The Genotypic Characterisation of Human Herpesvirus 8 in Different Groups.

Submitted in fulfilment of the conditions governing candidates for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The prevalence of human herpesvirus 8 (HHV-8) in patients with Kaposi's sarcoma (KS) or people at risk of developing KS is high, while that in low-risk populations remains unclear. Use of disparate serological assays for anti HHV-8 detection contributes to this uncertainty.

Hypothesising that in populations at low risk of HHV-8, the detection of HHV-8 genome enhances the specificity of and lends sensitivity to estimations of HHV-8 prevalence, studies were conducted to compare the genoprevalence and molecular epidemiology of HHV-8 in UK subpopulations at varying risk of HIV infection or KS: blood donors, human immunodeficiency virus (HIV)-infected individuals, bone marrow transplant (BMT) recipients, and patients with chronic fatigue syndrome (CFS).

A protocol for amplifying sub-genomic HHV-8 DNA was first developed using blood originating from HIV-seropositive patients, from which CD45+ cells were immunomagnetically separated and their extracted DNA submitted to nested PCR. It was determined that such an approach afforded greater sensitivity to HHV-8 DNA detection than approaches based on PCR applied to separated peripheral blood mononuclear cells.

Using the improved protocol, DNA from open reading frame (ORF) 26 of the HHV-8 genome could be amplified from 24% of blood donor samples. In a subsequent donor group, DNA from ORFs 26 and K1 was detectable in 8% and 9%, respectively. ORF K1 sequences could be classified as belonging to genotypes A1, A4 and C3. HHV-8 seropositivity in the second group was 12% and 24%, as determined by two antibody assays, and

herpes simplex virus-2 seropositivity was 0%. No associations were found between HHV-8 genome and anti HHV-8 detection.

The findings in the BMT and CFS groups further substantiate the hypothesis that HHV-8 infection is more widespread than previously thought, carriers may not mount antibody responses detectable by current serological assays, and the principal HHV-8 transmission route is not sexual.

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DECLARATION

The findings reported in this thesis result entirely from my own work. Colleagues who helped in the various aspects of the work are listed in Acknowledgments. The work has not previously been submitted, in part or in full, for a degree or diploma of this or any other University or examination board.

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ABBREVIATIONS / DEFINITIONS

AIDS	Acquired immunodeficiency syndrome
AKT	Proto-oncogene protein akt
AP-1	Activator protein-1
ART	Anti-retroviral therapy
Bad	Bad protein, promotes cell death by displacing Bax protein
Bak	Bak integral membrane protein, promotes cell death
Bax	Bax protein, 21 kDa homologous partner of Bcl-2
BCBL	Body cavity based lymphoma
BCP-1	Body cavity based / Primary effusion lymphoma cell line
Bcl-2	B cell leukaemia / lymphoma-2 genes
BHK	Baby hamster kidney
BFRF3	Epstein-Barr viral capsid antigen
BMT	Bone marrow transplant
bp	Base pair
CAM	Ca^{2+}/calmodulin-dependent protein kinase
CC	Subfamily of chemokines which have 2 adjacent cysteine residues
CCL	CC chemokines, previously designated MIP
CCR	CC chemokine receptor
CD	Castleman's disease
Cdks	Cyclin-dependent kinases
CFS	Chronic fatigue syndrome
CIs	Confidence intervals
CMV	Cytomegalovirus
CXC	Subfamily of chemokines which have 2 cysteine residues separated by a single amino acid
d	Days
DNA	Deoxyribonucleic acid
dNTPs	Deoxyncleotide triphosphates
ds	Double stranded
EBV	Epstein-Barr virus
EDTA	Disodium ethylenediaminethetra-acetate
EGS	External guide sequence
ELISA	Enzyme linked immunosorbant assay
FCS	Fetal calf serum

ABBREVIATIONS / DEFINITIONS contd.

FLICE	Fas-associated death domain-like interleukin-1 beta-converting enzyme
FLIP	FLICE inhibitory protein
GC	Guanine-cytosine
GPCR	G protein-coupled receptor
GSK-3	Glycogen synthase kinase 3
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HHV-8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HSCT	Haemopoietic stem cell transplant
HSV-1	Herpes simplex virus-1
HSV-2	Herpes simplex virus-2
HVA	Herpesvirus ateles
HVS	Herpesvirus saimiri
IFA	Immunofluorescence assay
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL-6	Interleukin-6
IL-8	Interleukin-8
IRF	Interferon regulatory factor
ISH	*In-situ* hybridisation
kb	Kilobase pair
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
LNA	Latency-associated nuclear antigen
LANA	Latency-associated nuclear antigen
MCD	Multicentric Castleman's disease
MCP	Major capsid protein
MHV-68	Mouse herpesvirus strain 68
MIP	Macrophage inhibitory protein
mon	Months
MPC	Magnetic particle concentrator
NaI	Sodium iodide

ABBREVIATIONS / DEFINITIONS contd.

NaN_3	Sodium azide
Na_2PO_4	Sodium Phosphate
NF	Nuclear factor
NFAT	Nuclear factor of activated T-cells
NRK	Normal rat kidney cells
ORF	Open reading frame
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PHYLIP	Phylogeny inference package
PKR	Protein Kinase
rSFVs	Semliki Forest viruses
Rb	Retinoblastoma tumour suppressor gene
RBC	Red blood cell
RNA	Ribonucleic acid
RRV	Rhesus rhadinovirus
Rta	Replication and transcription activator
RT-PCR	Reverse transcriptase polymerase chain reaction
SDF-1alpha	Stromal cell-derived factor-1alpha
SHV-2	Herpesviruses saimiri
Taq	*Thermus aquaticus*
Tat	Transactivator protein
TATA	A conserved A-T rich sequence which is contained in promoters for RNA polymerase II
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TPA	12-*O*-tetradecanoylphorbol 13 acetate
TR	Terminal repeat
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VZV	Varicella-zoster virus
yr	Year

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF HERPESVIRUSES

1.1.1 GENERAL FEATURES

Herpesviruses are a family of DNA viruses found commonly in humans and animals. Nearly 100 herpesviruses have been identified, with most animal species shown to be infected by at least one member of the family. They share four characteristic biological properties:

(i) Synthesis of viral DNA and assembly of their capsid occur in the nucleus of infected cells.

(ii) They encode their own enzymes involved in nucleic acid metabolism, DNA synthesis and protein processing.

(iii) Production of their infectious viral particles is usually accompanied by destruction of the infected cell.

(iv) Latent infection occurs which persists for the life of their natural hosts. The latent viral genomes usually take the form of circular episomes, with only a small subset of viral genes being expressed. The site of latency differs from one herpesvirus to another.

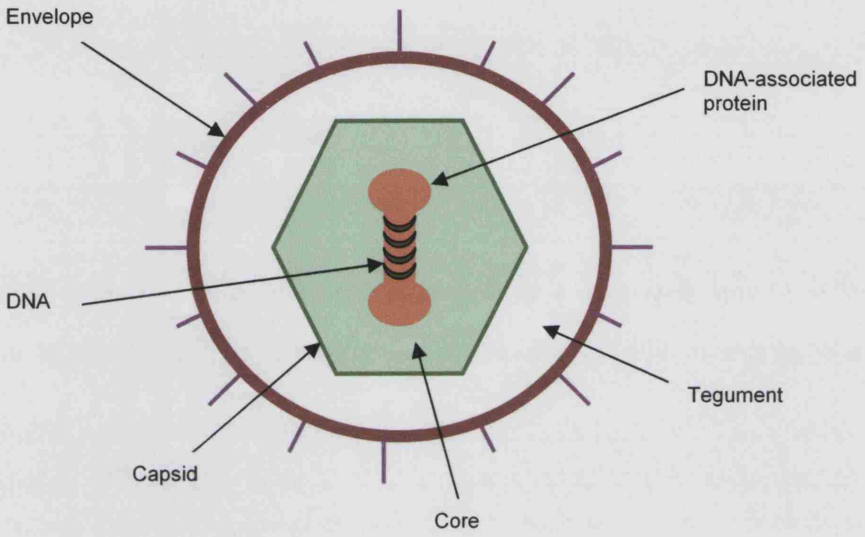
1.1.2. STRUCTURE OF HERPESVIRUSES

1.1.2.1 The virion

Herpesviruses have a common virion architecture consisting of a core containing linear double-stranded DNA, an icosahedral capsid, an amorphous tegument and a lipid envelope with viral glycoprotein spikes on its surface (Figure 1-1). The viral particles have a diameter of about 120 to

more than 200 nm and contain, in addition to viral DNA, 25-35 virus-encoded proteins and host-specific phospholipids derived from the nuclear membrane (Roizman, 1996).

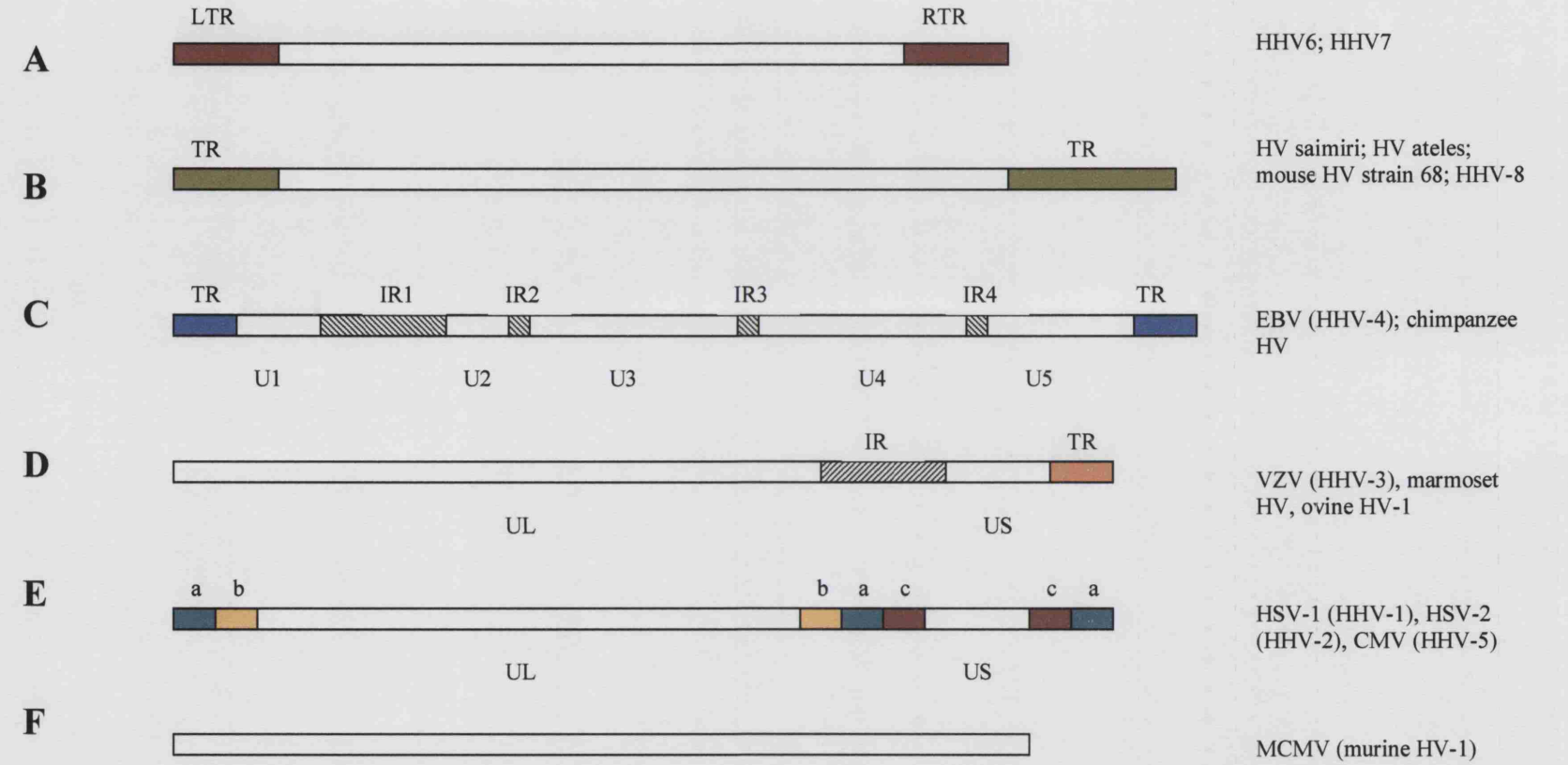
Figure 1.1 Schematic structure of a herpesvirus



1.1.2.2 Genomic organisation

Herpesviruses can be distinguished from each other not only by the size and base composition of their DNA, but also by the structural arrangement of their unique and repeated sequences. The length of the genome varies from approximately 120 to 230 kilobases (kb) and is characteristic for each genus. Variations in the number of internal and terminal repeat regions result in minor variations in the size of individual isolates. Herpesviruses can be divided into six structurally distinct groups on the basis of the presence and location of repeated sequences longer than 100 base pairs (bps) (Figure 1.2).

Figure 1-2 Architecture of herpesvirus genomes



Key: LTR, left terminal repeat; RTR; right terminal repeat; TR, terminal repeat; IR, internal repeat; U, unique sequence; UL; long unique sequence; US, short unique sequence; repeat sequences, a, b, c.

(i) Group A viruses possess a large sequence in one terminus which is directly repeated at the other terminus. They include human herpesviruses 6 and 7 (HHV-6 and HHV-7).

(ii) Group B viruses possess a terminal sequence that is directly repeated numerous times at both termini. Typically an area of low GC content (L-DNA) is flanked by repetitive regions of high GC content (H-DNA). Examples include human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), and the primate herpesvirus saimiri (HVS or SHV-2).

(iii) Group C viruses, for which both terminal and internal repeat sequences are present throughout the viral genome, and which further subdivide it into well-defined unique sequences. These include Epstein-Barr virus (EBV) and chimpanzee herpesvirus (pongine herpesvirus 1).

(iv) Group D viruses have a terminal region which is repeated in an inverted orientation internally. Examples include varicella-zoster virus (VZV) and a large number of viruses isolated from mammals and birds.

(v) Group E viruses have a more complex genomic structure, in which sequences at both termini are repeated in inverse orientation and juxtaposed internally. They include herpes simplex viruses types I (HSV-I) and 2 (HSV-2) and human cytomegalovirus (CMV).

(vi) Group F viruses have no repeat regions in the genome, and are exemplified by murine herpesvirus 1.

The herpesviruses vary significantly in terms of their base composition, with the guanine-cytosine (GC) content ranging from 46% in VZV to 69% in HSV (Roizman, 1996). An under-representation of the CpG dinucleotide pair and a relative excess of CpA and TpG dinucleotides have been observed in the lymphotropic herpesviruses, possibly due to methylation-dependent CpG suppression in rapidly dividing cells (Honess *et al.,* 1989). However, despite these major differences, comparison of herpesvirus DNA sequences shows large regions of distant co-linear homology at the predicted protein level. Relatively well conserved genes code for *trans*-activating factors and enzymes involved in viral replication, such as DNA polymerase, ribonucleotide reductase and thymidine kinase, and some structural glycoproteins, including the major spike components (Stewart *et al.,* 1996). The similarities are largely restricted to the early and late genes expressed during the productive cycle, while genes expressed in latently infected cells are usually unique, suggesting a possible origin from cellular DNA.

1.1.3. TAXONOMY OF HERPESVIRUSES

Herpesviruses are classified into three subfamilies - alpha, beta and gamma, each of which display different biological properties and tissue tropism (Murphy *et al.,* 1995). Further subdivision into genera is based on DNA sequence homology, similarities in genomic sequence arrangement and viral proteins demonstrable by immunological methods (Roizman *et al.,* 1981; Roizman, 1982) (Table 1.1).

Table 1.1 Taxonomy of herpesviruses

Alphaherpesvirinae:	
Simplexvirus	Human herpesvirus 1, 2 (HSV-1, HSV-2)
Varicellovirus	Human herpesvirus 3 (VZV)
Betaherpesvirinae:	
Cytomegalovirus	Human herpesvirus 5 (CMV)
Muromegalovirus	Mouse cytomegalovirus 1
Roseolovirus	Human herpesvirus 6, 7 (HHV-6, HHV-7)
Gammaherpesvirinae:	
Lymphocryptovirus	Human herpesvirus 4 (EBV)
Rhadinovirus	Human herpesvirus 8 (HHV-8)

1.1.3.1 Alphaherpesviruses

The subfamily Alphaherpesvirinae includes the genera *Simplexvirus* and *Varicellovirus*, within which are human HSV-1, HSV-2 and VZV (also referred to as HHV-I, 2 and 3, respectively). They have a characteristically short productive cycle in epithelial cells and are able to establish latent infection primarily, but not exclusively, in sensory ganglia.

1.1.3.2 Betaherpesviruses

The subfamily Betaherpesvirinae includes the genera *Cytomegalovirus* and *Muromegalovirus*, within which are human CMV (HHV-5) and murine CMV (MHV-1). Although HHV-6 and HHV-7 have also been classified in this subfamily on the basis of their genetic homology with human CMV, these viruses share several biological properties with the gammaherpesviruses. The betaherpesviruses replicate in a variety of cell types *in vivo*, including epithelial cells, while the host cell range is more restricted *in vitro*. Infection progresses slowly and is accompanied by cell enlargement (cytomegaly) and by the appearance of characteristic nuclear eosinophilic inclusion bodies, formed by the accumulation of defective particles containing enveloped viral proteins without DNA, (also referred to as assembled capsids). Latent infection has been confirmed in many tissues, although lymphoreticular cells, secretory glands and renal tissue are the more common sites.

1.1.3.3 Gammaherpesviruses

The subfamily Gammaherpesvirinae includes the genera *Lymphocryptovirus* and *Rhadinovirus*, with little nucleotide sequence homology or antigenic cross-reactivity existing between these two groups.

Gammaherpesviruses are characterized by their tropism for lymphoid cells and their capacity to induce cell proliferation *in vivo*, resulting in transient or chronic lymphoproliferative disorders, and *in vitro*, where many can immortalise the infected cells. They have a narrow natural host range which is restricted to the family or order to which the natural host belongs. Most gammaherpesviruses replicate inefficiently in haematopoietic cells, but some have efficient productive cycles in epithelial cells and fibroblasts. Latent virus is usually detected in lymphoid organs.

The lymphocryptoviruses (or gamma-1 herpesviruses) include EBV (HHV4) and related viruses of Old World primates such as chimpanzees (*Herpesvirus pan*), orangutans (*Herpesvirus orangutan*) and gorillas (*Herpesvirus gorilla*). These viruses share a tropism for B lymphocytes, and a genomic architecture characteristic of group B or C, and a similar gene organisation. Furthermore, several of their structural and nonstructural proteins are antigenically related, especially among the primate viruses, resulting in the presence of cross-reactive antibodies (Gerber and Birch, 1967; Chu *et al.*, 1971; Landon and Malan, 1971).

The rhadinoviruses (or gamma-2 herpesviruses) have a group B genome and are classically the herpesviruses of primates. They can be divided into two distinct lineages, RV1 and RV2. Examples include the ateles virus of spider monkeys (HVA), the saimiri virus of squirrel monkeys (HVS),

rhesus rhadinovirus (RRV), and some viruses of horses (equid herpesvirus 2) (Telford *et al.,* 1993) and mice (murine herpesvirus strain 68 (MHV-68) (Sunil-Chandra *et al.,* 1992a,b, 1994). The only human herpesvirus in this group is HHV-8 (Chang *et al.,* 1994). It has been classified in this genus due to its close similarity to the saimiri virus. HHV-8 is a RV-1 gamma-2 herpesvirus. There may be as yet undiscovered viruses of the RV-2 lineage capable of infecting humans (Lacoste *et al.,* 2001).

Within these three subfamilies of herpesviruses, eight are known to infect humans. These are summarised in Table 1.1 (Roizman, 1993).

Table 1.2 Human Herpesviruses

Designation	Common Name	Subfamily	Group
Human herpesvirus 1	Herpes simplex virus 1	α	E
Human herpesvirus 2	Herpes simplex virus 2	α	E
Human herpesvirus 3	Varicella zoster virus	α	D
Human herpesvirus 4	Epstein Barr virus	γ	C
Human herpesvirus 5	Cytomegalovirus	β	E
Human herpesvirus 6		β	A
Human herpesvirus 7		β	A
Human herpesvirus 8	Kaposi's sarcoma-associated herpesvirus	γ	B

1.2 PHYSICAL AND GENOMIC STRUCTURE OF HUMAN HERPESVIRUS 8

1.2.1 THE DISCOVERY OF HHV-8

In 1994, two small fragments of the HHV-8 genome (KS330Bam and KS631Bam) were discovered in Kaposi's sarcoma (KS) tissue by representational difference analysis and characterised to be related to gammaherpesviruses (Chang *et al.,* 1994). As it has been detected in virtually all cases of KS, it is also known as Kaposi's sarcoma-associated virus (KSHV).

Subsequent sequence analyses (Moore *et al., 1996a)* of a larger genomic region containing blocks of structural genes found in all herpesviruses confirmed that HHV-8 is a gamma-2 herpesvirus (rhadinovirus). The completion of the entire HHV-8 genomic sequence (Russo *et al.,* 1996; Neipel *et al., 1997a)* showed, in addition, that the organization of the HHV-8 genome is very similar to that of rhadinoviruses of the RV-1 lineage, in particular HVS and MHV-68, with which it shares a few genes not found in other rhadinoviruses (Efstathiou *et al.,* 1990; Albrecht *et al.,* 1992). HHV-8 is estimated to have separated from the HVS and HVA lineage approximately 35 million years ago, in the same epoch as the separation of Old and New World lineages of primates (McGeoch and Davison, 1999). The definition of HHV-8 genes discovered to date is summarised in Appendix 1.

1.2.2 MORPHOLOGY

The morphological characteristics HHV-8 are typical of herpesviruses (Figure 1-1). Before the discovery of HHV-8 (Chang *et al.,* 1994), herpesvirus particles described in short-term cultures from KS lesions (Giraldo *et al.,* 1972) were thought to be those of CMV (Giraldo *et al.,* 1980). The morphology of HHV-8 was quickly confirmed in electron micrographs of HHV-8-infected primary effusion lymphoma cell lines (Arvanitakis *et al.,* 1996; Renne *et al.,* 1996a; Said *et al.,* 1996a), an infected kidney-cell line (Foreman *et al.,* 1997) and biopsy samples of KS (Walter *et al.,* 1984; Orenstein *et al.,* 1997). HHV-8 appears as 100-150 nm particles, with an electrondense central core surrounded by a lipid envelope (Renne *et al.,* 1996a). Capsid substructures, such as ring-shaped capsomers of approximately 9 nm in diameter arranged in linear arrays, have also been observed (Arvanitakis *et al.,* 1996; Said *et al.,* 1996a).

The capsid composition of *Gammaherpesvirinae*, including HHV-8, has since been characterised by means of biochemical and imaging analyses (Nealon *et al.,* 2001). It has been confirmed that only a portion of the synthesised capsids undergoes viral DNA packaging during the lytic replication phase. As with the alpha- and betaherpesviruses, this leads to the formation of at least three capsid species, A, B, and C, with masses of approximately 200, 230, and 300 MDa, respectively. A capsids consist of an empty icosahedral shell; B capsids contain only scaffolding protein (ORF17.5); and C capsids contain packaged DNA and no scaffolding protein. It has been hypothesised that the B capsids may mature to C capsids, which in turn may function as the precursors of infectious virus

particles. The A capsids are probably the dead end products of B and C capsids.

The same group (Nealon *et al.,* 2001) confirmed that the hexameric and pentameric capsomers are composed of the major capsid protein (MCP) encoded by open reading frame (ORF) 25. The heterotrimeric complexes, forming the capsid floor between the hexons and pentons, are each composed of one molecule encoded by ORF 62 and two molecules of encoded by ORF 26. These three proteins (encoded by ORF 25, 62 and 26) have a significant amino acid sequence homology to capsid proteins of both alpha- and betaherpesviruses. A fourth small, basic, and highly antigenic protein, encoded by ORF 65, was also identified, and in contrast does not show any significant sequence homology to its structural counterparts from the other subfamilies. A fifth protein, encoded by ORF 17.5, is only found in B capsids.

1.2.3 GENOMIC CHARACTERISTICS

The size of the HHV-8 genome is calculated to be approximately 165 kb on the basis of studies of the genome banded from productive primary effusion lymphoma cells (Arvanitakis *et al.,* 1996; Renne *et al.,* 1996b) and confirmed by Gardella gel electrophoresis (Decker *et al.,* 1996) and mapping of the whole genome (Russo *et al.,* 1996, Neipel *et al.,* 1997a). The genome consists of multiple 801-bp tandem repeat regions of high GC content H-DNA (84.5%) at the terminal ends, with a 110-140 kb L-DNA segment of lower GC content (53.5%) centrally (Figure 1.3).

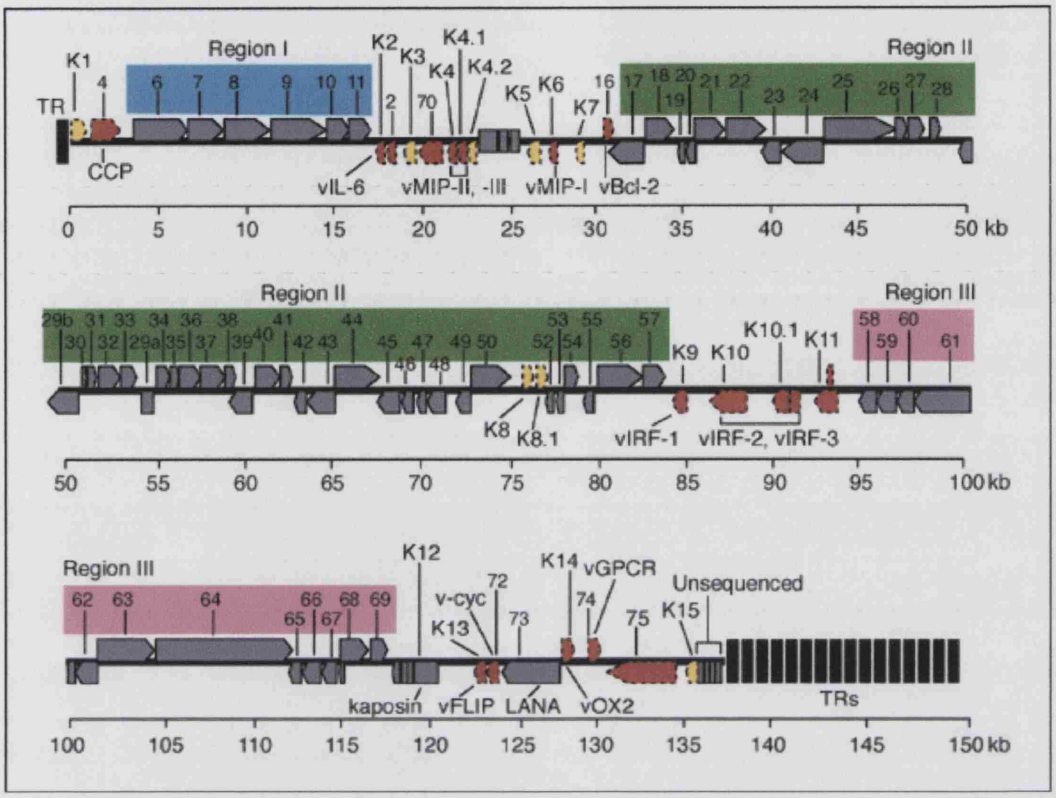
Larger estimates made earlier (- 270 kb) (Mesri *et al.,* 1996; Moore *et al.,* 1996a) were based on analyses of the primary effusion lymphoma (PEL)-derived BC-1 cell line, which contains a large (>30 kb) genomic duplication. This genomic duplication was also found in another cell line, independently derived from the same tumour, and may therefore have been present in the parental lymphoma (Russo *et al.,* 1996).

The linear HHV-8 genome can persist as a multicopy circular episome during latency. Gene expression of the episomal form is limited. The episome contains repetitive sequences that serve as multiple cooperative binding sites for the viral DNA binding protein latency-associated nuclear antigen (LANA), which is expressed during latency (Collins and Medveczky, 2002). The oligomerized protein associates with the viral genome and tethers it to host chromosomes, assuring continual lifelong persistence of the virus. Reactivation results in selective accumulation of linear genomic forms and infection of endothelial cells (Russo *et al.,* 1996).

1.2.3.1 The Terminal Repeat Region

The terminal-repeat region is a conserved feature of herpesviruses and is involved in packaging of the viral DNA into new virions during the lytic cycle of replication. HHV-8 has approximately 30 terminal-repeat units. In the BC-1 strain, insertions of long unique region fragments have been observed in the terminal repeats. None of the ORFs present in EBV have so far been identified within the terminal-repeat region of HHV-8. Hybridization with the terminal-repeat region used as a probe is a sensitive method for detecting viral DNA (Russo *et al.,* 1996). In EBV, for example, circularized

Figure 1.3 The human herpesvirus 8 (HHV-8) genome.



(From: Expert Reviews in Molecular Medicine, 2001 Cambridge University Press)

Note: The genome consists of a long unique region (140.5 kb) encoding for over 80 open reading frames (ORFs), surrounded by terminal repeat regions (TRs) consisting of 801 base pair direct repeat units with a high G+C content. Three large regions (I, II and III) contain genes conserved among the *Rhadinoviruses*, whereas the regions between them contain unique genes. Many of these unique genes encode homologues for host cellular proteins (shown in red with dotted outlines; genes without known homologues are shown in yellow with dotted outlines). Genes that are potentially important in the pathogenesis of KS are labelled.

Abbreviations: CCP, complement control protein; v-cyc, viral D-type cyclin; vFLIP, viral FLICE inhibitory protein; vGPCR, viral G-protein-coupled receptor; vIL-6, viral interleukin 6; vIRF, viral interferon regulatory factor; kb, kilobase; LANA, latency-associated nuclear antigen; vMIP (vCCL), viral macrophage inflammatory protein

viral genomes in latently infected cells maintain the length of their terminal repeats, and the presence of a clonal cell population arising from a single latently infected cell may be demonstrated by Southern blot analysis with restriction enzymes that do not cut into the terminal repeat (Raab-Traub and Flynn, 1986). The restriction enzyme *Thermus aquaticus* (Taq) 1 frequently cuts within the long unique region of the HHV-8 genome but not within the terminal repeat (Russo *et al.,* 1996).

The mechanism of replication of HHV-8 is probably similar to that of other herpesviruses (Roizman, 1993), in which the genome is replicated as a rolling circle, monomeric genomes being cleaved within the terminal-repeat region to form linear genomes. Sequences homologous to HVS packaging and cleaving sites are present in the terminal-repeat unit sequence (Russo *et al.,* 1996). Linear genomes are packaged into virions, as demonstrated by pulse-field and Gardella gel electrophoresis studies of viral particles (Renne *et al.,* 1996b), and most likely re-circularise at the terminal repeat after entry into the recipient cell.

1.2.3.2 The Long Unique Region

The 140.5-kb HHV-8 long unique region is larger than the corresponding regions of HVS and EBV, encoding at least 81 predicted ORFs (Russo *et al.,* 1996; Neipel *et al.,* 1997a). The ORFs are named according to the corresponding HVS genes with which they share a significant level of homology. Unique genes, not homologous to HVS, have a K prefix assigned to them (e.g. K.1), with newly discovered ORFs designated by the addition of a decimal point (e.g. K4.1). Seventeen HHV-8

genes have been identified as unique, with eight of these known to have mammalian homologues, presumed to be the sources of HHV-8 versions (Table 1-3) (McGeoch and Davison, 1999). Detailed analysis of conserved herpesvirus genes has confirmed that four genes previously considered unique to HHV-8 actually belong to the gamma-2-specific class of herpesviruses: these include K3 and K5 (both homologues of HVS gene 12), K8.1 (a probable homologue of HVS 51), and K13 (homologue of HVS 71). It has also been suggested that the saimiri transforming protein encoding gene of HVS is a possible counterpart of K1 (Jung *et al.,* 1991; McGeoch and Davison, 1999).

The long unique region is organised into three large regions of conserved genes, interspersed between blocks of non-conserved genes (Chee and Barrell, 1990; Chee *et al.,* 1990) (Figure 1-3). There are approximately 67 of these conserved genes, many of which encode herpesvirus structural proteins and replication enzymes. Homologues to several major herpesvirus glycoproteins (gB [encoded by ORF 8], gH [ORF 22], gM [ORF 39] and gL [ORF 47]) are encoded by HHV-8. Several conserved proteins are found in the long unique region, namely capsid proteins (major capsid protein [ORF 25], VP23 [ORF 26]), other capsid proteins encoded by ORFs 17, 43, 62, 65 and 17.5, tegument proteins encoded by ORFs 19, 63, 64, 67 and 75, replication enzymes (DNA polymerase [ORF 9] and helicases [ORFs 40, 41 and 44]), DNA replication proteins [ORFs 56 and 59]) and enzymes involved in nucleic acid metabolism (e.g. thymidylate synthetase [ORF 70], thymidine kinase [ORF

Table 1-3 Genes specific to the HHV-8 lineage (McGeoch and Davison, 1999)

HHV-8 gene	Encoded protein
K1	Transforming glycoprotein
K2*	Interleukin 6 homologue
K2.1*	Dihydrofolate reductase
K3.1*	Thymidylate synthetase
K4*	CC chemokine homologue
K4.1*	CC chemokine homologue
K4.2	
K6*	CC chemokine homologue
K7	T1.1 / PAN RNA
K8	
K9*	Homologue of transcription regulatory proteins, interferon regulatory factors (IRF) (*vIRF*-1)
K10	IRF related
K10.1	IRF related
K11	IRF related
K12	'Kaposin' transforming protein
K14*	Homologue of cell surface protein OX-2
K15	Latency associated membrane protein (LAMP2)

Note: * these genes have mammalian homologues

21], uracil glucosidase [ORF 46], dUTPase [ORF 54], and ribonucleotide reductases [ORFs 60 and 61]) (Russo *et al.,* 1996). These conserved proteins are likely to play an important role during the lytic replication cycle of HHV-8.

Non-conserved gene blocks in the HHV-8 genome contain some ORFs of genes that are either found only in rhadinoviruses or are unique to HHV-8 (Russo *et al.,* 1996). Many of these appear to have been pirated from the host genome during evolution, allowing the virus to replicate and evade host immune responses (Neipel *et al.,* 1998). Hence HHV-8 encodes a complement binding protein (encoded by *ORF 4)* that is related to a family of mammalian complement regulatory proteins and to a similar protein in HVS; an interleukin (IL)-6 homologue *(ORF K2)* which is unique to HHV-8; three chemokine homologues related to macrophage inflammatory protein (MIP)- Iα (viral [v]-CCL-I, vCCL-II and vCCL-III, encoded by ORFs K6, K4 and K4. 1 respectively); a bcl-2 homologue (ORF 16), a homologue of interferon regulatory factor (v-IRF; ORF K9); a homologue of a D-type cyclin (v-cyclin; ORF 72); an adhesion molecule homologue (OX-2; ORF K14); and a chemokine receptor homologue (ORF 74) (Russo *et al.,* 1996; Cesarman *et al.,* 1996a; Neipel *et al.,* 1997a,b; Nicholas *et al.,* 1997a,b). Unique to HHV-8 are the type-1 transmembrane protein encoded by ORF K1 and a putative small hydrophobic protein ('kaposin') encoded by ORF K12. Also found in some rhadinoviruses (e.g. HVS, bovine herpesvirus 4 [BHV-41], murine herpesvirus 68 [MHV-68]), but not others (e.g. equine herpesvirus 2), is the latency-associated nuclear antigen (LANA) protein which for HHV-8 is encoded by ORF 73 (Russo *et al.,* 1996; Neipel *et al.,* 1997a; Rainbow *et al.,*

1997). These genes may have a function in the virus life cycle and tumour formation.

1.2.3.3 Genomic Variation Amongst Strains

Studies comparing HHV-8 sequences from both KS biopsies and PEL cell lines confirm that the genome is highly conserved, with only 0.1% nucleotide variation between sequences. (Russo *et al.,* 1996; Moore *et al.,* 1996a; Nicholas *et al.,* 1997a; Neipel *et al.,* 1997a). Original studies to determine strain variation centred on ORFs 26 and 75. However, ORF 26 was soon found to be one of the more highly conserved genes with only 2% divergence over a 500-bp region and very few substitutions that result in a change at the amino acid level (Boshoff *et al.,* 1995a; Collandre *et al.,* 1995; Huang *et al.,* 1995; Moore and Chang, 1995; Marchioli *et al.,* 1996; Moore *et al.,* 1996a; Zong *et al.,* 1997). Limited sequence variation has also been found within two regions of ORF 75 (Zong *et al.,* 1997). In a combined analysis of several genomic regions, including ORFs 26 and 75, Zong *et al* (1997) found up to 1.5% overall nucleotide variation between isolates, and grouped these into three different main variants, provisionally termed A, B and C.

In contrast, ORF K1 displays up to 20% nucleotide and 40% amino acid variability, with sequences clustering into four main genotypes, I to IV, based on phylogenetic analysis and a distinctive deletion in the variable region-2 (VR-2) (Meng *et al.,* 1999). Zong *et al* (1999) also identified four main genotypes / subtypes, named A, B, C and D, and further divided these into 13 subgroups based on amino acid differences of at least 5% or greater

(i.e A1-A5, etc). Subtypes A and C are very similar, while subtype B is the most divergent subtype, displaying a 30% overall amino acid difference between A and C. The same group has more recently identified 24 ORF K1 genotypic variants based on 139 samples (Zong *et al.,* 2002), and confirmed that the subtypes correlate with ethnicity and geography. The B subtype is found predominately in sub-Saharan Africa or in persons of African heritage, and has only 3 subgroups (B1, B2 and B3) (Treurnicht *et al.,* 2002; Zong *et al.,* 2002). The A and C subtypes have a wide distribution throughout Europe, USA, Asia and the Middle East. American patients with acquired immunodeficiency syndrome (AIDS) are usually infected with subgroups A1, A4 and C3, while subgroup A5 has been found in some African samples (Zong *et al.,* 1999). The C2 variants predominate in both Saudi Arabian renal transplant-associated KS and in Scandinavian classic KS, although they have also been identified in classic KS from the USA. The D subtype appears to be rare and isolated to the Pacific Island region (Poole *et al.,* 1999). Sequences recovered from a Brazilian Amerindian population demonstrated a 25-35% variation in nucleotide sequence from types A to D. These have been assigned to a new variant termed 'E' (Biggar *et al.,* 2000).

1.3 HHV-8 GENE EXPRESSION AND HHV-8-ENCODED PROTEINS OF RELEVANCE

In common with other herpesviruses, HHV-8 is able to establish latency and hence has both a latent form and a lytic form (Russo *et al.,* 1996). The latent form is found in PEL cell lines and requires chemical

induction with 12-*O*-tetradecanoylphorbol 13 acetate (TPA) for full expression of lytic gene products (Sarid *et al.,* 1998). Three broad categories of gene expression, namely Class I, II and III, have been described based on this *in vitro* induction, although this may not reflect the true *in vivo* state.

Class I (latent) mRNAs, namely those encoded by ORF 71, ORF 72, and ORF 73, are constitutively expressed and created by the polycistronic transcription of two overlapping genes, LT1 and LT2 (Dittmer *et al.,* 1998; Kellam *et al.,* 1999; Sarid *et al.,* 1999). They are found under standard growth conditions and are not upregulated by TPA.

Class II transcripts, such as T0.7 (K12, Kaposin) and T1.1 (nut-1), are expressed at varying levels in latent cells. However they can be upregulated in the presence of TPA due to demethylation of the ORF 50 / replication and transcription activator (Rta) promoter region which initiates and controls the entire lytic cycle (Sun *et al.,* 1998; Lukac *et al* 1999; Gradoville *et al* 2000). Class II transcripts encode for an array of viral cytokines and chemokines, signal transducers, and regulatory proteins, many of which may contribute to viral pathogenesis.

Class III transcripts are expressed during the late lytic phase and encode for proteins involved in nucleic acid synthesis such as DNA polymerase (ORF 9), thymidine kinase (ORF 21), dihydrofolate reductase (ORF 2 / K2.1) and ribonucleotide reductase (ORF 60 and 61) (Russo *et al.,* 1996; Neipel *et al.,* 1997a; Nicholas *et al.,* 1998). Their function may be to enhance DNA synthesis thereby, aiding virion production.

The HHV-8 genome contains multiple non-conserved viral genes, some of which are strikingly similar to human genes involved in cellular growth control. These are briefly discussed below, as follows.

1.3.1 ORF 26

ORF26 is a late gene which encodes the structural protein TRI-2 (Chang and Ganem, 2000). It displays lytic expression between 24 to 48 hours post-induction, with peak expression at 36 hours (Jenner *et al.,* 2001, Paulose-Murphy *et al.*, 2001). TRI-2 joins with TRI-1 (ORF62) to form the heterotrimeric capsid triplexes (Nealon et al. 2001). The triplexes reside between the hexomeric and pentomeric MCP (ORF25) capsomers and make up the floor of the capsid. Two molecules of TRI-2 and one molecule of TRI-1 join to form each of the capsid's 320 triplexes (Nealon et al. 2001). TRI-2 is present in 640 copies per capsid and is the second most abundant and third largest structural component of the capsid. TRI-2 is 21% identical and 41% similar to the HSV-1 triplex component VP23 (Nealon et al. 2001).

1.3.2 ORF K1

The first gene at the left end of the HHV-8 genome, *ORF K1,* codes for a 46-kDa highly glycosolated transmembrane protein with similarities to the immunoglobulin receptor family (Lagunoff and Ganem, 1997; Neipel *et al.,* 1997a; Lee *et al.,* 1998a). It is expressed in the early lytic phase after induction by TPA in PEL cell lines and is thought to have a transforming potential, as demonstrated *in vivo* by Lee *et al.,* (1998b): expression of K1 induces focus formation and morphological changes in Rat-1 fibroblasts, and

can immortalize common marmoset lymphocytes to interleukin-2-independent proliferation to induce lymphomas *in vivo*.

Although ORF K1 is conserved at its N and C terminal ends, extensive polymorphism in the extracellular domain over an 840-bp region coding for 270 amino acids has been found (Meng *et al* 1999). The variability is greatest between amino acids 20 and 226, with two particular blocks of hyper-variability noted, namely variable region 1 (VR1), between amino acids 51 and 92, and variable region 2 (VR2), between residues 191 and 231. The cytoplasmic tail contains an immunoreceptor tyrosine-based activation motif (Lee *et al.,* 1998a). The K1 protein has been shown to initiate signalling pathways in B cells which result in the tyrosine phosphorylation of cellular proteins, mobilisation of intracellular calcium, and the activation of transcription factors such as nuclear factor of activated T-cells (NFAT) and activator protein-1 (AP-1) (Lee *et el.,* 1998a; Lagunoff *et al.,* 1999; Damania *et al.,* 2000). It has also been shown to downregulate B-cell receptor complex expression in the plasma membrane (Lee *et al.,* 2000). The amino terminus of the protein interacts with μ chains of B-cell receptor complexes to retain them in the endoplasmic reticulum, suggesting a role in the survival of HHV-8-infected cells. Samaniego *et al* (2001) proposed an additional role for the protein in mediating paracrine activation of endothelial cells. They demonstrated that cells expressing the protein showed increased NF-B-dependent promoter activity. NF-B is responsible for activating a number of inflammatory responses. The K1 protein may therefore activate uninfected endothelial cells in a paracrine manner through the activation of NF-B-dependent promoters and secretion of inflammatory cytokines. The identity

the K1 promoter has been clarified by Bowser *et al* (2002). Sequences upstream of the K1 gene were analysed by 5' rapid amplification of cDNA ends, as well as a nuclease protection assay to map the transcriptional start site of the K1 transcript. The K1 transcriptional start site lies 75 bp upstream of the translation start site. Sequences upstream of the K1 gene were characterized for their ability to activate a luciferase reporter gene in 293 epithelial cells, HHV-8-negative B cells (BJAB), HHV-8-positive B cells (BCBL-1), and KS tumour-derived endothelial cells (SLK-KS(-)). The 125-bp sequence upstream of the K1 transcript start site was sufficient to fully activate the luciferase reporter gene in all cell types tested.

1.3.3 KAPOSIN (ORF K12)

The expression of an abundant, latency-associated 0.7-kb transcript, T0.7 is found in all stages of KS and in the BCBL-1 cell line that is latently infected with HHV-8 (Renne *et al.,* 1996a; Zhong *et al.,* 1996; Staskus *et al.,* 1997). T0.7 is encoded by a unique HHV-8 open reading frame, K12, also known as kaposin. Muralidhar *et al* (1998) demonstrated the oncogenic potential of kaposin in transformed Rat-3 cell lines and suggested its possible role in the development of KS and other HHV-8-associated malignancies. The same group confirmed kaposin DNA was retained in transformed Rat-3 cells and expressed as predominantly cytoplasmic proteins of 16–18 kDa (Muralidhar *et al.,* 2000). They suggested that kaposin may be involved in the activation of cellular serine-threonine kinases, such as protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase (CAM) II and cyclin dependent kinase-2, which play an important role in cell proliferation.

Protein expression from the K12 transcript is complex and incompletely understood (Kliche *et al.,* 2001). Three kaposin proteins, termed A, B and C, have been identified (Sadler *et al.,* 1999). Kaposin A is encoded from the AUG condon at the 5' end of K12. Kaposins B and C are derived from CUG codons located in two reading frames within repetitive elements (DR1 and DR2) upstream of K12. Kaposin B is predominantly expressed in BCBL-1 cells lines. Kaposin A has multiple functions in single gene transfer assays: Rat-3 cells expressing kaposin A are tumorigenic in *nu/nu* mice (Muralidhar *et al.,* 1998); it induces reorganization of cellular F-actin and causes increased adhesion in Jurkat cells (Kliche *et al.,* 2001). The transforming and adhesion effects of kaposin A are mediated through its association with cytohesin-1, a guanine nucleotide exchange factor, which regulates integrin activity (Kliche *et al.,* 2001). Further characterisation of the PEL tumour HHV-8 K12 transcript has demonstrated that it contains additional complex nucleotide repeat elements compared to that of the BCBL-1 PEL cell line (Li *et al.,* 2002). The PEL tumour only encodes kaposins A and C, with the K12 transcript spliced. The 5' end of the K12 transcript was mapped by 5' RACE (rapid amplification of cDNA ends) and S1 nuclease protection assays and was at the site of a novel HHV-8 promoter.

1.3.4 VIRAL CHEMOKINES

Three HHV-8 genes have sequence similarity to members of the C-C-chemokine family, vCCL-1, vCCL-II, vCCL-III (previously designated vMIP-1, vMIP-II and vMIP-III) (Russo *et al.,* 1996; Boshoff *et al.,* 1997; Neipel *et al.,*

1997a; Nicholas *et al.,* 1997a,b). Two of these, encoded by *ORF 6* and 4 (vCCL-I and vCCL-II) are expressed at low levels in latently infected PEL cells, with expression increasing during the lytic cycle of viral replication (Moore *et al.,* 1996b; Nicholas *et al.,* 1997a,b). Viral CCL-I acts as an agonist, by binding selectively to CCR8 chemokine receptor expressed predominately on Th2 type T cells (Dairaghi *et al.,* 1999; Endres *et al.,* 1999). In contrast vCCL-II has a broad range of activity and can bind with chemokine co-receptors of the CC and CXC type. It has been shown to block viral entry of HIV-1 through its interaction with co-receptors C-CR5, CXCR4 and CCR3 (Boshoff *et al.,* 1997; Kledal *et al.,* 1997). Viral CCL-III (encoded by K4.1) acts as an agonist for CCR4 co-receptors and has also been shown to exert a chemotactic effect on cells displaying the CCR8 and CCR3 chemokine receptors (Boshoff *et al.,* 1997; Sozzani *et al.,* 1998; Stine *et al.,* 2000)

1.3.5 VIRAL INTERLEUKIN 6 (v-IL-6)

v-IL-6, which is encoded by *ORF K2,* is approximately 25% homologous at the amino acid level to human IL-6 (Moore *et al.,* 1996b;

Russo *et al.,* 1996; Neipel *et al.,* 1997b; Nicholas *et al.,* 1997a,b). It has been shown to support the growth of the IL-6-dependent mouse myeloma cell line, B9 (Moore *et al.,* 1996b; Nicholas *et al.,* 1997b), and transform NIH3T3 cells to cause tumour formation (Aoki *et al.,* 1999). It is expressed in latently infected lymphoma cell lines and PEL tissue, but not generally in KS tissues.

Initial studies demonstrated that human IL-6 and v-IL-6 differ in their cellular IL-6 receptor interaction in that whereas human IL-6 requires both interleukin co-receptor (IL-6R) and gp130 receptor protein for signal activation, v-IL-6 requires only gp130 (Molden *et al.,* 1997; Müllberg *et al.,* 2000; Aoki *et al.,* 2001; Chow *et al.,* 2001). The crystal structure of the vIL-6 signalling complex shows that two vIL-6 molecules homodimerize two gp130 receptors to form a tetramer (Chow *et al.,* 2001). Each vIL-6 molecule presents two topologically distinct binding epitopes termed sites II and III. Site II is used to engage the D2D3 domains of gp130, also known as the cytokine binding homology region or CHR, while site III binds the D1 or IGD domain of an opposing gp130 molecule completing the tetramer.

More recently, it has been confirmed that vIL-6 is indeed capable of utilizing IL-6R as part of an "enhanced" signalling complex (Boulanger *et al.,* 2004). The biochemical analysis of the assembly of the vIL-6 signalling complexes revealed both a "convergent" strategy utilizing IL-6R to produce a hexameric complex similar to the human cytokine, and the previously described "divergent" IL-6R-independent strategy.

1.3.6 Bcl-2 HOMOLOGUE

ORF 16 of HHV-8 encodes a homologue of the cellular anti-apoptotic protein bcl-2, with which it shares 16% sequence homology (Russo *et al.,* 1996; Cheng *et al.,* 1997; Neipel *et al.,* 1997a; Nicholas *et al.,* 1997a; Sarid *et al.,* 1997). These HHV-8 *v-bcl-2* transcripts can detected at low levels in KS lesions and PEL cell lines, and can be induced in PEL cell lines by TPA (Cheng *et al.,* 1997; Sarid *et al.,* 1997).

The Bcl-2 family of proteins act as either inhibitors or promoters of apoptosis, playing an important role in tissue homeostasis and the immune response (Chao and Korsmeyer, 1998). The prosurvival function of cellular Bcl-2 is a result of heterodimerization with proapoptotic family members such as Bak, Bax, and Bad. However, the mechanism by which *vbcl-2* protects against apoptosis remains unclear. Initial cellular assays indicated that *vbcl-2* does not heterodimerize with proapoptotic Bcl-2 family members such as Bax and Bak (Cheng *et al.,* 1997). However, *vbcl-2* does contain the Bcl-2 homology (BH) motifs BH1 and BH2, with the BH1 containing the signature "NWGR" sequence believed to be essential for the anti-apoptotic function. More recently, it has been confirmed that *vbcl-2* has its own distinct selectivity for binding BH3 peptides from the proapoptotic proteins Bak, Bax, and Bad (Huang *et al.,* 2002). *vbcl-2* may also protect virus-infected cells from apoptosis induced by the expression of viral cyclin (*v-cyclin*) (Ojala *et al.,* 1999, 2000). Expression of *v-cyclin* in cells with increased levels of cyclin-dependant kinase 6 (Cdk-6) induces apoptosis by a *v-cyclin*-Cdk-6-mediated inactivating phosphorylation of cellular Bcl-2. The phosphorylation of cellular Bcl-2 occurs in the unstructured loop of the proteins (amino acids 32–80). It has been confirmed that *vbcl-2* lacks this loop, and is therefore able to retain its anti-apoptotic activity even in the presence of v-cyclin-Cdk6 (Ojala *et al.,* 2000). Suppression of apoptosis may enhance the survival of HHV-8-infected cells so that the virus may replicate, spread, and persist in its host.

1.3.7 VIRAL INTERFERON REGULATORY FACTORS (v-IRFs)

The HHV-8 genome contains four tandemly arranged genes encoding viral interferon regulatory factors (*vIRF*-1 to 4) located between genes 57 and 58. Three of them, *vIRF*-1, *vIRF*-2, and *vIRF*-3, have been cloned and found to down-regulate the transcriptional activity of interferon type I gene promoters in infected cells by interfering with the transactivating activity of cellular IRFs (Burysek and Pitha, 2001). This in turn results in modification of the wide-ranging effects brought about by interferons (IFN), in particular in reducing the host's defence against viruses. Like HHV-8-encoded cytokine homologues, *V-IRFs* are expressed in latently infected primary effusion lymphoma cell lines, with expression markedly enhanced after lytic cycle induction (Moore *et al.,* 1996b).

ORF K9 encodes the homologue vIRF-1 (Moore *et al.,* 1996b; Russo *et al.,* 1996). This gene has low but significant homology to the IRF family of proteins responsible for interferon signal transduction. It can down-regulate the transcriptional activation induced by IFNs α, β, γ, thereby suppressing the host's IFN mediated innate immune response to viruses (Gao *et al.,* 1997; Li *et al.,* 1998). vIRF-1 can also bind to p53, a tumour suppressor gene, inhibiting its activity and preventing apoptosis in the infected cell (Nakamura *et al.,* 2001). It has been demonstrated that vIRF-1 has different transcriptional patterns during the latent and lytic phases (Chen *et al* 2000). In uninduced BCBL-1 cells, the promoter region of the minor transcript is mainly expressed, and does not contain a canonical TATA box, but a cap-like element and an initiator element flanking the transcription start site. In TPA-induced BCBL-1 cells, the promoter of the major transcript is

predominantly expressed, and contains a canonical TATA box. A luciferase reporter assay using a deletion mutant of the vIRF-1 promoter and a mutation in the TATA box showed that the TATA box was critical for the lytic activity of vIRF (Chen *et al.,* 2000).

vIRF-2 (ORF K11.1), is also latently expressed by PEL cell lines and has been shown to inhibit the IRF-1- or IRF-3-mediated transcriptional activation of the IFN-α gene promoter in infected cells (Burysek *et al.,* 1999). IRF-2 has oncogenic activities in NIH 3T3 cells which can be reversed by IRF-1 over-expression (Harada *et al.,* 1993). vIRF-2 physically interacts with protein kinase (PKR), thereby inhibiting autophosphorylation of double-stranded RNA-activated phosphate kinase and blocking phosphorylation of PKR substrates (Burysek and Pitha, 2001). Such inhibition allows the virus to overcome the interferon-mediated antiviral effect, which may contribute to the establishment of chronic infection.

vIRF-3 (ORF K10.5), also called LANA2, is a latently expressed nuclear protein. It is expressed in HHV-8-infected hematopoietic tissues, including PEL and CD but not KS lesions, and is abundantly expressed in the nuclei of cultured HHV-8-infected B cells. LANA-2 has been shown to be a potent inhibitor of p53-induced transcription in reporter assays, possibly contributing to HHV-8 tumorigenesis in hematopoietic tissues (Rivas *et al.,* 2001). It has also been demonstrated that vIRF-3 directly interacts with cellular IRF-3 and IRF-7, and the transcriptional co-activator CBP/p300 (Lubyova B., *et al* 2004).

1.3.8 VIRAL PROTEINS THAT INHIBIT FAS-ASSOCIATED DEATH DOMAIN PROTEIN INTERLEUKIN-10 CONVERTING ENZYME (FLICE)

HHV-8 encodes for a viral FLICE-inhibitory protein (vFLIP), designated K13, which resembles the prodomain of caspase-8 in structure and has been shown to protect cells against death receptor-induced apoptosis *in vitro* and *in vivo* (Thome *et al.,* 1997). vFLIP K13 also has the ability to transform by activation of the NF-kappaB pathway, suggesting that it has activity beyond its role as an inhibitor of death receptor signalling, and may play a causative role in the pathogenesis of HHV8-associated malignancies (Sun *et al.,* 2003).

1.3.9 VIRAL CYCLIN (v-cyclin)

Several gammaherpesviruses encode a viral homologue of cellular cyclin D, which may function to deregulate host cell cycle progression (Sharp and Boshoff, 2000). One of these is encoded by ORF 72 of HHV-8 and is called K cyclin or viral cyclin (*v-cyclin*). HHV-8 *v-cyclin* is expressed in latently infected spindle and PEL lines, in uncultured PELs and in KS tissues (Cesarman *et al.,* 1996a; Davis *et al.,* 1997a; Godden-Kent *et al.,* 1997; Reed *et al.,* 1998), suggesting that it may have a role in either the proliferation or the arrest of differentiation of these cells. It encodes a protein that has about 30% amino-acid identity with human cellular cyclin D2 (Cesarman *et al.,* 1996a; Russo *et al.,* 1996; Li *et al.,* 1997). Cellular D-typed cyclins are implicated in the pathogenesis of several human malignancies (Sherr, 1995, 1996).

v-cyclin contains a region of high sequence similarity to the cyclin box domain of cellular cyclins, and is responsible for interactions with cyclin-dependent kinases (Cdks) (Chang *et al.,* 1996a; Godden-Kent *et al.,* 1997; Li *et al.,* 1997; Verschuren *et al.,* 2004). The HHV-8 *v-cyclin* associates predominantly with Cdk 6, more weakly with Cdk 4 (Godden-Kent *et al.,* 1997; Li *et al.,* 1997), with the Cdk 6-*v-cyclin* complex able to phosphorylate the physiological target of D-type cyclins, namely the retinoblastoma tumour suppressor protein, Rb (Chang *et al.,* 1996a; GoddenKent *et al.,* 1997; Li *et al.,* 1997). Two-dimensional electrophoresis studies reveal that HHV-8 *v-cyclin* induces phosphorylation of Rb at authentic sites (Chang *et al.,* 1996a). The activation of Cdk 6 activity induced by the v-cyclins of both HVS and HHV-8 is much more pronounced than that by human cyclin D2: the two exhibit a broader specificity and can also phosphorylate histone H1.

A timely coordination of cellular DNA synthesis and division cycles is governed by the temporal and spatial activation of Cdks through binding to partner cyclin proteins. *v*-cyclin is able to interfere with the middle / end of phase G1 of the cell cycle via the inactivation of Rb and the Cdk inhibitors p16(Ink4a), p21Cip1 and p27Kip1 (Verschuren *et al.,* 2004). The inhibitor p27Kip1 is also known to be an effective inhibitor of cyclin E/Cdk2 activity (Ellis *et al.,* 1999). Hence *v-cyclin* appears to be able to activate both pathways necessary for G1/S-phase progression (i.e. cyclin D/Cdk6 and cyclin E/Cdk2), thereby enhancing the survival of HHV-8-infected cells (Ellis *et al.,* 1999; Swanton *et al.,* 1997).

1.3.10 LATENCY-ASSOCIATED NUCLEAR ANTIGEN (LANA)

ORF 73 of HHV-8 encodes a large nuclear protein which is identical (Rainbow *et al.,* 1997) to the previously described high-molecular-mass latent nuclear antigen (LNA) (224-236 kDa) (Gao *et al.,* 1996a) and a component of the LANA, defined by immunofluorescence (Gao *et al.,* 1996a; Kedes *et al.,* 1996). In addition to being a highly immunogenic protein, LANA assists in maintaining viral episomes by attaching viral DNA to the host chromatin through binding to the terminal repeat region of the HHV-8 genome (Cotter and Robertson, 1999; Ballestas and Kaye, 2001). It binds to the terminal repeat DNA binding domain as an oligomer at LANA residues 996–1139 (Komatsu *et al.,* 2004), and interacts with SUV39H1 histone methyltransferase, a key component of heterochromatin formation (Sakakibara *et al.,* 2004). This interaction creates the characteristic pattern of intra-nuclear dots observed under immunofluorescence. The LANA N terminus has been shown to have a key role in chromosome association, LANA-mediated DNA replication and episome persistence (Barbera *et al.,* 2004). LANA can also bind to and inhibit p53-mediated apoptosis, allowing HHV-8 infected cells to persist (Friborg *et al.,* 1999). Hence LANA is a key factor for maintaining viral latency.

A naturally occurring C-terminal truncated isoform of LANA (LANA-Delta76) has also been identified in HHV-8 positive PEL cells (BCP-1 and BC-3) (Canham and Talbot, 2004). This 3.2 kb transcript that does not associate with viral episomal DNA, and has the addition of a poly(A) tail at nt 3264 of orf73, resulting in an in-frame stop codon (TAA) effectively truncating

LANA by 76 amino acids (approximately 8 kDa). Its function is as yet unknown.

1.3.11 G PROTEIN-COUPLED RECEPTOR HOMOLOGUE

ORF 74 of both HHV-8 and HVS encodes a homologue of a G protein-coupled receptor (GPCR) belonging to the rhodopsin / β-adrenergic subfamily of G protein-coupled receptors, with seven membrane-spanning domains (Cesarman *et al.,* 1996a; Arvanitakis *et al.,* 1997). Transfection of HHV-8 GPCR has been shown to enhance the proliferation of rat kidney fibroblasts (NRK-49F), suggesting that it may contribute to tumour cell proliferation (Arvanitakis *et al.,* 1997) This receptor has the highest sequence homology to IL-8 chemokine receptors and is also related to the EBI-1 cellular protein induced by EBV infection (Birkenbach *et al.,* 1993).

HHV-8-GPCR is constitutively active, that is, it can signal in the absence of ligands (Arvanitakis *et al.,* 1997). It is capable of interacting with a much broader array of chemokines than most mammalian receptors, of both the CXC and CC families, and can be modified in its signalling activity by them (Couty and Gershengorn, 2004). Studies demonstrated that factors such as IL-8 and growth-related oncogene-α can activate HHV-8-GPCR above its constitutive signalling level, and are, therefore, considered full agonists of the receptor (Arvanitakis *et al.,* 1997; Rosenkilde and Schwartz, 2000). In contrast, factors such as the HHV-8-encoded CC chemokine vCCL-II and human stromal cell-derived factor-1alpha (SDF-1alpha) are inverse agonists of HHV-8-GPCR since they inhibit HHV-8-GPCR constitutive signalling, converting the receptor from an active to an inactive state (Geras-Raaka *et al.,* 1998). Other chemokines, including neutrophil activating

peptide-2 and epithelial cell-derived neutrophil-activating 78 (ENA78, CXCL5), are neutral antagonists for HHV-8-GPCR, that is, ligands that do not affect constitutive signalling but would compete for binding and inhibit the effects of agonists or inverse agonists (Rosenkilde and Schwartz, 2000).

HHV-8-GPCR utilizes a variety of signalling pathways to modulate the biology of its host. It activates the transcription factor nuclear factor (NF) kappa B in primary endothelial cells and in a KS-derived endothelial cell line (Pati *et al.,* 2001), and NFAT in activated T cells (Pati *et al.,* 2003). Activation of these two factors appears to be dependent upon the PI3-K/Akt pathway and inactivation of glycogen synthase kinase 3 (GSK-3) by Akt (Pati *et al.,* 2003). Both NF-κB and NF-AT activation induce secretion of several growth factors and proinflammatory and proangiogenic cytokines (interleukin-6 and IL-8), and increase the expression of adhesion molecules (e.g. VCAM-1, ICAM-1) (Pati *et al.,* 2001; Schwarz and Murphy, 2001; Pati *et al.,* 2003).

1.3 TROPISM OF HHV-8

HHV-8 exhibits a broad *in vivo* and *in vitro* cellular tropism. HHV-8 DNA and transcripts have been identified *in vivo* in peripheral blood mononuclear cells (PBMC) of patients with KS (Ambroziak *et al.* 1995; Whitby *et al.* 1995; Humphrey *et al.* 1996; Moore *et al.* 1996c; Uccini *et al.,* 1997) and multicentric Castleman's disease (MCD) (Kikuta *et al.,* 1997a), including B cells (Harrington *et al.,* 1996; Kikuta *et al.,* 1997a; Henry *et al.,* 1999; Monini *et al.,* 1999), T cells (Harrington *et al.,* 1996; Kikuta *et al.,* 1997a; Sirianni *et al.,* 1997a) and monocytes (Blasig *et al.,* 1997). HHV-8

has also been detected in endothelial cells (Boshoff *et al.,* 1995b; Li *et al.,* 1996; Orenstein *et al.* 1997; Sirianni *et al.,* 1997b; Staskus *et al.,* 1997; Sturzl *et al.,* 1997; Monini *et al.,* 1999), epithelial cells, including glandular prostate cells and foreskin keratinocytes (Diamond *et al.,* 1998; Cerimele *et al.,* 2001), and dendritic cells (Corbellino *et al.,* 1996a).

In the KS tissues, HHV-8 DNA is present in a latent form in the vascular endothelial and spindle cells (Boshoff *et al.,* 1995b; Decker *et al.,* 1996; Staskus *et al.,* 1997; Sturzl *et al.,* 1997; Schulz *et al.,* 1998; Dupin *et al.,* 1999), in addition to lytic virus present in a low number of infiltrating inflammatory monocytes (Blasig *et al.,* 1997). HHV-8 DNA has been also detected in PELs (Cesarman *et al.,* 1995b; Dupin *et al.,* 1999). PEL cell lines such as BCBL-1 and BC-3 carry HHV-8 in a latent form, and lytic replication can be induced by TPA (Dupin *et al.,* 1999; Renne *et al.,* 1996b; Schulz *et al.,* 1998).

In vitro, HHV-8 has been shown to infect a variety of human and animal cells, such as human B cells, epithelial cells (the 293 cell line), human endothelial cells, human foreskin fibroblast cells, human carcinoma cells (bladder, prostate, lung, and squamous), owl monkey kidney cells, and baby hamster kidney (BHK-21) cells (Flore *et al.,* 1998; Renne *et al.,* 1998; Moses *et al.,* 1999; Vieira *et al.,* 2001). Indeed, Bechtel *et al* (2003) confirmed that most adherent cell lines, irrespective of species of origin or tissue lineage, are permissive for HHV-8 viral entry and the establishment of latency. This indicates a wide distribution of receptors, possibly integrins, capable of binding to the virus (Akula *et al.,* 2002). However, there are some cultured

cells that lack susceptibility to HHV-8 infection. Although primary B cells can be infected *in vitro* (Mesri *et al.,* 1996; Kliche *et al.,* 1998), paradoxically established B cell lines that lack susceptibility (Renne *et al.,* 1998; Blackbourn *et al.,* 2000; Bechtel *et al.,* 2003).

It appears that the cellular tropism of HHV-8 varies with the lesion involved. Staskus *et al.,* (1999) used IL-6 expression as a marker of HHV-8 involvement in KS, PEL and MCD. They demonstrated that although PEL parallels KS in the pattern of latent and lytic cycle viral gene expression, the predominant infected cell type in PEL is the B cell, compared to spindle and endothelial cells in KS. MCD differed from KS not only in the infected cell type (B-cell and T-cell lineage) but also in the pattern of viral gene expression, with only a few cells in the lesion infected and all of these cells expressing lytic-cycle genes.

1.4.1 PERSISTENCE IN INFECTED ENDOTHELIAL CELLS

Experiments with polymerase chain reaction (PCR) *in-situ* hybridization reveal that HHV-8 can infect the atypical endothelial cells lining the vascular spaces in KS lesions and endothelial tumour (spindle) cells of fully developed, nodular KS lesions, but is not generally present in normal endothelial cells (Boshoff *et al.,* 1995b; Li *et al.,* 1996). This has been confirmed by microdissection studies (Boshoff *et al.,* 1995b). *In-situ* hybridization (Staskus *et al.,* 1997) and immunohistochemical studies localising with LANA (Rainbow *et al.,* 1997) have confirmed HHV-8 gene expression in KS spindle cells within tumours. HHV-8 establishes a persistent infection in most of these spindle cells, as demonstrated by

studies of the expression of genes ORF K12, ORF 72 and ORF 73 in latently infected PEL cell lines (Rainbow *et al.,* 1997; Staskus *et al.,* 1997; Sturzl *et al.,* 1997).

The atypical flat endothelial cells that are found in nodular KS and in the early stages of the disease were found to express only the latent *ORF KI2 T0.7* transcript (Rainbow *et al.,* 1997; Staskus *et al.,* 1997; Sturzl *et al.,* 1997); however, *ORF KI2 T0.7* transcripts are particularly abundant in both PEL cell lines and KS lesions (Renne *et al.,* 1996a; Zhong *et al.,* 1996). A subpopulation (approximately 10%) of KS spindle cells also expresses a polyadenylated nuclear noncoding RNA, T1.1 (nut-1), and is abundantly expressed in PEL cell lines induced lytic replication (Renne *et al.,* 1996a; Sun *et al.,* 1996; Zhong *et al.,* 1996; Staskus *et al.,* 1997). The distribution pattern of T1.1-expressing cells in KS tissue is similar to that of a few lytically infected spindle cells, which can be defined by their expression of mRNA for the major capsid protein (encoded by *ORF 25)* (Staskus *et al.,* 1997). Expression of T1.1 may therefore be indicative of lytic replication within KS lesions. This suggests that a subpopulation of the spindle cells can produce HHV-8 virions, as suggested by three reports describing the presence of intranuclear herpesvirus-like particles of 120 nm in diameter or intranuclear inclusions characteristic of herpesviruses in KS tissues (Walter *et al.,* 1984; Ioachim, 1995; Orenstein *et al.,* 1997).

Several independent lines of evidence therefore suggest that HHV-8 infects and persists in spindle cells *in vivo.* In contrast, all primary cell cultures established from KS lesions lose detectable HHV-8 (Ambroziak *et al.,* 1995; Lebbe *et al.,* 1995; Aluigi *et al.,* 1996; Flamand *et al.,* 1996), and

few cultures have been reported to maintain detectable HHV-8 for several passages (Lebbe *et al.,* 1995; Aluigi *et al.,* 1996). Two permanent KS cell lines that are tumorigenic in severe combined immunodeficiency and nude mice and contain chromosomal abnormalities (Siegal *et al.,* 1990; Lunardi-Iskandar *et al.,* 1995) also lack detectable HHV-8 (Flamand *et al.,* 1996).

1.4.2 PERSISTENCE IN HAEMATOPOIETIC CELLS

The most highly expressed HHV-8 transcripts in primary effusion lymphoma cell lines are polyadenylated transcripts that encoded by ORF K12 (T0.7) and ORF K7 (T1.1) (Renne *et al.,* 1996a; Sun *et al.,* 1996). Proteins encoded by ORFs 16 (bcl-2), 72 (CV-cyc), 74 (vGCR), K2 (vIL-6), K4 (vCCL-II), K6 (vCCL-I) and K9 (vIRF) are also expressed in PEL cell lines but at lower levels (Cesarman *et al.,* 1996a; Moore *et al.,* 1996b; Rainbow *et al.,* 1997; Sarid *et al.,* 1997). A polyclonal antibody specific for v-IL-6 has been used to demonstrate expression of viral cytokine in HHV-8-infected haematopoetic cells in lymph nodes and in un-induced PEL cell lines. Expression of vIL-6, vCCL-I, vCCL-II in PEL cell lines can also be induced by treatment with phorbol esters (Moore *et al.,* 1996b).

Limited data are available on the persistence of HHV-8 in PBMCs, although viral DNA and RNA have been demonstrated in cells of haemopoietic origin (Ambroziak *et al.,* 1995; Whitby *et al.,* 1995; Humphrey *et al.,* 1996; Moore *et al.,* 1996bc; Sirianni *et al.,* 1997b). Such results suggest that blood cells may represent a target for the virus *in vivo*. The presence of circular and linear HHV-8 genomes in PBMCs was reported in

one study, reflecting the presence of both latently and productively infected cells (Decker *et al.,* 1996).

1.4.3 PERSISTENCE IN OTHER TISSUES

HHV-8 has been found in a wide variety of other tissues, although the data are limited. The presence of HHV-8 DNA in genital tissues, namely prostatic tissues and semen of men with KS, has been reported (Monini *et al.,* 1996a; Corbellino *et al.,* 1996b; Howard *et al.,* 1997; Staskus *et al.,* 1997), although it cannot be excluded that HHV-8-infected mononuclear cells may represent the source in the semen. Studies by PCR have not shown that prostatic tissue from men without Kaposi's sarcoma is infected with HHV-8 (Corbellino *et al.,* 1996c; Blackbourn and Levy, 1997; Tasaka *et al.,* 1997; Rubin *et al.,* 1998). The genital tissues of women also harbour HHV-8, with the virus found in cervical and vaginal swabs from HIV positive African women (Lampinen *et al.,* 2000; Taylor *et al.,* 2004).

HHV-8 has also been detected in the dorsal root ganglia, sputum, saliva, mouth swabs, throat washing and bronchoalveolar lavage fluid, predominantly in patients with Kaposi's sarcoma (Howard *et al.,* 1995; Boldogh *et al.,,* 1996; Corbellino *et al.,* 1996a; Koelle *et al.,* 1997; Blackbourn *et al.,* 1998; Lampinen *et al.,* 2000; Pauk *et al.,* 2000; Duus *et al.,* 2004; Taylor *et al.,* 2004).

1.4.4 THE EMERGING ORAL TROPISM OF HHV-8

The human herpesviruses are generally ubiquitous among human populations, are generally shed in the oral mucosa, and are primarily

transmitted via the salivary route. Numerous studies have confirmed the presence of HHV-8 in the oral cavity (Di Alberti *et al.,* 1997; Blackbourn *et al.,* 1998; Lampinen *et al.,* 2000; Pauk *et al.,* 2000; Taylor *et al.,* 2004; Triantos *et al.,* 2004). In addition, epidemiological studies suggest that oral transmission of HHV-8 does occur among healthy populations (Cook *et al.,* 2002 a,b) .

It has recently been confirmed that wild type HHV-8 is not only present in the oral cavities of healthy individuals, but that it is also capable of supporting permissive infection of primary oral epithelial cells *in vivo*, with these cells capable transferring HHV-8 infection *in vitro* (Duus *et al.,* 2004). HHV-8 virions in buccal cells from healthy donors were demonstrated by transmission electron microscopy, with both lytic and latent viral proteins detected. These findings strongly suggest that oral epithelial cells may constitute an infected reservoir of HHV-8, and as such shares similarities with EBV and murine herpesvirus homologues (Peacock and Bost 2000; Sitki-Green *et al.,* 2003).

1.5 PRIMARY HHV-8 INFECTION

Clinical manifestations of primary infection with HHV-8 have been described but are not thought to be associated with significant concurrent morbidity in immunocompetent populations (Kasolo *et al.,* 1997; Kikuta *et al.,* 1997b; Luppi *et al.,* 2000; Plancoulaine *et al.,* 2000; Andreoni *et al.,* 2002). In immunocompetent children, HHV8 may be associated with a febrile maculopapular rash (Plancoulaine *et al.,* 2000), while bone marrow failure,

splenomegaly and fever have been reported in adult patients upon organ transplantation (Luppi *et al.,* 2000). Transient angiolymphoid hyperplasia was found to occur as part of an HHV8-seroconversion syndrome in an HIV-infected adult (Plancoulaine *et al.,* 2000).

The outcome of primary HHV-8 infection in immunosuppressed hosts can be variable, and it is also more difficult to directly attribute symptoms to HHV-8 rather than the immunosuppression. However, a case report of an HIV-1-seropositive male noted HHV-8 seroconversion five weeks prior to the sudden onset of symptoms, which included fever, arthralgia, cervical lymphadenopathy, and splenomegaly (Oksenhendler *et al.,* 1998). PCR of the cervical mass specimen revealed HHV-8 DNA, vascular hyperplasia, and "intense" activation and proliferation of B cells which was negative for EBV gene expression. The febrile episode was prolonged but resolved spontaneously within 2 months. It has also been reported that HIV positive patients who had evidence of subsequent HHV-8 seroconvertion, showed a greater relative risk for progression to KS than did those who had seroconverted for HHV-8 prior to HIV-1 infection (Goudsmit *et al.,* 2000). Hence it appears that primary HHV-8 infection has a higher morbidity in those who are HIV-1-infected than in those who reactivate a previous, latent HHV-8 infection. Conversely, primary HHV-8 infection during iatrogenic immunosuppression following organ transplantation carries a lower risk for KS than does reactivation of a previous infection (Jenkins *et al.,* 2002).

1.6 HHV-8 ASSOCIATED DISEASE

1.6.1 KAPOSI'S SARCOMA

1.6.1.1 Clinical Features and Epidemiology

In 1872, Dr Moritz Kaposi, a Hungarian dermatologist, first described an idiopathic, multiple pigmented sarcoma, now called 'classic' or sporadic KS (Kaposi, 1872; Breimer, 1994). For many years, KS was thought to be a lesion that affected predominantly elderly men of Mediterranean and eastern European origin, with a particularly high incidence in Italy, Greece, Turkey, and Israel (Landman *et al.,* 1984; Franceschi and Geddes, 1995; Iscovich *et al.,* 2000). Classic KS lesions tend to remain confined to the lower extremities and preferentially afflict men rather than women (15:1), with those affected typically living with the disease for 10 years or more (Hengge *et al.,* 2002). Epidemiologic studies in Italy have confirmed that the incidence of KS is highest in the South, with two particular southern Italian hot spots on the islands of Sardinia and Sicily (Whitby *et al.,* 1998; Santerelli *et al.,* 2001). However, there are also hot spots in low-incidence regions such as the Po Valley in northern Italy (Ascoli *et al.,* 2001). Various environmental risk factors have been proposed to account for the variable geographic predisposition to KS in Italy. It has been proposed that the occupation of cereal farming increases exposure to the aluminosilicate-rich volcanic soils specific to southern Italy and may contribute to localized immune suppression in the extremities and increased KS risk (Cottoni *et al.,* 1997).

The presence of KS was first noted in Africa in the 1920s (Williams, 1992). In the 1960s, it was reported to comprise up to 8% of malignancies,

with endemic foci in parts of Africa (Oettle, 1962; MacLean, 1963; Hutt and Burkitt, 1965; Williams, 1975). 'Endemic' KS affects two main age groups: young men with an average age of 35 years and children with an average age of 3 years (Hutt and Burkitt, 1965; Williams, 1975; Ziegler and Katongole-Mbidde, 1996). The highest prevalence of endemic KS prior to AIDS was found in a broad band crossing equatorial Africa, with particularly high rates in northeastern Zaire and western Uganda and Tanzania. This geographic pattern conformed to areas of frequent podoconiosis, a lymphatic disease of the legs presenting as lymphedema etiologically associated with chronic, barefoot exposure to volcanic soils, and is also linked to exposure to aluminosilicates (Ziegler, 1993). Current clinicoepidemiologic studies of endemic KS as an independent disease entity are now virtually impossible with the advent of AIDS. However four forms of KS have been described in Africa. One form has an indolent course, similar to classic KS, but affects young adults. The other three forms are more aggressive and are similar to AIDS-associated KS in their progression: however one of these, remains cutaneous with local tissue invasion, while another occurs most often in young children with a mean age of 3 years, is aggressive with visceral progression, but often lacks the cutaneous involvement (Hengge *et al.*, 2002).

In the 1960s and 1970s, a third epidemiological type of KS associated with immunosuppression was identified among organ transplant patients, constituting approximately 5% of tumours (Penn, 1979, 1983; Qunibi *et al.*, 1988). 'Iatrogenic' KS can be induced by immunosuppressive therapy, with subsequent regression on removal of immunosuppression (Hengge *et al.*,

2002). It can present either chronically or with rapid progression (Hengge *et al.,* 2002), and exhibits distinct ethnogeographic associations, occurring in only about 0.4% of transplant patients in the United States and Western Europe (Penn, 1979; Farge *et al.,* 1993) but in about 4.0 to 5.3% of renal transplant patients in Saudi Arabia (Qunibi *et al.,* 1988).

A fourth variant of KS, the 'epidemic' type, was identified in the early 1980s. It heralded the AIDS epidemic (Hymes *et al.,* 1981), and was later recognised as an AIDS-defining condition in HIV-infected individuals. Whereas the median age of KS-affected individuals in developed countries before the AIDS epidemic was over 70 years, it was the late thirties in the West. The disease is extremely aggressive and displays more frequent mucosal progression than in the other epidemiologic forms (Levine, 1993; Schwartz, 1996). It commonly presents multifocally and frequently on the upper body, head, and neck (Hengge *et al.,* 2002), and evolves quickly, both in local progression of lesions to tumours and in visceral dissemination, leading to organ dysfunction and high mortality. The risk for KS varies greatly among the different groups at risk for HIV transmission, being particularly high in homosexual and bisexual men (IARC, 1996), for whom KS is the most common neoplasm (Goedert, 2000). This elevated risk is seen even among men aged 13-24 yr, suggesting a rapid increase in risk after homosexual contact. Furthermore, women with AIDS who are sexual partners of bisexual men are more likely to have KS than women who are partners of intravenous drug users (Peterman *et al.,* 1993; Serraino *et al.,* 1995). Men who are more sexually active and have sexual partners from epicentres of the AIDS epidemic are also at increased risk of developing KS.

These data suggested that an infectious sexually transmitted agent (independent of HIV) is associated with KS. Transmission of such an agent via the blood is apparently less common, since KS occurs in only 3% of people who acquire HIV through blood transfusion (Dawkins *et al.,* 1998).

1.6.1.2 HHV-8 Detection in Kaposi's Sarcoma Tissues

HHV-8 DNA has been found in nearly all KS tissues, despite differences in detection methods and in the quality or preservation of tumour material, and the detection rate is similar whether the patients are HIV-infected or HIV-negative (Chang *et al.,* 1994, 1996a; Boshoff *et al.,* 1995a; Dupin *et al.,* 1995a; Moore and Chang, 1995; Schalling *et al.,* 1995; Su *et al.,* 1995; Buonaguro *et al.,* 1996; Chuck *et al.,* 1996; Dictor *et al.,* 1996; Gaidano *et al.,* 1996a; Luppi *et al.,* 1996a; Noel *et al.,* 1996; Ziegler and Katongole-Mbidde, 1996; Lebbe *et al.,* 1997). However, the clinical disparities between the individual forms of KS suggest a role for cofactors in the outcome of HHV-8 infection. For example, coinfection by HIV-1 is clearly the major risk factor unique to AIDS-associated KS and distinguishes its aggressive course from the other forms. Although there is no indication of significant differences in the HHV-8 detection rate among the four variants of KS, several groups have reported a higher detection rate of HHV-8 by PCR in late-plaque or nodular stages than in early or patch-stage KS (Luppi *et al.,* 1996a; Noel *et al.,* 1996). The amount of viral DNA detected by Southern blot in KS lesions averages from undetectable to an estimated 10-20 viral genome copies per cell equivalent. While HHV-8 DNA is readily detectable by PCR in DNA extracted from fresh tissue, nested PCR is often required to

obtain positive results from fixed, paraffin-embedded tissue. These conditions probably play a role with respect to the differences in positivity rate observed by different investigators.

1.6.1.3 HHV-8 seropositivity and the development of KS

Seroconversion for HHV-8 has been proposed as a strong predictor of KS (Gao *et al.,* 1996b; Kedes *et al.,* 1996; Moore *et al.,* 1996c; Renwick *et al.,* 1998). Extensive epidemiologic studies in Italy have confirmed that high HHV-8 seropositivity rates are associated with areas where the incidence of classic KS is the highest, such as southern Italy, Sardinia, Sicily, and the Po Valley (Whitby *et al.,* 1998; Ascoli *et al.,* 2001; Santerelli *et al.,* 2001). Similarly, despite the clinical heterogeneity of African KS, all cases are associated with HHV-8 seropositivity (Gao *et al.,* 1996b; Lennette *et al.,* 1996). However, African populations that have similar HHV-8 seroprevalences may have significant variations in KS incidences (Grossman *et al.,* 2002). Such data suggest the close correlation between HHV-8 seroprevalence and the incidence of KS in Western countries, but in Africa, other co-factors may be involved, such as exposure to volcanic soil (Ziegler, 1993).

HHV-8 seropositivity has also been associated with the development of iatrogenic KS. Indeed the high frequency of iatrogenic KS in Saudi Arabia may reflect the 7% endemic seroprevalence of HHV-8 reported in healthy Saudi donors or patients with non-KS malignancies (Qunibi *et al.,* 1998). Interestingly, transplant-associated KS is seen predominantly in kidney

allograft recipients and not other solid-organ or bone marrow transplant recipients (Cattani *et al.,* 2001). This may be associated with differences in immunosuppressive therapy which may favour HHV-8 reactivation in the kidney recipients, for example cyclosporine (Penn, 1987). Furthermore, the kidney may be a site of latent HHV-8, as proposed by Sarid *et al.,* (2001), who reported two HHV-8-seronegative patients who received infected kidney tissue, seroconverted, and subsequently developed KS. Studies have also confirmed that most iatrogenic KS patients are HHV-8 positive prior to transplantation, suggesting that reactivation of latent viral infection leads to disease (Parravicini *et al.,* 1997; Frances *et al.,* 2000; Cattani *et al.,* 2001; Jenkins *et al.,* 2002).

1.6.2 MUCOCUTANEOUS LESIONS OTHER THAN KS

HHV-8 DNA sequences have been detected in one sample of skin with scabies, and in skin lesions related to glomerulonephritis from two immunosuppressed patients (Noel *et al.,* 1996). HHV-8 sequences have also been detected in occasional angiosarcomas (Gyulai *et al.,* 1996a; McDonagh *et al.,* 1996) and tissue affected by angiolymphoid hyperplasia with eosinophilia (Gyulai *et al.,* 1996b), skin carcinomas (squamous cell carcinoma and Bowen's disease) in immunosuppressed individuals (Rady *et al.,* 1995). However, HHV-8 DNA was not detected in immunosuppression-associated dermatofibromas, despite their many histologic similarities with AIDS-related KS lesions (Foreman *et al.,* 1997). HHV-8 sequences were also sequenced in lesions of a patient with pemphigus vulgaris without HIV infection or KS, (Memar *et al.,* 1997; Jang *et al.,* 2000). The aetiological

connection between HHV-8 infection and most of these disorders, is suspect, however.

1.6.3 LYMPHOPROLIFERATIVE DISORDERS

1.6.3.1 Primary effusion lymphoma (PEL)

PEL (also known as body cavity based lymphoma) is another neoplastic condition associated with HHV-8. It was first described among AIDS patients (Knowles *et al.,* 1988), in whom it usually occurs during an advanced stage of the disease (Komanduri *et al.,* 1996). However it is rare even in AIDS patients, constituting only 0.13% of all AIDS-associated lymphomas in the United States, with previous KS diagnosis conferring an increased risk of PEL relative to all other AIDS-associated non-Hodgkin's lymphomas (Mbulaiteye *et al.,* 2002). PEL has also been reported in HIV-negative individuals, in whom it has been termed 'classic PEL': although extremely rare, the incidence is higher in HIV-negative elderly patients of Eastern European / Mediterranean and Jewish descent, two populations with high seropositivity for *HHV-8* and elevated incidence of classic KS. (Nador *et al.,* 1995; Ascoli *et al.,* 2002). In addition, like KS, PEL is seen primarily in homosexual men and seldom in other groups at risk for HIV infection (Jaffe, 1996; Nador *et al.,* 1996). In patients with AIDS, PEL is a fulminant lymphoproliferative disease, and the median survival time is less than six mon (Komanduri *et al.,* 1996; Nador *et al.,* 1996); however, a more indolent course has been documented in immunocompetent patients (Strauchen *et al.,* 1996).

PEL is a distinct subtype of non-Hodgkin's lymphoma that has morphological features shared by large-cell immunoblastic lymphomas and anaplastic large-cell lymphoma (Ansari *et al.,* 1996; Carbone *et al.,* 1996a,b; Cesarman *et al.,* 1996b). It is characterised by distinctive clinical, immunophenotypic and molecular genetic features (Cesarman *et al.,* 1995a). It presents predominantly as malignant effusions in the pleural, pericardial or peritoneal cavities, usually without significant tumour mass or lymphadenopathy; however, lymphomatous infiltration of serosal surfaces adjacent to the site of the primary malignant effusion is sometimes seen (Komanduri *et al.,* 1996). Morphologically, the cells bridge the features of large-cell immunoblastic and anaplastic large-cell lymphomas, and are usually large and irregularly shaped, with abundant cytoplasm and variably chromatic and pleomorphic nuclei (Ansari *et al.,* 1996). PEL cells have indeterminate (null) immunophenotypes, lacking expression of any lineage-associated B- or T-lymphocyte antigens but usually express the common leucocyte antigen CD45. A B-cell lineage is suggested by the presence of clonal immunoglobulin gene rearrangement (Knowles *et al.,* 1989; Cesarman *et al.,* 1995b; Komanduri *et al.,* 1996). The B-cell derivation is also supported by the monoclonal nature of primary effusion lymphoma, as demonstrated by a consistent rearrangement of the immunoglobulin genes.

Co-infection with EBV is common in PELs; EBV monoclonality has been established in most cases (Cesarman *et al.,* 1995a; Komanduri *et al.,* 1996; Nador *et al.,* 1996). It is therefore of interest to observe that several cell lines derived from these lymphomas, with genetic and immunological markers similar to those of the original lymphomas, were latently infected

with HHV-8 but not EBV (Arvanitakis *et al.,* 1996; Gao *et al.,* 1996b; Renne *et al.,* 1996a; Said *et al.,* 1996a). HHV-8 particles are not identified in the cytoplasm, but nuclear particles measuring 110 nm have been observed (Renne *et al.,* 1996a; Said *et al.,* 1996a,b).

Attempts to establish aetiological associations between PEL and HHV-8 have largely relied on the on the HIV-positive population, in whom PEL is more common. Nador *et al* (1996) reported the original cases of PELs associated with HHV-8: effusions from the 30 patients all contained HHV-8 and in 26 of these the PCR product was confirmed by Southern blot hybridization. Twenty-five of the described cases occurred in HIV-infected homosexual men and three in uninfected elderly men who did not belong to any established HIV risk group. Two cases of primary effusion lymphomas have been described in HIV-negative women (Said *et al.,* 1996b).

1.6.3.2 Castleman's disease

Castleman's disease (CD), also referred to as angiofollicular or giant lymph node hyperplasia, is a rare, usually polyclonal, non-neoplastic disorder of unknown aetiology (Castleman *et al.,* 1956). Two distinct histopathological variants with different clinical characteristics have been described: the hyaline vascular type and the plasma-cell type. The more common hyaline form presents primarily as a solitary mass, most frequently in the mediastinum or retroperitoneum, is asymptomatic and is usually curable surgically. The rare plasma-cell type is typically characterized by generalised lymphadenopathy, immunological abnormalities and type B symptoms.

The systemic variety, also designated multicentric Castleman's disease (MCD), is primarily of the plasma-cell type, but the hyaline type has occasionally been reported in a multicentric clinical appearance (Herrada and Cabanillas, 1995; Shahidi *et al.,* 1995). MCD has an aggressive clinical course with a poor prognosis, and such patients are at increased risk for KS and lymphomas (Peterson and Frizzera, 1993). About 70% of patients with all forms of CD under 30 years of age, and men are affected more often than women. Patients with MCD often tend to be in their fifties or sixties and to be at increased risk of developing non-Hodgkin's lymphoma and KS (Peterson and Frizzera, 1993; Oksenhendler *et al.,* 1996).

Unlike KS and PEL, not all CD has been found to contain HHV-8 DNA (Dupin *et al.,* 1995b; Soulier *et al.,* 1995; Barozzi *et al.,* 1996; Corbellino *et al.,* 1996d; Gessain *et al.,* 1996). Soulier *et al* (1995) detected HHV-8 in all of 14 HIV-positive lesions from French patients with MCD, comprising six plasma-cell type, seven mixed and one hyaline vascular type. Seven of the patients also had KS in the same tissue sample, and an additional two at another site; 64% showed KS both in the same tissue and elsewhere. Of 17 HIV-negative MCD lesions, seven (three plasma cell, two mixed, two hyaline type) contained HHV-8. KS was diagnosed in one of these subjects. Whereas the vast majority of cases among HIV-positive patients were found to contain the virus by Southern blot, only two of the seven cases in HIV-negative patients found to be positive by PCR were positive by Southern blot. To evaluate the significance of the positivity rate in the HIV-negative patients, reactive lymph nodes from 34 HIV-seronegative control patients were analysed: only one HHV-8-positive case was found. Such findings were

confirmed by other groups (Dupin *et al.,* 1995b, Corbellino *et al.,* 1996d, Gessain *et al.,* 1996; Oksenhendler *et al.,* 2000).

1.6.3.3 Other lymphoproliferative disorders

HHV-8 sequences have been found in non-HIV-infected patients with angioimmunoblastic lymphadenopathies (Luppi *et al.,* 1996b). A distinct benign, non-HIV-related, lymphadenopathy histologically characterised by a predominantly follicular lesion with giant germinal centre hyperplasia and increased vascularity (Luppi *et al.,* 1996b) is one of these. A similar disease entity with histological appearance has been reported in one HIV-infected patient (Soulier *et al.,* 1995). Furthermore, HHV-8 has been found in HIV-infected patients with otherwise unexplained interstitial pneumonitis, although the pathogenic role of HHV-8 in this condition remains unclear (Luppi and Torelli, 1996; Trovato *et al.,* 1999; Muller *et al.,* 2000).

Other lymphoproliferative disorders have also been found to harbour HHV-8 DNA, albeit rarely. These include other non-Hodgkin's lymphomas, Hodgkin's disease, reactive lymphadenopathies (Bigoni *et al.,* 1996), cutaneous lymphoma in AIDS (Corbellino *et al.,* 1996e), AIDS-associated immunoblastic lymphoma (Engels *et al.,* 2001), primary central nervous system lymphoma (Gomez-Brouchet *et al.,* 2001), post-transplantation lymphoproliferative disorders (Kapelushnik *et al.,* 2001), and pulmonary inflammatory myofibroblastic tumour (Gomez-Roman *et al.,* 2001). A condition of immunocompetent patients named germinotropic lymphoproliferative disorder, in which HHV-8- and EBV-coinfected B-cell

plasmablasts invade lymphoid follicle germinal centres, has also been described (Du *et al.*, 2002).

The viral load is significantly higher in lymphoid tissue from HIV-infected persons as compared to HIV-seronegative individuals (Bigoni *et al.*, 1996), although it is still lower than in splenic tissue or peripheral blood mononuclear cells (PBMCs) from the same patients. This suggests that the presence of HHV-8 in these lesions may be a reflection of HHV-8 carriage by non-neoplastic B cells (Corbellino *et al.*, 1996e). Detection of HHV-8 in mature T-cell lymphoproliferative disorders has been reported (Sander *et al.*, 1996), but this has not been confirmed (Cesarman *et al.*, 1995a, Pastore *et al.*, 1995, Pawson *et al.*, 1996).

1.6.4 MULTIPLE MYELOMA

The connection between HHV-8 infection and multiple myeloma (MM) is controversial. Rettig *et al.*, (1997) demonstrated the presence of HHV-8 DNA by PCR and in-situ hybridization in the cultured bone-marrow dendritic cells of 15 patients with MM but not in plasmablasts. The authors also demonstrated by RT-PCR the expression of *v-IL-6* in three of three cultured myeloma bone marrow dendritic cells, suggesting that HHV-8-v-IL-6 contributes to the mechanism whereby bone-marrow dendritic cells infected with HHV-8 promote myeloma growth. Said *et al.*, (1997) confirmed these findings. However, HHV-8 could not be detected by other groups either by PCR in DNA samples from myeloma specimens (Cesarman *et al.*, 1995a; Pastore *et al.*, 1995; Gessain *et al.*, 1997) or bone marrow samples (Rettig *et al.*, 1997). Similarly, only 1 of 15 serologic studies has demonstrated

increased anti-HHV-8 reactivity associated with MM patients (Gao *et al.,* 1998; Tarte *et al.,* 1999).

1.6.5 OTHER TUMOURS

In studies of angiosarcoma in HIV-negative individuals, Gyulai *et al.,* (1996a) reported one HHV-8-positive case. McDonagh *et al.,* (1996) found HHV-8 by PCR in seven cases in the United States. These findings have not been confirmed by other investigators (Chang *et al.,* 1994; Boshoff *et al.,* 1995a,b; Dictor *et al.,* 1996; Jin *et al.,* 1996a,b).

HHV-8 has also been found in lesional tissue of patients with squamous cell or basal cell carcinomas (Rady *et al.,* 1995; Inagi *et al.,* 1996; Nishimoto *et al.,* 1997). Rady *et al.,* (1995) reported the widespread presence of HHV-8 by PCR in various skin tumours from four immunosuppressed patients, but in studies in Austria, Germany, Sweden and the United Kingdom, this association could not be confirmed (Adams *et al.,* 1995; Boshoff *et al.,* 1996; Dictor *et al.,* 1996; Uthman *et al.,* 1996). More recently HHV-8 sequences have been found by PCR and ISH in multiple eruptive dermatofibroma, a rare disorder seen in immunocompromised patients, simulating KS (Kasakov *et al.,* 2003).

1.6.6 SARCOIDOSIS

Di Alberti *et al* (1997) demonstrated the presence of HHV-8 in sarcoid lesional tissue by nested PCR. However subsequent studies have failed to confirm this finding (Belec *et al.,* 1998a; Lebbe *et al.,* 1999; Maeda *et al.,* 2000; di Gennaro *et al.,* 2001). Furthermore Haburchak *et al* (2001) found patients with sarcoidosis to be HHV-8-seronegative.

1.6.7 PULMONARY HYPERTENSION

Primary pulmonary hypertension has recently been reported in two patients with HHV-8–associated Castleman's disease, with lung tissue from one of these patients being positive for LANA-1 (Bull *et al.,* 2003). Cool *et al* (2003) observed that the plexiform lesions of the disorder showed histological similarities to the endothelial abnormalities of cutaneous KS. They demonstrated that lung tissue from 10 of 16 patients with primary pulmonary hypertension (62 percent) were positive for LANA-1 immunohistochemically. Furthermore viral cyclin was detected following PCR analysis of tissue from the same 10 patients. No LANA-1 was detected in lung tissue from patients with secondary pulmonary hypertension, although one such patient had PCR evidence of viral cyclin. Antibodies against a structural HHV-8 glycoprotein, K8.1, have also been detected a patient with primary pulmonary hypertension (Henke-Gendo *et al.,* 2004). Such data suggest that HHV-8 infection may have a pathogenetic role in primary pulmonary hypertension.

1.6.8 CARDIAC DISEASE

Individuals with PEL have been reported to have a high incidence of congestive heart failure, a condition shared with classic KS patients (Ascoli *et al.,* 2002). It has also been reported that Italian patients with cardiovascular disease have a higher HHV-8 seroprevalence than the general population (Carletti *et al.,* 2002). Furthermore, HIV-1-infected patients with KS had an increased odds ratio of 3.35 for developing atheroma relative to those without KS (Grahame-Clarke *et al.,* 2001), raising the possibility that HHV-8 may be

associated with heart disease. Interestingly, a HHV-8-encoded chemokine, vCCL-I is chemotactic for endothelial cells expressing its receptor, the CC chemokine receptor 8 (CCR8) (Haque *et al.,* 2001). CCR8 is itself expressed in KS spindle cells, as well as in endothelial cells found in atherotic plaques; thus, vCCL-I may be mimicking the action of its cellular counterpart, the chemokine I-309, which is released by endothelial cells in response to the atherogenic apolipoprotein A.

1.7 DETECTION OF HHV-8 IN NON KS-AFFECTED TISSUE

1.7.1 SKIN OTHER THAN KS

HHV-8 DNA has been detected in normal skin of HIV-infected patients with KS (Dupin *et al.,* 1995a; Gaidano *et al.,* 1996a; Lebbe *et al.,* 1997); and more rarely, in the normal skin of patients with classic and endemic KS (Ambroziak *et al.,* 1995; Lebbe *et al.,* 1995; Boshoff *et al.,* 1996; Uthman *et al.,* 1996), or iatrogenic KS (Dictor *et al.,* 1996). The HHV-8 infection load appears to be lower in the normal skin than in KS lesions (Dupin *et al.,* 1995a; Rady *et al.,* 1995).

1.7.2 GASTROINTESTINAL TRACT

Early reports suggested that HHV-8 DNA is rarely detected in throat swabs and sputum of HIV-infected patients with KS (Whitby *et al.,* 1995). Initial studies also suggested that HHV-8 DNA was not present in saliva of patients with HIV-related KS (Ambroziak *et al.,* 1995) but subsequent workers have found HHV-8 DNA in the saliva of up to 33% of HIV-infected

persons but not in HIV-negative individuals (Boldogh *et al.,* 1996). Vieira and colleagues (1997) provided data on the potential infectivity of HHV-8 in saliva, demonstrating that infectious HHV-8 can be present in saliva, as evidenced by the persistence of HHV-8 DNA in cell cultures inoculated with cell-free saliva fluid and the induction of viral-specific RNAs in cells inoculated with cell-free saliva fluid. The virus has been identified not only in oral KS lesions (Jin *et al.,* 1996b; Flaitz *et al.,* 1997) but also in the normal oral mucosa of HIV-seropositive individuals (Triantos *et al.,* 2004)

HHV-8 DNA has also been amplified from duodenal aspirates and intestinal biopsy tissue of HIV-infected individuals (Thomas *et al.,* 1996). Stool samples could not be found HHV-8 DNA positive in one study (LaDuca *et al.,* 1998).

1.7.3 NEURONAL TISSUE

There are limited data available as yet about the possible neurotropism of HHV-8. It has been suggested in one report that dorsal root ganglia in patients with AIDS and KS harbour viral DNA (Corbellino *et al.,* 1996a). HHV-8 has also been detected in the cerebrospinal fluid from HIV-infected patients (Brink *et al.,* 1998). Furthermore, HHV-8 DNA has been demonstrated in cerebral tissue from patients with multiple sclerosis and also in healthy brain tissue (Merelli *et al.*, 1997). Chan *et al* (2000) have also reported that HHV-8 DNA could be detected in brain tissues of a majority (63.3%) of the adult population in Hong Kong.

1.7.4 PERIPHERAL BLOOD

The rate of detection of HHV-8 in PBMCs from KS patients varies widely. However, most of the larger studies suggest that about 50% of PBMC samples from KS patients give positive results when tested by nested PCR under standard conditions, e.g. using 100-500 ng of PBMC DNA (Whitby *et al.,* 1995; Lefrere *et al.,* 1996; Moore *et al.,* 1996c; Lebbe *et al.,* 1997). When assaying for the presence of HHV-8 DNA in PBMC, the importance of using sufficient DNA to detect a low copy number of viral DNA has been noted (Decker *et al.,* 1996; Blackbourn *et al.,* 1997).

Several groups have attempted to detect HHV-8 in PBMC from healthy individuals, usually control groups to be compared to patients with conditions linked to infection with HHV-8. When PBMCs from healthy individuals in countries with a low prevalence of KS (e.g. France, United Kingdom, USA) were studied, no HHV-8 genomes could be detected by nested PCR (Ambroziak *et al.,* 1995; Whitby *et al.,* 1995; Lefrere *et al.,* 1996; Marchioli *et al.,* 1996). In Italy, a country where classic KS is relatively frequent, HHV-8 was detected in 9% of PBMCs and lymphoid tissues from HIV-uninfected individuals (Bigoni *et al.,* 1996). In Uganda, where endemic KS is common, HHV-8 was detected in 14% of patients with tumours other than KS (Chang *et al.,* 1996b). These studies are consistent with finding a high prevalence of HHV-8 in PBMC in populations at high risk of developing KS.

1.7.5 GENITAL TISSUE AND FLUIDS

Several studies suggest the presence of HHV-8 in prostatic tissues of some HIV-infected men (Corbellino *et al.,* 1996b; Monini *et al.,* 1996a; Staskus *et al.,* 1997). HHV-8 was found to be preferentially detected in semen rather than spermatocytes, suggesting secretion into seminal fluids (Monini *et al.,* 1996a; Howard *et al.,* 1997), although it cannot be excluded that HHV-8-infected mononuclear cells occasionally represent the source of HHV-8 in semen. In a survey of tissues from AIDS patients with KS, Corbellino *et al.,* (1996b) found that prostate tissues harboured the viral genome, suggesting that the prostate is a major site of infection in these patients. *In-situ* hybridization studies probing for latent HHV-8 gene expression showed that viral gene expression in prostatic glandular epithelium is common in people without KS (Staskus *et al.,* 1997), lending support to the supposition that the virus is widely disseminated in the healthy male population. However, studies by PCR have not identified that prostatic tissue from men without KS to be infected with HHV-8 (Corbellino *et al.,* 1996c; Tasaka *et al.,* 1996, Blackbourn and Levy, 1997; Rubin *et al.,* 1998).

The presence of HHV-8 in the semen of healthy men is controversial. It may be detected in some semen samples from HIV-infected patients with or without KS, but the reported detection rates in the United Kingdom and the United States vary from 0% (of only four samples tested) to 33% (Ambroziak *et al.,* 1995; Gupta *et al.,* 1996; Marchioli *et al.,* 1996; Monini *et al.,* 1996b; Howard *et al.,* 1997). The results obtained in semen samples from healthy, HIV-seronegative donors are even more controversial in view of these implications for the spread of HHV-8 in the general population.

Samples from Italian semen donors were initially reported to be 91% positive (Monini *et al.,* 1996a) but later to be 23% positive (Monini *et al.,* 1996b), whereas no positive samples were found among 115 semen donors in the United Kingdom (Howard *et al.,* 1997) or in 20 in Milan, Italy (Corbellino *et al.,* 1996c). Some of these discrepant results probably reflect regional differences in HHV-8 prevalence (as shown by serological studies), selection of semen donors, or both. A high detection rate was reported in HIV-negative semen donors in the United States (Lin *et al.,* 1995).

HHV-8 has so far not been found in vulval mucosa or cervical malignancies of females without a history of HIV disease (Tasaka *et al.,* 1997).

1.8 SEROEPIDEMIOLOGY OF HHV-8

1.8.1 SEROLOGICAL METHODS FOR THE DETECTION OF HHV-8 ANTIBODIES

Many serological assay formats have been developed to test for the presence of HHV-8 antibodies and to complement testing by PCR. However, due to the phenomenon of immunodominance, the ability of various HHV-8 epitopes to trigger the immune response is uncertain, and the sensitivity and specificity of the assays are variable.

First generation assays were developed using HHV-8 infected PEL cells lines as the sources of antigen. PEL cell lines express latent antigens, predominantly LANA. Treatment of PEL cells with TPA induces expression of lytic antigens. Hence most of the early immunofluorescence assays (IFAs) for HHV-8 are based on B-cell lines derived from PELs. (Cesarman *et al.,*

1995b; Arvanitakis *et al.,* 1996; Gaidano *et al.,* 1996b; Gao *et al.,* 1996a; Renne *et al.,* 1996a; Said *et al.,* 1996a). These cell lines are latently infected with HHV-8. In the first IFA report, the cell line used (BC-1) was dually infected with HHV-8 and EBV (Moore *et al.,* 1996a), requiring absorption of EBV-specific antibodies to minimise cross-reactivity. Lytic replication of HHV-8, but not EBV, could be induced in these cell lines with sodium butyrate, allowing the detection of antibodies to a prominent, 40-kDa, lytic structural antigen (Miller *et al.,* 1996). Although of limited use for determining the seroprevalence of HHV-8 in the general population, the results obtained with these early assays indicated that, not surprisingly, most individuals with AIDS-associated KS and a much smaller proportion of HIV-infected individuals without KS carried antibodies to HHV-8 (Miller *et al.,* 1996; Moore *et al.,* 1996c).

When the nuclei of BC-1 were examined by western blotting for the presence of HHV-8-specific nuclear antigens, a nuclear protein of high molecular mass (226/234 kDa) was found to react specifically with sera from KS patients or those at increased risk for KS (Gao *et al.,* 1996b): 80% of AIDS-associated KS patients carried antibodies to this 'latent nuclear antigen', whereas no sera from United States blood donors or HIV-infected patients with haemophilia were reactive. This antigen is not cross-reactive with EBV-specific antibodies.

Widespread screening of groups at risk for KS and of the general population became possible when the first PEL cell lines infected with HHV-8 alone were established, e.g. BCP-1. BCP-1 cells express LANA, which is characterized by a typical speckled nuclear pattern (Gao *et al.,* 1996a; Kedes

et al., 1996). Detection of LANA by IFA correlates closely with reactivity to the 226/234-kDa nuclear antigen on western blots (Gao *et al.,* 1996b).

Examination of panels of sera from populations at high and low risk for KS suggests that antibodies to LANA predict the likelihood of KS developing in AIDS patients. Only 0%-3% of blood donors in the United States and United Kingdom were found to have antibodies to this latent nuclear protein (Gao *et al.,* 1996a,b; Kedes *et al.,* 1996; Lennette *et al.,* 1996; Simpson *et al.,* 1996), but 80-90% of sera from AIDS patients with KS and about 95% of sera from (non-immunosuppressed) 'classic' cases of KS reacted with it under optimal circumstances (Gao *et al.,* 1996a; Kedes *et al.,* 1996; Simpson *et al.,* 1996). Non-specific cross-reactive antibodies to cytoplasmic antigens interfere with the immunofluorescence at low serum dilutions, requiring either the isolation of whole nuclei (Kedes *et al.,* 1996) or the use of diluted sera, usually at least in a 1 in 100 or 1 in 160 ratio (Gao *et al.,* 1996b; Simpson *et al.,* 1996).

Lytic cycle (structural) HHV-8 antigens have also been found to react with sera from KS patients. In addition to the 40-kDa structural protein discussed above, recognized by 67% of the sera from patients with KS, some patients carry antibodies to other lytic HHV-8 proteins of approximately 27 and 60 kDa (Miller *et al.,* 1996; 1997). A 19-kDa capsid-related protein encoded by *ORF 65* has been used as a recombinant protein in enzyme-linked immunosorbent and western blot assays and is recognized by about 80% of sera from AIDS patients with KS and by 85-90% of those from patients with classic KS (Simpson *et al.,* 1996; Lin *et al.,* 1997). About 3%-5% of blood donors in the United Kingdom and United States show reactivity

to this protein. Its immunogenic determinants are located within the 80 amino acid stretch in the carboxy terminal, and this region is 21% identical to the corresponding region in the EBV homologue *BFRF3.* Although vp19/ORF 65 is thus not recognized by most sera from EBV-positive individuals and does not react with a set of high-titre EBV-positive sera, the question of whether there may be occasional cross-reactivity with EBV is not completely resolved. Concordance between the recombinant vp19/ORF 65 antigen and latent IFA antigen is high (around 80%-85%) in sera from patients with, or at risk of KS but lower in sera from blood donors in non-endemic regions and Africa. A combination of two or more antigen assays is thought to be required for optimal sensitivity (Simpson *et al.,* 1996).

Other recombinant lytic-phase proteins such as the minor capsid protein vp23, encoded by ORF 26, has been expressed as a recombinant protein and used as a serological antigen (Andre *et al.,* 1997; Davis *et al.,* 1997b). Significantly more sera from AIDS patients with KS than from HIV-negative controls react with this antigen. As this protein is recombinant, the antigen reacts with only about one-third of sera from AIDS patients with KS (Andre *et al.,* 1997); however, a synthetic peptide from this region was subsequently reported to be recognized by 60% of sera from this group of patients (Davis *et al.,* 1997b). The recombinant ORF 26 antigen was reported to react with the sera of only a few German blood donors (Andre *et al.,* 1997), whereas the ORF 26-derived synthetic peptide was reactive with 20% of sera from United States blood donors (Davis *et al.,* 1997b). A recombinant carboxy-terminal fragment of the major capsid protein encoded

by *ORF 25* has also been investigated and shown to cross-react with high-titre EBV antibodies (Andre *et al.,* 1997).

Other assays involve the detection of antibodies to unknown lytic antigens. These include an IFA using another PEL cell line, BCBL-1, treated with phorbol esters to induce the lytic replication cycle (Lennette *et al.,* 1996; Ablashi *et al.,* 1997; Smith *et al.,* 1997). With these assays, antibodies can be detected in nearly 100% of KS patients and in 20% of United States blood donors. While Lennette *et al.,* (1996), using a serum dilution of 1: 10, found antibodies to lytic HHV-8 antigens in 20% of United States blood donors, Smith *et al.,* (1997), using a 1:40 serum dilution and Evan's blue to reduce nonspecific background staining, found no antibodies in 52 blood donors. Smith *et al.,* (1997) noted that sera that are reactive by immunofluorescence at a dilution of <1:40 cannot be confirmed as reactive by radioimmunoprecipitation. The demonstration that cytoplasmic seroreactivity to PEL cells infected with HHV-8 can be cross-adsorbed by EBV-containing cell lines not infected with HHV-8 (Gao *et al.,* 1996a) suggests that nonspecific reactivity in lytic antigen assays may pose a specificity problem. Whole virion antigens may be of use in the design of competitive assays that are less sensitive to cross-reactive antibodies.

Although assays using these lytic-phase PEL cell lines are the most sensitive and widely used, the dilemma of possible cross reactivity to other herpesvirus antibodies remains, particularly as no HHV-8 negative PEL cell lines exist to act as controls for non-specific reactions. This consideration has led to the development of second generation assays such as that described by Inoue *et al* (2000). In this IFA assay, recombinant ORF K8.1

antigen expressed by Semliki forest virus is used. This antigen has no homologue to other herpesviruses, hence cross-reactivity does not occur. The availability of negative controls lends specificity to this assay. An ELISA based on a four-branch, multiple antigenic peptide derived from K8.1 likewise demonstrated high sensitivity and specificity (Lam *et al.,* 2002).

The specificity of immunofluorescence in induced primary effusion lymphoma cells varies with the assay protocol used, and the optimal conditions for these assays need to be established. Although the correlation among these different assays is good for sera from KS patients, there is considerable variation with regard to those from other groups, particularly those not at high risk of developing KS. In view of this Schatz *et al.* (2001) comprehensively compared six PEL-based IFA assays and eight ELISAs (with whole-protein and optimized peptide antigens). They concluded that a dual, anti-lytic / anti-latent IFA assay showed a good balance of specificity (89.1%) and sensitivity (94.9%). A whole virus ELISA followed by a PEL-based IFA assay also showed specific and sensitive results (Carletti *et al.,* 2002).

1.8.2 HHV-8 SEROPREVALENCE AND GEOGRAPHICAL DISTRIBUTION

The use of different serological assays for HHV-8-specific antibodies can profoundly influence the quantitation of seroprevalence, resulting in uncertainty about the exact seroprevalence of this virus in different populations and geographical areas (Rabkin *et al.,* 1998; Chatlynne *et al.,* 1999; Spira *et al.,* 2000). However, the relative seroprevalences estimated for geographically distinct populations remain consistent if similar antigens

are compared between groups (Rabkin *et al.,* 1998), with seroprevalence rates generally higher if assays detecting anti-lytic antibodies are used, as opposed to anti-latent antibodies (Chatlynne *et al.,* 1999). For example, 20% of one U.S. cohort of blood donors were seropositive for anti-lytic antibodies but none of the donors were seropositive for anti-latent antibodies (Lennette *et al.,* 1996). Similarly, a Swedish cohort showed 33% anti-lytic but only 6% anti-latent seroreactivity (Enbom *et al.,* 2002).

Nevertheless, it is generally agreed that antibodies to HHV-8 are found in most, if not all, patients with KS, are more common in individuals at risk for this disease than in the general population, and that the virus is not as widespread in the West as, for example, EBV. Antibodies to the latent nuclear antigen are found in about 85% of AIDS patients with KS and in more than 90% of individuals with classic KS (Gao *et al.,* 1996a; Kedes *et al.,* 1996; Simpson *et al.,* 1996). Among individuals studied in Denmark, the United Kingdom and the United States, HHV-8 antibodies to same antigen were detected in one-third of HIV-infected homosexual men without KS (Simpson *et al.,* 1996; Melbye *et al.,* 1998a,b), 8% of HIV-uninfected persons attending sexually transmitted disease clinics, 0%-3% of HIV-uninfected blood donors, 0%-3% of patients with haemophilia and none in intravenous drug users (Gao *et al.,* 1996a,b; Kedes *et al.,* 1996; Simpson *et al.,* 1996). Women in the United States, who are at low risk for AIDS-associated KS, yield correspondingly low LANA antibody titres, regardless of HIV status (Kedes *et al.,* 1997). Antibody positivity to vp19 / ORF 65 shows a very similar distribution: 81% of patients with AIDS-associated KS, 94% of those with 'classic' KS, 31% of HIV-infected homosexual men without KS, 2%-5%

of HIV-negative blood donors, 1% of patients with haemophilia and 3% of intravenous drug users (Simpson *et al.,* 1996). Therefore, the distribution of antibodies to both these antigens would suggest that HHV-8 is an uncommon infection in the general populations of those countries. Whether infection in the general populations is associated with sexually transmission remains unclear.

The phorbol ester-induced lytic IFAs may yield more sensitive results than either vp19/ORF 65 or LANA assays. Lennette *et al.,* (1996) examined the prevalence of antibodies to this antigen in the adult North American population. They found that HHV-8 seropositivity ranged from 16% to 28% in the general adult population, 96-100% for patients with AIDS-associated KS, 90% for HIV-infected homosexual men, and 23% for intravenous drug users. However, it is possible that the higher values may be not only due to increased sensitivity, but as a result of greater cross-reactivity to EBV or other human herpesviruses. Nonetheless, results from all of these assays broadly concur in suggesting that in Europe and North America HHV-8 is markedly more common among homosexual men than in other risk groups for HIV transmission. Thus, the distribution of HHV-8 seropositivity mirrors that of KS, which has long been known to occur more frequently among HIV-infected homosexual men than among patients with haemophilia, transfusion recipients or intravenous drug users (Beral *et al.,* 1990).

The relative seroprevalence of HHV-8 is also reported to be associated with the incidence of epidemic KS in HIV-infected populations. For example, in Thailand, where there is a high prevalence of HIV but a low

prevalence of AIDS-associated KS, only 2 to 12% of HIV-1-positive homosexual men were found to be HHV-8 seropositive (Ayuthaya *et al.,* 2002). HIV-1 probably exacerbates HHV-8 pathogenesis at multiple levels, including through immunosuppression, by priming of target cells and the tissue microenvironment for HHV-8 infection and replication, and by exerting direct effects on HHV-8 gene expression and viral replication. For example, T cells infected by HIV-1 have been shown to induce lytic reactivation of HHV-8 in PEL cells (Mercador *et al.,* 2000), and HIV-1 Tat and Vpr proteins can induce HHV-8 gene expression (Huang *et al.,* 2001).

The HHV-8 seropositivity rates in the general population are much less clear, with great variability reported in different geographic areas worldwide (Casper *et al.,* 2002b; Pellet *et al.,* 2003). HHV-8 seropositivity in Africa is variable geographically, ranging from 36 to 100% in different countries and populations in sub-Saharan Africa (Chang *et al.,* 1996c). Seroprevalence to both latent and lytic antigens is much higher (>50%) in countries of East, Central and West Africa (Gao *et al.,* 1996a; Lennette *et al.,* 1996; Simpson *et al.,* 1996). This suggests that infection with HHV-8 may approach near universal levels in some African populations (50%-70% seroprevalence). Indeed over 50% of children over the age of 6 years have been reported to be positive for HHV-8 antibodies in a cohort of children studied in Egypt (Andreoni *et al.,* 2002). Similarly, Mayama *et al* (1998) found that HHV-8 antibodies could be detected in 50% of children tested in Uganda. African populations that have similar HHV-8 seroprevalences may yield significant variations in KS incidences, however (Grossman *et al.,* 2002).

Lower seroprevalences for HHV-8 antibodies are found in the general populations of northern Europe and North America (Lennette *et al.,* 1996; Edelman *et al.,* 2000) than in several Mediterranean countries, such as Italy. The exception is Texas where the prevalence is substantially higher (15%) than other parts of North America (Baillargeon *et al.,* 2001; Hudnall *et al.,* 2003). In Europe, HHV-8 in the general population appears to be more common in regions known for their higher incidence of KS, although a careful comparison of the incidence of KS with HHV-8 seroprevalence in southern Europe is required before definitive conclusions can be reached. Particularly high HHV-8 seropositivity rates of over 24% have been reported in southern Italy, Sardinia, and Sicily (Gao *et al.,* 1996a; Whitby *et al.,* 1998; Calabro *et al.,* 1998; Ascoli *et al.,* 2001; Santerelli *et al.,* 2001), with the age distribution in seroprevalence corresponding to that of classic KS incidence (Calabro *et al.,* 1998). In the Po Valley, an area in northern Italy where classic KS is relatively uncommon, the seroprevalence among blood donors was found to be 4% by latent antigen IFA and western blotting (Gao *et al.,* 1996a). In Greece, 12% of HIV-seronegative surgical patients without KS were seropositive for ORF 65/vp19 and/or ORF 73 / LANA (Simpson *et al.,* 1996). Elsewhere in Mediterranean Europe, a group of blood donors in Spain yielded a low HHV-8 seropositivity of 6.5%, similar to that in northern Italy (Gambus *et al.,* 2001). The high prevalence of HHV-8 in the Mediterranean contrasts with non-Mediterranean European countries such as Latvia, where a recent screening of 150 healthy blood donors by PCR detected CMV, HHV-6, and HHV-7 but failed to detect HHV-8 (Kozireva *et al.,* 2001). The seroprevalence rate in German blood donors (3.0%) is similar to that found

previously in Western European countries (Preiser *et al.,* 2001). These findings suggest that the HHV-8 seroprevalence among the general population of KS-endemic countries may indeed be much higher than in northern Europe or the United States.

Reports from other parts of the world are limited. The prevalence rates in the Caribbean and Central America have been found to be between 0% as determined by latent antigen assays, and up to 29% by lytic antigen assays (Lennette *et al.,* 1996). More recently, the use of a lytic antigen assay in blood donors from Central and South America resulted in an HHV-8 seroprevalence of between 1.9% to 6.7%, with the variability thought to be linked to variation in immune status and exposure to HIV patients in the five cohorts tested (Perez *et al.,* 2004). In Brazil, seroprevalences among Amerindians (53%) (Biggar *et al.,* 2000) and urban communities (16%) (Freitas *et al.,* 2002) have been reported to be higher in the northern states than those from southern states. In blood donors from Brazilian southern states, a prevalence of 4.6% has been reported (Zago *et al.,* 2000). In Asia, the seropositivity rate of the general population is very variable: 1.4% (Satoh *et al.,* 2001) in Japan, 19.2% in Taiwan (Huang *et al.,* 2000), and 46.6% in the Xinjiang area, China (Dilnur *et al.,* 2001).

Most studies showed that HHV-8 seroprevalence in the general population of various countries was almost equally distributed between men and women but was increased in the older age groups (Calabro *et al.,* 1998; Huang *et al.,* 2000; Juhasz *et al.,* 2001b; Hudnall *et al.,* 2003). However, more recently Pellet *et al.* (2003) in the United States found no significant relationships between HHV-8 seropositivity and demographic characteristics.

1.9 ROUTES OF HHV-8 TRANSMISSION

1.9.1 ORAL

The primary mode of HHV-8 transmission remains unresolved. Extensive evidence exists for both sexual (Casper *et al.,* 2002a; Howard *et al.,* 1997; Lin *et al.,* 1995) and oral (Viera *et al.,* 1997; Blackbourn *et al.,* 1998; Lampinen *et al.,* 2000; Pauk *et al.,* 2000; Duus *et al.,* 2004; Triantos *et al.,* 2004) transmission in immunosuppressed individuals. However, recent epidemiological studies suggest that oral transmission of HHV-8 occurs among healthy populations (Cook *et al.,* 2002a,b). The virus has been detected in saliva, although the identities of the cell types harbouring HHV-8 *in vivo* in the oral cavity and producing virus particles is unclear. HHV-8 antigens have been detected in oral epithelial cells initiating from an early KS lesion (Webster-Cyriaque, 2002). In addition, the ability of the virus to infect primary human keratinocytes has also been demonstrated (Cerimele *et al.,* 2001; Bechtel *et al.,* 2003; Duus *et al.,* 2004).

1.9.2 SEXUAL

Risk factors for HHV-8 transmission may be evaluated by serological testing. Several studies have indicated that, irrespective of the type of antigen used, HHV-8 infection is more common among people attending sexually transmitted disease clinics than among blood donors (Kedes *et al.,* 1996; Lennette *et al.,* 1996; Simpson *et al.,* 1996). Although a number of sexual transmission routes have been hypothesized, no single sexual

behaviour has been clearly identified as most risky for HHV-8 transmission (Martin *et al.,* 2000). A detailed analysis of the behavioural risk factors among Danish homosexual men revealed that variables such as promiscuity and receptive anal intercourse, but not oral-anal contact, were associated with an increased the risk of HHV-8 seropositivity (Melbye *et al.,* 1998b). However, a subsequent study reported that participation in orogenital sex is a predictor for HHV-8 seroconversion (Dukers *et al.,* 2000). Furthermore, in the United States in the early 1980s, contact with homosexual men is associated with a markedly enhanced the likelihood of having or acquiring antibodies to HHV-8, suggesting that HHV-8 was introduced into that community in the late 1970s or early 1980s (Melbye *et al.,* 1998b). These findings suggest that HHV-8 is sexually transmitted in countries of low prevalence, consistent with the findings by PCR that infectious virus is secreted into the semen of infected men (Monini *et al.,* 1996a; Corbellino *et al.,* 1996b; Staskus *et al.,* 1997; Howard *et al.,* 1997). It is also apparent that the behavioural risk factors that were previously shown to increase the risk for KS (Beral *et al.,* 1990) also increase the likelihood of being infected with HHV-8, providing further evidence that HHV-8 is indeed the postulated 'KS agent'.

1.9.3 HOUSEHOLD

There is gathering evidence that sexual transmission is not the primary route of infection with HHV-8 in countries of high prevalence. In Africa, HHV-8 seroprevalence is relatively high in children, with seroprevalence reaching adult levels before adolescence (Mayama *et al.,*

1998; Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000). These data suggest transmission via close, non-sexual routes. Intrafamilial transmission has been suggested by the finding that children are more likely to be HHV-8 seropositive if they have mothers or siblings who are seropositive (Plancoulaine *et al.,* 2000). However extrafamilial non sexual transmission has also been identified (Cook *et al.,* 2002a,b). Although the precise mode of nonsexual transmission has not been clearly established, the relatively frequent carriage of HHV-8 in oral fluids suggests that transmission may occur via shedding from the oral cavity.

1.9.4 BLOOD

HHV-8 also appears to be transmissible, although less frequently, via exposure to blood. In the past, bloodborne transmission of HHV-8 has been discounted because HHV-8 transmission via blood transfusions has not been demonstrated and because groups with a risk of blood exposure (e.g., patients with hemophilia and injection drug users) have lower rates of KS than homosexual men. However, in a large cohort of women, HHV-8 seropositivity increased with increasing injection drug use, even after controlling for sexual behavior, and it was significantly associated with hepatitis C virus (HCV) infection, a surrogate marker for injection drug use (Cannon *et al.,* 2001). These results suggest that HHV-8 is transmitted via needle sharing and, thus, via exposure to blood.

1.9.5 VERTICAL

Vertical transmission of HHV-8 from mother to child before or during birth is uncommon. HHV-8 DNA was found in the PBMCs of only 2 infants of 89 infants born to HHV-8-seropositive mothers, (Mantina *et al.,* 2001). It has also been demonstrated that seropositive infants born to seropositive mothers, later become seronegative, suggesting that positive results are due to the transfer of maternal antibodies (Gessain *et al.,* 1999). Furthermore, in countries where HHV-8 seroprevalence is high, most infections occur after age 24 months (Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000; Andreoni *et al.,* 2002).

1.9.6 ORGAN TRANSPLANTATION

The route of HHV-8 transmission in Italian organ transplant recipients was investigated in one study. Parravicini *et al.,* (1997) found that 10 of 11 patients who developed KS were seropositive before receiving the allograft, in comparison with two of 17 transplant recipients who did not develop the disease. This suggests that, in HHV-8 endemic areas, most cases of transplant-associated KS are due to reactivation of a pre-existing HHV-8 infection; however, the authors also documented one case in which transmission of HHV-8 occurred from the allograft. In less KS-endemic regions, post-organ transplantation HHV-8 infection and KS are much rarer. A study from Scandinavia found only two cases of KS among 5000 transplant patients (Birkeland *et al.,* 1995). It has also been observed that

patients who develop KS after a renal transplant in North America are often of Jewish or Mediterranean ancestry (Harwood *et al.*, 1979).

1.10 THERAPEUTIC OPTIONS AGAINST HHV-8

Many of the currently available antiherpesvirus drugs that have been evaluated are DNA polymerase inhibitors, which are active against lytic but not latent herpesviral infection. *In vitro* sensitivity studies of antivirals at pharmacological concentrations in the PEL cell line BCBL-1, have demonstrated HHV-8 resistance to aciclovir and penciclovir but sensitivity to ganciclovir, cidofovir, adefovir, and foscarnet (Kedes and Ganem, 1997; Medveczky *et al.*, 1997). However, antiviral drugs did not inhibit episomal viral DNA synthesis, suggesting that no benefit can be expected from antiviral drugs in HHV-8-positive lymphomas or during latency (Medveczky *et al* 1997). This antiviral sensitivity of HHV-8 has recently been confirmed by the use of a real-time quantitative PCR to assess antiviral activity of molecules against HHV-8 (Friedrichs *et al.*, 2004), and by a flow cytometric assay (Long *et al.*, 2003). The *in vivo* evidence to support these data is controversial. Casper *et al.*, (2004) reported the effect of ganciclovir on the clinical and virologic course of MCD in 3 patients. Two patients experienced a reduction in the frequency of episodic flares of MCD and detectable HHV-8 DNA with intravenous or oral ganciclovir, whereas the third patient recovered from an acute episode of renal and respiratory failure with intravenous ganciclovir therapy. However, Senanayake *et al.*, (2003) found antiviral

treatment clinically ineffective in the treatment of an HIV-seronegative patient with MCD, with no consistent change in HHV-8 levels during therapy.

Clinical reports have also demonstrated that highly active antiretroviral therapy (HAART) containing protease inhibitors, used for the treatment of HIV infection, also produces a positive clinical response in KS patients and a reduction in HHV-8 viraemia (Lebbe *et al.,* 1998; Gill *et al.,* 2002). Furthermore, Gill *et al.*, (2002) demonstrated that both protease inhibitor-based and nonnucleoside reverse transcriptase inhibitor-based antiretroviral treatment combinations resulted in an undetectable HHV-8 load and KS regression. There was no clear association between CD4 cell count response and the KS response to HAART, but there was a significant relationship between HIV load response to HAART and clinical improvement of KS.

Novel approaches in anti-HHV-8 therapy include the use of the anti-CD20 monoclonal antibody rituximab, which has been reported to induce remission of clinical symptoms MCD and reduce HHV-8 viraemia (Corbellino *et al.,* 2002). Interferon alpha (IFN-α) has been shown to inhibit lytic viral replication to protect micro-endothelial cells from infection with HHV-8, thereby restricting proliferation of the virus within these cells (Krug *et al.,* 2004). Furthermore the transient expression of high levels of vIRF-1 is inadequate to interfere with antiviral effects of IFN-α (Pozharskaya *et al.,* 2004). Phosphonoformic acid has been observed to block production of infectious virus but not inhibit the rapid expansion of latently infected endothelial cells (Krug *et al.,* 2004). A nucleic acid-based gene interference approach, using ribonuclease P (RNase P) complexed with external guide

sequence (EGS) to target the immediate-early transcription activator, Rta, has been shown to be a promising strategy to block HHV-8 gene expression and growth (Zhu *et al.*, 2004). A reduction of 90% in Rta expression and a reduction of approximately 150-fold in viral growth were observed in cells treated with a functional EGS.

1.11 DETECTION OF HHV-8-CARRYING CELLS IN PERIPHERAL WHOLE BLOOD

Many different blood processing techniques are used to separate fractions of blood cells from peripheral blood prior to DNA isolation. These approaches may affect both the quality and quantity of cells available for DNA extraction and subsequent PCR amplification. It is also important to consider which fraction of blood cells will provide the best source of target DNA. Finally, the practicalities of different techniques may influence decisions in selecting which approach is most suitable for the research planned. The impact of different approaches of isolation of cells from peripheral blood on subsequent PCR amplification of HHV-8 sub-genomic DNA has not been well characterized with respect to sensitivity and reproducibility.

1.11.1 RELIABILITY OF METHODS TO DETECT SMALL AMOUNTS OF HHV- 8 GENOMES IN PBMCS

HHV-8 DNA has been identified by PCR in PBMCs of individuals with and without KS, and in some healthy donors, as summarized in Table 1.4.

The detection rate of HHV-8 in PBMCs of individuals with KS is generally reported to >50% and appears to be correlated with the stage and extent of KS (Camera Pierotti *et al.*, 2000; Campbell *et al.*, 2000). However, in individuals without KS, the HHV-8 detection rate in PBMCs is more variable, particularly in healthy individuals without HIV infection. The rate has ranged from 0% - 80% (Belec *et al.*, 1998b; Cattani *et al.*, 1998; Dupon *et al.*, 1997; Huang *et al.*, 1997; Kikuta *et al.*, 1997b; Lefrere *et al.*, 1996; Spira *et al.*, 2000). This marked variability may be related to the low copy number of viral DNA present in PBMCs (Blackbourn *et al.*, 1997; Decker *et al.*, 1996) and the differing sensitivity levels achievable in detecting small amounts of HHV-8 DNA. Many groups have attempted to improve sensitivity by employing nested PCR, and a wide a range of approaches have been employed (Belec *et al.*, 1998b; Cattani *et al.*, 1998; Min *et al.*, 1999; Spira *et al.*, 2000).

1.11.2 HHV-8 IN DIFFERENT CELL POPULATIONS IN PERIPHERAL BLOOD

HHV-8 can be carried by a variety of peripheral blood cell types, as summarized in Table 1.5. Consistent with the classification of HHV-8 as a gamma-2-herpesvirus, HHV-8 has been found in CD19-positive peripheral blood mononuclear cells, namely B cells, of KS patients and asymptomatic carriers (Ambroziak *et al.*, 1995; Blackbourn *et al.*, 1999; Harrington *et al.*, 1996; Henry *et al.*, 1999; Kikuta *et al.*, 1997a; Mesri *et al.*, 1996; Monini *et al.*, 1999). However, monocytes, circulating endothelial cells, and even T cells can also be shown to carry HHV-8 DNA (Blasig *et al.*, 1997; Harrington

Table 1.4 Detection of HHV-8 DNA in PBMCs

Country	Detection rate of HHV-8 DNA in PBMCs (total positive / total tested)	Patient subsets	Reference
USA	100% (10/10)	Patients with KS	Ambroziak *et al.*, 1995
	0% (0/6)	HIV-infected patients	
	0% (0/14)	Healthy HIV-uninfected controls	
United Kingdom	52% (24/46)	Patients with KS	Whitby *et al.*, 1995
	0% (0/26)	HIV-uninfected hospital controls	
	0% (0/143)	Blood donors	
Italy	10% (2/20)	Patients with AIDS	Bigoni *et al.*, 1996
	5% (2/38)	HIV-infected patients without AIDS	
	9% (5/56)	Healthy controls	
USA	89% (8/9)	Patients with KS	Decker *et al.*, 1996
	80% (4/5)	Allograft recipients	
	60% (3/5)	Healthy donors	
Italy	15% (10/66)	HIV-infected patients	De Milito *et al.*, 1996
	1% (1/89)	HIV-uninfected controls	
USA	35% (34/98)	HIV-infected patients with KS	Humphrey *et al.*, 1996
	19% (12/64)	HIV-infected patients without KS	

Table 1.4 Contd.

Country	Detection rate of HHV-8 DNA in PBMCs (total positive / total tested)	Patient sub-sets	Reference
France	91% (10/11)	AIDS patients with KS	Lefrere *et al.*, 1996
	2% (1/45)	Asymptomatic HIV-infected patients	
	0% (0/20)	Healthy controls	
USA	57% (12/21)	Homo / bi-sexual AIDS patients with KS	Moore *et al.*, 1996
	9% (2/23)	Homo / bi-sexual AIDS patients without KS	
	11% (2/19)	Haemophilia patients with AIDS	
France	47% (16/34)	Homo / bi-sexual males with KS	Dupon *et al.*, 1997
	27% (8/30)	Homo / bi-sexual males without KS	
	8% (2/24)	Blood product recipients	
	0% (0/25)	Healthy controls	
France	100% (3/3)	HIV-infected patients with MCD	Grandadam *et al.*, 1997
USA	25% (3 /12)	Patients with AIDS / KS	Huang *et al.*, 1997
	0% (0/2)	HIV-uninfected patients with KS	
	0% (0/4)	HIV-infected homosexual men	
	0% (0/5)	HIV-infected IVDU	
	0% (0/5)	Healthy blood donors	

Table 1.4 Contd.

Country	Detection rate HHV-8 DNA in PBMCs (total positive / total tested)	Patient subsets	Reference
Japan	64% (36/56) 80% (12/15) 100% (10/10)	Children with acute febrile illnesses Healthy adults Renal transplant recipients	Kikuta *et al.*, 1997b
France	81% (13/16) 20% (2/10)	HIV-infected patients with KS HIV-infected patients with clinical remission of KS	Poggi *et al.*, 1997
Uganda	84% (31/37)	Patients with KS	Purvis *et al.*, 1997
Italy	92% (12/13) 0% (0/18) 8% (1/12)	Patients with KS Relatives of KS patients Patients with dermatological disorders (non-KS)	Uccini *et al.*, 1997
France	42% (13/31)	HIV-uninfected patients with KS	Lebbe *et al.*, 1997
Central Africa Paris	22% (11/49) 2% (1/45)	Blood donors In-patients (HIV/HBV/HCV seronegative)	Belec *et al.*, 1998b
Italy	65% (17/26) 86% (6/7) 24% (7/29) 23% (3/13)	Patients with classic KS Patients with iatrogenic KS Patients with dermatological disorders (non-KS) Healthy volunteers	Cattani *et al.*, 1998

Table 1.4 Contd.

Country	Detection rate of HHV-8 DNA in PBMCs (total positive / total tested)	Details	Reference
USA	71% (5/7) 13% (18/135)	HIV-infected patients with KS HIV-infected patients without KS	Min *et al.*, 1999
Brazil	33% (4/12)	Patients with AIDS / KS	Camera Pierrotti *et al.*, 2000
USA	41% (12/29) 0% (0/20)	HIV-infected patients with KS Healthy controls	Spira *et al.*, 2000
Germany	13% (30/237)	HIV-infected patients	Lorenzen *et al.*, 2002

Table 1.5 HHV-8 in Peripheral Blood Sub-Populations

Cell type	CD Marker	Details	Reference
B cells	CD19	3 HIV-infected patients with KS	Ambroziak *et al.*, 1995
	CD19	2 KS positive patients	Harrington *et al.*, 1996
	CD19	B cells infected with HHV-8 from BC-1 cell line	Mesri *et al.*, 1996
	CD19	2 HIV negative patients with MCD and 1 patient with LCD	Kikuta *et al.*, 1997a
	CD19	1 blood donor	Blackbourn *et al.*, 1999
	CD19	Patient with KS	Henry *et al.*, 1999
	CD19	3 HIV-infected men with and without KS	Monini *et al.*, 1999
T cells	CD3	2 KS positive patients	Harrington *et al.*, 1996
	CD2	2 HIV-uninfected patients with MCD and 1 patient with LCD	Kikuta *et al.*, 1997a
	CD2	Patient with KS	Henry *et al.*, 1999
	CD8 and CD4	3 patients with AIDS / KS	Sirianni *et al.*, 1997a
	N/S	Late stage AIDS-KS patient	Monini *et al.*, 1999

Note. N/S: not specified

Table 1.5 Contd.

Cell type	CD Marker	Details	Reference
Monocytes	CD14	Patient with KS	Henry *et al.*, 1999
	N/S	5 HIV-infected men with and without KS	Monini *et al.*, 1999
Circulating spindle cells	N/S	Patients with KS	Sirianni *et al.*, 1997b
Haemopoietic stem cells / progenitor endothelial cells, fibrocytes	CD34	Patient with KS	Henry *et al.*, 1999

Note. N/S: not specified

et al., 1996; Henry *et al.*, 1999; Kikuta *et al.,* 1997a; Monini *et al.,* 1999; Sirianni *et al.,* 1997a)

From these findings it is evident that HHV-8 may be tropic for blood cell types other than B cells. Furthermore, the distribution of HHV-8 in these various cell sub-populations may vary with the stage of infection (latent or lytic), and the concurrent disease, if any. For example, Browning *et al*, (1994) demonstrated the presence of peripheral blood-derived spindle cells expressing a variety of endothelial cell markers, and confirmed that their numbers were significantly increased in HIV-infected individuals with KS as compared to HIV-negative controls. Indeed these circulating KS-like spindle cell progenitors have been shown to be persistently infected with HHV-8, suggesting that they may act as vehicles of dissemination of KS and HHV-8 throughout the body. (Sirianni *et al.,* 1997b).

1.11.3 APPROACHES TO OBTAIN CELLS FROM PERIPHERAL BLOOD FOR DNA EXTRACTION

Peripheral whole blood consists of cellular material (99% red blood cells [RBCs], with white blood cells [WBCs] and platelets making up the remainder), water, amino acids, proteins, carbohydrates, lipids, hormones, vitamins, electrolytes, dissolved gases (oxygen, carbon dioxide, and nitrogen), and cellular wastes. Plasma is composed of approximately 92% water, with plasma proteins, mainly albumins, globulins, and fibrinogens, as the most abundant solutes.

As RBCs are anucleate, WBCs constitute the primary target in protocols to extract nucleic acid from blood. RBCs are a rich source of heme and porphyrin which can bind to and inhibit *Taq* DNA polymerase (Higuchi, 1995). As little as 1% of whole blood (v/v) in an amplification reaction can inhibit PCR. Consequently, removal of RBCs from WBCs before nucleic acid extraction is critical when preparing material for downstream applications involving PCR.

The most common method of separating cells in peripheral blood is by centrifugation. Following centrifugation, three layers are visible in centrifuged blood: the plasma at the top, the buffy coat (which comprises WBCs and platelets) in the middle, and the RBCs at the bottom. If blood is allowed to stand and clot, the plasma is depleted of clotting factors such as fibrinogen, and turns to serum. Although centrifugation is an efficient method of obtaining RBCs, plasma and serum, the isolation of WBCs from the buffy coat layer is technically difficult due to the ease with which contamination with platelets, RBCs blood cells and plasma occurs.

Better techniques for fractionating WBCs have been developed. PBMCs are conventionally isolated by centrifuging whole blood in a liquid density step gradient. In so doing, lymphocytes and monocytes are separated from RBCs and granulocytes (eosinophils, basophils, and neutrophils). A double gradient can be used to fractionate granulocyte and monocytes simultaneously (Toth *et al.*, 1992). Alternatively, RBC lysis solutions can be used to remove contaminating RBCs, allowing WBCs to be isolated as relatively a pure pellet upon subsequent centrifugation. Lysing reagents include agents such as ammonium chloride and sodium phosphate.

They are generally hypotonic solutions which may lyse all cells, but RBC's, being more fragile, are less able than WBCs to tolerate hypotonicity for long periods.

The use of monoclonal antibodies that bind specifically to cell lineage-specific antigens has made it is possible to fractionate specific cell types from mixed populations in blood. Immunomagnetic technology has greatly facilitated this process, by allowing cellular subtypes to be separated with higher purity for further investigations. The concept of using immunomagnetic beads to isolate proteins was first introduced in the late 1970s. Guesdon *et al.* (1978) described the use of magnetically responsive polyacrylamide agarose beads for the purification of various serum proteins. Currently, immunomagnetic beads are used as a quick and easy way to isolate whole cells from a mixed cell population - a simple alternative to flow cytometry and cell sorting (FACS). A wide variety of immunomagnetic beads directed towards different cell surface antigens are available.

Cell types predominantly isolated by the techniques discussed above and their relative representation in blood (approximate number in of blood) are summarized in Table 1.6.

1.12. SEROPREVALENCE AND TRANSMISSION OF HUMAN HERPESVIRUSES

1.12.1 HSV-1 and HSV-2

HSV-1 and HSV-2 are amongst the most common causes of human viral infections. Each subtype is generally associated with infection at different mucosal sites. HSV-1 is the principal cause of oro-labial herpes, and

Table 1.6 Cell Types in Blood isolated by various separation techniques

Cell Type	Approximate number in 1 µL of blood (Fauci *et al.*, 1998)	Blood cell isolation / separation techniques		
		Red Cell Lysis	Density gradient centrifugation	Immuno-magnetic cell separation
Red blood cell	5.15 – 4.9 million			
Platelets	130,000 – 400,000	✓		
White blood cell	4,300 - 10,800			
Granulocytes	4,500-8,300	✓	✓	Any specific subset of cells
- neutrophils	0-20			
- basophils	0-700			
- eosinophils				
Mononuclear cells		✓	✓	
- lymphocytes	1,600-4,500			
- monocytes	40-100			

HSV-2 of genital herpes. Type-specific seroepidemiological studies have confirmed that, globally, the prevalence of HSV-2 infection is correlated with sexual activity, whereas the most important correlate of HSV-1 seropositivity is increasing age, with transmission occurring horizontally in childhood (Nahmias *et al.,* 1990; Cowan *et al.,* 1994; Obasi *et al.,* 1999). Improvements in socioeconomic conditions in some western countries have resulted in declining rates of childhood infection (Nahmias *et al.,* 1990). A serological survey of over 5000 samples collected as part of the PHLS Serosurveillance Programme revealed that the rate of infection with HSV-1 among 10 to 14-yr olds had dropped from 34% in 1986-1987 to 24% in 1994-1995 (Vyse *et al.*, 2000). An increase in the proportion of first episode genital herpes cases due to HSV-1 has also been observed (Scoular *et al.,* 1990; Ross *et al.,* 1993).

The prevalence of HSV-1 and HSV-2 in the U.K. was comprehensively investigated by a cross-sectional survey of 869 sexually transmitted disease clinic attendees and 1594 blood donors in London (Cowan *et al.,* 1996). This study reported that among clinic attendees, the prevalence of HSV-1 infection was 59.5% and that of HSV-2 infection was 22.7%, and among blood donors it was 44.6% and 7.6%, respectively.

1.12.2 EBV

EBV is an ubiquitous herpesvirus that targets the mucosal epithelium and is spread through contact with oral secretions (Rickinson and Kilpatrick, 2001). Primary infection with EBV is followed by the transformation of B cells leading to immortalization and the establishment of a latent infection, permitting the virus to persist for life (Anagnostopoulos *et al.,* 1995). EBV

acquired in childhood is usually mild or asymptomatic, whereas infection in adulthood results in infectious mononucleosis in 30–75% of cases (Straus *et al.,* 1993a). In addition, EBV is linked to several cancers including Burkitt's lymphoma, nasopharyngeal carcinoma and non-Hodgkin's lymphoma. A large seroprevalence study of EBV undertaken among individuals aged 0–30 yr in England and Wales, using sera collected through a PHLS sero-surveillance network in 1994–1995 showed that EBV seropositivity was 35% in those between 1 to 4 yr of age, 54% in the 10 to 14 yr-olds, 72% in the 15 to 19-yr olds, and >80% in those over 20 yr (Morris *et al.,* 2002).

1.12.3 CMV

CMV may cause illness through primary infection, but having remained latent within the host may be reactivated, especially if host immunity is impaired (Alford and Britt, 1993). It is the viral agent most frequently associated with congenital infections in humans. Although 90% of CMV congenitally infected infants are asymptomatic at birth, approximately 10% develop cytomegalic inclusion disease, which confers a 20–30% mortality rate (Demmler *et al.,* 1988). Primary infection in individuals who are immunocompetent is usually subclinical or causes mild symptoms similar to infectious mononucleosis. However, in neonates, individuals with impaired immunity and a small proportion of immunocompetent patients, primary infection may be severe, resulting in hepatitis, pneumonia, retinitis, encephalitis, and Guillain-Barré syndrome (Alford *et al.,* 1990; Rubin, 1990; Schooley 1990; Winston *et al.,* 1990; Ryan *et al.,* 1995; Wreghitt *et al.,* 2003).

In the UK, the seroprevalance of CMV in adults is ~ 50%, ranging from 40% to 100% depending on ethnicity and social class (Griffiths, 2000). Spread is enhanced by the intermittent shedding of CMV from multiple sites that continues for years after congenital, neonatal or postnatal infection (Reynolds *et al.,* 1973). The most common routes for neonatal and postnatal transmission of CMV to infants are the ingestion of breast milk and of cervicovaginal secretions (Dworsky *et al.,* 1983). Infection can also occur in the early years of childhood, following infection at nurseries or day-care centres, as young children can excrete the virus in respiratory secretions and urine for many months (Hutto *et al.,* 1985). Furthermore, sexual transmission to seronegative individuals can occur at the onset of sexual activity, and parenteral transmission through blood products has also been reported (Ho, 1990).

1.12.4 HHV-8

As alluded to in Sections 1.7 and 1.8, there is conflicting evidence regarding the seroepidemiology and primary transmission routes of HHV-8 in the general population residing in non KS-endmeic regions. Reliable estimates of HHV-8 prevalence in low risk populations such as blood donors from non-KS endemic regions are not available due to the uncertainty surrounding the specificity and sensitivity of the diverse serologic methods used. Furthermore, evaluations of the performance of the various assays have been hindered by the lack of pedigreed specimens that represent infected and noninfected immune-competent individuals from low seroprevalence populations (Rabkin *et al.,* 1998; Enbom *et al.,* 2000; Schatz

et al., 2001). However, there have been an increasing number of studies which support the premise that the virus is shed orally and may thus be more prevalent than if the predominant route of spread is sexual transmission (Viera *et al.,* 1997; Blackbourn *et al.,* 1998; Lampinen *et al.,* 2000; Pauk *et al.,* 2000; Duus *et al.,* 2004; Triantos *et al.,* 2004). In order to clarify the uncertainties, HHV8 seroprevalence needs to be compared to that of the other herpesviruses for which seroepidemiological and other data have more clearly delineated the routes of transmission. The presence of HSV-2 antibody is a marker of high-risk sexual activity, while antibodies for HSV-1 and EBV indicate contact involving exchanges of nasopharyngeal secretion and saliva. The presence of antibody to CMV reflects spread by multiple routes, from contact with cervicovaginal secretion, respiratory secretions, urine, and blood.

1.13 GENOPREVALENCE OF HHV-8

HHV8 DNA is readily detected by Southern blotting or PCR, in KS lesions, PELs and some lymphoid tissue from patients with multicentric Castleman's disease. Only small amounts of viral DNA are present in non-neoplastic tissue from HHV8-infected individuals, in particular in PBMCs, semen, saliva and breast milk. Consequently, more sensitive PCR techniques for detection have been used to amplify HHV-8 genomes (Decker *et al.,* 1996; Blackbourn *et al.,* 1997; La Duca *et al.,* 1998; Dedicoat *et al.,* 2004). The reported HHV-8 genoprevalence in healthy individuals without HIV infection as determined by HHV-8 DNA detection in PBMCs is very variable (ranging from 0% to 80%) (Lefrere *et al.,* 1996; Decker *et al.*, 1996;

Dupon *et al.,* 1997; Huang *et al.,* 1997; Kikuta *et al.,* 1997b; Belec *et al.,* 1998b; Cattani *et al.,* 1998; Spira *et al.,* 2000). Furthermore, studies have suggested that the HHV-8 DNA detection rate in peripheral blood of KS patients and in patients subsequently progressing to KS is higher than in patients who do not develop KS or are at low risk of KS (Moore and Chang, 1995; Whitby *et al.,* 1995; Lefrere *et al.,* 1996; Moore *et al.,* 1996c; Smith *et al.,* 1997; Min and Katzenstein, 1999; Cannon *et al.,* 2003; Engels *et al.,* 2003). Viraemia, measured as viral DNA in serum or plasma, has also been estimated (Bourboulia *et al.,* 2004). However, it has been reported that HHV-8 DNA in plasma in patients with KS may only be measured when the viral load in PBMCs is particularly high (Harrington *et al.,* 1996; Boivin *et al.,* 2002).

1.14 COMPARISON OF HHV-8 SEROPREVALENCE AND GENOPREVALENCE

The absence of gold standard serological tests and sensitive methods for detecting low copy numbers of the HHV-8 genome in PBMCs has contributed to the uncertainty over the true prevalence of this virus in the general population of non-KS-endemic regions. The use of improved methods to determine HHV-8 genoprevalence may better mirror the true prevalence in these individuals. Furthermore, a comparison of genoprevalence and seroprevalence may assist in clarifying the specificity and sensitivity of serological methods. Such data may also allow for assessment of the stage of HHV-8 infection: primary, latent, or lytic. In general, people who are HHV-8 seropositive without detectable DNA in the

peripheral blood may be considered to be latently infected (Gao *et al.,* 1996b; Simpson *et al.,* 1996), seronegative people who are only DNA-positive to be hosting an early primary infection (Andreoni *et al.,* 2002), and those who are positive in both tests to be undergoing reactivated infection or late primary infection.

1.15 BONE MARROW TRANSPLANTATION AND HHV-8

Despite recent advances in transplantation techniques, herpesvirus infections remain a major cause of morbidity and mortality in transplant recipients. Improvements in immunosuppressive drug regimens have decreased the risk of graft-versus-host disease and rejection in bone marrow transplant (BMT) / haemopoietic stem cell transplant (HSCT) recipients and solid organ transplant recipients. All such drugs carry with them an increased risk of herpesvirus reactivation.

KS has been diagnosed in patients receiving immunosuppressive treatment following solid organ transplantation (Penn, 1995, Gotti and Remuzzi, 1997; Shepherd *et al.*, 1997) and BMT / HSCT (Porta *et al.*, 1991; Helg *et al.*, 1994; Vivancos *et al.*, 1996; Erer *et al.*, 1997; de Medeiros *et al.*, 2000; Tamariz-Martel *et al.*, 2000; Palencia *et al.*, 2003). HHV-8 DNA is regularly detectable in post-transplant KS lesions (Chang *et al.,* 1994; Boshoff *et al.,* 1995a; Dupin *et al.,* 1995a; Moore and Chang, 1995; Schalling *et al.,* 1995; Su *et al.,* 1995; Buonaguro *et al.,* 1996; Chang *et al.,* 1996a; Chuck *et al.,* 1996; Dictor *et al.,* 1996; Gaidano *et al.,* 1996a; Luppi *et al.,* 1996a; Noel *et al.,* 1996; Ziegler and Katongole-Mbidde, 1996; Lebbe *et al.,* 1997). HHV-8 infection may be as a result of reactivation due to

immunosuppression, although it is also possible that HHV8 may be transmitted during transplantation, either by blood products or from allogeneic transplant tissue. Complications associated with HHV-8 infection, other than KS, in BMT recipients have also been described, ranging from fever and marrow aplasia with plasmacytosis (Luppi *et al.*, 2000) to bone marrow failure (Cuzzola *et al.*, 2003).

1.16 CHRONIC FATIGUE SYNDROME AND HHV-8

HHV-8 may be linked to other diseases for which the aetiology has yet to be determined, and for which immunosuppression is a prominent feature. An example of such a disease is chronic fatigue syndrome (CFS).

CFS is a distressing chronic condition for which there is currently no satisfactory management. It has a female preponderance and may be precipitated by severe emotional stress or acute severe infection, resulting in neurological and muscular fatigue of variable severity (Evengard *et al.*, 1999; Kakumanu *et al.*, 1999). The criteria for diagnosis of CFS are somewhat controversial. The American criteria include a requirement for several physical symptoms, reflecting the consideration that CFS has an underlying immune or infectious pathology (Figure 1.4) (Fakuda *et al.*, 1994). The UK Oxford criteria for diagnosis differ slightly in that mental fatigue is included (Table 1.7) (Sharpe *et al.*, 1991). The aetiology of chronic fatigue syndrome remains unknown but a number of factors have been implicated, namely infectious agents, environmental toxins and psychoneurologic disorders (Klonoff, 1992).

Reactivation of latent herpesviral infection, in particular, HHV-6 and EBV, has been proposed as contributing to CFS, (Bond, 1993, Straus, 1993b, Manian, 1994). Studies have confirmed that the antibody profiles of CFS patients reflect a chronic reactivation of latent EBV infection (Jones *et al.*, 1985; Straus *et al.*, 1985). However, rather than indicating a primary role for EBV in producing the symptoms of CFS, the activation of EBV may reflect instead a state of chronic immune dysregulation. HHV-6 is also reactivated more often in patients with CFS (Buchwald *et al.*, 1992; Yalcin *et al.*, 1994; Patnaik *et al.*, 1995; Zorzenon *et al.*, 1996). HHV-6 is an attractive potential pathogenic agent in CFS, since it has a remarkably wide tissue tropism, and is associated with clinical encephalitis and demyelinating diseases in immunosuppressed individuals, and multiple sclerosis in immunocompetent individuals (Caserta *et al.*, 1994; Carrigan *et al.*, 1996; Challoner *et al.*, 1995). As with EBV, however, infection with HHV-6 is ubiquitous, so it is possible that the reactivation of HHV-6 in patients with CFS is an epiphenomenon, reflecting immune dysregulation. The association of HHV-8 with CFS has not been investigated previously.

Immunological studies of CFS patients have reported the marked increase in the circulation of markers of immune activation, particularly cytokines (Vollmer-Conna *et al.*, 1998; Zhang *et al.*, 1999; Vollmer-Conna *et al.*, 2004). Research examining the release of cytokines by peripheral blood mononuclear cells *in vitro* has documented excessive IFN-alpha production in response to exogenous antigens (Lever *et al.*, 1988), in addition to increased endotoxin-stimulated release of IL-1 beta, IL-6, and TNF-alpha (Chao *et al.*, 1991). Cytokines have been proposed as the mediators of this

Figure 1.4 Revised Case Definition of Chronic Fatigue Syndrome And Idiopathic Chronic Fatigue: An Algorithm For Evaluation (Fukuda *et al.*, 1994)

Severe fatigue that persists or relapses for > 6 months

Exclude if patient found to have:

1. Active medical condition that may explain the chronic fatigue, such as untreated hypothyroidism, sleep apnoea, narcolepsy
2. Previously diagnosed medical conditions that have not clearly fully resolved, such as previously treated malignancies or unresolved cases of hepatitis B or C virus infection
3. Any past or current major depressive disorder with psychotic or melancholic features; bipolar affective disorders, schizophrenia, delusional disorders, dementias, anorexia nervosa, or bulimia nervosa
4. Alcohol or other substance abuse within two years before the onset of chronic fatigue and at any time afterward

*Classify as **chronic fatigue syndrome** if:*

1. Sufficiently severe: of new or definite onset (not lifelong)
2. Not substantially alleviated by rest
3. Results in substantial reduction in previous levels of occupational, educational, social or personal activities
4. Four or more of the following symptoms are concurrently present for > 6 months:
    - Impaired memory or concentration
    - Sore throat
    - Tender cervical or axillary lymph nodes
    - Muscle pain
    - Multi-joint pain
    - New headaches
    - Unrefreshing sleep
    - Post-exertional malaise

*Classify as **idiopathic chronic fatigue** if fatigue severity or symptom criteria for chronic fatigue syndrome are not met.*

Table 1.7 Oxford (British) Criteria for Chronic Fatigue Syndrome

Severe disabling fatigue of at least a 6-month duration that: • Affects both physical and mental functioning • Is present for more than 50% of the time Other symptoms, particularly myalgia and sleep and mood disturbances, may be present.
Exclusion criteria: • Active, unresolved, or suspected disease that is likely to cause fatigue • Psychotic, melancholic, or bipolar depression (but not uncomplicated major depression) • Psychotic disorders • Dementia • Anorexia or bulimia nervosa

CFS due to their potent CNS and cardiovascular effects.

It is has been considered that a microbial agent involved in the aetiology of CFS is one that produces or is capable of inducing the production of cytokines. In this context, it may be significant that the HHV8 genome encodes a protein homologous to human IL-6 (Moore *et al.*, 1996a; Russo *et al.*, 1996; Neipel *et al.*, 1997b; Nicholas *et al* 1997a,b). Moreover, HHV8 has also been shown to encode for virus proteins homologous to human MIP-1α and IRF (Russo *et al.*, 1996; Boshoff *et al.*, 1997; Burysek and Pitha, 2001).

1.17 STUDY HYPOTHESES

Evidence has gathered to suggest that HHV-8 is a virus that is shed orally in the absence of disease, and not one that is predominantly sexually transmitted. Accordingly, the prevalence of infection in the general population residing in non KS-endemic regions would approach that of other oral human herpesviruses such as EBV, CMV and HSV-1, rather than that of HSV-2. However, the prevalences reported in the literature result exclusively from serological detection of HHV-8 antibodies, which have given very conflicting figures for non KS endemic regions, reflecting the poor specificity of currently used antibody detection assays. The principal hypotheses investigated in this study are:

1. The HHV-8 prevalence in the general population of non KS-endemic region is higher than expected from a sexually transmitted agent

2. HHV-8 seroprevalence data underestimate true prevalence.
3. HHV-8 genoprevalence provides an estimate that more closely mirrors true prevalence.
4. Conventional methods for the isolation of WBCs from peripheral whole blood do not provide the optimal cellular substrates for HHV8 DNA sub-genomic amplification.
5. Genoprevalence studies may increase the specificity of, and assist in lending sensitivity to serological estimations of HHV-8 prevalence in populations at low risk of KS.
6. HHV-8 is reactivated in people with: immunosuppressive conditions but not at high risk of either HIV infection or KS, such as UK BMT recipients; and diseases in which immune dysfunction is a prominent feature, such as CFS. In these patient groups, HHV-8 is carried in peripheral blood subsets different from those in non-immunosuppressed people.

1.18 STUDY AIMS

In accordance to the hypotheses outlined above, the following aims were pursued:

1. The development of highly sensitive protocols to allow HHV-8 genoprevalence to be estimated.

2. Comparison of HHV-8 genoprevalence with seroprevalence in study populations at varying risk of HIV infection or KS: blood donors, HIV-infected genitourinary clinic attendees, BMT recipients and CFS patients.
3. Comparison of HHV-8 geno- and sero-prevalences in these populations with seroprevalences of antibodies to EBV, CMV, HSV-1 and HSV-2.

1.19 STUDY DESIGN

This thesis details the investigations which were undertaken in three stages to test the hypotheses outlined in section 1.17 and fulfill the aims outlined in section 1.18:

1.19.1 OPTIMISATION OF METHODS FOR FRACTIONATING HHV-8-CARRYING CELLS IN PERIPHERAL WHOLE BLOOD

In order to develop a highly sensitive protocol for HHV-8 sub-genomic amplification, conventional methods of WBC isolation from peripheral whole blood, namely RBC lysis and density gradient centrifugation, were compared with immunomagnetic bead cell isolation. The impact of these techniques on the subsequent PCR amplification of HHV-8 sub-genomic DNA from whole blood was determined.

1.19.2 GENOPREVALENCE AND SEROPREVALENCE OF HHV-8 IN SUB-POPULATIONS AT LOW RISK OF KS: UK BLOOD DONORS

The extent of HHV-8 infection in UK blood donors was estimated using various combinations of genome- and antibody-based assays. The rates thus obtained were compared with the seroprevalence rates of other human herpesviruses (HSV-1, HSV-2, EBV and CMV), and with the HHV-8 genome-, anti HHV-8-, anti HSV-1-, anti HSV-2-, anti EBV- and anti CMV-detection rates in an HIV-1-infected patient group. The immunomagnetic method of fractionating CD45+ cells from peripheral blood, determined to be the most optimal method to provide cellular material for HHV-8 DNA amplification (as determines by stage 1 of the investigation), was applied to samples of both study groups.

1.19.3 GENOPREVALENCE AND DISTRIBUTION OF HHV-8 IN PERIPHERAL BLOOD OF BMT RECIPIENTS AND PATIENTS WITH CFS

The extent of HHV-8 genoprevalence in BMT recipients and individuals with chronic fatigue syndrome was determined, using the immunomagnetic method of fractionating CD45+ cells from peripheral blood. Furthermore, the distribution of HHV-8 genome in leukocyte sub-populations, derived from immunomagnetically-fractionated CD2+, CD14+, CD19+ and CD31+ cells from these 2 groups of patients, was studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 PROCESSING OF BLOOD SAMPLES

Approximately 8 ml of peripheral blood from study individuals collected in EDTA-treated vacutainers were stored if necessary for no more than 7 d at 4°C. After low speed centrifugation, 1 ml of plasma was separated and stored at 4°C for later serological studies, and the remaining blood resuspended.

2.2 CELLULAR ISOLATION

Four procedures were investigated to determine which would be optimal for subsequent DNA extraction and PCR: red blood call (RBC) lysis, density gradient centrifugation, immunomagnetic cell separation, and density gradient centrifugation followed by immunomagnetic cell separation.

2.2.1 RBC Lysis

1. The EDTA vacutainer containing whole blood was inverted several times to mix thoroughly before 1 ml was removed.
2. This was placed into a sample preparation tube and 2 ml Specimen Wash Solution, which contains sodium phosphate, added (Amplicor, Roche Diagnostics, USA).
3. The tube was mixed by gentle inversion (5-10x) and allowed to incubate at room temperature for 5 min before being centrifuged at 4500 rpm for 3 min at room temperature.
4. The supernatant was carefully aspirated and a further 1 ml of wash solution added and gently vortexed before centrifuging again for 3 min.

5. This was procedure was repeated for a total of two washes.
6. The resultant dry cell pellet was stored at -70°C prior to DNA extraction.

2.2.2 Density Gradient Centrifugation

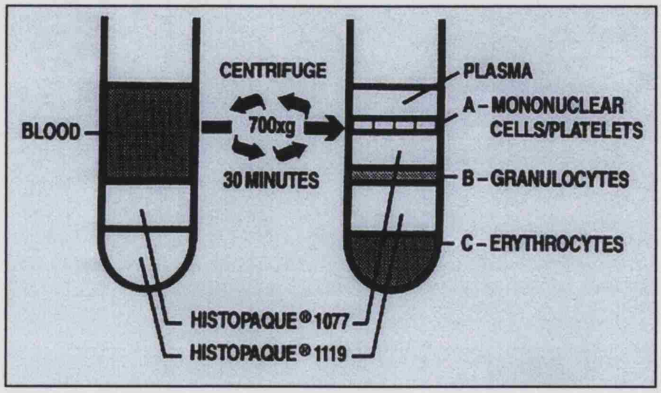
A gradient density centrifugation method was used for isolation of mononuclear cells and granulocytes from blood samples. Solutions used for this purpose consist of a polysucrose and sodium diatrizoate (HISTOPAQUE-1077, Sigma Diagnostics, St. Louis, USA) which was adjusted to a density of 1.077 +/- 0.001 g/ml. When blood is layered onto HISTOPAQUE-1077 and subjected to centrifugation, mononuclear cells are held at the plasma-HISTOPAQUE interphase, while erythrocytes and granulocytes gravitate to the bottom. HISTOPAQUE-1119 can be used in conjunction with HISTOPAQUE-1077, to ensure that cells of the myeloid series can also be harvested employing a one-step procedure, as outlined below:

1. A double gradient was formed by layering 1.5 ml of HISTOPAQUE-1077 over an equal volume of HISTOPAQUE 1119, in a 10 ml-conical centrifuge tube.
2. Subsequently, 3 ml of whole blood was carefully layered onto the upper HISTOPAQUE-1077 medium.
3. The tubes were centrifuged at 700 g for 30 min at room temperature. Two distinct opaque layers would then be observed (layers A and B in Figure 2.1). Cells of the granulocytic series would be found at the 1077/1119 interphase, whereas lymphocytes, other mononuclear

cells, including endothelial cells (Sbarbati *et al.,* 1991; Slifkin and Cumbie, 1992; Kas-Deelan *et al.,* 1998), and platelets would be found at the plasma / 1077 interphase (Toth *et al.,* 1992).

4. The plasma was then aspirated carefully to within 0.5 cm of layer 'A' and discarded.
5. Layer 'A' was then aspirated and transferred to a tube labelled 'mononuclear'.
6. Subsequently, the next layer of fluid was aspirated to within 0.5 cm of layer B and discarded.
7. Layer 'B' was then aspirated and transferred to a tube labelled 'granulocytes.'
8. The cells isolated in each tube were washed by addition of 10 ml PBS prior to centrifugation for 10 min at 200 g. The supernatant was aspirated and discarded.
9. The washing procedure was repeated a further two times.
10. The resultant clean cell pellets were resuspended in 250 µl of nuclease-free water.

Figure 2.1 Density gradient centrifugation method



2.2.3 Immunomagnetic Cell Separation

Dynabeads (Dynal A.S., Oslo, Norway) are spherical, supermagnetic, polystyrene beads with diameters between 2.8 μm (M-280) to 4.5 μm (M-450). They can be directly coated with monoclonal antibodies that recognise a specific cell type within the target cell suspension, or be coated first with any mouse, rat or rabbit polyclonal or monoclonal antibody. The cell types targeted in the current investigation and their cell surface cluster of differentiation (CD) markers are summarised in Table 2.1.

2.2.3.1 Positive Isolation of Leucocytes

Positive isolation of leucocytes was performed using Dynabeads M-450 CD45 (mAb clone T29/33) (Manyonda *et al.*, 1992). CD45 antigen is the most common leucocyte marker and is expressed in all cells of haematopoietic origin except erythrocytes. The cytoplasmic part of CD45 exhibits tyrosine phoso-kinase activity and the expression of the CD45 antigen appears to be necessary for antigen-induced T cell proliferation (Battifora and Trowbridge, 1983).

2.2.3.2 Positive Isolation of Endothelial Cells

Positive isolation of endothelial cells was undertaken using secondary coated Dynabeads, namely M450 sheep anti-mouse IgG, coated with purified mouse anti-human CD31 monoclonal antibody (Pharmingen, clone WM-59) (Hewett and Murray, 1993). Also known as platelet endothelial cell adhesion molecule (PECAM-1), CD31 has wide tissue distribution and is highly expressed on endothelial cells, but is also found on platelets, monocytes, granulocytes.

Table 2.1 Cluster of Differentiation (CD) markers on the cell surfaces

CD Antigen	Other names	M. W.	Expression	Functions
Cd2	T11, LFA-2	45-58	Thymocytes (95%), Mature Peripheral T Cells (Almost all), NK Cells (80-90%), Thymic B Cells (50%)	Adhesion molecule, binding CD58 (LFA-3); Can activate T cells
Cd14		53-55	Macrophages/Monocytes (90%), Granulocytes (30%), Langerhans Cells, Dendritic Cells, B Cells	Receptor for complex of LPS and LPS binding protein
Cd19		95	Pre B, B Cells, Follicular Dendritic Cells	Co-receptor for B cells
Cd31	Pecam-1	130-140	Endothelium, Platelets, Macrophages, Kupffer Cells, Granulocytes, T / NK Cells, Lymphocytes, Megakaryocytes, Fibroblasts, Osteoclasts, Neutrophils	Possibly an adhesion molecule
Cd45	Leukocyte Common Antigen (LCA), T200, B220	180-240	All haemopoietic cells, stronger in lymphocytes	Tyrosine phosphatase, augments signalling through antigen receptor of B and T cells

From: 1st International Workshop and Conference on Human Leukocyte Differentiation Antigens (HLDA), 1982.

2.2.3.3 *Positive Isolation of Monocytes / Macrophages*

Dynabeads M-450 CD14 were used to isolate human myelomonocytes of the monocyte, macrophage and one granulocyte subset (Theodorsen *et al.*, 1995). These Dynabeads were coated with a mouse IgG2a monoclonal antibody (clone RM052) against CD14 antigen. The CD14 antigen is a receptor for the lipopolysaccharide complex (LPS) and the LPS-binding protein (Wright *et al*, 1990).

2.2.3.4 *Positive Isolation of B Lymphocytes*

Dynabeads M-450 CD19 were used to isolate B lymphocytes from whole blood (Rasmussen *et al.*, 1992). CD19 is the most widely expressed surface marker for B cells. The mouse IgM mAb (clone AB1) is coated directly to the beads (Ling *et al*, 1987).

2.2.3.5 *Positive Isolation of T Lymphocytes*

CD2 Dynabeads were used to isolate T lymphocytes from whole blood (Wiesneth *et al.*, 1996) The CD2 antigen is a 50kDa single chain transmembrane glycoprotein and acts as a receptor for CD58 (Breitmeyer *et al*, 1987) and CD59 (Hahn *et al.*, 1992). It is mainly expressed on T cells, but is also found on NK cells, thymocytes and various T cell lines.

2.2.3.6 *The Positive Isolation Procedure*

Positive isolation allows isolation of predefined subsets directly from heterogeneous cell suspensions, such as whole blood. The antibody coated Dynabeads are added directly to the cell suspension, where they bind the

target cells. The complex of beads-cells are then positively isolated magnetically, as outlined below:

1. The Dynabeads are supplied as a suspension containing $4xl0^8$ beads/ml in 0.1 M Na_2PO_4 buffer, pH 7.4, containing 0.1 % bovine serum albumin (BSA) and the cytotoxic agent, 0.02% NaN_3. Before use, the beads were washed with a washing buffer (2% fetal calf serum (FCS) in PBS.
2. The desired amount of Dynabeads was transferred into a washing tube. The washing tube was then placed on a magnetic particle concentrator (MPC) for 60 s and the fluid removed with a pipette.
3. After the tube was removed from the concentrator, three volumes of washing buffer were added and the beads resuspended.
4. The procedure was repeated a further two times.
5. One ml of whole blood was pipetted into a 1.5 ml Eppendorf tube containing $2x10^7$ of Dynabeads M-450 CD45 (50 μl). This was placed in a Dynal Sample Mixer (Dynal A.S., Oslo, Norway), to provide gentle tilting and rotation, and incubated at 4°C to prevent non-specific attachment of phagocytic cells to the Dynabeads. An incubation time of 20 min was used in order to achieve a cellular yield of over 90% from whole blood.
6. The tube was then removed and placed in a MPC (Dynal A.S., Oslo, Norway) for 3 min to collect the rosetted cells (Figure 2.2). The supernatant was pipetted off carefully to leave the cells attached to the wall off the tube.

7. The tube was removed from the concentrator, 1 ml of washing buffer added, and the rosetted cells gently resuspended. The tube was repositioned into the concentrator and the supernatant removed.
8. The rosetted cells were washed five times and then eluted in 250 μl of nuclease-free water.

The technique was essentially similar for the other Dynabead subsets used, minor variations to the procedure being summarised in Table 2.2.

2.2.4 Density Gradient Centrifugation followed by Immunomagnetic Cell Separation

The gradient density centrifugation method described in Section 2.2.2 was used for separate isolation of mononuclear cells and granulocytes from whole blood. The Histopaque granulocyte and monocyte isolates were each divided into 2 equal fractions, one of which was used to undertake CD45+ immunomagnetic cell separation, and the other, CD31+ cell isolation (as described in section 2.2.3).

Figure 2.2 Immunomagnetic cell separation

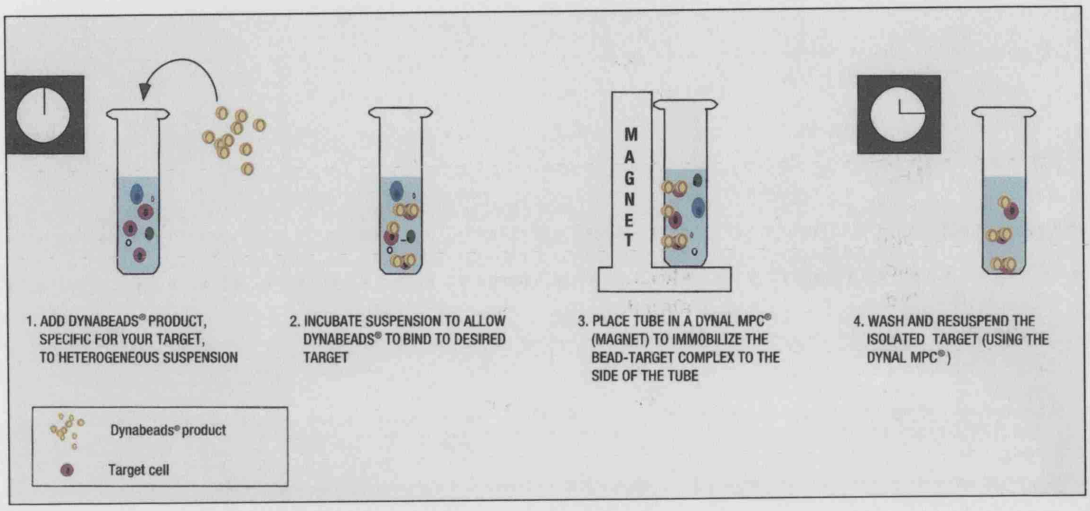


Table 2.2 Variations to the Positive Isolation Procedure

Dynabead product	Procedural notes
M-450 CD31 Endothelial Cells	• Secondary coated beads M-450 sheep anti-mouse IgG antibodies were coated with mouse CD31 (PECAM-1) IgG1 (Pharmingen MoAb, clone WM-59) using 2μg Ab per mg of beads (~33μl). • These were left to incubate on a MPC at 4°C for 24 hours prior to use • The whole blood was diluted with one half volume of cold PBS/FCS prior to addition of the Dynabeads
M-450 CD14 Monocytes / Macrophages	• The whole blood was diluted with one half volume of cold PBS/FCS prior to addition of the Dynabeads
M-450 CD19 Pan-B Cells	• The whole blood was diluted with one half volume of cold PBS/FCS prior to addition of the Dynabeads
M-450 CD2 Pan-T Cells	• The whole blood was diluted with one half volume of cold PBS/FCS prior to addition of the Dynabeads

2.3 EXTRACTION OF DNA FROM SAMPLES

A procedure based on the lysing and nuclease-inactivating properties of NaI, together with the nucleic acid binding properties of silica particles (EZ-Glassmilk) was employed for DNA extraction (Geneclean Kit III, BIO 101, La Jolla, California):

1. The cellular isolates, prepared by one of the methods described in Section 2.2, were thawed to room temperature.
2. Three volumes of lysis buffer (750 µl 6 M NaI) and 20 µl of silica particles were added and the tubes vortexed.
3. This was incubated at room temperature for 5 min with frequent tube inversions to ensure that the nucleic acids bound to the silica particles.
4. The samples were vortexed again, then centrifuged in a fixed angled Eppendorf microcentrifuge (30 sec at 12,000g). An extended fine pipette was used to carefully remove the supernatant, leaving the pellet intact.
5. The pellets then underwent three washes with 500 µl of New Wash (NaCl, Tris, EDTA, ethanol) (BIO 101, La Jolla, CA), with 5 s of centrifugation between washes to separate the supernatant.
6. After removal of the last New Wash solution, the microcentrifuge tubes were placed in an Eppendorf heating block at 56 °C, with open lids for 5 min to allow the pellets to dry.
7. The pellet was resuspended in 20 µl of Elution Solution (RNase / DNase / pyrogen-free water) (BIO 101, La Jolla, CA) and incubated at

56 °C for 5 min prior to centrifugation for 30 sec.

8. The supernatant containing the eluted DNA was carefully removed and placed in a new tube. In excess of 80% of bound DNA is obtained by this first step. A further 10-20% of DNA was recovered using a second elution step with 20 µl of Elution Solution.
9. The appropriate volume of DNA extract was use as template in the first round of PCR, and the remaining extract was stored at -20°C.

2.4 PCR AMPLIFICATION OF HUMAN β-GLOBIN SUB-GENOMIC DNA

The presence of DNA in extracts from whole peripheral whole blood was verified by amplifying a 110-bp fragment of the β-globin gene using as the sense primer 5'CAACTTCATCCACGTTCACC3' and antisense primer 5' ACACAACTGTGTTCACTAGC3' (Saiki *et al.*, 1985). The PCR was carried out in a 25 µl reaction mixture containing:

1. nuclease-free water
2. 1.5mM $MgCl_2$
3. 10 nM each of dNTP
4. 1 unit Taq DNA polymerase (Invitrogen, Paisley U.K.)
5. 20 pmol of each pair of primers
6. 2.5 µl of the DNA extract

Amplification was undertaken using a 480-thermal cycler. Samples were heated for 94°C for 5 min, followed by 35 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 1 min (extension), followed by a 5-min extension period at 72°C.

2.5 PCR AMPLIFICATION OF HHV-8 SUB-GENOMIC DNA

All the DNA extracts underwent detection of HHV-8 DNA using a modification of the PCR method described by Chang *et al.*, (1994). A nested PCR to amplify a 171 bp fragment of ORF 26 DNA was undertaken using as sense inner primers 5'TTCCACCATTGTGCTCGAAT3' and antisense inner primers 5'TACGTCCAGACGATATGTGC3' (Di Alberti 1997). The PCR conditions had been optimised by previous co-workers and were carried out in a 25 µl reaction mixture containing:

1. nuclease-free water
2. 1.5mM $MgCl_2$
3. 10 nM each of dNTP
4. 1 unit Taq DNA polymerase (Invitrogen, Paisley U.K.)
5. 20 pmol of each pair of primers
6. 2.5 µl of the extract.

A 213 bp segment from the VR1 region of ORF K1 of HHV-8 was also amplified by a nested PCR, using as first round primers 5'CCCTGGAGTGATTTCAACGC3' (sense) and 5'ACATGCTGACCACAAGTGAC3' (antisense), and as second round primers 5'GAGTGATTTAACGCCTTAC3' (sense) and 5'TGCTGACCACAAGTGACTGT 3' (antisense) (Zong *et al.*, 1999). The PCR conditions had been optimised by previous co-workers using the Opti-prime kit (Stratagene Europe, Amsterdam, The Netherlands), and were carried out in a 50 µl reaction mixture containing:

1. nuclease-free water
2. Buffer 8 (Stratagene Europe, Amsterdam, The Netherlands)
3. 3.2 mM $MgCl_2$
4. 10 nM each of dNTP
5. 1 unit Taq DNA polymerase
6. 20 pmol of each pair of primers
7. 5 µl of extract, reduced to 2 µl of the first round PCR product for the second round PCR.

The extreme sensitivity of PCR can result in problems with DNA contamination, resulting in false positives. In view of this there was a strict adherence to a set of routine precautions, and both positive and negative controls were used for each PCR amplification undertaken (Kwok, S. and Higuchi, R., 1989). The positive control was obtained from a diluted BCBL-1 cell line, kindly provided by Dr. E. Cesarman (Cornell University, New York). PCR detection of HHV8 DNA was repeated at least twice from each sample extract.

Amplification of both regions was carried out under the same PCR thermocycling conditions, using a PTC-100 thermal cycler. Samples were heated for 94°C for 5 min, followed by 35 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 1 min (extension), followed by a 5-min extension period at 72°C.

2.6 DETECTION OF PCR PRODUCTS

PCR products were detected as follows:

1. Seven μl of PCR product was mixed with 2 μl of loading buffer (Tris-disodium ethylenediaminetetracetate (EDTA), pH 8.0, containing 40% sucrose and 0.25% bromophenol blue) and electrophoresed through a 2% composite agarose gel (3:1, NuSieve:Seakem, Flowgen, Lichfield, Staffs) in 1 x Tris-borate-EDTA-buffer (TBE).
2. A 1-kb ladder molecular weight marker (Gibco BRL, Paisley, Glasgow) was run either side of the test samples to assess the size of the amplified product.
3. Gels were then stained in an ethidium bromide solution (concentration of 5 μg / ml).
4. The DNA fragments were visualised using a short wave uv transilluminator and photographed using an instant Polaroid camera.

2.7 PURIFICATION OF PCR PRODUCTS FOR SEQUENCING

The PCR products were purified using Geneclean III (Bio101, La Jolla, California) as follows:

1. Products were loaded onto a 2% composite agarose gel and electrophoresed, as described in Section 2.6. Alternate wells were used to allow adequate area for cutting out each band from the gel without risk of contamination from other products.
2. Short wave transillumination was used to visualize the bands so that they could be carefully cut out with a scalpel, and transferred to separate 1.5 ml Eppendorfs.

3. The Eppendorfs were then weighed and the appropriate quantity of TE added to equalize their weight.
4. A half volume TBE modifier (Bio101, La Jolla, California) and 4.5 volumes of NaI was added to the Eppendorfs and the tubes incubated at 56°C until the agarose had melted (approximately 5 min). The TBE modifier was required to correct the pH of the solution to enable the nucleic acid to bind to the silica matrix.
5. Once the agarose had melted, 5 µl of silica particles was added to each Eppendorf and vortexed.
6. The solution was incubated at room temperature for 5 min, mixing every min to ensure the silica remained suspended.
7. The samples were vortexed again, then centrifuged in a fixed angled Eppendorf microcentrifuge (5 seconds at 12,000g) to pellet the silica matrix.
8. The supernatant was removed with an extended fine pipette, and the pellets washed three times using 500 µl New Wash (Bio101, Vista, CA) with centrifugation (5 s at 12,000 g) and removal of the supernatant between each wash.
9. The tubes were placed in an Eppendorf heating block at 56 °C, with open lids for 5 min to allow the pellets to dry.
10. The pellet was resuspended in 20 µl of Elution Solution and incubated at 56 °C for 5 min prior to centrifugation for 30 sec.
11. The supernatant containing the eluted DNA was carefully removed and placed in a new tube.
12. To confirm that the cleaning had been successful and to roughly

estimate the quantity of DNA recovered, 1 μl of this supernatant was taken and run on a 2% agarose gel alongside a 1μg of I-kb ladder (Invitrogen), stained and visualised.

2.8 AUTOMATED SEQUENCING OF PCR PRODUCTS

All the purified PCR products underwent sequencing of both strands of the DNA product. Cycle sequencing was applied, using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit with AmpliTaq™ FS DNA polymerase (Perkin Elmer, Forest City, CA):

1. A 20-μl sequencing reaction mix was prepared, consisting of 8 μl of the sequencing mix, 3.2 pmol of one of the primers and purified DNA at approximately 100 ng for the HHV-8 PCR product (as determined by the intensity of the band visualised on the agarose gel run after DNA purification).
2. This was placed in a PTC-100 thermal cycler and underwent 25 cycles of 30 s at 95^{O}C, 30 s at 50^{O}C and 4 min at 72^{O}C.
3. After cycling the sequencing reaction was placed in 50 μl of 95% ethanol containing 2 μl of 3 M sodium acetate (pH 4.5), and kept in ice (at -20^{O}C) for a minimum of 10 min.
4. The reaction vessels were then spun in a microcentrifuge for 20 min at maximum speed, following which the supernatant was removed and the pellets washed with 250 μl of 75% ethanol, then respun for a further 5 min.
5. The supernatant was aspirated and the DNA pellets dried (these

pellets are stable for 3 months if kept in the dark at -20^{o}C).

6. The sequencing pellets were then electrophoresed.
7. Following this, the raw sequence data were processed and analysed using the programmes Analysis, SeqEd (applied Biosystems, Foster City, CA), Lasergenne Navigator and Megalign (DNAstar Inc., Madison, WI, USA).

2.9 ANALYSIS OF SEQUENCE DATA

The programme "Analysis" (Applied Biosystems) was used to display the gel image after each sequencing run. Grey tracker lanes indicated the position of each sample and the sample files are generated from these positions. Each sample file could be viewed in one of four ways: file information, which is details about the run; raw data; analysed data; sequence data, (i.e. coded A,G,C,T,N).

The raw data were reanalysed by base calling using the programme 'Analysis,' as the computer often assigns the first and last bases incorrectly which may result in the contig assembly programme being unable to recognise overlapping sequences. The custom tool on the controller was used to locate the beginning of the data, normally located after the dye terminator peaks at the start of the raw data (these are produced from the unincorporated dye terminators). The X-axis number for the first base, found in the lower left hand corner of the Raw Data window, and the number of the last base were recorded. This produced a new sample file but the raw data remained unaltered. Using the tools on the controller it was possible to look at the chromatogram and check to ensure that the correct bases had been called. If

there any ambiguous data were generated, the raw data were checked, and if necessary, the base in question changed to an "N," until data were available from the sequencing of the other strand. The final sequence data were available in two formats: the sample file and ASCII text files.

The SeqEd programme version 1.03 allowed contigs to be assembled from the ABI sequence data. The sequences of interest are imported and any anti-sense sequences made to read by reversing the complement sequence. The sequences could now be aligned by highlighting the sequences of interest and compared for mismatches, with chromatograms displayed and closely inspected to resolve any bases that were indeterminate (labelled as "N"). The unanimity sequence was then exported in the form of a text file and used to determine the amino acid sequence.

The EDITSeq programme of *Lasergene Navigator* enabled sequence data to be converted into a format easily recognisable to the *Lasergene Navigator* suite of programmes. Subsequently, the MegAlign programme of *Lasergene Navigator* allowed the degree of similarity between different sequences to be analysed by comparing nucleotides and/or amino acids. Alignment of multiple sequences was performed either by the Clustal method The alignment was viewed in four different formats: the Alignment Report, Sequence Distances, Residue Substitution and Phylogenetic Tree. Phylograms and sequence alignments were generated using the Clustal X, Bioedit, Phylip, Treeview and Tree explorer programmes.

2.10 SEROLOGICAL DETECTION OF HUMAN HERPESVIRUSES

Serum samples prepared as described in Section 2.1 were coded and underwent serological detection for a variety of human herpesviruses.

2.10.1 HHV-8 SEROLOGY

Two IFAs were applied for the serological detection of antibodies to HHV-8:

2.10.1.1 IFA based on the KS-1 cell line

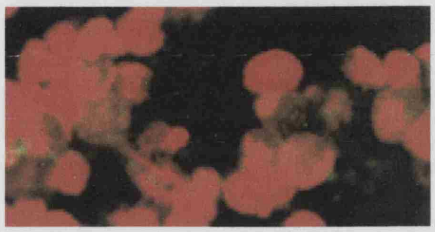
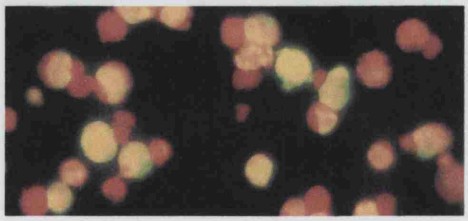
HHV-8 seropositivity was determined by a mouse monoclonal antibody-enhanced IFA (IFA-1) produced by Biotechnologies Incorporated, Maryland, USA for research use. This was based on the KS-1 cell line which is derived from a PEL of an HIV-negative patient; it produces lytic virus and contains a broad array of viral proteins (Said *et al*, 1996a).

The procedure was carried out in two basic reaction steps: in the first, the plasma to be tested was brought into contact with wells lined with fixed cells infected with HHV-8. The second step involved adding fluorescin isothiocyanate-conjugated (FITC)-labelled anti-human antibody:

1. The plasma was first diluted 1:20 in PBS, pH 7.4 and 20 µl applied to each well, with a positive control applied to one well, a negative control to another, and wash buffer in yet another.
2. The slides were then incubated in a moist chamber for 30 min at 37°C.

3. After the incubation step, the slides were removed from the moist chamber and washed for 5 min in wash buffer, while slowly stirring on a magnetic stir plate. Antibody, if present in the test sample, would form a complex with the antigen in the cellular substrate.
4. The surface of the slide was carefully dried with the provided blotter, and 20 μl of FITC anti-human IgG conjugate were dispensed on each well.
5. The slides were then incubated in a moist chamber for 30 min at 37^{O}C before the washing step was repeated and approximately 10 μl of mounting solution per well carefully applied.
6. If the antibody to HHV-8 was present (a positive reaction), bright apple-green fluorescence would be seen with the aid of a fluorescence microscope fitted with a FITC excitation filter KP490, using 200-500X magnification. Uninfected cells, stained red by the counterstain, provided a contrasting background.
7. The fluorescence reaction was graded from -1 to +4 as outlined in Table 2.3. Positive reactivity ranged in fluorescence intensity from brilliant to weak. The internal controls on each microscope slide contained both HHV-8 infected and uninfected cells and provided direct comparison for wells containing sample test sera.
8. The IFA slides were read independently by me and Dr. C.G.Teo. In order to minimise the risk of non-specific reactions with test sera, only those samples rated as 2+ or above were recorded as positive. Inter-reader reproducibility was recorded as 97%, and where there was disagreement, the test serum was re-tested.

Table 2.3 Interpretation of Results of IFA-1

Grade	Interpretation	Percentage of cells fluorescent on the slide
-1	No fluorescent staining	0% e.g. 
0	Background fluorescent staining (no cellular fluorescence)	0%
+1	Weak	0-25%
+2	Moderate	25 – 50%
+3	Bright	50 – 75% e.g. 
+4	Brilliant	75 – 100% e.g. 

2.10.1.2 IFA based on the ORF K8.1 expressing BHK-21 cell line

A second HHV-8 serology test was undertaken using the anti-orf K8.1 IFA (IFA-2), as described by Inoue *et al.*, 2000. This assay has been proposed as more reliable than IFAs based on PEL cell lines, as the K8.1-based IFA provides sensitivity similar to that of lytic PEL-based IFAs but improved specificity. Furthermore, it has been shown to be more sensitive than an ELISA based on the same open reading frame (Corchero *et al.*, 2001). The assay uses recombinant BHK-21 cells infected with Semliki Forest viruses (rSFVs) expressing the HHV-8-specific protein K8.1. Expression of this HHV-8-specific protein at very high levels by the rSFV system allowed easy scoring for IFA and thereby increased specificity. The rSFV system also allowed detection of antibodies against glycosylation-dependent epitopes of K8.1.

The IFA-2 method involved the following steps:

1. Approximately 10^4 cells were applied to each 5-mm-diameter well of Teflon-coated slides (12 wells per slide), dried, and then fixed in cold acetone for 10 min.
2. Fixed cells on the slides were incubated for 1 h at 37°C with twofold dilutions of human sera beginning at 1:20.
3. After three washes with PBS, the slides were incubated for 30 min with FITC goat anti-human IgG.
4. The slides were counterstained with Evans blue, prior to viewing.

2.10.2 CMV SEROLOGY

The CMV Diamedix Microassay serological test (Miami, Florida) is an enzyme immunoassay of the ELISA type that provides semi-quantitative results with a single dilution of test specimen (Doern *et al.,* 1994). The use of a standardised strong positive control permits uniformity of reporting.

1. The calibrator, control and test sample were diluted by adding 200 µl of sample diluent to 5 µl of each solution.
2. Diluted samples were placed in wells coated with CMV antigen (derived from the AD 169 strain) and incubated at room temperature for 20 min. If antibodies to CMV were present in the samples, they will combine with the antigen in the well.
3. The wells were washed three times with wash solution (Diamedix, Miami, Florida) to remove all residual sample, and 100 µl of conjugate, containing antibodies to human IgG labelled with alkaline phosphatase, added. The conjugate binds immunologically to any IgG anti-CMV that has combined with the antigen in the well during the first incubation.
4. The wells were washed three times again, 100 µl of the enzyme substrate (p-nitrophenyl phosphate) added and the wells allowed to stand at room temperature for a further 20 min before a stop solution was added.
5. The substrate was hydrolyzed by any bound alkaline phospatase to form a yellow end product (p-nitro-phenol). The intensity of the colour was read in a photometer (absorbance 405 nm) and was proportional to the concentration of antibodies to CMV present in the test

specimen.

2.10.3 EBV SEROLOGY

Plasma samples were diluted 1 in 10 and tested using a standard indirect IFA protocol for the detection of antibodies to the EBV viral capsid antigen complex using the EBV producer cell line P3HR-1 without prior chemical induction (Lennette, 1988). A 5/95 dilution of FITC was used to was used to label positive samples.

2.10.4 HSV-1 AND HSV-2 SEROLOGY

HSV-1 and 2 are antigenically very closely related. Specialist assays have been developed for the authentic discrimination of type-specific HSV humoral responses (Ashley, 1993). The ELISAs used in the current study were modified from a radioimmunoassay based on the blocking of type-specific epitopes by test sera (Slomka *et al,* 1995).

The methods for the HSV-1 and HSV-2 specific antibody ELISAs were identical except where stated.

1. HSV-1 and HSV-2 infected cell lysates were prepared as described by Slomka *et al.,* 1995, and used to separately coat 96-well microtiter plates (Greiner) overnight at 4 °C with 100 μl antigen diluted 1:25 in PBS and washed with PBS.
2. HSV-2 coated plates were subjected to detergent-treatment where 150 μl PBS containing 1.5% Triton X- 100 and 0.5% Nonidet P40 was added to each well and incubated at room temperature for 30 min followed by PBS washing.

3. Plates were then incubated for 2 hours at 37°C with 150µl of 10% FCS / PBS.
4. After PBS washing, 100 µl of test serum diluted 1:4 in 10% FCS in 0.2% Tween 20 PBS (PBS-T) was added to each well and incubated at 37°C for 1 hr.
5. After washing with 0.05% PBS-T, 100 µl of the appropriate HSV type specific Mab (Slomka *et al.,* 1995) diluted 1: 16,000 in 10% FCS 0.2% PBS-T was added to each well and incubation continued at 37°C for 1 hr.
6. Plates were washed with 0.05% PBS-T and incubated for a further hr at 37°C with 100 µl of goat anti-mouse horseradish peroxidase conjugate (Tago) diluted 1: 1000 in 10% FCS 0.2% PBS-T.
7. Plates were washed with 0.05% PBS-T followed by PBS (x1) and 100 µl of TMB added to each well and incubated at room temperature.
8. The colour reaction was stopped after 15 and 30 min for the HSV-1 and HSV-2 ELISAs respectively, by the addition of 50 µl of 2 M H_2SO_4 and absorbance measured at 450 nm. Results were quantified by calculating the percentage blocking of MAb binding to its homologous antigen based on the mean absorbance of quadruplicate wells containing a strong positive control serum and diluent.

2.11 OPTIMISATION OF METHODS FOR FRACTIONATING HHV-8 CARRYING CELLS IN PERIPHERAL WHOLE BLOOD

2.11.1 STUDY SUBJECTS

Sixty three patients were recruited to the study from the Department of Genitourinary Medicine at Charing Cross Hospital, London. All were HIV-infected homosexual men. In order to minimize variables such as gender, age, and health status, analyses comparing different techniques of cellular isolation from peripheral blood were performed on split samples from the same individuals. Peripheral blood samples were collected in EDTA vacutainers as outlined in Section 2.1. Informed consent and ethical committee approval were obtained prior to the study.

2.11.2 STUDY DESIGN

The study was undertaken in three phases as outlined below:

2.11.2.1 Phase 1

The first stage of the investigation involved 8 men. Three of these individuals were manifesting active KS. The demographic and clinical details of these patients are summarised in Table 2.4. Peripheral blood samples from three of this group were spilt and processed by four different methods to isolate white blood cells, namely:

1. Red blood lysis
2. Density gradient centrifugation to fractionate monocytes and granulocytes
3. Immunomagnetic cell separation to fractionate leukocytes (which are CD45+) and circulating endothelial (CD31+) cells
4. Density gradient centrifugation followed by immunomagnetic cell separation

The samples from the remaining five patients were processed by the first 3 methods only.

2.11.2.2 Phase 2

Peripheral blood samples were collected from five other HIV-infected homosexual individuals with no signs of clinical KS. These were used to compare the rate of detection of HHV-8 DNA by PCR, following immunomagnetic separation of 5 different cell fractions, namely CD45+, CD31+, CD19+, CD14+ and CD2+. The details of these patients are summarised in Table 2.5

2.11.2.3 Phase 3

Finally, peripheral blood samples were obtained from fifty HIV-infected homosexual men, 2 with a history of KS. These were used to directly compare the rate of detection of HHV-8 sub-genomic DNA from WBC isolates obtained using red cell lysis to that obtained from CD45+ immunomagnetic cellular isolates.

Table 2.4 Study Patients whose blood samples were used to compare the effect of different methods of peripheral blood cellular isolation on subsequent PCR for HHV-8 sub-genomic DNA

Patient	Unique Identifier	Age	CD4 count	CD4 %	Antiretroviral Therapy	Presence of KS
1	CCH85	39	420	30	Nil	
2	CCH89	49	24	6	zalcitabine, saquinavir, lamivudine	Yes
3	CCH104	26	96	16	stavudine, lamivudine	Yes
4	CCH87	54	140	10	zidovudine, zalcitabine, saquinavir	
5	CCH88	29	255	17	Nil	
6	CCH90	54	40	4	zidovudine, lamivudine, indinavir	
7	CCH91	25	477	9	Nil	
8	CCH92	41	437	19	zidovudine, zalcitabine, saquinavir	Yes

Note: All patients HIV-infected homosexuals; patients CCH92 and CCH88 are partners

Table 2.5 Study Patients whose blood samples were used to compare the effect of different methods of peripheral blood cellular isolation on subsequent PCR for HHV-8 sub-genomic DNA

Patient	Unique Identifier	Age	CD4 count	CD4 %	Antiretroviral Therapy	Presence of KS
1	CCH85	39	420	30	Nil	
2	CCH89	49	24	6	zalcitabine, saquinavir, lamivudine	Yes
3	CCH104	26	96	16	stavudine, lamivudine	Yes
4	CCH87	54	140	10	zidovudine, zalcitabine, saquinavir	
5	CCH88	29	255	17	Nil	
6	CCH90	54	40	4	zidovudine, lamivudine, indinavir	
7	CCH91	25	477	9	Nil	
8	CCH92	41	437	19	zidovudine, zalcitabine, saquinavir	Yes

Note: All patients HIV-infected homosexuals; patients CCH92 and CCH88 are partners

2.11.3 METHODS

2.11.3.1 Isolation of WBCs from peripheral blood

Red blood lysis was performed as detailed in Section 2.2.1, density gradient centrifugation as in Section 2.2.2, immunomagnetic cell separation as in Section 2.2.3 and density gradient centrifugation followed by immunomagnetic cell separation as in Section 2.3.4. In the current investigation a reduced volume of blood was available for analysis (3ml as compared to 6 ml) and some blood samples were processed up to 7 d after venepuncture.

2.11.3.2 Extraction of DNA from samples.

This is described in Section 2.3.

2.11.3.3 PCR amplification of the human beta-globin gene.

This is described in Section 2.4.

2.11.3.4 PCR amplification of HHV-8 sub-genomic DNA.

This is detailed in Section 2.5.

2.11.3.5 Detection of PCR products.

This is detailed in Section 2.6.

2.11.3.6 Purification of PCR products and automated sequencing.

These are described in Sections 2.7 and 2.8

2.12 GENOPREVALENCE AND SEROPREVALENCE OF HHV-8 IN SUB-POPULATIONS AT LOW-RISK OF KS: UK BLOOD DONORS

2.12.1 STUDY SUBJECTS

Two groups of subjects were recruited to the study: UK blood donors, representing a population considered to be at low risk of developing KS; and, for comparison with the blood donor group, a group of UK HIV-infected individuals, representing a sub-population that is known to be at high risk of KS.

2.12.1.1 Blood donors

The blood donors comprised 192 randomly selected individuals from 2 blood transfusion centres in the UK: 25 samples obtained from Leeds and 167 from North London. Of these, 92 samples were obtained in 1996/7, with a further 100 samples collected in 2001. All individuals selected had already been screened by the blood donor units and had found seronegative for HIV, *Treponema pallidum*, and hepatitis B virus.

The group consisted of 93 males (48%) and 99 females (52%), with an age distribution of 24–62 yr, mean 41.5 yr (Table 2.6), The characteristics of the donors recruited in 1996/7 compared to 2001 were not significantly different (Tables 2.7 and 2.8), as were the characteristics of the Leeds donors compared to the London donors (Table 2.7).

Table 2.6 Characteristics of the Total Blood Donor Group

Group Characteristics			
Total number of patients	**Mean age (yr)**	**Male donors (no.)**	**Female donors (no.)**
192	41.5	93	99

Table 2.7 Characteristics of Blood Donors Recruited in 1996/7

	Group Characteristics			
	Number of patients	**Mean age (yr)**	**Male donors (no.)**	**Female donors (no.)**
London	67	39	31	36
Leeds	25	43	11	14
Total group	92	40.5	42	50

Table 2.8 Characteristics of Blood Donors Recruited in 2001

Group Characteristics			
Total number of patients	**Mean age (yr)**	**Male donors (no.)**	**Female donors (no.)**
100	42	51	49

2.12.1.2 HIV-infected individuals

The second group comprised 103 serologically confirmed HIV-infected individuals, recruited from the Genitourinary Medicine Clinic of Charing Cross Hospital, London over a 14-mth period between 1997 and 1997. The group consisted of 101 males and 2 females, with an age distribution of 23-66 yr, and mean age of 41 yr (Table 2.9). Risk factors identified for their HIV infection were: men having sex with men (100 individuals), intravenous drug use (1 individual) and being of African origin (2 individuals). At the time of inclusion to the study, 19 (18%) of the HIV-infected group were affected by a disease indicative of AIDS, with 5 of these individuals having clinical KS. Fifty individuals (49%) had a CD4 count of <200 cells/ml, 22 (21%) between 200-400 cells/ml and 31 (30%) >400 cells/ml. The percentage of circulating CD4 cells detected was <13% in 40 (39%), and >13% in the remaining 63 individuals (61%). In total, 39 patients (38%) were on anti-retroviral therapy.

2.12.2 METHODS

2.12.2.1 Isolation of WBCs from peripheral blood

Immunomagnetic cell separation was used to isolate CD45+ (pan-leucocyte) cells from peripheral whole blood as described in Section 2.2.3.

2.12.2.2 Extraction of DNA from samples

This is described in Section 2.3

Table 2.9 Characteristics of HIV-infected Group

Group Characteristics			
Total number of patients	**Mean age (yr)**	**Male donors (no.)**	**Female donors (no.)**
103	41	101	2

2.12.2.3 PCR amplification of HHV-8 sub-genomic DNA

This is described in Section 2.5. PCR for amplifying ORF K1 in the 1996/1997 blood donor samples was carried out 4 yr after PCR for ORF 26 DNA. This was because during the time when the samples were collected, the primer sequences and conditions for PCR amplification of ORF K1 DNA had not been optimised.

2.12.2.4 Detection of PCR products

This is described in Section 2.6

2.12.2.5 Purification of PCR products and automated sequencing

This is described in Sections 2.7 and 2.8

2.12.2.6 Serological detection of human herpesviruses HHV-8, CMV, EBV, HSV-1 and HSV-2

This is described in Section 2.10

2.12.3 STATISTICAL EVALUATION

The statistical package SPSS was used to explore the characteristics of the individuals recruited to this study. Association between given variables was calculated using logistic regression with confidence intervals set at 95%.

2.13 GENOPREVALENCE AND DISTRIBUTION OF HHV-8 IN PERIPHERAL BLOOD OF BMT RECIPIENTS AND PATIENTS WITH CFS

2.13.1 STUDY SUBJECTS

Two groups of subjects were recruited to this stage of the investigations: U.K. BMT recipients and a group of individuals diagnosed with CFS.

2.13.1.1 BMT recipients

The BMT recipients comprised 30 randomly selected BMT recipients from the Hammersmith Hospital in the UK, sampled between 3 and 18 mth after transplantation. The group consisted of 19 males (63%) and 11 females (37%), with an age distribution of 11-39 yr, and mean age of 19.5 yr. A second peripheral blood sample was obtained from 4 of these patients, between 3-5 mth after the first sample was tested.

2.13.1.2 CFS patients

Blood samples were obtained from 39 patients attending Coppetts Wood Hospital in the U.K., who fulfilled the Oxford criteria for the diagnosis of CFS. All were heterosexual and of European descent. The group consisted of 10 males (26%) and 29 females (74%), with an age distribution of 24 - 62 yr, and mean age of 40.7 yr. The mean age of onset of fatigue was 34.1 yr, and the mean duration, 7.3 yr. A second peripheral blood sample

was obtained from 7 of these patients, between 1 and 4 mth after the initial sample was tested.

2.13.2 METHODS

2.13.2.1 Isolation of WBCs from peripheral blood

Immunomagnetic cell separation was used to isolate CD45+ (pan-leucocyte) cells from peripheral whole blood as described in Section 2.2.3. Immunomagnetic separation of CD31+, CD19+, CD14+ and CD2+ cell fractions was applied to samples from 5 patients in the BMT group and 18 patients in the CFS group.

2.13.2.2 Extraction of DNA from samples

This is described in Section 2.3

2.12.2.3 ORF26 PCR amplification of HHV-8 sub-genomic DNA

This is described in Section 2.5.

2.13.2.4 Detection of PCR products

This is described in Section 2.6

2.13.2.5 Purification of PCR products and automated sequencing

This is described in Sections 2.7 and 2.8.

2.13.2.6 Serological detection of human herpesviruses HHV-8, CMV, EBV, HSV-1 and HSV-2

This is described in Section 2.10, and was undertaken for 12 patients in the BMT group and all 39 patients in the CFS group.

CHAPTER 3

RESULTS

3.1 OPTIMISATION OF METHODS FOR FRACTIONATING HHV-8 CARRYING CELLS IN PERIPHERAL WHOLE BLOOD

The presence of DNA in all the extracts from whole peripheral whole blood was confirmed by amplification of a 110-bp DNA fragment from the β-globin gene prior to HHV-8 sub-genomic DNA amplification.

3.1.1 PHASE 1

For each of the 3 patients (Patients 1-3) whose blood samples underwent cellular isolation by all 4 methods detailed in Section 2.2, nine different cellular substrates were available for subsequent HHV-8 DNA PCR, with the results summarised in Table 3.1. RBC lysis did not permit any successful PCR. Following density gradient centrifugation, the monocyte cell fractionated subset fractionated from one of the 3 patients (Patient 3) was nested PCR-positive but not after first-round PCR. Immunomagnetic cell separation of both CD45+ and CD31+ cellular isolates consistently provided a 100% detection rate following PCR with samples from all 3 patients positive after first round PCR. For the samples subjected to density gradient centrifugation followed by immunomagnetic separation, better detection rates were obtained than for those subjected to density gradient centrifugation only. For Patient 1, immunomagnetic separation allowed HHV-8 DNA in CD45+ and CD31+ cells of the monocyte but not the granulocyte fraction to be amplified by nested PCR (Figure 3.1). For Patients 2 and 3 (who had active KS at the time of sampling), HHV-8 DNA could be amplified by nested PCR from CD45+ and CD31+ cells of both monocyte and granulocyte fractions to be amplified, and variably so by non nested PCR (figure 3.2).

In the remaining 5 patients whose blood samples were processed by the 3 rather than 4 methods, immunomagnetic separation resulted in successful PCRs: HHV-8 DNA could be detected by nested PCR in CD45+ cells of all the samples and in CD31+ cells in 4 samples, and for one sample (from Patient 6), HHV-8 DNA was amplifiable by first-round PCR in both CD45+ and CD31+ cells.

3.1.2 PHASE 2

Nested PCR following immunomagnetic separation of specific leukocyte subsets showed that the detection rates for HHV-8 sub-genomic DNA by PCR were as follows (in descending order): CD45+ cells (4/5 samples positive); CD31+ cells (1/5); and CD19+, CD14+ and CD2+ cells (0/5) (Table 3.2; Figures 3.3 and 3.4).

3.1.3 PHASE 3

Of the 50 other patients tested for HHV-8 sub-genomic DNA by nested PCR, RBC lysis permitted successful amplification in 4 (8%). This rate compared with 19 (38%) patients who were positive when PCR was undertaken on DNA isolated from cells obtained by CD45+ immunomagnetic cell separation.

3.1.4 SEQUENCING

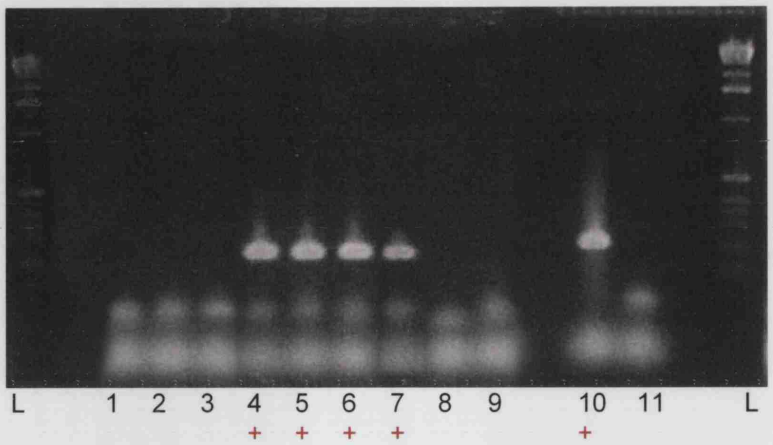
All 31 HHV-8 PCR positive products amplified from the CD45+ cell fractions from the 3 phases of this study were sequenced and confirmed to belong to the ORF 26 of the virus (Figure 3.5). Of these sequences, 21 were identical, and 10 different from the majority sequence by 1 bp to 4 bps.

Table 3.1 PCR Detection of HHV 8 from peripheral blood, following different methods of WBC isolation

	RBC Lysis		Density Gradient Centrifugation				Immunomagnetic Cell Separation				Density Gradient Centrifugation Followed by Immunomagnetic Cell Separation							
			Monocytes (M)		Granulocytes (G)		CD45+		CD31+		M / CD45+		M / CD31+		G / CD45+		G / CD31+	
Patient	FR	N	FR	N	FR	N	FR	N	FR	N	FR	N	FR	N	FR	N	FR	N
1 (CCH85)	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	-	-	-
2* (CCH89)	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+
3* (CCH104)	-	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	+	+
4 (CCH87)	-	-	-	-	-	-	-	+	+	+	ne	ne	ne	ne	ne	ne	ne	ne
5 (CCH88)	-	-	-	-	-	-	-	+	+	+	ne	ne	ne	ne	ne	ne	ne	ne
6 (CCH90)	-	-	-	-	-	-	+	+	+	+	ne	ne	ne	ne	ne	ne	ne	ne
7 (CCH91)	-	-	-	-	-	-	-	+	-	-	ne	ne	ne	ne	ne	ne	ne	ne
8* (CCH92)	-	-	-	-	-	-	-	+	-	+	ne	ne	ne	ne	ne	ne	ne	ne

Note: ne: not evaluated; FR: first round PCR; N: nested PCR; * individuals with clinical KS

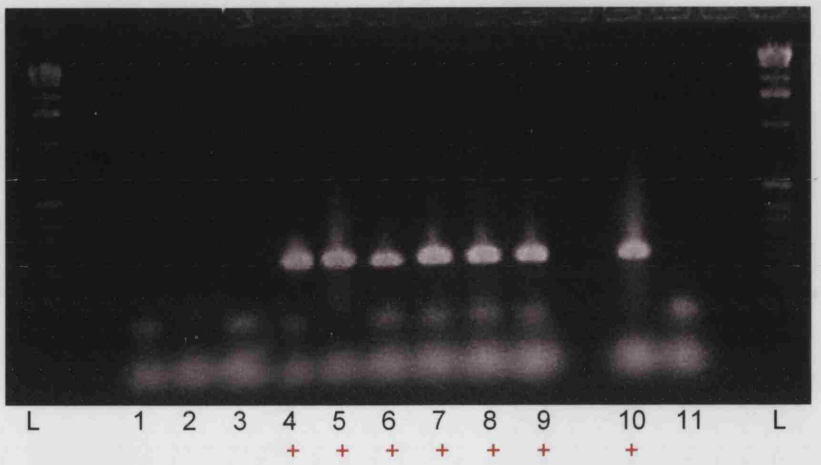
Figure 3.1 **Gel Image of HHV-8 ORF 26 Nested PCR, following isolation of cells by different methods from the peripheral blood of Patient 1 (CCH85)**



Gel lanes:

L: ladder
1: RBC lysis
2: density gradient centrifugation – monocytes
3: density gradient centrifugation – granulocytes
4: immunomagnetic bead separation – CD45
5: immunomagnetic bead separation – CD31
6: density gradient centrifugation – monocytes / immunomagnetic bead separation – CD45
7: density gradient centrifugation – monocytes / immunomagnetic bead separation – CD31
8: density gradient centrifugation – granulocytes / immunomagnetic bead separation – CD31
9. density gradient centrifugation – granulocytes / immunomagnetic bead separation – CD31
10: positive control
11: negative control
L: ladder

Figure 3.2 **Gel Image of HHV-8 ORF 26 Nested PCR, following isolation of cells by different methods from the peripheral blood of Patient 2 (CCH89)**



Gel lanes:

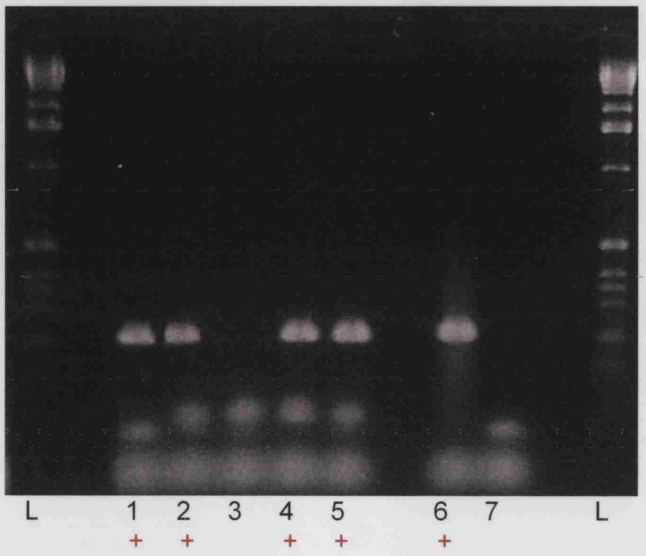
L: ladder
1: RBC lysis
2: density gradient centrifugation – monocytes
3: density gradient centrifugation – granulocytes
4: immunomagnetic bead separation – CD45
5: immunomagnetic bead separation – CD31
6: density gradient centrifugation – monocytes / immunomagnetic bead separation – CD45
7: density gradient centrifugation – monocytes / immunomagnetic bead separation – CD31
8: density gradient centrifugation – granulocytes / immunomagnetic bead separation – CD31
9. density gradient centrifugation – granulocytes / immunomagnetic bead separation – CD31
10: positive control
11: negative control
L: ladder

Table 3.2 PCR detection of HHV-8 following immunomagnetic cell separation of different cellular subsets

Patient	Immunomagnetic Cell Separation				
	CD45	CD31	CD19	CD14	CD2
1 (CCH93)	+	+	-	-	-
2 (CCH94)	+	-	-	-	-
3 (CCH95)	-	-	-	-	-
4 (CCH96)	+	-	-	-	-
5 (CCH97)	+	-	-	-	-

Note: All patients HIV-infected homosexuals, with no clinical evidence of active KS

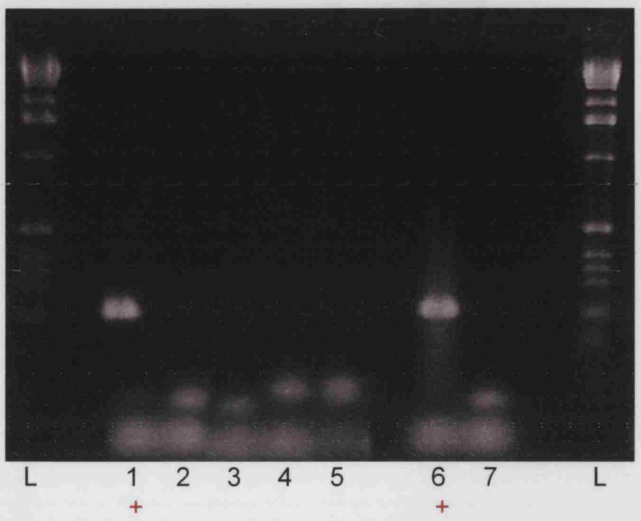
Figure 3.3 **Gel Image of HHV-8 ORF 26 Nested PCR, following immunomagnetic cell separation of CD45 from the peripheral blood of HIV-infected individuals**



Gel lanes:

L: ladder
1: Patient 1 – CCH93
2: Patient 2 – CCH94
3: Patient 3 – CCH95
4: Patient 4 – CCH96
5: Patient 5 – CCH97
6: positive control
7: negative control
L: ladder

Figure 3.4 **Gel Image of HHV-8 ORF 26 Nested PCR, following immunomagnetic cell separation of CD31 from the peripheral blood of HIV-infected individuals**

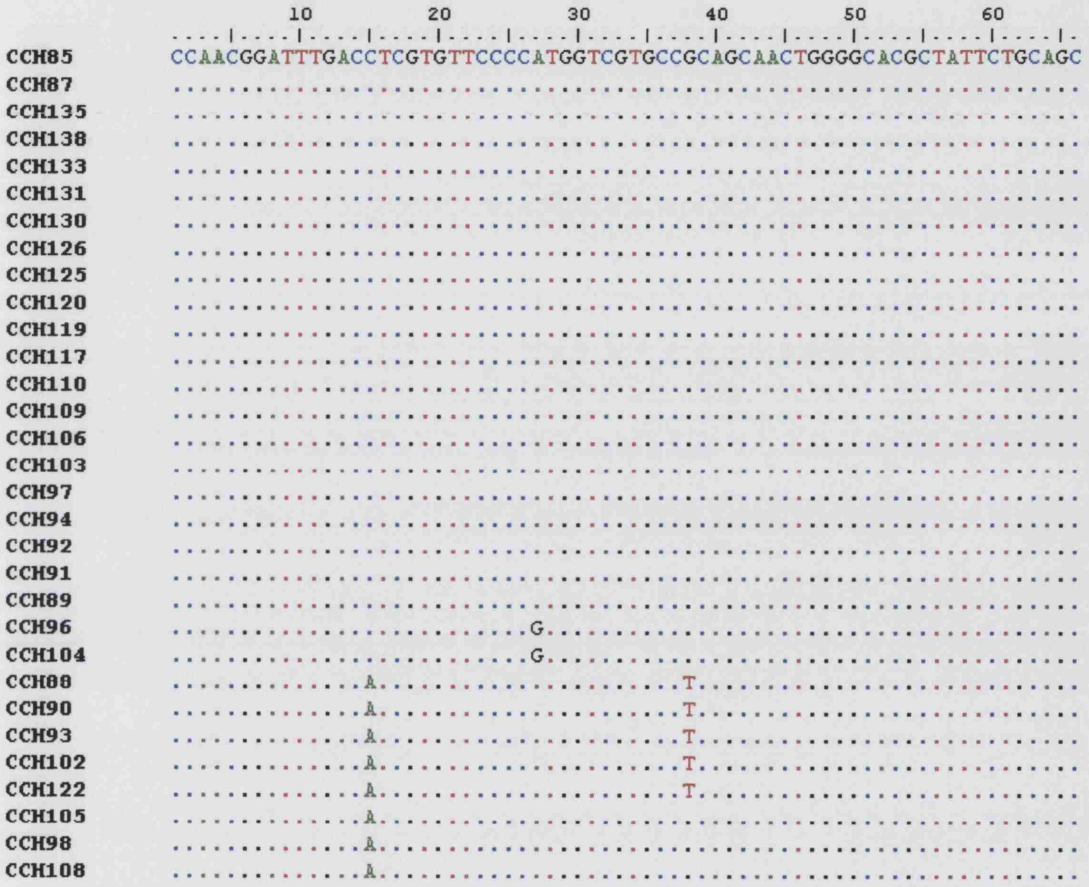


Gel lanes:

L: ladder
1: Patient 1 – CCH93
2: Patient 2 – CCH94
3: Patient 3 – CCH95
4: Patient 4 – CCH96
5: Patient 5 – CCH97
6: positive control
7: negative control
L: ladder

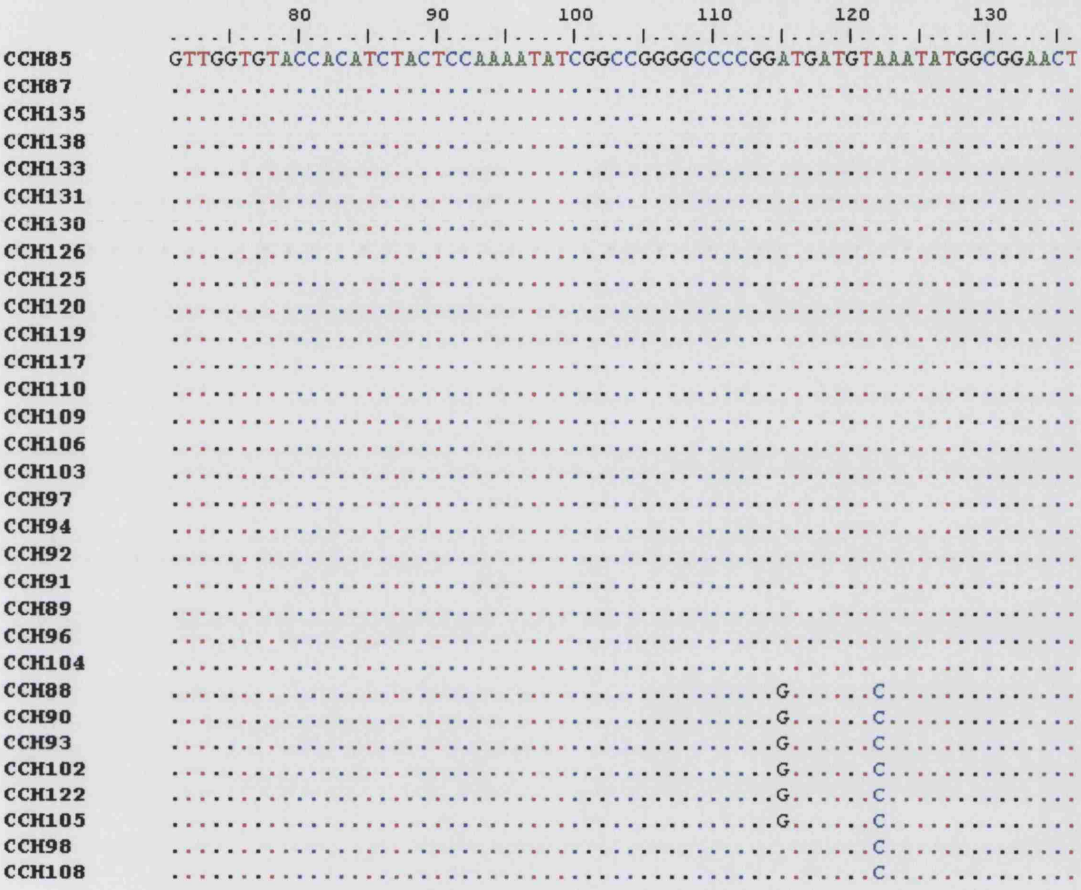
Figure 3.5 Diversity of HHV-8 ORF 26 DNA sequences derived from HIV-infected individuals in optimisation study

a. Sequence Alignment



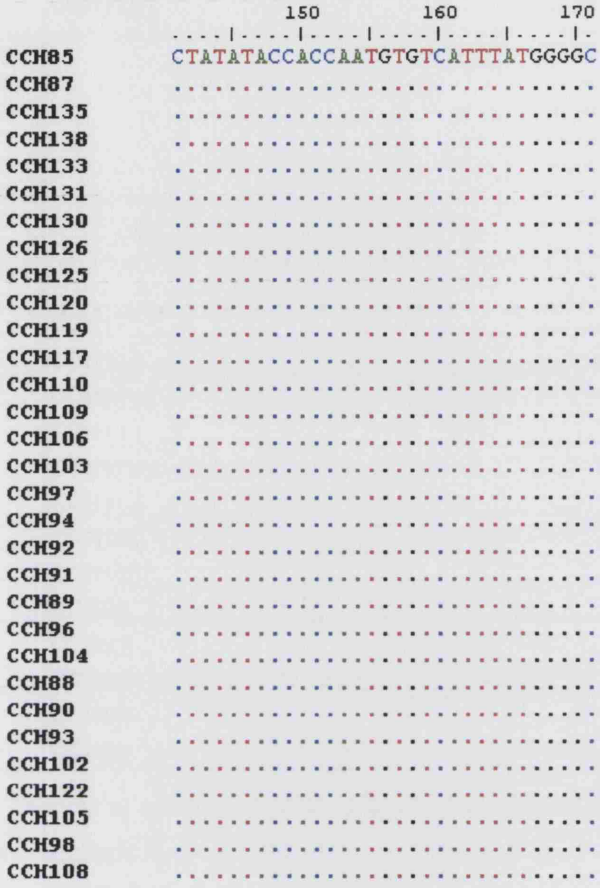
Note: generated using the Clustal X and Bioedit sequence analysis programmes

a. Sequence Alignment contd.



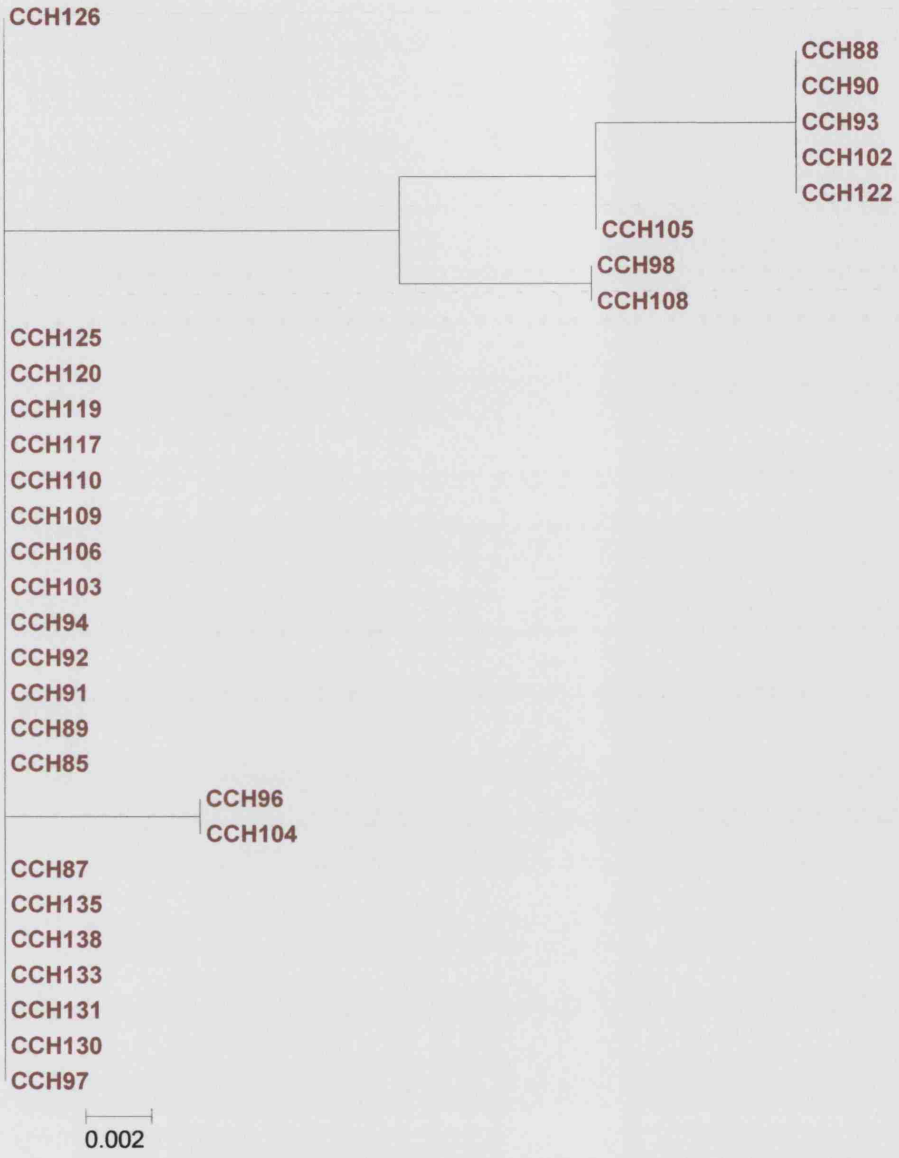
Note: generated using the Clustal X and Bioedit sequence analysis programmes

a. Sequence Alignment contd.



Note: generated using the Clustal X and Bioedit sequence analysis programmes

b. Phylogram



Note: The tree was generated using the Clustal X, Bioedit, Phylip, Treeview and Tree explorer programmes

3.2 GENOPREVALENCE AND SEROPREVALENCE OF HHV-8 IN SUB-POPULATIONS AT LOW-RISK OF KS: UK BLOOD DONORS

3.2.1 BLOOD DONORS

3.2.1.1 Total blood donor samples

Samples from the total blood donor group were tested for ORF26 DNA, ORF K1 DNA and anti HHV-8 as detected by IFA-1. Analysis revealed that 9 blood donors (9/192; 5%) were positive for all 3 markers (Table 3.3). The overall ORF 26 DNA positivity rate for all 192 samples was 16% (30/192) and for ORF K1 DNA was 7% (14/192) (Table 3.4). One sample was ORF K1 DNA-positive only. Hence in total 16% (31/192) of samples were HHV-8 genome-positive.

The 30 ORF 26 DNA-positives were confirmed by sequencing, with 21 sharing identical sequences, and 9 different from the majority sequence by 1 bp to 4 bps (Figure 3.6). The sequences which varied by a single bp were verified by repeat sequencing. The sequences of the 14 ORF K1 DNA-positives were also confirmed, of which 8 belonged to the A1 subtype, 4 to A4 and 2 to C3 (Figure 3.7). Only three ORF K1 DNA positives had the same sequence (C63, C116 and C122), with the others diverging from this by up to 26 bps. Seropositivity for anti-HHV-8 was 34% (66/192) by IFA-1. Detection of HHV-8 DNA was accompanied by serological detection of HHV-8 antibodies in 17 of the 31 PCR-positive samples (55%). The association between ORF26 DNA-positivity and IFA-1-seropositivity was significant ($p=0.020$; 95% confidence interval (CI) 1.161-5.646) (Tables 3.5 a, b). The association between ORF K1 DNA positivity and IFA-1-positivity was also statistically associated ($p = 0.006$, 95% CI 1.637-18.117 (Tables 3.6 a, b).

Table 3.3 Summary of findings obtained from blood donor samples relating to ORF26 and K1 DNA-positivity, and anti HHV-8 positivity as detected by IFA-1

Period of recruitment	3 HHV-8 markers		Total
	negative	positive	
1996/7	87 (94.6%)	5 (5.4%)	92 (100%)
2001	96 (96%)	4 (4%)	100 (100%)
Total	183	9	192
	(95.3%)	(4.7%)	100%

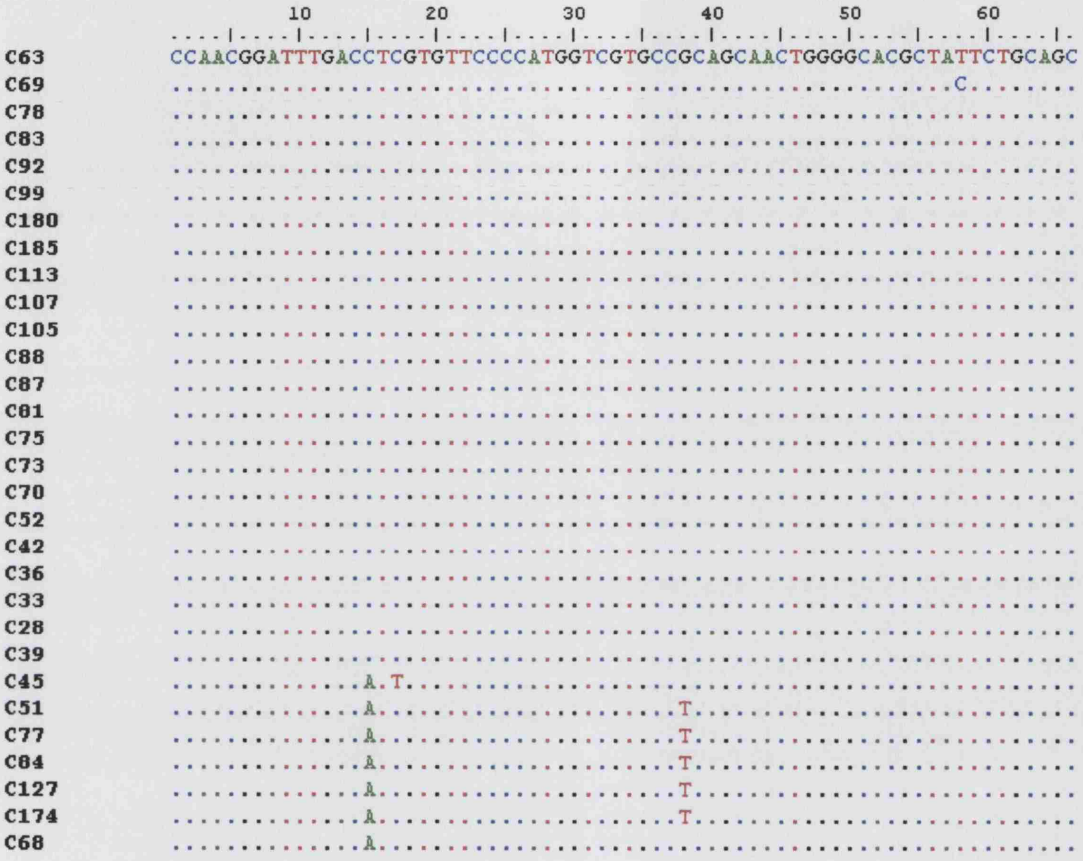
Table 3.4 Summary of Results for Total Blood Donor Group

Group Characteristics				No positive serological results (%)						No positive PCR results (%)	
Total no patients	Mean age (yr)	No male donors	No female donors	Anti HSV-1	Anti HSV-2	Anti EBV	Anti CMV	Anti HHV-8 (IFA-1)	Anti HHV-8 (IFA-2)	HHV8 ORF 26 DNA	HHV8 ORF K1 DNA
192	41.5	93	99	39% (74)	1% (2)	ne	ne	34% (66)	ne	16% (30)	7% (14)

Note: ne: not evaluated

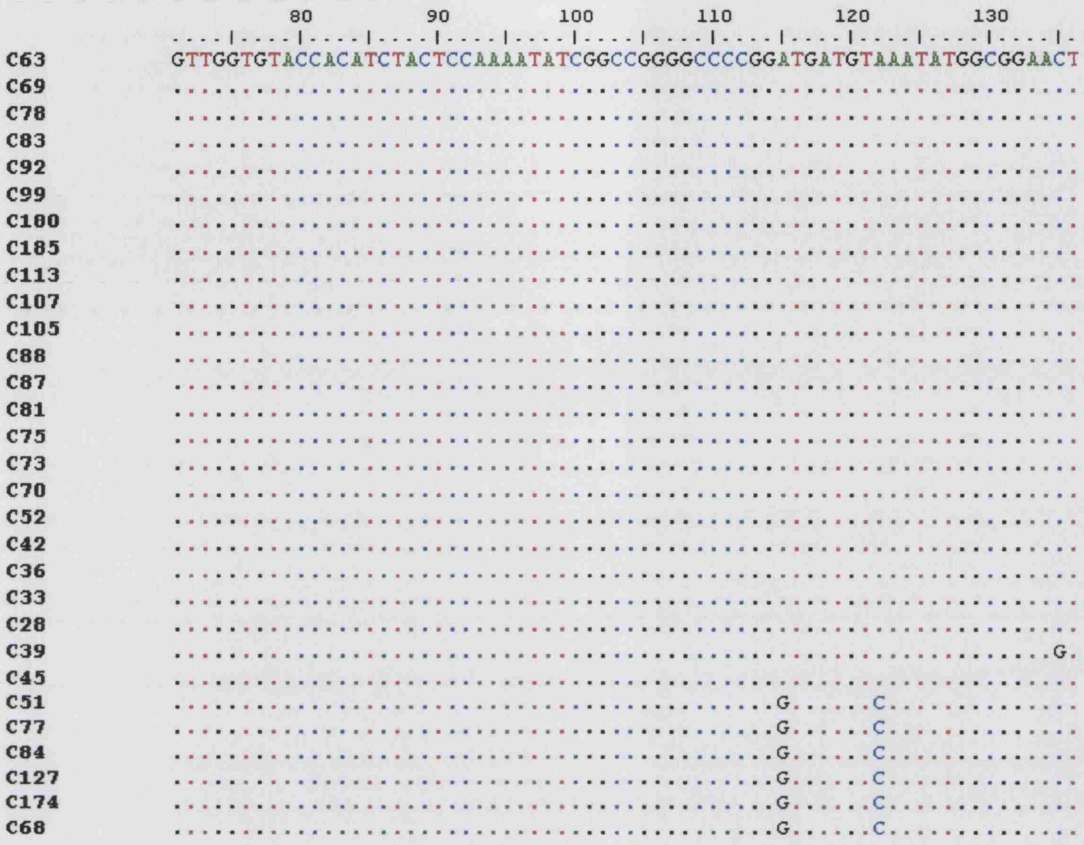
Figure 3.6 Diversity of HHV-8 ORF 26 DNA sequences derived from blood donors

a. Sequence Alignment



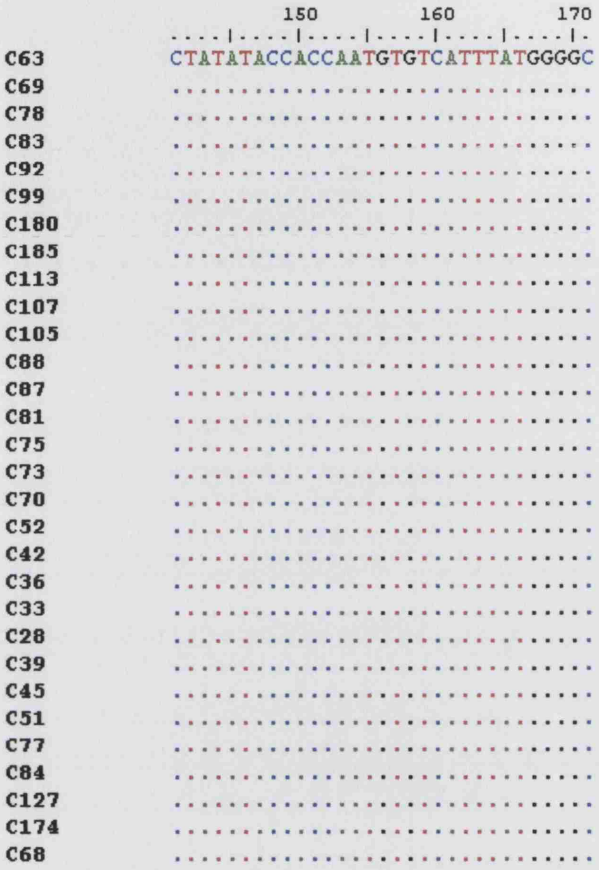
Note: generated using the Clustal X and Bioedit sequence analysis programmes

a. Sequence Alignment contd.



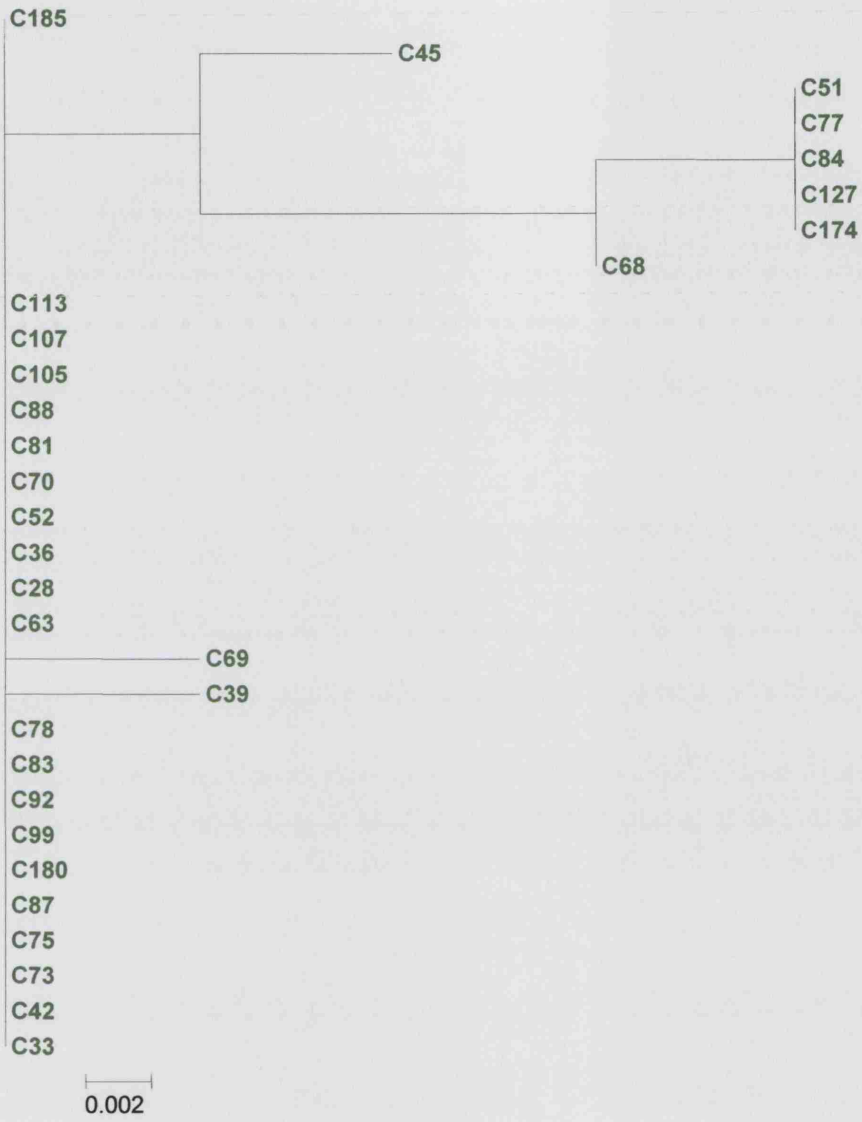
Note: generated using the Clustal X and Bioedit sequence analysis programmes

a. Sequence Alignment contd.



Note: generated using the Clustal X and Bioedit sequence analysis programmes

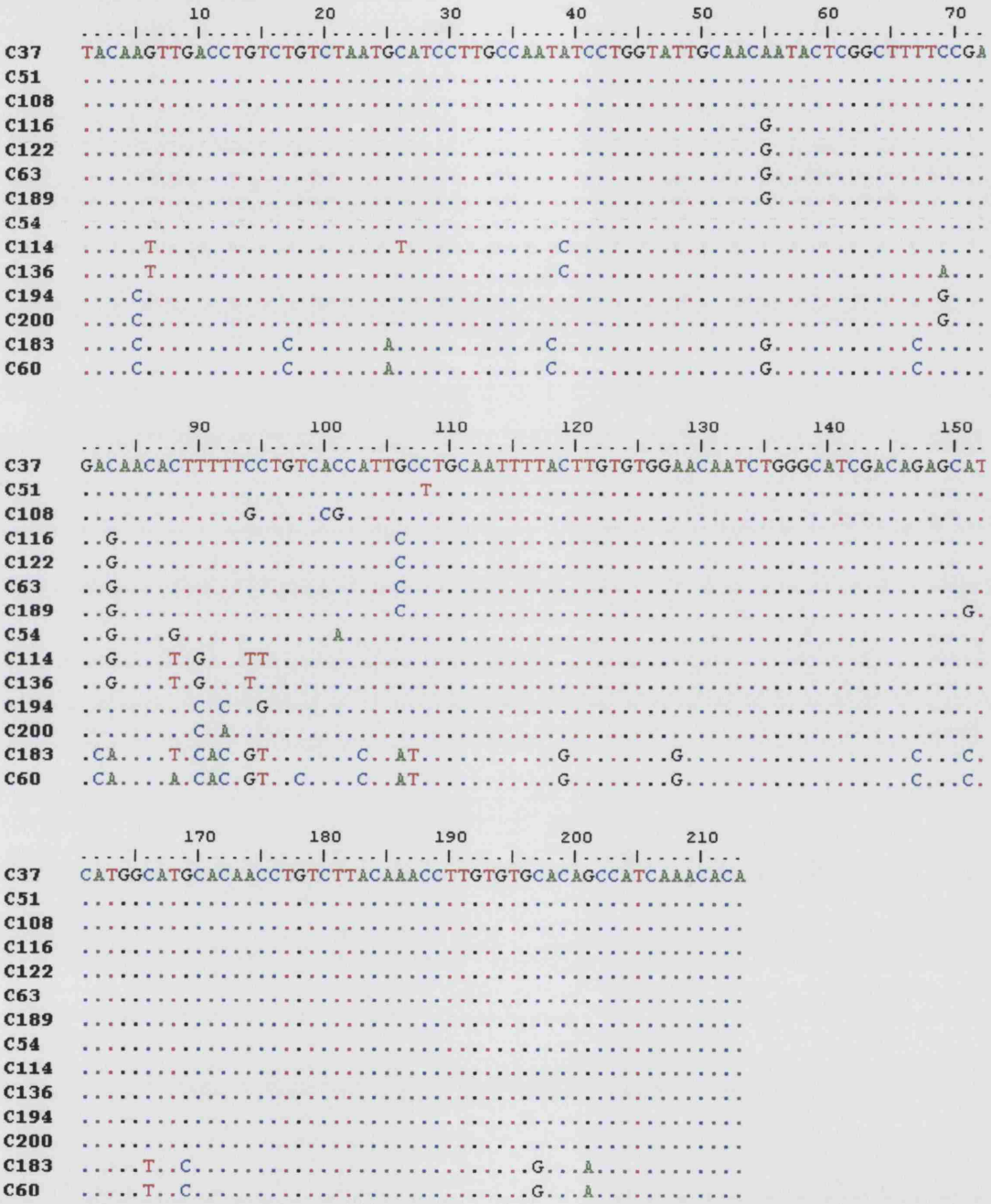
b. Phylogram



Note: The tree was generated using the Clustal X, Bioedit, Phylip, Treeview and Tree explorer programmes

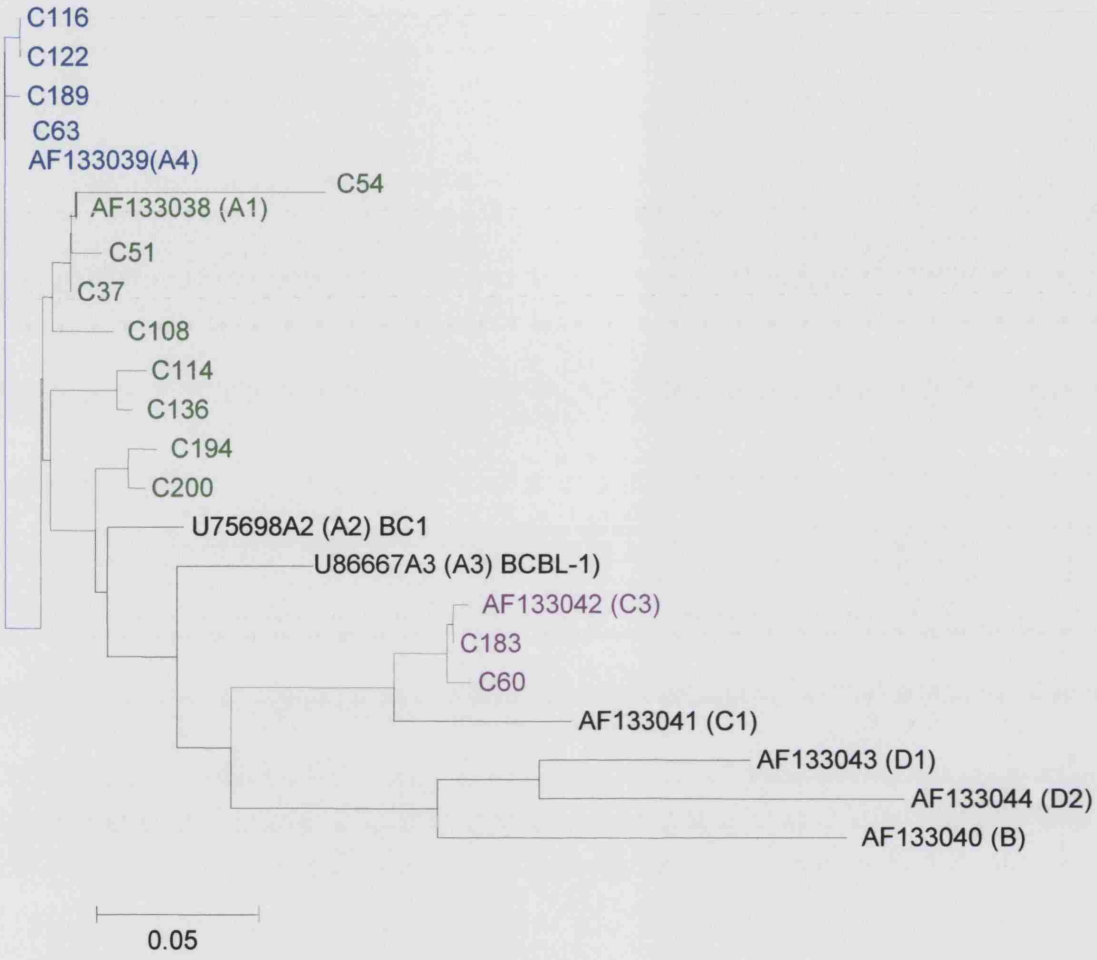
Figure 3.7 Diversity of HHV-8 ORF K1 DNA sequences derived from blood donors

a. Sequence Alignment



Note: generated using the Clustal X and Bioedit sequence analysis programmes

b. Phylogram



Note: The tree was generated using the Clustal X, Bioedit, Phylip, Treeview and Tree explorer programmes; previously published sequences added from Genbank for comparison

Tables 3.5 a, b. HHV-8 ORF 26 DNA and anti HHV-8 (IFA-1) in total blood donor group

a. HHV-8 DNA vs anti HHV-8

HHV-8 ORF 26 DNA	**HHV-8 serology**		Total
	negative	**positive**	
negative	112 (69.1%)	50 (30.9%)	162 (100)
positive	14 (46.7%)	16 (53.3%)	30 (100)
Total	126 (65.6%)	66 (34.4%)	192 (100%)

b. Logistic regression

		Sig.	Exp(B)	95.0% C.I.for EXP(B)	
				Lower	Upper
Step 1(a)	HHV-8 IFA-1	.020	2.560	1.161	5.646
	Constant	.000	.125		

a Variable(s) entered on step 1: ORF26 / HHV-8 IFA-1

Tables 3.6 a, b. HHV-8 ORF K1 DNA and anti HHV-8 (IFA-1) in total blood donor group

a. HHV-8 DNA vs anti HHV-8

HHV-8 ORF K1 DNA	HHV-8 serology		Total
	negative	positive	
negative	122	56	178
	(68.5%)	(31.5%)	(100)
positive	4	10	14
	(28.6%)	(71.4%)	(100%)
Total	126	66	192
	(65.6%)	(34.4%)	(100%)

b. Logistic regression

		Sig.	Exp(B)	95.0% C.I.for EXP(B)	
				Lower	Upper
Step 1(a)	HHV-8 IFA-1	.006	5.446	1.637	18.117
	Constant	.000	.033		

a Variable(s) entered on step 1: K1 / HHV-8 IFA-1

Tables 3.7 a, b. Anti HHV-8 (IFA-1) and anti HSV-1 in total blood donor group

a. Anti HHV-8 vs anti HSV-1

Anti HHV-8	HSV-1 ELISA		Total
	negative	positive	
Negative	101 (80.2%)	25 (19.8%)	126 (100%)
positive	17 (25.8%)	49 (74.2%)	66 (100%)
Total	118 (61.5%)	74 (38.5%)	192 (100%)

b. Logistic Regression

		Sig.	Exp(B)	95.0% C.I.for EXP(B)	
				Lower	Upper
Step 1(a)	HHV-8 IFA-1	.000	11.644	5.758	23.551
	Constant	.000	.248		

a Variable(s) entered on step 1: HHV-8 IFA-1 / HSV-1 ELISA

Seropositivity rates for anti HSV-1 and HSV-2 were 39% (74/192) and 1% (2/192), respectively. Seventy-four % (49/66) of the samples positive for HHV-8 antibody were also positive for anti HSV-1. The association between anti HHV-8 as tested by IFA-1 and anti HSV-1 positivity was significant ($p<0.001$; 95% CI 5.758-23.551) (Tables 3.7 a, b).

3.2.1.2 Blood donor samples assembled in 1996 and 1997.

Samples from the blood donors recruited in 1996-7 were tested for the presence of ORF 26 DNA, ORF K1 DNA and anti HHV-8 as detected by IFA-1. Analysis revealed that 5 blood donors (5/92; 5%) were positive for all 3 markers. The ORF 26 DNA-positivity rate was 24% (22/92) (Table 3.8), i.e., higher than for the total blood donor group (16%). Five samples (5.4%; 5/92) were ORF K1 DNA-positive. All of these were also positive for ORF26 DNA. The group showed an anti HHV-8 seropositivity rate (as detected by IFA-1) of 46% (42/92), an anti HSV-1 seropositivity rate of 50% (46/92), and an anti HSV-2 seropositivity rate of 2% (2/92). Furthermore, 71% (30/42) of samples positive for anti HHV-8 were also positive for anti HSV-1, confirming the association between the 2 markers ($p< 0.001$; 95% CI 2.17-13.003). The seroprevalence of EBV (87%, 80/92) as well as CMV (42%, 39/92) were not significantly associated with HHV-8 seropositivity. There was no significant difference between the Leeds and London donors in terms of markers for HHV-8 infection and for anti-HSV-1, anti-HSV-2, anti-EBV, and anti-CMV (Table 4.2).

Table 3.8 Summary of Results for Blood Donors Recruited in 1996/7

	Group Characteristics				No positive serological results (%)						No positive PCR results (%)	
	Total patient no	Mean age (yr)	No male donors	No female donors	Anti HSV-1	Anti HSV-2	Anti EBV	Anti CMV	Anti HHV-8 (IFA-1)	Anti HHV-8 (IFA-2)	HHV8 ORF 26 DNA	HHV8 ORF K1 DNA
London	67	39	31	36	54% (36)	0% (0)	84% (56)	42% (28)	43% (29)	ne	25% (17)	1% (1)
Leeds	25	43	11	14	40% (10)	8% (2)	96% (24)	44% (11)	52% (13)	ne	20% (5)	16% (4)
Total group	92	40.5	42	50	50% (46)	2% (2)	87% (80)	42% (39)	46% (42)	ne	24% (22)	5% (5)

Note: ne: not evaluated

3.2.1.3 Blood donors assembled in 2001

The blood donors sampled in 2001 were tested for ORF26 DNA, ORF K1 DNA, anti HHV-8 as detected by IFA-1, and anti HHV-8 by IFA-2. No samples were positive for all 4 markers. Four blood donors (4/100; 5%) were positive for 3 markers, namely ORF26 DNA, ORF K1 DNA and anti HHV-8 as detected by IFA-1. The HHV-8 ORF 26 DNA positivity rate was 8% (8/100) (Table 3.9), i.e., significantly lower than for the rate (24%) in donors sampled in 1996/7 (p=0.04, 95% CI 0.116-0.658). The ORF K1 DNA positivity rate was 9% (9/100), with one sample being ORF K1 DNA-positive but not ORF 26 DNA-positive. Although this was higher than the rate for blood donors sampled in 1996/7 (5%), the difference was not statistically significant. Within the 2001 group, the anti HHV-8 seropositivity rate for IFA-1 was 24% (24/100) compared to 12% (12/100) for IFA-2; the difference was significant (p=0.001; 95% CI 2.412-33.584). Although the association between ORF26 DNA-positivity and IFA-1-seropositivity was weak (p=0.08), the association between ORF K1 DNA positivity and IFA-1-positivity was statistically associated (p = 0.03, 95% CI 1.158 – 19.375). There was no association between HHV-8 ORF26 or K1 DNA positivity and IFA-2 seropositivity. The IFA-1 seropositivity rate of 24% was significantly lower than that of the blood donors sampled in 1996/7 (46%) (p=0.02; 95% CI 0.203-0.696).

The anti HSV-1 seropositivity rate was 28% (28/100). Seventy-nine % (19/24) of samples positive for anti-HHV-8 IFA-1 were also positive for anti HSV-1; this association was significant (p<0.001; CI 8.469-94.496). Furthermore, 58% (7/12) samples positive for anti-HHV-8 IFA-2 were also

Table 3.9 Summary of Results for Blood Donors Recruited in 2001

Group Characteristics				No positive serological results (%)						No positive PCR results (%)	
Total no patients	Mean age (yr)	No male donors	No female donors	Anti HSV-1	Anti HSV-2	Anti EBV	Anti CMV	Anti HHV-8 (IFA-1)	HHV-8 IFA-2	HHV8 ORF 26 DNA	HHV8 ORF K1 DNA
100	42	51	49	28% (28)	0% (0)	ne	ne	24% (24)	12% (12)	8% (8)	9% (9)

Note: ne: not evaluated

positive for anti HSV-1; this association was also significant. No anti HSV-2 was detected.

3.2.2 HIV-INFECTED INDIVIDUALS

The overall positivity rate for HHV-8 ORF 26 DNA in all 103 samples was 37% (38/103) (Table 3.10), notably higher than in the blood donors ($p<0.001$, 95% CI 1.806-5.519). The 38 ORF 26 DNA-positives were confirmed by sequencing, with 24 sharing identical sequences, and 14 different from the majority sequence by 1 bp to 4 bps (Figure 3.8). There was no significant difference in the rate of detection among the risk groups (MSM, IVU and African), although the sizes of the last 2 groups were too small to allow definitive conclusions to be made.

Seropositivity for HHV-8 was 85% (87/103) as detected by IFA-1, again significantly higher than the blood donor group ($p<0.001$; 95% CI 5.636-19.118). Concordant ORF 26 DNA and anti HHV-8 results were obtained in 33 (87%) of 38 ORF 26 DNA-positive samples. Both HHV-8 DNA detection and seropositivity were not statistically associated with CD4 count, CD4 %, or the presence of AIDS indicator disease. Of the five patients with clinical KS, all were positive for anti-HHV-8 as determined by IFA-1. Three of these individuals were also positive for HHV-8 ORF26 DNA.

Seropositivity rates for the other herpesviruses examined, namely, anti HSV-1, HSV-2, CMV and EBV, were also significantly higher for the HIV-infected individuals than the blood donor group ($p<0.001$). Anti HSV-1 was present in 86% (89/103) and anti HSV-2 in 66% (67/101) of HIV-infected individuals. Eighty-seven % (75/87) of the samples that were positive for anti HHV-8 were also positive for anti HSV-1, but the association was not

Table 3.10 Summary of results for HIV-infected study group

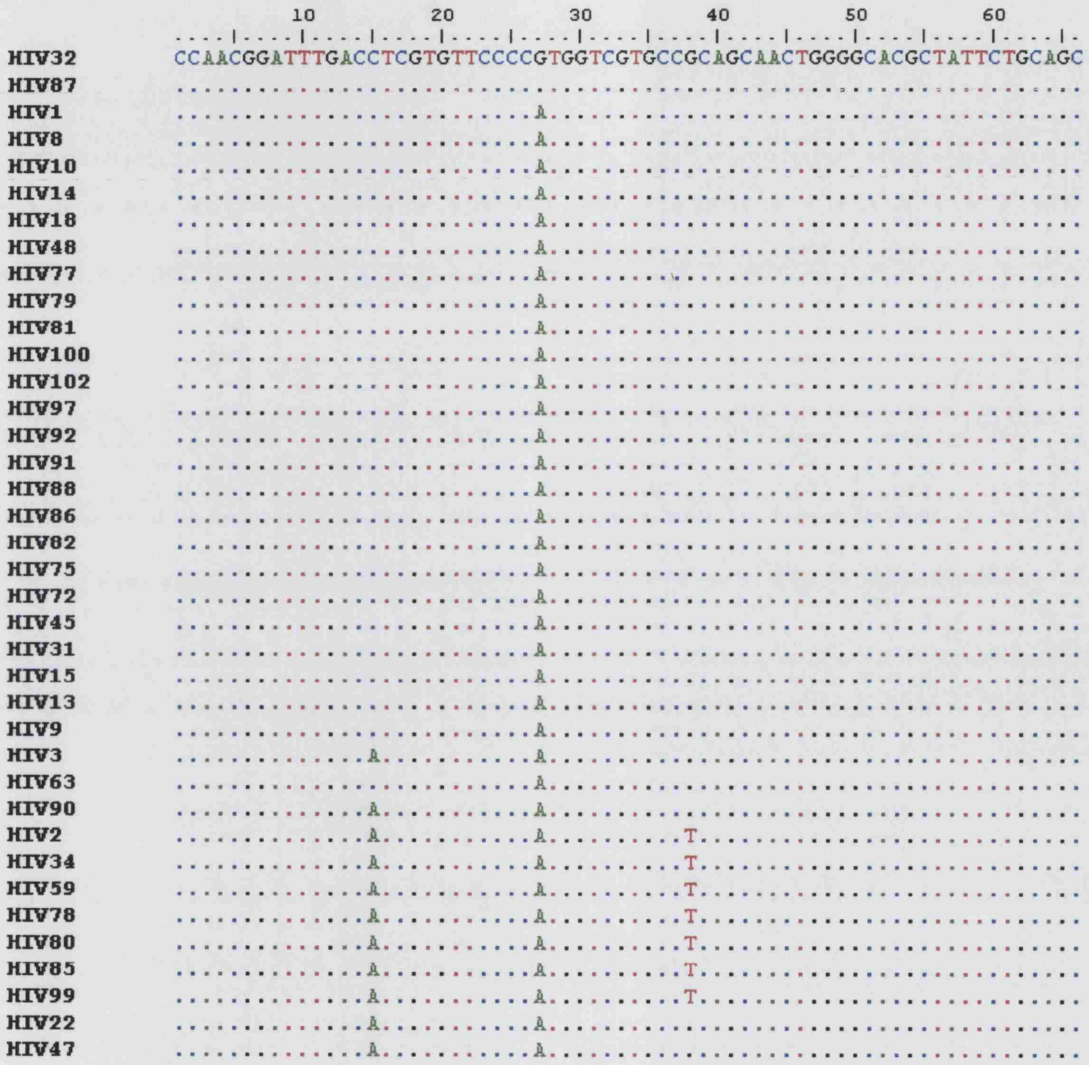
Group Characteristics				No positive serological results (%)						No positive PCR results (%)	
Total no patients	Mean age (yr)	No male patients	No female patients	Anti HSV-1	Anti HSV-2	Anti EBV	Anti CMV	Anti HHV-8 (IFA-1)	Anti HHV-8 (IFA-2)	HHV8 ORF 26 DNA	HHV8 K1 DNA
103	41	101	2	86% (89)	66%* (67)	100% (103)	99% (102)	85% (87)	ne	37% (38)	ne

Note: ne: not evaluated

* 2 HSV-2 results were equivocal and excluded from analysis; hence 63% is calculated from a sample group of 101.

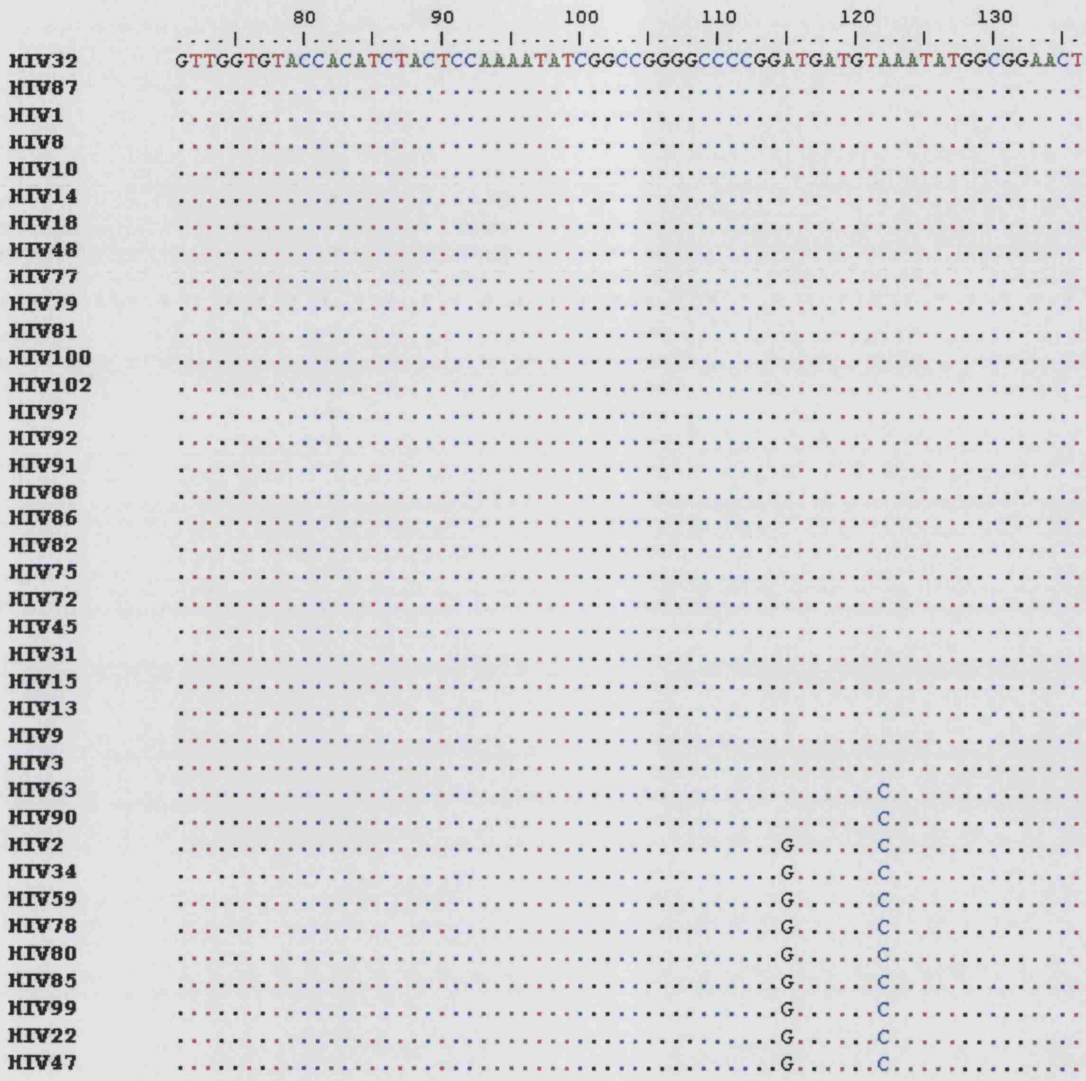
Figure 3.8 Diversity of HHV-8 ORF26 DNA sequences derived from HIV-infected individuals in genoprevalence study

a. Sequence Alignment



Note: generated using the Clustal X and Bioedit sequence analysis programmes

a. Sequence Alignment contd.



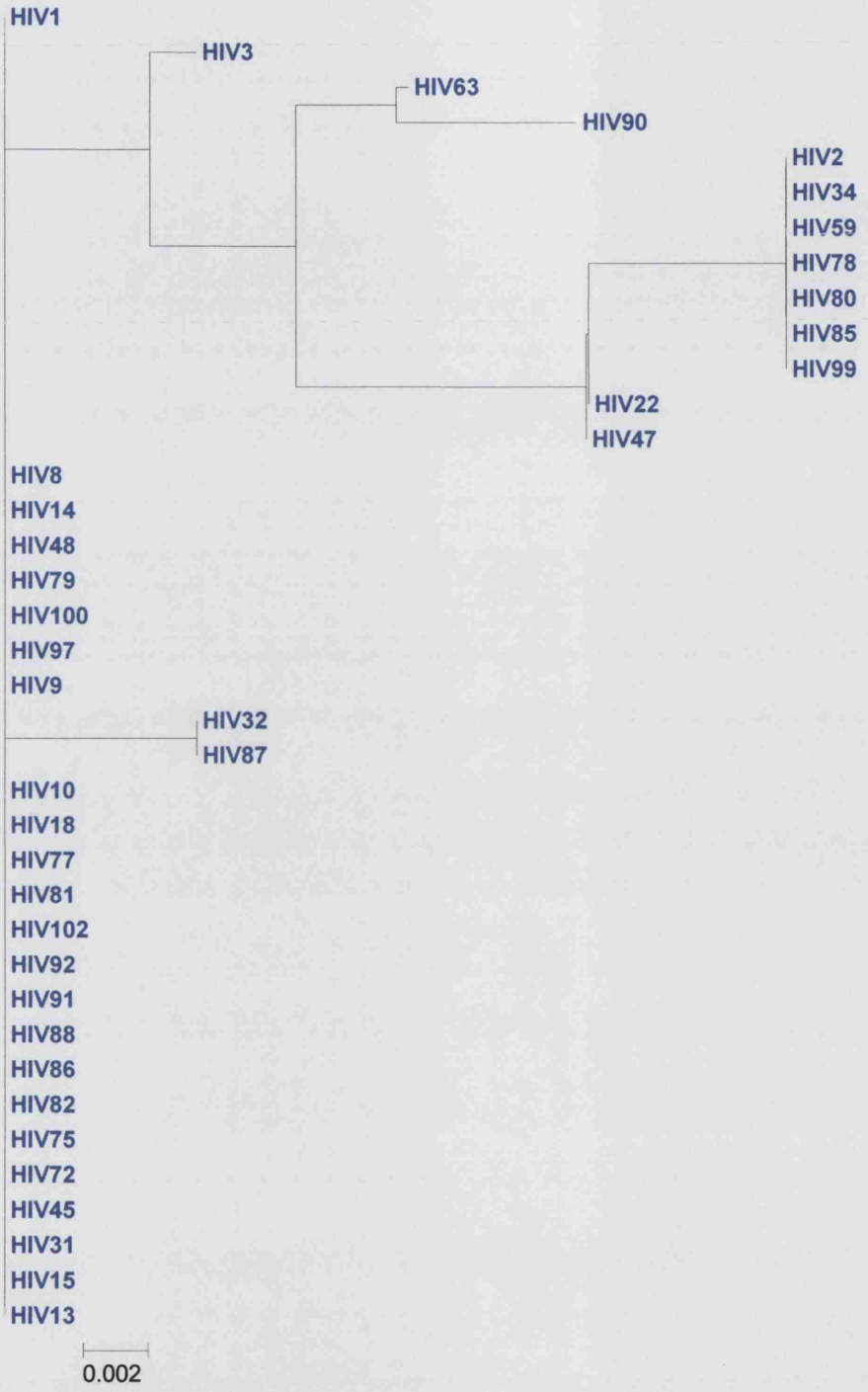
Note: generated using the Clustal X and Bioedit sequence analysis programmes

a. Sequence Alignment contd.

```
                  150         160         170
                  ....|....|....|....|....|....|.
HIV32             CTATATACCACCAATGTGTCATTTATGGGGC
HIV87             ...............................
HIV1              ...............................
HIV8              ...............................
HIV10             ...............................
HIV14             ...............................
HIV18             ...............................
HIV48             ...............................
HIV77             ...............................
HIV79             ...............................
HIV81             ...............................
HIV100            ...............................
HIV102            ...............................
HIV97             ...............................
HIV92             ...............................
HIV91             ...............................
HIV88             ...............................
HIV86             ...............................
HIV82             ...............................
HIV75             ...............................
HIV72             ...............................
HIV45             ...............................
HIV31             ...............................
HIV15             ...............................
HIV13             ...............................
HIV9              ...............................
HIV3              ...............................
HIV63             ...............................
HIV90             ...............................
HIV2              ...............................
HIV34             ...............................
HIV59             ...............................
HIV78             ...............................
HIV80             ...............................
HIV85             ...............................
HIV99             ...............................
```

Note: generated using the Clustal X and Bioedit sequence analysis programmes

b. Phylogram



Note: The tree was generated using the Clustal X, Bioedit, Phylip, Treeview and Tree explorer programmes

significant. The anti EBV detection rate was 100% and that of anti CMV 99% (102/103), with either not significantly associated with anti HHV-8 positivity.

3.3 GENOPREVALENCE AND DISTRIBUTION OF HHV-8 IN PERIPHERAL BLOOD OF BMT RECIPIENTS AND PATIENTS WITH CFS

3.3.1 BMT RECIPIENTS

3.3.1.1 HHV-8 ORF 26 DNA prevalence

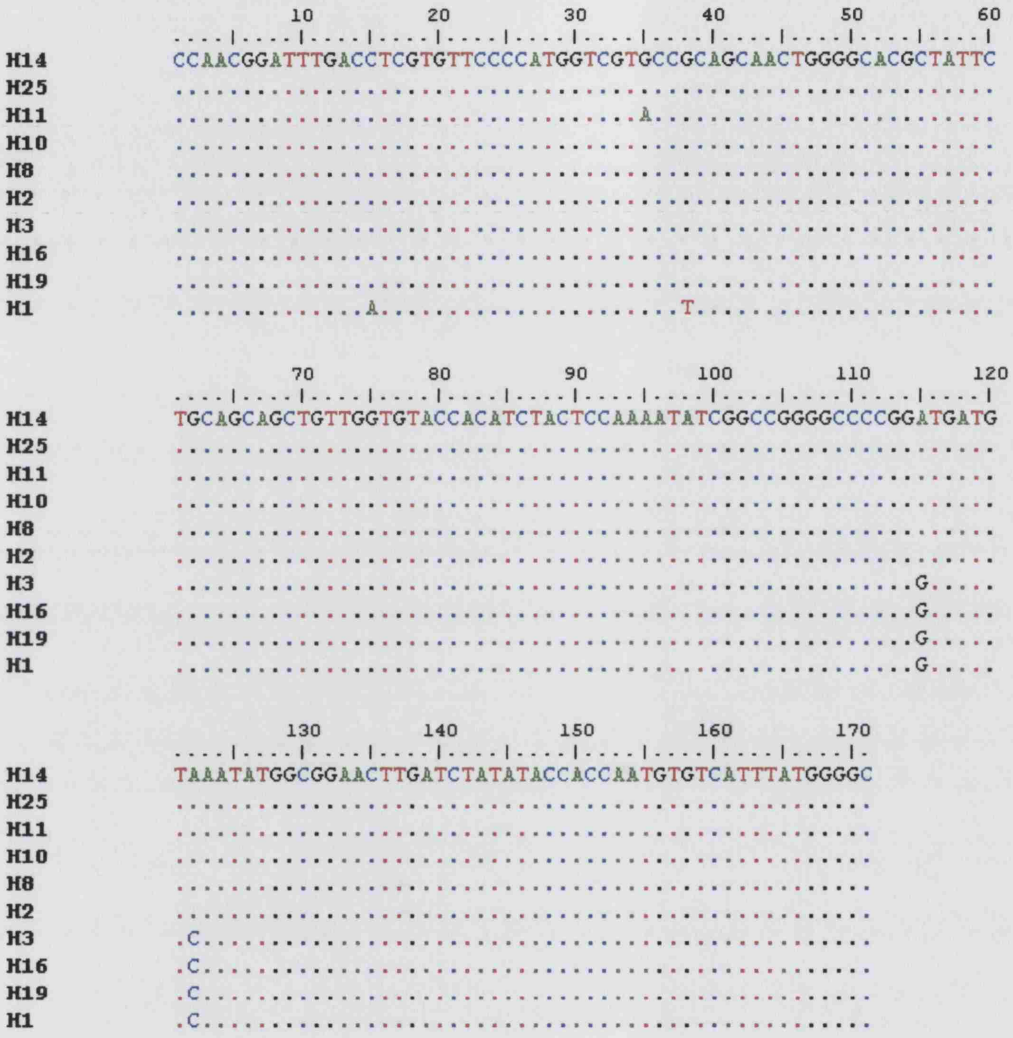
The total BMT group (n=30) was tested for HHV-8 ORF26 DNA, following isolation of CD45+ cells from their peripheral blood samples. Ten patients (10/30; 33%) were positive. These 10 ORF 26 DNA-positives were confirmed by sequencing, with 5 sharing identical sequences, and 5 different from the majority sequence by 1 bp to 4 bps (Figure 3.9).

3.3.1.2 Herpesvirus serology

Herpesvirus serology was undertaken for 12 of the BMT recipients (Table 3.11). Four patients (4/12; 33%) were positive for anti HHV-8 as detected by IFA-1. Of these, 3 were also positive for HHV-8 ORF26 DNA. The overall detection of HHV-8 ORF26 DNA was higher (6/12; 50%) than that of anti-HHV-8 (4/12; 33%). Seropositivity rates for anti HSV-1 and HSV-2 were 67% (8/12) and 17% (2/12), respectively. Two patients (2/4; 50%) positive for anti HHV-8 antibody were also positive for anti HSV-1. No patients were both anti HHV-8 and HSV-2 positive. The seropositivity rate for anti EBV was 92% (11/12), and that for anti CMV 42% (5/12).

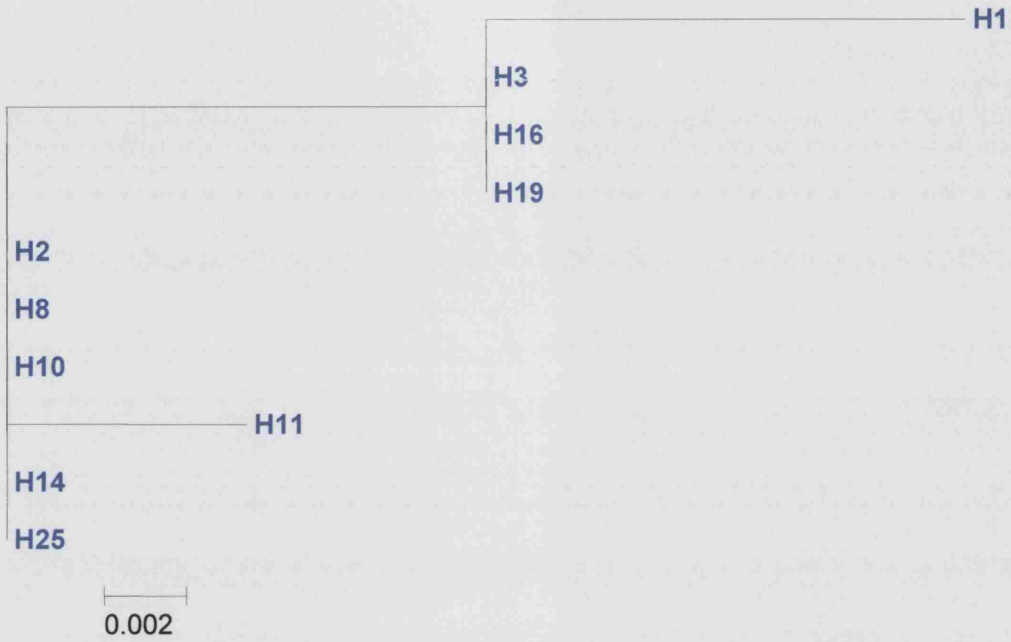
Figure 3.9 Diversity of HHV-8 ORF26 DNA sequences derived from BMT recipients

a. Sequence Alignment



Note: generated using the Clustal X and Bioedit sequence analysis programmes

b. Phylogram



Note: The tree was generated using the Clustal X, Bioedit, Phylip, Treeview and Tree explorer programmes

Table 3.11 ORF 26 HHV-8 DNA-positivity, anti HHV-8 positivity as detected by IFA-1, and anti HSV-1, HSV-2, CMV and EBV in BMT patients (n=12)

Patient I.D.	Anti HSV-1	Anti HSV-2	Anti EBV	Anti CMV	Anti HHV-8 (IFA-1)	HHV8 ORF 26 DNA
H1	-	-	+	+	+	+
H2	+	-	+	-	-	+
H3	+	-	+	-	+	+
H4	-	-	+	-	-	-
H5	+	+	+	-	-	-
H6	+	-	+	-	-	-
H7	+	+	+	+	-	-
H8	+	-	+	-	-	+
H9	+	-	+	+	-	-
H10	-	-	+	+	-	+
H11	+	-	-	-	+	+
H12	-	-	+	+	+	-
Total no. positive (%)	8 (67%)	2 (17%)	11 (92%)	5 (42%)	4 (33%)	6 (50%)

Note: + = positive result; - = negative result.

3.3.1.3 Persistent ORF 26 DNA detection

Repeat testing for HHV-8 ORF26 DNA was undertaken for 4 patients (Table 3.12). Two of these individuals (H2 and H3) were positive for both tests: Patient H2 was negative for anti HHV-8 as detected by IFA-1 for both tests, whereas Patient H3 was positive for the first test only. The other two patients (H4 and H5) were ORF26 DNA-positive and seropositive for anti-HHV-8 only on the second test.

3.3.1.4 HHV8 ORF 26 DNA distribution in leukocyte subpopulations

Nested ORF26 PCR following immunomagnetic separation of specific leukocyte subsets showed that the detection rates for HHV-8 sub-genomic DNA by PCR were as follows (in descending order): CD45+ cells (3/5 samples positive); CD19+ cells (2/5); and CD31+, CD14+ and CD2+ cells (0/5) (Table 3.13).

3.3.2 CFS PATIENTS

3.3.2.1 HHV-8 ORF 26 DNA prevalence

Samples from the total CFS patient group were tested for HHV-8 ORF26 DNA, following isolation of CD45+ cells from their peripheral blood samples. Analysis revealed that 9 patients (9/39; 23%) were positive (Table 3.14). The 9 ORF 26 DNA-positives were confirmed by sequencing, with 7 sharing identical sequences, and 2 different from the majority sequence by 4 bps (Figure 3.10).

Table 3.12 Repeat testing in BMT patients for ORF 26 HHV-8 DNA-positivity, anti HHV-8 positivity as detected by IFA-1, and anti HSV-1, HSV-2, CMV and EBV (n=4)

Patient I.D.	Repeat Tests	Anti HSV-1	Anti HSV-2	Anti EBV	Anti CMV	Anti HHV-8 (IFA-1)	HHV8 ORF 26 DNA
H2	Test 1	+	-	+	-	-	+
	Test 2	+	-	+	-	-	+
H3	Test 1	+	-	+	-	+	+
	Test 2	+	-	+	-	-	+
H4	Test 1	-	-	+	-	-	-
	Test 2	-	-	+	-	+	+
H5	Test 1	+	+	+	-	-	-
	Test 2	+	+	+	-	+	+

Note: + = positive result; - = negative result.

Table 3.13 Distribution of HHV-8 ORF 26 DNA in peripheral blood sub-populations of BMT patients

Patient	Peripheral blood sub-population				
	CD45	CD31	CD19	CD14	CD2
H8	+	-	+	-	-
H9	-	-	-	-	-
H10	+	-	+	-	-
H11	+	-	-	-	-
H12	-	-	-	-	-

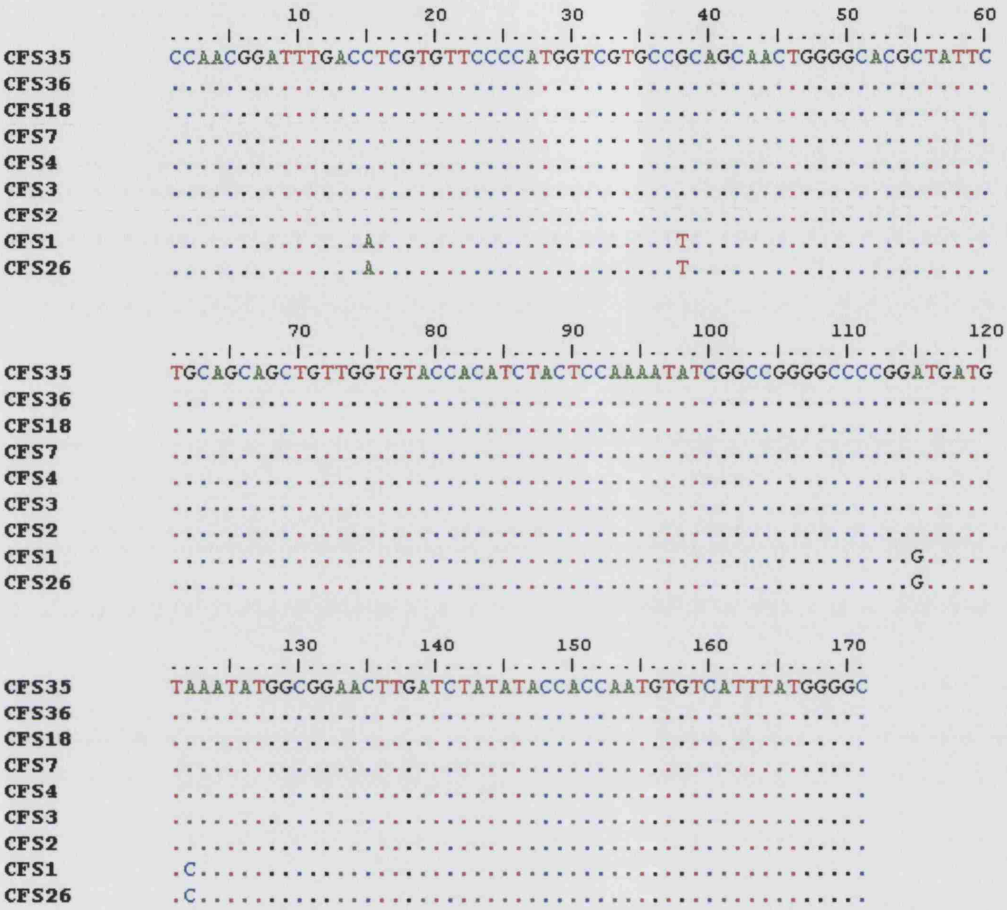
Note: + = positive result; - = negative result.

Table 3.14 ORF 26 HHV-8 DNA-positivity, anti HHV-8 positivity as detected by IFA-1, and anti-HSV-1, HSV-2, CMV and EBV in CFS patients (n=39)

	Anti HSV-1	Anti HSV-2	Anti EBV	Anti CMV	Anti HHV-8 IFA-1	HHV8 ORF 26 DNA
Total no. positive (%)	24 (62%)	2 (5%)	38 (97%)	17 (44%)	3 (8%)	9 (23%)

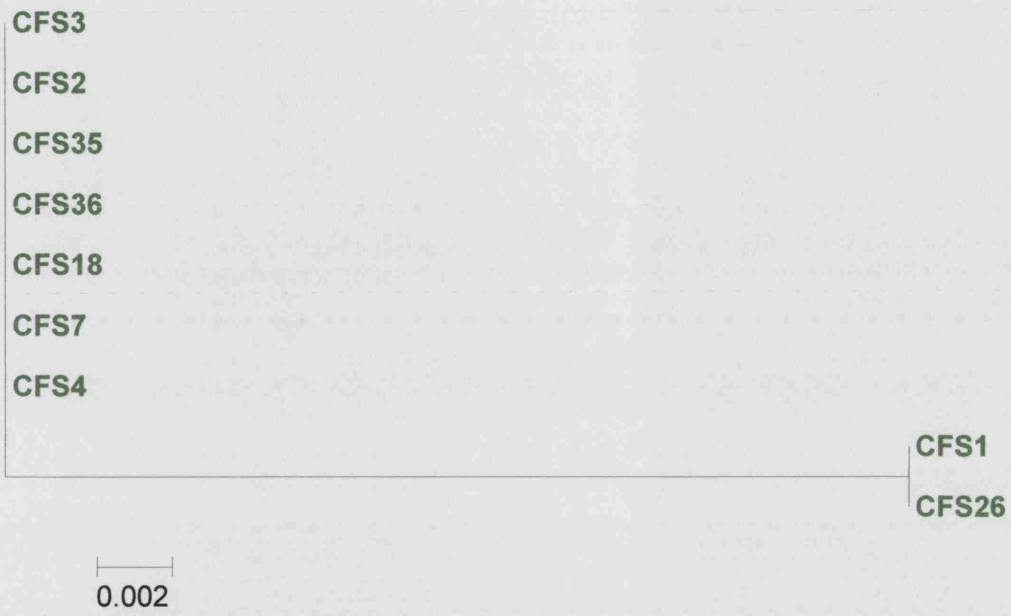
Figure 3.10 Diversity of HHV-8 ORF26 DNA sequences derived from CFS patients

a. Sequence Alignment



Note: generated using the Clustal X and Bioedit sequence analysis programmes

b. Phylogram



Note: The tree was generated using the Clustal X, Bioedit, Phylip, Treeview and Tree explorer programmes

3.3.2.2 Herpesvirus serology

Only three patients (3/39; 8%) were positive for anti HHV-8 as detected by IFA-1, of whom one was also ORF26 DNA-positive. Seropositivity rates for HSV-1 and HSV-2 were 62% (24/39) and 5% (2/39), respectively. Two patients (2/3; 75%) positive for anti HHV-8 antibody were also positive for anti HSV-1. No patients were both anti-HHV-8 and HSV-2 positive. The seropositivity rate for anti EBV was 97% (38/39) and for anti CMV 44% (17/39).

3.3.2.3 Persistent ORF 26 DNA detection

Repeat testing for HHV-8 ORF26 DNA was undertaken for 7 patients (Table 3.15). Three of these individuals (CFS-2, CFS-3 and CFS-26) were persistently positive. However, Patients CFS-3 and CFS-26 were negative for anti HHV-8 as detected by IFA-1 for all tests, whereas Patient CFS-2 was positive on their second test only. Two patients (CFS-5, CFS-28) were persistently negative for both ORF26 DNA and anti-HHV-8. Patient CFS-1 was persistently positive for anti-HHV-8 but only ORF26 DNA positive on the first test. The final patient, CFS-4, was positive for HHV-8 DNA and negative for anti-HHV-8 on the first test, and conversely, HHV-8 DNA negative and anti-HHV-8 positive on the second test.

Table 3.15 Repeat testing in CFS patients for ORF 26 HHV-8 DNA-positivity, anti HHV-8 positivity as detected by IFA-1, and anti-HSV-1, HSV-2, CMV and EBV

Patient I.D.	Repeat Tests	HSV-1 ELISA	HSV-2 ELISA	EBV IFA	CMV ELISA	HHV-8 IFA-1	HHV8 ORF 26 PCR
CFS-1	Test 1	-	-	+	-	+	+
	Test 2	-	-	+	-	+	-
CFS-2	Test 1	-	-	+	-	-	+
	Test 2	-	-	-	-	+	+
	Test 3	-	-	-	-	-	+
CFS-3	Test 1	+	-	+	-	-	+
	Test 2	+	-	+	-	-	+
	Test 3	+	-	+	-	-	+
CFS-4	Test 1	-	-	+	-	-	+
	Test 2	-	-	+	+	+	-
CFS-5	Test 1	+	-	+	-	-	-
	Test 2	-	-	+	-	-	-
CFS-26	Test 1	-	-	+	-	-	+
	Test 2	-	-	+	-	-	+
CFS-28	Test 1	+	-	+	+	-	-
	Test 2	+	-	+	+	-	-

Note: + = positive result; - = negative result.

3.3.2.4 HHV8 ORF 26 DNA distribution in peripheral blood subpopulations

Nested PCR for ORF 26 DNA following immunomagnetic separation of specific leukocyte subsets showed that in 9 of 18 patients (50%), the DNA could be amplified from CD45+ cells, and in none of the CD31+, CD19+, CD14+ and CD2+ fractions.

3.4 SUMMARY OF RESULTS FOR THE BLOOD DONOR, HIV-INFECTED, BMT RECIPIENT AND CFS PATIENT STUDY GROUPS.

Nested PCR for ORF 26 DNA following immunomagnetic separation of CD45+ cells from the peripheral blood of the study groups, demonstrated a higher HHV-8 DNA-positivity in the HIV-infected (37%; 38/103) and BMT recipient (33%; 10/30) (50%; 6/12) study groups, as compared with the blood donor (16%; 30/192) and CFS (23%; 9/39) study groups. These are summarised in Table 3.16, along with the data on the seropositivity results in the same groups for anti-HSV-1, anti-HSV-2, anti-EBV, anti-CMV, and anti-HHV-8 as determined by IFA-1.

Table 3.16 Comparison of HHV-8 DNA positivity, anti HHV-8 positivity as detected by IFA-1, and anti HSV-1, HSV-2, CMV and EBV in all the study groups.

	Group Characteristics				No positive serological results (%)					No positive PCR results (%)
	Total number	Mean age (yr)	Male	Female	Anti HSV-1	Anti HSV-2	Anti EBV	Anti CMV	Anti HHV-8 (IFA-1)	HHV8 ORF 26 DNA
BLOOD DONORS										
London 1995/6	67	39	31	36	54% (36)	0% (0)	84% (56)	42% (28)	43% (29)	25% (17)
Leeds 1995/6	25	43	11	14	40% (10)	8% (2)	96% (24)	44% (11)	52% (13)	20% (5)
1995/6	92	40.5	42	50	50% (46)	2% (2)	87% (80)	42% (39)	46% (42)	24% (22)
2001	100	42	51	49	28% (28)	0% (0)	ne	ne	24% (24)	8% (8)
TOTAL	192	41.5	93	99	39% (74)	1% (2)	ne	ne	34% (66)	16% (30)
HIV – INFECTED INDIVIDUALS										
TOTAL	103	41	101	2	86% (89)	66%* (67)	100% (103)	99% (102)	85% (87)	37% (38)
BMT RECIPIENTS										
TOTAL	30	19.5	11	19	67% (8/12)	17% (2/12)	92% (11/12)	42% (5/12)	33% (4/12)	50% (6/12) 33% (10/30)
CFS PATIENTS										
TOTAL	39	40.7	10	29	62% (24)	5% (2)	97% (38)	44% (17)	8% (3)	23% (9)

CHAPTER 4

CONCLUSIONS AND DISCUSSIONS

4.1 OPTIMISATION OF METHODS FOR FRACTIONATING HHV-8 CARRYING CELLS IN PERIPHERAL WHOLE BLOOD

4.1.1 CONCLUSIONS

Amplification of HHV-8 sub-genomic DNA by nested PCR of DNA isolated from immunomagnetically separated CD45+ (pan-leukocyte) peripheral blood cells potentially provides a highly sensitive means of HHV-8 genoprevalence estimation. Undertaking density gradient centrifugation prior to immunomagnetic cell separation offers no advantage over immunomagnetic cell separation alone.

The high prevalence HHV-8 DNA found in CD31+ (endothelial) peripheral blood cells, as compared to CD19+, CD14+, and CD2+ cells, suggests that, in HIV-infected homosexuals, HHV8 preferentially resides in endothelial cells rather than in B, T or monocyte subpopulations. Circulating endothelial cells may have a role in the dissemination of the virus in HIV-infected homosexual men.

4.1.2 DISCUSSION

The lack of a gold standard technique to detect HHV-8 infection in peripheral blood has hindered the determination of the true epidemiology of this virus. Evaluation of the extent of infection in epidemiological studies is

traditionally determined by quantitative serological tests, such as IFAs, and ELISAs. However as discussed in Section 1.7.1, the sensitivity and specificity of these assays for a wide array of antibodies to HHV-8 antigens are variable. This has resulted in uncertainty about the true prevalence of HHV-8 in different groups, particularly populations at low risk of KS. An alternative approach to attempt to clarify the prevalence of HHV-8 is by PCR detection of viral DNA in the peripheral blood. However, the small quantity of HHV-8 genomes carried in PBMCs (Decker *et al.*, 1996; Blackbourn *et al.*, 1997) has resulted in great variation in the reported genoprevalence of HHV-8 (Table 3.1). Such variability is despite the use of nested PCRs devised to increase sensitivity (Belec *et al.*, 1998b; Cattani *et al.*, 1998; Min *et al.*, 1999; Spira *et al.*, 2000).

The initial investigation described in this thesis provides a systematic evaluation of the effect of different techniques of cellular isolation on the subsequent detection rate of HHV-8 sub-genomic DNA from peripheral blood. Furthermore, it sheds light on the prevalence of this virus in different subpopulations of peripheral blood cells in HIV-infected homosexual individuals. The findings show that use of CD45+ immunomagnetic cell separation from peripheral blood prior to DNA extraction and nested PCR for HHV-8 sub-genomic DNA provide a highly sensitive means of estimating HHV-8 genoprevalence. Overall, the HHV-8 genoprevalence in all 63 of the HIV-infected homosexual subjects studied here, as estimated by nested PCR of DNA extracted from immunomagnetically separated CD45+ cells is 49% (31/63). This rate compares with 6% (4/63) in DNA extracted from WBCs left

after RBC lysis. Other studies have reported a genoprevalence in similar study subjects of 0% to 27% (Ambroziak *et al.*, 1995; Bigoni *et al.*, 1996; De Milito *et al.*, 1996; Humphrey *et al.*, 1996; Lefrere *et al.*, 1996; Moore *et al.*, 1996c; Dupon *et al.*, 1997; Huang *et al.*, 1997; Min *et al.*, 1999; Lorenzen *et al.*, 2002).

Although HHV-8 is widely accepted to be involved in the aetiology of KS, a wide range of HHV-8 genoprevalence rates (25% to 100%) has been reported in HIV-infected patients with KS (Ambroziak *et al.*, 1995; Whitby *et al.*, 1995; Decker *et al.*, 1996; Humphrey *et al.*, 1996; Lefrere *et al.*, 1996; Moore *et al.*, 1996c; Dupon *et al.*, 1997; Huang *et al.*, 1997; Poggi *et al.*, 1997; Purvis *et al.*, 1997; Uccini *et al.*, 1997; Min *et al.*, 1999; Camera Pierrotti *et al.*, 2000; Spira *et al.*, 2000; Lorenzen *et al.*, 2002). In the current investigation, all 5 patients in the study with clinical evidence of KS (Patients CCH89, CCH92, CCH104, CCH122; CCH124) were positive for HHV-8 DNA in extracts prepared from CD45+ cells isolated immunomagnetically (100%). However, none of these 5 patients were positive for HHV-8 DNA from samples generated following RBC lysis. There is some evidence that anti-retroviral therapy can lead to a reduction in the level of HHV-8 viraemia, particularly after 12 months of therapy (Gill *et al.*, 2002; Bourboulia *et al.*, 2004). Such reduction may lead to undetectable levels of HHV-8 by conventional methods of cellular isolation. In this context, it is notable that all the five patients were receiving high dose antiretroviral therapy for their HIV infection at the time of HHV-8 testing.

The CD31+ (endothelial) cellular subset, as immunomagnetically purified, was positive for HHV-8 DNA in 8 out of the 13 HIV-infected homosexual

patients tested (62%). This relatively high rate of detection is consistent with the finding that circulating KS-like spindle cell progenitors from the blood may be persistently infected with HHV-8 (Sirianni *et al* 1997b). These cells may be responsible for viral dissemination throughout the body, accounting for the multicentricity of KS.

The CD19+, CD14+ and CD2+ cell populations were not found to carry HHV-8 DNA. The data for the isolation of HHV-8 from these subsets in other studies are sparse (Table 3.2), despite the initial reports suggesting the exclusive B cell tropism of HHV-8 (Ambroziak *et al.*, 1995; Blackbourn *et al.*, 1999). The evidence accrued from this study shows that amplifying for HHV-8 DNA in these subsets will not lead to accurate estimations of HHV-8 genoprevalences.

Evidence is shown here that immunomagnetic separation of CD45+ cells from peripheral blood prior to HHV-8 nested PCR results in high rates of HHV-8 DNA detection when compared to separation methods utilising RBC lysis or density gradient centrifugation. The lower rate of HHV-8 detection from buffy coat WBC pellets generated using the RBC lysis solution may be related to lysis of WBCs in the course of RBC lysis, particularly as WBCs may become more vulnerable if samples are processed 48 hr after venepuncture sampling. Furthermore, the WBCs in the cellular pellet are contaminated by platelets. The performance of the lysing procedure may also be inconsistent, as shown in a previous study investigating performances in multiple tubes of peripheral blood taken from the same individual (Pacifici *et al.*, 1998). Such inconsistency can

produce considerable discrepancies in the yield of leukocyte subsets isolated, which is particularly important for HIV-infected patients who may have depleted levels of some of the lymphocyte subsets (Tiirikainen, 1995).

Although density gradient centrifugation prior to immunomagnetic cell separation increased the sensitivity of HHV-8 DNA detection as compared to RBC lysis or density gradient centrifugation alone, it did not improve the sensitivity or consistency of HHV-8 detection when compared to immunomagnetic cell separation alone. Furthermore, given that the density gradient centrifugation approach is cumbersome, and is unsuitable for blood samples of small volumes (<5 ml), its routine use for HHV-8 DNA detection in peripheral blood is not promising.

4.2 GENOPREVALENCE AND SEROPREVALENCE OF HHV-8 IN SUB-POPULATIONS AT LOW-RISK OF KS: UK BLOOD DONORS

4.2.1 CONCLUSIONS

The genoprevalence and seroprevalence of HHV-8 in blood donors in the UK have hitherto not been reported. The findings described in this study indicate that the genoprevalence of HHV-8 in blood donors is as high as 16%, and the seroprevalence 34%. The high rate of genome positivity may be a reflection of the immunomagnetically fractionated CD45+ cells used as substrates for HHV-8 DNA amplification, and of the nested PCR. The high

detection rate is unlikely to be due to PCR cross-contamination as the DNA sequences derived from the hypervariable ORF K1 were diverse, reflecting natural genetic drift. Moreover, HHV-8 genome positivity was not reliably concordant with anti HHV-8 seropositivity, as determined by 2 IFA methods. HHV-8 genome positivity as determined by ORF 26 (8%) and K1 (9%) DNA detection were concordant when PCR was undertaken contemporaneously (blood donors sampled in 2001). Degradation of stored DNA samples derived from the 1996/1997 blood donor group may account for the lower HHV-8 genopositivity as determined by PCR detection of ORF K1 DNA (5%; undertaken after 4 years) as compared to ORF 26 DNA (24%).

Lastly, the seroprevalence of HHV-8 mirrors that of HSV-1, rather than HSV-2. The 1996/7 blood donors showed a higher rate of positivity for ORF26 DNA, anti HHV-8 as detected by IFA-1, and anti HSV-1. Possible reasons for these variations were considered. The criteria for blood donor selection in the period between 1996/7 and 2001 were enquired into. However, no confirmed policy changes were identified, although this was the period where public health concern regarding the spread of prion disease via blood products had become an important issue. The only factor identified as different between the sampling of the 2 groups was the time of year that the blood donors were sampled. The 1996/7 donors were sampled in winter (between November and January), whereas the 2001 donors were recruited in spring (April).

4.2.2 DISCUSSION

Previous HHV-8 prevalence studies vary considerably according to the population type and geographical areas represented. Particularly in populations at low risk of KS, such as blood donors in industrialised countries, the wide variation in seroprevalence figures is attributed to differences in specificity and sensitivity of the detection method used. Findings in the literature of HHV-8 seroprevalence rates in blood donors are summarised in Table 4.1, other general populations and healthy individuals in Table 4.2, patients with KS in Table 4.3, HIV-infected, KS-negative people in Table 4.4, HIV-negative, KS-negative people with other risk factors for KS in Table 4.5, and HIV-negative and HIV-positive patients with high-risk behaviour in Table 4.5.

The reported seroprevalence of HHV-8 in blood donors varies widely not only in different geographical regions (Table 4.1), but is also dependant on the type of antibodies to be detected, with assays detecting anti-latent antibodies generally yielding lower estimates of HHV-8 seroprevalence than those detecting anti-lytic antibodies (Chatlynne *et al.,* 1999). Within Europe, high blood donor seroprevalence has been reported not only in regions endemic for KS, such as Sardinia (21-35%) (Calabro *et al.,* 1998; Santarelli *et al.,* 2001), but also in non-KS endemic regions such as Rome (28%) (Rezza *et al.,* 1998) and Sweden (20%) (Enbom *et al.,* 2000). The findings described in this study show a HHV-8 seroprevalence in UK blood donors to be between 12% to 34%. Such a higher-than-expected range of figures is supported by the corresponding

genoprevalence data. The only previous estimate of the HHV-8 seroprevalence in UK blood donors, using both an IFA to latent antigens and and ORF 65 based ELISA, reported the rate to be between 2% to 3% (Simpson *et al.,* 1996). The study described in this thesis markedly extends the seroprevalence range in the UK context. HHV-8 seroprevalence in blood donors from the USA shows wide variations, ranging from 0% - 23%, with higher detection rates tending to originate from Texas (Baillargeon *et al.,* 2001; Hudnall *et al.,* 2003), and assays based on a BCBL-1 derived lytic IFA (Lennette *et al.,* 1996; Hudnall *et al.,* 2003). Reports from other parts of the world also show wide variations in blood donors: between 1% and 8% seropositivity in the Caribbean (Ablashi *et al.,* 1999; Challine *et al.,* 2001), 0.2% - 23% in Asia (Fujii *et al.,* 1999; Wang *et al.,* 2002), and 1% - 74% in parts of Africa (Ablashi *et al.,* 1999; Sitas *et al.,* 1999; Kakoola *et al.,* 2001; Enbom *et al.,* 2002).

Studies reporting the seroprevalence of HHV-8 in general populations and healthy individuals (Table 4.2) are more numerous than those based on blood donors. The consensus from these studies is that while the seroprevalence in young children may be low (Chandran *et al.,* 1998; Hudnall *et al.,* 1998; Enbom *et al.,* 1999; Gambus *et al.,* 2001; Martro *et al.,* 2004) it rises steadily with age, and is not correlated with puberty and sexual activity (Enbom *et al.,* 1999; Gessain *et al.,* 1999; Huang *et al.,* 2000; Martro *et al.,* 2004). They indicate that the virus is spread by close, casual, non sexual contact. The findings of the current study also show an association of anti HHV-8 seroprevalence to be more concordant to that of anti HSV-1 rather than anti

HSV-2, further indicating the predominance of non sexual rather than sexual contact in the transmission of HHV-8 in populations at low risk of developing KS.

There are currently no data on the seroprevalence of HHV-8 in the U.K. general population. The reported HHV-8 seropositivity in general populations around the world varies considerably, even in a given region (Casper *et al.,* 2002b; Pellet *et al.,* 2003). In Europe, a seroprevalence of 0.3% by latent anti-HHV-8 IFA and 2.4% by lytic anti-HHV-8 IFA of 666 serum samples from individuals without signs of acute disease (0-65+ yrs) in the Czech Republic (Suchankova *et al.,* 2003), contrasts with one of 54% in 200 elderly adults from an outpatient clinic in Malta (Engels *et al.,* 2000). Accordingly, it is not possible to compare the blood donor seropositivity rates reported in this study with those in adult European populations.

There are much more data on seroprevalence of HHV-8 in general populations in the US. However, the reported seropositivity of HHV-8 is also very variable, with many studies reporting on sub-populations or specific age groups, making comparison of data difficult. Furthermore, the highest seroprevalence rate of 11%, reported in healthy adults using a sVCA (small capsomere interacting protein, ORF65) western blot (Lin *et al.,* 1997), is lower than that of 23% reported in Texan blood donors (Hudnall *et al.*, 2003), a group considered to be at lower risk of herpesvirus infections than the adult general population.

The data on HHV-8 seropositivity in African general populations are considerable, and have often been based on samples previously used to determine HIV seroprevalence. Furthermore, KS, a lesion associated with HHV-8, is endemic in many regions of Africa. In view of these considerations, high HHV-8 seropositivity rates reported from this part of the world are expected. Thus, many studies report >40% seropositivity rates (Lennette *et al.,* 1996; He *et al.,* 1998; Oslen *et al.,* 1998; Andreoni *et al.,* 1999; Enbom *et al.,* 1999; Gessain *et al.,* 1999; Engels *et al.,* 2000; Baeten *et al.,* 2002). Indeed, rates of 87% have been reported using a K8.1 EIA on serum samples from the Bantus people of Botswana, South Africa (Engels *et al.,* 2000), suggesting that HHV-8 infection may approach near universal levels in some African populations. In comparison, the findings described in this study demonstrate that in a population considered to be at low risk of HIV and KS, namely UK blood donors, HHV-8 seroprevalence is lower (12% to 34%).

Data on HHV-8 seroprevalence from Asia and the Middle-East are more sporadic, often with single study seropositivity rates available (Table 4.2). Rates as low as 0.6% in Thailand (Ayuthaya *et al.,* 2002) contrast with those of 47% in Xinjang, China (Dilnur *et al.,* 2001). Due to the paucity of comparative studies, it is not yet possible to determine what the true prevalences are in this part of the world.

In individuals with KS, the reported HHV-8 seropositivity rates are far more consistent, both in given regions and worldwide (Table 4.3). Although Katano *et al.,* (1999) detected antibodies to HHV-8 in only 32% of patients with

KS sampled in Japan, the vast majority of other studies from other parts of the world show the seroprevalence rate to exceed 80% (Gao *et al.,* 1996a; Lennette *et al.,* 1996; Simpson *et al.,* 1996; Lin *et al.,* 1997; Smith *et al.,* 1997; Calabro *et al.,* 1998; Chandran *et al.,* 1998; Chatlynne *et al.,* 1998; Dupin *et al.,* 1998; Quinibi *et al.,* 1998; Raab *et al.,* 1998; Rezza *et al.,* 1998; Couty *et al.,* 1999; Enbom *et al.,* 2000; Quinlivan *et al.,* 2001; Wang *et al.,* 2002). The findings described in this study confirm the high HHV-8 seropositivity in individuals with KS, although the number of such patients in my study group is small.

In HIV-infected individuals worldwide HHV-8 seroprevalence is generally reported to be higher than in general populations or blood donors, although in many cases the data for direct comparison are not available (Table 4.4). Furthermore, the HHV-8 seropostivity rate tends to be higher in homosexual HIV-infected individuals as compared to heterosexual HIV-infected individuals (Kedes *et al.,* 1996; Regamey *et al.,* 1998a; Challine *et al.,* 2001; Gambus *et al.,* 2001). Indeed, Gambus *et al.,* (2001) found that the seroprevalence of HHV-8 among Spanish homosexual HIV-infected individuals was 87%, as compared to 18% in heterosexual HIV-infected men. The majority of the HIV-infected individuals recruited in the current investigation were homosexual men (100/103). The high rate of HHV-8 seropositivity of 85% (87/103) as detected by IFA-1 provides further data to support the hypothesis that male homosexual behaviour in HIV-infected people groups predisposes to HHV-8 infection. Other studies have shown that unprotected sexual intercourse and sexually

transmitted disease predisposes to HHV-8 infection (Kedes *et al.,* 1996; Sosa *et al.,* 1998; Fujii *et al.,* 1999; Whitby *et al.,* 1999).

Attempts have also been made to assess the possible risk factors for HHV-8 infection in HIV-negative / KS-negative groups (Table 4.5). However, comparisons are often hindered by a lack consensus about the true prevalence of HHV-8 in given populations considered to be 'low-risk.' Nevertheless there is some evidence that, as observed in HIV-infected individuals by the present study, homosexual behaviour in HIV-negative individuals is associated with higher HHV-8 seropositivity as compared to heterosexual behaviour (Rezza *et al.,* 1998; Blackbourn *et al.,* 1999; Enbom *et al.,* 2000; Osmond *et al.,* 2002). Furthermore, high risk sexual behaviour may also increase the risk of HHV-8 seroprevalence (Bestetti *et al.,* 1998; Enbom *et al.,* 1999; Challine *et al.,* 2001). Quinibi *et al,* (1998) demonstrated a relatively high seroprevalence of HHV-8 in renal transplant recipients from Saudi Arabia. However, the data on the risk of HHV-8 infection in blood product recipients are not coherent, with some studies demonstrating increased risk (Challine *et al.,* 2001), and other failing to demonstrate an association (Lennette *et al.,* 1996; Theodossiades *et al.,* 2003). Concurrent malignant disease (excluding KS, PEL and CD) does not appear to be associated with an increased risk of HHV-8 infection (Lennette *et al.,* 1996; Quinibi *et al.,* 1998; Couty *et al.,* 1999; Sitas *et al.,* 1999; Wang *et al.,* 2002).

Further studies focusing on high risk behaviour in mixed populations of HIV-positive and HIV-negative individuals demonstrate variable associations with both high risk sexual behaviour and injecting drug use (Table 4.5), with

some reporting low HHV-8 seropositivity rates (Hjalgrim *et al.,* 2001; Greenblatt *et al.,* 2001), while others showing much higher ones (Cannon *et al.,* 2001; Marcelin *et al.,* 2002; Renwick *et al.,* 2002).

In summary, review of the HHV-8 seroprevalence data has confirmed that reliable estimates of HHV-8 prevalence, particularly in low-risk populations such as blood donors, are not yet available. Hence genoprevalence data may increase the specificity of, and assist in lending sensitivity to serological estimations of HHV-8 prevalence. The findings reported in this study indicate that the seroprevalence of HHV-8 in U.K. blood donors is as high 34%. HHV-8 genoprevalence in the same group is as high as 16%, using an optimised method for detecting low copy numbers of the HHV-8 genome in PBMCs as detailed in Section 1.11.1. However, despite statistical association between both ORF26 and K1 DNA-positivity and IFA-1-seropositivity in the total blood donor group, genome positivity was not concordant with anti HHV-8 seropositivity, when each of the blood donor groups, namely those recruited in 1996/7, and those in 2001, was considered separately. Furthermore, in the blood donors recruited in 2001 for whom a second serological test for HHV-8 (IFA-2) was undertaken, no association was found when between HHV-8 genome positivity and IFA-2 seropositivity. This lack of concordance means that it is not possible to precisely evaluate the specificity and sensitivity of either of the 2 IFA methods used, or to assess the stage of HHV-8 infection, namely primary, latent, or lytic, by comparing HHV-8 genoprevalence with HHV-8 seroprevalence. However, it is possible that the significantly higher HHV-8 seroprevalence rate detected

using the IFA-1 method (24%) as compared to the IFA-2 method (12%) may be a reflection of the wider array of antigens expressed by the KS-1 cell line from which the IFA-1 method is derived. The KS-1 cell line, which is infected with HHV-8 but not EBV, produces lytic virus and therefore contains a broad range of viral proteins (Said *et al.*, 1996a). The IFA-2 method uses BHK-21 cells infected with rSRV expressing only the HHV-8 K8.1 antigen (Inoue *et al.*, 2000). Although K8.1 is a lytic phase glycoprotein and one of the most immunogenic HHV-8 proteins, (Raab *et al.*, 1998), the sensitivity of the IFA-2 method which uses this antigen exclusively may not be as high as the IFA-1 method, where several more lytic HHV-8 antigens are expressed.

In view of the uncertain specificity and sensitivity of serologic methods used determine HHV-8 prevalence, the use of optimised methods to ascertain HHV-8 genoprevalence may provide a better indication of the true prevalence of this virus in low-risk general populations of non-KS-endemic regions. The findings of this study report that the HHV-8 genoprevalence in U.K. blood donors is 16% as detected by PCR for ORF26 PCR DNA 7% for K1 DNA. Previous data on the genoprevalence of HHV-8 in the U.K. blood donors is limited to one study by Whitby *et al.*, (1995), who were unable to detect HHV-8 in PBMCs from any of the 143 blood donors tested. Furthermore, there are limited data for comparison on the genoprevalence of the virus in healthy individuals without HIV infection from other countries, with a wide range of genoprevalence reported (0% to 80%) (Lefrere *et al.*, 1996; Decker *et al.*, 1996; Dupon *et al.*, 1997; Huang *et al.*, 1997; Kikuta *et al.*, 1997b; Belec *et al.*, 1998b;

Cattani *et al.,* 1998; Spira *et al.,* 2000). Such wide variances may be due to the use of poorly optimised methods for fractionating HHV-8-carrying peripheral blood cells. Accordingly, it is not possible to compare the blood donor genoprevalence rates reported in this study with those in other Western populations at low risk of KS.

Table 4.1 Anti HHV-8 seropositivity in blood donors: summary of the literature

Country	Region	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Europe					
France	Paris	0.3%	(1/300)	LNA immunoperoxidase technique	Bestetti *et al.*, 1998
	Paris	2%	(2/100)	LNA-1 IFA	Dupin *et al.*, 1998
	Paris	2%	(6/399)	KS-1 IgG IFA	Challine *et al.*, 2001
Germany		4%	(2/50)	K8.1 Western blot	Raab *et al.*, 1998
Greece		4%	(2/50)	BCP-1 LANA IFA	Theodossiades *et al.*, 2003
Hungary		3%	(5/180)	K8.1 ELISA	Juhasz *et al.*, 2001a
Italy		4%	(4/107)	BCP-1 IFA	Gao *et al.*, 1996a
		4%	(4/107)	LNA immunoblot	
		18%	(138/779)	LANA IFA	Calabro *et al.*, 1998
		19%	(146/779)	ORF 65 ELISA	
	South	25%	(69/280)	LANA-1 IFA	Whitby *et al.*, 1998
	South-east	18%	(26/146)	LANA IFA	Calabro *et al.*, 1998
		16%	(24/146)	ORF 65 ELISA	
	Central (Tuscany / Florence)	16%	(16/101)	LANA IFA	Calabro *et al.*, 1998
		14%	(14/101)	ORF 65 ELISA	
	North / Central	7%	(34/467)	LANA-1 IFA	Whitby *et al.*, 1998
	North (Conegliano)	12%	(13/105)	LANA IFA	Calabro *et al.*, 1998
		10%	(10/105)	ORF 65 ELISA	
	North /Rome	28%	(26/94)	BCL-1 lytic IFA	Rezza *et al.*, 1998
		2%	(2/94)	BCL-1 latent antigen	
	North (Po Valley / Padova)	21%	(64/299)	LANA IFA	Calabro *et al.*, 1998
		20%	(61/299)	ORF 65 ELISA	
	Sardinia (Sassari)	21%	(27/128)	LANA IFA	Calabro *et al.*, 1998
		23%	(29/128)	ORF 65 ELISA	
	Sardinia	35%	(79/226)	BC-3 Latent / Lytic IFA	Santarelli *et al.*, 2001

Table 4.1 Contd.

Country	Region	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Spain		7%	(40/613)	BCP-1 Latent IFA	Gambus *et al.*, 2001
Sweden		0.6% 20%	(1/162) (33/162)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Enbom *et al.*, 2000
Switzerland		5%	(9/178)	ORF 65.2 ELISA	Regamey *et al.*, 1998a
United Kingdom		3% 2 %	(4/150) (3/174)	Latent IFA ORF 65 ELISA	Simpson *et al.*, 1996
The Americas		0% 0%	(0/122) (0/122)	BCP-1 IFA LNA immunoblot	Gao *et al.*, 1996a
		1%	(2/141)	BCBL-1 IFA	Kedes *et al.*, 1996
		0% 20%	(0/44) (9/44)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Lennette *et al.*, 1996
		0% 5%	(0/117) (6/117)	Latent IFA ORF 65 ELISA	Simpson *et al.*, 1996
	Maryland	20%	(6/30))	Minor capsid protein ELISA	Davis *et al.*, 1997b
		0%	(0/50)	BCBL-1 Latent / Lytic IFA	Smith *et al.*, 1997
		12%	(5/40)	KS-1 Latent / Lytic IFA	Verbeek *et al.*, 1998
		11%	(10/91)	Whole virus ELISA	Chatlynne *et al.*, 1998
	Texas	4% 2% 6%	(2/50) (1/50) (3/50)	BCP-1 IFA LNA immunoblot ORF 65 immunoblot	Gao *et al.*, 1998
		6%	(7/110)	Whole virus ELISA	Ablashi *et al.*, 1999
		13% 0% 0% 0% 0%	(4/30) 7% (2/30) (0/30) (0/30) (0/30) (0/30)	Lytic mIFA K8.1 IFA K8.1 ELISA ORF 65 ELISA Latent mIFA ORF 73 IFA	Inoue *et al.*, 2000
	Brazil	5%	(34/747)	LANA IFA	Zago *et al.*, 2000
	Texas	10% 13%	(153/1534) (203/1534)	BCP-1 IFA ORF 65 (lytic) ELISA	Baillargeon *et al.*, 2001

Table 4.1 Contd.

Country	Region	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
The Americas contd.	Texas	5%	(5/100)	BCBL-1 latent IFA	Hudnall *et al.*, 2003
		23%	(23/100)	BCBL-1 lytic IFA	
	South America	3.7%	(92/2470)	BCBL-1 lytic IFA	Perez *et al.*, 2004
Caribbean					
Jamaica		4%	(10/250)	Whole virus ELISA	Ablashi *et al.*, 1999
Trinidad		1%	(2/151)	Whole virus ELISA	Ablashi *et al.*, 1999
Guadeloupe	(French West Indies)	8%	(12/160)	KS-1 IgG IFA	Challine *et al.*, 2001
Africa					
South Africa					
Zambia		38%	(15/40)	Whole virus ELISA	Ablashi *et al.*, 1999
Johannesburg	Black	20%	(17/85)	ORF 73 LNA-1 IFA	Sitas *et al.*, 1999
	White	5%	(11/224)		
East Africa					
Uganda		38%	(22/58)	Whole virus ELISA	Ablashi *et al.*, 1999
		74%	(84/114)	ORF 65 / ORF 73 ELISA	Kakoola *et al.*, 2001
Tanzania		1%	(2/152)	Latent BCP-1 IFA	Enbom *et al.*, 2002
		25%	(38/152)	Lytic BCP-1 IFA	
		25%	(38/152)	Lytic BCP-1 IFA	
West Africa					
Ghana		36%	(36/100)	Whole virus ELISA	Ablashi *et al.*, 1999
Asia					
Japan		0.2%	(2/1000)	LANA IFA	Fujii *et al.*, 1999
Taiwan		23%	(40/174)	ORF 16 / 57 / 71 immunoblots	Wang *et al.*, 2002

Table 4.2 **Anti HHV-8 seropositivity in general populations / healthy individuals: summary of literature**

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Europe					
Czech Republic		0.3%	(2/666)	BC-1 Latent IFA	Suchankova *et al.*, 2002
		2%	(16/666)	BC-1 Lytic IFA	
France	Healthy subjects in Paris	2%	(2/100)	BCP-1 LANA IFA	Marcelin *et al.*, 1998
Greece	HIV neg / KS neg	11.5%	(3/26)	ORF 65 ELISA	Simpson *et al.*, 1996
		11.5%	(3/26)	Latent IFA	
Israel	HIV negative Ethiopian immigrants	39%	(79/202)	Western blot	Grossman *et al.*, 2002
Italy	Sardinia	11%	(10/91)	sVCA immunoblot	Angeloni *et al.*, 1998
	Sicily	12%	(112/970)	BCBL-1 Latent / Lytic IFA	Perna *et al.*, 2000
Malta		54%	(108/200)	ORF 65 EIA	Engels *et al.*, 2000
		16%	(32/200)	LANA IFA	
		9%	(18/200)	K8.1 EIA	
Spain	Children	0%	(0/100)	BCP-1 Latent IFA	Gambus *et al.*, 2001
Sweden	Children (< 9 y)	0%	(0/100)	Latent BCP-1 IFA	Enbom *et al.*, 1999
	Children (< 9 y)	1%	(1/100)	Lytic BCP-1 IFA	
	Infants: 6-38 m	4%	(3/75)	ORF 65 / K8.1 ELISAs and whole virus lytic IFA	Martro *et al.*, 2004
	Children / adolescents 38 m -17 y	3%	(5/160)		
Switzerland	Heterosexual	7%	(5/68)	ORF 65.2 ELISA	Regamey *et al.*, 1998a

Table 4.2 Contd.

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
The Americas	Age range: 6m - >61 yrs	0% 9%	(0/437) (43/437)	Lytic BCBL-1 IFA Latent BCBL-1 IFA	Lennette *et al.*, 1996
	Healthy adults	11%	(3/28)	sVCA Western blot	Lin *et al.*, 1997
	Healthy adults	7%	(2/29)	BCBL-1 IFA	Hudnall *et al.*, 1998
	Women	1%	(1/84)	LANA IFA	Kedes *et al.*, 1997
	Healthy men	3% 8%	(4/120) (9/120)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Chandran *et al.*, 1998
	Infants	0%	(0/158)	LANA BCBL-1 IFA	Goedert *et al.*, 1997
	Children: 1-5 y	0% 0%	(0/110) (0/110)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Chandran *et al.*, 1998
	Infants: 6-12m	0%	(0/14)	BCBL-1 IFA	Hudnall *et al.*, 1998
	Children: 4-11 y	26%	(32/123)	Latent IFA and ORF 65 ELISA	Baillargeon *et al.*, 2002
	Infants: 6-38m Children / adolescents 38 m -17 y	6% 3%	(5/88) (15/464)	ORF 65 / K8.1 ELISAs and whole virus lytic IFA	Martro *et al.*, 2004
	U.S.A.-born pregnant women Haitian-born pregnant women	1% 13%	(1/80) (8/63)	LANA BCBL-1 IFA	Goedert *et al.*, 1997
	Individuals of African origin in French Guiana	13%	(177/1337)	KS-1 Latent / Lytic IFA	Plancoulaine *et al.*, 2000
	Cuba	17%	(64/379)	Lytic IFA	Fernandez *et al.*, 2002
	Villagers in French Guiana	12%	(214/1819)	KS-1 Latent / Lytic IFA	Plancoulaine *et al.*, 2002

Table 4.2 Contd.

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Africa					
East Africa					
Eritrea	Children (< 1 y)	0%	(0/29)	Latent BCP-1 IFA	Enbom *et al.*, 1999
		7%	(2/29)	Lytic BCP-1 IFA	
	Children (2-5 y)	0%	(0/48)	Latent BCP-1 IFA	
		15%	(7/48)	Lytic BCP-1 IFA	
	Children (>5 y)	2%	(2/84)	Latent BCP-1 IFA	
		20%	(17/84)	Lytic BCP-1 IFA	
	Rashaida tribe	26%	(11/42)	Latent BCP-1 IFA	Enbom *et al.*, 1999
	Rashaida tribe	45%	(19/42)	Lytic BCP-1 IFA	
Kenya	Heterosexual trucking company employees	43% (456/1061)		Whole virus lysate ELISA	Baeten *et al.*, 2002
Uganda		11%	(9/82)	Latent BCBL-1 IFA	Lennette *et al.*, 1996
		77%	(63/82)	Lytic BCBL-1 IFA	
	HIV neg / KS neg	51%	(24/47)	BCP-1 IFA	Gao *et al.*, 1996a
	HIV neg / KS neg	62%	(29/47)	LNA immunoblot	
		53%	(9/17)	Latent IFA	Simpson *et al.*, 1996
		35%	(6/17)	ORF 65 ELISA	
	Children / young adults (<24 y)	33%	(71/215)	LANA IFA	Mayama *et al.*, 1998
	Children / young adults (<24 y)	43%	(90/212)	ORF 65 ELISA	
West Africa					
Cameroon	Newborn (0-6 m)	46%	(17/37)	Latent / lytic KS-1 IFA	Gessain *et al.*, 1999
	7-12 m	13%	(4/32)		
	13-24 m	14%	(4/28)		
	3-4 y	14%	(5/36)		
	5-8 y	24%	(8/34)		
	9-11 y	25%	(9/36)		
	12-24 y	39%	(11/28)		
	15-20 y	48%	(13/27)		
	Children / adolescents total	28%	(71/258)		

Table 4.2 Contd.

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Nigeria		6%	(3/52)	Latent BCBL-1 IFA	Lennette *et al.*, 1996
		56%	(29/52)	Lytic BCBL-1 IFA	
	Children 6-38 m	14%	(25/184)	ORF 65 / K8.1	Martro *et al.*, 2004
	Infants: 6-16 m	6%	(3/47)	ELISAs and whole	
	Children 17-27 m	13%	(10/75)	virus lytic IFA	
	Children 28-38 m	19%	(12/62)		
Central Africa					
Congo		79%	(255/321)	ORF 65 EIA	Engels *et al.*, 2000
		69%	(221/321)	LANA IFA	
		82%	(263/321)	K8.1 EIA	
Southern Africa					
Botswana	Semi-nomadic	61%	(95/155)	ORF 65 EIA LANA	Engels *et al.*, 2000
	San people	86%	(133/155)	IFA	
		87%	(134/155)	K8.1 EIA	
	Bantus	49%	(79/161)	ORF 65 EIA	Engels *et al.*, 2000
		80%	(129/161)	LANA IFA	
		75%	(122/161)	K8.1 EIA	
Central Africa					
Democratic republic of		81%	(13/16)	Lytic BCBL-1 IFA	Lennette *et al.*, 1996
Congo (Zaire)		25%	(4/16)	Latent BCBL-1 IFA	
North Africa					
Dominican Republic		0%	(0/40)	Latent BCBL-1 IFA	Lennette *et al.*, 1996
		13%	(5/40)	Lytic BCBL-1 IFA	
Egypt	Children – young adults	8.5%	(21/246)	BCBL-1 Latent IFA	Andreoni *et al.*, 1999
		45%	(110/246)	BCBL-1 Lytic IFA	
Haiti		29%	(15/52)	Lytic BCBL-1 IFA	Lennette *et al.*, 1996
		0%	(0/52)	Latent BCBL-1 IFA	

Table 4.2 Contd.

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Southern Africa					
Zambia	Pregnant women	47%	(130/275)	BCBL-1 IFA	He *et al.*, 1998
		58%	(122/211)	LANA IFA / ORF 65 WB	Olsen *et al.*, 1998
Zimbabwe		11%	(4/37)	Latent BCBL-1 IFA	Lennette *et al.*, 1996
		32%	(12/37)	Lytic BCBL-1 IFA	
Asia / Middle East					
China	Xinjiang	46.6%	(34/73)	TY-cell line Latent IFA	Dilnur *et al.*, 2001
India	Healthy individuals	4%	(4/108)	Whole virus ELISA	Ablashi *et al.*, 1999
Israel	Jewish	10%	(238/2403)	BCP-1 LANA IFA	Davidovici *et al.*, 2001
Japan	HIV negative patients / blood donors	2%	(6/263)	ORF 59 ELISA	Katano *et al.*, 1999
Malaysia	Healthy individuals	4%	(6/159)	Whole virus ELISA	Ablashi *et al.*, 1999
Papua New Guinea	Urban	21%	(20/94)	BCBl-1 Latent / Lytic IFA	Rezza *et al.*, 2001
	Rural	30%	(17/56)		
Saudi Arabia	Healthy individuals	7%	(1/15)	P40 / sVCA immunoblot	Qunibi *et al.*, 1998
Sri Lanka	Healthy individuals	4%	(3/80)	Whole virus ELISA	Ablashi *et al.*, 1999
Thailand	Healthy individuals	3%	(2/75)	Whole virus ELISA	Ablashi *et al.*, 1999
	Healthy individuals	0.6%	(6/1018)	Latent / Lytic IFA + mixed recombinant ORF protein ELISA	Ayuthaya *et al.*, 2002

Table 4.2 Contd.

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Taiwan	Children < 5 y	3%	(6/200)	BCBL-1 IFA	Huang *et al.*, 2000
	Children 6-10 y	4%	(4/100)		
	Children / adolescents 11-20 y	12%	(16/132)		
	Adults 21-30 y	16%	(26/167)		
	Adults 31-40 y	19%	(23/120)		
	Adults 41-50 y	8%	(9/115)		
	Adults 51->70 y	12%	(31/259)		
	Pregnant women	24%	(26/134)		

Table 4.3 Anti HHV-8 seropositivity in Individuals with KS: summary of the literature

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Europe					
France	HIV negative	100%	(16/16)	LNA-1 IFA	Dupin *et al.*, 1998
	AIDS	100%	(10/10)	BCP-1 LANA IFA	Marcelin *et al.*, 1998
	HIV negative	78%	(14/78)	BCP-1 LANA IFA	
	AIDS	83%	(24/29)	Latent / lytic BCP-1 IFA	Couty *et al.*, 1999
	AIDS	83%	(24/29)	Latent / lytic BC-3 IFA	
Germany	AIDS	89%	(17/19)	K8.1 Western Blot	Raab *et al.*, 1998
	HIV negative	100%	(2/2)	K8.1 Western Blot	
Greece	Classic KS	94%	(17/18)	Latent IFA	Simpson *et al.*, 1996
		94%	(17/18)	ORF 65 ELISA	
Hungary	Classic KS	80%	(8/10)	K8.1 ELISA	Juhasz *et al.*, 2001a
Italy	AIDS	71%	(10/14)	BCP-1 IFA	Gao *et al.*, 1996b
		79%	(11/14)	LNA immunoblot	
	HIV negative	100%	(11/11)	BCP-1 IFA	Gao *et al.*, 1996b
		100%	(11/11)	LNA immunoblot	
	Classic KS (Sardinia)	95%	(1/20)	sVCA immunoblot	Angeloni *et al.*, 1998
	AIDS	100%	(12/12)	LANA IFA / ORF 65 ELISA	Calabro *et al.*, 1998
	Classic KS	100%	(3/3)	LANA IFA / ORF 65 ELISA	
	KS Post-transplantation	100%	(2/2)	LANA IFA / ORF 65 ELISA	
	Rome	67%	(8/12)	BCL-1 latent antigen	Rezza *et al.*, 1998
		83%	(10/12)	BCL-1 lytic IFA	

Table 4.3 Contd.

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Sweden	AIDS	88%	(7/8)	Latent BCBL-1 IFA	Enbom *et al.*, 2000
	AIDS	88%	(7/8)	Lytic BCBL-1 IFA	Enbom *et al.*, 2000
Switzerland	AIDS	74%	(26/35)	BCBL-1 HHV-8 nuclear antigen IFA	Quinlivan *et al.*, 2001
		83%	(29/35)	BCBL-1 HHV-8 membrane antigen IFA	
		49%	(17/35)	ORF 65 ELISA	
	AIDS	92.3%	(24/26)	ORF 65.2 ELISA	Regamey *et al.*, 1998
United Kingdom	AIDS	81%	(83/103)	Latent IFA	Simpson *et al.*, 1996
The Americas	AIDS	88%	(35/40)	BCP-1 IFA	Gao *et al.*, 1996b
		80%	(32/40)	LNA immunoblot	
	AIDS	82%	(37/45)	BCBL-1 IFA	Kedes *et al.*, 1996
	AIDS	54%	(47/87)	Latent BCBL-1 IFA	Lennette *et al.*, 1996
		96%	(84/87)	Lytic BCBL-1 IFA	
	AIDS	67%	(32/48)	BC-1 P40 immunoblot	Miller *et al.*, 1996
		65%	(31/48)	BC-1 IFA	
	AIDS	60%	(21/35)	Minor capsid protein ELISA	Davis *et al.*, 1997b
	AIDS	89%	(42/47)	sVCA Western blot	Lin *et al.*, 1997
	AIDS	100%	(7/7)	BCBL-1 Latent / Lytic IFA	Smith *et al.*, 1997
	AIDS	92%	(57/62)	Whole virus ELISA	Chatlynne *et al.*, 1998

Table 4.3 Contd.

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
	AIDS	93%	(41/44)	Latent BCBL-1 IFA	Chandran *et al.*, 1998
	AIDS	98%	(43/44)	Lytic BCBL-1 IFA	
	AIDS	100%	(27/27)	Lytic mIFA	Inoue *et al.*, 2000
		100%	(27/27)	K8.1 IFA	
		85%	(23/27)	K8.1 ELISA	
		85%	(23/27)	ORF 65 ELISA	
		67%	(18/27)	Latent mIFA	
		63%	(17/27)	ORF 73 IFA	
Brazil	AIDS	80%	(32/40	LANA IFA	Zago *et al.*, 2000
	HIV negative	93%	(67/72)	Whole virus ELISA	Chatlynne *et al.*, 1998
	Classic KS	100%	(6/6)	BCBL-1 IFA	Hudnall *et al.*, 1998
Africa					
	HIV-negative	100%	(28/28)	Latent BCBL-1 IFA	Lennette *et al.*, 1996
		100%	(28/28)	Lytic BCBL-1 IFA	
Uganda	AIDS	82%	(14/17)	ORF 65 ELISA	Simpson *et al.*, 1996
	AIDS	78%	(14/18)	BCP-1 IFA	Gao *et al.*, 1996b
		89%	(16/18)	LNA immunoblot	
	HIV-negative	100%	(21/21)	Latent BCBL-1 IFA	Lennette *et al.*, 1996
		100%	(21/21)	Lytic BCBL-1 IFA	
	HIV-negative	100%	(1/1)	BCP-1 IFA	Gao *et al.*, 1996a
		100%	(1/1)	LNA immunoblot	
	HIV negative	79%	(63/80)	BCP-1 IFA	Ziegler *et al.*, 2003
South Africa	HIV-positive	83%	(42/51)	ORF 73 LNA-1 IFA	Sitas *et al.*, 1999

Table 4.3 Contd.

Country	Details	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Asia / Middle-East					
Japan	AIDS KS	32%	(7/22)	ORF 59 ELISA	Katano *et al.*, 1999
Saudi Arabia	Renal transplant recipients	93%	(13/14)	P40 / sVCA immunoblot	Qunibi *et al.*, 1998
Taiwan	HIV negative	83%	(5/6)	ORF 16 / 57 / 71 immunoblots	Wang *et al.*, 2002

Table 4.4 **Anti HHV-8 seropositivity in HIV-infected / KS unaffected patients: summary of the literature**

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Europe					
Czech Republic		11% 35%	(14/129) (45/129)	BC-1 Latent IFA BC-1 Lytic IFA	Suchankova *et al.*, 2002
Denmark	Homosexual	30%	(6/14)	LANA IFA / ORF 65 ELISA/WB	Melbye *et al.*, 1998b
France		27% 27%	(6/22) (6/22)	Latent / lytic BCP-1 IFA Latent / lytic BC-3 IFA	Couty *et al.*, 1999
	Homosexual Heterosexual	70% 24%	(97/139) (9/37)	KS-1 IgG IFA	Challine *et al.*, 2001
Greece	Haemophilia	2%	(1/50)	BCP-1 LANA IFA	Theodossiades *et al.*, 2003
Israel	Ethiopian immigrants	57%	(27/47)	Western blot	Grossman *et al.*, 2002
Italy					
Rome		15% 61%	(10/69) (42/69)	BCL-1 latent antigen BCL-1 lytic IFA	Rezza *et al.*, 1998
Veneto	Homosexual / Bisexual IVDU Other	62% 11% 14%	(8/13) (2/18) (3/21)	LANA IFA / ORF 65 ELISA	Calabro *et al.*, 1998
Spain	IVDU Heterosexual Homosexual	11% 18% 87%	(29/254) (23/125) (157/181)	BCP-1 Latent IFA	Gambus *et al.*, 2001
Switzerland	Homosexual Heterosexual IVDU	30% 25% 0%	(19/63) (2/8) (0/12)	ORF 65.2 ELISA	Regamey *et al.*, 1998a

Table 4.4 Contd.

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
United Kingdom	Haemophilia	0%	(0/28)	Latent IFA	Simpson *et al.*, 1996
		0%	(0/26)	ORF 65 ELISA	
	IVDU	0%	(0/38)	Latent IFA	Simpson *et al.*, 1996
		5%	(2/38)	ORF 65 ELISA	
	STD clinic / HIV positive women	22%	(17/79)	Latent IFA	Whitby *et al.*, 1999
The Americas		20%	(11/54)	sVCA Western blot	Lin *et al.*, 1997
		13%	(7/54)	BC-1 P40 immunoblot	Miller *et al.*, 1996
		13%	(7/54)	BC-1 IFA	Miller *et al.*, 1996
		33%	(6/18)	BCBL-1 Latent / Lytic IFA	Smith *et al.*, 1997
		27%	(9/33)	Minor capsid protein ELISA	Davis *et al.*, 1997b
		50%	(7/14)	Whole virus ELISA	Chatlynne *et al.*, 1998
		73%	(29/40)	BCBL-1 IFA	Hudnall *et al.*, 1998
		65%	(15/23)	K8.1 IFA	Inoue *et al.*, 2000
		30%	(7/23)	K8.1 ELISA	
		30%	(7/23)	ORF 65 ELISA	
		26%	(6/23)	Latent mIFA	
		26%	(6/23)	ORF 73 IFA	
	AIDS	30%	(12/40)	BCP-1 IFA	Gao *et al.*, 1996
	AIDS	18%	(7/40)	LNA immunoblot	
	AIDS / children	0%	(0/51)	Latent IFA / ORF 65 ELISA	Blauvelt *et al.*, 1997
	Bi-/homosexual	35%	(13/37)	BCBL-1 IFA	Kedes *et al.*, 1996

Table 4.4 Contd.

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
The Americas contd.	Homosexual	20%	(19/94)	Latent BCBL-1 IFA	Lennette *et al.*, 1996
		93%	(87/94)	Lytic BCBL-1 IFA	
	Homosexual	18%	(7/40)	LNA p226/9234 immunoblot	Gao *et al.*, 1996b
	Homosexual	56%	(42/75)	KS-1 Latent / Lytic IFA	Verbeek *et al.*, 1998
	Homosexual	34%	(16/47)	Latent BCBL-1 IFA	Chandran *et al.*, 1998
		60%	(28/47)	Lytic BCBL-1 IFA	
	Homosexual	58%	(15/26)	Latent / lytic BCBL-1 IFA	Blackbourn *et al.*, 1999
San Francisco	Bi-/homosexual (1978-1980)	49%	(49/101)	Whole virus EIA	Osmond *et al.*, 2002
	Homosexual (1984-1985)	47%	(38/81)		
	Homosexual (1995-1996)	52%	(42/81)		
	Heterosexual	0%	(0/9)	BCBL-1 IFA	Kedes *et al.*, 1996
	Haemophilia	3%	(9/300)		
	Haemophilia	0%	(0/20)	LNA p226/9234 immunoblot	Gao *et al.*, 1996b
	IVDU	23%	(3/13)	Lytic BCBL-1 IFA	Lennette *et al.*, 1996
		0%	(0/13)	Latent BCBL-1 IFA	
	VDRL+ / STD clinic	28%	(13/46)	BCBL-1 IFA	Kedes *et al.*, 1996
	Women	3%	(10/302)	LANA IFA	Kedes *et al.*, 1997

Table 4.4 Contd.

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
	U.S.A-born pregnant women	2%	(2/118)	LANA BCBL-1 IFA	Goedert *et al.*, 1997
	Haitian-born pregnant women	4%	(1/28)		
	Infants	0%	(0/26)		
	Pregnant women	5%	(47/887)		Goedert *et al.*, 2003
	Infants: ~12m	0.3%	(3/900)		
Honduras	Men / women	24%	(39/160)	Lytic IFA	Sosa *et al.*, 1998
	Women	23%	(25/110)		
	Men	28%	(14/50)		
	Homosexual	35%	(7/20)		
	Female sex workers	36%	(9/25)		
	Drug use history (not I.V.)	26%	(23/89)		
	History of STDs	24%	(18/76)		
	Never use condoms	21%	(17/80)		
	Abnormal Pap smear	30%	(13/43)		
Africa					
East Africa					
Uganda		47%	(16/34)	ORF 65 ELISA	Simpson *et al.*, 1996
		53%	(18/34)	Latent IFA	
		51%	(18/35)	BCP-1 IFA	Gao *et al.*, 1996a
		71%	(25/35)	LNA immunoblot	
		46%	(16/35)	Whole virus ELISA	Chatlynne *et al.*, 1998
Southern Africa					
Zambia		43%	(9/21)	Whole virus ELISA	Chatlynne *et al.*, 1998
Zambia	Pregnant women	51%	(53/103)	BCBL-1 IFA	He *et al.*, 1998

Table 4.4 Contd.

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Asia / Middle East					
India		2%	(1/42)	Whole virus ELISA	Chatlynne *et al.*, 1998
Israel	HBV seropositive Jews	22%	(362/1648)	BCP-1 LANA IFA	Davidovici *et al.*, 2001
Japan	Unprotected sexual intercourse / homo/bi-sexual	27%	(23/85)	LANA IFA	Fujii *et al.*, 1999
	Unprotected sexual intercourse / heterosexual	7%	(4/54)		
	Haemophilia / other coagulopathies	0%	(0/118)		
		26%	(15/57)	ORF 59 ELISA	Katano *et al.*, 1999
Taiwan		40%	(4/10)	ORF 16 / 57 / 71 immunoblots	Wang *et al.*, 2002
Thailand		11%	(22/196)	Whole virus ELISA	Chatlynne *et al.*, 1998
Other					
Trinidad		0%	(0/9)	Whole virus ELISA	Chatlynne *et al.*, 1998
		0.7%	(7/1018)	Latent / Lytic IFA and mixed recombinant ORF protein ELISA	Ayuthaya *et al.*, 2002

Table 4.5 **Possible Risk factors for anti HHV-8 seropositivity in HIV uninfected / KS unaffected people: summary of the literature**

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Europe					
Denmark	Homosexual	20%	(46/180)	LANA IFA / ORF 65 ELISA/WB	Melbye *et al.*, 1998b
France	Dermatological disease	4%	(3/83)	LNA-1 IFA	Dupin *et al.*, 1998
	Myeloma	0%	(0/18)	Latent / lytic BCP-1 IFA	Couty *et al.*, 1999
		0%	(0/18)	Latent / lytic BC-3 IFA	
	Pregnant women	2%	(1/50)	Latent / lytic BCP-1 IFA	Couty *et al.*, 1999
		2%	(1/50)	Latent / lytic BC-3 IFA	
	Paris STD clinic	13%	(67/512)	BCP-1 IFA	Janier *et al.*, 2002
	Before heart transplant	3%	(4/150)	BC-3 Latent IFA	Emond *et al.*, 2002
	3 months after heart transplant	3%	(5/150)		
	Pregnant women	5%	(9/183)	KS-1 IgG IFA	Challine *et al.*, 2001
	Organ donors	8%	(8/100)	KS-1 IgG IFA	
	IVDU	2%	(2/82)	KS-1 IgG IFA	
	Thalassaemia patients	0%	(0/61)	KS-1 IgG IFA	
	Sickle Cell disease patients	17%	(26/154)	KS-1 IgG IFA	
	Kidney transplant recipients	15%	(15/99)	KS-1 IgG IFA	
	High risk sexual behaviour	29%	(5/17)	KS-1 IgG IFA	
Greece	Haemophilia	2%	(1/50)	BCP-1 LANA IFA	Theodossiades *et al.*, 2003
Italy	Haemophilia	25%	(4/16)	LANA IFA	Calabro *et al.*, 1998
		31%	(5/16)	ORF 65 ELISA	

Table 4.5 Contd.

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Rome	Homosexual	26%	(29/112)	BCL-1 latent antigen	Rezza *et al.*, 1998
		64%	(72/112)	BCL-1 lytic IFA	
Rome	IVDU	9%	(6/65)	BCL-1 latent antigen	Rezza *et al.*, 1998
		45%	(29/65)	BCL-1 lytic IFA	
Sardinia	Relatives of KS patients	39%	(14/36)	sVCA immunoblot	Angeloni *et al.*, 1998
Spain	IVDU	12%	(15/128)	BCP-1 Latent IFA	Gambus *et al.*, 2001
	Heterosexual	16%	(24/148)		
	Homosexual	28%	(42/150)		
Sweden	High risk sexual behaviour	3.5%	(4/114)	Latent BCBL-1 IFA	Enbom *et al.*, 2000
	High risk sexual behaviour	30%	(34/114)	Lytic BCBL-1 IFA	
	Pre-BMT	0%	(0/34)	Latent BCBL-1 IFA	Enbom *et al.*, 2000
		24%	(8/34)	Lytic BCBL-1 IFA	
	Post BMT	0%	(0/34)	Latent BCBL-1 IFA	Enbom *et al.*, 2000
		29%	(10/34)	Lytic BCBL-1 IFA	
Switzerland	Homosexual	20%	(11/54)	ORF 65.2 ELISA	Regamey *et al.*, 1998a
	Renal transplant recipient – before transplant	6.4%	(14/220)	ORF 65.2 ELISA / LANA IFA	Regamey *et al.*, 1998b
	Renal transplant recipient – 1 year after transplant	18%	(39/220)		
United Kingdom	Haemophilia	2%	(1/56)	ORF 65 ELISA	Simpson *et al.*, 1996
	IVDU	0%	(0/25)		
	STD clinic / HIV negative women	16%	(14/90)	Latent IFA	Whitby *et al.*, 1999

Table 4.5 Contd.

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
The Americas	Haemophilia	0% 12%	(0/83) (10/83)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Lennette *et al.*, 1996
	Bone marrow recipients	0% 11%	(0/38) (4/38)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Lennette *et al.*, 1996
	Hodgkins disease	0% 21%	(0/52) (11/52)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Lennette *et al.*, 1996
	Non-Hodgkin's Lymphoma	0% 5%	(0/37) (2/37)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Lennette *et al.*, 1996
	Nasopharyngeal Carcinoma	0% 10%	(0/20) (2/20)	Latent BCBL-1 IFA Lytic BCBL-1 IFA	Lennette *et al.*, 1996
	Bi-/homosexual	13%	(3/23)	BCBL-1 IFA	Kedes *et al.*, 1996
	Homosexual	43%	(23/53)	Latent / lytic BCBL-1 IFA	Blackbourn *et al.*, 1999
	MSM	16%	(76/474)	BCBL-1 IFA	Casper *et al.*, 2002
San Francisco	Bi-/homosexual (1978-1980)	26%	(76/297)	Whole virus EIA	Osmond *et al.*, 2002
	Homosexual (1984-1985)	14%	(24/171)		
	Homosexual (1995-1996)	22%	(105/476)		
	Multiple myeloma	11% 52% 81%	(3/27) (14/27) (22/27)	BCP-1 IFA LNA immunoblot ORF 65 immunoblot	Gao *et al.*, 1998

Table 4.5 Contd.

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
	Non-myeloma cancer patients	0% 26% 22%	(0/27) (7/27) (6/27)	BCP-1 IFA LNA immunobiot ORF 65 immunoblot	Gao *et al.*, 1998
	High titres to EBV capsid proteins	0%	(0/22)	LNA p226/9234 immunoblot	Gao *et al.*, 1996b
	Renal transplant	50%	(29/58)	BCBL-1 IFA	Hudnall *et al.*, 1998
Honduras	Men / women Women Men Female sex worker Drug use history (not I.V.) History of STDs Never use condoms	11% 11% 12% 15% 17% 19% 13%	(19/166) (16/110) (3/25) (9/60) (14/82) (7/36) (8/63)	Lytic IFA	Sosa *et al.*, 1998
South Africa	Cancer patients (non-KS)	32%	(1055/3298)	ORF 73 LNA-1 IFA	Sitas *et al.*, 1999
Africa					
East Africa					
Eritrea	Female sex workers	6% 15%	(6/107) (16/107)	Latent BCP-1 IFA Lytic BCP-1 IFA	Enbom *et al.*, 1999
	Pregnant women	0% 1%	(0/100) (1/100)	Latent BCP-1 IFA Lytic BCP-1 IFA	Enbom *et al.*, 1999
Uganda	Cancers other than KS	50%	(302/607)	BCP-1 IFA	Ziegler *et al.*, 2003 / Newton *et al.*, 2003

Table 4.5 Contd.

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
West Africa					
Cameroon	Female sex workers	53%	(72/135)	LNA immunoperoxidase technique	Bestetti *et al.*, 1998
	Pregnant women	37%	(90/246)	LNA immunoperoxidase technique	Bestetti *et al.*, 1998
	Children born to HHV-8 positive mothers: 0-2 months	100%	(12/12)	Latent / lytic KS-1 IFA	Gessain *et al.*, 1999
	Same children born to HHV-8 positive mothers: 12-15 months	22%	(2/9)	Latent / lytic KS-1 IFA	
Asia / Middle East					
Saudi Arabia	Renal transplant recipients	28%	(5/18)	P40 / sVCA immunoblot	Qunibi *et al.*, 1998
	Chronic renal failure	29%	(4/14)	P40 / sVCA immunoblot	Qunibi *et al.*, 1998
	Oncology patients	7%	(1/15)	P40 / sVCA immunoblot	Qunibi *et al.*, 1998
Taiwan	Cancer patients (non-KS)	13%	(8/62)	ORF 16 / 57 / 71 immunoblots	Wang *et al.*, 2002

Table 4.6 Anti HHV-8 seropositivity in sub-populations with high risk behaviour (HIV-infected and uninfected)

Country	Risk group	% anti HHV-8 seropositive (no. positive / no. tested)		Test format	Reference
Denmark	Copenhagen STD clinic attendees; young adults, 1976-1977	4%	(27/641)	K8.1 ELISA	Hjalgrim *et al.*, 2001
Djibouti	Non-prostitute women	17%	(7/41)	BC-3 Latent IFA	Marcelin *et al.*, 2002
	Street prostitutes	26%	(11/43)		
	Bar prostitutes	20%	(24/123)		
	Non-prostitute men	36%	(27/75)		
Holland	Amsterdam IVDU	2.5%	(30/1179)	ORF 65 / ORF 73 EIA	Renwick *et al.*, 2002
USA	Women with high risk behaviour	4%	(102/2483)	BCBL-1 LANA IFA	Greenblatt *et al.*, 2001
		12%	(298/2483)	BCBL-1 Lytic IFA	
	Women with high risk behaviour	16%	(208/1295)	ORF 65 / K8.1 ELISAs	Cannon *et al.*, 2001
	Daily IVDU	36%	(10/28)		
	Seropositive for syphilis	26%	(32/123		

4.3 GENOPREVALENCE AND DISTRIBUTION OF HHV-8 IN PERIPHERAL BLOOD OF BMT RECIPIENTS AND PATIENTS WITH CFS

4.3.1 CONCLUSIONS AND DISCUSSION

The only published data on the seroprevalence of HHV-8 in the U.K. population not at risk of HIV or KS are those relating to UK blood donors, reported to be between 2% and 3% (Simpson *et al.*, 1996). Furthermore, previous data on the genoprevalence of HHV-8 in the low risk UK populations are also limited to one study of blood donors (Whitby *et al.*, 1995) which found no HHV-8 DNA in PBMCs from any of the 143 donors tested. Accordingly, it is not possible to compare the HHV-8 seroprevalence and genoprevalence rates of BMT recipients and CFS patients reported in this thesis with other published UK data.

However, the findings described in this thesis for U.K. blood donors provide a more direct comparison, as the methods used to determine HHV-8 prevalence are identical. The comparisons made here have to be descriptive, however, as the sample sizes in respect of BMT and CFS patients are considerably smaller than the blood donor sample, and tests of statistical associations are not possible. Notwithstanding, some observations may be made. The HHV-8 seroprevalence in blood donors as determined by IFA-1, shown to be up to 34% in this study, is very similar to that reported for the BMT

recipients (33%). The HHV-8 genoprevalence rate in the BMT recipient group (33%) appears higher than that of the blood donors (16%). This higher rate may be due to reactivation of the virus in this immunosuppressed group, thereby substantiating the hypothesis in Section 1.17. The higher HHV-8 genoprevalence (50%) as compared to HHV-8 seroprevalence (33%) in the BMT group is also as expected, since these individuals are immunosuppressed and hence unable to mount a full immune response to infection.

It may be postulated that the high rate of HHV-8 infection in the BMT group may be related to an increased risk of HHV-8-associated disease, such as KS. However, although KS has been described in patients who receive intensive immunosuppressive treatment for solid organ transplantation, this neoplasm appears to be very rare after BMT, particularly in regions where KS is not endemic. Both HHV-8 primary infection and reactivation have been associated with the development of KS in patients who undergo solid organ transplantation (Parravicini *et al.*, 1997; Regamey 1998b; Farge *et al.*, 1999). In contrast, the association between HHV-8 infection and the development of KS in patients who undergo BMT / HSCT is not clear. The lack of HHV8-associated KS in this immunosuppressed population suggests that occurrence of this disease involves secondary mechanisms possibly linked to the continued activation of HHV-8 genes. Gluckman *et al.*, (1995) reported only one case of KS possibly transmitted by an HHV-8 DNA-positive donor in a group of more than 1500 allogeneic BMT recipients from France. A few case reports of KS in BMT recipients have emerged. A case of KS after allogeneic BMT in a black

child, resident in Spain, with active HHV-8 infection was recently reported (Tamariz-Martel *et al.*, 2000). Furthermore, a woman from the USA with chronic myeloid leukemia who had received an allogeneic HSCT previously from an HLA-identical sibling developed HHV-8-associated KS following continuous immunosuppressive therapy (de Medieros *et al.*, 2000). Of the other five cases of post BMT KS reported to date from Italy, Spain and Switzerland, two of them following reinfusion of autologous marrow (Porta *et al.*, 1991; Vivancos *et al.*, 1996), and the other three after an allogeneic BMT (Helg *et al.*, 1994; Erer *et al.*, 1997; Palencia *et al.*, 2003); in the latter 3, 2 had received ciclosporin A and prednisone (Helg *et al.*, 1994; Palencia *et al.*, 2003) and one with ciclosporin A, methotrexate and methylprednisolone (Erer *et al.*, 1997) as prophylaxis against graft-versus-host disease.

HHV-8 infection may also have an impact on the outcome of allogeneic BMT, and this may be a more frequent complication than the development of KS. Rosenwajg *et al.*, (1999) examined 200 BMT donor/recipient pairs for the risk of HHV8 transmission at BMT and found that the HHV-8 seroprevalence in both groups was not markedly dissimilar. Among the donors, 14.5% were HHV-8 seropositive as determined by an IFA to LANA, while 10% of the recipients were positive before, and 18% after BMT. Of note are 10 patients HHV-8 seropositive prior to BMT who became seronegative post-BMT. In the current investigation, Patients H3, H4 and H5 were variably HHV-8 seropositive on repeat testing post BMT, with lack of concordance between HHV-8 genome positivity and IFA-1 seropositivity. This lack of concordance means that, as

observed for the blood donors in this study, it is not possible to precisely evaluate the specificity and sensitivity of the IFA-1 method used, or to assess the stage of HHV-8 infection, namely primary, latent, or lytic, by comparing HHV-8 genoprevalence with HHV-8 seroprevalence. Rosenwajg *et al.* (1999) also determined that HHV-8 seropositivity of blood donors from the Paris area was comparable to that of BMT donors and recipients before BMT, suggesting that the BMT recipients had not been at increased risk of HHV-8 by blood products received before BMT. Furthermore, no association was found between the HHV-8 seropositivity before or after transplantation, to chronic graft-versus-host disease, or to overall BM transplantation survival. No KS was observed in the HHV-8 positive recipients. These data indicate that, if post-BMT infection with HHV8 occurs, it probably results from contamination by blood transfusions rather than from BMT donors, and that HHV8 prior seropositivity, seroconversion or seroreversion is not linked to the outcome of BMT. Using the IFA-1 test format, the current investigation confirms that HHV-8 seropositivity in UK bone marrow recipients (33%) is not dissimilar to that of UK blood donors (34%) reported in this study. Furthermore, among the BMT recipients that underwent repeat testing, neither HHV-8 seropositivity or HHV-8 DNA positivity were consistently higher for tests undertaken nearer to the transplantation date, when levels of immunosuppression would be expected to be greater.

There are no published studies on the detection of HHV-8 with CFS patients. Hence, it is also not possible to compare the seroprevalence and genoprevalence rates directly with those reported in this thesis. Although

definitive conclusions regarding the association of HHV-8 with CFS are not possible, the relatively high rate of detection of HHV-8 DNA (in 23%) of these patients may be related to reactivation due to immune dysfunction related to stressors, be it environmental, biological or psychological. However, it is noted that the HHV-8 genoprevalence rate (23%) in these individuals is similar to that of the 1996/7 blood donors reported in this study, and the seroprevalence rate (8%) is lower than that of the blood donors. Accordingly, the risk of HHV-8 reactivation in the CFS patients does not appear to be substantially greater than that in other populations at low risk of HIV infection / KS, such as blood donors. Therefore, the findings from this patient group do not substantiate the hypothesis that HHV-8 is activated to a wide extent in patients with immune dysfunction.

That HHV-8 has a tropism for B cells is confirmed by the finding in 2 of the BMT cases studied here. Previous studies have reported the presence of HHV-8 in B cells of KS patients and asymptomatic carriers (Ambroziak *et al.*, 1995; Harrington *et al.*, 1996; Mesri *et al.*, 1996; Kikuta *et al.*, 1997a; Blackbourn *et al.*, 1999; Henry *et al.*, 1999; Monini *et al.*, 1999). Furthermore, primary B cells can be infected with HHV-8 *in vitro* (Mesri *et al.*, 1996; Kliche *et al.*, 1998), and the K1 protein of HHV-8 has been shown to initiate signalling pathways in B cells (Lee *et el.*, 1998a; Lagunoff *et el.*, 1999; Damania *et el.*, 2000), and to downregulate B-cell receptor complex expression in the plasma membrane (Lee *et el.*, 2000).

While the B-cell tropism of HHV-8 has been amply demonstrated, the principal finding remains the dramatically high amplification rate of HHV-8 sub-genomic DNA by nested PCR of DNA in CD45+ peripheral blood cells compared to the rates in other cell populations. The cell surface marker CD45+ is present on all cells of hematopoietic origin, except erythroid cells, platelets and their precursor cells. HHV-8 has been detected not only in B cells as detailed above, but also in monocytes (Henry *et al.*, 1999; Monini *et al.*, 1999), circulating endothelial cells (Henry *et al.*, 1999) and even T cells can also be shown to carry HHV-8 DNA (Harrington *et al.*, 1996; Kikuta *et al.*, 1997a; Sirianni *et al.*, 1997a; Henry *et al.*, 1999; Monini *et al.*, 1999). What remains puzzling from my studies in blood donors, BMT recipients and CFS patients is that HHV-8 DNA could not be amplified from the cells that carry CD14, CD31 and CD2 antigens, and could be amplified in B cells in only a small proportion of samples. I therefore have to postulate that the principal cell types in peripheral blood carrying HHV-8 are not B cells, T cells, monocytes or endothelial cells, and they bear the CD45+/CD19-/CD2-/CD14-/CD31- phenotype. They remain to be characterised fully.

It remains that the isolation of CD45+ cells from peripheral blood ensures that HHV-8 may be found in as wide a variety of cell types as possible. Its continuing use should lead to high sensitivity to the detection of HHV-8 genomic DNA in future HHV-8 genoprevalence studies.

CHAPTER 6

SUMMARY

AND

SUGGESTIONS FOR FURTHER WORK

The search for a new virus associated with KS was motivated by epidemiologic studies that pointed to the involvement of a sexually transmitted factor other than HIV in KS tumorigenesis (Beral *et al.*, 1990). Hence when HHV-8 was discovered in KS lesions and linked to its aetiology, the virus was presumed to be predominantly sexually transmitted, particulary between homosexual men. Early supporting evidence for this hypothesis was provided by Kedes *et al.*, (1996) who found that antibodies to HHV-8 were relatively common in HIV-negative individuals who attended sexually-transmitted disease clinics compared to HIV-negative blood donors. In addition, and consistent with the epidemiology of KS, HHV-8 infection was reported to be common in homosexual men infected with HIV, but much rarer in other groups of HIV-positive individuals, such as haemophiliacs or recipients of HIV-infected blood transfusions, who rarely develop KS. Furthermore, this same group of investigators also reported that the risk of becoming infected with HHV-8, as defined by the presence of antiviral antibodies, is positively correlated to the number of sexual partners.

However, there is now gathering evidence that sexual transmission is not the primary route of HHV-8 infection, particularly in countries of high HHV-8 prevalence. In Africa, HHV-8 seroprevalence levels are high in children and approach adult levels before adolescence (Mayama *et al.,* 1998; Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000). Andreoni *et al.* (1999) have also reported that HHV-8 antibodies are highly prevalent in Egyptian children, confirming that HHV-8 infection may be acquired early in life. Furthermore, Baillargeon *et al.*

(2002) have reported a high prevalence of HHV-8 infection among children in south Texas, suggesting that transmission via close, non-sexual routes may also occur in areas of low KS / HIV prevalence. Although the precise mode has not been clearly established, extensive evidence has emerged for oral transmission of HHV-8 (Di Alberti *et al.,* 1997; Koelle etal., 1997; Viera *et al.,* 1997; Blackbourn *et al.,* 1998; Lampinen *et al.,* 2000; Pauk *et al.,* 2000; Cook *et al.,* 2002a,b; Duus *et al.,* 2004; Triantos *et al.,* 2004). These data imply that the prevalence of HHV-8 is wider than previously thought.

Although most seroprevalence studies in populations not at risk of HIV / KS suggest that HHV-8 infection is not common, studies in blood donors from the U.S.A have reported notably higher rates of up to 23% (Lennette *et al.*, 1996; Verbeek *et al.*, 1998; Chatlynne *et al.*, 1998; Inoue *et al.*, 2000; Baillargeon *et al.*, 2001; Hudnall *et al* 2003). Such disparities suggest that assays formulated and constructed to study populations at high risk of KS cannot be applied to study low-risk populations without loss of specificity (Inoue *et al.*, 2000; Spira *et al.*, 2000).

In view of the uncertain specificity and sensitivity of serological estimations of HHV-8 seroprevalence, genoprevalence studies may enhance the specificity of and lend sensitivity to estimations of HHV-8 prevalence in populations at low risk of HIV or KS. 'Specificity' because samples which are false anti HHV-8 positives would not show positivity for HHV-8 DNA, and 'sensitivity' because samples taken during early acute infection may be negative for anti HHV-8 but positive for HHV-8 DNA. This thesis describes the work done

to compare the genoprevalence with the seroprevalence of HHV-8 in UK subpopulations at varying risk of HIV infection or KS: blood donors, genitourinary clinic attendees infected by HIV, BMT recipients and CFS patients.

A protocol for amplifying sub-genomic HHV-8 DNA was first optimised. This protocol was developed using blood originating from HIV-infected patients from which CD45+ cells were immunomagnetically separated and DNA extracted therefrom submitted to nested PCR. It was determined that such an approach afforded greater sensitivity to HHV-8 DNA detection than conventional methods of separation of PBMCs, such as red blood cell lysis and density gradient centrifugation. While endothelial cell tropism was demonstrable in HIV-infected individuals, and B-cell tropism in BMT recipients, the amplification rate of HHV-8 sub-genomic DNA by nested PCR of DNA in CD45+ peripheral blood cells compared to the rates in other cell populations was consistently higher. These data suggest that the principal cell types in peripheral blood carrying HHV-8 are not B cells, T cells, monocytes or endothelial cells. Hence further studies should be undertaken to further characterise which CD45+/CD19-/CD2-/CD14-/CD31- cell type carries HHV-8. These may involve the selection of HHV-8 infected blood donors and the use of larger blood volumes from them to undertake immunomagnetic cell separation for a wider array of cell sub-types. Furthermore, the use of flow cytometric methods coupled with staining for HHV-8 antigens or *in-situ* hybridisation for HHV-8 mRNA or DNA may be undertaken.

Using the improved protocol developed in the current investigation, DNA from ORF 26 of the HHV-8 genome could be amplified from 24% of London

blood donor samples. In a subsequent donor group, DNA from ORFs 26 and K1 was detectable in 8% and 9%, respectively. A wide difference in HHV-8 seropositivity of 12% and 24% was obtained using the two antibody assays IFA-1 and IFA-2. Furthermore, a lack of concordance was observed between HHV-8 seropositivity and genome detection, which further emphasises the poor specificity of assays for anti-HHV-8 in low-risk populations. Evidence for oral transmission of HHV-8 is suggested by the association of HHV-8 seropositivity with that of HSV-1, rather than HSV-2. However, the disparity in serological and ORF 26 DNA results between the 1996/7 and the 2001 groups, the retrospective nature of ORF K1 DNA testing should be considered when interpreting the findings reported. Furthermore, sensitivity (in contrast to specificity) cannot be assured by genome-based studies, since individuals can persistently carry anti HHV-8 after clearing HHV-8 DNA from their blood. In view of these limitations, it is suggested that further studies be done to improve the estimation of HHV-8 prevalence in low-risk populations. This would involve a prospective approach of blood donors with larger sample size, with HHV-8 genome detection undertaken shortly after processing of samples. Furthermore, comparisons between blood donors from different regions in the U.K. would provide data on possible geographical variations in HHV-8 prevalence, as has been suggested by data from the U.S.A. A multi-centre approach to testing, with application of quality-assured testing protocols should also generate better reproducibility of the results.

The HHV-8 seroprevalence in blood donors as determined by IFA-1,

shown to be up to 34%, is very similar to that reported for the BMT recipients (33%). Hence, the findings in the BMT group substantiate the hypothesis that HHV-8 infection is more widespread in low risk populations than previously estimated. Furthermore, the extent of infection may be even more widespread in immunosuppressed people not at risk of HIV or KS. Further work to substantiate these hypotheses would involve larger sample sizes, more varied immunosuppressed patient groups, such as organ transplant patients and renal dialysis patients, and longitudinal studies of HHV-8 prevalence both prior to and following immunosuppression. The extent of HHV-8 detection and lytic cycle replication in association with different immunosuppressive regimes could also be explored (Hudnall *et al.,*1999).

The data reported on the prevalence of HHV-8 in CFS patients appears to suggest a relatively high rate of detection of HHV-8 DNA (23%), which may be related to reactivation due to immune dysfunction. However, the similarity of the HHV-8 genoprevalence rate in these individuals to that of the 1996/7 blood donors reported indicates that the risk of HHV-8 reactivation in the CFS patients is not substantially greater than that in other populations at low risk of HIV infection / KS, such as blood donors. Indeed, there is controversy as to whether CFS patients are immunosuppressed, with much data on other possible aetiologies, such as psychological, or environmental factors (Moutschen *et al.,* 1994; Evengard *et al.,* 1999). Furthermore, a recent systematic review by Lyall *et al.,* (2003), failed to find a consistent pattern of immunological abnormalities associated with this perplexing disorder. In view of

these disparities, interpretation of the reported HHV-8 prevalence rate in this group is problematic. Further work to clarify the prevalence of HHV-8 detection in patients with CFS should involve categorising patients into groups according to the risk factors and immunological markers detected, larger sample sizes, and longitudinal studies of HHV-8 prevalence during the course of the disease.

In summary, the findings reported in this thesis substantiate the hypothesis that HHV-8 infection in populations at low risk of KS, is more widespread than previously thought. Furthermore, the lack of concordance between HHV-8 genome positivity and IFA-1 seropositivity suggests that carriers may not mount antibody responses detectable by current serological assays. Hence HHV-8 genoprevalence may provide an estimate that more closely mirrors the true prevalence of this virus in low risk populations. Comparison of HHV-8 seroprevalence with seroprevalences of antibodies to EBV, CMV, HSV-1 and HSV-2, revealed an association between of HHV-8 seropositivity with that of HSV-1, rather than HSV-2, implying that HHV-8 is a virus that is shed orally, and not one that is predominantly sexually transmitted. Review of the published HHV-8 seroprevalence data, reveals the paucity of studies reporting the prevalence of this virus in the UK, particularly in low-risk groups. Further comparitive investigations of HHV-8 prevalence, using the optimised protocol for amplifying sub-genomic HHV-8 DNA developed in this study, would be invaluable to confirm the prevalence of the virus not only in blood donors but also in other low risk populations from different geographical areas within the U.K.

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APPENDIX 1 –

DEFINITION OF HUMAN HERPES VIRUS 8 GENES

Table A1: Definition of HHV-8 Genes

Gene ID	Name(s)	Definition
KSHV001	K1	K1 glycoprotein
KSHV002	vCBP ORF4	complement binding protein cellular homolog
KSHV003	SSB MDBP ORF6	single-stranded DNA binding protein
KSHV004	ORF7	transport and capsid assembly protein
KSHV005	gB ORF8	glycoprotein B
KSHV006	ORF9 DNA pol	DNA polymerase cellular homolog
KSHV007	ORF10	unknown
KSHV008	ORF11	unknown
KSHV009	vIL-6 K2	interleukin-6 cellular homolog
KSHV010	vDHFR ORF2	dihydrofolate reductase cellular homolog
KSHV011	K3	unknown cellular homolog
KSHV012	vTS thyA ORF70	thymidylate synthase cellular homolog
KSHV013	vCCL2 vMIP-II K4	CC chemokine cellular homolog
KSHV013.1	K4.1 vBCK vCCL3 vMIP-III	CC chemokine cellular homolog
KSHV014	K5	unknown cellular homolog
KSHV015	vCCL1 vMIP-1A vMIP-I K6	CC chemokine cellular homolog
KSHV016	T1.1 PAN RNA nut-1 K7	polyadenylated nuclear RNA
KSHV017	vBCL-2 ORF16	anti-apoptotic factor cellular homolog
KSHV017.1	AP ORF17.5 SCAF	scaffolding protein (assembly protein)
KSHV018	ORF18	unknown
KSHV019	ORF17 PRO	protease
KSHV020	ORF19	tegument protein
KSHV021	TK ORF21	thymidine kinase
KSHV022	ORF20	unknown
KSHV023	ORF22	glycoprotein H
KSHV024	ORF23	unknown
KSHV025	MCP ORF25	major capsid protein
KSHV026	ORF24	unknown

Table A1: Definition of HHV-8 Genes *contd.*

Gene ID	Name(s)	Definition
KSHV027	TRI-2 ORF26	triplex subunit 2 (minor capsid protein)
KSHV028	ORF27	unknown
KSHV029	ORF28	unknown
KSHV030	ORF29b	DNA packaging protein; terminase
KSHV031	ORF30	unknown
KSHV032	ORF31	unknown
KSHV033	ORF32	unknown
KSHV034	ORF33	unknown
KSHV035	ORF34	unknown
KSHV036	ORF29a	packaging protein
KSHV037	ORF35	unknown
KSHV038	PT ORF36	serine protein kinase, phosphotransferase, weak cellular homolog
KSHV039	ORF37	alkaline exonuclease
KSHV040	ORF38	unknown
KSHV041	ORF39	glycoprotein M
KSHV042	ORF40	component of DNA helicase-primase complex
KSHV043	ORF41	component of DNA helicase-primase complex
KSHV044	ORF42	unknown
KSHV045	ORF44	component of helicase-primase complex
KSHV046	ORF43	capsid protein
KSHV047	ORF45 KIE-2	blocks cellular IRF-7
KSHV048	vUDG ORF46	uracil DNA glycosylase cellular homolog
KSHV049	ORF47 gL	glycoprotein L
KSHV050	ORF48	unknown
KSHV051	ORF49	unknown
KSHV052	Rta LytA ORF50 ART	replication and transcription activator
KSHV053	K8 bZIP	unknown

Table A1: Definition of HHV-8 Genes *contd.*

Gene ID	Name(s)	Definition
KSHV053.1	K8.1A	glycoprotein, longer alternatively spliced glycoprotein
KSHV053.2	K8.1B	glycoprotein, shorter alternatively spliced glycoprotein
KSHV054	ORF52	unknown
KSHV055	ORF53	unknown
KSHV056	dUTPase ORF54	deoxyuridine triphosphatase, dUTPase
KSHV057	ORF56	component of DNA helicase-primase complex
KSHV058	ORF55	unknown
KSHV059	ORF57	post-transcriptional regulator
KSHV060	K9 vIRF-1	interferon regulatory factor cellular homolog
KSHV061	K10	unknown
KSHV061.1	K10.5 LANA2 vIRF3	latency-associated nuclear antigen 2, weak cellular homolog
KSHV062	K11	unknown
KSHV062.1	vIRF-2 K11.1	interferon regulatory factor cellular homolog
KSHV063	ORF58	unknown
KSHV064	ORF59 PF-8	DNA replication protein, polymerase processivity factor
KSHV065	ORF60	ribonucleotide reductase small subunit cellular homolog
KSHV066	ORF61	ribonucleotide reductase large subunit cellular homolog
KSHV067	TRI-1 ORF62	triplex subunit 1 (capsid assembly and DNA maturation protein)
KSHV068	ORF63	tegument protein
KSHV069	ORF64	tegument protein
KSHV070	VP26 sVCA SCIP ORF65	small capsomere interacting protein
KSHV071	ORF66	unknown
KSHV072	ORF67	tegument protein
KSHV073	ORF68	major envelope glycoprotein
KSHV074	ORF69	unknown

Table A1: Definition of HHV-8 Genes *contd.*

Gene ID	Name(s)	Definition
KSHV075	K12	kaposin
KSHV076	vFLIP K13	FLICE-inhibitory protein cellular homolog
KSHV077	vCyc v-Cyclin k-Cyclin ORF72	cyclin D cellular homolog
KSHV078	LANA LNA-1 LNA ORF73 LANA1	latency-associated nuclear antigen
KSHV079	vOX-2 vAdh K14	OX-2 membrane glycoprotein, adhesion molecule cellular homolog
KSHV080	ORF74 vGCR vGPCR	G protein coupled receptor cellular homolog
KSHV081	ORF75	tegument protein, FGARAT/FGAM synthase cellular homolog
KSHV082	K15 LAMP	latency-associated membrane protein

APPENDIX 2 –

PUBLICATIONS AND PRESENTATIONS RELATED TO THE THESIS

Publications

Porter SR, Di Alberti L, **Kumar N**. Human Herpes Virus 8 (Kaposi's sarcoma herpesvirus). Oral Oncology 1998; 34: 5-14.

Leao JC, **Kumar N,** McLean K, Porter SR, Scully CM, Swan AV, Teo CG. The effect of Human Immunodeficiency Virus-1 protease inhibitors on the clearance of Human Herpesvirus 8 from blood of human immunodeficiency virus-1 patients. J Med Virol. 2000 Dec;62(4):416-420.

Kumar N, Mclean K, Porter SR, Teo CG. Optimisation of Methods for Fractionating HHV8-Carrying Cells in Peripheral Whole Blood (paper in preparation)

Kumar N, Mclean K, Ward K, Porter SR, Teo CG. The Genotypic characterisation of Human Herpes Virus 8 and cellular tropsim of HHV-8 in Different Groups. (paper in preparation)

Oral and poster presentations

Kumar N. The discovery of a new Human herpesvirus – HHV-8. Oral presentation at Postgraduate Education Meeting. Charing Cross Hospital. December 1996.

Kumar N. Human herpes virus type 8 – yet another cause of human disease? Lecture at Infection Update Meeting. Coppetts Wood Hospital. February 1997.

Kumar N. An update on Human herpes virus 8. Lecture for MSc postgraduates in Oral Medicine. Eastman Dental Institute for Oral Healthcare Sciences. November 1997 and November 1999

Leao JC, Di Albert L, **Kumar N**, Porter SR, Scully CM, Teo CG. Relative restriction of HHV-8 subgenomic sequence heterogeneity in KS tissues compared to peripheral blood of HIV-infected patients. The first annual meeting on Kaposi's sarcoma associated herpesvirus (KSHV) and related agents – **University of California, Santa Cruz. July, 1998**

Kumar N, Di Albert L, Beynon H, McLean K, Porter S, Teo CG. Differential peripheral blood-cell tropism of HHV-8. The first annual meeting on Kaposi's sarcoma associated herpesvirus (KSHV) and related agents – **University of California, Santa Cruz. July, 1998**

Kumar N, Porter S, Teo CG. Prevalence of HHV-8 in Healthy Blood Donors. 6th International Conference on Malignancies in AIDS and other Immunodeficiences: Basic, Epidemiologic and Clinical Research. **Bethesda, USA. April 2002**