LITERATURE REVIEW

CHAPTER TWO: LITERATURE REVIEW

2.1 HUMAN HERPESVIRUS-6

2.1.1 The discovery of a novel herpesvirus

The discovery of the sixth human herpesvirus in 1986 was as a consequence of research aimed at detecting viruses that infect lymphoid cells. Salahuddin *et al.* (1986) isolated a previously unrecognized virus from co-cultures of peripheral blood mononuclear cells (PBMC) and cord blood mononuclear cells of patients with AIDS and lymphoproliferative disorders. The novel virus was tentatively named Human B Lymphotropic Virus (HBLV) based on preliminary studies which indicated infection of B lymphocytes.

Subsequent morphological descriptions revealed features typical of a herpesvirus (Josephs *et al.*, 1986; Lusso *et al.*, 1987) and primary tropism for $CD4^+$ T lymphocytes. The virus was consequently renamed HHV-6 (Ablashi *et al.*, 1987) based on the proposal of the International Committee on Taxonomy of Viruses (Roizman *et al.*, 1981). Since the isolation of HHV-6 by Salahuddin and co-workers, detection of HHV-6 has been reported in other laboratories from patients with AIDS (Downing *et al.*, 1987; Tedder *et al.*, 1987; Agut *et al.*, 1988; Lopez *et*

al., 1988), chronic fatigue syndrome (Ablashi *et al.*, 1988a) and infants with exanthem subitum (Yamanishi *et al.*, 1988).

2.1.2 Ultrastructure

Examination of HHV-6 particles in infected cells by electron microscopy revealed that the viral morphology was characteristic of the herpesvirus group (Biberfeld *et al.*, 1987), and on DNA hybridization it was easily distinguished from the known herpesviruses (Josephs *et al.*, 1986; Salahuddin *et al.*, 1986).

The viral nucleocapsid containing double-stranded DNA genome is composed of 162 capsomeres forming an icosahedral symmetry surrounding an electron-dense core. HHV-6 viral DNA is associated with the core, a smooth torus of uniform thickness approximately 65 nm in diameter (Biberfeld *et al.*, 1987). A prominent tegument approximately 20-40 nm thick surrounds the capsid. The whole structure is enveloped by a lipid membrane with glycoprotein spikes which places the diameter of the mature virion as approximately 160-200 nm (Biberfeld *et al.*, 1987; Yoshida *et al.*, 1989).

All the characteristics above classify HHV-6 as a herpesvirus with distinct features but compared to the other herpesviruses, it shares some features with HCMV. Both HHV-6 and HCMV have teguments, but

unlike HHV-6, HCMV appears as a torus of varied thickness (Biberfeld *et al.*, 1987).

2.1.3 Morphogenesis

HHV-6 nucleocapsids are assembled in the nuclei of infected cells. Newly replicated viral DNA are inserted into capsids. By electron microscopy, empty and DNA-filled nucleocapsids were observed in the nucleus. Fully tegumented nucleocapsids were observed only in the cytoplasm, however, partially tegumented nucleocapsids were occasionally found in the nucleus. Empty capsids were rarely seen in the cytoplasm (Biberfeld *et al.*, 1987).

A model for virion maturation was proposed by Roffman *et al.* (1990) based on electron microscope observations. It was noted that nucleocapsids in the nuclei of infected cells were not surrounded by tegument. Instead these unenveloped capsids were found coated with tegumental material of varied thickness in the lumen of double-membraned compartments. These intranuclear compartments called tegusomes, which are islets of cytoplasm invaginated into the nucleus, aid in the maturation process. Full capsids bud through the tegusomal membranes in a step involving the envelopment of the capsid which soon loses its' envelope as it buds at the membrane and gradually acquires a complete tegument in the

tegusome. The tegusome then fuses with the nuclear membrane and releases the tegumented nucleocapsid into the cytoplasm (Roffman *et al.*, 1990). These particles then bud into cytoplasmic vacuoles and acquire envelopes. The vacuole containing the virion fuses with the cell membrane and releases the mature virion by exocytosis.

Nii *et al.* (1990) observed two sites for HHV-6 envelopment in the infected cell. Budding of capsids through the inner nuclear membrane gave rise to enveloped viral particles approximately 150 nm in diameter without a prominent tegument. Alternatively, by fusing with the outer nuclear membrane, virions with distinct tegument lose their envelopes and bud into vacuolar membranes or cytoplasmic vesicles to once again acquire envelopes with surface spikes, in the cytoplasm (Nii *et al.*, 1990; Kramarsky and Sander, 1992).

All extracellular HHV-6 virions outside the infected cell had a distinct tegument around their capsids, however capsids enveloped at the inner nuclear membrane were found to have indistinct tegument or none at all. The fact that enveloped intranuclear capsids are not tegumented and do not have spikes but extracellular virions have both, suggest that the pathway via tegusomes and cytoplasmic vacuoles is a more plausible viral transport mechanism for infectious virus.

2.1.4 Replicative Cycle

A schematic representation of the replication cycle of HHV-6 based on the various studies on cellular tropism and prevalence in body fluids is given in Figure 2.1. Upon primary infection, herpesviruses establish latent infections (Lusso, 1992). Primary infection with HHV-6 normally occurs in early childhood, causing exanthem subitum (ES) (Yamanishi *et al.*, 1988) but the virus persists through adulthood being shed intermittently in the saliva. Reactivation or asymptomatic chronic infection may account for persistent viral shedding (Levy *et al.*, 1990).

Takahashi *et al.* (1989) isolated HHV-6 from $CD4^+$ mature T lymphocytes of ES patients. In the acute phase of disease, both $CD4^+$ cells and monocytes/macrophages contained HHV-6 DNA. Following recovery, viral DNA was found only in the monocytes/macrophages suggesting HHV-6 persists in a latent state. In a study by Kondo *et al.* (1991), it was found that HHV-6 may establish a latent infection in monocytes/macrophages *in vivo* and using an *in vitro* latency system,

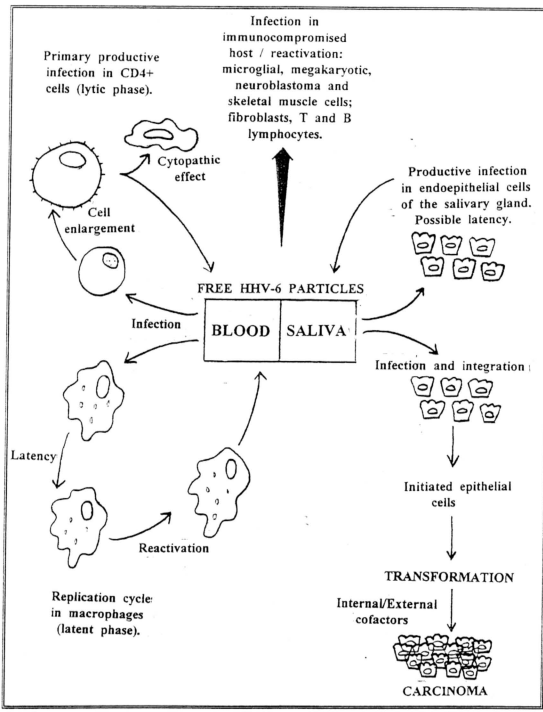

Figure 2.1 : Schematic representation of the replicative cycle of HHV-6

(Yadav, personal communication)

showed that reactivation was possible by phorbol ester treatment. The same was also noted by Kikuta *et al.* (1991).

The main site of acute viral replication is the $CD4^+$ T lymphocytes in which the infected cell becomes enlarged due to viral production. However, CD4 is not the surface receptor for HHV-6, in fact the process for selection is unknown (Lusso *et al.*, 1989a). Mature virions are initially released by exocytosis. Subsequently, the cell dies as a result of cytopathic effect, releasing free virus particles (Fig. 2.1) which infect cells via endocytosis (Cirone *et al.*, 1992).

HHV-6 DNA and proteins were observed in high proportions in endoepithelial cells of salivary gland tissue. HHV-6 may hibernate in a latent state in these cells, occasionally producing free virus once reactivated, into the saliva (Fox *et al.*, 1990; Krueger *et al.*, 1990). This is in accord with the study by Levy *et al.* (1990) in which HHV-6 was shed intermittently in the saliva.

Recent studies on epithelial carcinomas revealed HHV-6 infection within the transformed cells. Figure 2.1 presents a hypothesis on the replicative cycle of HHV-6 in these tissues (Yadav *et al.*, 1994). HHV-6 DNA was found in cervical carcinoma by PCR but the location of the virus in the carcinoma was not known (Wang, *et al.*, 1994a). However Chen *et al.* (1994a) observed that HHV-6 could infect HPV-transformed

epithelial cells and persist in a latent state. Similarly, HHV-6 specific antigens and DNA were noted in transformed epithelial cells of oral carcinoma (Yadav *et al.*, 1994).

It has been hypothesized that HHV-6 particles in the saliva readily establish latent infection in endoepithelial cells. Its' reactivation may contribute to immunosuppression and transactivate cellular and/or HPV viral oncogenes in the cell (Chen *et al.*, 1994a; 1994b). The virus genome also contains transforming sequences that may contribute towards neoplasia (Razzaque, 1990; Razzaque *et al.*, 1993). HHV-6 most likely acts in synergy with cofactors such as carcinogens and other viruses to transform epithelial cells.

2.1.5 Interstrain Heterogeneity

Biological, immunological and molecular analysis of HHV-6 isolates revealed distinct differences, in which at least 2 groups were distinguished, that is, variant A and variant B. Variant A (GS-type) strains were in general, isolated from patients with lymphoproliferative disorders and immunocompromised adults while variant B (Z29-type) strains were isolated from children with primary infection (Ablashi *et al.*, 1991; Aubin *et al.*, 1991; Schirmer *et al.*, 1991; Dewhurst *et al.*, 1992; Aubin *et al.*,

1993) except in two patients, from which both variants were isolated (Dewhurst *et al.*, 1992).

Based on *in vitro* growth studies of HHV-6 in continuous cell lines, it was found that susceptibility to viral infection varied between isolates. GS-type isolates infected HSB-2, J JHAN, Sup T1 cells, while Z29-type strains grew well in MOLT-3 cells (Wyatt *et* al., 1990; Ablashi *et al.*, 1991; Dewhurst *et al.*, 1992). Variant A strains were more lytic, disclosing a higher cytopathic effect than variant B strains (Ablashi and Salahuddin, 1992; Berneman and Ablashi, 1992).

Of the seven monoclonal antibodies (MAbs) raised against HHV-6 GS strain, four reacted with 31 GS-type and Z29-type isolates, however, three MAbs reacted only with isolates similar to GS and U1102 (Wyatt *et al.*, 1990; Ablashi *et al.*, 1991; Schirmer *et al.*, 1991; Chandran *et al.*, 1992; Dewhurst *et al.*, 1992; Aubin *et al.*, 1993). Non-reactivity with a MAb suggests differences in the antigenic make up of the strains. The strains could be divided into 2 groups containing group-specific epitopes (Balachandran, 1992).

Isolates could also be distinguished based on hybridization patterns and restriction fragment length polymorphisms of the entire genome. These isolates were found to segregate into two groups, consistent

with MAb studies and growth properties (Wyatt *et al.*, 1990; Ablashi *et al.*, 1991; Aubin *et al.*, 1991; Schirmer *et al.*, 1991).

Nucleotide sequence analysis of polymerase chain reaction (PCR) products and plasmid clones further supports the presence of two groups of isolates closely related to each other (Aubin *et al.*, 1991; Teo *et al.*, 1991; Gompels *et al.*, 1995). Aubin *et al.* (1991) however considered the classification of HHV-6 into 2 variant groups too limiting as most isolates within the same group can be differentiated from one another. Chou and Marousek (1994) went further to segregate the variant B strains into two clusters, group 1 and 2 based on nucleotide and translated peptide sequence homology in a putative immediate-early region of HHV-6. This subdivision was first proposed by DiLuca *et al.* (1992).

2.1.6 Molecular Biology - Genome

HHV-6 has a linear, double-stranded DNA genome with a mean G+C content of 43-44% based on CsCl density gradient studies. The genome size varies between strains, ranging between 160-170 kb as estimated by summation of restriction enzyme fragment lengths and pulsed-field gel electrophoresis (Lopez and Honess, 1990; Lindquester and Pellett, 1991). The viral genome structure is composed of an A+T rich unique long sequence (UL) of 141-142 kb flanked by G+C rich terminal direct

repeats (DR) varying from 10 to 13 kb at each end of the genome (Pellett *et al.*, 1990; Lindquester and Pellett, 1991; Martin *et al.*, 1991a; Neipel *et al.*, 1991).

Head to tail genomic concatemers were found in infected cell nuclei; similar to other herpesviruses, viral replication appears to be carried out via the rolling circle mechanism in which head to tail concatemers are generated, then cleaved to unit length linear genomes prior to capsid assembly (Martin *et al.*, 1991a).

Both the left (DR_L) and right (DR_R) direct repeat elements of HHV-6 genome contain a region of interstrain and intrastrain length heterogeneity (het) located at the left end of the DRs (Fig. 2.2) (Pellett *et al.*, 1990; Lindquester and Pellett, 1991). A 6 base repeat sequence GGGTTA was mapped to the right end of DR elements, present as a tandem repeat with 60 copies (Kishi *et al.*, 1988; Martin *et al.*, 1991a). This repeat motif is similar to human telomeric sequences (Meyne *et al.*, 1989) which was also noted in other lymphotropic herpesviruses, including Marek's disease virus (MDV) sequences (Kishi *et al.*, 1988) and HHV-7 sequences.

Cross -hybridization was observed between HHV-6 and MDV at this region (Kishi *et al.*, 1988). Various roles have been suggested to

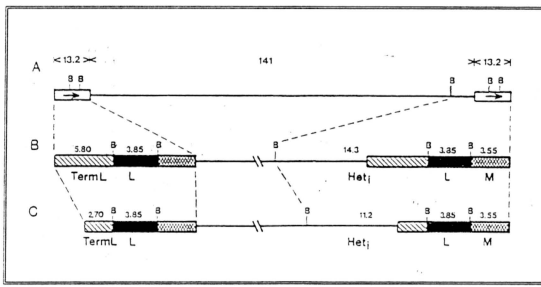

Figure 2.2 : Model of HHV-6 genome architecture

(A) Longer form of the genome. (B) and (C) Enlargements of the direct repeat elements in the longer and shorter forms of the genome, respectively.
The location of BamH1 sites, **B** and relevant fragments are identified. Fragment lengths are in kb (Lindquester and Pellett, 1991).

account for the presence of the GGGTTA sequence in HHV-6. The sequence may function in a similar manner as the DR_2 sequence of HSV-1, in the cleavage of concatemeric genomes or play a role in efficient viral chromosome segregation during cell division (Thomson *et al.*, 1994a). It has been suggested that integration of viral DNA into the host genome occurs with this sequence (Luppi *et al.*, 1993). Torelli and co-workers (1995) have located an integration site for HHV-6 in the human telomeric region of chromosome 17.

Additionally, the termini of DR_L and DR_R contain *cis*-acting sequences, ***pac*-1** and ***pac*-2**, which are involved in DNA packaging (Gompels and Macaulay, 1995). The junction between the DR termini of concatemerized genome consists of ***pac*-2.cs.*pac*-1** in which **cs** is the cleavage site (Thomson *et al.*, 1994a).

The HHV-6 genome contains an origin of lytic replication, *ori*-Lyt in the UL, upstream of the gene encoding the major DNA binding protein (MDBP). HHV-6 encodes an origin of replication binding protein (OBP) which binds to sequences in the *ori*-Lyt (Gompels *et al.*, 1992; Dewhurst *et al.*, 1993; Inoue *et al.*, 1994). Thomson *et al.* (1994a) suggested that a separate *ori* in the DRs may be responsible for plasmid maintenance in latency.

The UL sequence of HHV-6 contains 3 clusters of major repeats designated R_1, R_2 and R_3 by Gompels *et al.* (1995). R_1 which are the SR repeats, encode an SR domain (Nicholas, 1994), R_2 is a 1.5kb array of T(C/G) sequence and R_3 is an internal repeat (IR) array of approximately 30 copies of a repeat unit containing consensus binding sites for the transcription factors AP2 and $NF_\kappa B$ (Martin *et al.*, 1991b). The IR array in the HHV-6 U1102 features tandem repeat units of 110bp with a *Kpn*1 site (Martin *et al.*, 1991a), however in the B variant strain Z29 genome, the length of the repeat unit is 105 bp with a *Sac*2 site instead of a *Kpn*1 site (reviewed in Inoue *et al.*, 1993).

Cross-hybridization studies between HHV-6 and other human and animals herpesviruses were initially negative (Josephs *et al.*, 1986; Lopez *et al.*, 1988) however, Efstathiou *et al.* (1988) showed that a 5.5 kb cloned fragment of HHV-6 hybridized to HCMV under stringent conditions. This cross reactivity is due to a 22 kb region in the HHV-6 genome that is homologous and encodes for several conserved genes among other herpesviruses. It was found that the organization of the open-reading frames (ORFs) in the cross-hybridizing region is closely analogous and shares 66% amino acid sequence homology to HCMV (Lawrence *et al.*, 1990).

While HHV-6 has some lymphotropic properties, based on amino acid sequence homology and gene organization, HHV-6 is more

closely related to HCMV, a betaherpesvirus. An overall colinearity was observed between HHV-6 and HCMV, with HHV-6 being classified as a betaherpesvirus (Lawrence *et al.*, 1990; Neipel *et al.*, 1991). A closer homology has been observed between HHV-6 and HHV-7, sharing similar genome organizations and a tropism for T lymphocytes (Frenkel *et al.*, 1990; Berneman *et al.*, 1992; 1995).

Nucleotide sequence analyses have been carried out on various HHV-6 strains, including U1102, GS, Z29, SIE, TAN, MBE, HST and AJ (Inoue *et al.*, 1993). Of these, strain U1102, a representative of HHV-6 variant A (Ablashi *et al.*, 1993), has been sequenced completely by Gompels and colleagues (1995). It was found that nucleotide sequence homology of corresponding regions of HHV-6A and B variants are 90-95% and between isolates within a variant, 99% (Inoue *et al.*, 1993).

Genes encoded by HHV-6 strain U1102 were identified by ORFs greater than 80 bp, 75% non-overlapping, containing initiating methionine codons (ATG); amino acid sequence similarity to other herpesvirus or cellular proteins, and transcriptional initiation/termination signals (Gompels *et al.*, 1995).

Spliced genes were also identified for ORFs without an initiation codon in the first 30% of the sequence. One hundred and nineteen ORFs were designated DR1 to DR8 in the DRs, U1 to U100 in

the UL segment and LT1, LJ1 and RJ1 spanning the telomeric repeats (Fig. 2.3). Of these, 102 ORFs seem to be distinct genes likely to encode proteins (Gompels *et al.*, 1995).

Analysis of the immediate-early (IE) locus to the left of IR, has identified spliced genes, among them U90 which is spliced to U89 and other sequences. Other spliced genes may be identified in this region with further analyses (Martin *et al.*, 1991b; Nicholas, 1994; Schiewe *et al.*, 1994). The HHV-6 IE locus corresponds to the HCMV major IE genes based on its location, and low CpG dinucleotide frequency. At least 2 ORFs within the HHV-6 IE locus can transactivate the HIV long terminal repeat (LTR) *in vitro* (Martin *et al.*, 1991b).

Comparative analyses of encoded amino acid sequences revealed genes conserved in all herpesviruses. These 'core' genes encode proteins essential for enzymatic, structural and regulatory functions for viral entry, DNA synthesis and virion assembly. Seven discrete gene blocks represent regions in which the number, order and transcriptional polarity of genes within each gene block is conserved among the herpesviruses to some degree (Chee and Barrell, 1990).

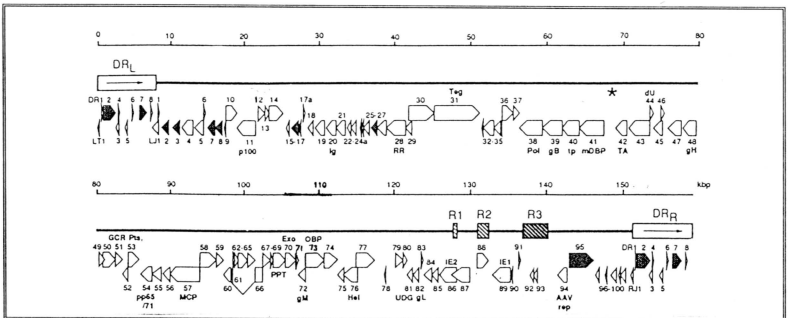

Figure 2.3 : Predicted HHV-6 gene organization

Protein coding regions are indicated by open arrows, *ori*-lyt by a star. GCR (G-protein coupled receptor), RR1 (large subunit of ribonucleotide reductase), Teg (tegument protein), POL (DNA polymerase), tp (transport protein), TA (conserved herpesvirus transactivator), Pts (protease/assembly protein), exo (alkaline exonuclease), UDG (uracil DNA glycosylase), Hel (helicase) (Gompels *et al.*, 1995).

The order in which these 7 gene blocks are located in the genome and the orientation of each block vary among the herpesvirus subfamilies, namely alpha, beta and gamma (Inoue *et al.*, 1993).

The arrangement of genes in HHV-6 is similar to HCMV, that is, the betaherpesvirus gene arrangement. HHV-6 has a core of 86 kb, extending from U27 to U81, which is the most compact of herpesviruses. The region spanning U2 to U86 (116,175 bp) in HHV-6 is colinear to the region in HCMV between UL23 to UL122 (143,012 bp).

A general feature of betaherpesviruses are the presence of gene families, some gene families in HHV-6 are related to those in HCMV. One such example is the HCMV US22 gene family which is closely related to HHV-6 DR7 in an inverse orientation. The HHV-6 DR region is related to the HCMV short region. Both regions are located at the genome ends, contain **pac-1** and **pac-2** sites and encode members of the HCMV US22 gene family (Chee *et al.*, 1990; Thomson *et al.*, 1994a, Gompels *et al.*, 1995).

Highly conserved ORFs encoding major capsid protein (MCP) and alkaline phosphatase were found in the region studied by Lawrence *et al.* (1990) now known as U53 to U71 in the UL segment. The organization of ORFs U56, U57 and U58 (formerly designated 3L, 4L and 5R) in HHV-6 is in the same orientation as HCMV homologs, and

homologs of HHV-6 U59, U62 and U68 (formerly designated 6R, 8R and 14R) are found only in HCMV (Lawrence *et al.*, 1990; Gompels *et al.*, 1995). This further strengthens the relationship between HHV-6 and HCMV to the likeness of HSV-1 and VZV (Davison and McGeoch, 1986; Lawrence *et al.*, 1990).

An interesting find in the nucleotide sequence of HHV-6A (U1102) was the transformation suppressor (*ts*) gene encoding a protein similar to the *rep* 68/78 gene product of the adeno-associated virus-2 (AAV-2) (Thomson *et al.*, 1991; Araujo *et al.*, 1995). The HHV-6B (Z29) *rep* 68/78 homolog was located analogous to the HHV-6A *ts* gene with more than 96% homology (reviewed in Inoue *et al.*, 1993; reviewed in Araujo *et al.*, 1995). Similar to the AAV *rep* 68/78, HHV-6A *ts* suppressed H-*ras* transformation of NIH3T3 cells at the transcriptional level and inhibited HIV-1 LTR expression (Araujo *et al.*, 1995).

2.1.7 Molecular Biology - Proteins

HHV-6 proteins are determined from experimental data or extrapolated from homologs of other sequenced herpesviruses. Glycoproteins, gB and gH are conserved in all herpesviruses, their gene homologs in HHV-6 are U39 and U48 (Gompels *et al.*, 1995). The major envelope glycoprotein, gB has an important role in viral entry and in the fusion of

infected cell membranes in syncytial formation. Located on the virion surface, HHV-6 gB induces neutralizing antibodies in the presence of complement (Chou and Marousek, 1992; Foa-Tomasi *et al.*, 1992).

Glycoprotein B undergoes antigenic variations, segregating HHV-6 isolates into two groups, variant A and B. MAb 2D10 immunoprecipitated glycosylated spesies of 112 kDa, 64 kDa and 58 kDa from HHV-6A-infected cells and spesies of 102 kDa, 59 kDa and 50 kDa from HHV-6B-infected cells. Variant-specific MAb 2D9 reacted only with HHV-6A gB (Foa-Tomasi *et al.*, 1992; Campadelli-Fiume *et al.*, 1993; Ellinger *et al.*, 1993).

The similarity in the electrophoretic profiles of proteins immunoprecipitated by MAbs 2B9, 2D9, 2D10 and 6A5G3 suggest that these MAbs react with the same glycoprotein, that is gB (Campadelli-Fiume *et al.*, 1993). This is indicative that the glycoprotein designated gp116/64/54 immunoprecipitated by MAb 6A5G3 is actually gB (Balachandran *et al.*, 1989; Campadelli-Fiume *et al.*, 1993).

Glycoprotein H, a transmembrane envelope glycoprotein, has a similar role as gB in herpesviruses, involved in cell fusion and virus penetration. HHV-6 gH found on the virion surface, contain neutralizing antibodies that inhibit cell to cell fusion and virus infectivity (Foa-Tomasi *et al.*, 1991; Liu *et al.*, 1993a). MAb 2E4 reacted with gp100 and its'

cleavage products of apparent molecular weight 80 kDa and 32.5 kDa (Foa-Tomasi *et al.*, 1991). Other immunoprecipitation reactions revealed a 102K phosphoprotein as the glycoprotein gH of both HHV-6 variants suggesting conserved functions of gH among the variant groups (Qian *et al.*, 1993). Glycoprotein H forms a heterodimer membrane glycoprotein complex with gL (Liu *et al.*, 1993b).

Nucleotide sequence analysis of HHV-6 (U1102) has identified an ORF, 18L (designated U72 by Gompels *et al.*, 1995) that encodes an integral multiple membrane spanning hydrophobic glycoprotein, gM, conserved in all herpesviruses. (Lawrence *et al.*, 1995). The major virion envelope glycoprotein complex gp82/105 is encoded by spliced genes located at the DRs and the right end of the UL segment of HHV-6A genome. MAbs against these proteins neutralize HHV-6A isolates, suggesting that gp82/105 has a role in viral infectivity (Balachandran, 1992; Pfeiffer *et al.*, 1995).

While the membrane proteins mentioned above play a role in viral entry and spread, immediate-early (IE) proteins control the expression of delayed early genes and late genes in a 'cascade' manner. Many HHV-6 IE genes act as transcriptional activators, including U27, U41 and U94 which encode p41, MDBP and *ts* respectively (Thompson *et al.*, 1994a; Zhou *et al.*, 1994; Araujo *et al.*, 1995; Gompels *et al.*, 1995).

HHV-6 p41 is a 41 kDa phosphoprotein conserved among both the variant groups, recognized by MAb 9A5D12 (Chang and Balachandran, 1991). Produced early in the replicative cycle, HHV-6 p41 DNA-binding protein is the positional homolog to HCMV ICP36, a processivity factor which stimulates viral replication by association with viral DNA polymerase. Thus, p41 may have a role in viral replication similar to ICP36 (Agulnick *et al.*, 1993; Thompson *et al.*, 1994a).

The major immunoreactive phosphoprotein designated p100 in HHV-6A and 101K in HHV-6B is weakly homologous to HCMV pp150, a virion tegument protein (Neipel *et al.*, 1992; Pellett *et al.*, 1993). Analysis of an 8.9 kb *Hin*d III fragment HHV-6 clone, pZVH14 identified the putative large tegument protein (LTP) in which the predicted product of U31 was homologous to HCMV LTP (Josephs *et al.*, 1986; 1991; Gompels *et al.*, 1995). The MCP homolog of HHV-6 encoded by U57 is closely homologous to the HCMV MCP. Both HHV-6 variants contained similar sized proteins, that is, 135 kDa as determined by MAb reactivity (Balachandran *et al.*, 1989; Littler *et al.*, 1990).

Proteins necessary for replication are conserved in HHV-6 including, DNA polymerase, MDBP and origin binding protein (OBP) (Gompels *et al.*, 1995). HHV-6B *ori*-Lyt contains two binding sites for the alphaherpesvirus origin-binding protein homolog (OBP_{H6B}). Similar to

alphaherpesvirus origin regions, the two OBP_{H6B} binding sites lie within 23 bp segments, separated by an AT-rich region and have an imperfect dyad symmetry (Dewhurst *et al.*, 1994; Inoue *et al.*, 1994). OBPs of both HHV-6 variants are functionally interchangeable. Based on the similarity between HHV-6B *ori*-Lyt and HSV-1 ori_s, it was deduced that efficient functioning of HHV-6B *ori*-Lyt in the initiation of DNA replication requires the two OBP_{H6B} binding sites (Dewhurst *et al.*, 1994; Inoue *et al.*, 1994).

2.1.8 Seroepidemiology and Transmission

Seroepidemiological studies have revealed that HHV-6 is highly prevalent in human populations in the various continents namely Asia (Yamanishi *et al.*, 1988; Balachandra *et al.*, 1989; Okuno *et al.*, 1989; Yadav and Ablashi, 1990; Shanavas *et al.*, 1992), Australia (Pietroboni *et al.*, 1988), Africa (Downing *et al.*, 1987; Tedder *et al.*, 1987; Agut *et al.*, 1988; Lopez *et al.*, 1988), Britain (Briggs *et al.*, 1988), Europe (Krueger and Ablashi, 1987; Linde *et al.*, 1990) and the United States (Brown *et al.*, 1988; Lopez *et al.*, 1988).

Seroconversion occurs early in life, with primary infection commonly observed between six and 24 months of age after the waning of maternal antibodies (Briggs *et al.*, 1988; Balachandra *et al.*, 1989). More than 90% of children between one and four years of age are HHV-6

seropositive (Horwitz and Beneke, 1993). Reinfection may occur with different strains of the virus in which various strains may be present in an individual at different sites. Following primary infection, the virus persists in the PBMCs and the central nervous system (Hall, 1995).

Based on detection techniques and cut-off values, the IgG seropositivity in older children and adults ranged between 60 to 85 percent (Ablashi *et al.*, 1988a; Saxinger *et al.*, 1988; Yadav *et al.*, 1991). It was noted that HHV-6 seropositivity declined with age (Levy *et al.*, 1990; Yanagi *et al.*, 1990) while females had slightly higher titers than males (Briggs *et al.*, 1988; Clark *et al.*, 1990).

High seroprevalence and early age of primary infection both indicate that HHV-6 is ubiquitous, in which transmission is believed to occur horizontally in the home environment. While the mother is believed to be the main source of infection, transmission may occur in the postnatal period. Serological studies on pregnant women, mothers of children with ES and control women of similar ages harboured no significant differences. Also, the seropositivity of pregnant women in their first and third trimesters were unchanged suggesting no HHV-6 reactivation (Balachandra *et al.*, 1989; Yoshikawa *et al.*, 1990).

Transmission of HHV-6 via breast milk has been ruled out following seroconversion in infants who did not receive breast milk and the

failure to detect viral genetic sequences in breast milk (Takahashi *et al.*, 1988; Dunne and Jevon, 1993).

The main route of transmission appears to be via secretions from the upper digestive or respiratory tract. HHV-6 is shed intermittently in the saliva of healthy and immunocompromised individuals. The virus has been isolated from the saliva of more than 80% of these individuals (Pietroboni *et al.*, 1988; Gopal *et al.*, 1990; Jarrett *et al.*, 1990; Levy *et al.*, 1990). With saliva as a source of transmission (Takahashi *et al.*, 1988; Levy *et al.*, 1990), strong suggestions implicate the salivary gland (Fox *et al.*, 1990; Krueger *et al.*, 1990) and bronchial gland (Krueger *et al.*, 1990) as sites of HHV-6 replication and persistence.

Apart from saliva, other routes of transmission include organ transplantation (Ward *et al.*, 1989), blood transfusion (Asano *et al.*, 1991b), urine (Luka *et al.*, 1990) and transplacental transmission (Aubin *et al.*, 1992).

2.1.9 Cellular Tropism

The establishment of optimized culture conditions for the propagation of HHV-6 in human mononuclear cells were achieved by Lusso *et al.* (1987). Consequently, it was shown that while other cell lineages are susceptible to infection, HHV-6 shows primary tropism for $CD4^+$ T lymphocytes (Lusso, 1987a). The identity of the HHV-6 cellular receptor is unknown, glycoprotein CD4 is not a receptor even though the virus infects $CD4^+$ cells (Lusso *et al.*, 1989). The cellular host range of the virus includes cells of both lymphoid and nonlymphoid origin, however only lymphoid cells allow a lytic viral replication cycle. HHV-6 infection of cells *in vitro* induces a cytopathic effect leading to massive cell death (Lusso, 1992).

HHV-6 infects mature $CD8^+$ T lymphocytes, natural killer (NK) cells and γ/δ T lymphocytes inducing *de novo* expression of CD4, thus predisposing these cell types to HIV-1 infection (Lusso *et al.*, 1991, 1993, 1995) as the CD4 glycoprotein is a receptor component for HIV (Dalgliesh *et al.*, 1984).

B lymphocytes, previously immortalized by EBV, are also susceptible to HHV-6 infection (Ablashi *et al.*, 1988a). It has been suggested that EBV infection induces HHV-6 surface receptors or the

presence of EBV within the B cell overcomes intracellular restriction factors and encourages infection by HHV-6 (Lusso, 1992).

Limited HHV-6 replication has been noted in fibroblasts (Luka *et al.*, 1990), neuroblastoma cells (Levy *et al.*, 1990), megakaryoblasts, glioblastoma cells (Ablashi *et al.*, 1988a) and recently, skeletal muscle cells (reviewed in Lusso and Gallo, 1995).

Latent HHV-6 infection has been observed in mononuclear phagocytes in which no productive infection was seen in the monocytes (Kondo *et al.*, 1991). HPV-transformed cervical epithelial cells have also been found to harbour latent HHV-6 (Chen *et al.*, 1994a). Reports of HHV-6 in salivary gland and bronchial epithelial tissue (Fox *et al.*, 1990, Krueger *et al.*, 1990) further strengthen the observation that HHV-6 is capable of persistent, latent infection in human epithelial cells (Chen *et al.*, 1994a).

Both HHV-6 A and B exhibit differences in cellular tropism, suggesting that the variants recognize different receptor complexes on the cell membranes. Selective growth of HHV-6 isolates in established cell lines are a major criteria in the identification of the variants (Table 2.1). Variant A strains productively infect HSB-2, Jurkat and SupT1 while variant B strains grow best in MOLT-3 (Lusso, 1992).

It is quite clear that HHV-6 is an immunotropic virus, infecting directly cells of the immune system, thus evading the hosts' antiviral defence mechanism and ensuring its' survival *in vivo* (Lusso and Gallo, 1995).

Table 2.1 Characteristics of HHV-6 variant A and B

Characteristics	**Variant A (GS type)**	**Variant B (Z29 type)**
Cellular tropism	Pan-T, NK cells HSB-2 cell line	$CD4^+$ T cells MOLT-3 cell line
Cytopathic effect	+	+
Syncytia formation	+ (SupT1 cells)	–
Immunological reactivity	Distinct	Distinct
Genetic restriction pattern	Distinct	Distinct
Prevalence in humans	Low (?)	High

(modified from Lusso and Gallo, 1995)

2.1.10 Clinical Manifestations

Exanthem Subitum

Primary infection with HHV-6 causes exanthem subitum (roseola infantum or sixth disease) (Yamanishi *et al.*, 1988), a distinct clinical syndrome described by Zahorsky in 1910 (reviewed in Caserta and Hall, 1993). A disease of early childhood, exanthem subitum (ES) is characterized by abrupt fever (40°C) lasting between 3-5 days, rapid defervescence followed by the appearance of a faint morbilliform rash which diminishes without sequelae (Krugman *et al.*, 1985). The disease is almost always benign with no specific antiviral treatment required. While only 30% of infants display symptomatic ES (Knowles and Gardner, 1988; Yamanishi *et al.*, 1988), a large number show inapparent primary infection with HHV-6 (Takahashi *et al.*, 1988).

HHV-6 viremia has been noted in young children with febrile illness, associated with otitis (Asano *et al.*, 1989; Suga *et al.*, 1989; Pruksananonda *et al.*, 1992). Often, the degree of viremia correlates with the severity of clinical symptoms (Asano *et al.*, 1991b). Febrile convulsions have been observed in a proportion of ES cases in which HHV-6 was found in the cerebrospinal fluid (Kondo *et al.*, 1993). Rare complications arising from primary HHV-6 infection include meningo-encephalitis,

fulminant hepatitis and fatal multiple organ failure (Asano *et al.*, 1990a; Ishiguro *et al.*, 1990; Prezioso *et al.*, 1992).

HHV-6 variant B isolates appear to be responsible for ES (Ablashi *et al.*, 1991; Aubin *et al.*, 1991; Dewhurst *et al.*, 1992; DiLuca *et al.*, 1992; Pruksananonda *et al.*, 1992) however a mixture of both A and B variants has been observed by Dewhurst *et al.* (1992) in two cases.

Infectious mononucleosis

Infectious mononucleosis (IM) is classically an illness induced by primary EBV infection in young adults. Within two weeks of infection, heterophile antibodies are formed. HHV-6-associated IM also observed in young adults, resemble EBV-induced IM, with the exception of heterophile antibodies and exclusion of EBV and HCMV infection. Clinical manifestations include acute fever, sore throat and systemic lymphadenopathy accompanied by hepatosplenomegaly (Krueger and Sander, 1989).

In Malaysia, IM is usually caused by HHV-6 although HCMV is sometimes responsible (Tan and Henle, 1972; Yadav, 1991) because EBV infection occurs in early life and by adolescence most are EBV-positive. In Western societies, HHV-6 accounts for 10-15% of IM,

while a double infection with EBV and HHV-6 causes 58% of IM (Krueger and Sander, 1989).

Chronic Fatigue Syndrome

Chronic fatigue syndrome (CFS) is a disease which manifests as a combination of symptoms, including fatigue, fever, pharyngitis and impaired cognition (Komaroff *et al.*, 1992). The sudden onset of 'flu-like' symptoms marking the beginning of CFS implicate viruses in its' pathogenesis, among them human herpesviruses and retroviruses (Ablashi *et al.*, 1995a).

Compared to other human herpesviruses, HHV-6 seems to be more strongly associated with CFS. HHV-6 reactivation rates in CFS patients are higher than in healthy individuals. Reactivation of HHV-6 together with other viral agents could contribute to CFS by damaging the immune system (reviewed in Ablashi *et al.*, 1995a).

Organ Transplants

Significantly higher anti-HHV-6 IgG titers have been observed in immunosuppressed organ transplant recipients, including cardiac, renal and liver transplant patients (Irving *et al.*, 1988; Ward *et al.*, 1989; Chou and Scott, 1990; Okuno *et al.*, 1990). Similar observations were noted by

Asano *et al.* (1991a) in bone marrow transplant recipients. Reactivated HHV-6 may cause severe morbidity in immunosuppressed patients, especially after transplantation, being associated with fever, rash, encephalitis and interstitial pneumonitis (Irving *et al.*, 1988; Chou and Scott, 1990; Okuno *et al.*, 1990, Asano *et al.*, 1991a; Carrigan *et al.*, 1991; Yoshikawa *et al.*, 1991; Cone *et al.*, 1993; Drobyski *et al.*, 1994).

It has been observed that rejection rates are higher in kidney recipients demonstrating HHV-6 viremia post-transplantation (Okuno *et al.*, 1990). Also, poor graft function in bone marrow transplant recipients was seen in patients with HHV-6 infection. Both kidney rejection and poor bone marrow engraftment seem to be associated with HHV-6 infection (Okuno *et al.*, 1990; Carrigan *et al.*, 1991).

Acquired Immunodeficiency Syndrome

HHV-6 may be an opportunistic virus in immunosuppressed HIV positive individuals, similar to its' role in organ transplantation. In light of recent developments, HHV-6 might as well be a cofactor in the progression towards Acquired Immunodeficiency Syndrome (AIDS). The virus has a primary tropism for $CD4^+$ T lymphocytes similar to HIV, replicating in and subsequently destroying these cells. Double infection of

$CD4^+$ cells with HHV-6 and HIV-1 resulted in accelerated cytopathic effect (Lusso *et al.*, 1987; 1989b).

HHV-6 upregulates CD4 expression and induces *de novo* expression of the HIV receptor, CD4 in previously negative cells as mentioned earlier, thus increasing susceptibility to HIV infection (Lusso *et al.*, 1991; 1993; 1995).

HHV-6 contains sequences with the ability to transactivate HIV-1 LTR, including pZVB70, pZVH14 and *Sal*1-L sequences (Horvat *et al.*, 1991; Thompson *et al.*, 1994b). ORF-1 within *Sal*1-L encodes a protein which directly transactivates the HIV-1 LTR (Kashanchi *et al.*, 1994). HHV-6 also induces the release of cytokines, tumour necrosis factor α and interleukin 1β which regulate HIV expression in AIDS (Flamand *et al.*, 1991).

HHV-6 DNA and antigens have been found in various tissues in AIDS patients. A recent study of an HIV-infected infant revealed HHV-6 infection in the central nervous system resulting in fulminant encephalitis (Knox *et al.*, 1995). HHV-6 and HIV were detected in the retina, brain, lung, kidney and lymph nodes of AIDS patients (Qavi *et al.*, 1989; Corbellino *et al.*, 1993; Knox and Carrigan, 1994). HHV-6 infection is active and disseminated in AIDS patients, especially during symptomatic stages of the disease.

All these observations suggest an important role for HHV-6 as a cofactor in which the virus may reduce the $CD4^+$ T cell population in AIDS patients, transactivate HIV regulatory genes, stimulate HIV activatory cytokines, induce *de novo* expression of CD4 and affect the function of NK cells (Lusso and Gallo, 1994).

Neoplasia

Since the isolation of HHV-6 from patients with lymphoproliferative disorders (Salahuddin *et al.*, 1986), studies have been conducted to establish a role for the virus in various lymphomas. Conflicting reports further complicate the possibility of a causal role for the virus.

Earlier reports suggest a role for HHV-6 in Hodgkin's disease (HD) based on serological data (Clark *et al.*, 1990; Torelli *et al.*, 1991; Levine *et al.*, 1992) and antigenic studies (Iyengar *et al.*, 1991). Sumiyoshi *et al.* (1993) suggest that HHV-6 DNA detected in malignant lymphomas (ML), including Hodgkin's disease (HD), B cell and T cell lymphomas, were derived from latent infection.

Table 2.2 gives an overall view of possible HHV-6 associations with a variety of diseases. HHV-6 has also been found in

other lymphomas however the mere presence of the virus does not indicate a pathogenic role (Sumiyoshi *et al.*, 1993).

Recent detection of HHV-6 in solid tumours add to mounting interest of the possible link between virus and transformed cell. HHV-6 has been reported in oral and cervical carcinoma (Chen *et al.*, 1994b; Wang *et al.*, 1994a; Yadav *et al.*, 1994). It has been observed that HHV-6 has transforming potential (Razzaque, 1990; Razzaque *et al.*, 1993) and is capable of transactivating HPV in cervical carcinoma cell lines (Chen *et al.*, 1994a) suggesting that this novel virus plays a role in carcinogenesis.

Table 2.2 Proposed associations of HHV-6 with diseases

Disease	Reciprocal of Titer (IgG)	Comments	References
Exanthem subitum	80-2540 (strong)	Causative agent: primary HHV-6 infection	Yamanishi *et al.*, 1988
Infectious mononucleosis	80-10240 (strong)	Causative agent in the absence of EBV and HCMV infection: HHV-6	Niederman *et al.*, 1988; Krueger and Sander, 1989; Yadav, 1991
Chronic fatigue syndrome (CFS)	640-2540 (contributory)	Contributory role for HHV-6 in association with other human herpesviruses	Komaroff *et al.*, 1992; Ablashi *et al.*, 1995a
Fulminant hepatitis	-	Increased anti-HHV-6 IgG and IgM titers, absence of other hepatitis-causing viruses	Asano *et al.*, 1990a
Kikuchi	320-1280 (moderate)		Kikuchi, 1972; Eizuru *et al.*, 1989
Sarcoidosis	160-2540 (moderate)	Reactivated HHV-6 infection	Biberfeld *et al.*, 1988
Sjogren's syndrome	640-2540 (strong)	No distinct difference in HHV-6 seroprevalence between patients and controls	Krueger and Ramon, 1988; Ranger-Rogez *et al.*, 1995
Rosai-Dorfman	-		Levine *et al.*, 1992
Multiple sclerosis	160-2540 (strong)		Challoner *et al.*, 1995
Atypical polyclonal lymphoproliferation (APL)	-	Active dual infection by HHV-6 and EBV induce APL to malignancy (lymphoma)	Krueger *et al.*, 1989
Malignant lymphoma (Hodgkin's disease, Non-Hodgkin's disease)	160-5120 (weak)		Jarrett *et al.*, 1988; Josephs *et al.*, 1988; Clark *et al.*, 1990, Iyengar *et al.*, 1991; Torelli *et al.*, 1991
Kaposi's sarcoma	-	Associated with HHV-6 and the recently found Kaposi's sarcoma-associated herpesvirus (KSHV)	Bovenzi *et al.*, 1993; Ensoli *et al.*, 1995
Acquired immunodeficiency syndrome (AIDS)	80-5120 (contributory)	HHV-6 may be a cofactor in the development of AIDS	Lusso *et al.*, 1989b; Horvat *et al.*, 1991; Kashanchi *et al.*, 1994; Lusso and Gallo, 1994; Thompson *et al.*, 1994b;
Oral carcinoma	320-10240 (strong)	Elevated antibody levels and DNA in oral carcinoma suggest a cofactorial role in multistep carcinogenesis	Shanavas *et al.*, 1992; Yadav *et al.*, 1994
Cervical carcinoma	80-2540 (moderate)	HHV-6 transactivates HPV transforming genes, infects cervical epithelial cells, found in cervical carcinoma	Chen *et al.*, 1994a; b; Wang *et al.*, 1994; Yadav *et al.*, (in press).

2.1.11 Antiviral Therapy

The potential pathological role of HHV-6 in various diseases (Table 2.2) hastens the search for effective antiviral agents. It was found that phosphonoformate (PFA), phosphonoacetic acid (PAA) and ganciclovir (GCV) inhibit viral replication. Acyclovir, however is significantly ineffective against HHV-6, suggesting that HHV-6 does not encode viral thymidine kinase (Streicher *et al.*, 1988; Agut *et al.*, 1989; DiLuca *et al.*, 1990; Black *et al.*, 1991). While PFA, PAA and GCV are effective antivirals against HHV-6 replication, these pyrophosphate analogues are considerably toxic.

Recent evidence implicates Ampligen, a synthetic, double stranded RNA and Kutapressin™ (KU), a prescription drug as potent, antiviral agents. Both Ampligen and KU may inhibit viral attachment to cellular receptors and inhibit intracellular virus maturation *in vitro* exerting relatively low toxicity (Ablashi *et al.*, 1994a; 1994b). Strayer and colleagues (1995) have observed that Ampligen therapy in CFS patients yields significant improvements. Ampligen may prevent the release of HHV-6 from infected cells suggesting that continuous use of Ampligen could prevent reactivation (Ablashi *et al.*, 1994b).

Clinical trials using Ampligen and KU in patients with reactivated HHV-6 infection would be necessary to determine the efficacy of these drugs in relieving disease symptoms.

2.2 DIAGNOSTIC METHODS

Various diagnostic methods have been employed in the detection of HHV-6 infection based on immunology and microbiology. These include serodiagnostic tests, molecular detection of viral nucleic acids and proteins, and virus isolation by cell culture techniques.

2.2.1 Serodiagnostic Tests

HHV-6 has been studied using standard tests such as indirect immunofluorescence assay (IFA) (Salahuddin *et al.*, 1986; Tedder *et al.*, 1987, Aubin *et al.*, 1993), anticomplement immunofluorescence test (ACIF) (Lopez *et al.*, 1988, Yamanishi *et al.*, 1988) and enzyme-linked immunosorbent assay (ELISA) (Saxinger *et al.*, 1988; Asano *et al.*, 1990b; Iyengar *et al.*, 1991). Other tests include radioimmunoprecipitation (RIP) (Ablashi *et al.*, 1988a; Balachandran *et al.*, 1989), neutralization (Suga *et al.*, 1990; Aubin *et al.*, 1993) and immunoblotting (IB) (Ablashi *et al.*, 1988a; Iyengar *et al.*, 1991).

The specificity and sensitivity of HHV-6 detection vary among tests. While IFA allows quick identification, non-specific fluorescence poses difficulties in the accurate reading of results. To reduce this occurrence, ACIF has been used to detect anti-HHV-6 antibody,

yielding intense and specific signal even with low antibody titer values (Yamanishi *et al.*, 1988).

The ELISA, a highly specific and extremely sensitive test, can be used to detect antigen from blood samples (direct ELISA) and antibodies in human sera (indirect ELISA). Most laboratories utilize the indirect ELISA to detect human antibodies to HHV-6 proteins (Saxinger *et al.*, 1988; Asano *et al.*, 1990b; Iyengar *et al.*, 1991).

The use of monoclonal antibodies (MAbs) directed against HHV-6 proteins (Balachandran *et al.*, 1989) has resulted in a mild controversy. While no significant antigenic cross-reactivity has been observed between HHV-6 and most human herpesviruses, increased antibody titers to HHV-6 have been noted following primary HCMV infection (Chou and Scott, 1990; Irving *et al.*, 1990; Sutherland *et al.*, 1991; Ward *et al.*, 1991; Adler *et al.*, 1993). This observation may be a result of simultaneous HHV-6 and HCMV infection, reactivation of HHV-6 induced by HCMV or cross-reactive antigens (Adler *et al.*, 1993).

Most investigators however found no evidence for cross-reactivity following absorption studies with HHV-6 or HCMV antigens, concluding that both infections were genuine (Saxinger *et al.*, 1988; Buchbinder *et al.*, 1989; Irving *et al.*, 1990; Linde *et al.*, 1990). In addition, no correlation was observed between antibody titers to HHV-6

and titers to other human herpesviruses in IFA and ELISA tests (Pellett *et al.*, 1992).

2.2.2 Polymerase Chain Reaction

Of all major molecular biology techniques, none may have stirred as much attention as the polymerase chain reaction (PCR). PCR is an *in vitro* method of nucleic acid synthesis in which a defined DNA sequence is amplified via repeated cycles of heat denaturation, primer annealing and primer extension (Saiki, 1990). The reaction requires deoxynucleotide triphosphates (dNTPs) to provide energy and nucleosides for DNA synthesis; DNA polymerase, oligonucleotide primers, template and buffer containing magnesium.

Double-stranded DNA template is heat denatured at high temperatures to separate the strands and cooled to allow primers to anneal to their complementary strands forming primer-target DNA duplexes. DNA polymerase carries out synthesis by extending the primers in the 5' to 3' direction across the region between the primers (Fig. 2.4). Initial synthesis extends beyond the sequence complementary to the other primer but with each PCR cycle, the region between the primers including the primers, that is, the target region increases exponentially. Each amplification cycle

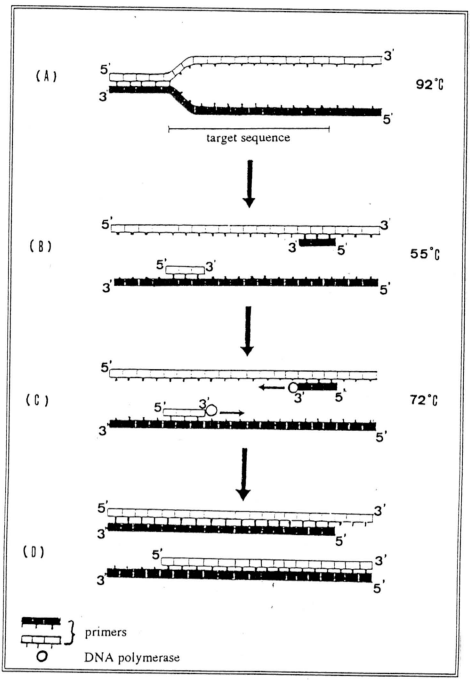

Figure 2.4 : The PCR amplification cycle of a specific target sequence

A) Double-stranded DNA is separated by heat denaturation.
B) Two oligonucleotide primers anneal to their complementary recognition sequences in the 5' to 3' orientation.
C) *Taq* DNA polymerase initiates synthesis by primer extension.
D) Two copies of the original target DNA sequence are synthesized after 1 cycle. Each of the four strands initiate the second cycle of DNA synthesis.

literally doubles the content of the original template DNA resulting in 2^n accumulation of the target sequence in which n is the number of cycles. After 30-40 cycles, the predominant PCR product will be the target region whilst the longer sequences, negligible (Saiki *et al.*, 1985; Mullis *et al.*, 1987).

The efficiency and specificity of a PCR assay relies heavily on its' components. *Taq* DNA polymerase isolated from *Thermus aquaticus* works at the optimum temperature of 72°C and withstands the high denaturation temperature of 92-94°C unlike its' predecessor, the Klenow fragment of *E.coli* DNA polymerase I. The *Taq* enzyme requires Mg ions which are supplied in a buffer (Saiki *et al.*, 1988).

The design of PCR primer pairs follows several basic rules. An oligonucleotide should be 17-25 bases in length. Longer primers impair mobility and increase annealing time. Primer-target hybrid stability is dependent upon primer length and G+C content. Primer pairs with high G+C content of approximately 50% are desirable. The 3' termini of primer pairs should be non-complementary to avoid 'primer-dimer' artifacts as complementary termini may self-anneal and act as substrate for DNA polymerase, hence the formation of 'primer-dimers' (Saiki, 1990).

PCR for the detection of HHV-6 DNA sequences has been utilized extensively by various laboratories (Lawrence *et al.*, 1990;

kb fragment and pHC5, an 830 bp BamHI region homologous to pZVH14 (Table 2.3). Other suitable primer sequences have also been described (Qavi *et al.*, 1989; Gopal *et al.*, 1990; Jarrett *et al.*, 1990; Kido *et al.*, 1990; Kondo *et al.*, 1990; Aubin *et al.*, 1991; Wilborn *et al.*, 1994).

The detection of HHV-6 by PCR is greatly influenced by the amount of template present in each reaction. Although the PCR is highly sensitive, sufficient amounts of the sample containing amplifiable template is required. Jarrett *et al.* (1990) suggested that between 5×10^4 and 5×10^5 infected PBMCs were needed to allow reliable HHV-6 detection. The inability to detect HHV-6 in healthy HHV-6 seropositive individuals was also attributed to the small amount of template used as a higher concentration yielded better results (Kondo *et al.*, 1991).

Table 2.3: Primer and probe sequences in HHV-6 detection

Set	Oligonucleotide sequence (5'→3')	Amplified product (bp)	Strain	Reference
H6-6	AAGCTTGCACAATGCCAAAAAACAG	223	U1102	Gopal *et al.*, 1990
H6-7	CTCGAGTATGCCGAGACCCCTAATC			
H6-6/7 (probe)	AACTGTCTGACTGGCAAAAACTTTT			
A	GATCCGACGCCTACAAACAC	830	SIE	Collandre *et al.*, 199
				Aubin *et al.*, 1991
C	CGGTGTCACACAGCATGAACTCTC	249 (nested region within the 830 bp region)		
B	TACCGACATCCTTGACATATTAC			
S (probe)	GGCTGATTAGGATTAATAGGAGA			
HS6AE	CGGCCATTTAACGGAACCCTAG	~750 (nested region within the 830 bp region)	SIE	Dewhurst *et al.*, 199
HS6AF	TCCAGAGAAAGGGTGTTGCG			
O22	GCGTGAATCAAACCTCGCTCGA	380	U1102	Lawrence *et al.*, 199
				Aubin *et al.*, 1993
O23	GCCTTACTCGGAATCTACTGC			

PCR assays of HHV-6 using different sampling methods have given varying results. PCR of crude saliva lysates detected 90% HHV-6 positivity in healthy individuals (Jarrett *et al.*, 1990) compared to 63% positivity in saline gargles (Gopal *et al.*, 1990) and 3% in throat swabs (Kido *et al.*, 1990).

The ability to amplify archival paraffin-embedded tissue adds to the importance of the PCR technique in histopathology. However, the type and duration of fixation used on the tissue prior to paraffin-embedding, affects the sensitivity of the PCR. Tissue fixed in acetone or 10% buffered neutral formalin (Greer *et al.*, 1991) for less than 24 hours is ideal for analysis by PCR (Bresters *et al.*, 1994). In a less than ideal fixative condition for a tissue sample, the preservation of DNA is greatly compromised as the DNA degrades and fragments. Therefore, while PCR is able to amplify impure and fragmented DNA, only short target sequences of about 100 bp amplify better, thus reducing the possibility of false negative results (Honma *et al.*, 1993). The number of PCR cycles can vary between 20-40, whereby too few cycles give low yield while too many increase the amplification of nonspecific products, as a result of 'plateau effect' (Innis and Gelfand, 1990).

The efficiency of PCR can be significantly increased by subjecting a tissue sample to a 'nested' PCR protocol as it improves the

chances of detecting low copy DNA such as viral DNA (Pierre *et al.*, 1991). Nested PCR is a two-step amplification process of a single region. An external primer pair amplifies a targeted region in the first PCR experiment. A second PCR experiment is carried out on a small amount of PCR product from the first experiment using internal/nested primers (Mullis and Faloona, 1987; Jarrett *et al.*, 1990).

While PCR is extremely sensitive, this has proved to be its' undoing. Low level contamination that may arise at any stage in a PCR assay could distort the fine line between true and false positives (Kwok and Higuchi, 1989). Contamination of tissue samples may take place at any level, pre- or post-PCR, during sample collection, DNA extraction or while performing the PCR experiment. The source of contamination is frequently the carryover of previously amplified sequences (amplicons), positive control plasmids, or even shed DNA from skin or hair.

Amplicon contamination is by far the most serious as they are ideal targets for PCR amplification. Amplicon carryover can be avoided by following routine precautions listed below (Kwok and Higuchi, 1989).

- Physically separate areas for pre- and post-PCR amplifications
- Utilization of a laminar flow hood with a UV option, positive displacement pipettors and other equipment dedicated solely for PCR experiments.

- Disposable laboratory materials such as pipette tips and Eppendorf tubes are autoclaved before use in PCR experiments and discarded into detergent once used.
- Frequent changing of disposable gloves and protective clothing covering the head and face to minimize contamination risks (Kelley and McClain, 1994).
- Pre-aliquotted reagents in quality-controlled lots in amplicon-free environment.
- Minimum sample handling.

In the event of amplicon buildup, a few methods for amplicon sterilization have been described, the enzymatic dUTP/UNG method (Longo *et al.*, 1990), the photochemical Isopsoralen method (reviewed by Persing, 1991) and UV-mediated DNA crosslinking of PCR mix prior to the addition of template DNA (Jackson *et al.*, 1991). Other anti-contamination strategies include the use of nested PCR, which is more sensitive and specific. This method eliminates false-positive signals due to 'jumping PCR', the presence of contaminating fragments the same size in length as the target DNA (Jackson *et al.*, 1991).

Appropriate controls are important in PCR assays. Strategically placed negative controls throughout pre- and post- PCR studies

will help highlight contamination problems and also isolate the stage at which the contamination occurs. A negative control paraffin block processed in a similar manner to sample tissues and consequently extracted alongside the samples may monitor contamination at the pre-PCR level. A 'no DNA' control aliquotted for PCR at the same time as samples monitors sample to sample contamination (Jackson *et al.*, 1991).

A positive control should also be included to monitor the PCR assay: test the primers, *Taq* enzyme and the detection method. A substantially diluted positive control that amplifies weakly and consistently is sufficient (Kwok, 1990). An internal control for samples with regard to PCR should be carried out. Normally, β-globin gene sequences are amplified to determine the accessibility and integrity of DNA present in the samples (Saiki *et al.*, 1985).

Detection methods for PCR amplifications are necessary to confirm specificity. Often, gel electrophoresis followed by Southern blot hybridization with a known probe sequence complementary to the amplified fragment is performed to confirm PCR results. Hybridization is usually carried out with radioisotopes, however the use of non-isotopic probes are fast gaining popularity due to their relative simplicity in handling as compared to hazards in radioactive methods. Other non-isotopic detection methods include the reverse dot blot in which target DNA is amplified

with biotinylated primers, hybridized to a membrane containing oligonucleotide probes (Saiki *et al.*, 1989). While this method is specific and sensitive, it is time consuming. A colorimetric method involves target DNA which is amplified with modified primers that produce PCR products with a biotinylated end and a DNA binding protein recognition site on the other end. The amplicons adsorb to a moiety attached to a microtiter plate and is visualized by colour detection (Wahlberg *et al.*, 1990). It is a sensitive method, however there is no distinction between specific and nonspecific products.

2.2.3 Southern Blot Hybridization

Nucleic acid hybridization permits the detection of nucleic acid fragments complementary to a probe. Southern (1975) developed a procedure for the transfer of DNA from a gel onto a membrane filter. The Southern blot involves the detection of DNA, digested with restriction enzymes and electrophoretically separated and subsequently blotted onto a membrane filter by capillary flow. The immobilized DNA is then probed with a specific probe.

Cloned HHV-6 DNA fragments, such as pZVH14 have been used in Southern blot assays as probes (Josephs *et al.*, 1986; Pellett *et al.*, 1990; Aubin *et al.*, 1991). Initially nonradioactive probes were utilized,

however the expense, hazards and instability involved prompted the search for alternative labelling techniques. Boehringer Mannheim developed a nonradioactive DNA labelling and detection system. The advent of nonradioactive DNA labelling has introduced various probe-labelling options, 3'-end labelling, labelling by *Taq* DNA polymerase and others, all requiring the incorporation of digoxigenin (Boehringer Mannheim, 1993).

Restriction mapping of HHV-6 strains by Southern blotting carried out by Aubin *et al.* (1991) distinguished two groups which were later designated variant A and B. This was also observed in the PCR study of the strains, restriction analysis of the amplified fragments led to the same conclusion. While Southern blotting is sensitive, it is costly, time-consuming and requires a large amount of extracted DNA sample which is not always available for molecular studies (Low, 1989). The PCR accomplishes all this and more, as it amplifies DNA fragments that can be used for further studies such as restriction enzyme analysis and sequencing.

2.2.4 Dot Blot Hybridization

Dot blot hybridization is the same as Southern blot in principle but the transfer is much simpler, no elaborate electrophoresis, or capillary blotting needed to transfer DNA. Instead extracted denatured DNA is dotted onto a membrane and hybridized with a probe.

Dewhurst *et al.* (1993) used a dot blot procedure to confirm HHV-6 PCR results and differentiate the variants present at the same time. A drawback to this procedure is the lack of discrimination between specific and nonspecific hybridization (Low, 1989).

2.2.5 *In Situ* Hybridization

In situ hybridization (ISH) is a revolutionary technique designed to demonstrate nucleic acid sequences in heterogeneous tissues. ISH allows precise localization and identification of cells containing a specific nucleic acid sequence. Its' application in virology has provided means for the analysis of HHV-6 DNA sequences within formalin-fixed, paraffin-embedded tissue. ISH is a sensitive, specific and rapid method for establishing viral diagnosis (Stoler and Ratliff, 1990; Weiss *et al.*, 1990; Mitchell *et al.*, 1992).

ISH for the detection of HHV-6 has been carried out by researchers with the aim of establishing a replication site for HHV-6 in salivary glands and bronchial epithelial cells (Fox *et al.*, 1990; Krueger *et al.*, 1990). Studies using ISH as a viral detection procedure in various diseased tissues include atypical polyclonal lymphoproliferation and malignant lymphoma (Krueger *et al.*, 1989; 1992), Non-Hodgkin's disease (Shen *et al.*, 1993) and Kikuchi's disease (Sumiyoshi *et al.*, 1993).

While ISH is analogous in principle to immunohistochemistry, it is also similar to molecular hybridization in terms of interaction between a nuclei acid probe and complementary target. The success of an ISH experiment relies heavily on the type of tissue studied and fixation conditions, the size and type of probes and stringency of posthybridization washes. Overfixation of tissues in formalin prior to paraffin-embedding causes tissue loss from slides during hybridization and also reduces the intensity of ISH signal by 25% (Wilcox, 1993). Tissue fixed in 10% neutral buffered formalin for about 24 hours is suitable, further delay may result in the denaturation of target nucleic acids (Mitchell *et al.*, 1992).

ISH studies may use DNA, RNA or oligonucleotide probes. While DNA and RNA probes are derived from cloned sequences, oligonucleotides are synthesized. Of these, oligonucleotides have an advantage over DNA or RNA probes, in that they facilitate entry into sample tissue with minimum permeabilization due to their small size (20-35 bases). Not only are DNA probes too large, they can also cause a low signal to noise ratio (Mitchell *et al.*, 1992).

Radioactively labelled probes, though highly sensitive have given way to noradioactively labelled probes due to safety hazards. Biotinylated probes while popular, have a tendency to exhibit nonspecific binding of streptavidin in tissues. A more sensitive alternative is the

digoxigenin labelling of DNA probes via random primer labelling, 3'-end labelling or 3'-tailing (Boehringer Mannheim, 1993; Morris *et al.*, 1990; Stoler and Ratliff, 1990).

Reaction conditions during hybridization enhance hybrid formation, therefore any nonspecific binding between probe and non-target material is removed by high stringency washes. These washes maximize signal to noise ratio. The validity of ISH results can be established by the use of appropriate controls. Negative controls should include a 'no probe' control and a known negative sample. Positive signals should be interpreted cautiously however, as it is quite easy to mistake high background signals for positive (Mitchell *et al.*, 1992; Wilcox, 1993).

2.2.6 Immunohistochemistry

Immunohistochemistry (IHC) refers to the study of interactions between antigens and antibodies in cells and tissues. IHC is a method by which specific antigens within a tissue sample may be detected using monoclonal or polyclonal antibodies. Generally MAbs are preferred to polyclonal antibodies due to advantages such as high homogeneity, absence of nonspecific antibodies and no batch to batch non-variability (Boenisch, 1989a). IHC is popularly carried out with the avidin-biotin methods, that is, the avidin-biotin complex (ABC) and labelled avidin-biotin (LAB)

methods. These techniques require a biotinylated secondary antibody as a link to the MAb, which is then detected by the ABC or LAB substrate solution.

Monoclonal antibodies commonly used for antigen analysis include p41, gp116/64/54, gp82, gp102 among others (Balachandran *et al.*, 1989; Eizuru *et al.*, 1989; Okuno *et al.*, 1990). The presence of viral antigens have been reported in renal transplant biopsies (Okuno *et al.*, 1990), salivary gland and bronchial epithelial cells (Fox *et al.*, 1990; Krueger *et al.*, 1990) and lung tissue (Pitalia *et al.*, 1993).

Antibody cross-reactivity was observed between certain HHV-6 derived MAbs and some strains of HHV-7 (Wyatt *et al.*, 1991; Berneman *et al.*, 1992) however a study by Black *et al.* (1993) showed no cross-reactivity between those MAbs.

In IHC, incubation time and temperature, and antibody titer are interdependent. Optimal working dilutions of antibody titers should be determined by titration. On paraffin sections, this is obtained by intense specific staining with minimum background (Boenisch, 1989b). Formalin based fixatives are usually acceptable for immunostaining, however variation in fixation times may cause problems such as conformational changes in epitopes. Therefore to overcome such difficulties, MAbs should be chosen carefully with regard to the reactive epitope (Farmilo and Stead, 1989).

2.3 HHV-6 AND CANCER

One of the major causes of death is cancer. Cancer refers to a derangement in the control of cellular growth in which growth inhibition is overcome, and cells divide extensively forming large masses of transformed cells called tumours. Non-invasive tumours are usually benign, while invasive tumours, malignant. The abnormal growth of cells (dysplasia) in number and size displaces to neoplasia in which an abnormal mass of cells proliferate in persistent and progressive fashion. A loss of relationship between cells of irregular shape and size is observed. Loss of polarity and the presence of macronucleoli lead to malignancy.

2.3.1 Carcinogenesis

Cancer has a multifactorial aetiology; chemical carcinogens, physical agents, ionizing radiation, viruses and host factors have been implicated in the progression towards neoplasia. The process itself involves initiation, promotion and tumour progression (Scully, 1992). Initiation of cancer commonly occurs as a result of genetic changes in the cell caused by chemical or physical stimuli. The initiated cell may remain dormant or be promoted to cancerous condition and progress in the formation of a

tumour. Often chromosome aberrations in cancers are situated near oncogenes.

Proto-oncogenes are present in normal host cells and control cell growth, proliferation and differentiation, crucial to growth regulation. Carcinogenic agents like viruses, chemicals and radiation can activate oncogenes. However, oncogene activation alone does not cause malignancy, clearly other factors are also involved in carcinogenesis.

Carcinogenesis is a multistep process involving a series of oncogenes activated by chemical, biological or physical factors. A viral aetiology for human tumours is a concept that has been toyed with since the 1840s. It is now established that cancers such as Burkitt's lymphoma and nasopharyngeal carcinoma are strongly associated with EBV while cervical carcinoma is associated with HPV and HSV-2 (Brock and Madigan, 1991).

2.3.2 HHV-6 as an oncogenic virus

It has been observed that most human herpesviruses possess DNA with oncogenic properties, this reputation and the detection of HHV-6 in various malignancies have generated studies on the oncogenic potential of this virus. Assessment of oncogenic function is carried out by *in vitro* transfection and *in vivo* malignancy.

Research shows that intact HHV-6 genomic DNA and its' subclones, pZVH14 and pZVB70 can neoplastically transform nontumourigenic mouse fibroblast NIH3T3 cells and human epidermal keratinocytes RHEK-1. The transformed cell lines produced fast growing tumours in athymic nude mice. HHV-6 DNA was maintained in both the cell lines and the resultant tumours (Razzaque, 1990; Razzaque *et al.*, 1993).

In a separate study by Chen *et al.* (1994a), HHV-6 established a latent infection in HPV-transformed epithelial cells. It was suggested that reactivation of HHV-6 in these cells contribute to immunosuppression and consequently transactivate cellular/viral oncogenes.

The role of HHV-6 in oncogenesis is still subject to further studies. Preliminary research indicate that this is no ordinary virus, that merely causes the benign ES. The virus contains at least two transforming sequences and is able to transactivate other viruses such as HPV and HIV (Chen *et al.*, 1994a; Thompson *et al.*, 1994b). This is in line with multistep carcinogenesis with multiple aetiological factors that has been suggested by many researchers (Chen *et al.*, 1994a; Leach *et al.*, 1994; Yadav *et al.*, 1994).

2.3.3 Oral Cancer

Oral cancer ranks as the eighth most frequently occurring cancer in developed countries. In Asiatic and Pacific regions however, oral cancer remains a major type of cancer (Yadav *et al.*, 1994). Common histological types include squamous cell carcinoma (SCC), verrucous carcinoma and spindle cell carcinoma (Lucas, 1976).

Oral carcinoma is a disease of late adulthood with 78.5% occurring in individuals between 50 and 70 years of age. Based on a Malaysian study of oral cancer over a 25 year period (1967-1991), it was found that 51% of carcinoma cases were females while Indians constituted between 59.3-66% of reported cases (Kok and Chong, 1992). The racial preference of this cancer may reflect on oral habits, such as betel quid and tobacco chewing, smoking, snuff taking and alcohol.

The high incidence of oral carcinoma in India has been attributed to 'pan' (betel-leaf,-nut, tobacco dust, slaked lime) and tobacco chewing. Carcinogens produced by chewing betel or tobacco quid are exposed to the buccal sulcus, tongue and soft palate of the mouth cavity resulting in higher risk of developing cancer. Similarly tobacco smokers are at risk of developing cancer in the oropharynx (Jussawalla *et al.*, 1971; Sanghavi, 1981; Jayant and Notani, 1991).

Elevated p53 expression was seen in oral SCC patients with a history of heavy smoking (Field *et al.*, 1991). Elevated expression of oncogenes, *int*-2, c-*erbB*-1 and c-*myc* have been observed in the development of oral carcinoma which correlate with advanced malignancy and poor prognosis (Scully, 1992).

Elevated HHV-6 IgG antibody titers were observed in oral carcinoma patients (Shanavas *et al.*, 1992). Similarly HHV-6 DNA and proteins were found in oral carcinoma cases by PCR and immunohistochemistry respectively. Immunohistochemical analysis of these cases revealed the expression of HHV-6 proteins in transformed cells. It has been hypothesized that HHV-6 may be a cofactor in carcinogenesis in combination with HSV-2, HPV and carcinogens (Yadav *et al.*, 1994; Ablashi *et al.*, 1995b).

2.3.4 Cervical Cancer

Cervical cancer is the second most frequent malignancy in women all over the world (Ablashi *et al.*, 1995b). Once a leading cause of cancer death, the early detection of cervical carcinoma whilst in the beginning stages, by the Papinicolaou cytological test has greatly improved survival rates. Invasive carcinoma appears to have a peak incidence in

women between 30-40 years of age, with the mortality rate rising in the fourth decade (Kumar *et al.*, 1992).

Risk factors include smoking, early age at first intercourse and multiple sexual partners. Over 90% of cervical carcinoma cases contain high risk HPVs that is, HPV 16, 18 and 31 in which the majority have integrated HPV-16 and 18 DNA (Kumar *et al*, 1992). The aetiology of cervical carcinoma implicates these HPVs, however HPV infection alone is not sufficient for carcinogenesis. HPV may act in synergy with other co-carcinogens such as HSV-2 (Kumar *et al.*, 1992).

Recently the detection of HHV-6 in the female genital tract and in cervical carcinoma caused researchers to rethink the role of this virus in neoplasia (Chen *et al.*, 1994b; Leach *et al.*, 1994). HHV-6 is capable of infecting HPV-immortalized cervical epithelial cell lines and cervical carcinoma cell lines and establishing latency. The virus also contains transactivators that upregulate expression of HPV-18 E6 and E7 oncogenes, suggesting a co-carcinogenic role (Chen *et al.*, 1994a).

2.3.5 Breast Cancer

Carcinoma of the breast accounts for 20% of cancer deaths in the West while it is the leading cancer in Asia. Risk factors include genetic predisposition, long reproductive life, obesity, diet and hormone

imbalances. Genetic predisposition, for example the inherited mutation of p53 gene coupled with hormones may play a role in carcinogenesis. Breast cancer cells secrete growth factors, TGFα and PDGF, which are estrogen dependent (Kumar *et al.*, 1992). It has been suggested that circulating hormones, hormone receptors on transformed cells and these growth factors progress towards neoplasia. The existence of viruses in breast cancer has been studied extensively however no conclusive evidence has surfaced (Kumar *et al.*, 1992).