CHAPTER ONE

1.1 General Introduction

In 1986, in the laboratory of Robert Gallo at the National Cancer Institute in Betheseda, Salahuddin and colleagues reported the discovery of a novel herpesvirus that was present in peripheral blood lymphocytes (PBL) of patients with various lymphoproliferative diseases (Salahuddin *et al.*, 1986; Lusso, 1996). This virus was first designated as human B-lymphotrophic virus (HBLV) as it was thought to infect preferentially B-lymphoid cells. With the improvement of virus propagation systems *in vitro*, it was soon realised that the primary target cell of this virus was the T-lymphocyte (Lusso *et al.*, 1988). The virus was designated human herpes virus 6 (HHV6).

HHV6 is the sixth herpesvirus to be discovered. Currently 8 herpesviruses have been found, being herpes simplex virus 1 and 2, varicella-zoster virus, human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and human herpes virus 6, 7 and 8. All these viruses are ubiquitous in the human population.

In India, antibody prevalence of 76% was found among cancer patients (Shanavas *et al.* (1992) whereas in Africa, Ablashi *et al.*, (1988) found a prevalence of 52%. In Malaysia, several epidemiological studies have been carried out. Yadav *et al.*, (1990) reported a low seroprevalence of IgG-HHV6 in 34% of Kadazans in Sabah. This was followed by another report by the same group in 1991 whereby antibody prevalence between 49-76% was obtained. This study also revealed that indigenous (Iban, Kadazan, Bidayuh and Orang Asli) tribes had lower prevalence of HHV6 antibodies compared to

Indians, Malays and Chinese (Yadav *et al.*, 1991). Levine *et. al.,* in 1992 reported the presence of IgG HHV6 in Malaysian Chinese to be 41.5% and in Malaysian Indians to be 54.2%. A later study by Chua *et al.*, (1996), however found a higher seroprevalence of 83.7% among healthy Malaysians. In a study done in our lab (Chua., 1999) on subjects between 0-20 years, presence of IgG HHV6 was detected in 90.6%. Both Chua *et al.* (1996) and Chua, (1999) found no significant difference in seroprevalence of IgG-HHV6 between ethnic groups (Malay, Chinese and Indians) or between sexes.

Some patients from whom the HHV6 virus was isolated were also infected with human immunodeficiency virus 1 (Salahuddin *et al.*, 1986; Downing *et al.*, 1987) and later, HHV6 was proposed as a cofactor in the progression of AIDS (Lusso *et al.*, 1988, 1995; Ablashi *et al.*, 1998). HHV6 has also been isolated from children with exanthem subitum (Yamanishi *et al.*, 1988; Chua *et al.*, 1996), patients with other disorders (Krueger *et al.*, 1991) as well as healthy adults.

1.2 Human Herpes Virus 6 (HHV6)

HHV6 has been classified as a beta-herpesvirus subfamily on the basis of its genetic homology with human cytomegalovirus (hCMV). Along with HHV7, it is in the Roseola virus genus (Roizman *et al.*, 1992).

1.2a Morphology and Ultrastructure

The diameter of a mature HHV6 particle is about 200 nm. Its structure includes an internal core (capsid), consisting of 162 capsomers arranged into the typical icosahedral symmetry. This capsid encloses a double stranded DNA genome of approximately 160,000 base pairs. The core is uniform in thickness but varies in shape from perfectly round to ellipsoidal (Nii *et al.*, 1990) and consists of punctate and /or filamentous structures (Yoshida *et al.*, 1989).

A lipid-containing envelope surrounds the nucleocapsid by an amorphous tegument and externally with spikes projecting from its surface. Figure 1.1 shows a diagrammatic presentation of the human herpesvirus 6.

1.2b Genetic Characteristics

The genome of HHV6 is large and complex containing about 102 genes. (Gompels *et al.*, 1995). It consists of a single long unique (UL) element about 140,000 base pairs (bp), rich in A+T and flanked by two identical direct repeat (DR) segments of approximately 8,000 bp each. The direct repeats are believed to contain the essential elements for maintenance of the latency state (Thomson *et al.*, 1994; Gompels and Macauly, 1995). Restriction endonuclease polymorphism has been documented among different HHV6 isolates (Jarrett *et al.*, 1989; Kikuta *et al.*, 1989) and two major viral subspecies (variant A and B) have been defined.

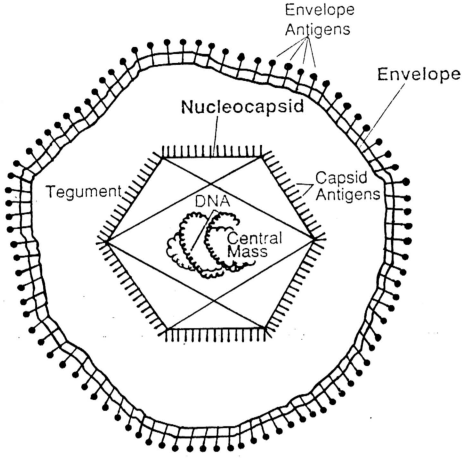

Figure 1.1 : HHV6 virion structure. The 180 to 200 nm enveloped virion contains a 100 nm nucleocapsid. Prominent tegument lies between nucleocapsid and outer envelope. Double stranded DNA viral genome is spooled around a nucleoprotein central mass within the nucleocapsid. (from Leach *et al.*, 1992)

The genome of HHV6 has coding regions on both DNA strands. An analysis of the complete nucleotide sequence of HHV6 (Gompels *et al.*, 1995) revealed the existence of 5 major families of genes. Some of the genes have been characterized based on homology or colinearity with other herpesviruses, especially hCMV. The genes include putative immediate early (IE) genes (Schiewe *et al.*, 1994), the DNA polymerase gene (Teo *et al.*, 1991), genes coding for a nucleophosphoprotein (Chang and Balachandran, 1991), a major immunogenic 101kDa structural protein (Neipel *et al.*, 1992; Pellett *et al.*, 1993) and the major capsid protein (Littler *et al.*, 1990).

The complete nucleotide sequence of strain U1102 (Downing *et al.*, 1987) which is closely related to the GS prototype (Salahuddin *et al.*, 1986) was obtained in 1995 (Gompels *et al.*, 1995). The G + C overall content is 43%, one of the lowest among the herpesviruses. HHV6 shares the highest degree of nucleotide sequence homology with HHV7, while both HHV6 and HHV7 are more distantly related to hCMV (Neipel *et al.*, 1991, Berneman *et al.*, 1992; Gompels *et al.*, 1995). The genomes of HHV6 and HHV7 have been found to be roughly colinear with that of hCMV.

1.3 Human Herpes Virus 6 Variants A and B

With the characterization of an increasing number of viral strains isolated throughout the world, it is evident that heterogeneity exists and that the different isolates segregate into two well- defined subgroups, referred to as HHV6A and HHV6B (Ablashi *et al.*, 1991; Aubin *et al.*, 1991; Schirmer *et al.*, 1991).

These two subgroups are clearly distinct, however, it is also clear that the two groups are genetically very closely related. Some segments of genomic sequences differ

by as little as 4% between the groups (Aubin *et al.*, 1993). Within a variant, genomic sequences have been found to differ by 1% to 3% (Aubin *et al.*, 1991; Chou and Marousek., 1992; Gompels *et al.*, 1993). This sequence similarity is reflected in the high degree of antigenic cross-reactivity between the variants. The most rapid and efficient methods currently available for subgroup identification are based on either the reactivity of a specific monoclonal antibody with productively infected cells, or on the restriction patterns of specific regions of the viral genome. After amplification by polymerase chain reaction (PCR), a consistent pattern is observed for each of the two subgroups upon digestion with specific restriction enzymes (e.g. Bgl II, Hind III). Further endonuclease restriction analysis has led to at least one further subdivision within subgroup B (B1 and B2) (Di Luca *et al.*, 1992).

In a report by Dewhurst, differentiation between the isolates was carried out by using a Hind III restriction site polymorphism (Aubin *et al.*, 1991; Dewhurst *et al.*, 1992). In the study of viral isolates, DNA was amplified by PCR, digested with Hind III and analyzed (Dewhurst *et al.*, 1992). The digestion results were subsequently confirmed by DNA dot blot analysis with variant of A or B specific oligonucleotide probes, which differ at a single residue. This base change creates the Hind III site which is used to differentiate the variants.

To date, no definitive link has been established between HHV6A and any human disease, but the fact that the A-type isolates were mostly derived from immunocompromised patients indicates a role of this virus in immunodeficient conditions. The prototype for this virus strain is GS.

HHV6 subgroup B is prototyped by strain Z29. It has been found responsible for most cases of primary HHV6 infection documented in early childhood (Dewhurst *et al.*, 1993b) and has been etiologically associated with exanthem subitum (Yamanishi *et al.*, 1988; Chua *et al.*, 1996), a usually benign febrile disorder, also referred to as Roseola infantum or sixth disease.

The two variants of HHV6 also differ in other aspects. Firstly, their geographical distribution might be distinct. In the United States, HHV6B was isolated in PBMC of 97% of children who had acute febrile illness (Dewhurst *et al.*, 1993b). Less than 3% were infected with HHV6A, whereas in Central Africa, variant A was present in 44% of PBMC samples collected from infants with a first febrile episode resulting from HHV6 (Qiu *et al.*, 1996). Secondly, the two variants may infect different tissues. For example, in North America, HHV6A has been commonly identified in living tissues of both healthy and diseased adults, even though it is rare in the peripheral blood mononuclear cells (PBMC) of this population (Cone *et al.*, 1996).

Thirdly, the variants might differ in their relative potential for pathogenicity or their reactivation properties. For example, the frequency of detection of HHV6A DNA in PBMC from adults with chronic fatigue immunodeficiency syndrome was found to be significantly higher than PBMC from healthy adults (Di Luca *et al.*, 1995a). Likewise, HHV6A was found to be the predominant HHV6 variant in the lymph nodes in HIV-1 infected adults (Knox and Carrigan., 1996; Lindquister *et al.*, 1997). The variants are also believed to have different immunobiologic courses and neurotropism (Hall *et al.*, 1998),

whereby in one case, variant A was found present in cerebrospinal fluid (CSF) only but when saliva and peripheral blood mononuclear cells were tested, only variant B was found.

1.4 Cell Tropism and Growth Properties of HHV6A and HHV6B

Isolates of HHV6 have been shown to replicate with varying efficiency in a wide array of host cell types including primary T-cell /monocytes / macrophages, natural killer (NK cells) plus astrocytes, as well as continuous cell lines of T and B cells, megakaryocytes and glial cell lineages (Pellett and Black., 1996). In general, the host cell range of laboratory adapted isolates of HHV6A appears to be broader than of laboratory adapted isolates of HHV6B.

There is a consensus that HHV6 has primary tropism for CD4 T-lymphocytes, both *in vitro* (Lusso *et al.*, 1988) and *in vivo* (Takahashi *et al.*, 1989). When activated lymphocytes of different tissue origin (e.g. peripheral blood, cord blood, thymus, lymph node) are exposed to HHV6 infection, the majority of infected cells display the phenotype of activated CD4 T cells. Although both variants A and B infect preferentially CD4+ T cells, differences do exist in their cellular tropism. They differ in their ability to infect established human T cell lines and B-lymphoblastoid cell lines (LCL). Furthermore, HHV6A has the ability to infect productively and cytopathically several types of cytotoxic effector cell, such as CD8+ T-lymphocytes (Lusso *et al.*, 1991a, b), and NK cells (Lusso *et al.*, 1993). As these cells are involved in the antiviral defence mechanism *in vivo*, HHV6 may counteract the protective immune surveillance of the host

and establish persistent infection (Lusso., 1996). In contrast, HHV6B is unable to attack cytotoxic effector cells efficiently.

To date, little information is available on the surface membrane receptors of HHV6. However, the difference in cellular tropism between the variants suggests that the composition of the receptor complex may be different for the two viral subgroups. There has been evidence too that the receptor for HHV6 is not the CD4 glycoprotein (Lusso *et al.*, 1988).

The lymphotrophic nature of HHV6, the *in vitro* observations that these viruses affect expressions of interferon (IFN), interleukin (IL)-8, IL-1β and tumour necrosis factor-α (TNF-α) (Kikuta *et al.*, 1990; Inagi *et al.*, 1996) and their ability to modulate other immunologically important cell surface molecules (CD3, CD4 and EB1) (Furukawa *et al.*,1994) suggest that these viruses may have immunomodulatory potential. It can he hypothesized that the activation of HHV6 variant A directly induces a status of immunodeficiency and maybe an important cofactor for the pathogenesis of various immunodeficiency syndromes including AIDS. In contrast, infection with HHV6 variant B may result in immunocompetent benign disorders, such as exanthem subitum (ES) and infectious mononucleosis like syndrome (Furukawa *et al.*, 1994).

1.5 Animal Models of HHV6 Infection

Animal models have not been extensively used to study HHV6 infection, transmission and pathogenicity. Several monkey species have been examined for the presence of HHV6 reactive serum antibodies, ability of their peripheral blood lymphocytes (PBL) to replicate the virus and appearance of clinical manifestations after injection with HHV6.

It has been observed that the HHV6 variants differ in cellular tropism, now it has been found differ in species tropism also. The strain A has restricted species range as it has been found to replicate *in vitro* in activated T cells from the chimpanzee (*Pan troglodytes*) (Lusso *et al.*, 1990) and the pig tailed macaque (*Macaca nemestrina*) (Lusso *et al.*, 1994). As for variant B, both cynomolgus macaque (*Macaca fascicularis*) and African green monkeys (*Cercophytecus aethiops*) PBL were successfully infected *in vitro* and *in vivo*.

1.6 Human Herpes Virus 6 Infection

Herpesvirus causes both productive and latent infections. In productive infection, the infected cells express most, if not all viral genes sequentially. The immediate early (IE) or α genes are expressed first, followed by early (β) and lastly, by late or γ genes. One of the HHV6 genes transcribed under IE conditions, that is, in the absence of prior protein synthesis, is U94 Open Reading Frames (ORFs) (Mirandola *et al.*, 1998). In the process of viral replication, the cell dies. In latent infection, only a small subset of

the viral genes is expressed, usually in the absence of prior infected cell protein synthesis, and the cell survives.

1.6a Primary Infection

Primary infection of HHV6B has been manifested as Roseola in 50% Japanese infants (Kusuhara *et al.*, 1992) however, less than 1/3 United States children (Pruksanonanda *et al.*, 1992) have reported roseola cases due to primary HHV6B infection. In Malaysia, primary infection was found to occur mainly in the pediatric age group. A study by Chua *et al.* (1996) revealed that from birth up to 1 year of age the seroprevalence was 82%.

The presence of HHV6 in saliva (Cone *et al.* 1993., Pietroboni *et al.*, 1988) and salivary glands (Fox *et al.*, 1990) suggests that primary infection occurs via the oropharynx. HHV6 has also been detected in a variety of cell types in pathologic specimens including lymphocytes, macrophages, endothelial and epithelial cells, but the site and target of primary infection within the oropharynx is unknown (Kondo *et al.*, 1991).

Clinical manifestations of the potential neurotropism of HHV6 are indicated by the occasional neurologic complications that accompany primary infection, such as meningoencephalitis and encephalopathy (Yoshikawa *et al.*, 1992a). A significant drop in circulating neutrophil and lymphocyte counts accompanies acute infection. Besides this, primary infections may also be associated with an elevated frequency of seizures in some children and with invasion of the central nervous system (CNS).

The frequency with which HHV6 is detected in the brains of individuals dying of varied causes, however, implies that its presence in the CNS is not necessarily pathologic. This is because HHV6 DNA has been detected in the brain autopsy specimens of adults without evidence of acute HHV6 infection or pathology (Luppi *et al.*, 1994). Thus, HHV6 has been delineated as a viral entity, with the potential to remain latent or persist in the CNS with subsequent reactivation leading to possible neuropathologic consequences. Tang *et al.* (1997) found that in the presence of two distinct herpesviruses in the same CSF specimen, being HHV6 and herpes simplex virus (HSV), coinfection could occur in the CNS

1.6b Persistent and Latent Infection

There are several indirect lines of evidence that support the view that HHV6 is maintained in a latent state in peripheral blood of most healthy adults. Firstly, a large number of healthy individuals harbour detectable amounts of HHV6 DNA in their peripheral blood mononuclear cells (PBMC) (Di Luca *et al.*, 1996) even though infectious virus cannot be isolated from the blood of these persons. Secondly, *in vitro* infection of PBMC specimens from HHV6 seropositive adults with HHV7 results in the active lytic replication of endogenous HHV6B (Katsafanas *et al.*, 1996).

The most direct evidence for persistent infection has been from the identical restriction endonuclease profiles obtained from pre and post-transplant specimens obtained from a bone marrow recipient (Yoshikawa *et al.*, 1992b) indicating the persistence of the virus in both specimens.

In 90% of specimens from healthy donors, HHV6 has been detected in lymphocytes with frequencies that vary with the sensitivity of the PCR assay (Cone *et al.*, 1993; Cuende *et al.*, 1994). Quantitative PCR revealed HHV6 DNA levels ranging from negative to 4,000 HHV6 genome equivalents per 10^6 PBMCs. HHV6 antigen has also been found in histologically normal sections of salivary glands (Fox *et al.*, 1990; Krueger *et al.*,1990) with benign tumours, lymph nodes and neurons and glial cells in the brain. In these cases, the frequency in which the virus was found are too high to be due to reinfection and is believed to be persistently active infections.

Recent sequence analysis of HHV6 and HHV7 genomes revealed all HHV6 genes have HHV7 counterparts except for two. One of the two is the U94 open reading frame (ORF) of HHV6, which encodes a product Rep H6. In latent infection, only small subsets of viral genes are expressed, and the cell survives (Rotola *et al.*, 1998).

It was found that PBMC of healthy individuals infected with HHV6 express the U94 gene, transcribed under immediate early (IE) conditions. To verify that U94 may play a role in the maintenance of the lethal state, lymphoid cell lines were derived that were able to stably express U94. HHV6 was able to infect these cells, however viral replication was restricted. No cytopathic effect developed. Viral transcripts present in the first days postinfection declined thereafter. Decline in the level of intracellular viral DNA was also observed. It was thus concluded that the U94 product of HHV6 regulates viral gene expression and enables establishment and maintenance of latent infection in lymphoid cells (Rotola *et al.*, 1998).

The T cell lines that stably express HHV6 U94 are permissive for HHV6 infection but viral replication is restricted. There is a hypothesis that U94 /rep H6 may have a function in the regulation of HHV6 latency (Rotola *et al.*, 1998).

A possibility for the function of U94 is that the U94 gene product might function as a sequence-specific DNA binding protein to allow latent-phase replication of the genome or that the U94 gene product is a repressor of the viral IE genes (Mador *et al.*, 1998). This hypothesis is consistent with the fact that this gene is a potent transcription regulator, which has been reported to suppress the expression of several genes (Araujo *et al.*, 1995).

Reports have stated that HHV6 could be isolated from immunosuppressed patients such as AIDS, leukaemia, lymphoma and organ transplant patients. Renal transplantation also showed reactivation (Asano *et al.*, 1989, Okuno *et al.*, 1990). These results suggest that the virus persists latently after primary infection and is reactivated under some circumstances such as immunosuppression.

Although HHV6 DNA could be found in salivary gland (Fox *et al.*, 1990), the actual site of latent infection is not clear yet.

1.7 Major Antigens of Human Herpes Virus 6

Using specific polyclonal or monoclonal antiserum, Balachandran *et al.* (1989) identified more than 30 immunoreactive polypeptides in HHV6 infected cells, ranging from 31 to 80kD (Lusso, 1996) . Approximately nine proteins were glycoproteins, 8 were associated with one infected cell membranes (Balachandran *et al.*, 1991b) and 6 were

associated with the viral envelope (Shiraki *et al.*, 1989). Table 1.1 shows proteins that have been found to be specific for HHV6 infected cells.

The progression of the humoral response induced shortly after primary infection was monitored by pairing immune precipitation profiles of acute- and convalescent - phase sera from exanthem subitum patients. For most HHV6-infected human sera samples, a major capsid protein (MCP) of 135kDa was identified as the immunodominant precipitable band. Littler *et al.*, (1990), who identified this MCP gene, cloned and expressed it in *E. coli* cells. However, the 135kDa band reacted poorly with human sera in immunoblot assay.

This showed that HHV6 positive sera have only low levels of antibody to this protein, suggesting that the reactivity in this test may not be a useful diagnostic response (Littler *et al.*, 1990). However, this observation does not eliminate the MCP as a major epitope in other assay systems such as IFA and immunoprecipitation, as in these systems, the protein may exist in a different conformation.

For both the strains of HHV6, antigenically reactive proteins were found. For strain U1102 (var. A), the antibodies against an internal structural protein of 100kDa (p100) were shown to be both sensitive and a specific marker for HHV6 infection (Neipel *et al.*, 1992). Sera that were found positive for HHV6 antibody by immunofluorescence invariably recognized at least one epitope in the carboxyl-terminal half of HHV6 p100. It was often detectable even in samples that reacted very weakly or were non-reactive in a standard immunofluorescence assay.

Table 1.1 : HHV6 proteins detected in infected cells by immunoprecipitation with monoclonal and polyclonal antibodies. (from Balachandran *et al.*, 1989)

Antibody	Proteins immunoprecipitated	References
Rabbit anti-HHV6 antibodies	180K, 135K, 125K, 120K, 116K, 105K, 82K, 77K, 72K, 68K, 64K, 50K, 41K, 33K	Balachandran *et al.*, 1989
Human serum N620 (serum in which the first HBLV (HHV6) virus was first isolated by Salahuddin *et al.*,1986)	180K, 165K, 155K, 135K, 130K, 125K, 120K, 116K, 112K, 110K, 105K, 102K, 98K, 95K, 92K, 90K, 88K, 86K, 84K, 82K, 77K, 74K, 72K, 68K, 64K, 62K, 60K, 54K, 50K, 48K, 47K, 44K, 41K, 38K, 33K, 31K	Balachandran *et al.*, 1989
Polyvalent rabbit serum	100K, 135K, 65K, 62K, 22K, 20K	Neipel *et al.*, 1992
Polyvalent mouse serum	70K, 85K, 101K, 143K	Yamamoto *et al.*, 1990
Monoclonals		
2D6, 2D4, 13D6	gp82k and gp105k	Balachandran *et al.*, 1989
6A5H7,6A5G3, 14B3	gp116k, gp64k, gp54k	
7A2	gp102k	
9A5D12	41K, 110K	
12B3G4	135K	
C3108-103	101K	Pellett *et al.*, 1993

- *143kDa, 41kDa, 101kDa - nucleocapsid protein*
- *135kDa - major capsid protein*

For the Z29 (var. B) strain, 20 proteins with molecular weight ranging from 30,000 to 200,000 were identified. Sera and monoclonal antibodies generated from immunized mice were found to react strongly with a 101kDa protein in immunoblot assay suggesting that the protein is a component of the nucleocapsid. This protein was found to be a specific serological marker for HHV6B infection as sera lacking HHV6B specific antibody and sera which were seropositive for one or more herpesvirus failed to react with this protein (Yamamoto *et al.*, 1990).

Pellett and co-workers, in 1993, confirmed the work of Yamamoto and group by proving that the 101kDa protein is indeed the major immunoreactive virion protein of the variant B. A dot matrix comparison between the immunoreactive protein of variant A and B (p100 and 101K) was done and it revealed that the two proteins are colinear. In the alignment of the amino acid sequence, 81% of the 101K residues are identical in p100, while 90% are similar. It was found that the 101K specific monoclonal antibody C3108-103 reacted with 8 HHV6B isolates but none of the HHV6A isolates, suggesting that the 101K protein is specific for variant B. These dissimilar regions of p100 and 101K may prove useful in the development of variant specific serodiagnostic assays (Pellett *et al.*, 1993). Figure 1.2 shows the alignment of the p100 and 101K amino acid sequences. The amino acid codes can be referred to in Appendix 1.

1.8 Mode of HHV6 Transmission

There are two possibilities in the mode of transmission of HHV6 i.e. vertical (transplacental) (Hall *et al.*, 1994) and horizontal transmission.

Horizontal transmission is indicated by isolation of viruses with nearly identical restriction endonuclear profiles from phases during exanthem subitum outbreak in an orphanage nursery (Okuno *et al.*, 1991) and from siblings and mothers of children with ES (Asano *et al.*, 1992).

Other modes of transmission have also been found. In 1994, Leach found that 10 - 20% of cervical secretion specimens were positive for HHV6 DNA suggesting the possibilities of both sexual and prenatal infection (Leach *et al.*, 1992). In another case, studies done by Aubin suggested intrauterine transmission of HHV6 (Aubin *et al.*, 1992; Adams *et al.*, 1998).

A few reports have described the detection of HHV6 DNA in saliva and throat swab by PCR (Gopal *et al.*, 1990; Jarrett *et al.*, 1990). HHV6 DNA was also detected in throat swabs from ES patients, so it was believed that HHV6 was secreted into throat mucus (Kido *et al.*, 1990).

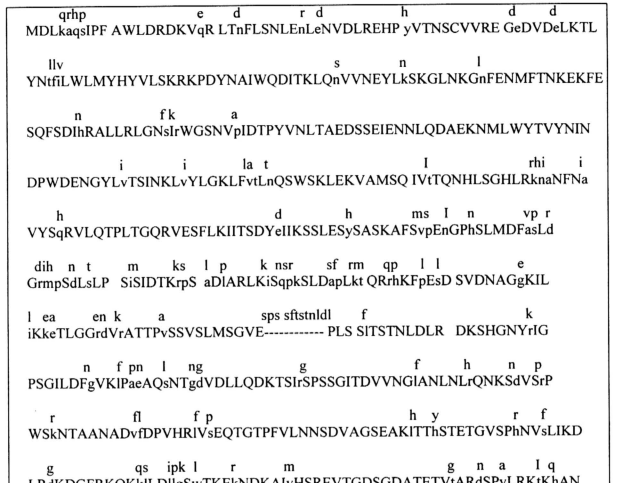

Figure 1.2 : Alignment of the p100 (variant A) and 101K (variant B) nucleocapsid protein amino acid sequences. The lower sequence is the 101K (p101) sequence. Only the nonidentical residues are shown for p100 (upper sequence shown in lowercase).

1.8a Transmission through Sera

In 1990, Farr reported that IgM could not be detected in PBL of newborns, thus eliminating vertical transmission as a mode (Farr *et al.*, 1990). Hall contradicted this when he found 30% healthy newborns having HHV6 in their mononuclear cells (Hall *et al.*, 1994). Dunne, in 1992 reported the rarity of HHV6 detection in cord blood (Dunne *et al.*, 1992). Earlier, Yoshikawa reported that HHV6 antibody titres in umbilical cord blood are even higher than in maternal blood (Yoshikawa *et al.*, 1990). In 1996, Chua *et al.*, found that there is high maternal transplacental transfer of antibodies to HHV6.

Because of its high seroprevalence, most new-borns have maternal antibodies to HHV6. The protective effect of the maternally derived antibody is evidenced by the low frequency of primary infections in babies, the high rate of infection after the antibody wanes (Asano *et al.*, 1989).

Virus neutralising IgM is detectable 5-7 days after the onset of ES, peaks after 2-3 weeks and is generally gone after 2 months (Suga *et al.*, 1992). Approximately 5% of adults may be IgM seropositive at any time (Suga *et al.*, 1992), but the trigger for the induction is not known. The IgG response develops 7-10 days following the febrile phase of ES (Balachandran *et al.*, 1991a). In most cases, IgG is present at detectable levels thereafter. Neutralising IgA was not detected (Suga *et al.*, 1992).

1.8b Transmission through Saliva

HHV6 has been recovered at high frequency (85%) from the saliva of both healthy, non-infected individuals and those infected with HHV6. Seroconversion occurred between 1-3 years of age, seroprevalence ranged between 80% to 100% among adults under 40 and decreased to 35% between ages 62 and 68 (Levy *et al.*, 1990).

Infectious cell-free HHV6 has been reported to be present in the saliva of nearly all adults (Pietroboni *et al.*, 1988; Levy *et al.*, 1990) and HHV6 antigens have been detected in salivary bronchial gland epithelium (Fox *et al.*, 1990; Krueger *et al.*, 1990). HHV6 DNA has also been detected in saliva from 90% of people tested by PCR (Cone *et al.*, 1993). The abundance of HHV6 in saliva has been estimated by quantitative PCR to average less than 100 (Jarrett *et al.*, 1990) or more than 50,000 HHV6 genomes per ml of HHV6 positive saliva (Cone *et al.*, 1993). Work done by Levy implies that saliva is the most likely source of infection. As most individuals acquire their HHV6 infection during childhood, the persistent viral shedding in healthy adults seems to represent reactivation or chronic infection not associated with any symptom (Levy *et al.*, 1990). Hence, saliva from silent shedders constitutes the most probable source of person-to-person transmission. With these as evidence, transmission via oral secretion remains the most likely possibility.

Levy also found that the virus is shed intermittently and contrary to previous assumptions, immunosuppression does not seem to be a pre-requisite for virus recovery. No substantial difference in the frequency of virus shedders was found between HIV-1 seropositive and HIV-1 seronegative subjects (Levy *et al.*, 1990).

When sera and saliva from 44 healthy young adults were tested for HHV6 variants A and B DNA by the nested PCR method, HHV6B infection was ascertained in 98% of the subjects and 95% were found to excrete variant B in their saliva. HHV6A was found in PBMC of 16%, but not detected in saliva (Aberle *et al.*, 1996). With this result, it was therefore confirmed that saliva is the predominant route of transmission of HHV6B (Aberle *et al.*, 1996). Studies investigating the difference in pathogenicity of the two variants demonstrated that variant B is the one usually detected in saliva (Jarrett *et al.*, 1990; Di Luca *et al.*, 1995b; Gautheret *et al.*, 1995) and in PBMC during primary infection (Dewhurst *et al.*, 1993a). Variant A was initially found only in PBMC of immunosuppressed patients.

Fox's finding showed that the salivary gland tissue is a site of HHV6 replication and perhaps its persistence . He also suggested mother to infant transmission of HHV6 with saliva as the vehicle. He speculated that HHV6 reactivation within the salivary gland in late pregnancy results in the shedding of large amounts of virus in the saliva, thereby facilitating transmission of HHV6 from mother to infant (Fox *et al.*, 1990).

1.8c Transmission through Breast Milk

Breast milk has been determined to be a major route by which infants become infected with hCMV in Western countries (Dworsky *et al.*, 1983) and Japan (Fujioka *et al.*, 1994; Minamishima *et al.*, 1994).

Work done by Kusuhara revealed that seroprevalence of HHV6 in breast-fed and bottle-fed children stood at 90.9% and 93.0% respectively. This result showed that breast

milk was not a significant source of early HHV6 infection in infancy (Kusuhara *et al.*, 1997).

Two others obtained negative results on the possibility of HHV6 infection via breast milk. Dunne failed to demonstrate the HHV6 genome in breast milk (Dunne and Jevon., 1993) and Takahashi reported the acquisition of HHV6 infection by 9 months of age in 11 of 12 bottle-fed infants (Takahashi *et al.*, 1988).

1.9 Detection of Human Herpes Virus 6 Infections

Most studies of HHV6 antibody prevalence have utilised indirect or anticomplementary IF for the detection of anti-HHV6 IgG. The subjective nature of IF has led to difficulties in interpretation of results, which probably caused published seroprevalence rates to vary greatly. Anti-HHV6 ELISA has been developed (Saxinger *et al*, 1988; Dahl *et al*, 1990), whereby sensitivity seems better than that of IF, though the ambiguity of sera with reactions at cut-offs remained.

To understand the reasons in the complexity of the diagnosis, one has to consider the characteristic life cycle of HHV6 in the host (Lusso, 1996). The virus enters the body during primary infection, which occurs almost universally in early childhood, the virus is probably never cleared from the organism and persists in selected anatomical sites (including blood leukocytes), most likely in a latent state, for the entire lifetime. Reactivation or exogenous reinfection may occur, particularly in concomitance with episodes of immune disregulation or deficiency. After such episodes, latency may be established again. With these to be considered, it is obvious that diagnostic methods which cannot discriminate between latent (clinically silent) and active (clinically

relevant) infection, such as serum IgG antibody testing or DNA-PCR on blood cells, may be inappropriate or even yield misleading results for the clinical diagnosis of HHV6 infection (Lusso , 1996).

Exanthem subitum can be confused with other febrile rash syndromes including those caused by measles, rubella and echovirus 16 (Asano *et al.*, 1991). Clinical diagnosis based on the age of the child, the fever followed by rash course and the maculopapular rash are reliable in 60-86% of cases (Balachandran *et al.*, 1991a; Rudin and Hirxch., 1991). The presence of viral DNA in healthy controls may represent asymptomatic reactivation, persistence of latent, viral genome or persistent low level viral replication (Chan *et al.*, 1997).

Rudin wrote that IgM by itself is not a reliable marker for HHV6 infection because most cases confirmed by culture or IgG seroconversions have no detectable IgM response (Rudin *et al.*, 1991) and approximately 5% of adults may be IgM positive at any time (Suga *et al.*,1992). This however contradicts other results that showed that unlike IgG anti-HHV6, specific IgM antibody may provide a reliable marker of active infection (Fox *et al.*,1990; Secchiero *et al.*,1995). The main limitations of detection of HHV6 IgM are its relatively low sensitivity and the possible cross reactivity with other human herpesviruses.

Radioimmunoprecipitation, Western blots, flow cytometry, reverse transcription PCR, immunohistochemistry (Knox and Carrigan ,1994) and *in situ* hybridization on RNA are suitable methods for the demonstration of active HHV6 infections in circulating cells or tissues. However, the method available now is the ELISA using recombinant proteins or infected cellular extracts as antigens (Saxinger *et al.*, 1988, Foa-Tomasi *et al.*,

1991). A new method which can be used as a sensitive indicator of active HHV6 infection in infants prior to their seroconversion, PCR-ELISA, detects amplified PCR products and the system has been found to be rapid, specific and sensitive (Osiowy *et al.*, 1998). A summary of the methods that have been used for the detection of HHV6 is shown in Table 1.2.

Much research has been directed towards identification of antigenic proteins of HHV6. To date several have been recognized (refer section 1.7) but only one has been found to be immunodominant, being the 101kDa nucleocapsid protein (Yamamoto *et al.*, 1990; Pellett *et al.*, 1993) . Through the identification of this protein, it is believed that a more accurate diagnosis of HHV6 would be possible. Through epitope recognition, a minimal risk of cross reactivity with other species of herpesviruses can be expected. An interesting possibility would be the eventual use of the peptides as vaccines.

Table 1.2 : Human herpes virus 6 antibody and virus detection methods

Method	Reference
Antibody Assays	
a) Immunofluorescence assay	
i) T-cell lines (MT-4, Molt-3, Molt-4, CCRF-CEM,HUT-78, H9, HPB-ALL	Asada *et al.*, 1989
ii) IgG	Irving *et al.*, 1990; Dahl *et al.*, 1990; Robert *et al.*, 1990; Coyle *et al.*, 1992; Sloots *et al.*, 1996
iii) IgM	Irving *et al.*, 1990; Dahl *et al.*, 1990
iv) Monoclonal antibodies (Mab)	Wyatt *et al.*, 1991., Ablashi *et al.*, 1991, Pellett *et al.*, 1993
v) Anticomplement IF assay (ACIF)	Robert *et al.*, 1990
vi) Complementary IF (CIA)	Coyle *et al.*, 1992
vii) Radioimmunoassay (RIA)	Coyle *et al.*, 1992
ELISA	
i) IgG ELISA with membrane antigen	Saxinger *et al.*, 1988; Dahl *et al.*, 1990; Foa-Tomasi *et al.*, 1991
ii) 60 min IgG ELISA commercial assay	Sloots *et al.*, 1996
Antigen Assays	
Polymerase chain reaction(PCR)	
i) Hybrid-based microtiter plate assay (PCR-ELISA)	Osiowy *et al.*, 1997
ii) Nested PCR	Kondo *et al.*, 1990
In situ immunohistochemistry	Fox *et al.*, 1990; Yamamoto *et al.*, 1990
Strain/variant detection	
i) PCR and Southern blot	Aubin *et al.*, 1991; 1993; Chou *et al.*, 1994
ii) Antigen capture ELISA	Marsh *et al.*, 1996

To date, 2 variants of herpesvirus have been identified. There is a possibility that proteins may have the capacity for variations and may be used to differentiate more herpesviruses that are being isolated around the world.

1.10 Epidemiology of Human Herpes Virus 6

Research results indicate that perinatal and congenital HHV6 infection was rare and mainly reflected reactivation of HHV6 in pregnancy (Balachandran *et al.*,1989). It was found that babies are born with a low anti-HHV6B titer and lose the antibodies within 6 months. After the protection is lost, they are easily infected by HHV6 since other members in the home consistently excrete HHV6B in the saliva (Pietroboni *et al.*,1988; Levy *et al.*,1990). The high chance (51%) of infection from 6-12 months of age may also be related to the frequent oral exploration of the environment by children of this age group, which allows contact with saliva or saliva -contaminated formula. Farr *et al.* (1990) found that in Western Australia, the HHV6B positive rate was evident in young children between 5-6 months and by 11-12 months the positive rate was approaching the level that was found in the adult population.

Although there has been no direct proof of protection by maternal HHV6 antibodies, several studies (Pietroboni *et al.*,1988; Balachandran *et al.*,1989; Okuno *et al.*,1989; Chua *et al.*, 1996) found that HHV6 infection increases after the first few months of life, suggesting a protective role for the maternal antibodies. In addition, findings that all patients were seronegative before they became infected strongly support the possibility that infection seldom occurs before maternal antibody disappears (Huang *et al.*, 1992).

1.11 Human Herpes Virus 6 Associated Diseases

A variety of diseases presumed associated with primary or reactivated HHV6 infection is classified into two groups. In the first group, there are diseases causally related to HHV6 infection. This includes exanthem subitum (Yamanishi *et al.*,1988; Enders *et al.*,1990) and heterophile antigen negative infectious mononucleosis (Steeper *et al.*, 1990)

The second group of diseases associated with elevated levels of HHV6 antibody IgG are suggestive of HHV6 reactivation This group includes certain autoimmune disorders, chronic fatigue syndrome (Ablashi *et al.*, 1991) and lymphoproliferative disorders.

Primary infection with HHV6 occurs most commonly in children following the waning of maternal antibodies approximately 3-6months after birth. At seven months, seroprevalence rises, with approximately 75% of infants acquiring antibodies. Antibody titers reach their highest at 3-4 years of age and thereafter decrease gradually with age (Briggs, *et al.*, 1988; Chou *et al.*, 1992).

The most common clinically defined entity associated with HHV6B is the childhood disease exanthem subitum, also known as roseola infantum or sixth disease. Yamanishi and co-workers provided the first compelling evidence that primary HHV6B infection might be associated with human disease when they showed that HHV6B could be isolated from PBMC of 4/4 Japanese infants with exanthem subitum (Yamanishi *et al.*, 1988). This was subsequently confirmed by others (Enders *et al.*, 1990). In North America and Europe, perhaps 60-70% of primary infection by HHV6 are asymptomatic (Breese-Hall., 1994) or result in febrile illness without rash (Portolani *et al.*, 1993), while

in Japan approximately 60% of children are diagnosed with exanthem subitum (Kusuhara *et al.*, 1992).

HHV6B associated exanthem subitum is characterised by high fever (>39°C) lasting 3-5 days (Asano *et al.*, 1991,1994; Okada *et al.*, 1993) that is followed by an erythematous and macular or macropapular rash of 1-3 days duration (Asano *et al.*, 1991,1994). Lymphocytosis and neutropenia are frequently seen (Balachandran *et al.*, 1991a). Symptoms in more severe cases include high fever (>40°C), respiratory tract inflammation, intestinal symptoms and rash. Sometimes infection is accompanied by seizures (Yoshikawa *et al.*, 1992a; Jee *et al.*, 1998), though the seizures may be due to the febrile illness and not due to the HHV6 infection (Hukin *et al.*, 1998). Others include hepatosplenomegaly (Irving *et al.*, 1990), aseptic meningitis, fatal fulminant hepatitis with HHV6 present in the brain and fatal disseminated infection in an apparently immunocompetant child. The variant B accounts for all exanthem subitum -associated HHV6 infections (Dewhurst *et al.*, 1992, 1993b; Pellett *et al.*, 1990; Schirmer *et al.*, 1991).

The virologic course of HHV6B infection during exanthem subitum is as follows. During the febrile phase, HHV6 is present in CD4+ cells in a readily culturable form (Yamanishi *et al.*, 1988; Takahashi *et al.*, 1989; Asano *et al.*, 1989,1991; Okada *et al.*, 1993) with some investigations reporting isolation rates above 90% (Asano *et al.*, 1989). The viremia is greatly reduced during the rash phase (and is normally gone by the time the disease resolves). HHV6 antibodies can be detected as soon as three days after the onset of fever and in most cases, by eight days (Asano *et al.*, 1989; Ueda *et al.*, 1989; Yoshiyama *et al.*, 1990).

Adults exhibited a mild afebrile illness with serological evidence of infection or reactivation and the HHV6 variant A is the strain isolated in the majority of these patients. Enlarged bilateral, non-tender, anterior and posterior cervical lymph nodes, clinically distinguishable from hCMV and EBV infections with up to three month persistence has been seen in 18-21 year old males (Niederman *et al.*, 1988; Dewhurst *et al.*, 1993b). Other conditions associated with HHV6 infection include CFS or myalgic encephalitis (Ablashi *et al.*, 1988; Buchwald *et al.*, 1992) but no more proof exists that HHV-6 causes CFS than for any other suspected virus (Pellett *et al.*, 1996, Buchwald *et al.*, 1996; Wallace *et al.*, 1999). HHV6 has also been linked with multiple sclerosis (MS). In a study carried out in Betheseda, Maryland, more than 70% of patients with the relapsing -remitting form of MS, showed an increased immune response to HHV6 and approximately 35% of all MS patients studied had detectable levels of active HHV6 is serum. This was contradicted by Martin *et al.* (1997) who did a study on 115 cerebrospinal fluid (CSF) samples and 116 serum samples of MS patients and found no presence of HHV6 and also by Mayne *et al.* (1998) who found no correlation between HHV6 infection in PBMC and development of multiple sclerosis. He concluded that continuous disseminated HHV6 infection in MS patients is not a possibility, but did not rule out the possibility of low-grade herpesvirus infection within the MS brain. It is believed that the central nervous system may act as a reservoir for HHV6 (Merelli *et al.*, 1997) and there may be a point in time during the progression of MS when the virus reactivates, accounting for its presence in the subset of MS patients.

In vitro studies have shown that HHV-6 inhibits HIV growth, possibly due to rapid lysis of CD4+ host cells for both viruses (Pietroboni *et al.*, 1988; Yasukawa *et al*,

1998), whilst others suggest decreased cytopathic effects and prolonged CD4+ survival with decreased HIV-1 replication (Levy *et al.*, 1990). These variations in studies may be due to strain or assay differences (Carrigan *et al.*, 1990). Morodi postulated that HHV6 contributes to immunosuppression, prior to AIDS, thus predisposing the organism to HIV infection (Morodi *et al.*, 1998) Another postulation is that the reactivation of HHV6 may dramatically amplify the immunological damage present in HIV-infected individuals. In turn, the increasing immunological imbalance in the host, combined with the synergistic effects of HHV6 and HIV, may accelerate the replication and the spread of HIV, leading to the final destruction of the immune system (Lusso and Gallo, 1995; Ablashi *et al*, 1998)

Many lymphoproliferative disorders including follicular lymphoma, diffuse lymphoma, Burkitt's lymphoma, sarcoidosis, Sjögren's syndrome and acute myeloid leukaemia (Ablashi *et al.*, 1988), have shown both elevated levels of specific antibody to HHV6 and the presence of HHV6 DNA sequences. However no causal relationship has been found between HHV6 and any of these conditions.

Recipients of kidney, bone marrow (HHV-6 demonstrating a bone marrow suppressive role (Drobyski *et al.*, 1993) or liver transplants (Lautenschlager *et al.*, 1998; Yoshikawa *et al.*, 1998) have demonstrated both seroconversion and viraemia with accompanying non-specific febrile illness (Drobyski *et al.*, 1994) although no primary infections have been conclusively diagnosed. One discovery indicated that seroconversion to HHV6 following liver transplant is an indication of cytomegalovirus disease (Dockrell *et al.*, 1997). There is a possibility that the seroconversion could be due to cross reaction between antibodies to cytomegalovirus and HHV6B. Chronic (Tajiri *et*

al., 1997) and fulminant hepatitis was associated with HHV6 infection in a fatal case from which virus was isolated. IgM antibody was present and HHV6 DNA was discovered by polymerase chain reaction (PCR) amplification of brain tissue with specific primers for HHV6 DNA detection (Asano *et al.*, 1990).

HHV6 has also been linked to Hodgkin's disease (HD) by IgG serology and PCR (Torelli *et al.*, 1992; Di Luca *et al.*, 1994), to non-Hodgkin's lymphoma by Southern blotting and PCR (Fillet *et al.*, 1995), to necrotizing encephalitis in Griscelli's Syndrome patients (Wagner *et al.*, 1997) to angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) by PCR (Luppi *et al.*, 1993a) , to Karposi's Sarcoma by PCR (Bovenzi *et al.*, 1993), to oral carcinoma by immunohistochemistry and *in situ* hybridization (Chen *et al.*, 1994; Vasudevan and Vijayakumar, 1998), to gastroduodenitis and pancreatis in immunosuppressed individuals (Randhawa *et al.*, 1997) and to anticonvulsant hypersensitivity syndrome and reactive haemophagocytic syndrome (Descamps *et al.*, 1997) . A summary of the diseases associated with HHV6 is shown in Table 1.3.

Table 1.3: Reported HHV6 isolations and associated diseases

HHV6 ASSOCIATED DISEASES	REFERENCES
Lymphoproliferative and malignant diseases and AIDS	Salahuddin *et al.*, 1986; Downing *et al.*, 1987; Lusso and Gallo, 1995, Knox and Carrigan, 1994
Chronic fatigue syndrome (CFS)	Ablashi *et al.*, 1991; Yalcin *et al.*, 1994; Di Luca *et al.*, 1995a; Wallace *et al.*, 1999
Exanthem subitum	Yamanishi *et al.*, 1988; Enders *et al.*, 1990
Aseptic meningitis, chronic and fulminant hepatitis, meningoencephalitis	Irving *et al.*, 1990; Tajiri *et al.*, 1997
Multiple sclerosis	Challoner *et al.*, 1995; Mercelli *et al.*, 1997
Organ transplant i) Bone marrow ii) Liver iii) Kidney iv) Cardiac	 Ward *et al.*, 1989; Chan *et al.*, 1997 Lautenschlager *et al.*, 1998; Yoshikawa *et al.*, 1998 Irving *et al.*, 1988; Okuno *et al.*, 1990 Irving *et al.*, 1988
Necrotizing encephalitis in Griscelli's syndrome	Wagner *et al.*, 1997
Angioblastic lymphadenopathy with dysproteinemia	Luppi *et al.*, 1994
Oral carcinoma	Chan *et al.*, 1994; Vasudevan and Vijayakumar, 1998
Gastroduodenitis and pancreatis	Randhawa *et al.*, 1997
Anticonvulsant hypersensitivity syndrome and reactive haemophagocytic syndrome	Descamps *et al.*, 1997
Kaposi's sarcoma	Bovenzi *et al.*, 1993
HIV infection	Downing *et al.*, 1987 ; Lopez *et al.*, 1988

1.12 Protein Analysis and Structure

Primary structure or amino acid sequence of a protein is the order of amino acids along the linear chain of the polymer. In the crystal structure of proteins, regions of repeating structural patterns known as secondary structure is found. The two most commonly found secondary structures in globular proteins are the right-handed alpha helix and the beta pleated sheet. Tertiary structures refers to how elements of secondary structure arrange themselves in 3 dimensions in the folded protein and how the amino acid side chain interacts with one another to form hydrogen bonds, electrostatic salt bridges and hydrophobic interactions. Proteins fold in the native (i.e., naturally occurring biologically active) tertiary structure because of a variety of thermodynamic factors. The tertiary structure gives a protein overall shape and dimensions and also provides a means of bringing into spacial proximity amino acid sequences that may be distant in the linear sequence of the protein, but that come together to form the catalytic site of an enzyme or a recognition site for the action of another protein (Copeland, 1994).

1.13 Immunogenic Epitope Analysis and Mapping

The increasing use of peptides for immunological research, diagnostics, pharmaceuticals and vaccines has led to the development of new immunodiagnostic and therapeutic agents. One of the advances that has led to this increase in peptide research is the improvements made in synthetic chemistry (Geysen *et al.*, 1984; Houghten *et al.*, 1985). Nature has discovered that it can control nearly all biological processes by various

kinds of molecule recognition and that peptides and proteins are uniquely suited for this because of their potential for diversity.

Although peptides often tend to be poor drug candidates because they do not typically have good oral or central activity, in many instances they can be modified to improve their activity. The use of synthetic peptides in the study of the immune response stems from the ability of peptides to mimic similar antigenic sequences in proteins.

Mapping of B cell epitopes (also called antigenic determinants, antigenic sites, antibody combining sites etc.) and T cell epitopes of a given protein is the essential part of the design of synthetic vaccines or diagnostics and an essential element for the understanding of the immune response and the pathology of major diseases. B cell epitopes of proteins have been studied using both monoclonal and polyclonal antibodies. The interaction between antibody and B cell epitope does not necessarily require the B cell epitope to be fully intact. Parts of the B cell epitope as linear peptides may bind antibody, although at lower affinity.

From a practical point of view, epitopes can be divided into three groups : (Figure 1.3)

i) Real linear epitopes. These epitopes are easily mapped using peptides, but probably do not occur very often.

ii) Discontinuous epitopes with relative large linear parts. The linear parts of these epitopes are easily mapped using peptides.

iii) Conformational epitopes. These epitopes probably form the majority of all existing epitopes. They are difficult to map with peptide- based methods. One has to rely on methods like site-directed mutagenesis or in the case of

replicating agents, on "escape mutations". Often the data will only produce a useful definition of the epitope if the structure of the protein carrying this epitope is known.

Epitopes can be mapped using numerous approaches: they can be

i) 'predicted' from the amino acid sequence of a protein

ii) inferred from the location of amino acid mutations which disrupt antibody binding

iii) assessed by amino acid sequencing of antibody -binding clones of genomic expression libraries

iv) can be assessed using peptides.

The approach based on synthetic peptide libraries (Geysen *et al.*, 1984, 1985; Lam *et al.*, 1991) has provided methods that provide sets of overlapping peptides of known linear amino acid protein sequence. The PEPSCAN (Geysen *et al.*,1984) method has been most useful in the systematic mapping of numerous B cell epitopes and T cell epitopes. Using the PEPSCAN, B cell epitopes have been mapped for proteins of viruses; such as foot-and-mouth disease virus (Geysen *et al.*, 1984, 1985), parvovirus (Brown *et al.*, 1992), herpesviruses (Middeldorp and Moleon, 1988) and the hormones include hCG and inhibin (Amerongen *et al.*,1994).

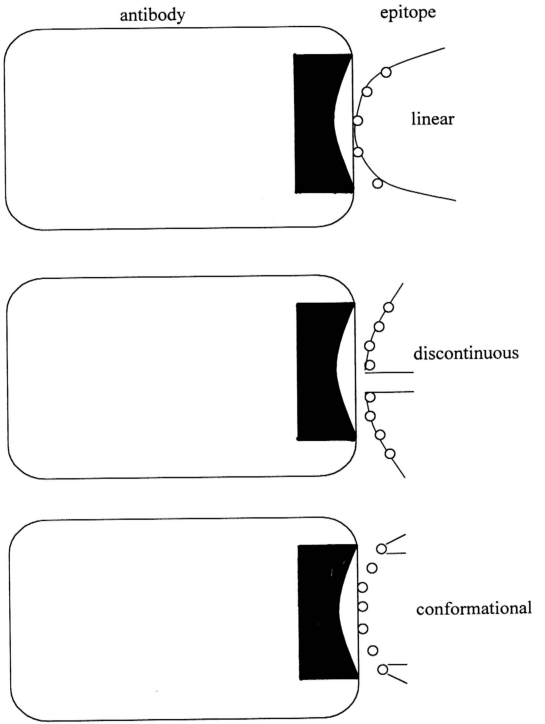

Figure 1.3: Schematic representation of the three theoretically possible epitope configurations. The antibody is represented as a rough outline of the Fab fragment. The darkened area indicates the part of the antibody in contact with the amino acids of the epitope. The amino acids are represented by small circles (adapted from Meloen *et al.*, 1991).

1.14 Multipin Peptide Synthesis

Multipin peptide synthesis (Geysen *et al.*, 1987) revolutionised the field of epitope mapping. In this technique, numerous overlapping peptides, based on known amino acid sequence of the protein of interest is synthesized on the tips of solid polyethylene pins. Once synthesized, a modified ELISA protocol is used to detect the interaction between an antibody and its specific antigens.

It is possible to synthesize peptides of any length using this technique but as most naturally occurring epitopes are 8-13 amino acids long, it is best to synthesize peptides within this length. The "overlap format" of a synthesis of a 10-mer with an overlap of 5 amino acid per peptide means that for a protein of interest of 50 amino acids, the first peptide would be from amino acid 1 to 10. The next would be from 5 - 15, 10 - 20 and so forth until the 10^{th} peptide, which would be amino acid 40 - 50 (Figure 1.4).

An attraction to the methodology is that highly specialised knowledge of peptide chemistry is unnecessary; the peptides produced are impure but sufficient for the screening of antisera for peptide binding activity. The peptides are covalently bound to the pin surface and so, are re-usable. Due to the strength of the covalent binding, the antibodies can be removed from the pins without breaking the bonds between pins and the peptides.

This strategy uses a base-labile protecting group (Fmoc) for the α-amino group and acid-labile side chain protecting groups. To make a peptide, amino acids are added one at a time, commencing from the carboxyl end of the sequence. i.e. in the reverse direction from biosynthetic peptide assembly. Pins are Fmoc deprotected in DMF to

reveal a free amino group ready for coupling of the carboxyl group of the first amino acid. The protected amino acid, dissolved in DMF is activated with a dehydrating agent such as a diimide (DIC), along with a catalyst such as hydroxybenzotriazole (HOBt).

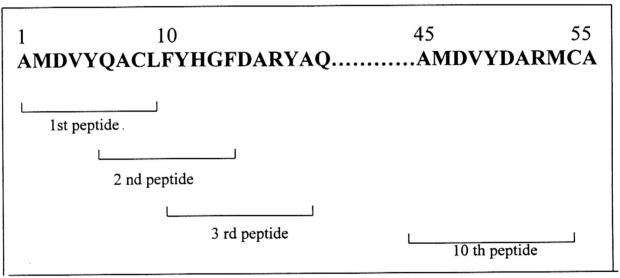

Figure 1.4 : The amino acid sequence of overlapping peptides based on a hypothetical 55 amino acid residue protein. The alphabets represent single letter abbreviations for amino acids (Appendix 1)

1.15 Prediction of Antigenicity by Hydropathy, Accessibility and Secondary Structure

Due to limitations on the number of peptides which can be readily synthesized and tested, the choice of peptides have often been made based on predictive algorithms which have been published (Hopp and Woods, 1981). Other limitations include cost, facilities requirement and peptide presentations as affected by coupling procedures during synthesis (Geysen *et al.*, 1987).

There have been attempts to predict the antigenic sites of proteins from their primary sequence structure or from properties of the protein related to polypeptide folding. Antigenicity is acknowledged as a surface property, and accessibility on the surface of the native structure is a minimum requirement for an antigenic B-cell site. Other structural properties that have been acknowledged are hydrophilicity (Hopp & Woods, 1981), occurrence of β-turns, protruding regions of proteins, segmental mobility and occurrence of specific amino acids in sequence (Milich., 1989). Each factor may contribute in enhancing or decreasing the activity of a site, since preference for one epitope implies discrimination against the other.

Historically, the first methods used for prediction of a protein B-cell antigens were based on hydrophilicity (Hopp and Woods, 1981). In their study, the 20 amino acids were partitioned and quantitated in a biphasic aqueous/organic solvent mixture. Preference to the aqueous solvent was scaled as hydrophilicity. Spans of the protein primary structure containing primarily hydrophilic amino acids are believed to lie on the surface of the molecule where they can interact with the solvent. The segments with the highest average hydrophilicity (lowest hydrophobicity) are then very likely to correspond with exposed surface regions and hence, they are potentially highly antigenic. Factors such as charges, shape and molecular chemistry could hinder binding to a hydrophilic region (Geysen, 1986). The hrdrophilicity model has been used to analyze more than 12 proteins with known antigenic sites : the most hydrophilic site of each protein was indeed one of the antigenic sites.

Like the protein themselves, the antigenic determinants of proteins consist of amino acid residues in a particular 3-D array. The residues that make contact in the

antibody combining site are called 'contact residues'. To make contact, of course these residues must be exposed on the surface of the protein, not buried in the hydrophobic core. (Berzofsky and Berkower, 1993). The hydrophilicity method is only about 50% accurate for monomer globular proteins. This is to say that only 50% of hydrophilic predicted surface regions of this type of protein would actually invoke a B-cell response in a typical experiment.

Before accepting a definition of antigenic residues as all those found on the interface with the antibody, it is worth considering that the essence of antigenicity involves binding energy, that is the interaction energy between two protein antigens. A peptide not containing residues of the antigenic determinant would have lower binding energy. But for cases in which all residues in the determinant are present in the peptide, affinity may be low because the topography of the residues in the peptide may not produce as complementary a fit in the antibody-combining site as the native conformation would. Another possibility for reduction in affinity may be because only a small fraction of the peptide molecules are in a nativelike conformation with at any time (Berzofsky and Berkower, 1993).

Among other limitations of the Hopp and Woods method is that the hydropathy profile does not give an unambiguous or detailed indication of protein topology. Besides that, there is also the possibility that a significant fraction of the surface region can be nonpolar (Conolly., 1983). It is also true that all hydrophilic regions of the protein do not necessarily correspond to epitopes, that not all epitopes are hydrophilic or even on the protein surface and that major antigenic regions are not necessarily the most hydrophilic.

Secondary structures could be helpful in determining the peptides for synthesis. However, it is not a dominant factor in the degree of antigenicity. A good example of the importance of secondary structure is the case of the loop peptide (sequence 64-80)of hen egg-white lysozyme (Maron *et al.*, 1971). This loop in the protein sequence is created by the disulphide linkage between cysteine residues 64-80 and has been shown to be a major antigenic determinant for antibodies to lysozyme (Maron *et al.*, 1971). The peptide containing the loop binds antibodies with high affinity, but opening of the loop of the disulphide bond destroys most of the antigenic activity for antilysozyme antibodies (Maron *et al.*, 1971).

Surface accessibility is yet another approach in predicting antigenic sites. The basis of accessibility is to examine the degree of protrusion from the protein surface of a protein. Usually, the most protruding sites are found to be part of antigenic sites bound by antibodies (Thornton *et al.*, 1986). These sites are identified using short synthetic peptides and so are sequential in nature. Surface accessibility cannot be predicted from acid amino sequence alone because it is very much a structure-dependant molecule. In the present study, the surface accessibility was predicted using the Protscale software (http:/expasy.hcuge.ch/cgi-bin/protscale.pl.). In a study by Novotny *et al.*, (1986), the exposed surface area was found to be lowest for the least immunoreactive residues, supporting a relationship between surface exposure and antigenicity. However, this trend did not continue as most antigenic residues did not have more exposed areas than those with average antigenicity.

Another factor suggested to play a role in immunogenicity of protein epitopes is mobility. Measurement of mobility is largely dependent on the availability of a high-

resolution crystal structure, thus its applicability is only to a small subset of proteins. Westhoff *et al.*, (1984) used a series of hexapeptides to determine the specificity of antibodies raised against native tobacco mosaic virus protein and found that 6 of the 7 peptides that bound antibodies to native protein correspond to peaks of high mobility in the native protein. The correlation was better than could be accounted for by just using surface accessibility parameters. More mobile segments are likely to be those on the surface and therefore more exposed.

In an analysis done by Parker *et al.* (1986), three parameters were chosen that individually showed the highest scores at predicting antigenic sites. These were the HPLC hydrophilicity, accessibility and flexibility parameters. Each scaled plot was superimposed on the other and the maximum value of each residue was used to give the composite profile value. Thus, it was apparent that no single-parameter set was able to predict all antigenic sites.

To date, there have been many sophisticated algorithms that are able to predict antigenic sites more accurately. One of it is a computer programme "ADEPT" especially for the prediction of antigenic determinants. this programme includes a literature-derived database of proteins in the SWISSPROT standard with experimentally determined antigenic determinants, so the predictive ability of new methods can be assessed within the programme and corresponding statistical information calculated (Maksyutov and Zagrebelnaya, 1993). Another is prediction through molecular modelling using the "FOLDING" software developed by Brasseur in 1995. This method has been successfully used to predict epitopes for the papilloma virus (Gallet *et al.*, 1990).

1.16 Objectives of Present Study

The present study has 4 main objectives:

1) To compare and study prevalence of serum IgA and IgG antibodies to HHV6 in subjects aged 0 – 20. This would help determine if IgA, like IgG, follows a particular seroconversion profile with age.

2) To carry out immunofluorescence assay using HHV6 infected human cord blood mononuclear cells on samples (IgA in saliva and breast milk and IgG for serum) to look for the presence of humoral response to HHV6.

3) To identify immunoreactive regions at the carboxyl terminal of the p101 nucleocapsid protein of HHV6. This is done by synthesizing overlapping amino acids sequences of p101 of HHV6 and testing for antibody reactivity, using the peptides as antigens in ELISA. The determination of antigenic regions would be based on epitope mapping as well as prediction analysis based on hydrophilicity, surface accessibility and secondary structure of the protein.

4) To compare the HHV6 reactive regions of IgG in sera and IgA in saliva and breast milk to determine if IgA and IgG recognize shared antigenic regions of the carboxyl terminal of the virus protein.