is deduced with the help of the ABI Prism 3100 AVANT (Applied Biosystems, USA) genetic analyzer that works based on the principle of Sanger dideoxy sequencing.

The amplified products with the dye at the terminated 3'end were subjected to capillary electrophoresis by an automated sample injection. The emitted flurorescence from the dye labels on crossing the laser area were collected in the rate of one per second by cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on the computer for processing.

3.2.3.1.4. Analysis of samples:

The sequences were then analysed by sequence analysis softwares such as Bio Edit sequence alignment software and Chromas software.

3.2.3.1.5. BLAST ANALYSIS:

BLAST analysis, using pubmed, www.ncbi.nlm.nih.gov/BLAST was done to confirm the sequenced data with the standard strains and to determine the percentage homology.

3.2.3.1.6. Translation of Nucleotide sequence to Protein Sequences:

The Nucleotide sequences obtained on sequencing were converted to their protein sequences using Emboss transeq protein converter.

3.2.3.2. RESULTS:

The protein sequence results were analyzed by www.ncbi.nlm.nih.gov/blast and found to have 96-100% homology with other isolates and standard strains. The sequences were found to have aminoacid changes based on multalin alignment. The multalin results of both HSV1 and 2 isolates with genbank sequences of strain F for HSV1 and strain 333 for HSV2 are shown in figures 3.4 and 3.5.

Fig:3.4 Multalin anlysis of HSV 1 isolates thymidine kinase gene protein sequence

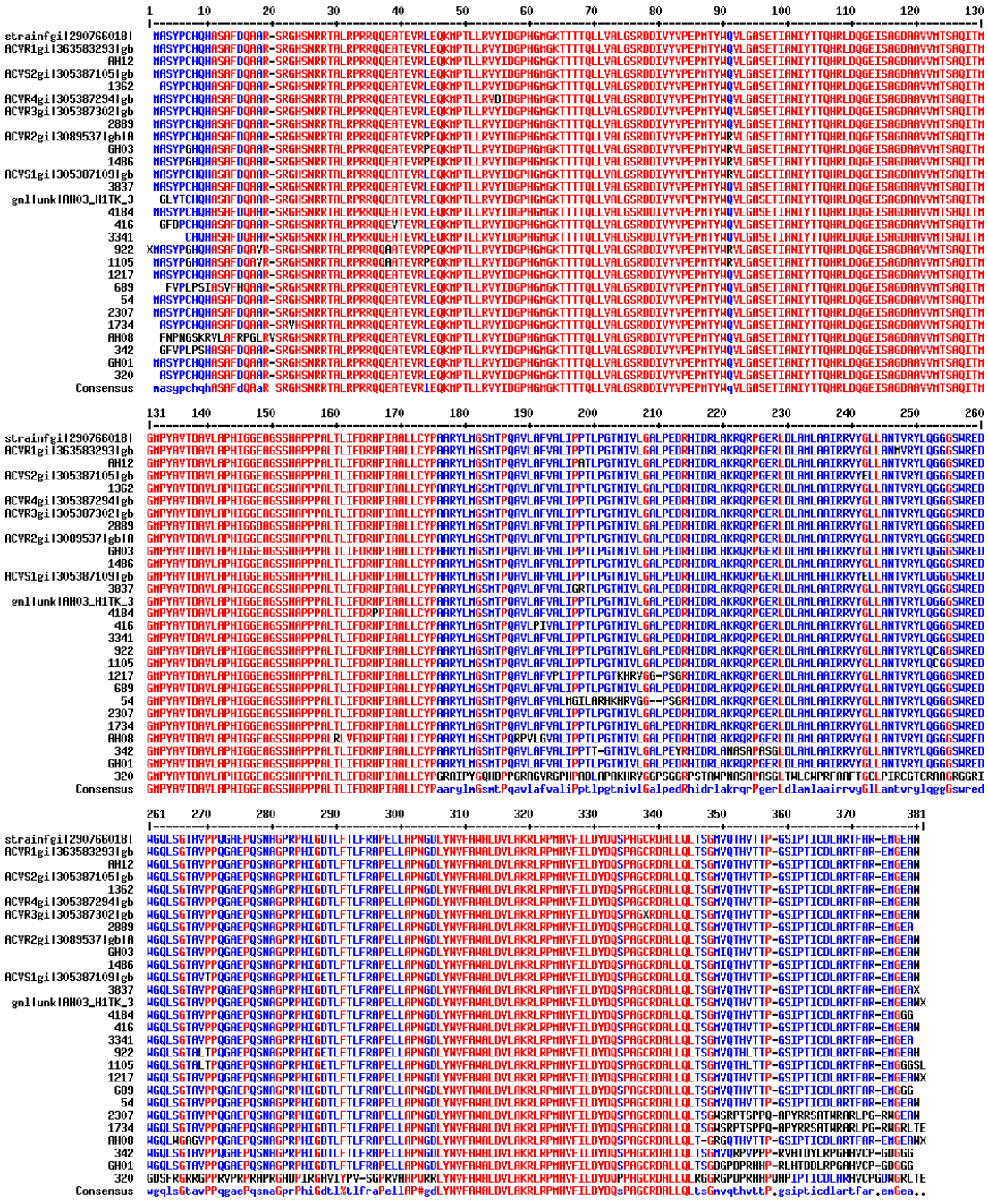
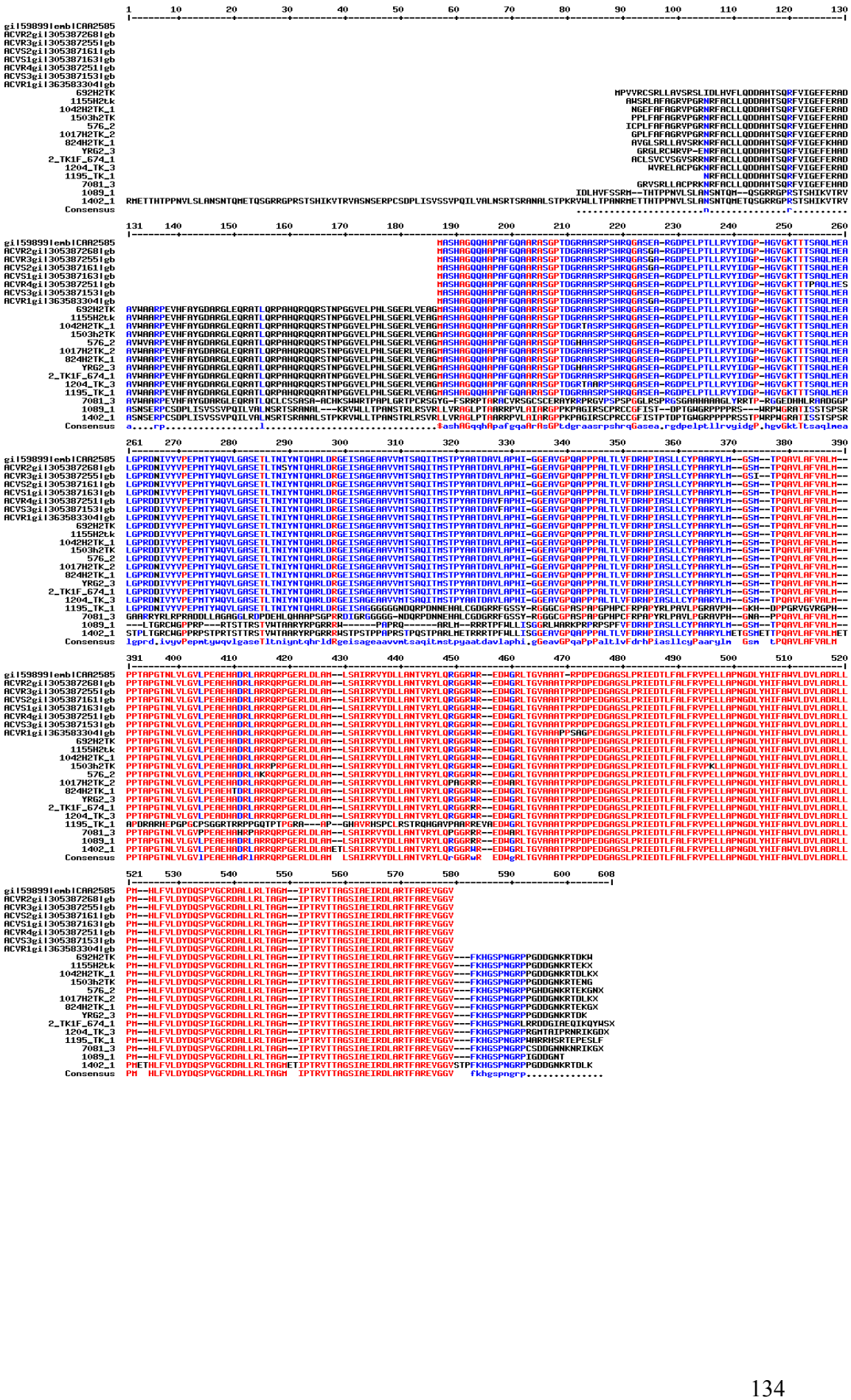


Fig 3.5: Multalin analysis of HSV 2 isolates thymidine kinase gene protein sequence



3.2.4.Objective:

To analyze the sequences for mutations responsible for the resistance to acyclovir by Herpes simplex viral isolates and molecular modeling of the wild type and mutant thymidine kinase gene.

3.2.4.1. Material and Methods:

3.2.4.1.1. Sequence and Sequence alignments:

The amino acid sequence of the wild Herpes simplex virus thymidine kinase – Strain F comprised of 376 amino acid residues was retrieved from NCBI (GI: 290766018) and the mutant sequence was obtained by transeq nucleotide to protein converter tool. .

Emboss pair-wise alignment was used to find the percentage identity and checking mismatches between the wild and mutant sequences using the Needle algorithm with standard settings at http://www.ebi.ac.uk/Tools/emboss/align.

3.2.4.1.2. Comparative Structure Prediction:

The homology model for the wild thymidine kinase was generated using MODELLER9v7[290]. The input to the program is an alignment of the target sequence with the related three dimensional structures. The complete crystal data of thymidine kinase is yet to be determined, and only partial structure information is available with a lot of missing residues. Therefore, in this study we attempted to predict the complete structure of thymidine kinase (UL23) of the WT and MT with the reported mutations, through structural bioinformatics approaches. The template structure for modeling the wild structure was obtained from I-TASSER which is a method based on structure threading [291]. Based on the alignment

comparative models of the target sequence were built by MODELLER applying the default model building routine. Energy minimization was done using the CHARMM force field using GROMACS and the resulting model was validated using SAVS Ramachandran plot (http://nihserver.mbi.ucla.edu/SAVES/). Since mutant type shared 97% identity with wild type, mutant structure was modeled using the modeled wild thymidine kinase structure as template.

3.2.4.1.3. Molecular dynamic Simulations:

Molecular dynamics simulation MD simulations were performed to demonstrate changes in structural behavior of the molecules with time (biological conditions) using GROMACS 3.2.2, with CHARMM force field [292]. The predicted structure of WT and the predicted structure of MT were used as the starting point for MD simulations. The protein was solvated with water molecules in a cubic box of 0.9nm dimensions. The simulation system was set up as an NPT ensemble, i.e. constant number of particles (N), constant pressure (P) and constant temperature (T). All protein atoms were at a distance equal to 1.0nm from the box edges. The system was subjected to energy minimization for 1000 steps by steepest descent to achieve native conformation. The equilibrated systems were then subjected to MD simulations for 1 ns at 300 K.

The trajectory files were analyzed using g rms and g rmsf GROMACS utilities in order to obtain the root mean square deviation (RMSD) and root mean square fluctuation (RMSF) values comparing backbone deviations, flexibility and residual fluctuations between WT and MT, as this helps in understanding the stability of the proteins in simulated conditions. XMGrace package (http://plasmagate.weizmann.ac.il/Grace/) was used to plot, compare and analyze the trajectories generated for WT and MT.

3.2.4.1.4. Docking of wild type and mutant type of thymidine kinase with Acyclovir.

Acyclovir (PubChem: 2022) three dimensional coordinates were obtained from NCBI PubChem in SDF format and were submitted to OPENBABEL to obtain the structure in pdb format. The obtained 3D structure was further geometry minimized using ProDRG SERVER (http://davapc1.bioch.dundee.ac.uk/prodrg/) [293]. Acyclovir was docked to the modeled wild and mutant structures of thymidine kinase using Autodock 4.0. Kollman United atom charges and polar hydrogens were added to the receptor molecules (WT and MT) for optimization of the docking process. In this docking simulation, semiflexible docking protocols were used, in which the protein structures were kept rigid and the Acyclovir being docked was kept flexible in order to explore an arbitrary number of torsional degrees of freedom. The Lamarckian Genetic Algorithm (LGA) was used for docking experiments [294]. AutoDock is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. AutoDock actually consists of two main programs: AutoDock performs the docking of the ligand to a set of grids describing the target protein; AutoGrid pre-calculates these grids. Graphical User Interface program of "Auto Dock 4.0 Tools" was used to prepare, run, and analyze the docking simulations. The best docked complexes based on the lowest binding energy were further analyzed for hydrogen bonding interactions using pymol and LIGPLOT. The complex was also scored for theoretical inhibitory constant (Ki) using AutoDock 4.0.

The molecular dynamics and docking protocol adopted in this study was based on similar work carried out in our lab for mutation analysis of KatG for *Mycobacterium Tuberculosis* [295].

3.2.4.2. RESULTS:

3.2.4.2.1. Results of Analysis of thymidine kinase gene:

The two standard strains and twenty eight HSV1 and twenty two HSV 2 isolates were sequenced after the amplification of the whole gene of UL23. The sequence results were multaligned using the multalin software along with those sequences of those resistant isolate sequences and standard reference strain sequences of strain F (HSV1) and strain 333(HSV2) that have already been published on Genbank. From the analysis 5 of HSV1 isolates and 9 of HSV 2 isolates were found to have mutations among the 50 isolates used. Similar mutations were observed in four HSV1 isolates at positions Gln89Arg, Leu42Pro which were exactly matching with the resistant sequence from Genbank. Two of these isolates HSV1 isolates showed mutations at Ala17Val, Ala36Glu, Gly251Cys, Pro268Thr. Apart from these mutations observed, three other mutations were observed in four of our sequences at positions Cys6Gly, Val267Leu, Asp286Glu, Asn376His in two isolates. Two mutations(Glu36Ala and Val352Leu) are novel mutations which have not been reported by others.

Seven of HSV2 isolates had a similar mutation at position Asp266Asn, two isolates at Thr213Ala, two isolates at His212Arg one isolate at Ala215Ser, one isolate at Lys415Arg and one isolate at Pro417Gln and Lys496Glu, one isolate at Thr410Ala and two isolates at Pro449Arg, Arg453Trp and Gly460Ala and one isolate at Ala450Gly .

Two of the isolates of HSV 1 (922 and 1105) showed similar nucleotide changes at various positions of the gene consistent with the other genes.

Of these one sequence (922) was further analyzed using molecular modeling and docking studies.

3.2.4.2.2. Results of molecular modeling of thymidine kinase gene:

The alignment between the wild and mutant sequences using EMBOSS pair-wise alignment showed a sequence identity of 97% and 11 mismatches between similar residues (Fig 3.6).

Fig 3.6: Pair-wise sequence alignment between mutant and wild sequence.

```
wild                1 MASYPCHQHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLEQKMPTLL     50
                      ||||||.||||||||||.||||||||||||||||||.||||||.||||||||
mutant              1 MASYPGHQHASAFDQAVRSRGHSNRRTALRPRRQQAATEVRPEQKMPTLL     50

wild               51 RVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWQVLGASETIANI    100
                      ||||||||||||||||||||||||||||||||||||||:||||||||||||
mutant             51 RVYIDGPHGMGKTTTTQLLVALGSRDDIVYVPEPMTYWRVLGASETIANI    100

wild              101 YTTQHRLDQGEISAGDAAVVMTSAQITMGMPYAVTDAVLAPHIGGEAGSS    150
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
mutant            101 YTTQHRLDQGEISAGDAAVVMTSAQITMGMPYAVTDAVLAPHIGGEAGSS    150

wild              151 HAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPG    200
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
mutant            151 HAPPPALTLIFDRHPIAALLCYPAARYLMGSMTPQAVLAFVALIPPTLPG    200

wild              201 TNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQ    250
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
mutant            201 TNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQ    250

wild              251 GGGSWREDWGQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPELLAP    300
                      .||||||||||||||:.||||||||||||||||:|||||||||||||||
mutant            251 CGGSWREDWGQLSGTALTPQGAEPQSNAGPRPHIGETLFTLFRAPELLAP    300

wild              301 NGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMVQT    350
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
mutant            301 NGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMVQT    350

wild              351 HVTTPGSIPTICDLARTFAREMGEAN    376
                      |:|||||||||||||||||||||||:
mutant            351 HLTTPGSIPTICDLARTFAREMGEAH    376
```

3.2.4.2.3. Structure prediction

The wild and mutant models were generated using MODELLER, we checked the structural quality using PROCHECK. In Ramachandran plot, the stereochemical quality of the protein model can be judged by the use of Φ , Ψ scatter plots. Since our model has no residues in the generously allowed and disallowed regions, it satisfies criteria as a good model.

Fig 3.7: Ramachandran plot of modeled wild thymidine kinase obtained by PROCHECK.

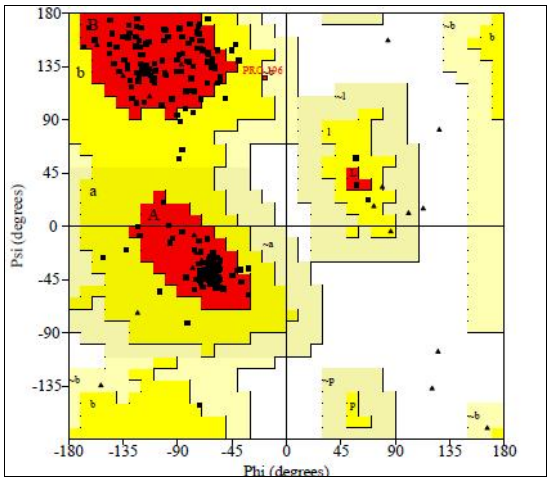
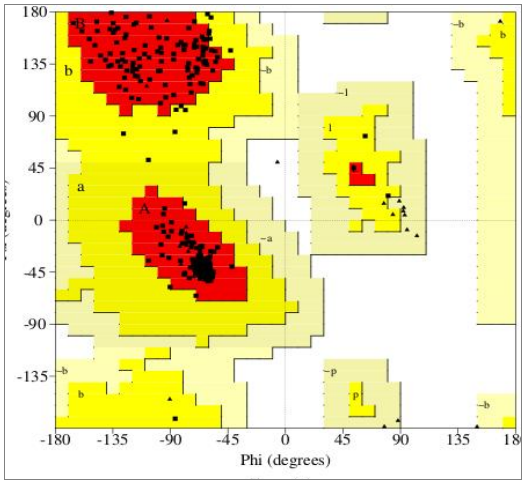


Fig 3.8: Ramachandran plot of modeled mutant thymidine kinase obtained by PROCHECK



3.2.5.3. Results of molecular dynamics simulation

The MD simulation results show that the combined mutations in mutant resulted in a significant change in acyclovir binding to the active site. RMSD and RMSF analyses were performed on WT and MT enzymes to study the protein stability and flexibility respectively. The RMSD comparison reveals the displacement of the MT trajectory with unstable higher deviations throughout the simulation process (1 ns), whilst WT exhibited a stable trajectory (Fig.3.9).

Comparison of RMSF trajectory reveals an overall increase of residual fluctuations in MT compared with WT that can be observed as peak deviations in (Fig. 3.10) .It was observed that some structural changes have taken place during the course of simulation of the mutant modelled structure (Fig 3.11). The structure with the lowest potential energy during the simulation was retrieved and used for further studies and analysis.

Fig 3.9: Time evolutions of backbone C-alpha root mean square deviation (RMSD)shown as a function of time of WT and MT structures at 300 K

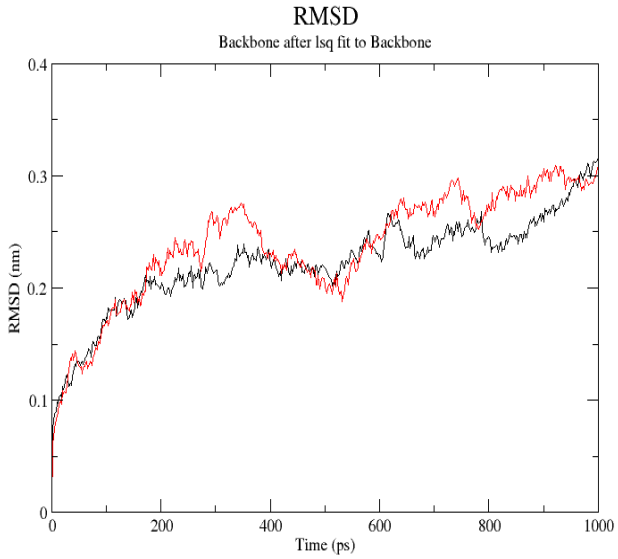


Fig 3.10: Root mean square fluctuation (RMSF) of the C-alpha backbone of individual residues over the entire simulation

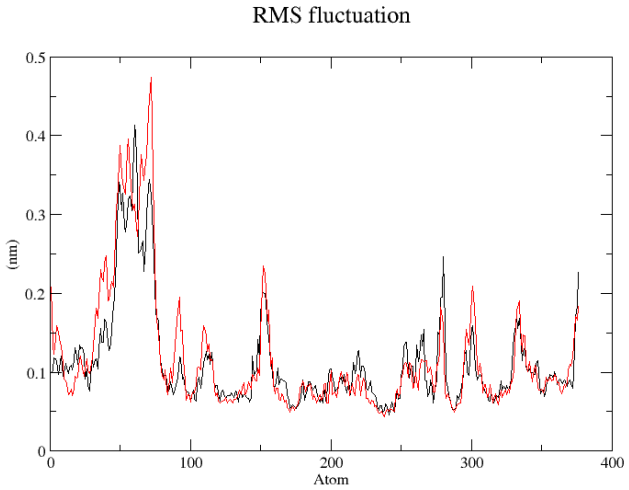
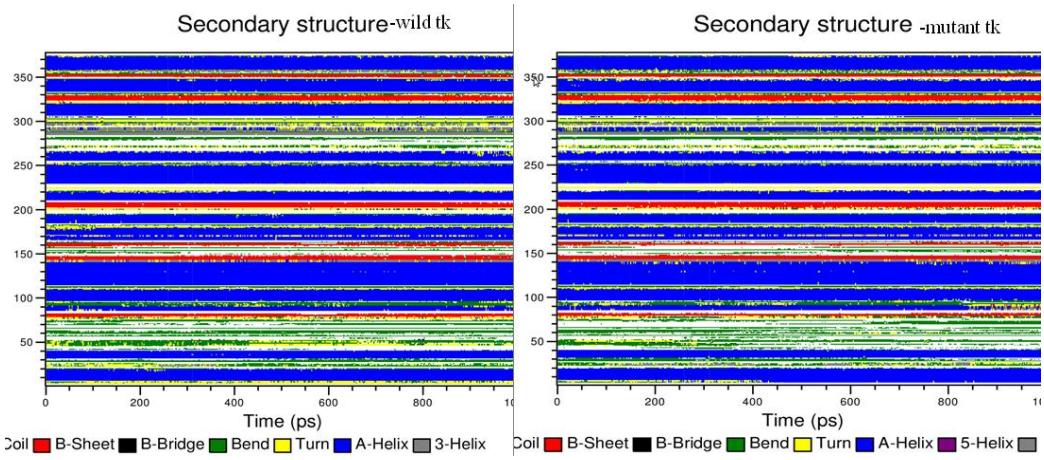


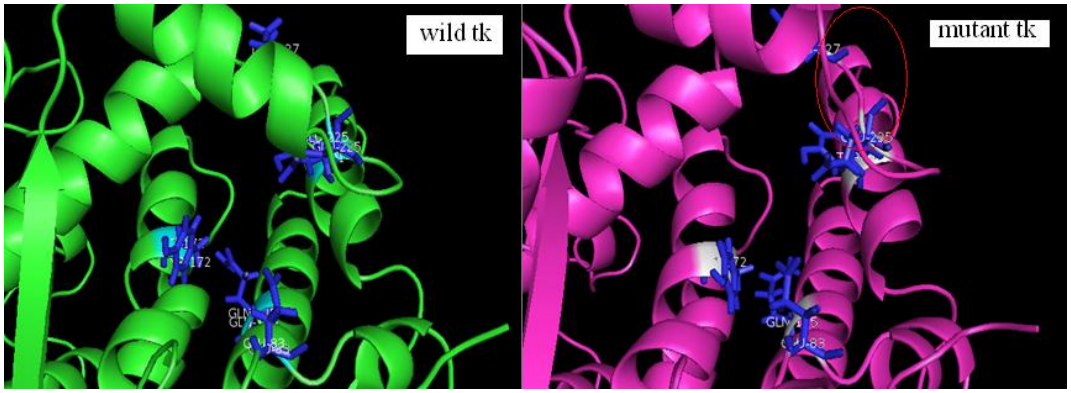
Fig 3.11: Secondary structural change during the course of simulation of wild and mutant thymidine kinase.



3.2.5.4. Analysis of the predicted wild and mutant thymidine kinase structure:

The structural comparison was done using Dalilite which showed a RMSD deviation of 3Å and a Z score of 40.9. The mutant structure is seen in a more open conformation compared to the wild structure and also there is a significant structural change in the binding site (Fig 3.12). It was further verified by calculating the solvent accessible surface area using GETAREA tool [296]. The wild and mutant structure has a assessable surface area of 21972.44 Å² and 22257.72 Å² resp. showing that mutant structure has more surface exposed to solvent or ligand molecules.

Fig 3.12: Structural differences in the binding site region of the mutant and wild structure, figure generated using pymol



3.2.5.5. Results of Docking

Docking studies with Acyclovir showed that the drug binds to both the WT and MT. Wild-type thymidine kinase – acyclovir complex and mutant thymidine kinase – acyclovir complex binding energies were found to be −5.04 kcal/mol and −4.58 kcal/mol, respectively (Table 3.7). The inhibitory constant (Ki) value, as calculated by AutoDock 4.0, showed a significant increase of (236.43 μm) in mutant tk complex compared with wild tk complex, indicative of relative drug resistance. Moreover, mutank tk- acyclovir complex (Fig. 3.13) showed a loss of hydrogen bonding and hydrophobic interactions in comparison with wild tk- acyclovir complex (Fig. 3.14), which indicates the decrease in binding affinity towards the drug. Normally, wild tk interacts with Acyclovir by hydrogen bonding with Tyr 172, Arg 176 and Gln 125 , as they are proven key active site residues involved in interactions with acyclovir from crystallized thymidine kinase co-crystallized with acyclovir(PDB ID: 2KI5), and the same was observed in the docking simulation, suggestive of predictive accuracy.

Table 3.7: Molecular interaction of Acyclovir with Herpes Simplex Virus – Strain F wild type thymidine kinase (WT) and mutant thymidine kinase (MT)

Receptor	Binding energy (kcal/mol)	Inhibition constant, Ki (μm)	Hydrogen interactions	Hydrophobic interactions
WT	-5.04	203.68	Glu83(3.01), Arg163(2.86), Glu228(3.21), Gln125(3.04), Arg176(3.13)	Trp88, Arg222, ile97, Met128, Tyr172, Met231, Ile100
MT	-4.58	440.11	Arg226(2.64),Tyr101(2.72), Glu225(2.91)	Ile97,Leu227, Tyr172, Leu217

Fig 3.13:a) Wild-type thymidine kinase. Acyclovir is shown as a stick model and coloured red, amino acid residues interacting with acyclovir are shown as lines and coloured by atom type (carbon is grey, oxygen is red, nitrogen is blue and hydrogen is white). Yellow dotted lines denote hydrogen bond. b) LIGPLOT showing the hydrophobic and hydrogen interaction.

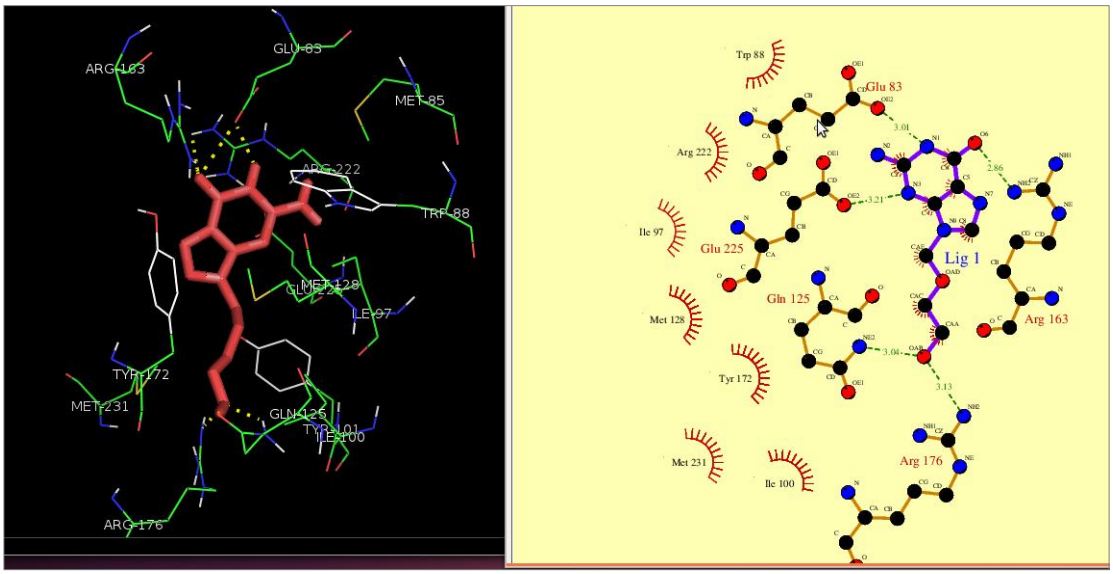
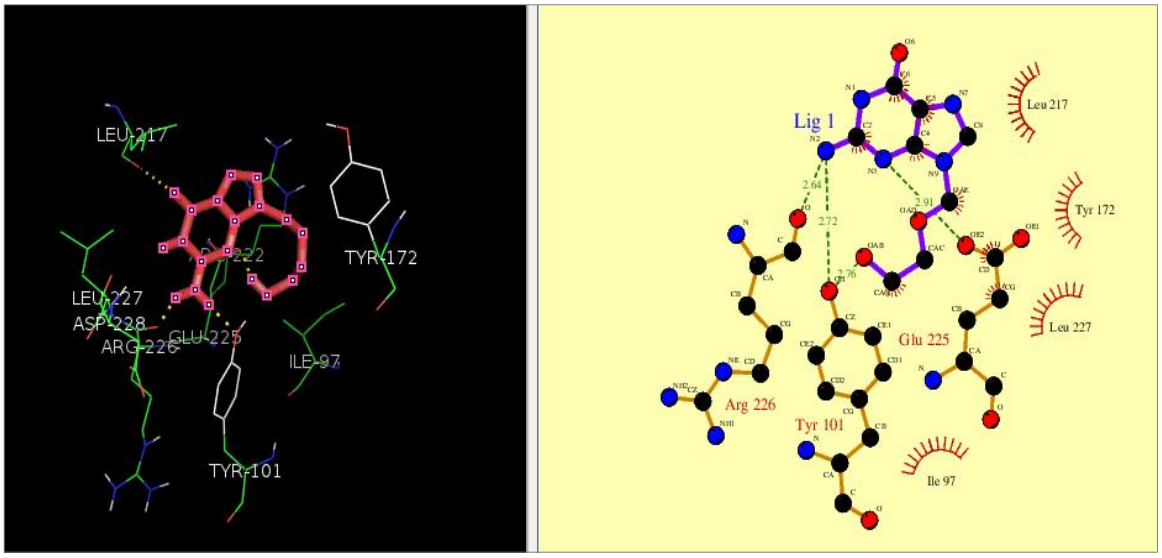


Fig 3.14:a) Mutant-type thymidine kinase. Acyclovir is shown as a stick model and coloured red, amino acid residues interacting with acyclovir are shown as lines and coloured by atom type (carbon is grey, oxygen is red, nitrogen is blue and hydrogen is white). Yellow dotted lines denote hydrogen bond. b) LIGPLOT showing the hydrophobic and hydrogen interaction.



3.3. Discussion:

Herpes infections are quite common among immunocompetent, neonates and immunocompromised patients. Drug resistance to acyclovir in HSV is becoming an important area of clinical importance. The prevalence of drug-resistant HSV in untreated individuals prior to the introduction of ACV was reported to be 0.3% of immunocompetent patients excreted ACV-resistant strains, in contrast to 3.6% of immunocompromised patients. The prevalence of resistance is highest (10.9%) in patients who have had a bone marrow transplant, particularly for those who have had allotransplants (18.4%)[297]. Recent surveys revealed that the prevalence of ACV-resistant HSV has remained low and virtually unchanged in the general population (0.1% to 0.7%) with no apparent differences between the ACV-treated and untreated groups[124]. Similar prevalence rates were reported for penciclovir and famciclovir (0.22%). The sporadic recovery of drug-resistant HSV from immunocompetent hosts only rarely correlates with clinical resistance.

Though phenotypic and genotypic characterization of acyclovir resistance have been described in detail in other parts of the world, in India this is the first study to have been performed on the phenotypic and genotypic characterization of acyclovir resistance in HSV based on thymidine kinase gene mutations. Abraham *et al*., has described the phenotypic ACV resistance among Indian strains of HSV using dye uptake method[298]. In this study we have tested the clinical isolates for ACV sensitivity and also characterized the UL23 gene for screening of mutations responsible for ACV resistance.

Molecular assays may allow a rapid and efficient identification of resistant viruses, as they facilitate detection of resistant virus directly in a clinical specimen. With the advances in molecular assays it is likely that genotypic tests

will achieve a more widespread diagnostic application. A detailed knowledge on the role of individual TK gene mutations in drug susceptibility is a prerequisite for diagnostic application of such tests. The relationship between a mutation discovered in the HSV TK gene and resistance to ACV is frequently difficult to establish because of the heterogeneity of mutations found in this gene. Some of these mutations are responsible for acquisition of resistance to ACV.

In our study mutations were observed based on sequencing of the entire TK gene. A total of 14 isolates of which 5(17.2%) were HSV1 isolates and 9(39.1%) HSV2 isolates were found to have mutations among the 52 isolates used. The sequence results were translated to its protein sequence using Transeq online software and multaligned using the multalin software along with those sequences of resistant isolate sequences(Genbank) and standard reference strain sequences of strain F (HSV-1) and strain 333(HSV-2) that have already been published on Genbank.

The protein sequences when multaligned with few other resistant sequences from the genbank along with the Strain F sequence showed 11 mutations overall. Nine mutations were identified to be those already reported polymorphisms occurring in the thymidine kinase gene. These are Cys6Gly, Ala17Val, Leu42Pro, Gln89Arg, Gly251Cys, Val267Leu, Pro268Thr, Asp286Glu, Asn376His. Two are novel mutations which have not been reported by others. These are Glu36Ala and Val352Leu.

Seven of HSV2 isolates had a similar mutation at position Asp266N, two isolates at Thr213Ala, two isolates at His212Arg, one isolate at Ala215Ser, one isolate at Lys415Arg and one isolate at Pro417Q and Lys496E, one isolate at Thr410Ala and two isolates at Pro449Arg, Arg453W and Gly460Ala and one

isolate at Ala450Gly. Two of the phenotypically resistant isolates of HSV-1 showed similar `nucleotide changes at various positions of the gene consistently. Both were ocular isolates.

The above mentioned mutations may be responsible for the phenotypic resistance exhibited by these isolates which is in complete concordance. Though mutations similar to that of those genbank sequences have been observed, mutations which have not been described by other workers have also been observed in our study. These mutations may also concordantly contribute to the resistance exhibited by these isolates.

Chapter 4

4.1. Hypothesis:

Phylogenetic analysis of the Herpes simplex virus 1 genome sequences targeting gG, gI, gC and thymidine kinase genes may also help in identifying new recombinants genotypes within our geographical location and to determine its relationship with regard to ACV resistant mutants.

4.2. Objectives:

- Genotyping of Herpes simplex virus 1 using PCR based RFLP method
  - To standardize and apply the Polymerase chain reaction for the amplification of gG gene of HSV 1 isolates
  - To standardize and apply the Polymerase chain reaction for the amplification of gI gene of HSV 1 isolates
  - To perform Restriction fragment length polymorphism analysis of gG using *PflMI* and *DdeI* and gI genes using *PleI* and *Sac1*
  - Analysis of the RFLP pattern to identify the genotypes

- Phylogenetic comparison of exonic US4 and US7 and UL44 regions of clinical Herpes simplex virus type 1 isolates for the establishment of association between the clinical specimen and its genotype
  - To standardize and apply the Polymerase chain reaction for the amplification of US4 gene of Herpes simplex virus 1 isolates
  - To standardize and apply the Polymerase chain reaction for the amplification of US7 gene of Herpes simplex virus 1 isolates

- ❖ To standardize and apply the Polymerase chain reaction for the amplification of UL44 gene of Herpes simplex virus 1 isolates
- ❖ To perform DNA Sequencing of the amplified products of US4, US7 and UL44
- ❖ To perfrom Phylogenetic analysis of the PCR based DNA sequences of US4, US7, UL44 and UL23 (thymidine kinase).

4.3. Methodology:

A total of 25 isolates from variegated clinical specimens, 15 ocular, 3 genital, 3 oral, 2 skin and 2 throat swab specimen isolates, were used for this purpose. The laboratory standard strain employed was HSV1-ATCC 733VR. Apart from these, seventeen strains each from Genbank for gG [Genbank: AY240815.1, AJ626513.1, AJ626519.1, AJ626525.1, AJ626520.1, AJ626500.1, AJ626507.1, AY240813.1, AY240803.1, AY240755.1, AY240729.1, AY240650.1, AY240818.1, AY240810.1, AY240804.1, AY240741.1, AY240738.1] and gI [Genbank: AJ626534.1, AJ626538.1, AJ626531.1, AJ626552.1, AJ626548.1, AJ626539.1, AJ626553.1, AJ626549.1, GU734771.1, AJ626555.1, AJ626537.1, AJ626550.1, AJ626540.1, AJ626556.1, AJ626541.1, AJ626536.1, AJ626535.1] genes were used as a part of the analyses. The isolates were grown on Vero cell line with Dulbecco's Modified Eagles Medium supplemented with 1% fetal bovine serum and antibiotics.

4.3.1. DNA Extraction:

The DNA was extracted using the QiAamp DNA mini kit (QIAGEN, Germany). The protocol followed was according to the manufacturer's manual (Appendix 2)

4.3.2. PCR Based RFLP for Genotyping HSV-1:

PCR was done targeting single nucleotide polymorphism within genes coding for glycoprotein G and glycoprotein I[32] .The primers used have been tabulated in Table 4.1.

Table 4.1: Primer sequences used for the amplification of gG and gI genes

GENE	PRIMER ORIENTATION	PRIMER SEQUENCE
gG	Forward	5' GACTCTCCCACCGCCATCAG 3'
	Reverse	5' TGTCTTCGGGCGACTGGTCT 3'
gI	Forward	5'CCTGCTTATTCTCGGGGAGCTTC 3'
	Reverse	5' AGCAGTTTCGGGTCGCAGGA 3'

4.3.2.1. Cocktail Preparation

The PCR was performed in a 50μL reaction volume containing 1X PCR buffer (10mM Tris with 15mM MgCl), 200mm of each dNTPs, 2.5 units of *Taq* DNA polymerase, 1mM each primer .

4.3.2.2. Thermal Profile:

This HSV glycoprotein specific PCR was carried out using a common profile for both the glycoprotein genes. The Thermal profile is described in Table 4.2:

Table 4.2: Thermal Profile for the amplification of gG and gI genes:

Temperature	Time	Cycles
Denaturation **96** ºC	**45 secs**	**40 cycles**
Annealing & **58** ºC	**45 secs**	
Extension **72** ºC	**45 secs**	

4.3.2.3. Sensitivity of the primers:

- DNA was extracted from standard strain of hsv-1 and quantified by UV – Visible spectrophotometer
- Amount of DNA present was 1.0394µg/µl, there was no protein contamination.
- Serial dilutions of the quantified DNA were made from 10^{-1} to 10^{-10} for gG and gI.
- 5µl were taken from each dilution for reaction.

4.3.2.4. Specificity of the Primers:

PCR reaction was set with extracted DNA of HSV-2, Adeno Virus, Cytomegalo virus, Varicella Zoster Virus, *Toxoplasma gondi*i, human leukocyte, *Candida albicans*, *Aspergillus flavus*, *Propionibacterium acnes* and also using Eubacterial DNA.

4.3.2.5. Restriction Fragment Length Polymorphism:

- The **269 bp** product of PCR amplification of gG were subjected to cleavage by restriction enzymes ***PflMI*** **and** ***DdeI.*** Table 4.3. describes the restriction sites of the enzymes;

Table: 4.3: Restriction sites of the enzymes *PflMI* and *DdeI*

ENZYME	GENOTYPE	RESTRICTION SITE
PflMI	**A**	**n.|CCAAGTATCGG|.n**
	B	**n.|CCAAGTATCGG|.n**
	C	**n.|CCAAGTATTGG|.n**[#]
DdeI	**A**	**n.|CCGGG|.n**
	B	**n.|CTGAG|.n**[#]
	C	**n.|CTGAG|.n**[#]

- site recognized by the enzyme.

4.3.2.5.1. Protocol for RFLP:

PflMI:

31µl reaction consisting of

Milli Q water	-18 µl
Buffer	- 2 µl
Restriction enzyme (1U)	- 1 µl
PCR amplified products	-10 µl

DdeI:

25µl reaction consisting of

Milli Q water	-12 µl
Buffer	- 2 µl
Restriction enzyme (1U)	- 1 µl
PCR amplified products	-10 µl

- The **410 bp** product of PCR amplification of gI were subjected to cleavage by restriction enzymes ***PleI* and *Sac1*** . Table 4.4. describes the restriction sites of tf the enzymes;

Table 4.4: Restriction sites of the enzymes *PleI* and *Sac1:*

ENZYME	GENOTYPE	RESTRICTION SITE
SacI	**A**	**n.| GAGCTG |.n**
	B	**n.| GAGCTG |.n**
	C	**n.| GAGCTC |.n***
PleI	**A**	**n.| TACTC |.n**
	B	**n.| GACTC |.n***
	C	**n.| GACTC |.n***

* site recognized by the enzyme.

PleI

29µl reaction consisting of

Milli Q water -16 µl

Buffer - 2 µl

Restriction enzyme (1U) - 1 µl

PCR amplified products -10 µl

SacI

25µl reaction consisting of

Milli Q water -12 µl

Buffer - 2 µl

Restriction enzyme (1U) - 1 µl

PCR amplified products -10 µl

4.3.2.5.2. Reaction Procedure for RFLP:

- The restriction enzyme digestion was done at 37˚C for 3 hours in PCR thermal cycler (Eppendorf).
- Then immediately snap frozen.

- The product of PCR-RFLP of gG will have been obtained as follows
  - Genotype A- amplicon not cleaved.
  - Genotype B- 2 fragments -97 and 172
  - Genotype C- 3 fragments-57, 97 and 115.

- The product of PCR-RFLP of gI gene will be obtained as follows
  - Genotype A- amplicon not cleaved.
  - Genotype B- 2 fragments -55 and 355
  - Genotype C- 3 fragments- 55, 133 and 222.

4.3.2.6. Visualization of RFLP products:

The RFLP products were subjected to electrophoresis on 4% agarose gel incorporated with 0.5 µg/ml ethidium bromide (Appendix 2). The entire product was mixed with 10µl of loading dye. For visualization by UV transilluminator (Vilber Lourmet, France) was used.

4.3.3. Amplification of gG, gI genes for DNA Sequencing:

The PCR based DNA amplification was done using three overlapping sets of primers covering the entire coding region of the glycoprotein G1[299,300].

4.3.3.1. PCR Reaction Mixture for gG, gI and gC:

a.	Dinitro triphosphates	8 µl
b.	10 X assay buffer	5 µl
c.	Forward Primer (1 in 25 dilution)	1 µl
d.	Reverse Primer (1 in 25 dilution)	1 µl
e.	*Taq* DNA polymerase enzyme	0.3 µl
f.	Milli Q water	30 µl

The primer sequences for gG are listed in the Table 4.5

Table 4.5: Primer sequences used for the amplification of gG

A:	**5' TGTTTCAACAGAAATGACCGCCC 3'**
B:	**5' CTCAAGATGTTCGCCGTCCC 3'**
C:	**5' ACGCCCGACCACACACCC 3'**
D:	**5' TATGTTGAGGCGTCGGAACC 3'**
E:	**5' AGTCGCCCGAAGACACCC 3'**
F:	**5' CCGCATGTGGGCTCTCCC 3'**

Table 4.6: Primer sequences used for the amplification of gI

S	**5' CTGTTCCGCCTGTCGCACCC3'**
AS*	**5'CGTAGTAGGTGGTGTGGGGGACC3'**
AS	**5'TTTGGCGCGTCACCGACCC3'**
S	**5'CGACCGACAGCACTCACAGCCC3'**
AS*	**5'GGGGCGGGTTGGTATACGCTC3'**
AS	**5'TCTGGGTCACCGTTAGCGCG3'**
S	**5'CGGCCTATGGCTCCTGCGACC3'**
AS	**5'ATGGGGGTTCGTGGTTGACCC3'**
S	**5'CCAAGCATCGACCACGCCCTTC3'**
AS	**5'CATTGGCGTGCGTGACGACC3'**
S	**5'TCAAATCGCATCCGAGCACCC3'**
AS	**5'GCCAGTGGCCGTGGACCTATACC3'**
AS*	**5'CGGCTTCCTGAACTCCTCCCATC3'**

*Primers used for DNA Sequencing only

Primers for gC gene:

Primers were designed using Primer 3 software to split the exonic gC gene into 5 overlapping sub regions labeled, for convenience, from C1 to C5. The primers are listed in the table 4.7.

Table 4.7: Primer sequences used for the amplification of gC

GENE SUB REGION	PRIMER ORIENTATION	PRIMER SEQUENCE (5' – 3')
gC1	Forward	CGTGTGGAGGTCGTTTTTCAGT
	Reverse	GTGGTGTTGTTCTTGGGTTTGG
C2	Forward	AAACCCCAACAATGTCACACAAAAC
	Reverse	CCAAGTAATACATTCCCTGGGTCG
gC3	Forward	GACCCAGGGAATGTATTACT
	Reverse	GTCCTCGAACCAGACAAACT
gC4	Forward	AGTTTGTCTGGTTCGAGGAC
	Reverse	GTCATCGGCAGGTGAAGGTC
gC5	Forward	ACCATCACCATGGAATTTGG
	Reverse	ATGACCTGAGGGGAGAGAGG

4.3.3.2. Thermal Profile:

Table 4.8. Thermal Profile For the amplification of gG gene:

Temperature	Time	Cycles
Initial denaturation **94** ºC	**2 mins 30 secs**	**1 cycle**
Denaturation **94** ºC	**1 min**	**35 cycles**
Annealing & Extension **70** ºC	**45 seconds**	

Table 4.9. Thermal Profile For the amplification of gI gene:

Temperature	Time	Cycles
Initial denaturation 95 ºC	**5 mins**	**1 cycle**
Denaturation 95 ºC	**45 secs**	**40 cycles**
Annealing 58 ºC	**45 secs**	
Extension 72 ºC	**45 secs**	
Final Extension 72 ºC	**5 mins**	**1 cycle**

Table 4.10. Thermal Profile For the amplification of gC gene:

Temperature	Time	Cycles
Denaturation **95** ºC	**30 secs**	**40 cycles**
Annealing **56** ºC (C1, C3-C5) **62** ºC (C2)	**60 secs**	
Extension **72** ºC	**60 secs**	

4.3.3.3. DNA Sequencing:

The amplicons were gel extracted using Qiagen Gel elution kit and used for sequencing.

4.3.3.3.1. Cycle Sequencing Reaction Protocol (Appendix 3):

- Ready reaction mix (RR mix) - 1 µl
- 5x sequencing buffer - 3 µl
- Forward primer (1pmol/µl) - 2 µl
- Milli Q water - 2 µl
- Amplified PCR product (without primer band) - 3 µl

Table 4.11. Thermal Profile for Cycle Sequencing:

Temperature	Time	Cycles
Initial denaturation **96** ºC	**1 min**	**1 cycle**
Denaturation **96** ºC	**10 Secs**	**25 cycles**
Annealing & **50** ºC	**5 secs**	
Extension **50** ºC	**4 mins**	

4.3.3.3.3. Purification of Extension Product and Sequencing:

- Same as described earlier**

4.3.3.3.4. PCR amplification and DNA sequencing of thymidine kinase gene:

- Already described in Chapter 3**

4.3.4. Construction of Phylogeny and Analyses of Tandem repeats:

Contig assembly was performed using DNA baser software. Multiple sequence alignment was done using ClustalW2 and Multalin Interface softwares. [301] Unrooted phylogenetic trees were constructed with maximum likelihood tool with bootstrap validation (500 replicates) using PHYLIP software. [302] Tandem repeats were detected using Etandem tool of Emboss package.

4.4. RESULTS:

4.4.1. Genotyping PCR For HSV-1 Typed Isolates And Specimen:

Genotyping PCR for HSV-1 was performed targeting the genes coding for glycoproteins G & I.

4.4.1.1. Sensitivity of the PCR:

GLYCOPROTEIN G:

The PCR was found to be sensitive till a DNA concentration of 0.5187μg (neat).

GLYCOPROTEIN I:

The PCR was found to be sensitive till a DNA concentration of 51.87pg (10^{-4}).

4.4.1.2. Specificity of the PCR:

GLYCOPROTEIN G:

The PCR was found to be highly specific to HSV-1.

GLYCOPROTEIN I:

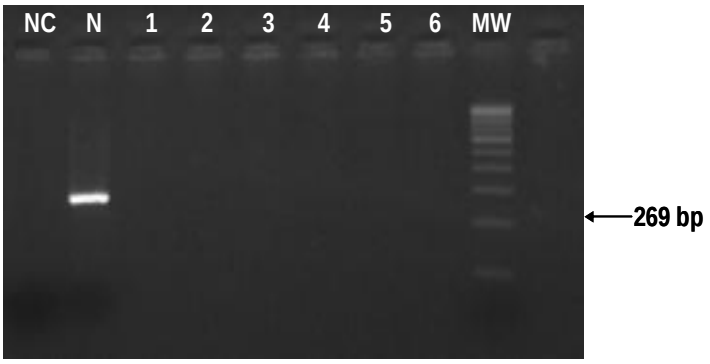
The PCR was found to be highly specific to HSV-1.

Fig. 4.1 gives electrophoretogram of Sensitivity of gG and gI PCR.

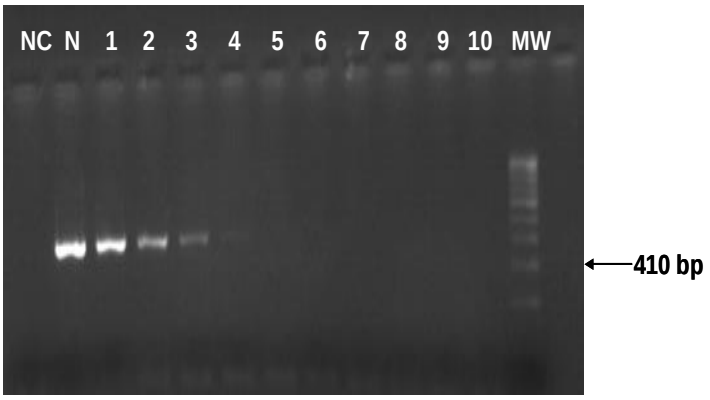
Fig. 4.2 gives electrophoretogram of specificity of gG and gI PCR.

The isolates and specimen typed HSV-1 were subjected to PCR for gG and gI. All of them showed positivity for both the sets of primers. Fig. 4.3 gives electrophoretogram of PCR on clinical isolates targeting gG and gI.

Fig 4.1: Electrophoretogram of Sensitivity of gG and gI PCR

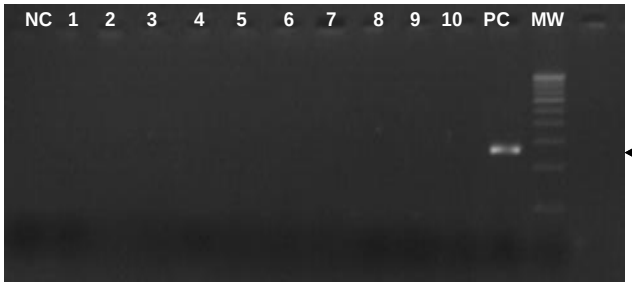


Glycoprotein G
NC: Neg control
Lane N : Neat DN
Lanes 1-6: 10 fold seri
dilutions of DNA of HSV-1
Lanes N: Positive
Lanes 1 – 6: Negativ
MW – *Phi X Hinf digest*



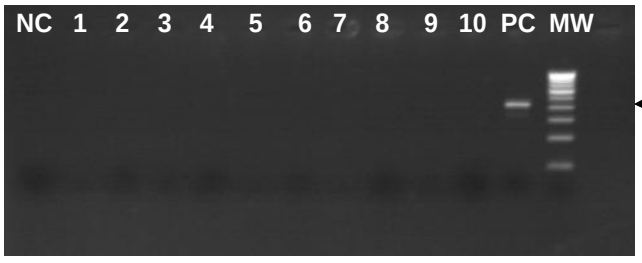
Glycoprotein I
NC: Neg control
Lane N : Neat DN
Lanes 1-10 : 10 fold ser
dilutions of DNA of HSV-
Lanes N – 4 : Positiv
Lanes 5 – 10 : Negativ
MW – *Phi X Hinf digest*

Fig. 4.2 Electrophoretogram of specificity of gG and gI PCR.



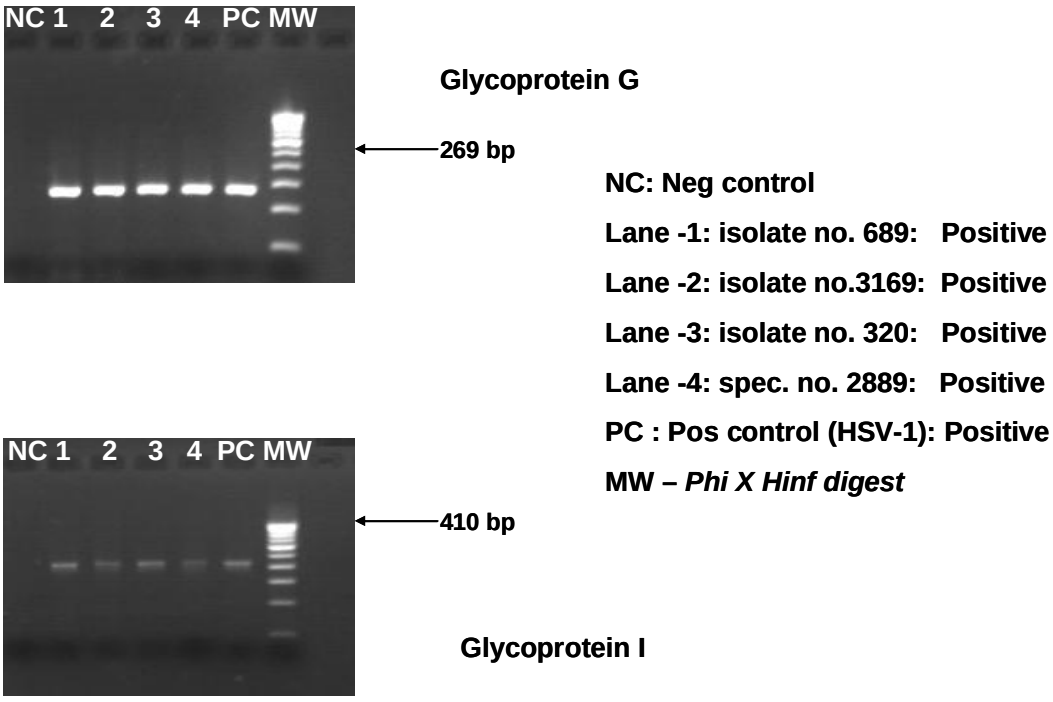
Glycoprotein G

NC: Neg control
Lanes 1-4: Viral DNA: Neg
Lane 5: Human DNA: Neg
Lane 6-7 : Bacterial DNA: Neg
Lane 8-9: Fungal DNA: Neg
LANE 10 : Parasitic DNA: Neg
PC: HSV-1 DNA: Pos
MW – *Phi X Hinf digest*



Glycoprotein I

Fig. 4.3: Electrophoretogram of PCR on clinical isolates targeting gG and gI.



All the PCR amplified products targeting gG and gI of HSV-1, were subjected to enzymatic digestion by restriction enzymes *PflMI* and *DdeI, SacI and PleI* respectively.

4.4.2.1. RFLP Analysis of gG with *PflMI* Enzyme:

Standard HSV-1 strain (ATCC 733 –VR) did not show any digestion with the enzyme. None of the 25 HSV-1 isolates showed any digestion with the enzyme.

4.4.2.2. RFLP Analysis of gG with *DdeI* Enzyme:

Standard HSV-1 strain (ATCC 733 –VR) did not show any digestion with the enzyme. None of the 25 HSV-1 isolates or the HSV-1 specimen showed any digestion with the enzyme. RFLP analyses of gG of standard strain, isolates and specimen showed that they all belonged to genotype –A.

4.4.2.3. RFLP Analysis of gI with *SacI* Enzyme:

Standard HSV-1 strain (ATCC 733 –VR) did not show any digestion with the enzyme. None of the 25 HSV-1 isolates or the HSV-1 specimen showed any digestion with the enzyme.

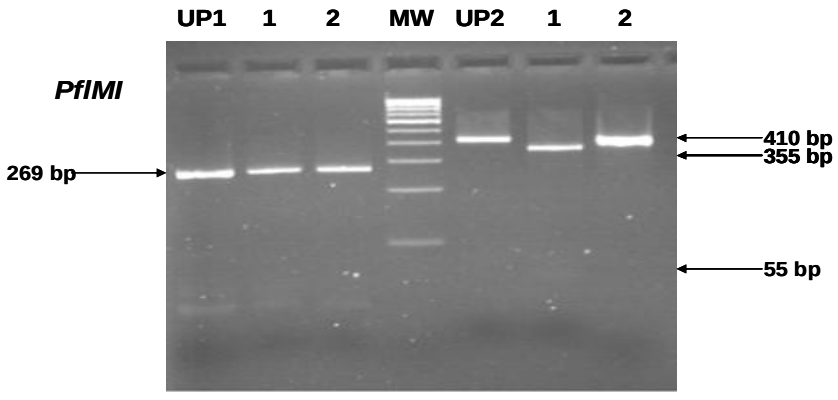
4.4.2.4. RFLP Analysis of gI with *PleI* Enzyme:

Standard HSV-1 strain (ATCC 733 –VR) did not show any digestion with the enzyme. Of the 25 isolates, 2 isolates showed complete digestion with 341bp and 69bp, 339 and 71 bp products respectively. HSV-1 specimen did not show any digestion.

RFLP analyses of gI of standard strain, isolates and specimen showed that standard strain, specimen and 22 isolates belonged to genotype A and 2 belonged to genotype B.

The two isolates found genotype B by RFLP analysis of gI and genotype A by RFLP analysis of gG were found to be recombinants.

Fig. 4.4. Electrophoretogram of RFLP analysis on clinical isolates.



UP1- Undigested product of gG
UP2- Undigested product of gI
Lane -1: isolate no. 342
Lane-2 : specimen no. 2889
MW – *Phi X Hinf digest*

4.4.3. Results of application of PCR for the amplification of gG, gI and gC for DNA Sequencing:

4.4.3.1. Analysis of gG:

The complete coding region of 717 bp was sequenced for 25 isolates and laboratory standard strain ATCC 733VR.

Alignment of the sequences showed the presence of various synonymous and non synonymous substitutions apart from addition of a codon (GGA) in five isolates and a codon deletion (AAC) in one ocular isolate.

Phylogenetic analyses were carried out along with 17 strains [Genbank: AY240815.1, AJ626513.1, AJ626519.1, AJ626525.1, AJ626520.1, AJ626500.1, AJ626507.1, AY240813.1, AY240803.1, AY240755.1, AY240729.1, AY240650.1, AY240818.1, AY240810.1, AY240804.1, AY240741.1, AY240738.1] classified as genotype A, from Genbank, using maximum likelihood method. The isolates clustered in 2 groups. The groups were arbitrarily labeled sub genotypes A1 and A2 respectively.

Seven out of 25 isolates and 6 out of 17 Genbank strains possessed a synonymous nucleotide transversion in the 613 and a non-synonymous transition in the 648 positions. Five of the 25 isolates and all six Genbank isolates had a codon addition (GGA) after 245th position, and a tandem repeat of 3 nucleotides-GGA repeated nine times, from position 234 to 262. These isolates along with 3 isolates from Genbank clustered together and fell into the A2 sub genotype category with moderate bootstrap values.

Ten isolates (variegated specimen type) and the laboratory standard strain had nucleotide substitutions in the 270, 401 and 655 positions, which were

exclusively seen among these isolates. None of these isolates possessed tandem repeats and branched out together as genotype A1.

The sub genotype classification was independent of the characteristic of the isolates. The other eight isolates did not fall into either category.

4.4.3.2. Analysis of gI:

The complete coding region of gI gene was sequenced for the 25 isolates and results were analysed.

Eight isolates (7/8 being ocular in origin) were intragenic recombinants, getting classified as type B, in this region. Phylogenetic analyses carried out along with 17 Genbank strains[Genbank: AJ626534.1, AJ626538.1, AJ626531.1, AJ626552.1, AJ626548.1, AJ626539.1, AJ626553.1, AJ626549.1, GU734771.1, AJ626555.1, AJ626537.1, AJ626550.1, AJ626540.1, AJ626556.1, AJ626541.1, AJ626536.1, AJ626535.1] using PHYLIP (Maximum likelihood method) revealed the separation of the intragenic recombinants into two separate clades. A third, larger clade contained a bifurcating smaller clade which divided the type A isolates of Genbank and laboratory isolates from the type B Genbank strains and four laboratory isolates with a similar variation pattern. The recombinants and the type A isolates did not conform to the sub genotype separation seen in gG gene and no distinct pattern of clustering was found among them, proving the sub genotypes were exclusively seen in gG gene.

The topological distributions of isolates 922 and 1105 (both ocular origin) gG and gI were similar.

4.4.3.3. Analysis of gC:

Sequence analysis was carried out for all the isolates along with 7 Genbank strains [Genbank: AJ421502.1, AJ421493.1, AJ421492.1, AJ421489.1, AJ421506.1, FJ593289.1 and AJ421494.1]. Phylogenetic tree was constructed using Maximum likelihood method by Phylip. Molecular phylogeny of exonic gC nucleotide sequences threw up existence of only 2 distinct genogroups, though previously 3 separate genogroups were deduced in this region.[20] The genotypes were arbitrarily labeled α and β, in this region. The nucleotide variations seen in positions – 381 C->G,669 G->A,724 AND 725 TA->AT among type β isolates and 781 G->T among type α isolates could be attributed to this cladic separation. Several synonymous and non synonymous substitutions were encountered, but not affecting the formation of α and β genotypes. Addition of a codon was encountered in 2 corneal isolates (416-ACG and AEH06-ACC) after position 1235. However, the evolution of the isolates with respect to gC genotypic classification was also without any predilection to the anatomical site of primary infection.

The ocular isolates 922 and 1105, though similar in the US region, were different genotypes in their UL region.

4.4.3.4. Analysis of thymidine kinase gene:

Sequence analysis was carried out for all the isolates along with strain F sequence from Genbank strains. Phylogenetic tree was constructed using Maximum likelihood method by Phylip. Molecular phylogeny of thymidine kinase gene nucleotide sequences threw up existence of only 2 distinct geno

groups. Several synonymous and non synonymous substitutions were encountered, but not affecting the formation of A and B genotypes. However, the evolution of the isolates with respect to thymidine kinase gene genotypic classification was also without any predilection to mutant strains and anatomical site of infection.

The topological distributions of isolates 922 and 1105 (both ocular origin) gG, gI and UL23 were similar but different in gC.

4.5. DISCUSSION

Genotyping is done to detect the frequency of a genotype in a population, to determine the extent of risk of reactivation/ re-infection occurring and the genotype responsible for it. It is useful in patient monitoring. Drug resistance of a genotype and identification of mutants[32]. Quantitative analysis of genomic polymorphism of HSV and its molecular evolution was determined using restriction enzymes[29].

Genotyping of HSV is more significant due to the fact that number of copies of virions present in the nerve ganglion affects the rate and extent of reactivation and the number of copies of virions varies according to the type of strain of virus causing primary infection[303,304].

Norberg *et al* determined the sequence diversity of the complete genes coding for glycoproteins G (gG), I (gI), and E (gE), comprising 2.3% of the HSV-1 genome and located within the unique short (US) region, for 28 clinical HSV-1 isolates inducing oral lesions, genital lesions, or encephalitis. Laboratory strains F and KOS321 were sequenced in parallel. Phylogenetic analysis, including analysis of laboratory strain 17, revealed that the sequences were separated into three

genetic groups. The identification of different genogroups facilitated the detection of recombinant viruses by using specific nucleotide substitutions as recombination markers[33].

Seven of the isolates and strain 17 displayed sequences consistent with intergenic recombination, and at least four isolates were intragenic recombinants. The observed frequency of recombination based on an analysis of a short stretch of the US region suggests that most full-length HSV-1 genomes consist of a mosaic of segments from different genetic groups. Polymorphic tandem repeat regions, consisting of two to eight blocks of 21 nucleotides in the gI gene and seven to eight repeats of 3 nucleotides in the gG gene, were also detected. Laboratory strain KOS321 displayed a frameshift mutation in the gI gene with a subsequent alteration of the deduced intracellular portion of the protein[33] .

Norberg, *et al*., further classified HSV-1 into three genotypes using RFLP, which clearly separated in the phylogenetic trees, described for clinical HSV-1 isolates. The genotypes were arbitrarily designated A, B, and C, and the classification was based on DNA sequencing of the US4, US7and US8 genes coding for the glycoproteins G (gG), I (gI), and E (gE), all localized in the unique short region of the genome. It was determined that RFLP is rapid and accurate method for genotyping of clinical HSV-1 isolates[32].

The results of PCR-RFLP Analysis showed that these isolates and standard strains belonged to genotype-A, and 2 of the isolates were found to be recombinants confirmed by DNA sequencing. One of the isolates marked genotype A (no restriction site) by RFLP of gG and gI was found to have a restriction site for *DdeI* at position 205 of gG by DNA sequencing.

The standard strain and other isolates were typed as genotype A and recombinants beyond any doubt. The significance of the mutations –additions seen at positions 377-379 of gI needs to be studied further to recognize possible associations between genotypes identity and functions of gG and gI proteins.

Quantitative evaluation of the genomic polymorphisms of HSV 1 strains from six countries – 3 Asian and 3 non Asian origin (Japan, Korea, China, Sweden, U.S.A. and Kenya) using restriction endonucleases, based on presence /absence of restriction sites concluded that the evolutionary pattern was similar among same ethnic groups and the variability encountered among the Asian strains was lesser compared to the Non-Asian ones and the overall mutation rate at $3.5*10^{-8}$/site/year was slow and host dependent.[305]

The purpose of this study was to detect if novel variants could preferentially infect tissues.

Phylogenetic analysis of 28 European clinical HSV-1 non ocular isolates in their glycoproteins G (gG), I (gI) and E (gE), comprising 2.3% of the HSV-1 genome and located within the unique short (US) region including laboratory strain 17, revealed that the sequences were separated into three genetic groups A,B and C. [33]

Nucleotide sequences of isolates in the genes G and I conformed to the European classification. Genotype A was the sole one encountered in all the 25 isolates, which included a majority of ocular isolates, in their gG gene; however some strains were genotype B in the gI region leading to them being labeled arbitrarily as recombinants.

Tandem repeats of GAA from nucleotides 234-262 were encountered in 7 isolates of gG and branched out as a separate clade, and hence assigned as a sub

genotype of A (A2) and the 10 other isolates possessed unique nucleotide variations seen exclusively in our isolates and classed as sub genotype A1.

A detailed analysis, by Norberg et al, of human corneal isolates pointed to the predominance of genotype B, in the European population. [306] However, all our strains were either Genotype A or intergenic recombinants, though novel sub genotypes exist in gG gene.

Phylogeny of gC threw up novel genotypes arbitrarily designated as α and β. Substitutions seen in positions 381 C->G,669 G->A,724 AND 725 TA->AT among type β isolates and 781 G->T could possibly have led to such a demarcation. The gC region is involved in the initial attachment of virus to the host cell, and since different host cells have varying amounts of heparan sulphate on their cell surface, it is possible that a genotypic variation could be anatomic site specific, which was not encountered, making the affinity purely quantitative.

The analysis of thymidine kinase gene has revealed that the isolates have clustered into two major groups. The clusters reveal the similar pattern as that with gC and gG except for a few isolates which have been described to be intragenic recombinants in gI analysis. Since it has been reported that in literature that thymidine kinase gene has large sequences which is prone to be polymorphism it may also be used as a region for phylogenetic analysis.

4.6. CONCLUSION:

On analyzing all four genes, most isolates have been found to be genotype A and only two isolates have been found to be genotype B based on analysis of gC and intragenic recombinants have been identified which are type A in gG and type B in gI and UL23. It is to be noted that neither the genotypic nor the sub genotypic classification was specific to a particular clinical specimen. Therefore, it is concluded that there is no significant association between novel genotypes and clinical entities and the existence of sub genotypes within the elucidated domain, again independent of clinical specimens is sans doubts.

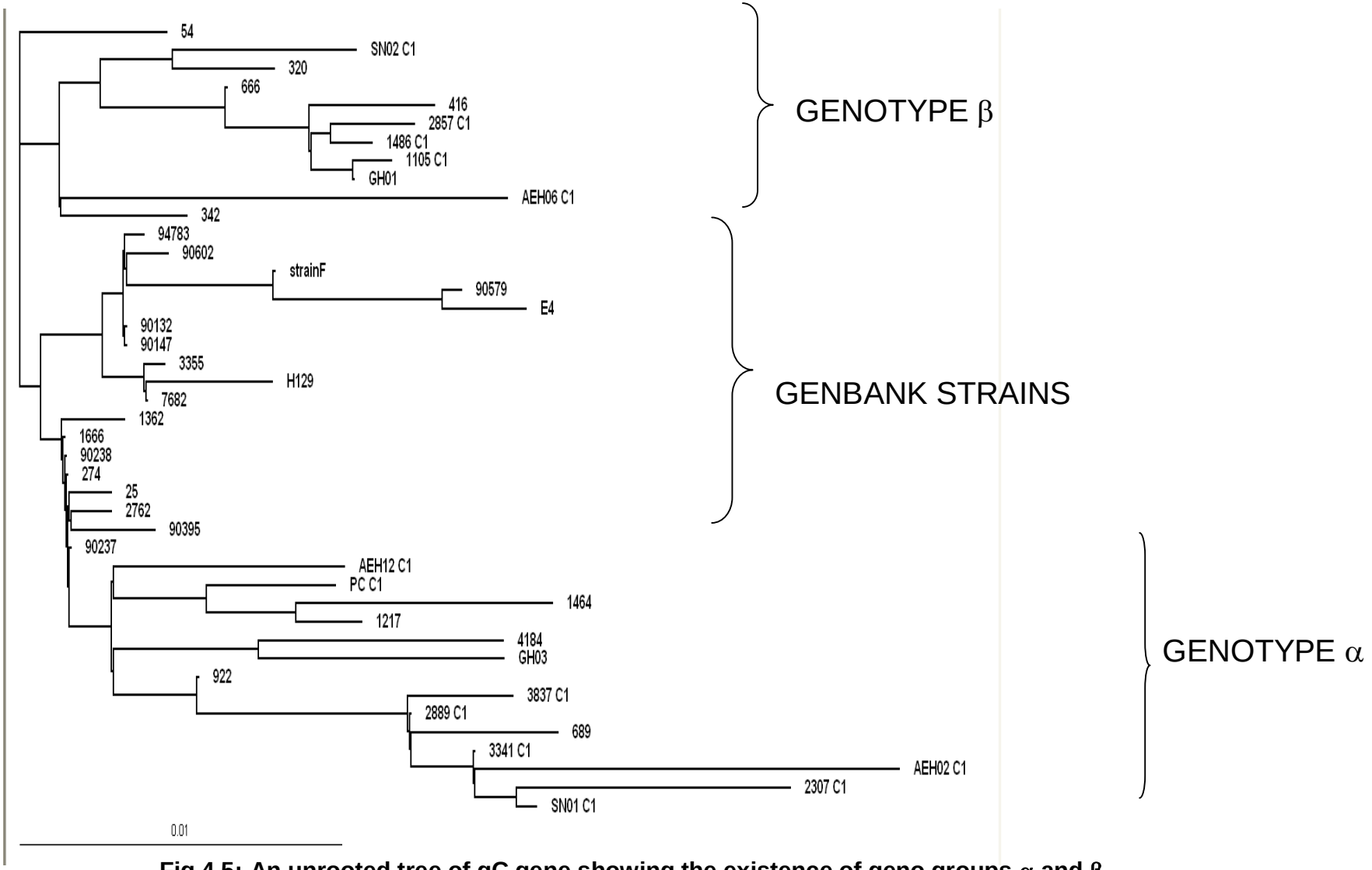


Fig 4.5: An unrooted tree of gC gene showing the existence of geno groups α and β.

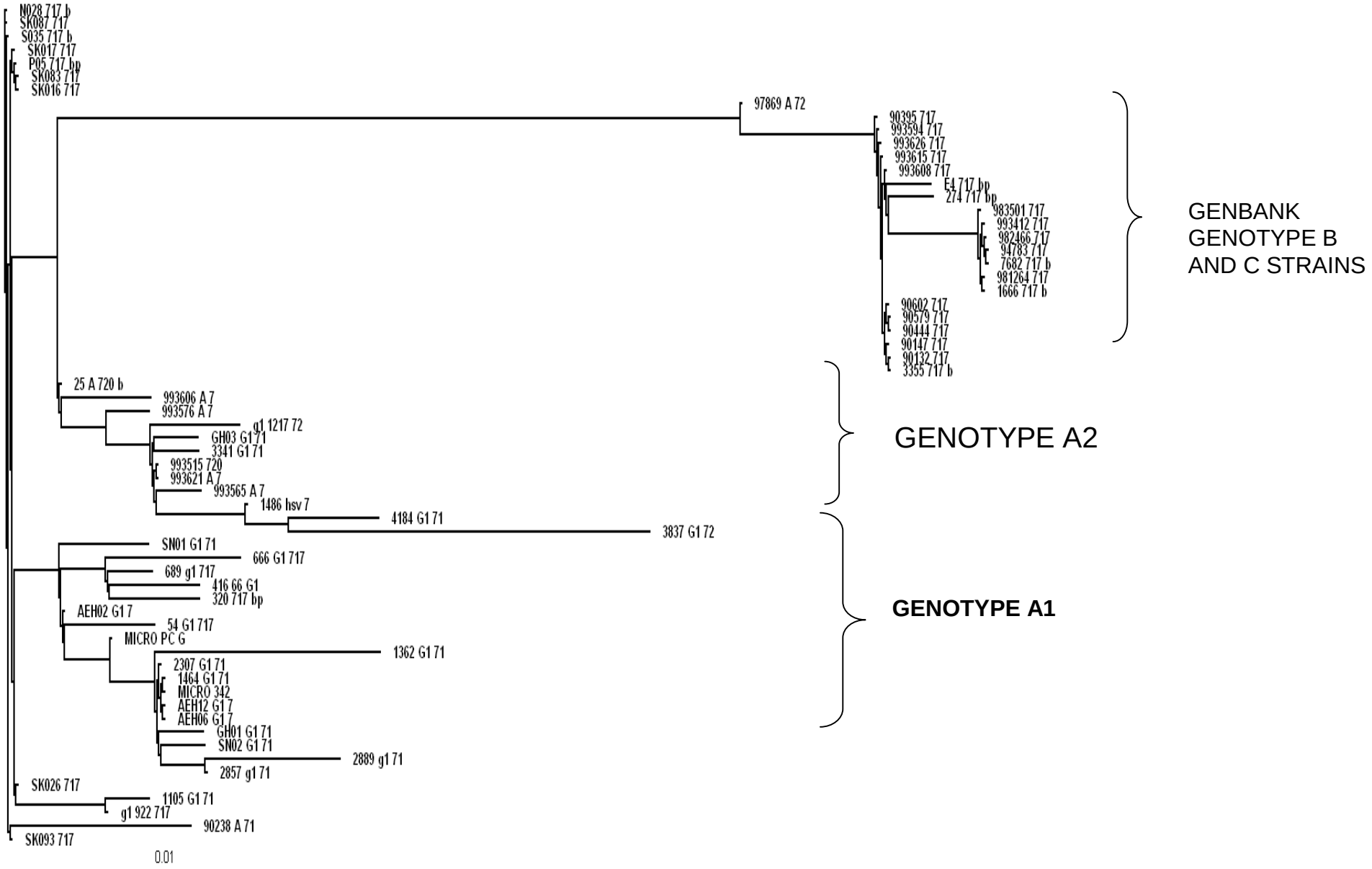


Fig 4.6: An unrooted phylogram of gG gene depicting classification of isolates into sub genotypes A1 and A2.

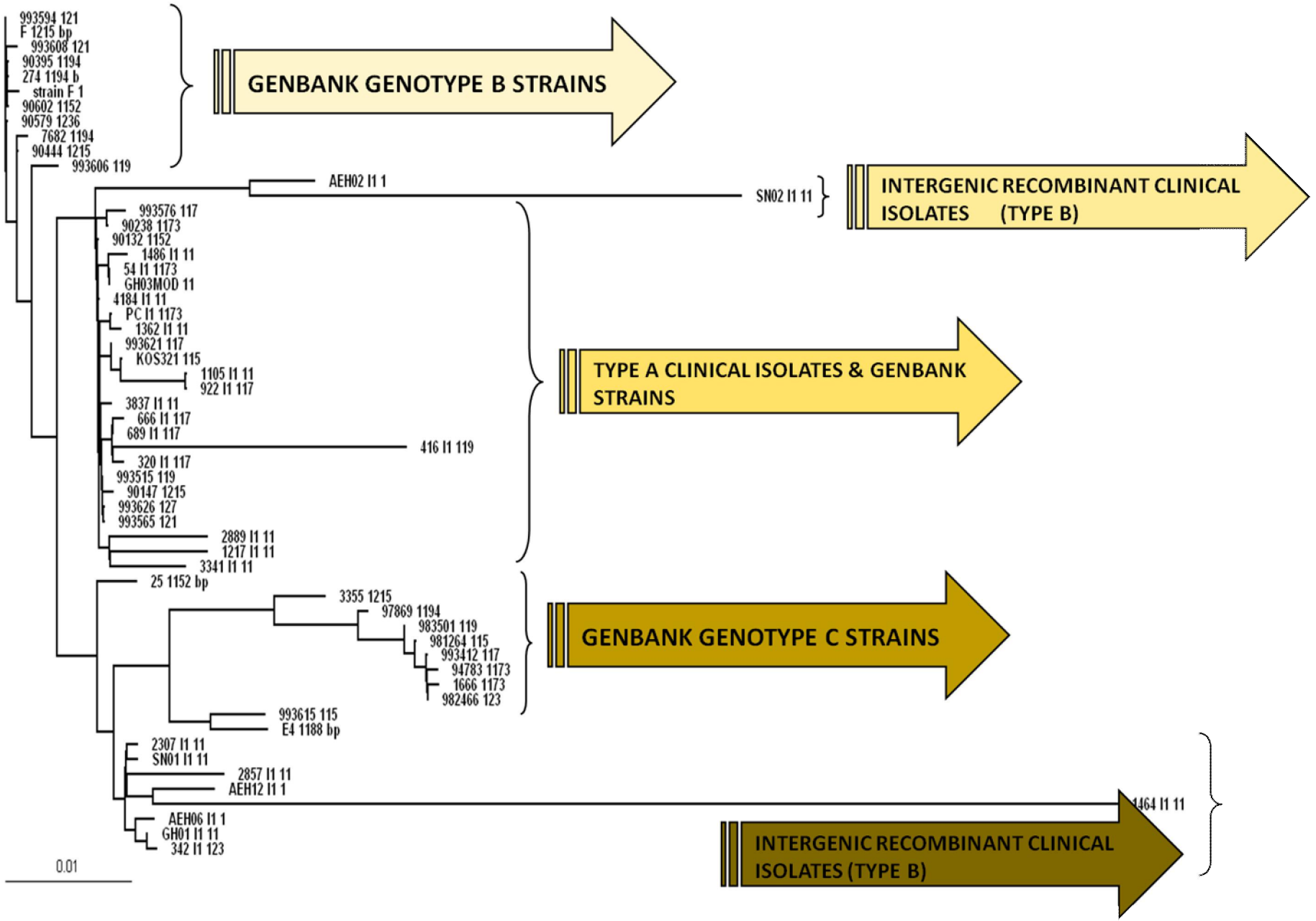


Fig 4.7: An unrooted phylogram of gI gene depicting classification of isolates into two genotypes

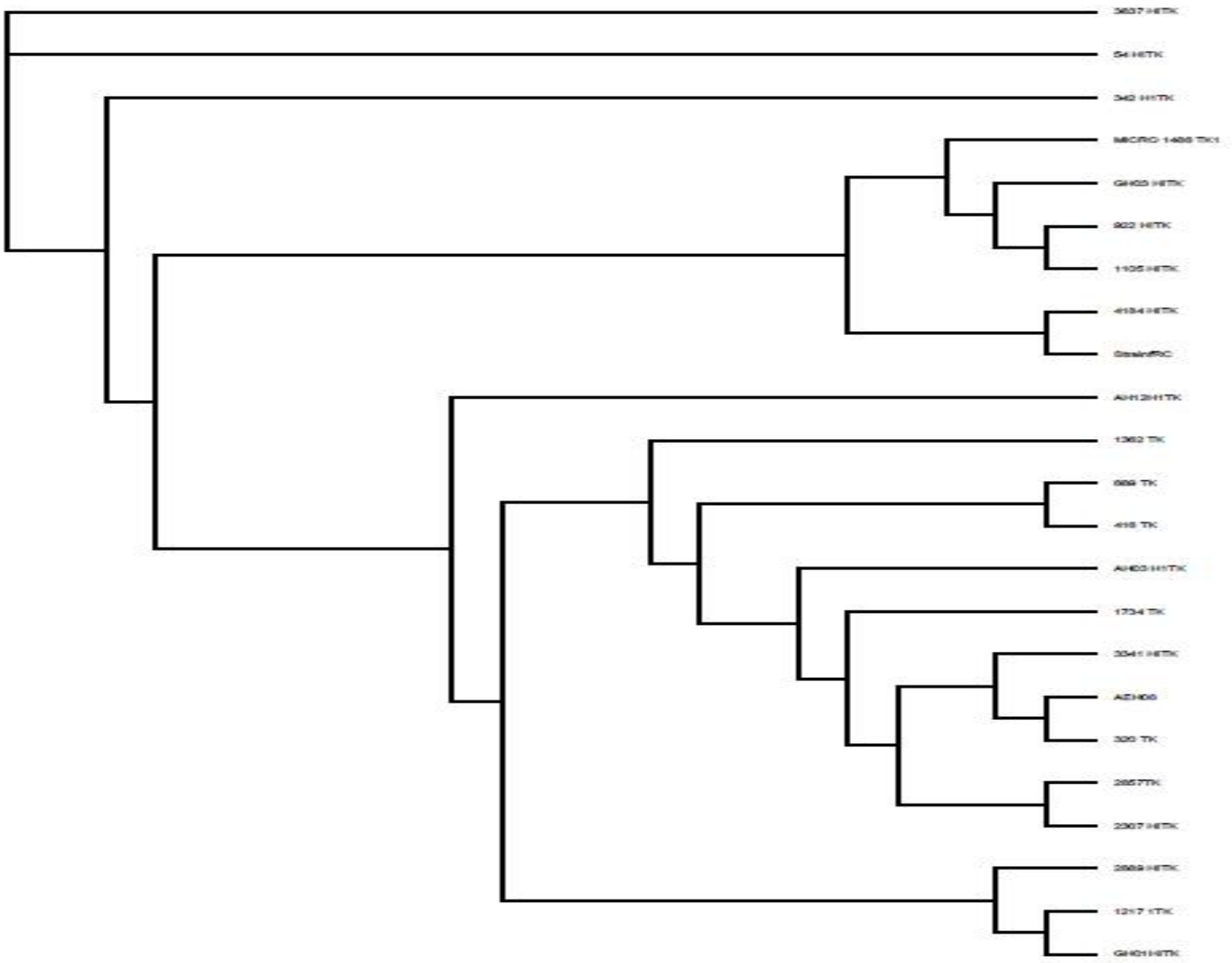


Fig 4.8: An unrooted phylogram of UL23 gene depicting classification of isolates into two genotypes

Chapter 5

5.1. Hypothesis:

Real time PCR assay is an effective and sensitive method as compared to pp65 antigenemia assay to monitor the treatment of Human Cytomegalovirus (HCMV) infections in renal transplant patients with HCMV disease.

5.2. Objectives:

- To standardize the real time Polymerase chain reaction(RTPCR) for the quantification of HCMV DNA in peripheral blood samples collected from renal transplant patients
- To perform pp65 antigenemia assay by immunoflourescence method for the semiquantitative detection of HCMV from leucocytes prepared from the peripheral blood samples of renal transplant patients
- To analyze the sensitivity of the RTPCR and pp65 antigenemia assay for the quantification of HCMV infection

5.2.1. Objective:

To Perform Real Time Polymerase Chain Reaction (RT-PCR) for the quantification of HCMV DNA in peripheral blood samples collected from renal transplant patients

5.2.1.1. Materials and Methods:

5.2.1.1.1. Collection of peripheral blood samples

- 52 peripheral blood samples from renal transplant patients having HCMV infection were collected

5.2.1.1.2. Separation of Leucocytes

- The blood was allowed to stand for 1-2 hours
- Plasma and the buffy were separated after centrifugation
- Leucocytes were separated and used for pp65 antigenemia assay

The buffy was used for DNA extraction

5.2.1.1.3. Clinical specimen:

A total of two hundred and forty eight peripheral blood samples were processed and a prospective study was carried out in three groups of donor-recipient pairs. Patients with neutropenia, high degree of renal dysfunction, were excluded from the study.

Group 'A' consisted of 39 kidney transplanted patients and the corresponding donors. One hundred and fifty six blood specimens were collected which included 117 samples from thirty-nine renal transplant recipients before and one month after transplantation and their corresponding donors samples pre-

transplantation. Thirty-nine more blood samples were the follow up samples collected from recipients after two months.

Group 'B' includes 18 more donors and the corresponding 18 recipients who could not be followed up one month after transplantation period. From this group fifty four samples were collected from donors pre-transplantation and their corresponding recipients prior to and post transplantation.

Group 'C' included 19 donors and 19 kidney transplant recipients who were unrelated. A total of 38 blood samples were collected one-month post transplant from the recipients and pre-transplant samples were collected from the donors. Pre-transplantation samples could not be collected from the recipients.

Samples were collected in 2 ml EDTA vacutainer tubes and were transported immediately to the laboratory from Kaliappa Renal Centre, Chennai. All specimens were transported in their naïve form without any transport medium. The blood specimens were processed immediately for pp65, and real time PCR for HCMV (qRT-PCR). The study was approved by both the institute's research and ethics committee and informed consents were obtained from the donors and the renal transplant recipients who participated in the study.

5.2.1.1.4. DNA Extraction:

Nucleic acid was extracted from 0.2 ml of EDTA-anticoagulated whole blood by using the QiaAmp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was eluted from the columns in a final volume of 50µl of elution buffer and was stored at -70°C until used. These extracted DNA samples were used for quantitative PCR assays (Appendix 2).

5.2.1.1.5. Real Time PCR Assay:

Real-time PCR targeting the morphologically transforming region (*mtr*) II sequence has been applied onto the DNA extracted from these specimens and Real Time PCR assay was carried out. Primers and probe sequence are listed in the Table 5.1

Table 5.1: Primer and probe sequence for Real Time PCR

Primer / Probe	**Sequence**
Forward primer	**5'TTACGCGACCAGATTGCAAGA3'**
Reverse primer	**5'TACCTACGTGACCTACCAACG 3'**
Probe	**5' (6FAM)-CTCCGCCTCACCTTTCATCGAGTAAA-TAMRA-3')**

Plasmid containing 74 bp target sequence was used as standard.

5.2.1.1.6. Thermal profile:

The thermal profile used for the real time amplification of the HCMV DNA has been described in Table 5.2.

Table 5.2: Thermal profile for the Real time amplification of HCMV *mtr II* gene.

Temperature	**Time**	**Cycles**
Initial Denaturation 95 ºC	**10 mins**	**1 cycle**
Denaturation 95 ºC	**15 seconds**	**45 cycles**
Annealing 55 ºC	**30 seconds**	
Extension 72 ºC	**20 seconds**	

For quantitation purposes 10^1 to 10^5 copies of the plasmid was included in each run along with a reagent control. The C_T values from unknown samples were plotted on the standard curve derived from the different dilutions of standards during the run, The HCMV genome copy number was calculated in copies/ml using Rotor gene 3000 sequence detector (Corbett Research, Australia).

5.2.1.2..RESULTS:

5.2.1.2.1. Results of real time PCR assay of Donor- Recipient groups:

A total of 57 donor recipient pairs were included in the study. Based on the availability of follow-up samples, they were classified into Group A and Group B. Recipients from whom peripheral blood samples could be obtained at one month and after were classified under Group A. Mean follow up days is 92.6 days. Group A was further classified into five subgroups I-V based on the pp65 and qRT- PCR values. Recipients from whom one month post transplant sample could be obtained were classified under group B. Nineteen more unrelated donors and kidney recipients were included under group C in the study. (Table 5.3)

In group A, sub-group I consisted of three donors positive for HCMV only by real time PCR whose copy numbers were 1, 144 and 289 copy/ml. The recipients were tested negative for HCMV before transplantation and by 30 days post transplantation they turned positive with, 2265 copies/ml, 119 copies/ml and, 25 copies/ml by real time PCR. Twenty six recipients (69.2%) whose pre-transplant samples along with the corresponding donor's blood, tested negative by RT-PCR were classified under sub-group II. But they became positive for HCMV one month after transplantation. Among the 26, all patients were positive by real-time PCR with highest copy number of 29,768 copies/ml and lowest was 4 copies/ml. Sub-group III consisted of a single patient whose pre-transplantation

sample had a real time count of 8 copies/ml which reduced to 7 copies/ml after a month of transplantation. The corresponding donor was also found positive for HCMV viremia with 11 copies/ml. Sub-group IV, consisted of two patients whose donors were tested positive for HCMV. Two donors were positive for real time PCR with 86 copies/ml and 537 copies/ml respectively. The corresponding recipient was negative for HCMV before and one month after transplantation.

Sub-Group V consisted of seven recipients (15.4%) whose donors, their pre-transplant specimens and three month post-transplant specimens were negative by real time PCR assay. Of the 18 donor-recipient pairs under Group B, six recipients were tested positive for HCMV DNA one month after transplantation by real-time PCR and 12 were negative by real-time PCR. One donor was positive by real-time PCR. Seventeen others were tested negative for HCMV DNA. Of the 19 unrelated donor-recipient pairs, all recipients were positive for the presence HCMV DNA the results of which copy numbers were ranging from 46 - 84,62,847 copies/ml respectively. Four of the donors were positive for the presence of HCMV DNA with copy numbers ranging from 14 - 458 copies/ml (Refer table 5.3).

5.2.1.2.2. Results on the follow up samples

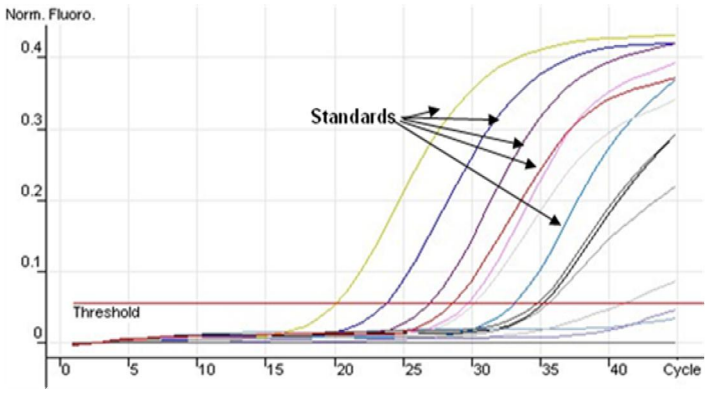
In sub-group I, all three recipients were followed up to two months and were tested negative for HCMV viremia. Fourteen among twenty seven patients in sub-group II were followed up and six of them were found to be negative. Three others remained positive with an increasing real time PCR count. Five others had varied HCMV copy numbers in the samples. One patient belonging to sub-group III was followed up to second month and the real time value increased to 106457 copies/ml. One among the two in-group IV, patient was followed up to three

months and remained negative. Yet another patient turned positive with 9092 copies/ml during the second month and could not be followed up further. Among the seven patients in group IV, one of them was turned positive with 246 copies of HCMV/ml. Overall a significant number of (66.6%) recipients whose donors were negative developed a post transplant HCMV infection (chi-square test p = 0.037%).

5.2.1.2.3. Valgancyclovir treatment

Among the seventy-six renal transplant recipients fifty seven were positive for HCMV after transplantation. Of the fifty-seven HCMV positives, thirty-three of them presented with symptoms of HCMV disease and were treated with Valgancyclovir (Valgan/Valcept-450 mg/ tablet) and twenty-four were not treated with Valgancyclovir.

Fig 5.1: Real Time Quantitation data for Cycling A.FAM/Sybr



5.2.2. Objective:

To perform pp65 antigenemia assay by immunoflourescence method for the semiquantitative analysis of HCMV from leucocytes prepared from the blood samples of renal transplant patients.

5.2.2.1. Materials and Methods:

5.2.2.1.1. Clinical specimen:

A total of two hundred and forty eight peripheral blood samples were processed and a prospective study was carried out in three groups of donor-recipient pairs. Patients with neutropenia, high degree of renal dysfunction, were excluded from the study.

Group 'A' consisted of 39 kidney transplanted patients and the corresponding donors. One hundred and fifty six blood specimens were collected which included 117 samples from thirty-nine renal transplant recipients before and month after transplantation and their corresponding donors samples pre-transplantation. Thirty-nine more blood samples were the follow up samples collected from recipients after two months.

Group 'B' includes 18 more donors and the corresponding 18 recipients who could not be followed up one month after transplantation period. From this group fifty four samples were collected from donors pre-transplantation and their corresponding recipients prior and one-month post transplantation.

Group 'C' included 19 donors and 19 kidney transplant recipients who were unrelated. A total of 38 blood samples were collected one-month post

transplant from the recipients and pre-transplant samples were collected from the donors. Pre-transplantation samples could not be collected from the recipients.

Samples were collected in 2 ml EDTA vacutainer tubes and were transported immediately to the laboratory from Kaliappa Renal Centre, Chennai. All specimens were transported in their native form without any transport medium. The blood specimens were processed immediately for pp65, and real time PCR for HCMV (qRT-PCR). The study was approved by both the institute's research and ethics committee and informed consent was obtained from the donors and the renal transplant recipients who participated in the study.

5.2.2.1.2. Antigenemia assay:

The pp65 antigenemia assay was carried out on smears containing 2 x 10^5 leucocytes prepared from 5 ml of EDTA anticoagulated blood within six hours of receipt of specimen. The smears were fixed in methanol for 10 minutes. Immunoflourescence staining was carried out on the smears using Immunoflourescence staining kit obtained from Argene SA, France. The smears were examined under fluorescent microscope (Optiphot, Nikon, Japan) with blue filter.

5.2.2.2. RESULTS:

5.2.2.2.1. Results of pp65 antigenemia assay on donor recipient pairs:

A total of 57 donor recipient pairs were included in the study. Based on the availability of follow-up samples, they were classified into Group A and Group B. Recipients from whom peripheral blood samples could be obtained at one month and after were classified under Group A. Mean follow up days is 92.6

days. Group A was further classified into five subgroups I-V based on the pp65 and qRT- PCR values. Recipients from whom one month post transplant sample could be obtained were classified under group B. Nineteen more unrelated donors and kidney recipients were included under group C in the study (Table 5.3). In group A, sub-group I consisted of three donors who were negative by pp65 antigenemia assay and the recipients both pre-transplant and one month transplant samples none were positive for pp65 antigenemia. Twenty six recipients (69.2%) whose pre-transplant samples along with the corresponding donor's blood, tested negative by pp65 antigenemia assay were classified under sub-group II. But they became positive for HCMV one month after transplantation. Among the 26, 15 patients were tested positive for both pp65 antigenemia. The highest positive pp65 cell count recorded among them was 78 cells and the lowest was 3 cells. In eleven others, the blood sample was negative by pp65 antigenemia assay. Mean pp65 antigenemia values/2 × 10^5 PBLs for this group was 13.8. Sub-group III consisted of a single patient whose sample was negative both pre- and post transplant. The corresponding donor was also found negative by pp65 antigenemia assay. Sub-group IV consisted of two patient whose donors were negative by pp65 antigenemia assay. The corresponding recipient was negative for pp65 antigenemia assay before and one month after transplantation.

Sub-Group V consisted of seven recipients (15.4%) whose donors, their pre-transplant specimens and three month post-transplant specimens were negative for pp65 antigenemia. Significantly a high value of pp65 antigenemia was observed among the recipients belonging to sub-group II (Table 5.2). Of the (table 5.2) 18 donor-recipient pairs under Group B, two recipients were positive by pp65 antigenemia assay and 16 were negative by pp65 antigenemia assay. All

Eighteen donor samples were tested negative by pp65 antigenemia. Of the 19 unrelated (table 5.3) donor-recipient pairs, all recipients were positive for the presence of both pp65 antigens the results of which were ranging from 5 - 230 cells/2 × 105 leucocytes. None of the donors were positive by pp65 antigenemia assay (Refer Table 5.3).

5.2.2.2.2. Results on the follow up samples

In sub-group I, all three recipients were followed up to two months and they tested negative for pp65 antigen. Fourteen among twenty seven patients in group II were followed up. All fourteen were found to be negative. One patient belonging to group III was followed up to second month and the pp65 count had increased to 37 cells. One among the two in group IV, patient was followed up to three months and remained negative. Among the seven patients in group IV, none were positive by pp65 antigenemia assay.

5.3. Analysis the sensitivity of the RTPCR and pp65 antigenemia assay for the detection of HCMV infection

In our study among the seventy six donor recipient pairs a significant number of the transplant recipients (66%) developed HCMV infection post transplant. Fifty seven (75%) of them tested positive for HCMV post transplant by real time PCR while thirty seven (49%) of them were found to be positive for HCMV by pp65 antigenemia assay. Of the seventy six donors, eleven (14.47%) of them tested positive for HCMV by real time PCR while one (1.3%) of them was positive for the virus by pp65 antigenemia assay.

The above results have proven that real time PCR is very sensitive in the detection of HCMV disease and its transmission from donors to the recipients.

The exact source of HCMV detected in the three recipients of sub-group I could assumed to be the donor as they were negative prior transplantation where the donors have been observed to have HCMV DNA only by real time PCR assay.

The HCMV infection observed among twenty six recipients of sub-group II is probably due to the reactivation of the latent virus present in them or other invasive procedures that lead to transmission of HCMV, wherein the viral DNA has been detected in all recipients but only 57% of the recipient group has been detected to have HCMV antigen in their blood. In the sub-group III of Group A since the donor's and the recipient's pre-transplantation sample were tested positive only by real time PCR assay for HCMV viremia the assumption that post-transplant the recipient would develop a aggressive disease course did not occur. Results analyzed from the various groups, substantiate the fact that the HCMV DNA (RT-PCR) was present among kidney donors and the rate of detection in our study was 11.8%.

It is recommended that donors be screened for HCMV serostatus to prevent adverse outcome of HCMV disease in renal transplant patients. But the lower survival of donor positive recipient positive (D+R +) transplants rather than donor positive, recipient negative (D+R-) and the absence of relationship between HCMV infection and acute cellular rejection have been reported [307-309]. In our study we observed the detection of HCMV DNA in large number of patients who along with their donor did not have HCMV DNA prior transplantation. HCMV detection in donors and recipients will be a better method to assess the status as DNA appears even before sero-conversation takes place.

Among the fifty seven HCMV positive recipients, only thirty-three (58%) of them were treated with valgancyclovir since they had symptoms of active HCMV disease, while twenty four of the recipients (42%) were not treated though they had HCMV DNA.

Twenty two (39%) of the transplant recipients with HCMV infection did not develop symptomatic disease any time in the study period. Of the thirty nine recipients who were followed up, only fourteen recipients with disease were put on treatment (36%) while twenty five (64%) of the recipients were not. As a result of the increased use of Gancyclovir prophylaxis, the incidence and severity of HCMV disease is significantly reduced. However, there is an increasing incidence of Gancyclovir resistant CMV infection. Therefore treatment may be restricted to symptomatic patients alone as the presence of HCMV DNA and low count of pp65 antigenemia does not always lead to development of disease process which was made evident from the study. And also, the importance of giving preemptive therapy to donors and transplant recipients before transplantation may not be needed. As reported earlier, patients belonging to sub-group II of Group A had significantly more pp65 antigenemia positive count when compared to patients belonging to other groups [310]. Bossart *et al* has shown an 84% agreement between pp65 antigenemia and quantitative assays and pointed out an episode of active infection missed out by pp65 and other instance where the patient showed significant DNA-emia with very low pp65 antigenemia positivity [311]. The advantages and disadvantages of pp65 and real time PCR in diagnosing HCMV infection and disease were well described by Carini *et al* 2007 [312]. A median of 11 vs 30 cells pp65 positive cells were reported among symptomatic and symptomatic patients respectively [312].

The utility of the real time PCR technique for HCMV infection and usefulness of both the technique in detection of symptomatic patients were highlighted. The cut-off of 13 cells/2 × 105 PBML cells was considered a predictor of symptomatic infection [312]. The significant positive pp65 antigenemia value quoted varies from equal to or more than 5 to 30 cells. In our study predominantly a pp65 positive count of equivalent and above 10 cells/2 × 10^5 and also accompanied positive high DNA copy number correlated well with the disease symptoms as observed among the 33 symptomatic patients who were treated with valganciclovir. In conclusion, the study observed the presence of HCMV DNA among kidney donors. The recipients develop HCMV infection even if the donors are negative for HCMV. pp65 antigenemia and real time PCR, can be applied as a qualitative measure to assess the HCMV infection among renal transplant patient and to monitor the prognosis. Treatment should be restricted to those with HCMV disease.

Infection with CMV is a major cause of disease and death in immunocompromised patients, including organ transplant recipients, patients undergoing hemodialysis, patients with cancer, patients receiving immunosuppressive drugs, and HIV-infected patients. Exposing immunosuppressed patients to outside sources of CMV should be minimized to avoid the risk of serious infection. Whenever possible, patients without CMV infection should be given organs and/or blood products that are free of the virus. Patients without CMV infection who are given organ transplants from CMV-infected donors should be given prophylactic treatment with valganciclovir (ideally) or ganciclovir and require regular serological monitoring to detect a rising CMV titre, which should be treated early to prevent a potentially life-threatening infection becoming established. The analysis of results revealed that

the donor recipient groups can be classified into 5 groups (Table 5.3) based on the results of pp65 antigenemia assay and real time PCR assay performed on the donor (D), pre transplant recipient (PRR) and post transplant recipient (PSR) samples. The positivity was taken into consideration by either of these methods. Group I comprised of 3 pairs who did not show HCMV infections (D-/PSR-/PRR-), group II comprised of 15 pairs where the only PSR samples showed the presence of HCMV infection and PRR and D were negative(D-/PSR-/PRR+), group III comprised of 1 pair wherein all donor and the recipient (PRR & PSR) samples showed the presence of HCMV infection(D+/PSR+/PRR+), group IV comprised of 3 pairs where in the donors and the PSR samples showed the presence of HCMV infection and the PRR samples did not show the presence of HCMV infection(D+/PSR-/PRR+) and group V wherein the only the D samples showed the presence of HCMV infection and the recipients were healthy(D+/PSR-/PRR-). The study reveals that 62.5% of the recipients in the study population acquired the HCMV infection for which the transmission history of the infection is unknown, 12.5% of the recipients showed the probable chances of acquiring the disease from the donors upon renal transplantation, 8.3% remained healthy despite the donors showing the presence of HCMV infection and in 4.1% of the recipients the history of the HCMV infection could not traced since the recipient (both PRR & PSR) and the donor samples showed the presence of HCMV. In all these patient groups real time PCR assay has been found to be more sensitive in the detection of HCMV disease. The same has been proven in the recipient groups who were followed up for monitoring the HCMV disease treatment may be restricted to symptomatic patients alone as the presence of HCMV DNA was detected by this assay.

Table 5.3: Analysis of results of tests performed for the detection of HCMV with donor-renal recipient patients

Groups	Subgroups	Donor/Recipient	No of pairs Positive by Real time PCR	No of pairs positive be pp65 antigenemia assay
Group A	Subgroup I	*D+/PRR-/PSR+	3/3	0/3
	Subgroup II	D-/PRR-/PSR+	26/26	15$^{\$}$/26
	Subgroup III	*D+/PRR+/PSR+	1/1	0/1
	Subgroup IV	*D+/PRR-/PSR-	2/2	0/2
	Sub group V	D-/PRR-PSR-	0/7	0/7
Group B		D-/PRR-/PSR+	6$^{\#}$/18	2/18
		D-/PRR-/PSR+	1/18	0/18
Group C		PSR+	19^/19	0/19
		D+	4/19	0/19

In group A

*- Donor (D), Pre-transplantation recipient (PRR) and Post-transplantation recipient(PSR) samples

-Post transplant recipients were tested for HCMV infections by pp65 antigenemia and viraemia by real-time PCR.

- All the donors were pp65 antigenemia negative indicating that they were not infective.

- * Six donors belonging to sub-groups I, III and IV were positive for HCMV by real time - PCR with copy numbers ranging from 1 to 537 copies/ml were all

Group B consisted of donors from whom sampling was not possible after one month of renal transplantation.

Group C consisted of unrelated donors and recipients.

- # Two among the recipients were positive for pp65 antigenemia also.

- ^ All 19 positive for HCMV DNA were also positive for pp65 antigenemia also.

- $ All 15 patients samples positive for pp65 antigenemia were also positive for real time PCR also.

- Eleven more were positive only by real time PCR assay.

Chapter 6

6.1 Hypothesis:

Resistance to ganciclovir exhibited by Human cytomegalovirus is most often due to mutations occurring in UL97 gene of the virus.

6.2. Objectives

- To standardize and apply the PCR on blood samples from Renal transplant patients and to sequence the amplified products
- To analyze the sequenced products for mutations responsible for the ganciclovir resistance

6.2.1. Objective:

To standardize and apply the PCR on blood samples from Renal transplant patients and to sequence the amplified products

6.2.2. Material and Methods:

5.2.2.1. DNA Extraction

- The pp65 count of the specimens selected for DNA extraction had already been conducted in the laboratory
- Those specimens having high pp65 count were selected for DNA extraction
- DNA extraction was done using QIAamp DNA mini kit (Appendix 2)

6.2.2.2. Polymerase Chain Reaction Amplification

- The DNA extracted was subjected to PCR amplification targeting the UL 97 gene
- For convenience 2 separate regions of the gene that were prone for mutations as already described in literature were selected for amplification.
- Two optimized primer sets were used for amplifying these regions of UL 97 gene[313]. The primer sequences are tabulated in Table 6.1.

Table 6.1: Primer sequence for the amplification of UL97 gene.

	Primer Sequences		Amplified product size	Amplified region
1	Forward	5'-TGGCCGACGCTATCAAATTT-3'	354 bp	1st region
	Reverse	5'-CCAGCGCCGACAGCTCCGAC-3'		
2	Forward	5'-ATGTCGGAGCTGTCGGCG-3'	285 bp	2nd region
	Reverse	5'-CGACACGAGGACATCTTG-3'		

6.2.2.3 Cocktail:

i. dNTPs - 8 µl

ii. 10X buffer - 5 µl

iii. FP UL97 I/III -1 µl

iv. RP UL97 II/1V -1 µl

v. Milli Q water - 30 µl

vi. Taq Polymerase - 0.3 µl

- 10 µl of DNA was added to 45 µl of the cocktail

6.2.2.4. Thermal cycle profile:

The thermal profile used for the amplification of UL 97 gene is described in Table 6.2.

Table 6.2: Thermal profile for the amplification of UL97 gene

Temperatures	Time	Cycles
Initial denaturation **95 ºC**	**5 mins**	**1 cycle**
Denaturation **95 ºC**	**1 min**	**40 cycles**
Annealing **55ºC**	**2 mins**	
Extension **72ºC**	**2 mins**	
Final extension **72ºC**	**10 mins**	
Final hold **22ºC**	∞	

6.2.2.5 Determination of sensitivity of the primers targeting the UL 97 gene

- The PCR was performed with the specific thermal cycle profile
- Serial dilutions(10^1 to 10^5) of the positive control were used for determination of sensitivity of the primers

6.2.2.6 Determination of specificity of primers targeting the UL 97 gene

The specificity of primers was tested using the DNA extracted from the following organisms: HSV 1(ATCC 733 VR), HSV 2 (sp 753167), VZA (oka vaccine strain), *Chlamydiae trachomatis* Serotype A *(ATCC VR 517B),Toxoplasma gondii, Mycobacterium tuberculosis* (H_{37} RV), *Staphylococcus* aureus (ATCC 25293), *Candida albicans* and AD 169 (Positive control)

6.2.2.7 Agarose Gel Electrophoresis:

The amplified product was subjected to electrophoresis on 2% agarose gel incorporated with 0.5 µg/ml ethidium bromide (Appendix 3) for visualization by UV transilluminator (Vilber Lourmet, France).

6.2.2.8 Gel Elution:

Gel elution was performed using the Axyprep gel elution kit (Axygen, USA)

- The desired band was cut out with a scalpel after electrophoresis in TBE buffer
- The gel slice was then transferred into a 1.5 ml clean sterile microcentrifuge tube and weighed (X mg)
- 3X sample volume of buffer DF-A was added to the tube
- The gel was resuspended in buffer DF-A by vortexing. It was incubated at 75ºC for 2-6 minutes until the gel was completely dissolved. Intermittent vortexing accelerates gel solubility
- 0.5X buffer DF-A volume of buffer DF-B was added
- 1X sample volume of Isopropanol was added. Isopropanol is needed if the DNA fragment is less than 400bp
- An Axy Prep column was placed to a 2ml microfuge tube and dissolved agarose is transferred into the column
- It was centrifuged at 12000rpm for 1 minute
- The filtrate was discarded and the column replaced
- 500 µl of wash buffer 1 was added
- It was centrifuged at 12000 rpm for 30 seconds

- 700 µl of wash buffer 2 was added
- Centrifuged at 12000 rpm for 30 seconds
- 30 µl of eluent was added. It is preferred to prewarm the eluent at 65ºC for its efficiency
- It was kept at room temperature for 1 minute
- It was centrifuged at 12000 rpm for 1 minute and stored at -20ºC

6.2.2.9. DNA Sequencing

- The products which showed bright specific bands by agarose gel electrophoresis was subjected to DNA sequencing

6.2.2.9.1. Cycle Sequencing:

6.2.2.9.1.1. Cocktail:

i. Ready reaction (RR) mix - 1 µl

ii. Buffer - 3 µl

iii. Milli Q water - 2 µl

iv. Forward primer - 2 µl

v. Amplified products - 2 µl

6.2.2.9.1.2. Thermal Profile:

The thermal cycle profile used for cycle sequencing is described in Table 6.3.

Table 6.3: Thermal profile for Cycle sequencing

Temperature	Time	Cycles
Initial denaturation 96 ºC	**1 min**	**1 Cycle**
Denaturation 96 ºC	**10 secs**	**25 Cycles**
Annealing 55 ºC	**5 secs**	
Extension 62 ºC	**4 mins**	
Final hold 4ºC	∞	

6.2.2.10. Purification (Appendix 3):

- The extension products were purified to remove the unincorporated dye terminators before the samples were analyzed

6.2.2.11. Loading of Samples

- The amplified products with the dye at the terminated 3'end were subjected to capillary electrophoresis by an automated sample injection

- The emitted flurorescence from the dye labels on crossing the laser area were collected at the rate of one per second by cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on the computer for processing.

6.2.2.12. BLAST analysis

After DNA sequencing and purification, BLAST analysis, using Pubmed, www.ncbi.nlm.nih.gov/BLAST was done to confirm the sequenced data with the standard strains and to determine the percentage homology.

6.2.2.13. MULTALIN analysis

Multiple sequence alignment tool developed based on "Multiple sequence alignment with hierarchical clustering", F.Corpet *et al*, 1988. Multalin analysis, using genbank ,www.ncbi.nlm.nih.gov/genbank was done to identify the presence of any substitution, addition, deletion or frame shift mutations.

- The PCR was specific to detect UL97 gene of AD 169 (positive control) and other organisms were negative. Figure 5 shows result of UL 97 gene specificity

6.3. RESULTS:

6.3.1. Results of PCR amplification of clinical specimens targeting the UL97 gene

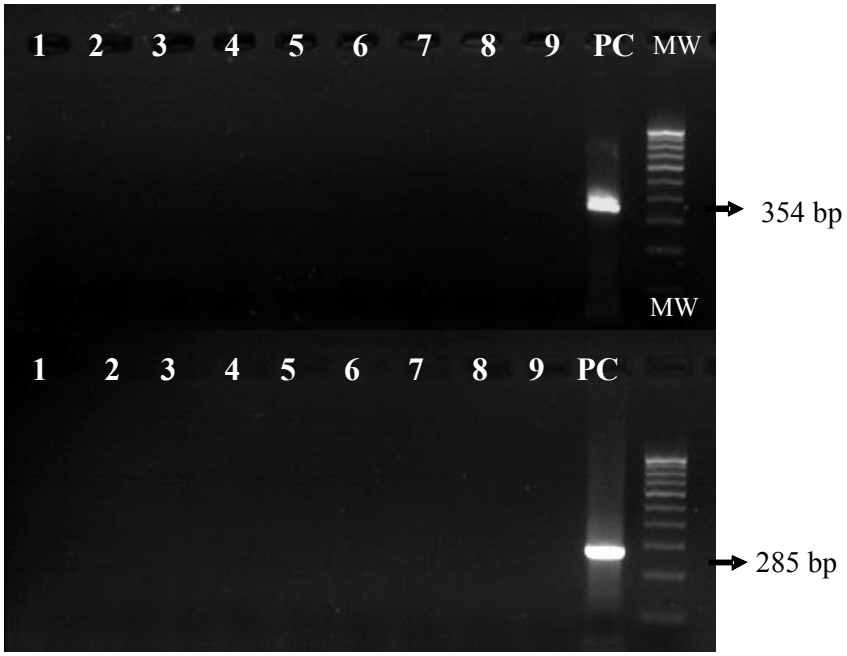
Fifty two peripheral blood samples of renal transplant patients having HCMV infection were subjected to PCR targeting UL97 gene. The results were obtained and are tabulated in the Table 6.4. The result showed that 15 specimens were positive for 1st region and 2nd region. Another 5 specimens were positive for 2nd region alone. Figure 6.1 shows results of PCR on clinical specimens targeting the UL97 gene

TABLE 6.4: Results of Amplification of UL97 gene by PCR

Clinical conditions	Samples	No. of specimens processed for PCR	No. of positive results	
			UL 97 1st region	UL 97 2nd region
Renal transplant recipients	Peripheral blood samples	52	15 (28.8%)	20*(38.46%)
Total		52	15	20

* Out of 20, 5 positive for 2nd region were negative in the 1st region.

Figure 6.1: Electrophoretogram of specificity of the primers for UL 97 Gene



Lane 1: Negative Control

Lane 2: HSV -1(ATCC 733 VR)

Lane 3: HSV- 2 (sp 753167)

Lane 4: VZA (oka vaccine strain)

Lane 6: *Toxoplasma gondii*

Lane 7: M*ycobacterium tuberculosis* (H $_{27}$ RV)

Lane 8: *Staphylococcus aureus* (ATCC 25293)

Lane 9: *Candida albicans*

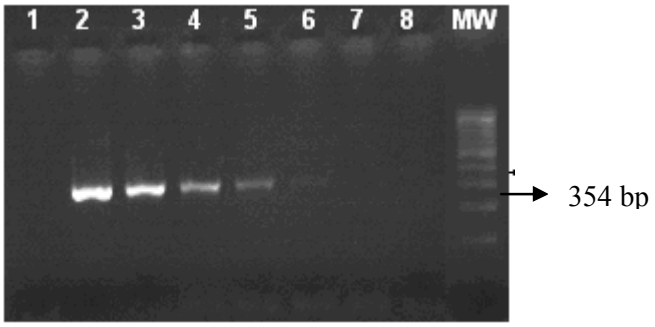
Lane PC: Human Cytomegalovirus AD 169 (Positive control)

Lane MW: Molecular weight marker 100bp ladder

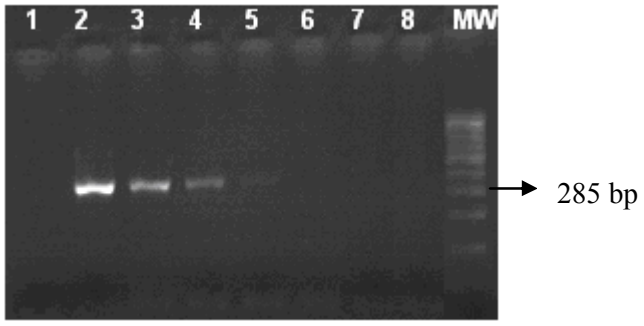
6.3.2. Results of sensitivity of PCR for primers targeting UL 97 gene

The PCR was sensitive to detect 0.0895 ng of DNA of HCMV with primers targeting UL 97 1st region and 0.895 ng of DNA of HCMV with primers targeting UL 97 2nd region. Figure 6.2 shows sensitivity of UL 97 gene

Figure 6.2: Electrophoretogram of sensitivity of the primers for UL 97 gene



Sensitive to detect 0.0895 ng amount of HCMV DNA



Sensitive to detect 0.895 ng amount of HCMV DNA

Lane 1: Negative control

Lane 2: Neat DNA conc of HCMV

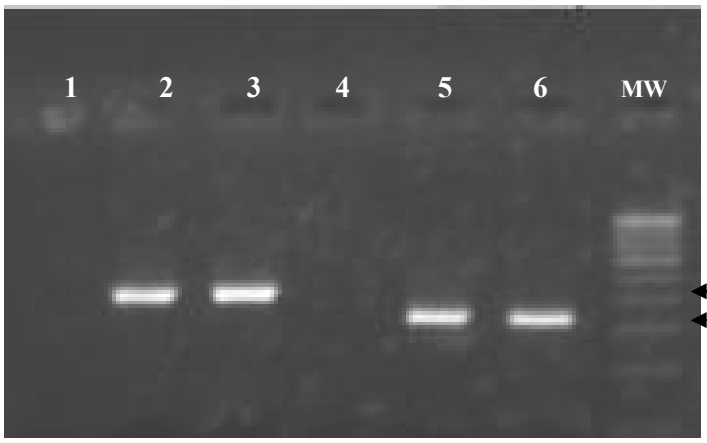
Lanes 3-8: 10 fold serial dilutions of DNA of HCMV

Lanes 2- 6: Positive

Lanes 1, 7, 8: Negative

Lane MW: *Phi X Hinf digest*

Figure 6.3: Electrophoretogram of PCR on clinical specimens targeting the UL 97 gene



Lane 1: Negative control

Lane 2: Specimen (plasma)

Lane 3: Positive control (HCMV AD 169)

UL 97 2nd region

Lane 4: Negative Control

Lane 5: Specimen (plasma)

Lane 6: Positive control (HCMV AD 169)

UL 97 1st region

Lane MW: Molecular Weight marker 100bp ladder

6.3.3. DNA Sequencing

- 15 specimens amplified in both 1st and 2nd regions and 5 specimens which were positive only for 2nd region were subjected to DNA sequencing. The results are tabulated in the Table 6.5. It is seen that from 15 specimens amplified in 1st and 2nd region 11 were positive for 1st region and 2nd region. 3 specimens were positive for 2nd region alone.

Figure 6.4: Sequencing result visualized by BioEdit Sequence Alignment Editor

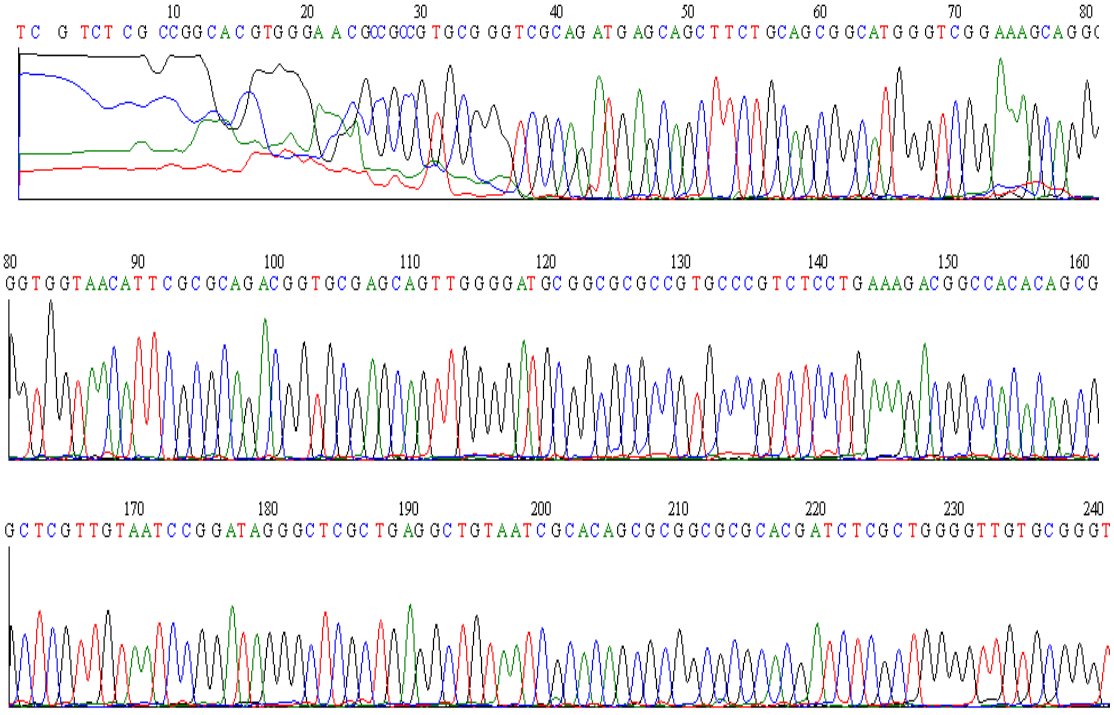


TABLE 6.5: Results of DNA Sequencing of UL97 gene

Clinical conditions	Samples	No. of positive results for PCR		No. of positive results for sequencing	
		UL97 1st region	UL972nd region	UL 97 1st region	UL 972nd region
Renal transplant recipients	Peripheral blood samples	15	20	11	14*
Total	52	15	20	11	14

* Out of 14 sequenced, 3 were positive for 2nd region were not sequenced in 1st Region

6.3.4. Results of Blast Analysis

- The percentage homology of sequenced product was analyzed. It was found that 95-100% homology was found in the specimens

6.3.5. Multalin Analysis

The specimens were multaligned along with Ganciclovir resistant and sensitive specimens. Mutations were observed in these specimens. The mutations were confirmed by reverse sequencing of the specimens. All specimens showed mutations in UL 97 1st and 2nd region. Silent mutations that did not result in amino acid change observed were V452V, P468P, A477A, S485S, A505A, R507R, N558N, V559V, R571R and L600L. Mutations that resulted in amino acid substitution were also observed in a few clinical specimens. Novel mutations were observed at positions where mutations have already been reported. These were Glu487Lys, Asn471His, His521Arg, Phe524Leu, Lys586His, Lys586Asn, Lys599Arg, Gly623Cys and Ser642Pro.

Figure 6.5: MULTALIN analysis of protein sequences of specimens in UL 97 1st region with resistant and sensitive sequences from genbank

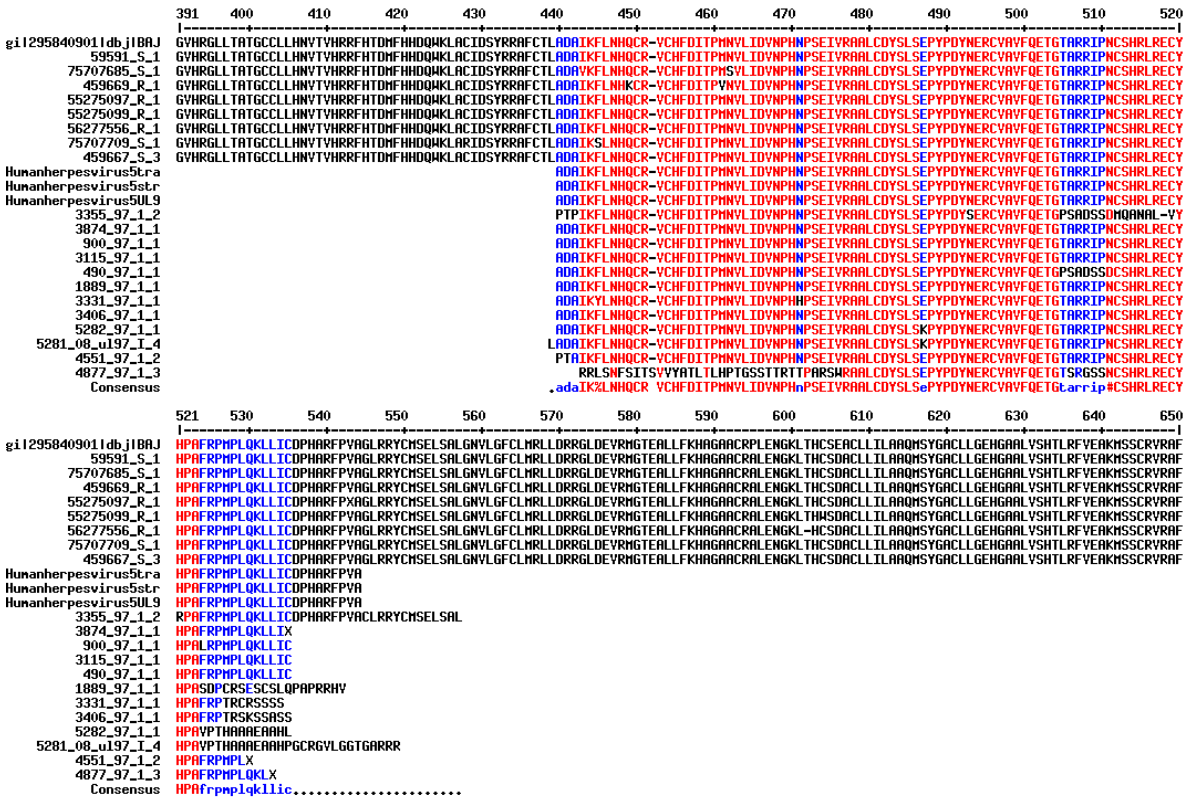
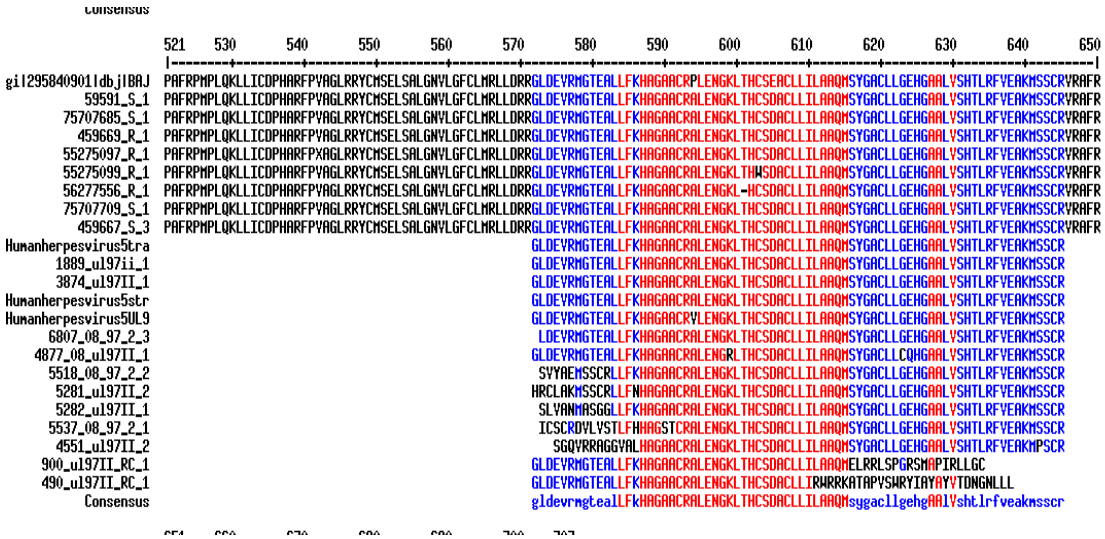


Figure 6.6: MULTALIN analysis of Protein sequences of specimens in UL 97 2nd region with resistant and sensitive sequences from genbank



6.4. Aanalysis of the sequenced products for mutations responsible for the ganciclovir resistance:

Ganciclovir is the antiviral drug most widely used in the management of severe and life-threatening human cytomegalovirus (HCMV) systemic infections in patients with terminal AIDS[314]. The isolation of ganciclovir-resistant HCMV strains is reported with increasing frequency in this patient group, and it is becoming an emerging clinical problem [245,246,315]. Prolonged antiviral maintenance therapy with suboptimal dosages and frequent discontinuation because of the toxic effects of the drug favor the emergence of ganciclovir-resistant HCMV strains.

Recently, it has been shown that ganciclovir phosphorylation in infected cells is controlled by the product of the HCMV UL97 open reading frame, a protein kinase homologue by sequence analysis [234,239]. Regions in the predicted product corresponding to conserved catalytic subdomains implicated in nucleotide binding, phosphotransfer, and substrate recognition have been identified on the basis of sequence homology [238,316], and a mutation conferring resistance to ganciclovir has been mapped to the UL97 gene in a laboratory-derived strain[234]. However, the development of mutations within the UL97 region have not been demonstrated to occur in HCMV strains isolated from persons receiving ganciclovir therapy.

Most of these mutations occur within the putative ATP-binding (codons 460 to 520) and substrate recognition (codons 590 to 607) sites of UL97[162]. At least two UL97 mutations associated with ganciclovir resistance outside these regions have been reported[317, 318], but these mutations appear to be rare, and

at least one appeared in the context of an accompanying mutation within the substrate recognition site. Hence these regions were focused in our study for mutations conferring resistance to ganciclovir.

The study was conducted to identify mutations in UL97 gene of Human cytomegalovirus by PCR based DNA sequencing methods. The PCR was standardized for the amplification of UL97 gene. The sensitivity and specificity of the primers were determined. The PCR was specific to detect UL97 gene of AD169, laboratory strain of HCMV and other organisms were negative. The PCR was sensitive to detect 0.0895 ng of DNA of HCMV with primers targeting UL97 1st region and 0.895 ng of DNA of HCMV with primers targeting UL97 2nd region. The standardized PCR targeting UL97 gene was applied to 52 peripheral blood samples from renal transplant patients. Out of 52 specimens, 15 were positive (28.8%) for 1st region and 20 were positive (38.46%) for 2nd region. 11 specimens from 1st region and 14 specimens from 2nd region were subjected to sequencing. The sequenced products were found to have 95-100% sequence homology with that of standard strains. Multalin analysis was performed in the sequenced data obtained and various nucleotides changes were detected. These nucleotide changes were confirmed by reverse sequencing of the specimens. Novel mutations were observed at positions Glu487Lys, Asn471His, His521Arg, Phe524Leu, Lys586His, Lys586Asn, Lys599Arg, Gly623Cys and Ser642Pro were confirmed based on sequencing which may contribute to resistance to ganciclovir. These mutations need to be further analyzed based on docking studies for confirmation of ganciclovir resistance.

Chapter 7

7.1. Hypothesis:

pp65 antigenemia assay is an important test for defining the antiviral therapy in Human cytomegalovirus diseases among renal transplant patients by way of isolation of the virus from peripheral blood leucocytes.

7.2. Objectives:

- To determine the pp65 count of the peripheral blood leucocytes collected from renal transplant patients.
- To isolate the HCMV from corneal fibroblast cells
- Analysis- Comparison of pp65 antigenemia assay and viral isolation

7.2.1. Objective:

Determination of pp65 count of the peripheral blood leucocytes collected from renal transplant patients.

7.2.1.1. Materials and Methods:

7.2.1.1.1. Samples:

Peripheral blood specimens from renal transplant recipients referred to our laboratory for pp65 antigenemia collected in 2 ml EDTA vacutainer tubes were transported immediately to the laboratory. All specimens were transported in their native form without any transport medium. The blood specimens were processed immediately for pp65 as requested for.

7.2.1.1.2. pp65 Antigenemia assay:

The pp65 antigenemia assay was carried out on smears containing 2×10^5 leucocytes prepared from 5 ml of EDTA anticoagulated blood within six hours of receipt of the specimen. Smears were fixed in methanol for 10 minutes. Immunofluorescence staining was carried out on the smears using pp65 staining kit obtained from Argene SA, France. The smears were stained with mouse monoclonal antibody (Argene SA, France). The smears were examined under fluorescent microscope (Optiphot, Nikon, Japan) with blue filter.

7.2.1.2. RESULTS:

Results of pp65 antigenemia assay:

Among the 51 prepared PBL's, 49 specimens were positive for pp65 antigenemia. Based on the results, the specimens were grouped into four (Group A, B, C and D). Patients under Group A had a cell count ranging from 2 to 10

cells. Group B had a cell count ranging from 11- 25 cells. Group C had a count ranging from 26 – 50 cells. Group D had a cell count >50 cells.

7.2.2: Objective

Isolation of Human cytomegalovirus from corneal fibroblast cells.

7.2.2.1 Materials and Methods:

7.2.2.1. Establishment of corneal fibroblast cultures:

The stromal layer of the donor was cut into large pieces and placed in tissue culture flasks. The tissues were nourished with Dulbeccos' minimum essential medium and F12 medium combination (Gibco, Invitrogen, New York, USA). The medium was supplemented with 15% fetal calf serum (Hi-Media, India), and fibroblast growth factor (Sigma, USA). At the end of 24 hours and 48 hours the outgrowth of fibroblast from the tissue was noted. Upon confluency the cells were trypsinized onto 16 well tissue culture plates for growth of cells for subsequent inoculation of leucocytes for isolation of HCMV.

7.2.2.2. Viral Culture:

Cultured Human corneal fibroblast cell line established from one donor eye and not more than 3 – 5 passages maintained in DMEM were used for the study. The leucocytes isolated from blood samples for pp65 antigenemia positive were inoculated onto monolayer of cells on 16 well tissue culture plates (BD falcon, USA) for the isolation of Human cytomegalovirus. The plates were rocked gently placing the tissue culture plate on the rocking machine for one hour at room temperature. The cells maintained with 1% FBS MEM and incubated at

37ºC in a CO_2 incubator. The cultures were observed for cytopathic effect (CPE). The cultures showing CPE typically of HCMV, replicates were harvested using cell scrapers and stored at -80º C. Fifty one consecutive pp65 positive peripheral blood leucocytes were tested for culture of HCMV. Un-inoculated corneal fibroblast cultures were also maintained to rule out nonspecific changes.

7.2.2.3. DNA Extraction:

Nucleic acid was extracted from 0.2 ml of culture harvest by using the QiaAmp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was eluted from the columns in a final volume of 50µl of elution buffer and was stored at -70°C until used. These extracted DNA samples were used for quantitative PCR assays (Appendix 2).

7.2.2.4. Real Time PCR Assay

Real-time PCR targeting the morphologically transforming region *mtr II* sequence was applied onto the DNA extracted from the culture isolates in Rotor gene Real time PCR machine (QIAGEN, 5 Plex) using primers and thermal profile described earlier [27]. The intra-assay and inter-assay 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, 2.5×10^7 copies/ml per reaction in the same and four independent runs respectively. Real Time PCR for the quantification of CMV was performed on the viral isolates propagated on corneal fibroblast culture. Un-inoculated corneal fibroblast culture DNA was used as the culture control to rule out the presence of viral markers in each lot of the cell lines. Reagent controls were also included in each run of the Real time PCR assay.

7.2.2.2. RESULTS:

7.2.2.2.1. Results of Isolation of HCMV:

Cultures showed megaloblastic changes in 21 (42.86 %) of the 49 specimens inoculated onto HCF cells (Figure 7.1).

7.2.2.2.2 Results of Real Time PCR Assay:

The Real Time PCR assay performed on the DNA extracted from the culture harvests revealed copy numbers ranging from 10 copies to 7.4 lakh copies/ml of DNA. The range of copy numbers varied for each group (Group A between 10- 431 copies/ml, Group B between 369- 7.1 lakh copies/ml, Group C between 328 – 7.04 lakh copies/ml, Group D between 1734 – 3.2 lakh copies/ml).

7.2.3. Comparison of pp65 antigenemia assay and viral isolation:

Among the 49 peripheral blood specimens used for inoculation, the cultures showed megaloblastic changes in 21 (42.86 %) specimens (Figure 1). Patients under Group A had a cell count ranging from 2 to 10 cells. The mean cell count of this group was 6.3. This group of patients had an isolation rate of 20%. Group B had a cell count ranging from 10- 25 cells with mean cell count of 15.2 and there were 7 (28%) isolates from this group of patients. Group C had a count ranging from 25 – 50 cells with a mean cell count of 39.3 and 4 (66.67%) specimens of these virus was isolated. Group D had a cell count >50 cells with a mean count of 229.11 and 8 (88.89%) from all the specimens the virus was isolated from the culture of fibroblasts (Table 7.1). The isolation of the virus was further proven by the real time PCR assay performed on the cell culture isolates.

The inactivation of the virus was ruled out as they were all stored at -80^{0}C until processed. Each lot of fibroblast cell culture without exposure to leucocyteswas harvested and DNA extracted from them were subjected to rule out presence of endogenous HCMV infection.

7.2.4. Statistical calculation:

The mean value of all four groups, were correlated with the positivity of HCMV in culture. The results were not statistically significant. Whereas when the actual pp65 antigenemia value of the 49 patients was correlated with the corresponding culture positivity and negativity, by paired "t" test the results were statistically significant indicating a strong correlation between the pp65 antigenemia value and the culture result (p= 0.0029).

7.3. Discussion:

Human fibroblasts have represented the conventional cell substrate for recovery of HCMV from clinical samples since the beginning of medical virology. Due to the increasing need for recovery of HCMV from the blood of immunocompromised patients, in our study we have demonstrated the successful isolation of HCMV from human corneal fibroblast cultures. In this study we have also demonstrated the correlation of pp65 antigenemia count of renal transplant patients and isolation of HCMV from the leucocytes of the same group.

Human corneal fibroblast cultures have proven to be the best substrate for the isolation of the virus in this study. In this study it has been proved that HCMV can be isolated from 42.85 % of the pp65 antigenemia positive

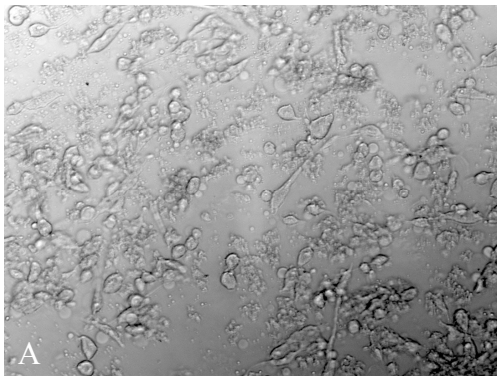
individuals, but in our laboratory set up the cut off value were 23 cells /2 $X10^5$ cells

From the literature it is evident that the cutoff pp65 value of 10 is considered to be the significant value in the treatment of renal transplant patients [226,319,320] except a report made by Gerna et al., in which it is suggested a pp65 antigenemia count of 100 was considered significant to treat the patient [321]. This information is based on western literature. In India, the seroprevalence of HCMV is reported very high among normal population and renal transplant patients [10,322,323]. In our earlier report we reported the rate of positivity of Human cytomegalovirus to be very high among the transplant patients [324]. Therefore a pp65 antigenemia count of 10 reported based on western literature [312,325-328] may not reflect the true situation of replication of HCMV in PBL's in Indian patients. It should also be born in mind that pp65 antigenemia assay is subjective as it is prone to the visual errors of individuals reporting on the smears. Therefore we attempted to estimate the significant pp65 antigenemia count that would have viable HCMV in the peripheral blood. We evaluated the most likely value of pp65 antigenemia that yields culture positive results. It was found that a count of 22.5 and above has more probability of HCMV isolation in our renal transplant patients. Therefore, a count of 23 should be considered significant pp65 count in patients in our laboratory set up and probably so in India.

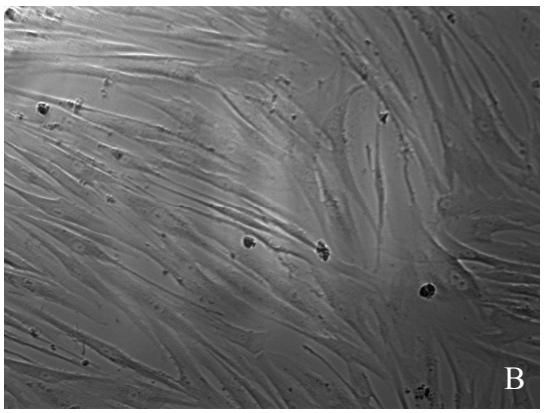
Despite the fact that pp65 antigenemia is the most valuable tool in the diagnosis of HCMV infections the viral isolation has also been a useful tool in diagnostics of HCMV infections.

In conclusion, HCMV was successfully cultivated for the first time in India on human corneal fibroblast culture. pp65 count of 23 was determined to be the value of clinical significance as it gives culture positive results among renal transplant patients which indicates the value of the test to be helpful in the therapy of these patients.

Fig 7.1: Images of Uninfected and infected cells of Human corneal Fibroblast cells:



A: HCMV Infected Human corneal fibroblast cells



B: Uninfected Human corneal fibroblast cells

8.0. CONCLUSIONS

Herpes viruses (Herpes simplex, Cytomegalovirus) are the causative agents of a wide variety of infections in humans. Though the infections caused by HSV are not severe and fatal in immunocompetent individuals, its causes morbidity in bone marrow graft recipients, whereas it causes much fewer problems in renal transplant recipients. AIDS carriers suffer greatly from HSV disease. Similarly HCMV is a significant cause of morbidity and mortality in immunocompromised patients. Early treatment prevents morbidity caused by these viral infections. The

innate antiviral response is thought to play a pivotal role in determining the outcome of an HSV infection. Accordingly, the production of type I interferon (IFN), comprised largely of IFNα and IFNβ, has been linked to protection against disease in both mouse models and in human studies. Hence we studied the innate immune response to HSV infection especially ocular based on the expression of TLR's in ARPE cells, whereby we have described the expression of TLR 4 which has not been reported earlier in HSV infections.

Isolation of drug-resistant HSV from immunocompetent patients has been reported infrequently (approx 0.3%) and, with very rare exceptions, resistant HSV is cleared normally with no adverse clinical outcome. The isolation of resistant HSV from immunocompromised patients is more common (4% to 7%). We have also characterised the resistant strains of HSV phenotypically and genotypically based on plaque reduction method and PCR-based DNA sequencing of the Thymidine kinase gene where we have described two novel mutations which have not been described earlier. We have also done the molecular modeling and

docking studies to prove that the mutations observed contribute to the ACV resistance exhibited by these clinical isolates.

Genotyping has been done based on the genetic variation among strains and the existence of intra-typic polymorphism of human herpes viruses, provides useful information for defining molecular epidemiology of infection. The unique short region of the genome which comprise of the genes coding for the glycoproteins gG, gI and gE has been used to study the phylogenetic relationship of the clinical isolates in various studies. In our study we used gG, gI and gC genes which have been used to studied to know the phylogenetic relationship between clinical isolates and the site of infection as well between resistant strains. We conclude that there is no relationship between genotypes and resistant strains and genotypes and the site of infection.

pp65 antigeneimia has been an important diagnostic test which has been used for monitoring active HCMV infections mainly in transplant patients. Application of molecular methods for such purposes may serve to be sensitive and rapid tools in monitoring of the disease. Monitoring the DNA levels on a real time basis may serve to be a clinically useful test in antiviral therapy. Hence we studied the usefulness of Real time PCR assay in detection and monitoring of HCMV infections in comparison with the pp65 antigenemia assay which is considered the 'Gold standard' in the diagnosis of HCMV. We have proved that Real time PCR has been highly sensitive in the detection and monitoring of the antiviral therapy in transplant patients.

Drug resistance in HCMV has also been posing to be a problem in HCMV infections. The UL97 gene has been described to have majority (94%) of

mutations which confers resistance to the commonly used drugs in the treatment for HCMV infections. Hence we have screened the UL97 gene for the presence of mutations and have described few novel mutations which have not been described earlier.

pp65 antigenemia has been used for the treatment of HCMV infections. A count of ten (10 cells per 2,00,000 cells) and above has been taken to be the threshold for the treatment of the disease. Hence we attempted to study the isolation of HCMV from blood leukocytes (CMV viremia), which is considered to be the most reliable marker of disseminated CMV infection and predicts invasive CMV disease. In our study we have described that a mean of 23 HCMV positive leucocytes is required for the isolation of HCMV *in-vivo*. Though many reports have been described in the western literature, this is the first such report to describe the threshold for the treatment based on isolation of HCMV from PBL of renal transplant patients in Indian scenario.

SPECIFIC CONTRIBUTIONS

- ✓ Toll Like Receptor 4 expression in HSV intra-ocular infections has been reported for the first time ever.
- ✓ Novel mutations at positions Glu36Ala and Val352Leu of Thymidine kinase gene of Herpes simplex virus 1 has been detected for the first time and proven to be responsible for resistance to Acyclovir in two of the ocular isolates.
- ✓ The above mutations have been characterized by molecular modeling and docking studies which has been reported for the first time in our study.
- ✓ Two sub groups of A1 and A2 based on glycoprotein G region of Herpes simplex virus has been reported for the first time based on our study.
- ✓ Our study helped determine 'No association' between genotypes of HSV and the site of infection as well as resistant strains.
- ✓ Molecular method namely Real time PCR has been found more sensitive in the detection of HCMV infections as well monitoring drug therapy in renal transplant patients.
- ✓ Novel mutations responsible for resistance to ganciclovir, has been reported in our study.
- ✓ Significant pp65 count of 23 has been reported for therapeutic purposes in HCMV infections in Indian scenario for the first time in our study.

FUTURE SCOPE OF WORK

- ✓ In case of HSV infections (ocular, CNS) the TLR expression could be studied to monitor the immune response of the patients.

- ✓ Based on the novel mutations described in the Thymidine kinase gene, a rapid diagnostic PCR kit could be made available for screening of Resistant mutant strains.

- ✓ Real time PCR assay can be applied as a routine diagnostic test instead of pp65 antigenemia assay in monitoring the disease in HCMV infected patients.

- ✓ Based on the novel mutations described in the UL97 gene, a rapid diagnostic PCR kit may be made available for screening of mutant strains for administering pre-emptive therapy.

- ✓ The cut-off value for the treatment of HCMV infections may be reconsidered to be 23 per 2,00,000 cells for Indian patients.

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APPENDIX 1

Reverse Transcriptase Polymerase Chain Reaction

RNA Extraction (QIAamp Viral RNA Mini Kit- Cat No: 52904):

- To 280µl of AVL buffer reconstituted with 2.8µl of carrier RNA was taken and 70 µl of the culture harvest was added.
- The solution was vortexed and incubate at Room temperature (RT) for 20 minutes.
- To the solution 280 µl of 100% ethanol was added and mixed gently and the solution is transferred to the column.
- The column was centrifuged at 8000 rpm for 1 minutes
- The column was replaced with a fresh collection tube and was washed with 500 µl of wash buffer 1 (AW1) by centrifuging at 8000 rpm for 1 minutes
- The column was replaced with a fresh collection tube and was washed with wash buffer 2 (AW2) by centrifuging at 14000 rpm for 3 minutes
- The eluted solution is discarded and the column is centrifuged at 14000 rpm for 1 minutes
- The column was replaced with a sterile vial and to the column 60 µl of the elution buffer (AVE) is added and incubated at room temperature for 1 minute
- The column is centrifuged at 8000 rpm for 1 minutes. The eluted RNA was stored at -80ºC.

Addition of Carrier RNA to Buffer AVL :

310 µl of Buffer AVE is added to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1µg/µl. The Carrier RNA is dissolved thoroughly and divided into convenient aliquots and stored at -20ºC.

Calculation of Volume of Buffer AVL- Carrier RNA mix:

n X 0.56 ml= y ml

y ml X 10 µl/ml= z µl

n =Number of samples to be processed simultaneously

y = Calculated volume of Buffer AVL

z = Volume of carrier RNA –buffer AVE to add to buffer AVL.

AW1 Buffer: To be reconstituted with 25 ml Ethanol(96-100%).

AW2 Buffer: To be reconstitute with 30 ml Ethanol(96-100%).

APPENDIX 2

DNA EXTRACTION

- To 20µl of proteinase K, 200 µl of the specimen is added along with 200 µl of the Lysis buffer (AL)
- The solution was vortexed and incubate at 56ºC in a water bath for 10 minutes
- To the solution 200 µl of 95-100% Ethanol is added and mixed gently and the solution is transferred to the column.
- The column was centrifuged at 8000 rpm for 1 minute
- The column was replaced with a fresh collection tube and was washed with 500 µl of wash buffer 1 (AW1) by centrifuging at 8000 rpm for 1 minute
- The column was replaced with a fresh collection tube and was washed with wash buffer 2 (AW2) by centrifuging at 14000 rpm for 3 minutes
- The eluted solution is discarded and the column is centrifuged at 14000 rpm for 1 minute
- The column was replaced with a sterile vial and to the column 200 µl of the elution buffer (AE) is added and incubated at room temperature for 1 minute
- The column is centrifuged at 80000 rpm for 1 minute. The eluted DNA was stored at -80ºC.

Reconstitution of Buffer:

AW1 Buffer: To be reconstituted with 25 ml Ethanol(96-100%).

AW2 Buffer: To be reconstitute with 30 ml Ethanol(96-100%).

APPENDIX 3

PCR AND DNA SEQUENCING

AGAROSE GEL ELECTROPHORESIS- REAGENT PREPARATION

10X TBE buffer: (pH 8)

Tris- Hcl – 54.1g

EDTA – 3.65g

Boric acid – 27.8g

Milli Q water – 500ml

Added Tris- HCl to 200ml of Milli Q water and dissolved completely. Then EDTA and boric acid was added. Mixed well and the volume was then made up to 500 ml with Milli Q water and then pH was checked. The stock 10X TBE buffer was diluted to 1X concentration before use.

GEL LOADING DYE:

Solution A - 0.1% Bromophenol blue in 1x TBE buffer

Solution B - 40% sucrose in 1x TBE buffer

Prepare solution A and B separately and equal volume was mixed.

PREPARATION OF GEL AND VISUALIZATION OF PCR PRODUCTS:

Agarose- 0.5 g was weighed and dissolved in 25ml of 1X TBE buffer, by heating in microwave oven until no granules were seen. Then added 8µl (12.5µg) of ethidium bromide mixed well and poured to the gel tray with appropriate combs and allowed to solidify. The gel was then placed in the tank

with 1X TBE buffer. 10µl amplified products were mixed with 2 - 3µl loading gel and loaded onto wells along with a molecular weight marker in each run.

The electrophoresis was run at 100 Volts for 30 minutes, and the results were documented in the gel documentation system (Vilber Lourmat, Marne La Valle, Cedex, France).

PREPARATION OF PURIFICATION REAGENTS

- **500mM EDTA:** 18.6 g of EDTA in 100 ml Milli Q water
- **125mM EDTA:**1µl of 500mM EDTA + 3 µl Milli Q water
- **3 M sodium acetate: (pH 4.6) :** 2.46g of sodium acetate was first dissolved in 5 ml of water and then pH was adjusted to 4.6 with acetic acid. Then the volume was made upto 10ml with water.

Procedure :

- To the 10 µl of cycle sequenced products, added 10 µl of Milli Q water in a 0.5ml microfuge vial.
- Then added 2 µl of 125mM EDTA.,to this added 2 µl of 3M-sodium acetate (pH 4.6) and 50 µl of chilled ethanol, Kept at room temperature for 15 minutes.
- Centrifuged at 12,000 rpm for 20 minutes.
- Discarded the supernatant and the pellet was washed twice with 200 µl of 70% ethanol at 12,000 rpm for 10 minutes.
- The vials were then dried at 37˚C and added 20 µl of formamide.
- Again denatured at 90˚C for 3 minutes and immediately loaded.

List of Publication:

1. **Samson Moses,** Malathi J, Nishi Rani Singha, R. Bagyalakshmi, Madhavan H.N. Evaluation of significance of pp65 antigenemia against culture of Human Cytomegalovirus in the peripheral Blood among Renal transplant patients. Indian Journal of Nephrology, Under issue preparation for October 2012
2. Hajib N Madhavan, Moses **Y Samson**, Murali Ishwarya, Ramanathan Vijayakumar, Malathi Jambulingam, pp65 antigenemia and real time polymerase chain reaction (PCR) based-study to determine the prevalence of human cytomegalovirus (HCMV) in kidney donors and recipients with follow-up studies. *Virology Journal,* 2010, 7:322-328.
3. Shyamala. G, Malathi. J, **Samson Moses. Y**, Lily Therese. K, Madhavan HN. Application of Nested Reverse Transcription Polymerase chain reaction for the detection of Rubella virus in clinical specimens. *Indian Journal Medical Research,* 2007, Jan; 125: 73 – 78.
4. Anusha H, Malathi J, Raajaram G, Annapoorni V, Umashankar V, Sathyabaarathi R, **Samson Moses Y**, H N Madhavan.. Phylogenetic comparison of exonic US4, US7 and UL44 regions of clinical herpes simplex virus type 1 isolates showed lack of association between their anatomic sites of infection and genotypic/sub genotypic classification. *Virology Journal,* 2012, 9:65

Presentations:

- Workshop on Diagnostic Methods for Leptospirosis 'LEPTOSHOPPE-2007' organized by The Indian Association of Medical Microbiologists, Tamilnadu & Pondicherry Chapter in association with The Tamilnadu Dr. M. G. R Medical University, Chennai & Institute of Microbiology, Madurai Medical College, Madurai.
- Presented a Poster on "Genotyping of Herpes Simplex Virus -1 detected/isolated in ocular specimens" at the Indian Eye Research Group conference at organized by Aravind Eye Hospitals, Madurai, 2007.
- Presented a Poster on "A review on the outbreaks of epidemic conjunctivitis caused by viral etiologic agents in Chennai"at International conference, Chennai, 2008.
- Presented a Poster on "Comparison of pp65 antigenemia assay with Real Time Polymerase Chain Reaction for the detection of HCMV infections in renal Transplant patients" at IAMM conference held at AFMC, Pune, 2008
- Presented a Poster on **"**Genotypic and phenotypic characterization of the acyclovir resistance among Herpes simplex virus 1 isolates" at BITS, Pilani, 2009.
- Presented a oral presentation on **"**Genotyping of Rubella virus" at the International Asia ARVO conference, Hyderabad, 2009.

BRIEF BIOGRAPHY OF THE CANDIDATE

Mr. Y. Samson Moses, completed his under graduation B.Sc Microbiology from M.G. R. College of Arts & Science, Hosur in the year 2000. He completed his Diploma in Medical Microbiology, from Christian Medical College & Hospital, Vellore in the year 2003. Thereafter he worked as a Junior Microbiologist in the same campus for a period of 5 months. He joined the off campus MS Medical Laboratory Technology conducted by BITS, Pilani and graduated in the year 2006. He worked as a Junior Scientist at VRF Referral Laboratory (a unit of Vision Research Foundation), Chennai from August 2006 to January 2007. He joined as a Senior Research Fellow at L&T Microbiology Research Centre in January 2007. Meanwhile He registered for PhD in BITS, Pilani in November 2006 under the guidance Dr. J. Malathi. He has attended a Workshop on Diagnostic Methods for Leptospirosis 'LEPTOSHOPPE-2007' organized by The Indian Association of Medical Microbiologists, Tamilnadu & Pondicherry Chapter in association with The Tamilnadu Dr. M. G. R Medical University, Chennai & Institute of Microbiology, Madurai Medical College, Madurai. He has made 1 oral and 4 poster presentations in National and International conferences. He has 4 publications. He has been responsible for conducting practical and theory classes in microbiology and immunology for the under graduate(BS Optometry) and post graduate (MSMLT) students registered under off campus courses of BITS Pilani. He was also involved in training the post graduate students from other institutes in various molecular biological techniques. He was awarded the Best performance in Clinical Microbiology, He has been awarded the travel fellowship at the IERG conference held at Madurai. He is presently working as a Junior Executive at VRF Referral Laboratory. He is responsible for the Molecular diagnostics of infectious diseases. He is also also responsible for conducting Microbiology classes for Under-graduate (B.Sc MLT) students registered under Tamilnadu Dr. M.G.R Medical University.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. J. Malathi completed her PhD from Birla Institute of Technology and Science in 2003. At present she is the Reader in the Department of Microbiology, L & T Microbiology Research Centre, Vision Research Foundation, Chennai. She has 13 years of rich experience in Medical Microbiology with 14 year postgraduate teaching in Medical microbiology. She is a recognized Ph. D guide in BITS-Pilani, and SASTRA. She had been involved in the initiation of Reverse Transcriptase PCR and Real Time PCR) development of nucleic acid–based molecular biological methods in L&T Microbiology Research Centre, Vision Research Foundation, Chennai. She is Principal Investigator of 2 and Co- Principal Investigator of 4 Research projects sanctioned by ICMR and DBT. Her areas of special interest are development of rapid diagnostic tests based on molecular techniques for rapid detection of infectious agents and diagnostic DNA chip for infectious diseases and to study the Molecular epidemiology of specific infectious diseases particularly related to ophthalmic infections, studying viral pathogenic mechanism and drug resistance. She has 38 publications in peer reviewed National and International journals. Two patents have been applied. She had done 25 presentations in National & International conferences, Invited academic lectures, National & International Symposia; CME programmes. She is a Life Member of Indian Association of Medical Microbiologists.