GENETIC AND SEROLOGIC CHARACTERIZATION OF HUMAN HERPESVIRUS TYPE 8 (HHV-8) IN SOUTH AFRICA

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A thesis submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the Degree of Master of Science in Medicine

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This thesis is dedicated to my loving family. I thank you for all the years of endurement.

Σας το αφιερωνο με πολλη αγαπη

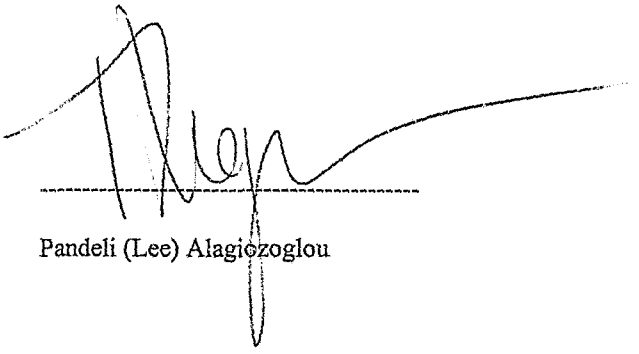
Abstract

Human herpes virus type 8 (HHV-8) is strongly implicated as the etiological agent of Kaposi's sarcoma (KS). The incidence of KS in South Africa is increasing in parallel with the HIV-1 epidemic. Molecular and serological prevalence of HHV-8 in HIV-1 infected individuals with and without KS was investigated. DNA fragments from ORF26 (capsid, $330BAM_{233}$) and ORF75 (tegument) regions were used to determine the prevalence of HHV-8 DNA in peripheral blood mononuclear cells (PBMC) from 429 HIV-1 infected individuals, 95 of whom had histologically confirmed KS. Of those without KS, 14 (4.2%) were PCR positive for HHV-8 DNA. In the individuals with KS, the proportion of HHV-8 DNA positive PBMC samples was 11 times higher (46/95, 48%). Similarly, an immunofluorescence assay showed that 78% of KS patients had antibodies to HHV-8 compared to 16% of KS negative individuals. Among the KS group, 93% of PCR positive samples were also HHV-8 antibody positive compared to only 66% of PCR negative samples indicating that viremia is associated with good antibody responses. Matched lymph node and PBMC samples were available for 8 patients. HHV-8 DNA was more frequently detected in the lymph node (3/8) than in the blood (1/8), suggesting that the lymph nodes are a reservoir for HHV-8. These data confirm the association between HHV-8 and KS and suggest that there is a high background prevalence of HHV-8 infection in HIV-1 infected individuals in South Africa.

The ORF 75 gene of 40 HHV-8 strains was sequenced and the phylogenetic relationships between South African and already published sequences were investigated. The majority (n=29) of strains overlapped with the published A and B subgroups and were termed A/B variants. Three strains were classified as subgroup C while 8 sequences did not cluster with any of the previously classified subgroups and were termed novel (N) group. The DNA distance of this novel group differed from the A, B and C subgroups by 4.7%, 3.8% and 4.5% respectively although within the N group there was only 0.4% variation. The addition of this group significantly increased the number of subgroup-specific polymorphisms from 17 to 47 over a 804 bp region. There was sufficient inter-subgroup genetic diversity that single strand conformational polymorphisms (SSCP) could be used to rapidly identify them. Thus, based on the analysis of the ORF75 gene, a unique HHV-8 sub-group is present in South Africa which accounts for 20% of circulating strains. Further studies are required to determine the extent of evolutionary phylogeny, distribution and pathogenic potential of this novel group.

DECLARATION

I, Pandeli (Lee) Alagiozoglou, declare that this thesis is my own unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, South Africa. It has not been submitted before for any degree or examination at this or any other University.



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Finally, I would like to thank my family for their encouragement and their faith in me. If I can do it Alex, so can you.

The gift of AIDS

I saw you coming towards me with a gift.
You wore slippers made of Kaposi's,
The gown of night and soaking sweats. You moved
As if you had been photographed you blurred
The trees you passed before. You held the box
Inside your chest somehow, the ribbons were
Your arteries, its corners were your spine
And ribs. I pleaded with you, pleaded that
You give me what you hid from me; you laughed
Like it was not as painful as it was for you,
And suddenly the ground was silver clouds
And we were so in love it was impossible
That you were dead. I saw you coming close,
I saw you and you had a gift for me,
The gift of AIDS and blood that was your heart,
You beating heart, your beating, beating heart.

Rafael Campo

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LIST OF ABBREVIATIONS USED IN THE TEXT

Abbreviation	Name
µg	Microgram
µl	Microlitre
µM	Micromolar
AIDS	Acquired Immunodeficiency Syndrome
BCBL	Body-cavity Based Lymphomas
bp	Base Pair
CMV	Cytomegalovirus
dATP	Deoxy-adenosine Triphosphate
dCTP	Deoxy-cytidine Triphosphate
ddATP	Dideoxy-adenosine Triphosphate
ddCTP	Dideoxy-cytidine Triphosphate
ddGTP	Dideoxy-guanosine Triphosphate
ddNTP	Dideoxy Nucleotide Triphosphate
ddTTP	Dideoxy-thymidine Triphosphate
dGTP	Deoxy-guanosine Triphosphate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
dTTP	Deoxy-thymidine Triphosphate
EBV	Epstein - Barr Virus
EDTA	Ethelenediaminetetra-acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
GCPR	G-coupled Protein Receptor
HHV-8	Human Herpesvirus 8
HIV	Human Immunodeficiency Virus
HMA	Heteroduplex Mobility Assay
HSV 2	Herpes Simplex Virus Type 2
HVS	Herpesvirus Saimiri
IFA	Immunofluorescence Assay
IgG	Immunoglobulin G
IFN	Interferon
IL	Interleukin
IPTG	Isopropylthio-β-d-galactoside
IRF	Interferon Regulatory Factor
JAK	Janus Tyrosine Kinase
kD	Kilodalton
KS	Kaposi's Sarcoma

KSHV	Kaposi's Sarcoma-associated Herpesvirus
LANA	Latency Associated Nuclear Antigen
LB	Luria Broth
M	Molar
MCD	Multicentric Castleman's Disease
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
MHV-68	Murine Herpesvirus-68
MIP	Macrophage Inflammatory Protein
ml	Millilitre
mM	Millimolar
NaCl	Sodium Chloride
nm	Nanometre
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phoshate Buffered Saline
PCR	Polymerase Chain Reaction
PEL	Primary Effusion Lymphoma
Rb	Retinoblastoma
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulphate
SSCP	Single Strand Conformation Polymorphism
STAT	Signaling Transducers and Activators of Transcription
STP	Saimiri Transforming Protein
TEMED	N,N,N',N'-tetraethylmethylethylenediamine
TR	Terminal Repeat
UV	Ultraviolet
VP	Viral Protein
WB	Western Blot
X-gal	5-bromo-4-chloro-3-idolyl-β-d-galactoside
ZA	South Africa

PUBLICATIONS ARISING FROM THIS STUDY

Lee Alagiozoglou, Helba Bredell, Henry Carrara, Rosana Pacella-Norman, Des Martin, Freddy Sitas and Lynn Morris. **Molecular and serological prevalence studies of Human herpes virus - 8 in HIV-1 infected patients in South Africa.** In preparation

Lee Alagiozoglou, Freddy Sitas, and Lynn Morris. **Phylogenetic analysis of HHV-8 in South Africa and identification of a novel subgroup.** Submitted to the Journal of General Virology.

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First annual Meeting of Kaposi's Sarcoma associated Herpesvirus (KSHV) and Related Agents. University of California, Santa Cruz, USA. 23 July - 1 August, 1998

Jian-Chao Zong, Dolores M. Ciufo, Lee Alagiozoglou and Gary S. Hayward. **Genetic diversity in KSHV: Observations and implications about evolutionary history, virus spread and potential biological diversity.**
The Third International Conference on Human Herpesviruses 6, 7 and 8. Clearwater Beach, Florida, USA. May 13 - 15, 1999

1. LITERATURE REVIEW

1.1 Kaposi's sarcoma

Kaposi's sarcoma (KS) was first described in 1872 by Moritz Kaposi as an indolent skin cancer commonly found in elderly Italian men (Kaposi, 1872). In 1972, virus-like particles resembling herpes virions were observed under the electron microscope in cell lines derived from KS tissues (Giraldo *et al.*, 1972b). These particles showed similarities to Epstein Barr Virus (EBV) and Cytomegalovirus (CMV) and so it was believed that a herpesvirus of the B subgroup was responsible for KS (Giraldo *et al.*, 1972a). In 1974, KS was described in organ transplant recipients undertaking immunosuppressive therapy (Myers *et al.*, 1974), but it was not until a decade later with more wide-spread immunosupression associated with the AIDS epidemic, that KS gained full prominence. In 1994, human herpesvirus 8 (HHV-8) was identified and implicated as the etiological agent of KS (Chang, 1994).

The different forms of KS are epidemiologically distinct, occurring in different groups of individuals (Wahman *et al.*, 1991). In general, classic KS is found in elderly Mediterranean or eastern European men (Oettle, 1962), endemic-KS in HIV-negative individuals in equatorial Africa (Martin III *et al.*, 1993), iatrogenic KS in solid organ transplant recipients (Fife & Bower, 1996) and AIDS-related KS in HIV-positive individuals (Wahman *et al.*, 1991). Except for AIDS KS, which is now the dominant form, the other forms of KS have remained confined to small niche groups. Although KS was found in central Africa before the AIDS epidemic, it is now the most frequently occurring cancer in HIV- infected people in this region (Wabinga *et al.*, 1993). The risk of acquiring KS is 20,000-fold higher in HIV-infected patients than in normal individuals (Beral *et al.*, 1990) and KS is now considered an AIDS-defining condition (Cesarman & Knowles, 1997, Hermans *et al.*, 1997). Among HIV-infected individuals in developed countries, homosexual or bisexual males have the greatest risk of developing KS compared to individuals with haemophilia, intravenous drug users or blood transfusion recipients (Beral *et al.*, 1990, Hermans *et al.*, 1996).

The four forms of KS are histologically indistinguishable from one another (Boshoff *et al.*, 1995b, Cesarman, 1995, Chang, 1994, Dupin *et al.*, 1995, Schalling *et al.*, 1995). KS presents as a variable mixture of irregularly shaped, round capillary and slit-like endothelial lined vascular spaces and spindle cells. The tumour is composed of inflammatory cells of predominantly lymphocytes, macrophages, red blood cells and hemosiderin pigment. The spindle cells eventually become the predominant cell type in the population forming fascicles that compress the vascular slits and the lesions become progressively nodular. KS tumours are usually multifocal and appear initially on the extremities of the skin such as the face and genitalia (Tappero *et al.*, 1993). The lesions usually involve the oral mucosa, lymph nodes and various organs such as lung, spleen, liver and gastrointestinal tract. However, any organ and major vessel can be involved. In immunocompetent hosts, KS is an indolent localised tumour suggestive of a low malignant potential. The clinical behaviour of African endemic KS often exhibits aggressively and in children it is fulminant and often fatal (Chang *et al.*, 1996). In AIDS patients, KS lesions disseminate to the lymphoid tissues and viscera and occur at sites of traumatic injury and can be disfiguring or even fatal (Cesarman & Knowles, 1997, Ganem, 1997). The appearance of oral or perioral lesions is often an indication of severe HIV disease (Porter & Scully, 1994).

1.2 Discovery of HHV-8

Although KS was first described over a century ago, it was not until 1994 that the etiological agent was discovered. Unique DNA sequences were identified in lesional tissues from a patient with AIDS using a technique called representational difference analysis (Chang, 1994, Lisitsyn, 1993). These sequences were present in infected KS tissue, but absent or present in low copy number in non-diseased tissue from the same individual. This method based on polymerase chain reaction (PCR) kinetic enrichment and subtractive hybridization identified two Bam HI restricted sequences which were subsequently cloned and sequenced and termed KS330*Bam* and KS631*Bam* (Chang, 1994). KS330*Bam* has 51% amino acid homology to ORF26, the open reading frame of the VP23 capsid protein of the herpesvirus saimiri (HVS) which causes fulminant lymphoma in squirrel monkeys (Albrecht *et al.*, 1992). Although a virus was not yet isolated, this set of unique DNA sequences with close homology to other herpesviruses was

considered to be a putative herpesvirus and given the name Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus type 8 (HHV-8). There was a strong association between the presence of HHV-8 sequences and KS: ORF26 fragments were detectable in over 90% of KS lesions (Chang, 1994). KS631*Bam* is homologous to ORF75, the tegument protein of HVS and both fragments show homology to EBV. Since HHV-8 shows close homology to EBV and HVS, which are both members of the *gammaherpesvirinae* subfamily of herpesvirus or rhadinovirus, HHV-8 was subsequently placed in this genus and is the first member of the rhadinoviruses genus known to infect humans (Moore *et al.*, 1996b) (Russo *et al.*, 1996) (Fig. 1). Both EBV and HVS are involved in tumorigenesis. EBV is a B-cell immortalising virus that causes malignant lymphomas whereas HVS immortalises T cells and is involved in the pathogenesis of T cell lymphomas. Homology of HHV-8 to viruses with transforming potential led to the idea that this novel herpesvirus is oncogenic and may play a role in the pathogenesis of KS.

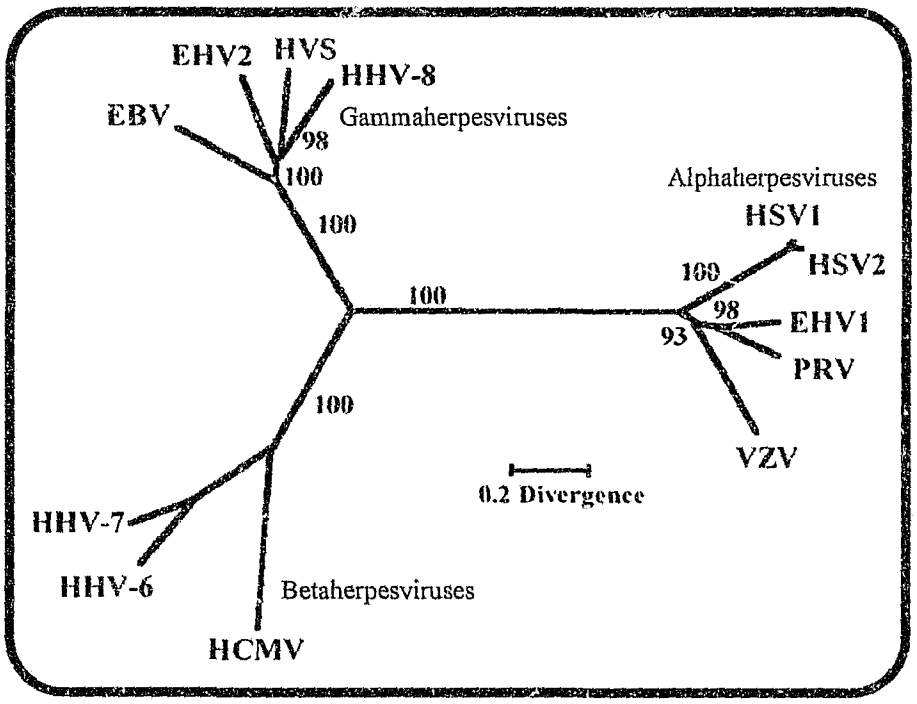


Figure 1: Phylogenetic comparison of 13 representative herpesviruses. The tree shows the amino acid sequences of the major capsid proteins and clearly shows that HHV-8/KSHV is a gamma-herpesvirus belonging to the genus *Rhadinovirus.*

Reprinted from Moore *et al.* (Moore *et al.*, 1996b)

HHV-8 DNA has also been found in 2 other neoplastic disorders, namely primary effusion lymphoma (PEL), also termed body-cavity-associated lymphomas (BCBL) (Cesarman & Knowles, 1997, Cesarman *et al.*, 1995), and Multicentric Castleman's disease (MCD) (Soulier *et al.*, 1995). PEL is a rare form of non-Hodgkin's lymphoma (NHL), characterised by malignant effusions in the abdominal cavity frequently found in AIDS patients. This malignancy is known to contain heavy and light chain immunoglobulin gene rearrangements indicative of a B-cell origin and lacks most cell lineage surface markers. PEL is usually coinfected with EBV and HHV-8, but HHV-8-positive/EBV-negative forms have been reported. These lymphomas grow mainly in the pleural, pericardial and peritoneal cavities. MCD is an atypical form of lymph node hyperplasia that presents with generalised lymphadenopathy and can occur in AIDS and less often in HIV-uninfected patients and presents as two variants: the hyaline-vascular variant and the plasma cell variant in which HHV-8 is more frequently detected (Schulz, 1998). The association between HHV-8 and MCD remains controversial. Only 13-41% of HIV-negative individuals with MCD have HHV-8 DNA although it is higher in patients with HIV-associated MCD (75-100%) (Peterson & Frizzera, 1993, Soulier *et al.*, 1995).

1.3 The virology of HHV-8

1.3.1 Virus isolation and propagation

HHV-8 was first isolated in 1996 using a BC-1 cell line which produces infective herpesviral particles (Mesri *et al.*, 1996), but serial propagation of HHV-8 was only achieved in 1997 using the 293 cell line (Foreman *et al.*, 1997). 293 cells are derived from adenovirus-transformed human embryonal-kidney epithelial cells and are EBV-free indicating that HHV-8 does not require EBV as a helper virus for replication (Foreman *et al.*, 1997). Further studies of HHV-8 have also been facilitated by the establishment of BCBL-1 and BC-3 cell lines from PEL which contain HHV-8, but lack EBV. Although spindle and endothelial cells in KS tissue contain HHV-8 genomes, these are lost during passage in cell culture suggesting that viral replication in these cells is limited. BCBL cell lines, on the other hand, maintain latent HHV-8 genomes which can be induced into a lytic state of replication using phorbol-esters (Renne *et al.*, 1996b). This has allowed visualisation of the virus by electron microscopy which is approximately 100-150 nm in size (Foreman *et al.*, 1997, Renne *et al.*, 1996b, Said *et al.*, 1997). HHV-8 DNA is

enclosed by a protein capsid and enveloped by a lipid membrane. This was shown by treating the viral particles with DNAse-1, which were resistant unless pretreated with detergents and proteolytic enzymes to disrupt the lipid bilayer of the viral envelope (Foreman *et al.*, 1997, Renne *et al.*, 1996b). Fractionation of PEL cells established that the HHV-8 genome is localised to the nuclear compartment of the cell (Foreman *et al.*, 1997). HHV-8 does not integrate into the human genome as a provirus but exists as an episome (Cesarman *et al.*, 1995). One copy of the HHV-8 genome has been detected per KS lesional cell, whereas, B-cells of BCBL's contain 40-60 copies suggesting a pathogenic role of this virus in these lymphomatous effusions (Cesarman, 1995, Cesarman *et al.*, 1995).

1.3.2 Latent and lytic viral replication cycles

HHV-8 causes both latent and lytic infections. Lytic infection results in production of virions which are released from the infected cell leading to cytolysis. Latent infection is characterised by maintenance of a low copy number of genomes in the nucleus; reduction of viral gene expression and no viral progeny production. Patterns of viral gene expression indicate that the endothelial (spindle) cells contain HHV-8 DNA. The majority of these KS cells are latently (persistently) infected as they express only the latent T0.7 transcript, consistent with the idea that HHV-8 exists as an intranuclear episome (Renne *et al.*, 1996a, Staskus *et al.*, 1997). However, approximately 0.5-1% of the latently infected cells also express T1.1 lytic transcripts suggesting that it may be this subpopulation of the infected cells that sustain viral infection (Zhong *et al.*, 1996).

1.3.3 Latent and lytic genomes

The size of the genome differs in virions and infected cells. DNA from HHV-8 virions is a linear molecule migrating about 150-200 kb in a pulse-field electrophoretic gel. BCBL-1 cell lines maintain a latent HHV-8 infection and kinetic analysis has shown their genome to be an extrachromosomal episome of 250-270 kb. HHV-8 can be induced by phorbol esters or butyrate to undergo lytic viral infection. The genome of the induced cells migrates in electrophoretic gels consistent with it being a linear DNA form and indicating that phorbol esters induce the latent

episomal virus to undergo lytic replication (Decker *et al.*, 1996). The latent and lytic DNA bands of HHV-8 co-migrate similar to the EBV and HVS genomes as expected given that these genomes show colinearity (Renne *et al.*, 1996a). The linear and episomal DNA forms of HHV-8 can be distinguished by running *in situ* lysis agarose (Gardella) gels which are resolved on the basis of differential migration.

1.3.4 Viral tropism

HHV-8 particles from PEL cell lines and KS lesions are infectious for human peripheral blood mononuclear cells (PBMC's) (Harrington *et al.*, 1996). The CD19+ cell fraction (B cells) were shown to be infected suggesting viral tropism for B cells (Mesri *et al.*, 1996). B cells are productively infected by HHV-8 as linear genomes have been isolated from these cells, however, monocytes/macrophages express the circular genome suggesting latent infection (Blasig *et al.*, 1997, Decker *et al.*, 1996). HHV-8 also infects the 293 human embryonal kidney epithelial cell line when cocultured with KS biopsy specimens. These cells exhibit nuclear and chromatin changes as well as syncytial cell formation indicating productive infection (Foreman *et al.*, 1997). Differences in gene expression between KS spindle cells and HHV-8 infected B-cells have also been documented with lymphatic B-cells exp sing an inducible viral-derived interleukin-IL 6 (v-IL6) gene absent in the KS spindle cells (Boshoff *et al.*, 1995a, Moore *et al.*, 1996a, Staskus *et al.*, 1997). These cytokines may contribute to the pathogenesis of KS (see 1.6).

1.4 Genomic organisation

The viral genome is a large, covalently closed, circular DNA episome varying in length from 170-270 kb depending on the source of cells used to isolate the virus (Mesri *et al.*, 1996, Renne *et al.*, 1996a) (Decker *et al.*, 1996). In BC-1 cell lines, a 20.7 kb region of the genome has been sequenced and the genomic organisation was shown to be very similar to HVS and murine herpesvirus-68 (MHV-68) (Russo *et al.*, 1996). In Fig. 2 the 81 open reading frames (ORF's) are shown within a long coding region of 140.5 kb and flanked by two terminal repeats (TR's). The variability in genome size is a result of duplication of the TR's within strains. The TR's consist of multiple 801 bp repeat subunits with an 85% G+C content (Neipel *et al.*, 1997b,

Russo *et al.*, 1996). The HHV-8 gene nomenclature corresponds to the HVS ORF's except for 15 genes which are unique to HHV-8 and are assigned a K prefix. The genes conserved within the gammaherpesviruses are collinear and encode proteins or enzymes which are required for replication and asse nbly of new viral progeny. This includes the major capsid protein (ORF25), thymidine kinase (ORF21), DNA polymerase (ORF9), glycoproteins such as gB (ORF8) and viral protease and assembly protein (ORF17) (Moore *et al.*, 1996b, Russo *et al.*, 1996, Unal *et al.*, 1997). The non-conserved genes appear to have important pathophysiological functions in lymphoid immortalisation and transformation. These include growth factor homologues such as vIL-6 (K2), macrophage inflammatory protein (MIP)-1α (K4), a protein with an anti-apoptotic function (K13) and K1 and K12 which are believed to be involved in cell transformation (Neipel *et al.*, 1997b, Nicholas *et al.*, 1997a, Nicholas *et al.*, 1997b, Nicholas *et al.*, 1998, Russo *et al.*, 1996, Schulz, 1998).

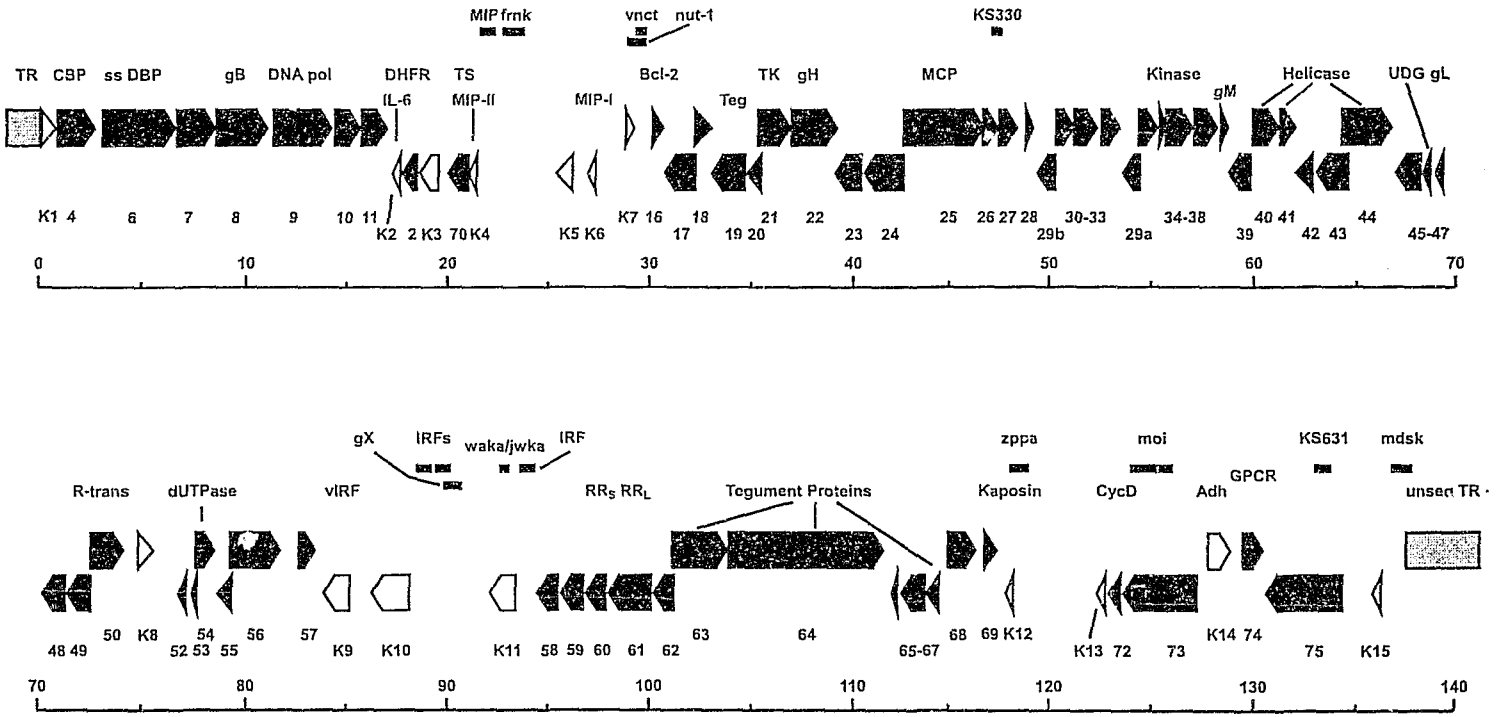


Figure 2: Genetic map of HHV-8. This map shows the 140.5 kb coding region of HHV-8 flanked by 2 terminal repeats (TR's). The dark areas represent conserved genes and the light areas are the 15 genes unique to this virus. The arrows indicate the direction of gene expression. Genes of interest are the ORF26 used in $KS330BAM_{233}$ amplification, ORF75 and ORF K1 used in subgroup determination. ORF K2 (vIL-6), ORF72 (vCyclin D) and ORF K4 (vMIP-2) are viral homologues to proteins involved in cell growth and angiogenesis.

Reprinted from Moore *et al.* (Moore *et al.*, 1996b)

1.5 Subtypic variation

HHV-8 has a highly conserved genome with only slight overall genetic variation between different isolates. A comparison of a 20 kb region from a KS lesion and a PEL cell line, encompassing regions ORF20 - ORF35, showed only 0.1% variation (Russo *et al.*, 1996). In a 190 kb genomic region from an AIDS BCBL cell line and a KS lesion, a 0.4% variation was noted (Poole *et al.*, 1999). However, the genome contains regions, such as ORF26 and ORF75, which show a 1.5% overall sequence variation when combined (Zong *et al.*, 1997). Subtyping of this virus has been done using these two regions and it was shown that three narrow, but distinct subgroupings of HHV-8 exist. Subgroups B and C predominate in Africa, whereas, subgroup A is associated with Mediterranean KS. All three subgroupings were found in AIDS patients in the USA, but the majority presented with very little variability, suggesting that they originated from a single common isolate transmitted during the AIDS epidemic (Zong *et al.*, 1997). Recently, Meng and coworkers found that individuals from the USA were infected with both A and C subgroups [Meng, 1999 #2227]. These researchers also identified a new sub-group of 12 isolates from a Zambian cohort which they designated subgroup Z. The distribution and occurrence of these subgroups in South Africa is unknown and will form part of the research of this paper

The first translatable gene of the HHV-8 genome, ORFK1, has recently received much attention partly because amino acid variation of up to 62% and nucleic acid variation of 16% has been documented [Zong, 1999 #2189; Lagunoff, 1997 #406; Russo, 1996 #1283; Lee, 1998 #1438; Nicholas, 1998 #1659; Cook, 1999 #2218; Meng, 1999 #2227]. This region encodes a glycoprotein related to an immunoglobulin receptor and sequencing this gene has revealed four major subgroups, A-D, and thirteen variants or clades. The subgroups differ from one another by 15-30% and there appears to be geographical and ethnic group distinction among the various clades (Fig. 3). The B subgroup is predominantly found in KS individuals from Africa. The C subgroup is found in classic, iatrogenic and AIDS-related KS in the Middle East and Asia (mainly C2). AIDS-KS specimens from the U.S.A were predominantly clustered with the A1, A4 and C3 variants. The D subgroup has been found only in the Pacific Islands. There appear to be two levels of variability in the ORFK1 gene, namely between the A and C subgroups and the B and D subgroups, as shown by the clustering of these groups in Fig. 3 (Zong *et al.*, 1999).

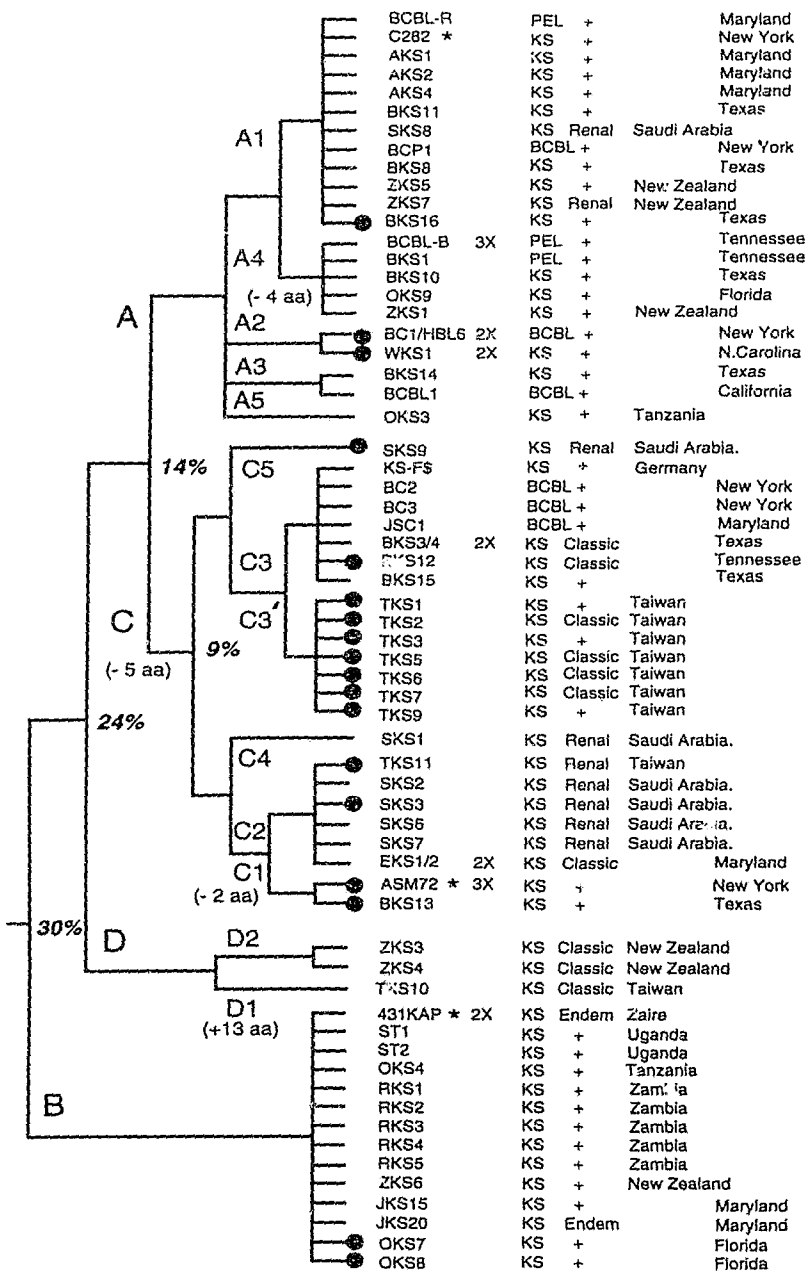


Figure 3: ORFK1 protein sequence phylogeny. Comparison of 63 ORF K1 protein sequences from USA, Germany, Taiwan, New Zealand, central and southern Africa and Saudi Arabia show four distinct groups delineating the A, B, C and D subgroups. Within the four major groups, numerous subgroupings have been defined. Group D is the latest cluster found comprising three patients from New Zealand and Taiwan. The different forms of KS associated with these isolates is also indicated. (+) indicates HIV-infected individuals with BCBL/PEL.

Reprint from Zong *et al.* (Zong *et al.*, 1999)

Although the authors state that these groupings are distinct, it should be noted, however, that the bootstrap values delineating these subgroups are very low bringing their significance into question. Recently, a different subtypic nomenclature has been established based on the ORFK1 and 2 glycoprotein regions (gB and gH) [Meng, 1999 #2227]. Four genotypes were classified in which genotype I, II and IV correspond to A, C and B of the Zong nomenclature respectively. Genotype III was unique as it was found only in Australia and believed to have originated from that continent. Since Australia contains a mixed population with numerous migrants, it could be possible that this genotype exists in other population groups.

Based on sequencing analysis of ORFK1, it is hypothesized that HHV-8 is an old virus transmitted familialy and associated with the migration of human populations out of Africa dating as far back as 35,000 to 60,000 years ago (Hayward, 1999). These migrations occurred in three waves; the first occurring about 100,000 years ago from Africa to the Middle East; the second migration occurred approximately 60,000 years ago from the Middle East and reached south Asia and Australia with the third migration branching about 35,000 years ago into northern Asia and southern Europe. The latest expansion from south Asia to the Pacific Islands occurred only 4,000 years ago. Based on this, it is suggested that the oldest subgroup is the B clade originating from Africa. The A and C subgroups are relatively new arising approximately 35,000 years ago in the middle East, Europe and Asia and possibly giving rise to the D subgroup in the Pacific Islands. This hypothesis allows for horizontal transmission to occur over time with selective pressure on the ORFK1 generating diversity.

At the extreme opposite side of ORFK1, within the coding region, are a further two highly divergent alleles, namely P (predominant) and M (minor), in the ORFK15 gene. There is a 29% amino acid identity between these 2 alleles which encode an integral membrane protein related to the LMP2 of EBV. The M and P alleles have proven useful for subtyping although they are not linked to the ORFK1 subgroup patterns. Unlike the hypervariability observed in the ORFK1 region these genes contain conserved regions believed to be tyrosine kinase signalling motifs. The M allele is most frequently found in classical and AIDS-KS patients, but has not been found in Africa or the Pacific Islands. Subgroup determination from various loci of the genome and how they compare to subject geographic locations is shown in Table 1 (Poole *et al.*, 1999).

OVERALL SUMMARY OF KSHV / HHV8 STRAIN VARIATION:

NAME	TYPE	SOURCE	HETEROGENEOUS LOCI									OVERALL SUBTYPE
			K1	ORF26	T0.7	LANA	ORF75E	UPS75	K14.1	K15	U/TTR	
BCBL-R	B+	U	A1	A	A/C		A/C	A/C	A/C	P	A/C	A1/P
C282	K+	U	A1	A	A/C	A/C	A/C	A/C	A/C		A/C	A1/P
AKS1	K+	U	A1	A	A/C		A/C	A/C	A/C			A1/P
AKS2	K+	U	A1	A	A/C		A/C	A/C	(P)			A1/P
AKS4	K+	U	A1	A			A/C	A/C	(P)			A1/P
BKS11	K+	U	A1	A	A/C		A/C		(P)			A1/P
SKS8	KR	S	A1	A	A/C		A/C		(P)			A1/P
BCP1	B	U	A1'	A	A/C		A/C		(P)	(P)		A1/P
BKS8	K+	U	A1'	A	A/C		A/C		(P)			A1/P
ZKS5	K+	Z	A1'	A	A/C		A/C		(P)			A1/P
ZKS7	KR	Z	A1'	A	A/C		A/C		(P)			A1/P
BCBL-B	B+	U	A4	A	A/C		A/C		(P)	(P)	A/C	A4/P
BKS1	B+	U	A4	A	A2		A/C		(P)			A4/P
BKS10	K+	U	A4	A	A/C		A/C		(P)			A4/P
OKS9	K+	F	A4	A	A/C		A/C		(P)		A/C	A4/P
ZKS1	K+	Z	A4	A	A/C		A/C		(P)			A4/P
HBL6/BC1	B+	U	A2	A	A2	A2	M	M	M	M	-	A2/M
HKS1	K+	U	A2						(M)			A2/M
BKS16	K+	U	A6	A	A2		M		M	(M)		A6/A2/M
BKS18	KC	U	A6						(M)			A6/??/M
BCBL1	B	U	A3	A3	A3	A3	A3	A/C	A/C	P	A3	A3/P
BKS14	K+	U	A3	A3	A3		A3		(P)			A3/P
MKS6	KC	SC	A3									A3/?
BKS20	KC	U	A7						(P)			A7/P
KS-F	K+	G	C3	A	A/C		A/C					C3/A/P
BKS12	KC	U	C3	A	A2		M		(M)			C3/A2/M
SKS7	KR	S	C2	A	A2		A/C		(P)			C2/A2/P
EKS1/2	KC	U	C2	A	A2		A/C	A/C	(P)			C2/A2/P
RGKS4	KC	SI	C3	A			A/C					C3/A/P
RGKS5	KC	SI	C3	A			A/C					C3/A/P
SKS9	KR	S	C5	C2	M		M		M			C5/M
BC2	B+	U	C3	C3	A/C	A/C	A/C	A/C	A/C	(P)	A/C	C3/P
BC3	B+	U	C3	C3	A/C		A/C	A/C	A/C	(P)	/C	C3/P
JCS1	B+	U	C3	C3	A/C		A/C		(P)			C3/P
BKS3/4	KC	U	C3		A/C				(P)			C3/P
BKS15	K+	U	C3	C3	A/C		A/C		(P)	(/)	A/C	C3/P
MKS2	KC	SC	C3	C3	A3				(M)			C3/A3/M
TKS1	K+	T	C3'	C2'	M		M	M	(M)	(M)	-	C3/C2/M
TKS2	KC	T	C3'	C2'	M		M	M	(M)	(M)	-	C3/C2/M
TKS3	K+	T	C3'	C2'	M		M		(M)	(M)	-	C3/C2/M
TKS5	KC	T	C3'	C2'	M		M		(M)	(M)		C3/C2/M
TKS6	KC	T	C3'						(M)	(M)		C3/C2/M
TKS7	KC	T	C3'	C2'	M		M		(M)	(M)		C3/C2/M
TKS9	K+	T	C3'	C2'	M		M		(M)	(M)		C3/C2/M
SKS1	KR	S	C4	C2	C4		A/C		(P)		A/C	C4/P
TKS11	KR	T	C2	C2	M		M		(M)	(M)		C2/M
SKS2	KR	S	C2	C2	A/C		A/C	A/C	(P)			C2/P
SKS3	KR	S	C2	C2	A3		M		(M)			C2/A3/M
SKS6	KR	S	C2	C2	A/C		A/C		(P)			C2/P
MKS1	KC	SC	C2	C3								C2/C3/?
MKS3	KC	SC	C2	C2								C2/?
MKS5	KC	SC	C2									C2/?
BKS21	KC	U	C2						(M)			C2/M
ASM72	K+	U	C1	C1	M	M	M	M	M	(M)	-	C1/M
BKS13	K+	U	C1	C2	M		M		M	(M)		C1/M
491KAP	KC	A	B	B1	B1	B	B	B1	B	P	B1	B/P
ST1	K+	A	B	B/C			A/C	A/C				B/A/P
ST2/3	K+	A	B	B2			B	B1				B/P
OKS3	K+	A	A5	B/C	B1		A/C		(P)		A/B	A5/B/A/P
OKS4	K+	A	B	B1	B2		B		(P)		B2	B/P
RKS1	K+	A	B	B2	B2		B		(P)		B2	B/P
RKS2	K+	A	B	B2	B1		A/C		(P)		A/B	B/A/P
RKS3	K+	A	B	B2	B2		B		(P)		B2	B/P
RKS4	K+	A	B	B2	B2	B	B		(P)		B2	B/P
RKS5	K+	A	B	B/C	B1		A/C		(P)		A/B	B/A/P
ZKS6	K+	A	B	B/C	B1		B		(P)			B/P
JKS20	KC	A	B	B/C	B1		A/C		(P)			B/A/P
SAKS1	K+	SA	B					B3				B/P
SAKS3	K+	SA	B					B2				B/P
SAKS5	K+	SA	B	B2	B3			B2				B/P
SAKS6	K+	SA	A5	B4	B4			Q				A5/B/Q
SAKS7	K+	SA	A5					Q				A5/B/Q
JKS15	K+	U	B	B2	B3		A/C	B4	(P)		A/B	B/A/P
OKS7	K+	F	B	B/C	B3		A/C	B4/M	(M)	(M)	-	B/A/M
OKS8	K+	F	B	B/C	B3		A/C	B4/M	(M)	(M)	-	B/A/M
ZKS3	KC	P	D2	D	D	D	D2	D	(P)			D2/P
ZKS4	KC	P	D2	D	D		D2		(P)			D2/P
TKS10	KC	P	D1	D	D		D1	D	(P)		D	D1/P

K=KAPOSI'S SARCOMA, C=CLASSIC, R=IATROGENIC, B=BCBL/PEL, +=HIV/AIDS-ASSOCIATED
A=AFRICA, F=FLORIDA, G=GERMANY, P=PACIFIC, S=SAUDI ARABIA, T=TAIWAN, U=USA,
SA=SOUTH AFRICA, SI=SICILY, Z=NEW ZEALAND. ( ) = Size Measurement Only

Table 1: Comparison of subtypic variation within 8 regions of the HHV-8 genome. The subgroup determination per region for each specimen is shown as well as the overall subgroup. Subgroup determination is dependent on the region of the genome sequenced. Although some overlap exists between the different loci.

Reprinted from Poole et al (Poole *et al.*, 1999)

1.6 Genetic pirating and pathogenesis

Rhadinoviruses are known to contain genes which are homologues of human cellular genes and which mimic human cytokines, cellular receptors and cell cycle enzymes (Nicholas *et al.*, 1997a). HHV-8 has pirated 15 cellular genes which have not been found in other herpesviruses. These gene homologues are believed to be involved in transformation and cell proliferation. ORF72 has high homology to the mammalian cyclin D family of proteins which are required for cellular division and proliferation (Chang, 1996, Peters, 1994). Human D1 cyclin has been implicated in development of parathyroid tumours (Arnold *et al.*, 1989) and hepatocellular carcinomas (Zhang *et al.*, 1993). These regulatory proteins activate cellular kinases to phosphorylate and inactivate the retinoblastoma-tumour suppressor protein (Murphy, 1997). HHV-8, therefore, has the potential to overcome G_1/S cell-cycle arrest and aid in tumour development (Fig. 4) (Li *et al.*, 1997).

vIL6 encoded by ORFK2 has been detected in BCP-1 cell lines, KS lesions, lymph nodes and areas rich in B-cells (Porter *et al.*, 1998). It has been reported that vIL-6 shares 25% amino acid identity with human IL-6 and is functionally active on human myeloma cell lines (Burger *et al.*, 1998, Moore *et al.*, 1996a, Neipel *et al.*, 1997a, Nicholas *et al.*, 1997b). vIL-6 triggers the Janus tyrosine kinase (JAK) and signalling transducers and activators of transcription (STAT) signalling pathways resulting in phosphorylation of signal transducers which enter the nucleus leading to transcriptional activation of target genes (Molden *et al.*, 1997). vIL-6 may play a role in the life cycle of HHV-8 by promoting viral growth through interaction with the IL-6 receptor (IL-6R) on KS spindle cells and in the pathogenesis of the virus as IL-6Rs are widely distributed in the body. As such, the viral version of this cytokine may be secreted and allowed to spread to adjacent cells providing a stimulus for the growth of cells latently infected with HHV-8. Alternatively, vIL-6 may recruit uninfected cells which become targets for viral infection (Fig. 5).

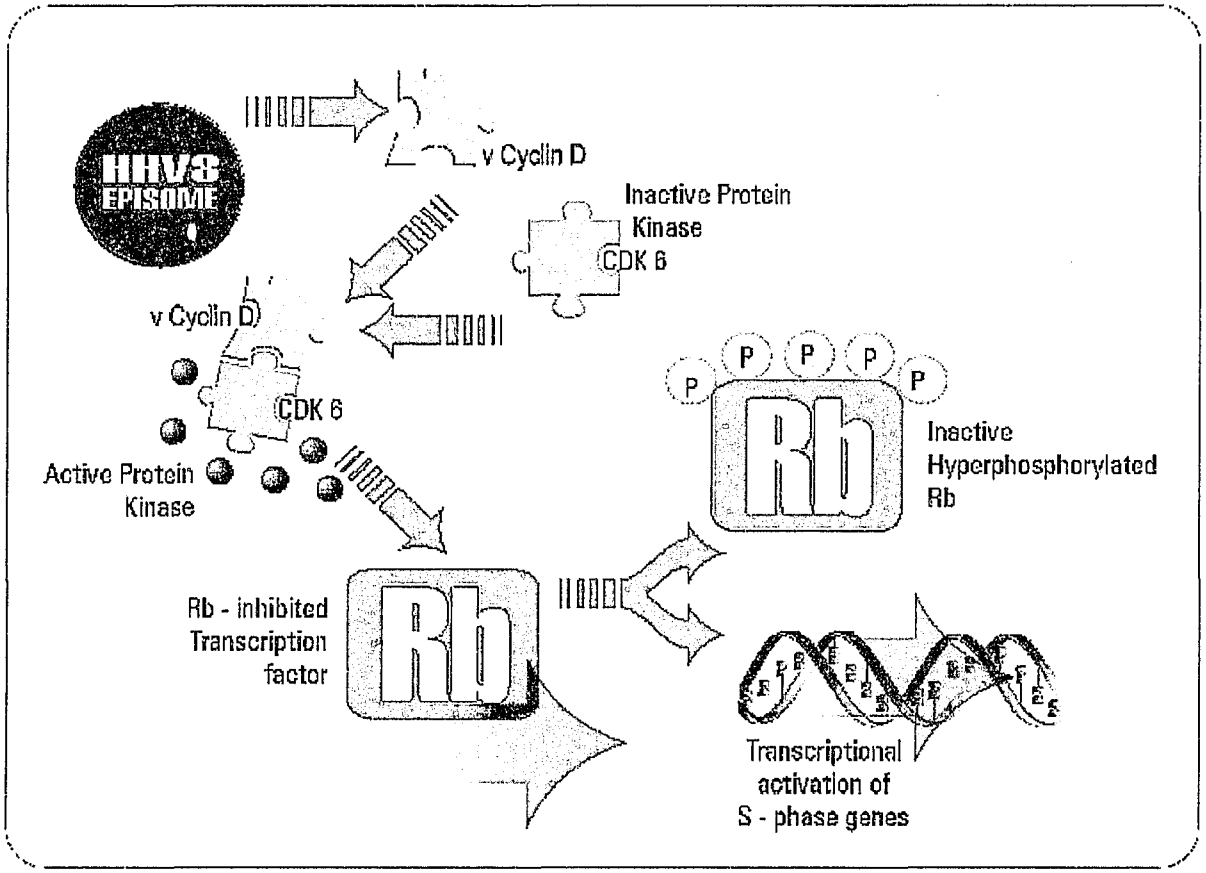


Figure 4: HHV-8 vCyclinD: effects in tumorigenesis. The retinoblastoma protein (Rb) is a tumour suppressor that regulates the G1 to the S phase of cell cycle progression. The vCyclin D, expressed by HHV-8, activates the cyclin-dependant kinase 6 (CDK 6). This kinase phosphorylates the Rb protein, preventing it from binding to transcription factors important for DNA synthesis and cell growth. This results in uncontrolled cell growth and viral-mediated tumorigenesis.

Redrawn from Gillison *et al.* (Gillison & Ambinder, 1997)

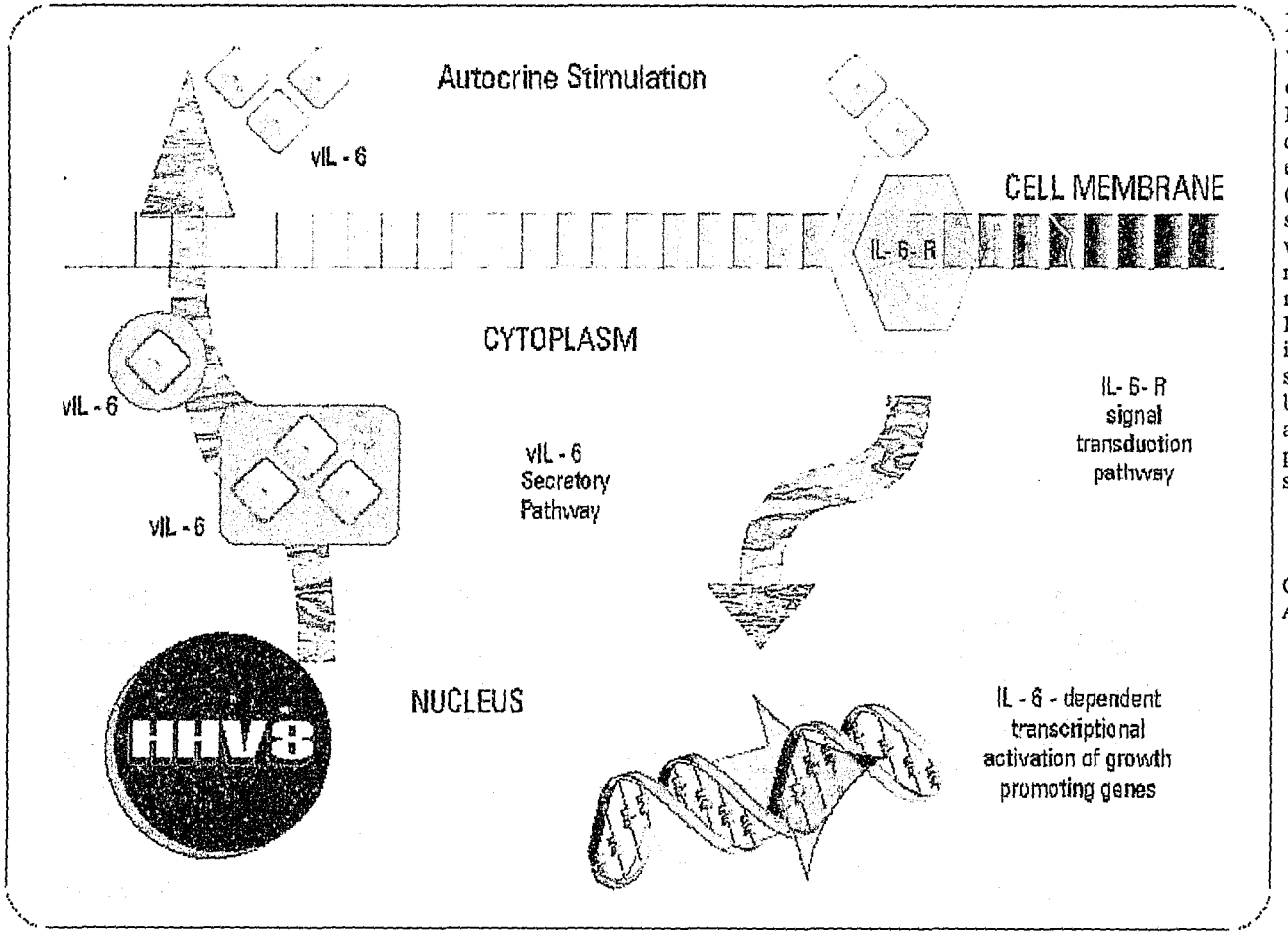


Figure 5: HHV-8 vIL-6: promotion of cell growth.
Both KS cells spindle cells and B lymphocytes express IL-6 receptors (IL6-R) on their cell surface. HHV-8 encoded vIL-6 is functional and may act in an autocrine manner by binding to the IL6-R on these cells. IL-6 induces the JAK/STAT signaling pathways leading to transcriptional activation of growth promoting genes and stimulation cell growth.

Redrawn from Gillison *et al.* (Gillison & Ambinder, 1997)

Apoptosis or programmed cell death is regulated by the proto-oncogene *bcl-2* whose homologue in HHV-8 is encoded by ORF16. This gene is involved in the pathogenesis of follicular lymphomas (Cleary *et al.*, 1986). HHV-8 v*bcl-2* can prevent early apoptotic cell death in cells infected with the virus allowing lytically infected cells to survive long enough to produce infectious viral progeny (Sarid *et al.*, 1997).

A cellular homologue to the interferon regulatory factor (IRF) family of transcription factors is ORFK9 (vIRF) which shares 13% amino acid identity to the cellular version (Moore *et al.*, 1996a). These transcription factors are DNA binding proteins that activate or suppress promoters containing variations on the IRF binding sequence (Harada *et al.*, 1994). It has been shown that vIRF can inhibit responses in endothelial cells to IFN-α, IFN-γ and IRF-1 at the transcriptional level. By interfering with gene induction mediated by IRF-1, HHV-8 inhibits cellular antiviral and inflammatory immune responses, such as IFN-α, IFN-β, IL-1β, RNA-activated protein kinase and expression of major histocompatibility complex class I molecules, allowing the virus to evade immune detection (Zimring *et al.*, 1998). Since vIRF can obstruct regulation of IRF-1, which is considered to be a tumour suppressor, the viral oncogene may play a role in neoplastic transformation (Gao *et al.*, 1997, Zimring *et al.*, 1998).

ORFK1, a transmembrane glycoprotein in HHV-8 is positioned at the same locus as saimiri transforming protein (STP) in HVS and LMP1 of EBV which are the main transforming oncogenes of these viruses. However, ORFK1 shows considerable amino acid differences from these oncogenes. While ORFK1 shows significant sequence and length variation between different isolates in the extracellular region, the cytoplasmic domain is entirely conserved (Nicholas *et al.*, 1998). When expressed in Rat1 fibroblasts the ORFK1 protein exhibited transforming potential. When the transforming gene (STP) of HVS was replaced by K1, marmoset CD8 T-cells were immortalised and lymphoproliferative disease occurred (Lee *et al.*, 1998). The cytoplasmic region of ORFK1 is probably the important domain for transforming activity. The variability in the extracellular domain may be due to its affinity for various receptors. However, the role of ORFK1 has yet to be established as it is not expressed in latently infected BCBL cell lines (Lagunoff & Ganem, 1997, Sarid *et al.*, 1998) and expression in spindle cells is not known.

Two human macrophage inflammatory protein (MIP) homologues have been found in HHV-8 encoded by ORFK6 (vMIP-1) and ORFK4 (vMIP-2). The vMIP-1 has been shown to inhibit entry of non-syncytial inducing (NSI) HIV-1 isolates into CD4+ cells via the CCR5 chemokine receptor (Moore *et al.*, 1996a). The K4 homologue (vMIP-2) acts as a powerful and efficient antagonist of chemotaxis and can inhibit HIV entry via the CCR3, CCR5 and CXCR4 coreceptors (Kledal *et al.*, 1997). The binding of vMIP-2 to cellular chemokine receptors have been shown to block responses to cellular chemokines and may, therefore, play a role in protecting HHV-8 infected cell populations from an immune response (Kledal *et al.*, 1997). Since viral - induced MIP proteins act as chemoattractants, new cells could serve as targets for HHV-8 infection. Alternatively, the recruited cells may produce chemokines or cytokines promoting growth and persistence of viral - infected cells. Both vMIP-1 and -2 have the potential to induce angiogenesis in chick chorioallantoic membranes (CAM) and may play a role in KS lesion formation. Angiogenesis involves endothelial cell proliferation and chemotactic migration, associated with an inflammatory response, leading to new blood vessel formation in CAM (Boshoff *et al.*, 1997).

Recently, the ORF50 gene product, Rta (transcriptional activator), has been associated with HHV-8 pathogenesis (Sun *et al.*, 1999). Rta is a homologue of the BRLF1 protein in EBV and is an immediate early gene product which initiates the lytic cascade. The reactivation of the lytic cycle facilitates virus production aiding its transmission to neighbouring cells. It is believed that the Rta controls the lytic cascade through vIL-6, vBcl-2, vMIP-1 and vMIP-2 early lytic cycle products. The anti-apoptotic role of these cytokines could increase survival of lytically infected cells in order for infectious virions to be produced. Alternatively, the chemoattractive capabilities of the cytokines could recruit new cells to the site of infection which would serve as targets for HHV-8 transmission. The cytokines may also create a favourable environment for the virus to undergo a change from latent to lytic replication and therefore, have the potential to assist in HHV-8 pathogenesis.

1.7 Involvement of HIV-1 Tat protein in KS pathogenesis

Although, it is generally believed that HIV promotes HHV-8 replication through impairing the host immune system, a more specific role for HIV-1 in KS pathogenesis has been postulated. The HIV-1 transactivating (Tat) protein, released by HIV infected cells, can enter a variety of cell types and *trans*-activate genes needed for growth. Tat may act as a paracrine growth factor, binding to the cell membrane and releasing a cascade of signalling events to control spindle cell growth. Tat treatment of KS cells activates c-Src, mitogen-activated protein (MAP) and Jun amino-terminal kinases (JNK) which are important in regulating cell proliferation (Ganju *et al.*, 1998). It has been shown that Tat augments hyperplastic KS cell growth *in vitro* and in xenotransplants in nude mice (Gallo, 1998b). Tat induces high levels of inflammatory cytokines, mainly IFN-γ, TNF-α, IL-1 and IL-6, which are integral to the production of microvascular inflammatory KS lesions. These cytokines promote endothelial cell growth, integrin expression, production of angiogenic factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), and up-regulate HHV-8 repl' .tion (Fife & Bower, 1996). High levels of bFGF and its receptor are produced in AIDS-KS cells which are believed to act in an autocrine fashion to promote growth and in a paracrine fashion to stimulate angiogenesis on endothelial cells. Integrin binds to receptors for extracellular matrix proteins on KS lesions. This binding induces cell adhesion and invasion which promote angiogenesis. Tat contains a basic region consisting of an RGD domain. This motif binds to endothelial cells and to integrins necessary for lesional development. This region is absent from Tat in simian immunodeficiency virus (SIV) and HIV-2. As SIV infected monkeys do not develop KS and HIV-2 infected individuals show a decreased association with KS, this suggests an important role for this Tat motif in KS pathogenesis (Ariyoshi *et al.*, 1998, Gallo, 1998a). Since HIV-1 is believed to upregulate HHV-8 replication, the implications are that new strains of HHV-8 may manifest due to the AIDS epidemic. Since South Africa is experiencing the fastest growth of HIV-infected individuals worldwide, it is important to research the association of HHV-8 subgroups in the HIV-infected sub-population in this country.

1.8 Methods of detection

1.8.1 Molecular methods

Detection of HHV-8 DNA has been performed primarily by amplification of the unique $KS330BAM_{233}$, the original fragment discovered in KS lesions. This is done by direct or nested PCR or Southern blot hybridization of HHV-8 associated DNA (Chang, 1994). Since contamination is a problem with PCR , Southern blot or dot-blot hybridization is preferable. Since hybridization systems lack the sensitivity of PCR they cannot be used for specimens containing low concentrations of virus or for fixed tissues (Moore & Chang, 1997). PCR is used to detect viral DNA sequences in a variety of cell lines, organs and tissues and has been shown to be highly specific and sensitive. It was through DNA amplification that a strong association between KS and HHV-8 was originally determined. HHV-8 DNA has also been detected in normal-appearing skin, semen and serum of patients with KS (Marchioli *et al.*, 1996)(Whitby *et al.*, 1995). PCR has been used to detect viral DNA in archival or paraffin wax embedded skin biopsy specimens (Cathomas *et al.*, 1996, Maiorana *et al.*, 1997).

1.8.2 Serological methods

A variety of serological assays have been used to detect anti-HHV-8 antibodies in blood. The most common assay detects the LNA-1 nuclear protein encoded by ORF73. This latent nuclear antigen has a molecular mass of around 224-236 kDa and appears to be highly immunoreactive (Gao *et al.*, 1996, Kellam *et al.*, 1997, Rainbow *et al.*, 1997). Cross-reactivity with proteins from EBV is always a potential problem as EBV is ubiquitous, has homology to HHV-8 and occurs frequently in HHV-8 infected persons. However, there is no homologue of ORF73 in EBV making it an ideal candidate for use in serological assays. Detection techniques commonly used are Western blots and immunofluorescence assays (IFA) using unstimulated PEL cell lines such as BCBL-1 or BCP-1 (Simpson *et al.*, 1996). The LNA protein also termed latency associated nuclear antigen (LANA) is the main antigen detected which appears as a speckled nuclear immunofluorescent pattern in the IFA. It remains unclear whether other HHV-8 antigens are detected in this assay (Kellam *et al.*, 1997). The presence of anti-HHV-8 antibodies is also predictive of KS especially in HIV-infected individuals. The hazards ratio for

KS in HHV-8 seropositive individuals that proceed to HIV infection is 3.15 and in HIV-infected individuals who later become HHV-8 seropositive, the risk is 6.64 (Renwick *et al.*, 1998). The sensitivity of these assays is high with over a 90% detection rate in some KS patient cohort studies (Table 2) (Schulz, 1998). The intensity of the fluorescent signal in the LANA IFA has also been shown to be strongly related to risk of developing KS. A high signal intensity has been associated with a 12-fold and 1,000 fold increase in risk of KS in HIV-negative and HIV-positive individuals respectively (Sitas *et al.*, 1999).

Detection systems based on lytic viral proteins have also been established (Table 2) as detecting only latent antigens may undermine the true prevalence of the virus (Chandran *et al.*, 1998, Miller *et al.*, 1996, O'Neill *et al.*, 1997, Smith *et al.*, 1997). Viral growth is induced by treatment with butyrate or phorbol esters and used in IFA, radioimmunoprecipitation assays and Western blots. This treatment promotes the expression of lytic or structural viral proteins. In blood donors, detection of HHV-8 antibodies using the lytic assay was shown to range from 0-25% compared to 0-3% by the LANA assay (Lennette *et al.*, 1996). These results suggest that the lytic assays are more sensitive although the precise antigens detected are unknown. However, the major capsid protein (ORF25) of HHV-8 has about 50% homology to the BcLF1 of EBV and so there may be some cross reactivity (Moore *et al.*, 1996b). There is also the possibility that HHV-8 antibodies may cross-react with proteins from other herpesviruses such as CMV and varicella-zoster virus.

Other serological assays have also been used with varying success. Western blots have been used to detect a 40 kDa butyrate-inducible antigen (Miller *et al.*, 1996) and the vp19 capsid-related protein encoded by ORF65 (Table 2) (Simpson *et al.*, 1996). The vp19 as well as the vp23 encoded by ORF26 have been used in IFA's, the former was shown to be more sensitive in detecting HHV-8 antibodies.

From Table 2 it can be seen that antibody detection rates are higher in individuals with classic KS, slightly lower in AIDS-KS and lowest among blood donors. The sensitivity of the different assays differ between countries and between cohorts. Prevalence studies show HHV-8 antibody detection ranging from 35-62% in Uganda and 13-31% in homosexual men, depending on the assay used (Table 2). In most cases, the detection rate of anti-HHV-8 antibodies in KS confirmed individuals is less than 100% suggesting that more sensitive tests need to be developed before it can be routinely used for diagnostic purposes.

A comparison of the different serological assays was done by Rabkin and coworkers (Rabkin *et al.*, 1998). Four of the tests were based on IFA's using two different cell lines and different serum dilutions, and three were ELISA's utilising recombinant ORF65 and ORF26 proteins or whole virus as antigen. The minor capsid protein ELISA (ORF26) lacked sensitivity and consistency and was not recommended. Interassay discrepancy was most obvious in the AIDS-related KS specimens. The latent and lytic IFA showed the best agreement suggesting that either assay could be used with confidence. There were, however, difficulties associated with the IFA method. Depending on the intensity of the weakest positive control used, different assay cut-off's make it difficult to determine the true prevalence in lower-risk populations such as normal blood donors or HIV-positive/KS-negative individuals. Scoring of the IFA results by different readers was also shown to be highly subjective. The final results obtained from these assays were as follows: 67%-100% detection rate in KS confirmed individuals, 27%-60% in HIV-positive patients and 0%-29% in blood donors. Although there is a wide variation in these figures, the current assays have strengthened the association between HHV-8 and KS. While these assays are useful for epidemiological studies, they are not recommended for individual patient diagnosis.

Table 2: Comparison of different serological assays

		Antibody detection rate (%) in:					
Antigen	Assay*	Classic KS	AIDS KS	Homosexual men	Haemophilia	Blood donors	Other groups
Latent antigen							
LANA (Latency associated nuclear antigen)	IFA	85-94	71-88	18-30	0-3 (UK/USA)	0-3 (UK/USA) 4-21 (Italy)	12-21 (Greece) 51 (Uganda)
LNA (226/234 kDa nuclear protein)	WB	100	80	18	0 (USA)	62 (Uganda)	
Structural (lytic) antigens							
40kDa protein	WB		67	13			
vp19 (ORF65)	ELISA/WB	86-94	75-84	31	2 (UK)	1.7-5 (UK/USA) 9-22 (Italy)	12 (Greece) 35 (Uganda)
vp23 (ORF26)	ELISA		~40 ~60			~11 (Germany) ~20 (USA)	
Other undefined antigens	IFA ELISA/IFA		96-100 100	90-100		20-25 (USA) 0-12 (USA)	32-100 (Africa)
	IFA		100			0 (USA)	

A variety of latent and lytic antigens have been used in first generation HHV-8 serological detection systems. Antibody detection rate is shown in different cohorts susceptible to the virus as well as in background population groups. The sensitivity and specificity of these assays require improvement as they do not detect antibodies in 100% of KS confirmed individuals (classic and AIDS KS) in most instances. * IFA = Immunofluorescence assay, ELISA = Enzyme linked immunosorbant assay WB = Western blot

Reproduced from Shultz *et al.* (Schulz, 1998)

1.9 Epidemiology

Human herpesviruses share a number of general properties. They maintain their latent episomal state throughout the life of the host and, with the exception of herpes simplex type 2 (HSV 2), they infect a relatively large portion of the population (Roizman, 1995). Given that HHV-8 has been shown to persist in infected individuals, there is considerable interest in determining the background prevalence of this new herpesvirus.

Evidence that HHV-8 is unlikely to be ubiquitous arises from studies looking at the frequency of HHV-8 DNA in blood from healthy donors. In a British prevalence study, HHV-8 DNA was not detected in PBMC's of normal blood donors, yet could be detected in 8% of AIDS patients without KS and in 52% of HIV-infected KS patients (Whitby *et al.*, 1995). Although these results are substantiated by other groups claiming that this virus is restricted to persons having an increased risk of developing KS (Weiss, 1996a), Bigoni and co-workers found that 9% of blood from healthy donors in Italy were HHV-8 PCR positive in blood (Bigoni *et al.*, 1996). Furthermore, HHV-8 prevalence in the Japanese populations appears to be high, with 64% of healthy children and 80% of healthy adults infected, suggesting that the infection occurs in early childhood (Kikuta *et al.*, 1997). In Africa, KS is increasingly common in both HIV-positive and HIV-negative individuals and is the most frequently reported cancer in parts of Africa (Chang *et al.*, 1996). According to the Kampala Cancer Registry in Uganda, KS is the most frequent cancer in males and second most frequent cancer in females (Wabinga *et al.*, 1993). In Gambia, HHV-8 has been reported in the blood of 10% of healthy individuals (Ariyoshi *et al.*, 1998) and although HHV-8 is not widely distributed, parts of Africa (Uganda) and southern Europe (Italy, Greece) have high prevalence rates, whereas it is still a relatively rare virus in Northern Europe and the USA (Schulz, 1998).

Since the frequency of HHV-8 DNA in the blood is low, various serological assays have been used to determine the prevalence of antibodies to this virus. Table 2 shows that the background sero-prevalence in Italian blood donors ranged from 4-21% (LANA) and 9-22% (vp19), whereas, in the USA and UK, antibodies to HHV-8 are as low as 0-3% (LANA) and 1.7-5% (vp19). In Italy, there is regional variation with HHV-8 antibodies detected more frequently in people from Sicily, Sardinia and the lower part of the Po valley which are regions with a high incidence of classic KS (Calabro *et al.*, 1998). In Uganda, reports of 32% (LNA/Western blot) and 51% (LANA) prevalence have been reported in areas where AIDS-related KS is common (Simpson *et al.*, 1996, Wabinga *et al.*, 1993). The incidence of HHV-8 in African countries has been shown to be highest in Nigeria, followed by Zimbabwe, Zaire and then Uganda (Ziegler, 1993). Before the HIV epidemic in Zambia, KS was uncommon in adults and even rarer in children. Now almost 50% of pregnant women without KS have antibodies to HHV-8 and KS manifests in 20%-25% of all pediatric malignancies (He *et al.*, 1998). In South Africa, the prevalence of HHV-8 in cancer patients was recently examined using the LANA IFA (Sitas *et*

al., 1999). The seroprevalence in KS negative cancer patients was found to be 32%, whereas, in KS subjects it was considerably higher at 83%. The seroprevalence was also found to increase with age and number of sexual partners. In this group, HHV-8 antibody titres did not show a significant association to HIV infection. However, the risk of KS in HIV-negative individuals with a high HHV-8 fluorescent signal was 12-fold, but increased to 1,000 fold in HIV-seropositive individuals. Since this assay is user-dependent and therefore subjective, we aim to conduct a similar survey using an in-house IFA supplemented by molecular assays.

1.10 Transmission of HHV-8

The routes of HHV-8 transmission still remain unclear, but distribution of anti-HHV-8 antibodies in various cohorts suggests that it may be sexually transmitted as it is most frequently found in populations with other sexually transmitted diseases, such as HIV and syphilis (Kedes *et al.*, 1996). Before the discovery of HHV-8 and the development of methods for viral detection, Beral and co-workers had reported a strong association between KS and anal-oral sexual practices (Beral *et al.*, 1992). Transmission has therefore been significantly linked to multiple homosexual/bisexual contacts and to occur via the oral-anal route (Blackbourn *et al.*, 1999, Friedman-Kein *et al.*, 1990, Martin *et al.*, 1998). Beral and co-workers further proposed that non-sexual transmission of KS in underdeveloped countries could occur through inefficient sanitation (Beral *et al.*, 1992). However, it is likely that there are other, as yet unknown, mechanisms of transmission in these regions. Determining the route of transmission of HHV-8 is important for designing and implementing intervention strategies to prevent further spread of this virus.

1.10.1 Digestive and gastrointestinal tract

There have been contradictory anecdotal reports regarding HHV-8 in saliva. Some studies have not detected HHV-8 in saliva of HIV-positive individuals with KS (Ambroziak, 1995), whereas other studies have reported HHV-8 in saliva of 33% of HIV-infected people, but not in HIV-negative persons (Boldogh *et al.*, 1996). Although HHV-8 has rarely been detected

in the sputum and throat swabs of HIV-infected individuals (Whitby *et al.*, 1995), DNA studies have shown that HHV-8 is tropic for the oral mucosa and is shed in saliva which may contribute to transmission (Di Alberti *et al.*, 1997, Koelle *et al.*, 1997). HHV-8 has also been detected in the duodenum and intestinal tissue from HIV-infected individuals (Thomas *et al.*, 1996), but rarely in stool samples. These data, therefore, do not lend support to the idea that the oral-faecal route is a major route of transmission in HIV-positive homosexual men (Whitby *et al.*, 1995).

1.10.2 Genital tract

There have also been contradictory reports regarding HHV-8 in the seminal compartment. HHV-8 is believed to be transmitted sexually mainly by homo- or bisexual practices (Dupon *et al.*, 1997) and HHV-8 DNA has been found in semen of HIV-infected homosexual men, but rarely in HIV-negative heterosexual men. Studies have detected HHV-8 in fractions containing urethral cells, but not in the spermatic heads (Monini *et al.*, 1996). Detection of HHV-8 in the seminal fluid is believed to be indicative of KS development (Lin *et al.*, 1995b) although other reports have not shown this relationship (Gupta *et al.*, 1996). Other studies show that HHV-8 is rarely present in semen (Ambroziak, 1995, Corbellino *et al.*, 1996, Marchioli *et al.*, 1996). In an Italian cohort of HIV unknown/KS negative individuals, HHV-8 was detected in 44% of prostrate tissue and 91% of semen samples (Monini *et al.*, 1996). Yet another study detected HHV-8 in only 14% of HIV-positive homosexuals (Gupta *et al.*, 1996). HHV-8 was also found in the foreskins and glans penis in 22% of these individuals as opposed to only 6.5% of specimens from the female genital tract (Monini *et al.*, 1996). In situ PCR has, however, detected HHV-8 transcripts in spermatozoa and mononuclear cells in the semen of AIDS-KS patients (Huang *et al.*, 1997). These cells were localised to the glandular epithelium and may have the potential to transmit the virus in prostatic secretions. However, in HIV-negative female patients, HHV-8 has not been detected in cervical tumours or vulval mucosa (Tasaka *et al.*, 1996). The discrepancy between these reports may be due to the type of cohorts studied. In certain areas of Italy for example, KS is more frequently diagnosed which may influence the differences in detection rate (Corbellino *et al.*, 1996, Diamond *et al.*, 1997, Koelle *et al.*, 1997)

1.10.3 Transmission of HHV-8 in South Africa

Vertical (mother to child) transmission of HHV-8 in South Africa has been reported with an estimated thirty percent of seropositive mothers transferring the infection to their infants (Bourboulia *et al.*, 1998). In this study, it was shown that 42% of the children were seropositive if their mothers were also HHV-8 seropositive compared to only 1% of children from HHV-8 seronegative mothers. Mothers with higher titres of anti-HHV-8 antibodies were also more likely to transmit the virus to their infants; only 1% of children were HHV-8 seropositive when mothers had low titres as opposed to 50% when mothers had high anti-HHV-8 titres (1:26,600 or greater) (Sitas *et al.*, 1999). The authors suggest that mothers with high HHV-8 viral load have a greater risk of transmitting the virus to their children.

Among black South Africans, HHV-8 seroprevalence has been shown to increase with age (24% in the 15-24 age group and 49% in those older than 65) (Sitas *et al.*, 1999, Wilkinson *et al.*, 1999). If the major route of transmission was sexual, it would be expected that the highest prevalence would be in the young adult population group and not in the older age group. The implication from these studies is that the virus is not heterosexually transmitted in a developing country setting. However, since HHV-8 DNA has been detected in saliva, transmission via this route may be important in developing countries due to overcrowding and lack of personal hygiene (Wilkinson *et al.*, 1999). The data also suggest that HHV-8 has been around for a long time in South Africa, but as a result of the HIV epidemic has become manifest as clinical KS.

1.11 Aims of this thesis

1. To determine the molecular and serological prevalence of HHV-8 in HIV-1 infected individuals in South Africa, compared to patients with clinically confirmed KS.

2. To define the HHV-8 subgroups circulating in Gauteng, South Africa and determine the phylogenetic relationships between South African isolates and those from elsewhere in the world.

3. To develop quick screening methods for different HHV-8 subgroups.

2. MATERIALS AND METHODS

2.1 Specimen collection

Peripheral blood was collected in EDTA from 429 individuals residing in Johannesburg. Of these, 95 were confirmed as having KS based on clinical and histological examinations. These KS samples were provided by Dr Freddy Sitas of the National Cancer Registry, Johannesburg, with all the relevant demographic data. Plasma samples were tested for antibodies to HIV-1/2 by ELISA. The remainder of the specimens (n=334) were from known HIV-positive individuals who had no visible signs of KS. These samples were drawn from local outpatient clinics and hospitals. For 8 of these patients, matched lymph node (LN) samples were also available. Ethical approval for this study was obtained from the University of the Witwatersrand Committee for Research on Human subjects (see Appendix).

2.2 Preparation of cell lysates

Plasma was separated from whole blood by centrifugation at 1,000 rpm for 10 min. Peripheral blood mononuclear cells (PBMC's) were isolated by discontinuous gradient centrifugation using Ficoll-Hypaque at 1,500 rpm for 30 min. Contaminating red blood cells which can affect *Taq* polymerase activity, were removed by incubating cells in 2 ml of 155 mM NH_4Cl (pH 7) for 3-7 minutes, followed by a wash at 1,000 rpm for 10 min in phosphate buffered saline (PBS). PBMC's were lysed in 50 µl lysis buffer (10 mM Tris-HCl pH 8.3, 0.5% Triton X-100, 0.001% sodium dodecyl sulphate) plus 3 µl 20 mg/ml Proteinase K overnight at 56°C. Proteinase K was denatured by heating at 95-100°C for 15 minutes. LN's were collagenase digested and isolated cells were lysed as for PBMC's (Gray *et al.*, 1996).

2.3 Detecting antibodies to HHV-8 latent antigens

Plasma specimens were screened for antibodies to the HHV-8 latency-associated nuclear antigen (LANA) expressed in the B-cell precursor-1 (BCP-1) cell line using an immunofluorescence assay (IFA) (Gao *et al.*, 1996, Simpson *et al.*, 1996). BCP-1 cells were cultured in RPMI 1640 growth medium containing 100 IU/ml penicillin and 100 µg/ml streptomycin with 20% fetal calf serum (FCS) and 1% L-glutamine. The cells were grown at 37°C in a humidified CO_2 incubator and split 1:2 every 2 days. For plating, $5x10^6$ cells were centrifuged at 1,000 rpm for 10 min and washed in PBS. The cells were centrifuged as before and fixed in 4% paraformaldehyde for 10 min at room temperature and then transferred to a 1.5 ml eppendorf tube. Cells were washed twice in a desk top microfuge for 15 sec at maximum speed with 1 ml PBS and permeabilised with 1 ml 0.5% Triton X-100 for 10 min at room temperature. After a further 2 washes in PBS the cells were finally resuspended in 1 ml PBS. Approximately 10,000 cells (5 µl) were placed in a 5 mm well of a 12 well HTC super-cured slide, allowed to dry and stored at -70°C until use.

Plasma samples were diluted 1:100 in 3% FCS and incubated on the wells in a humidified chamber for 30 min at room temperature. Slides were washed 5 times in 1% FCS. Mouse anti-human IgG-FITC conjugate was diluted 1:40 with Evans Blue counter stain and incubated in humidity for 20 min at room temperature. Cells were washed as before and analysed using a Microflex HFX-II fluorescent microscope at 400x magnification within 48 hours of preparation. Three known positive plasma samples with high, intermediate and low levels of anti-HHV-8 antibody, and one HHV-8 negative plasma sample, were included on each slide as controls. Negative control plasmas (n=3) were obtained from individuals who were KS and HIV-1/2 antibody negative. Specimens were scored as positive when punctuate flourescent spots were visible in the nucleus indicating the presence of latent nuclear antigens. Homogeneous cytoplasmic fluorescence was recorded as negative.

2.4 PCR amplification of HHV-8 DNA

The presence of intact amplifiable DNA in cell lysates was assessed by a β-globin PCR using primers PCO3 and PCO4 (see Table 3). The reaction volume was made up of 2-5 µl DNA template, 5 µl 10x buffer, 2.8 µl $MgCl_2$, 4 µl (1 M) dNTP mix, 2 µl (5 pmol/µl) PCO3, 2 µl (5 pmol/µl) PCO4 and 0.2 µl Supertherm *Taq* DNA polymerase in 50 µl sterile distilled water. The cycling conditions were: 94°C for 2 min (denaturation step) followed by one round of 94°C for 1.5 min, 50°C for 45 sec and 72°C for 45 sec. This was followed by 38 cycles of 94°C for 1 min, 55°C for 45 sec, 72°C for 45 sec ending with a 10 min extension step at 72°C.

Specimens which were positive for β-globin DNA were then screened for HHV-8 DNA by nested PCR using primers in 2 different regions. The $KS330BAM_{233}$ primers (KS1/KS2 as outer and KS3/KS4 as inner primers, see Table 3) were used as described (Chang, 1994). PCR reactions were done in a total volume of 50 µl in 30 cycles and products were run on a 1% agarose gel with 0.1 µg/ml ethidium bromide for detection. The second set of primers were in the ORF75 gene: first round KS1000 and KS1034 and second round KS2000 and KS2034 (see Table 3). The outer primers were designed, using KSHV long unique region (Genbank accession number U75698), to contain a GC ratio of 40-50% for annealing at a temperature range of 65-70°C. The inner pair correspond to LGH2000 and LGH2034 in Zong et al (Zong *et al.*, 1997) which amplify an 804 bp product. The following cycling conditions were used for first round replication: 1 cycle at 94°C for 2 min (denaturation step), 35 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min with a 5 min extension step at 72°C. Supertherm *Taq* DNA polymerase (1 U/µl) was used in the first round PCR and *Taq* polymerase (2.5 U/µl) from Boehringer Mannheim was used in the nested PCR. The nested PCR was identical to the first round PCR except that the annealing temperature was reduced to 55°C which was optimal for the KS2000/2034 nested primers. The amplified nested products were detected on a 1% agarose gel with 0.1 µg/ml ethidium bromide. A sample from a KS patient who was $KS330BAM_{233}$ PCR positive was used as a positive control and water was used as a negative control in all reactions.

Table 3: PCR and sequencing primers.

Primer	Use	Nucleotide sequence (5'-3')	No. Bases	Gene
KS1	PCR	ATAGGGAGCGTACTGCCG	720	ORF 26
KS2	PCR	CATCCTGGTGATGTCATC		
KS3	PCR	AGCCGAAAGGATTCCACCAT	230	ORF 26
KS4	PCR	TCCGTGTTGTCTACGTCCAG		
KS1000	PCR	CGGTTCGGTGGCATACAGGC	895	ORF 75
KS1034	PCR	CTGACTACAGAGGGTGTCCCCG		
KS2000	PCR	GGAAACAGGGTGCTGTG	804	ORF 75
KS2034	PCR	CATGGCCTACGACGTCAC		
PCO3	PCR	CGATCACTTGTGTCAACACA	110	B-globin
PCO4	PCR	CCACTTGCACCTACTTCAAC		
SP6	Sequencing	GTATTCTATAGTGTCACCTAAAT		Plasmid
M13 Reverse	Sequencing	GTCATAGCTGTTTCCTG		Plasmid
M13 Forward	Sequencing	CGACGTTGTAAAACGACGGCCAGT		Plasmid
T7	Sequencing	TAATACGACTCACTATAGGA		Plasmid

In case of PCR primers, the outer primers are listed first and then the inner primers.

2.5 Cloning

2.5.1 Ligation and transformation

PCR products amplified by thermostable polymerases, for example Taq from Boehringer Mannheim, preferentially add a single adenosine nucleotide at the 3' end of a double-stranded DNA product. These amplicons can then be cloned into a plasmid with a 5' Thymidine (T)-overhang. Plasmids were obtained from the pMOS*Blue* T-vector kit and pGEM®-T vector kit and cloning was performed as recommended by the manufacturers. The ligation reaction included 1 µl 10x ligase buffer, 1 µl pMOS*Blue* or pGEM®- T vector, 0.5 µl T4 DNA ligase (2-3 weiss units) and 4 µl PCR product. In addition, the pMOS*Blue* reaction contained 0.5 µl 100 mM DTT and 0.5 µl 10 mM ATP. The reaction was made up to a total of 10 µl with deionised sterile water and incubated overnight at 16°C (pMOS*Blue*) or 4°C (pGEM®).

The ligated product (2 µl) was transformed into 25 µl of competent *Escherichia coli* JM109 cells, kept on ice for 20 minutes and then heat shocked at 42°C for 45-50 sec. A second incubation on ice (2 min) was followed by a 1 hour incubation with 80 µl SOC medium at 37°C. 100 µl of the transformation mixture was added to Luria broth (LB) agar plates containing 60 µg/ml ampicillin, 40 µl 0.1 M IPTG and 35 µl of 25 mg/ml X-gal and incubated overnight at 37°C. Blue/white screening of the clones was possible due to the insertional inactivation of the lacZ gene.

2.5.2 Plasmid preparation

White colonies were selected and grown overnight at 37°C in an eppendorf tube containing 1 ml LB. Clones were screened by PCR, using 5 µl of bacterial culture, with the KS2000/KS2034 primers. Positive cultures were expanded and 4 ml were harvested, lysed and plasmid DNA isolated using the High Pure Plasmid Isolation Kit. The cell pellet was resuspended in 250 µl suspension buffer (50 mM Tris-HCl, 10 mM EDTA pH 8.0, 1 mg/ml RNASE A) and 250 µl of alkaline lysis buffer (0.2 M NaOH, 1% SDS) and incubated for 5 min at room temperature. After incubation, 350 µl chilled binding buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate pH 4.2) was added, inverted a few times and incubated on ice for 5 minutes. The solution was centrifuged for 10 min on a desk top microfuge at 14,000 rpm. DNA in the supernatant was loaded onto a filter column containing glass fibres which bind plasmid DNA. The column was spun at maximum speed (14,000 rpm) for 1 min to remove impurities and the DNA was washed with 500 µl wash buffer I (ethanol, 5 M guanidine hydrochloride, 20 mM TRIS-HCl pH 6.6). After spinning the buffer through the column, a second wash was performed with 700 µl wash buffer II (ethanol, 20 mM NaCl, 2 mM Tris-HCl pH 7.5) and the plasmid was eluted with 100 µl distilled water. DNA quantitation was determined by reading the optical density (OD) of the product at 260 nm. An OD_{260} value of 1 corresponds to approximately 50 µg/ml of double stranded DNA. The DNA was assumed to be free of protein when the OD ratio at 260 nm/280 nm was approximately 1.8 (Sambrook *et al.*, 1989).

2.6 Sequencing

2.6.1 Manual sequencing of PCR products

The first 3 KS330BAM$_{233}$ products were sequenced by the dideoxy-chain termination method using a Sequenase PCR product sequencing kit. 4 µl of the amplified product was added to 5 µl distilled water and 1 µl of the KS3 or KS4 primers (see Table 3). The mixture was denatured at 100°C for 3 min in a Biometra Trio-thermablock TB-1 cycler and placed immediately in an ice/water bath for 5 min. For each specimen, 4 tubes were made up with 2 µl of termination mix with each tube containing a different dideoxynucleotide triphosphate (dNTP). The labelling reaction was made up by adding 4 µl of cold DNA mixture (10 µl) together with 2 µl reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1 µl dithiothreitol (DTT, 0.1 m), 0.5 µl dATP labelled with ^{35}S (5 µCi) and 3.4 U DNA polymerase. The mixture was incubated for 5 min at 0°C and 3.5 µl was added to each of the termination tubes which had been pre-warmed at 50°C for 1 min. Sequencing commenced by heating the termination reactions at 50°C for 10 min in a thermal cycler after which 4 µl of stop solution (formamide/dextran blue) was added. Just before loading, the samples were denatured for 3 min at 80°C and 3 µl was added to each lane of a sequencing gel.

The sequencing gel was made up using 8% polyacrylamide (19:1 acrylamide: bis-acrylamide), 0.96 g/ml urea, 16% (v/v) 10x glycerol tolerant buffer (216 g Tris Base, 72 g Taurine, 4 g $Na_2EDTA{\cdot}2H_2O$, in 1L water), in a total of 50 ml deionised water. This was heated to approximately 60°C until the urea dissolved. Glycerol tolerant buffer was used to prevent distortion of the gel as a result of high glycerol content of the DNA polymerase. The solution was then filtered through No. 1 Whatman filter paper and the volume adjusted to 100 ml with distilled water. The gel was then polymerised by adding 500 µl 10% ammonium persulphate (APS) and 50 µl N, N, N', N' -Tetra-Methyl-Ethylenediamine (TEMED). After polymerisation, the gel was heated to 50°C by running at 60 W, 60 mA and 2500 V after which the samples were loaded and run for 3 hours. The loading buffer was 0.8x glycerol tolerant buffer and after 1 hour, 150 ml of 3 M sodium acetate (pH 4.8) was added to the 300 ml running buffer reservoir. Sodium acetate has a similar effect to a 'wedge' gel giving better band resolution and an increased number of nucleotides per run. After completion of the run the gel was removed from

the plates using 3MM paper and fixed in 15% methanol and 5% acetic acid for ½ hour. After fixing the DNA to the gel, it was covered with cellophane and dried at 80°C for 1 hour. The dried gel was then placed on a Hyperfilm βmax autoradiography film and exposed for 36 hours. The sequences were read manually.

2.6.2 Direct sequencing of cloned products

Amplicons cloned into a pMOS*Blue* T-vector or pGEM®-T vector contain universal primer binding sites which were used as sequencing primers. Two methods were used to sequence products on the ALF*express* DNA sequencer which detect dNTP's attached to the fluorescent molecule Cy5 Amidite (Cy5™). This molecule can be linked to a sequencing primer requiring the Cy5™ AutoRead™ Sequencing kit or to a dATP which requires a Cy5™-dATP labelling mix. The Cy5™-dATP labelling mix can be used in conjunction with the Cy5™ AutoRead™ kit. The volumes and solutions used with these 2 methods differed slightly and those used with the Cy5™-dATP reaction will be enclosed within curly brackets. The Cy5™-dATP product contains a mixture of deoxy- and dideoxynucleotides where the dGTP is replaced by 7-deaza dGTP to reduce problems associated with band compressions. For both kits/methods approximately 5-10 µg of cloned DNA was added to 8 µl of 2 M NaOH and the volume was made up to 40 µl with sterile distilled water. The reaction was then incubated for 10 min at room temperature after which 7 µl of 3 M sodium acetate (pH 4.8) and 4 µl distilled water was added. DNA was precipitated by adding 120 µl of pure ethanol at -70°C for 20 min after which the supernatant was discarded and the pellet rinsed with 70% ethanol. The remaining traces of ethanol were evaporated using a vacuum pump for 5-15 min. The pellet was resuspended in 10 µl {12 µl} distilled water and 2 µl of 10 pmol universal primer, bound to Cy5 {100 pmol unlabeled primer} plus 2 µl of annealing buffer. The annealing reaction tube was placed in a 3600 thermal cycler at: 65°C for 5 min followed by 37°C for 10 min. The cycler was then switched off to allow the reaction to reach room temperature after which 1 µl extension buffer containing DTT and 3 µl {3.5 µl} dimethyl sulfoxide (DMSO) was added (An additional 1 µl Cy5-dATP labelling mix was added when using the dATP method). 2 µl of 4 U/µl T7 DNA Polymerase was added and 4.5 µl {5.4 µl} of this cocktail was then added to 4 tubes containing 2.5 µl {3 µl} of the different dideoxy terminators, maintained at 37°C. After a 5 min incubation

period at 37°C, the sequencing reactions were halted with 5 µl {6 µl} stop solution (95% formamide, 5 mg/ml dextran blue). The termination reactions were heated to 80°C for 2 min and kept on ice until 8 µl was loaded onto each well of a gel. The gel formulation consisted of a 5% Long Ranger gel (19 g Urea, 24 ml Baxter water, 7.5 ml 10 x TBE and 50% Long Ranger). The solution was stirred for at least 30 min and filtered through a Millex HV 0.45 µl filter. Polymerisation occurred for 3 hours after addition of 25 µl TEMED and 250 µl 10% APS. The running buffer consisted of 0.5x TBE. The gel running conditions were programmed as follows: 1,000 V, 60 mA, 25 W, and 55°C. The sampling time was set for 2 min and the gel was run for 720 min.

2.6.3 Cycle sequencing of cloned products

A Thermosequenase cycle sequencing kit which relies on amplification of signal intensity by asymmetric PCR was used with the ALF*express*. This method uses 0.2 ml 96 well polycarbonate OmniPlate in place of individual 0.2 ml eppendorf tubes. Plasmids were diluted to 1-2 µg in 20 µl distilled water to which 2 µl of 2 pmol/µl Cy5 primer was added. From this mix, 6 µl was added to 2 µl of each of 4 sequencing formulations containing dideoxy termination nucleotides. The OmniPlate was then placed in a Hybaid touchdown thermal cycler with the following cycling parameters: an initial long denaturation step (96°C for 5 min); 30 cycles of denaturation (96°C for 45 sec) and elongation (60°C for 30 sec) and a final cycle of elongation (60°C for 5 min). Reactions were held at 4°C until 4 µl of stop solution was added. Samples were denatured at 90°C for 3 min before 5 µl was loaded onto a single well of a 5% Long Ranger gel.

Alternatively, a dRhodamine cycle sequencing ready reaction kit utilising a four dye-labelled primer chemistry was used. While the previous methods made use of one type of fluorescent molecule (Cy5), this kit utilises 4 different molecules attached to each of the dideoxy terminators, fluorescing at 540 nm, 570 nm, 600 nm and 620 nm, allowing for single tube reactions. For each reaction mixture, 8 µl of terminator ready reaction mix was added to 0.2 µg/µl plasmid DNA and 3.2 pmol primer made up to a final volume of 20 µl with distilled water. The tubes were then placed in a 2400 or 9600 GeneAmp PCR system with the following cycling

conditions: 25 cycles of denaturation (96°C for sec), annealing (50°C for 5 sec) and denaturation (60°C for 4 min). The reactions were kept at 4°C until purification. Sequencing products were purified using the Centri-Sep procedure. A Centri-Sep column was reconstituted with 800 µl of distilled water for 2 hours. The bubbles were tapped free and the column was allowed to drain. The remaining fluid was centrifuged at 3,000 rpm for 2 min and 20 µl of the Dideoxy terminator reaction was added to the column. The column was centrifuged as before and the specimen collected, vacuum dried and resuspended in 6 µl loading buffer (1 part 25 mM EDTA containing 50 mg/ml blue dextran and 5 parts deionised formamide) before it was denatured at 90°C for 2 min before loading in a well. The products were run on a 4% acrylamide gel (19:1 Acrylamide/Bis-acrylamide, 0.36 g/ml urea, 0.1 g/ml mixed bed resin, 0.1% 10x TBE, 2 µl/ml 10% APS and 0.7 µl/ml TEMED). The gel running conditions for the ABI Prism 377 DNA sequencer was as follows: 1680 V, 50 mA and 150 W. Sequences were run at 51°C for 7 hours. A variety of different universal sequencing primers were used with the kit: SP6 primer, M13 Reverse primer, M13 Forward primer and T7 primer (see Table 3).

2.7 Phylogenetic analysis

HHV-8 ORF75 sequences were analysed using the DNASIS for Windows version 2.5 software (Hitachi Software, genetic systems, USA) and compared with references representing the A, B and C subgroups. Sequence alignment was performed using Clustal W (Thompson *et al.*, 1994). A matrix of genetic distances between strains was determined by performing pairwise comparisons using the DNADIST programme from the PHYLIP phylogenetic inference package (Felsenstein, 1993). Genetic relationships between HHV-8 isolates were determined by constructing phylogenetic trees based on the FITCH-Margoliash and least-squares method with evolutionary clock using the KITSCH algorithm from PHYLIP. The programme was asked to select the best tree which graphically represents the amount of genetic variability between the strains and subgroups. The Kimura two-parameter model was used to generate pairwise distance matrices (Kimura, 1980) and the CONSENSE programme from PHYLIP was used to determine bootstrap values at each node. Analyses were performed on 100 consecutive replicates to calculate the probability that a group of strains would cluster together. Bootstrap values of >70% corresponding to a probability of ≥95%, were considered significant (Hillis & Bull, 1993).

2.8 Heteroduplex mobility assay

A heteroduplex mobility assay (HMA) was performed by adding 5 - 7.5 µl of the KS2000/KS2034 amplicon with 2.5 - 5 µl of similarly amplified product from the BCP-1 cell line (Fig. 6). Control reactions consisted of mixing 5 µl of amplified product with 5 µl of distilled water. A number of variations were tried in which 1.1 µl of annealing buffer (1 M NaCl, 0.1 M Tris pH 7.8, 20 mM EDTA) was added and/or the mixture was allowed to anneal slowly (on ice) or quickly (at room temperature) after thermal denaturation at 94°C for 2 min. Electrophoresis loading dye (3.5 µl of a 10x dye containing 0.25% bromophenol blue, 0.25% xylene cyanol and 25% Ficoll 400 diluted in distilled water) was then added to each specimen. The bands were resolved by running on a 1x mutation detection enhancement (MDE) gel or 5% polyacrylamide gel with or without 5 - 15% urea and/or 2% glycerol. The gels were set in a 160 X 160 cast with 1.5 mm thickness Bio-Rad chamber using 0.6 - 1x TBE as running buffer. The specimens were run at 100 - 200 V for 3 - 16 hours and electrophoresis stopped when the xylene cyanol dye front had migrated to the bottom of the gel. The gels were stained with 0.1 µg/ml ethidium bromide in TBE buffer for 1 hour and bands were visualised under UV light

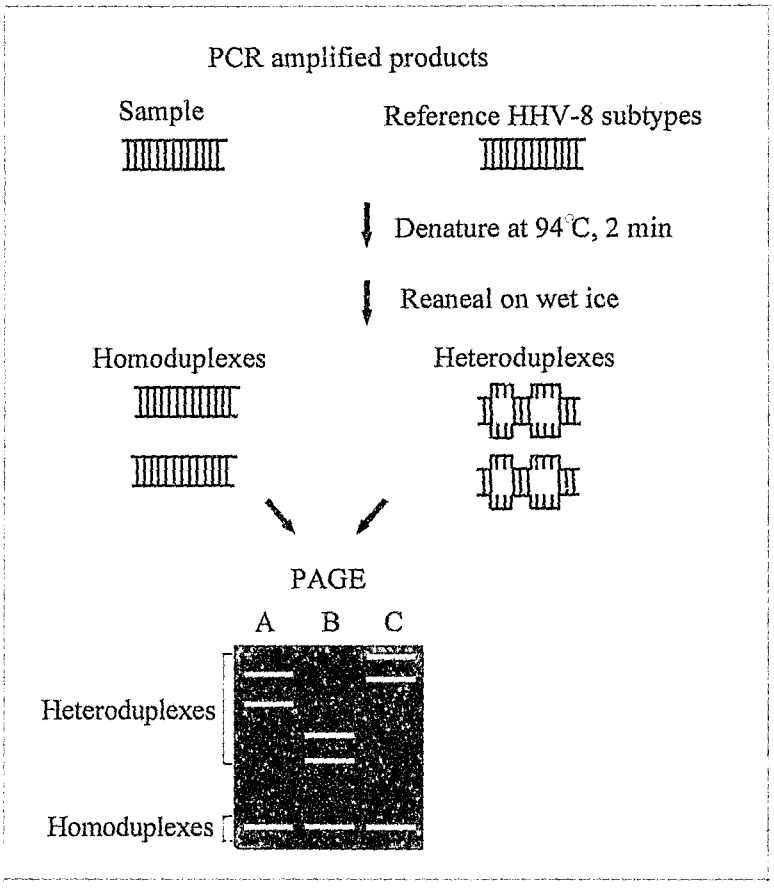


Figure 6: Schematic representation of heteroduplex mobility assay (HMA). Amplified PCR products are heat denatured and reannealed to a reference product amplified with the same primers. Due to mismatches created by the two heterologously annealed single strands of DNA, the isolate with the strongest identity to the reference product migrates closest to the homoduplexes. The degree of variation within a genome determines the mobility of heteroduplexes. Annealing of a DNA strand to its complementary strand results in homoduplex formation.

2.9 Single strand conformation polymorphisms

A diagrammatical representation of single strand conformation polymorphisms (SSCP) is shown in Fig. 7. SSCP was performed on specimens amplified by nested PCR of the ORF75

region. 5 µl of PCR product was added to 45 µl formamide and denatured by heating to 96°C for 2 minutes. The entire reaction volume (50 µl) was loaded onto a 5% polyacrylamide gel and run at 200 V for 6 hours in 1x TBE. Previously sequenced HHV-8 ORF75 regions belonging to different subgroups were included for comparison. Gels were stained with 0.1 µg/ml ethidium bromide in 1x TBE for 1 hour and single-stranded DNA bands were visualised under UV light.

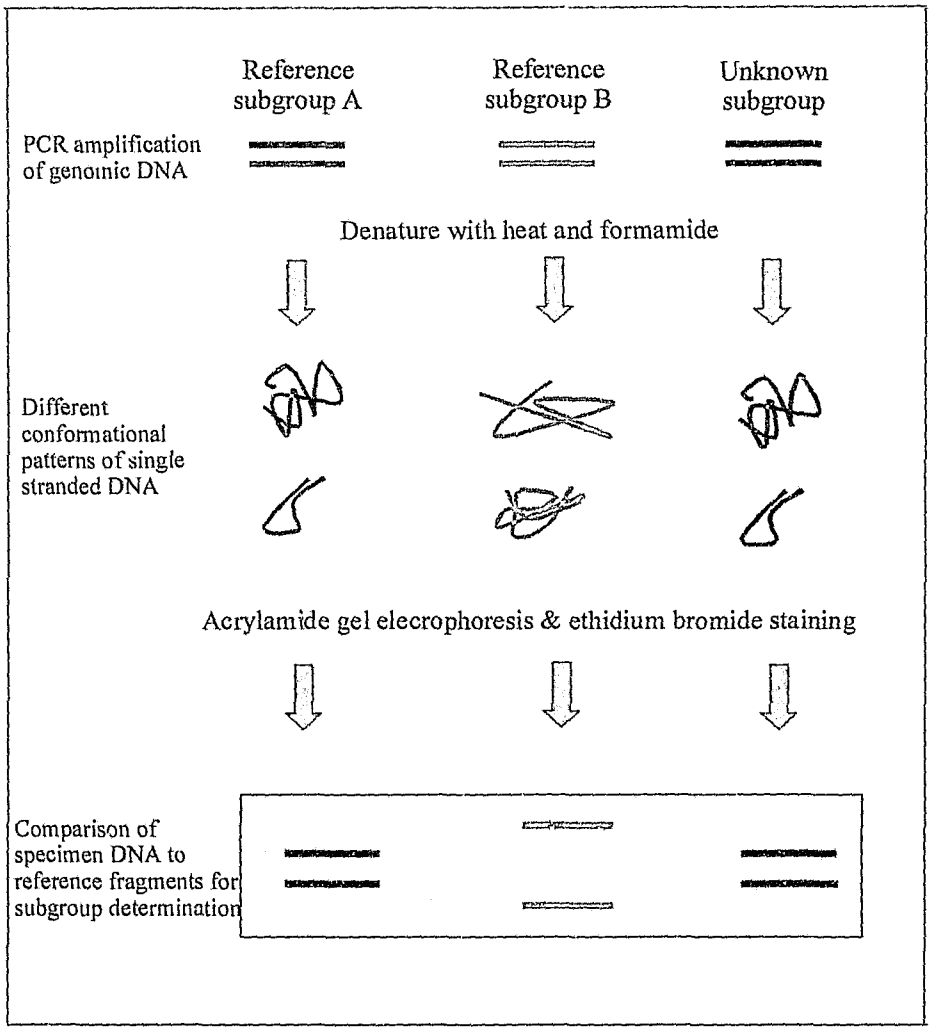


Figure 7: Schematic representation of single strand conformation polymorphisms (SSCP). Subtypic gene variations contain nucleic acid polymorphisms allowing for different folding of single-stranded structures upon cooling. These tertiary structures are unique for each subgroup and migrate at different rates through a non - denaturing gel. The high sensitivity of this assay is based on the radical change in migration due to a single base change. By comparing unknown specimen DNA to a reference fragment of known subgroup, the subgroup of the unknown isolate can be determined. In this diagram, the banding of the unknown specimen corresponds to that of subgroup A.

3. EPIDEMIOLOGY OF HHV-8 IN SOUTH AFRICA

3.1 INTRODUCTION

Both sero-epidemiological and molecular studies strongly implicate human herpesvirus 8 (HHV-8) as the infectious agent responsible for causing Kaposi's sarcoma (KS). HHV-8 DNA is consistently found in all forms of KS including AIDS-related (epidemic), iatrogenic, African (endemic) and Mediterranean (classic) KS. The presence of HHV-8 DNA in asymptomatic individuals predicts the subsequent development of KS (Chang & Moore, 1996, Gao *et al.*, 1996, Moore *et al.*, 1996c, Whitby *et al.*, 1995). Serological studies have shown that HHV-8 antibodies are detected in the majority (80-85%) of patients with classic KS. Unlike other human herpesviruses, HHV-8 is not ubiquitously present in the general population as shown by studies on blood donors in the USA and UK (0-3%) (Schulz, 1998). While the risk of acquiring KS in western countries is low, it is 20,000-fold greater in HIV - infected individuals (Gillison & Ambinder, 1997). Thus, HHV-8 appears to be under immunological control. HHV-8 sequences have also been identified in circulating spindle cells and it has been proposed that they localise in tissues and participate in the formation of KS lesions (Sirianni *et al.*, 1997). Since, HHV-8 is present many years prior to this event, the lymphoid system and other organs could pose as a reservoir of latently infected cells (Bigoni *et al.*, 1996).

While the incidence of KS is decreasing in Europe and the USA, it is on the rise on a course parallel to HIV infection in sub-Saharan Africa. KS is currently the most common tumour in some parts of Africa and the most common tumour among HIV-infected individuals [Wabinga, 1993 #650; Sitas, 1997 #2226]. In HIV-infected individuals, HHV-8 DNA is more likely to be found in PBMC's of patients with high HIV viral loads and lower CD4 cell counts (Min & Katzenstein, 1999). This suggests that HIV-1 may directly activate HHV-8 or may induce reactivation as a consequence of immunosuppression. The explosive heterosexual HIV-1 epidemic in sub-Saharan Africa, together with the associated immunosuppression, prompted us to examine the prevalence of HHV-8 in the HIV-infected population in South Africa and to compare this to a group of individuals with clinically confirmed KS.

3.2 RESULTS

3.2.1 PCR detection of HHV-8 DNA

Two nested PCR reactions in different gene regions were used to detect HHV-8 DNA in patient samples. The first was in the ORF26 region which amplified a fragment of 233 bp (Fig. 8). This fragment was sequenced manually and compared to a published sequence to confirm that HHV-8 DNA was being amplified (Fig. 9). The data confirmed that the amplified sequences were from the ORF26 region with mutations occurring at only 4 loci over 160 bases. This region is unrevealing in terms of genetic variability and was therefore not sequenced further. The second primer set amplified an 804 bp region in the ORF75 (Fig. 10). This region proved useful in determining phylogenetic relationships as it showed higher levels of genetic variation (Chapter 4).

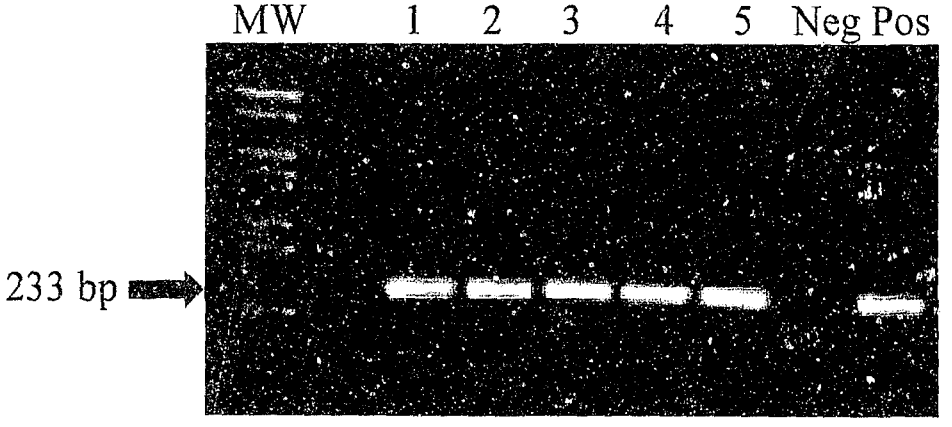


Figure 8: ORF26 PCR products. The $330BAM_{233}$ fragment amplified from 5 patients with KS. The molecular weight marker IV (Boehringer Mannheim) indicates the correct band size of 233 bp. Neg is a water control and Pos a known KS positive DNA sample.

```
KSHV330          1 TCGAATCCAA CGGATTTGAC CGCGTGTTCC CCATGGTCGT GCCGCAGCAA
LN2ZA            1 TCGAATCCAA CGGATTTGAC CGCGTGTTCC CCATGGTCGT GCCGCAGCAA
LN4ZA            1 TCGAATCCAA CGGATTTGAC CGCGTGTTCC CCATGGTCGT GCCGCAGCAA
LN7ZA            1 ---------- ---------- ---GTGTTCC CCATGGTCGT GCCGCAGCAA

KSHV330         51 CTGGGGCACG CTATTCTGCA GCAGCTGTTG GTGTACCACA TCTACTCCAA
LN2ZA           51 CTGGGGCACG CTATTCTGCA GCAGCTGTTG GTGTACCACA TCTACTCCAA
LN4ZA           51 CTGGGGCACG CTATTCTGCA GCAGCTGTTG GTGTACCACA TCTACTCCAA
LN7ZA           51 CTGGGGCACG CTATTCTGCA GCAGCTGTTG GTGTACCACA TCTACTCCAA

KSHV330        101 AATATCGGCC GGGGCCCCGG GTGATGTAAA TATGGCGGAA CTTGATCTAT
LN2ZA          101 AATATCGGCC GGGGCCCCGG GTGATGTCAA TATGGCGGAA CTTGATCTAT
LN4ZA          101 AATATCGGCC GGGGCCCCGG GTGATGTCAA TATGGCGGAA CTTGATCTAT
LN7ZA          101 AATATCGGCC GGGGCCCCGG GTGATGTCAA TATGGCGGAA CTTGATCTAT

KSHV330        151 ATACCACCAA
LN2ZA          151 ATACCACCAA
LN4ZA          151 ATACCACCAA
LN7ZA          151 ATACCACCAA
```

Figure 9: Sequence of the KS330BAM$_{233}$ PCR product. 160 bp from 3 South African isolates were compared to a published sequence (KSHV330). This reference is a portion of the ORF26 region obtained from the Genbank sequence (accession number: U75698). The polymorphic loci are highlighted in red.

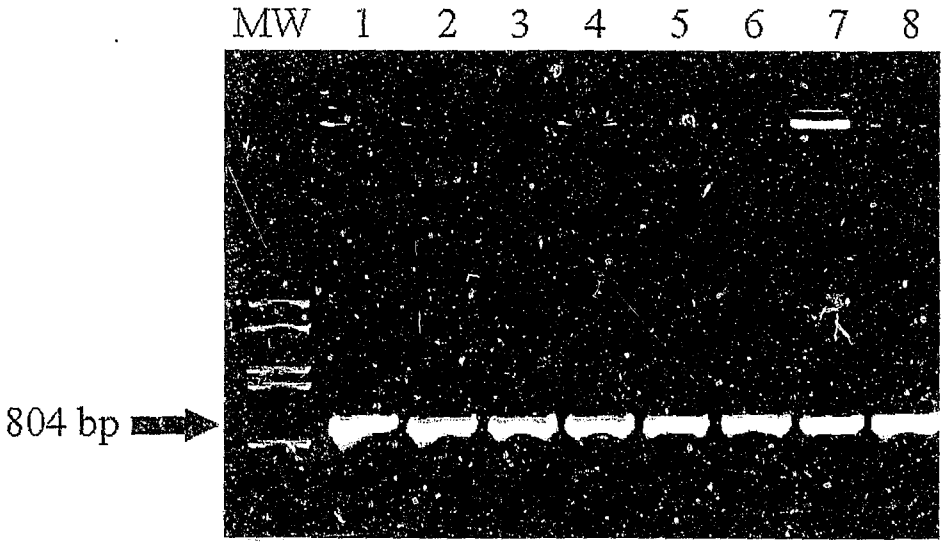


Figure 10: ORF75 PCR products. The KS2600/2034 fragment amplified from 8 specimens. The molecular weight marker VI (Boehringer Mannheim) indicates the correct band size of 804 bp.

3.2.2 Detection of HHV-8 DNA in peripheral blood cells from individuals with and without KS

Peripheral blood mononuclear cells (PBMC) from 334 HIV-infected individuals without KS and 95 individuals with KS were analysed for the presence of HHV-8 DNA by nested PCR in both ORF26 and ORF75. Individuals in the KS negative group were recruited from local HIV outpatient clinics (n=208) and from those who had been hospitalised (n=126)(see Table 4). The former were largely asymptomatic with a median CD4 count of 351 cells/μl while the latter were admitted with active opportunistic infections, mostly tuberculosis. The median CD4 count in this group was 218 cells/μl. Patients with KS were diagnosed clinically and this was confirmed by histological investigation. Of those whose age was known (n=90), 56 were male and 34 female and the median age was 36. The majority of these patients were HIV-infected (84/92, 88%) and, as such, were considered to be AIDS-KS cases.

	N	Males	Age*	PCR	IFA
KS negative, HIV-positive (n=334)					
Outpatients					
Private patients	147	81	36	147	121
Hillbrow Hospital AIDS Clinic	27	20	37	27	0
Johannesburg Hospital AIDS Clinic	26	15	38	26	0
Hemophiliac Clinic	8	ND	ND	8	8
Hospitalised patients					
Tuberculosis	74	74	ND	74	41
Meningitis	45	45	ND	45	33
Lymph node biopsies	7	7	34	7	0
KS confirmed (n=95)	95	56	36	95	88
TOTAL	**429**	**298**	**36**	**429**	**291**

Table 4: Details of KS negative and KS confirmed individuals.

The sex, average age and number of patients within each cohort studied in which PCR and IFA was done.
* Age, where known, is for the entire group.

KS patients were clinically diagnosed and confirmed histologically.

Table 5: Prevalence of HHV-8 DNA in PBMC's from individuals with and without clinically confirmed KS

	PCR positive		
	ORF26 region	**ORF75 region**	**Either/Both regions**
KS negative	14/334 (4.2%)	14/334 (4.2%)	14/334 (4.2%)
KS confirmed	42/95 (44%)	41/95 (43%)	46/95 (48%)
TOTAL	**56/429 (13%)**	**55/429 (13%)**	**60/429 (14%)**

The PCR positive results from both the KS negative and KS confirmed cohorts are shown. These results are shown for amplification of HHV-8 within 2 regions of the genome: ORF26 (330BAM) and ORF75 and the increased specificity of detection in both regions.

Amplification of HHV-8 gene sequences in PBMC was 10-fold higher among the KS confirmed cases (46/95, 48%, see Table 5) than among individuals who did not have KS (14/334, 4.2%). Similar results were obtained with both primer pairs indicating equivalent sensitivity of the two PCR reactions. Among the 14 individuals that were HHV-8 DNA positive, but without visible signs of KS, 9 were among outpatients and 5 were in the hospitalised cohort. The percentage of positive samples was similar in the two groups: 9/208 (4.3%) and 5/126 (4.0%) respectively. None of the PBMCs from the hemophiliac group were PCR positive. Overall, of the 60 samples which were HHV-8 DNA positive by PCR, 46 (77%) were among individuals with confirmed KS and 14 (23%) were among KS negative patients, confirming the association between HHV-8 DNA and KS.

3.2.3 HHV-8 DNA is more frequently found in lymph nodes compared to blood

Lymph node biopsies were available from 8 HIV-infected patients who were undergoing routine diagnostic investigation for suspected tuberculosis. One of these patients was found to have KS spindle cells in the lymph node on histological examination (Fig. 11), although he had no visible skin lesions at the time. This patient was HIV-seropositive and presented with splenomegaly, hepatomegaly and generalised lymphadenopathy. The remaining 7 patients were all KS negative clinically and on histological examination. PCR analysis using the KS330BAM$_{233}$ primers revealed that patient 7 harboured HHV-8 DNA in both PBMC and lymph nodes confirming his KS diagnosis (Fig. 12). Two of the KS negative patients were also found to be HHV-8 DNA positive in the lymph nodes, but were negative in the blood. All other patients were HHV-8 negative in both lymph node and blood samples.

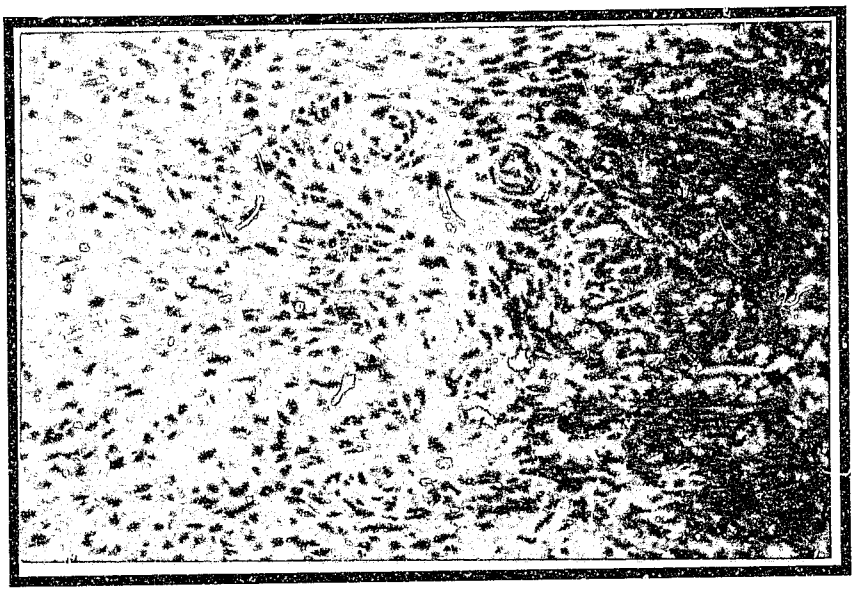


Figure 11a: Histopathology of KS in a lymph node. This picture shows a section from LN7ZA stained with hematoxylin and eosin (H&E) at 100x magnification. The LN shows an obvious change in structural integrity with a predominance of spindle-shaped cells. Surrounding these cells are irregular blood-filled vascular spaces. There is also a lymphocytic infiltrate which is a characteristic feature of KS.

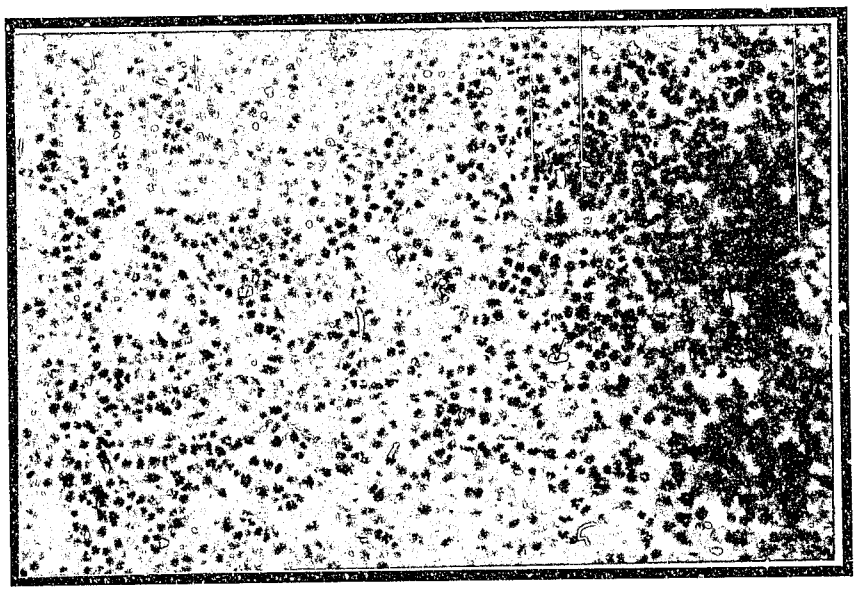


Figure 11b: Picture of a LN from an HIV-1 infected patient without KS for comparison. Normal histopathology with defined cortex and medullary regions are visible.

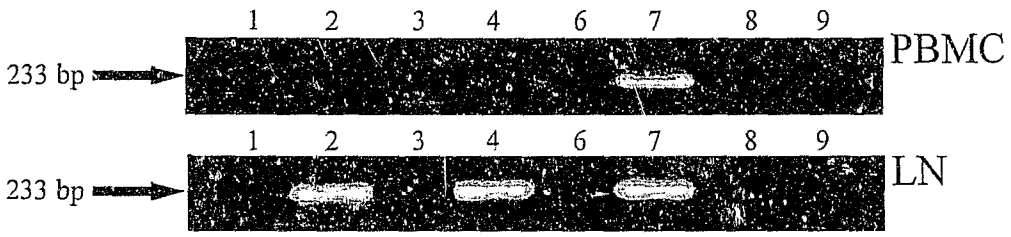


Figure 12: Matched PBMC and LN from 8 HIV-1 infected individuals. KS330BAM$_{233}$ PCR amplified HHV-8 in both LN and blood from a patient with histologically confirmed KS in LN biopsy (lane 7). HHV-8 DNA was detected in the LN's from 2 other patients (lanes 2 and 4) but not in their blood. None of these patients had visible KS lesions at the time of the investigation.

3.2.4 Prevalence of antibodies to HHV-8 in KS confirmed and HIV-positive individuals

Plasma samples from 88 of the 95 KS confirmed and 203 of the 334 HIV-infected individuals were available for serological analysis (see Table 5). Antibodies to the latency-associated nuclear antigen (LANA) were detected using the BCP-1 cell line in an indirect immunofluorescence assay (IFA). An example of a positively stained IFA is shown in Fig. 13. Results showed that 69 (78%) of KS patients had IgG antibodies to HHV-8 (Table 5). When patients who were positive for both anti-HHV-8 antibodies and DNA were analysed, this increased to 93%. Among the HIV-infected KS negative group 33 (16%) were antibody positive. Again a larger proportion of those who were PCR positive were also antibody positive although the numbers in this group were small. Of the 33 antibody positive samples, the majority came from hospitalised patients (22/74, 29.7%) compared to outpatient clinic attenders (10/121, 8.3%). One hemophiliac patient was HHV-8 antibody positive (1/8; 12.5%). Six samples were HHV-8 DNA PCR positive but were IFA negative.

Previous studies have shown that the signal intensity correlated with the titre of anti-HHV-8 antibodies (Sitas *et al.*, 1999). In order to determine if antibody titres correlated with viremia in blood, the 69 IFA positive samples from the KS confirmed individuals (Table 6) were scored as +, ++ or +++ depending on their fluorescence intensity and compared to the PCR

results (Table 7). There was no correlation between the antibody titres and the presence of viral DNA in blood. For both low and high titre samples approximately half of the patients had HHV-8 DNA in blood.

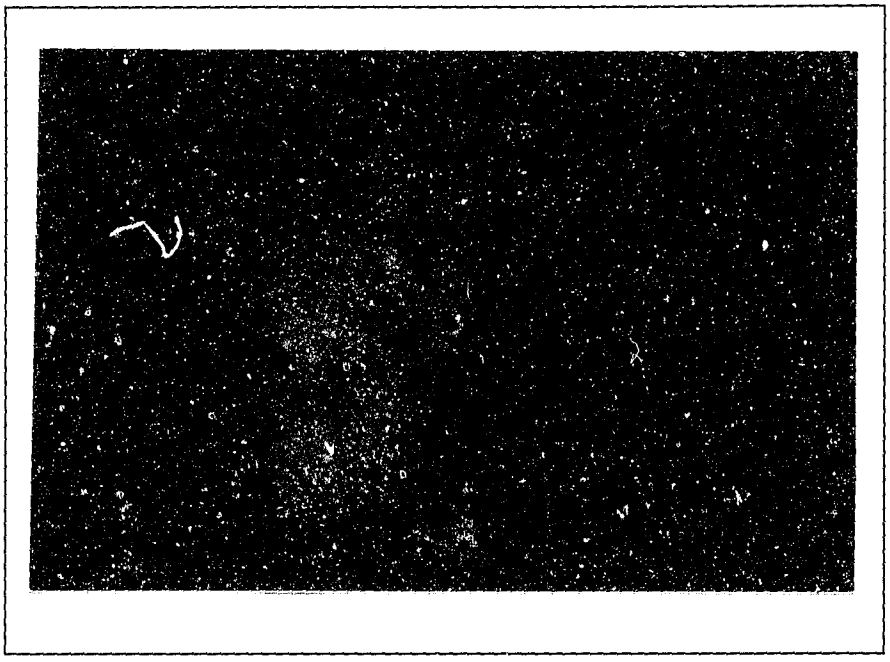


Figure 13: Detection of anti-HHV-8 antibodies using an IFA. LANA antigens appear as fluorescent green speckles in the nucleus of the BCP-1 cell line.

Table 6: Detection of anti-HHV-8 LANA antibodies in plasma from HIV-infected individuals with and without clinically confirmed KS.

		N	IFA positive	IFA negative
KS negative				
	PCR positive	8	5 (63%)	3 (38%)
	PCR negative	195	28 (14%)	167 (86%)
	Total	203	33 (16%)	170 (84%)
KS confirmed				
	PCR positive	41	38 (93%)	3 (7%)
	PCR negative	47	31 (66%)	16 (34%)
	Total	88	69 (78%)	19 (22%)

Table 7: IFA signal intensity in KS confirmed individuals

IFA intensity	PCR positive	PCR negative	Total	% PCR positive
+	23	18	41	56%
++	11	8	19	58%
+++	4	5	9	44%
Total	**38**	**31**	**69**	**55%**

3.3 DISCUSSION

HHV-8 DNA and anti-HHV-8 antibodies have been detected in the blood of South African individuals with and without KS, most of whom were also HIV-infected. A significantly higher proportion of individuals with KS were HHV-8 infected confirming the link between KS and HHV-8. However, HHV-8 was present in individuals without KS indicating that asymptomatic HHV-8 infection also occurs in this region.

There was a good correlation between the two non-overlapping nested primer sets indicating that positive PCR results were not due to contamination. Sequencing these fragments also confirmed that HHV-8 was being amplified. PCR is however, not adequate for diagnosis and epidemiology studies as the absence of HHV-8 DNA in a specific tissue does not exclude infection at other sites. For example, we and others have found that only ~50% of patients with KS have detectable HHV-8 DNA in blood (Whitby *et al.*, 1995) although it is present in almost all lesional material (Chang, 1994). Nevertheless PCR provides important information on the presence of potentially infectious virus in blood and other sites as well as pathogenesis and latency. Immunofluorescence techniques have been developed to detect circulating HHV-8 antibodies (Kedes *et al.*, 1996). These assays document exposure to the virus and have been useful in sero-prevalence studies. Varying sero-prevalence rates in different cohorts and countries are largely thought to reflect assay variability (Rabkin *et al.*, 1998). Current assays rely on the detection of latency associated nuclear antigens (LANA), the predominant antigen encoded by ORF73 (Lin *et al.*, 1995b). However, these assays are only 80-90% sensitive which would explain why some patients in this study were HHV-8 PCR positive but IFA negative.

Assays detecting lytic antigens in the cytoplasm have been reported to be more sensitive than LANA, but this awaits confirmation. In general, while current HHV-8 serologic assays require improvement both in terms of specificity and sensitivity, they are useful tools for sero-epidemiological studies although they should not be used in individual patient diagnosis (Rabkin *et al.*, 1998).

A higher proportion of KS patients had antibodies to HHV-8 as compared to HHV-8 DNA in their blood (78% versus 48%). Our sero-prevalence rates are similar to those reported in the USA, Italy and Uganda for AIDS-KS with 71-88% recorded [Gao, 1996 #2163]. Patients who were viremic (ie had HHV-8 DNA in blood) were more likely to be HHV-8 antibody positive (93% versus 66%). This indicates that viremia is associated with good antibody responses, possibly due to higher levels of replicating virus and antigen. However, there was no relationship between antibody titre and viremia (Table 7) indicating that the periphery is not the major site for antigen-specific B cell activation. Our finding that 48% of patients with KS had HHV-8 DNA in the circulation is similar to studies done previously in the USA (57%) (Moore *et al.*, 1996c), UK (52%) (Whitby *et al.*, 1995) and Europe (45%) (Quinlivan *et al.*, 1997). Why do not all KS patients have HHV-8 in blood? This could reflect virus levels that are below the level of detection by PCR or that the virus is no longer in the circulation after the establishment of lesions. HHV-8 is a latent virus appearing in the blood when immunological control is compromised such as in HIV-infection (Whitby *et al.*, 1995). Detection of HHV-8 DNA in blood is therefore probably associated with dissemination of the virus and the establishment of lesions. AIDS-KS is more severe than other forms of KS suggesting that HIV plays a role in the pathogenesis (Wahman *et al.*, 1991). Indeed HIV-1 Tat protein has shown to be a progression factor in AIDS-KS (Ferbeyre *et al.*, 1997, Fiorelli *et al.*, 1998, Harrington *et al.*, 1997).

Our data show the background sero-prevalence of HHV-8 in the HIV-infected population in Gauteng to be 16%. Similar to the KS infected individuals, anti-HHV-8 antibodies were more readily detectable than HHV-8 DNA and in this group the differential was higher (16% versus 4.2%). This probably reflects asymptomatic infection with antibody production, but little viral replication. The presence of virus in blood is a highly predicative marker with 50% of HHV-8 DNA positive individuals developing KS within 3-5 years (Moore *et al.*, 1996c, Whitby *et al.*,

1995). There is also a correlation between the levels of virus (viral load) and progression to KS (Gillison & Ambinder, 1997, Poggi *et al.*, 1997). Thus our data predict that 2.1% of HIV-infected individuals in this region will develop KS. In this study a higher proportion of patients with lower CD4 counts (hospitalised group) were antibody positive compared to those who were outpatients (27% versus 8.3%) indicating that patients with advanced HIV disease had higher HHV-8 infection rates. However, this was not the case with HHV-8 DNA which was equivalent in both groups (4% and 4.3%) although the numbers in these groups were small. Studies done elsewhere on advanced AIDS patients without KS have found 7% to be HHV-8 DNA positive in blood (Boshoff *et al.*, 1995a). This may reflect differences in the cohorts or the immaturity of the HIV epidemic in South Africa. However, we found that HHV-8 DNA was detected more frequently in the lymph nodes compared to blood suggesting that the lymph node is a latent site for HHV-8 infection. Thus, the prevalence of HHV-8 DNA in individuals with no visible signs of KS is likely to be higher than 4.2% as suggested by analysing blood.

Recently, the sero-prevalence of HHV-8 in KwaZulu-Natal, a rural province of South Africa, was reported (Wilkinson *et al.*, 1999). Among 136 hospitalised individuals, 34.6% were seropositive. In another study by Sitas and co-workers 32% of cancer patients in Gauteng, excluding those with KS, were found to be HHV-8 antibody positive (Sitas *et al.*, 1999). In the latter study, the percentage was similar between HIV-negative and HIV-positive persons and among black blood donors the sero-prevalence was reported to be 20%. Our finding of 16% sero-prevalence among HIV-infected individuals could reflect differences in the cohorts analysed or differences in the assays used to detect HHV-8 antibodies. Thus there may be regional differences with Kwa-Zulu Natal having a higher background sero-prevalence as is the case for HIV-infection (Health, 1999). Our data show that patients who were immunosuppressed had higher levels of anti-HHV-8 antibodies (27%) which may compare with individuals on immunosuppressive therapy for cancer treatment. Together results from our study, in addition to those of Sitas and Wilkinson, indicate a high background prevalence of HHV-8 in South Africa. Thus KS is likely to become a leading cause of cancer and an important HIV-related opportunistic infection in South Africa.

4. PHYLOGENETIC ANALYSIS OF HHV-8 ISOLATES

4.1 Introduction

KS is classified into four forms namely; classic (Mediterranean region), endemic (central Africa region), iatrogenic (immunosuppressed individuals), and epidemic (AIDS-associated). These different forms of KS are not histologically distinguishable, although genetic studies of HHV-8 have revealed that they are associated with different subgroups. The HHV-8 genome is 170 kb and shows little overall nucleotide variation [Decker, 1996 #589]. Recent studies have identified the ORF75 as useful in delineating different strains as it shows 1.5-1.9% variation (Zong *et al.*, 1997). Analysis of this region indicates that subgroup A predominates in classic KS, subgroups B and C in KS from Africa and all subgroups are found in AIDS-related KS. Strains within the A subgroup showed little variation suggesting that these viruses originated from a single source. The C variants were found in patients from Africa with aggressive disseminated disease. Another study confirmed the presence of A, B and C subgroups as well as a fourth variant, D, in the oral tissues of HIV-infected individuals from different regions (Di Alberti *et al.*, 1997). The A and D strains were found predominantly in Africa and the USA and the B and C strains in the UK and Italy. In another study, subtypes B and C were found in Sao Paolo, Brazil and shown to resemble variants found in the aggressive form of KS from Africa (Caterino-de-Araujo, 1998, Zong *et al.*, 1997). Using the ORF26 region, subgroups A, B and C were found in France although subgroup B was the predominant form (Boralevi *et al.*, 1998). Subgroup A strains were more frequently found in mucosal and/or visceral lesions in French AIDS patients. As subgroup A is also associated with AIDS BCBL lymphomas in the USA, this suggests that this subgroup may have a higher transforming potential. Together these data indicate that distinct HHV-8 variants based on analysis of the ORF75 or ORF26 may be geographically distributed. Whether genetic differences in the HHV-8 genome underpin differences in the clinical presentation still remain to be determined. The incidence of KS in South Africa has increased markedly with the onset of the HIV-epidemic and as such AIDS-KS is the predominant form. However, it is not known what the circulating HHV-8 subgroups in this region are.

4.2 Results

Among the 429 specimens analysed, 41 were found to be HHV-8 PCR positive (see Chapter 3) and were used for sequencing analysis. This included a LN and a blood sample from a single patient (LN7ZA and PB7ZA). Of the 40 patients, 25 were KS confirmed individuals and 15 had no visible signs of KS (Table 7). The majority of patients were males (29/40) and black with a median age for the cohort of 40. Those with clinically confirmed KS have been given a KS prefix. There is less information available on the 15 asymptomatic (KS negative) individuals as these were identified through screening of HIV-infected cohorts. A number of these (n=7) were migrant workers on mines (LN, MEN ant TS) and hence are all male. The remaining 8 were private patients (PP) or attended outpatient AIDS clinics (RS and DS). All patients were HIV-positive except for 2 (KS80ZA and KS82ZA) who had KS lesions, but were HIV-seronegative.

	Specimen	Gender	Ethnicity	Age	Born	Reside	HIV status
1	KS12ZA	M	B	33	Abroad	Gauteng	Pos
2	KS13ZA	F	B	31	Abroad	Abroad	Pos
3	KS18ZA	M	B	51	Gauteng	Gauteng	Pos
4	KS23ZA	M	B	30	KZN	KZN	ND
5	KS31ZA	F	B	44	Gauteng	Gauteng	Pos
6	KS33ZA	M	B	27	KZN	KZN	Pos
7	KS35ZA	M	B	58	Gauteng	Gauteng	Pos
8	KS37ZA	M	B	58	Gauteng	Gauteng	Pos
9	KS49ZA	M	B	44	NTVL	NTVL	Pos
10	KS61ZA	M	B	36	Abroad	Gauteng	Pos
11	KS64ZA	M	B	28	Abroad	Gauteng	Pos
12	KS65ZA	M	B	28	NTVL	Gauteng	ND
13	KS69ZA	F	B	56	NWCape	NWCape	Pos
14	KS70ZA	F	B	29	KZN	Gauteng	Pos
15	KS76ZA	M	B	50	Abroad	Gauteng	Pos
16	KS78ZA	M	B	41	Gauteng	Gauteng	Pos
17	KS80ZA	F	B	76	Gauteng	Gauteng	Neg
18	KS81ZA	M	B	34	Abroad	NTVL	Pos
19	KS82ZA	F	B	28	NTVL	Gauteng	Neg
20	KS83ZA	F	B	46	Gauteng	Gauteng	Pos
21	KS84ZA	F	B	25	Gauteng	Gauteng	Pos
22	KS85ZA	M	B	41	Gauteng	NTVL	Pos
23	KS88ZA	F	B	55	NTVL	Gauteng	Pos
24	KS89ZA	M	B	52	KZN	KZN	Pos
25	KS91ZA	F	B	33	Gauteng	Gauteng	Pos
26	LN2ZA	M	B	48	ND	Gauteng	Pos
27	LN4ZA	M	B	29	ND	Gauteng	Pos
28a	LN7ZA	M	B	37	ND	Gauteng	Pos
28b	PB7ZA	M	B	37	ND	Gauteng	Pos
29	MEN21ZA	M	B	ND	ND	Gauteng	Pos
30	MEN43ZA	M	B	59	ND	Gauteng	Pos
31	PP158ZA	M	W	40	ND	Gauteng	Pos
32	PP163ZA	F	B	40	Zambia	Gauteng	Pos
33	DS814ZA	M	W	37	SA	Gauteng	Pos
34	DS895ZA	M	B	40	Malawi	Gauteng	Pos
35	RS820ZA	M	Mixed race	ND	SA	Gauteng	Pos
36	RS901ZA	M	B	ND	ND	Gauteng	Pos
37	RS904ZA	M	Indian	45	SA	Gauteng	Pos
38	TS522ZA	M	B	ND	SA	Gauteng	Pos
39	TS652ZA	M	B	39	Mozambique	Gauteng	Pos
40	TS799ZA	M	B	ND	Transkei	Gauteng	Pos

Table 8: Demographic information on sequenced specimens. Patients from outside South Africa are marked in red. HIV-negative KS patients are shown in green. DNA from all samples were obtained from PBMC's except in the case of 3 LN's (blue). ND=not done/unknown, B=black, M=male, F=female.

4.2.1 Nucleic acid phylogenetic analysis

In order to determine the extent of genetic diversity among South African HHV-8 isolates, samples were sequenced and analysed phylogenetically using the ORF75 gene. The 804 bp fragment from the 41 samples were cloned and sequenced in both directions and compared to 14 distinct A, B and C subgroups used as reference strains (Zong *et al.*, 1997). The majority of the South African specimens (n=30) fell within an internal branch of the A and B subgroups and 3 grouped with C subgroup. However, 8 sequences did not cluster with any previously identified group and were termed novel (N) isolates (Fig. 14). The South African isolates in the C and N group were supported by high bootstrap values of 82% and 100% indicating that these two groups were significantly different and can be considered as 2 separate subgroups. Similarly, a bootstrap value of 100% at the nodes separating the C and A/B groups showed that these two groups were distinct. However, subgroups A and B were only separated by a bootstrap value of 42% indicating a low probability that they form 2 discrete subgroups although the A and B references were supported by high bootstrap values within these groups. This is more clearly shown using a star phylogeny where subgroups A and B form a single overlapping group separate from the A and B reference isolates (Fig. 15). Thus, the South African isolates are outliers of the reference A and B subgroups and are therefore termed A/B variants.

There was no characteristic difference among HHV-8 isolates from different regions within South Africa compared to individuals known to have originated from other countries, including Zambia, Malawi and Mozambique (see Table 8) as these sequences were dispersed among the local sequence strains. One patient (DS814ZA), a European homosexual man was shown to harbour a C strain which clustered with isolates from New York and showed 1 nucleotide difference from KSHV75, a USA strain. However this variant is probably also circulating locally as the viruses from 2 HIV-positive black females from Gauteng (KS70ZA and KS91ZA) were also significantly associated with this group (Fig. 14). HHV-8 isolates from the 2 HIV-negative specimens (KS80ZA and KS82ZA) did not show any unique groupings, but were similar to specimens from HIV-infected patients which were outliers of the A group. The N group appears to be unique to this region as it has not previously been described. It is, however, relatively common in that 20% (8/40) of isolates were shown to cluster with the N group. Individuals infected with N variants showed no unique demographic, clinical or histological features.

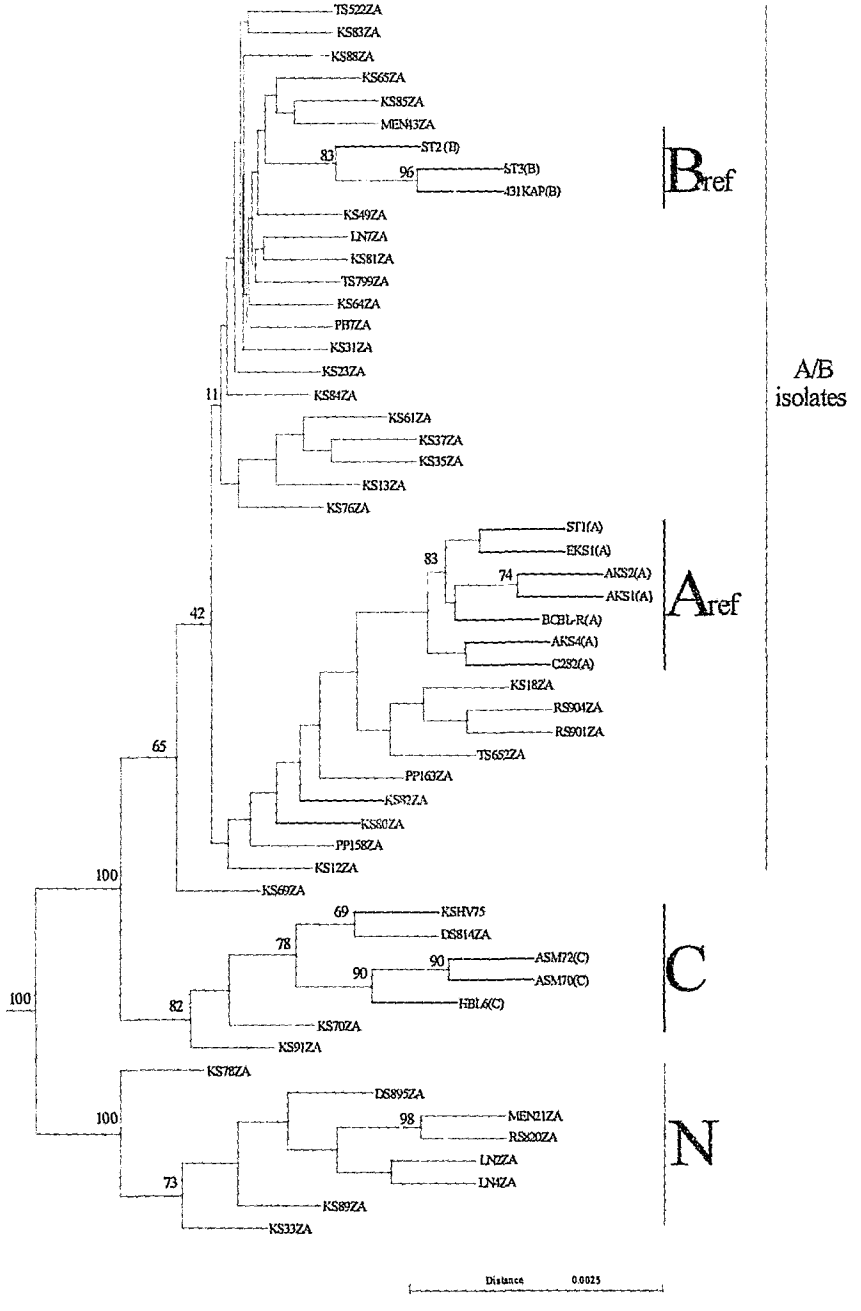


Figure 14: Phylogenetic analysis of the 804 bp ORF75 region of 41 South African HHV-8 isolates. All specimens were collected within South Africa. The best tree determined from 100 replicates in a KITSCH algorithm was used in the construction of the phylogenetic tree. Bootstrap values ≥70% are indicated as they represent significant branching. Three low bootstrap values (11%, 42% and 65%) are included to show that the South African A and B strains do not form significant groupings and are referred to as A/B variants. The 14 specimens in red are the reference samples and the blue writing denotes the subgroup. Two isolates highlighted in green are HIV-negative individuals. N = novel subgroup. PB7ZA and LN7ZA are matched PBMC and LN cells from the same individual.

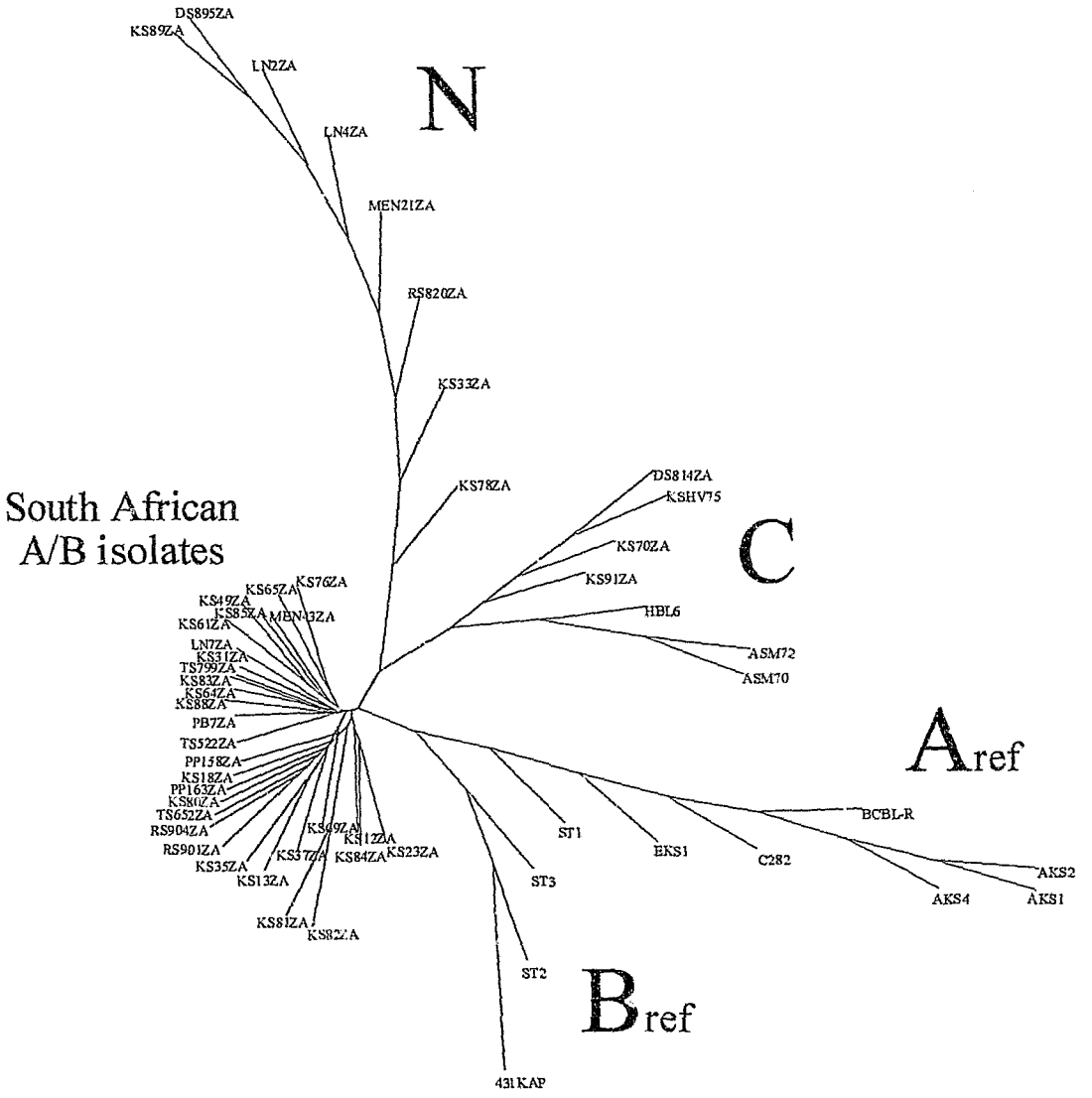


Fig 15: Star phylogeny showing the nucleic acid relationships of the 41 South African isolates. This tree was constructed as for Fig. 14 then using DRAWTREE from PHYLIP to create a star format. A clear distinction is visible between the N and C subgroups, whereas, the South African A and B variants cluster together. The A_{ref} and B_{ref} subgroups represent the reference strains highlighted in red and the HIV-negative individuals are shown in green.

4.2.2 Subtypic variation

In order to determine the degree of difference of the N group from the A, B and C groups, the inter-strain nucleotide distance was calculated using the distance matrix algorithm from PHYLIP. All eight group N strains were compared to subgroup A (n=7), subgroup B (n=3) and subgroup C (n=5) strains that were defined by phylogenetic analysis (see Fig. 14). The N group differed from A, B and C variants by 4.7%, 3.8% and 4.5% respectively (Table 9) indicating that it was equivalently different from each of the 3 established subgroups. The intra-strain variation for each of the subgroups was also determined. Within the reference A and B strains no intra-subgroup differences were observed although there was a 1% variation between strains that were classified as A/B (not shown). An average intra-subgroup variation of 0.4% was observed for both the C and N groups (Table 10).Thus, while the N group showed significant variation from the established subgroups, strains within the N subgroup showed limited variation relative to each other, similar to subgroups A, B and C.

Table 9: Inter-strain variation between the novel (N) and the A, B and C subgroups.

Inter-strain variation between N and A subgroups

N\A	ST1	EKS1	AKS2	AKS1	BCBL-R	AKS4	C282
KS78	0.049	0.049	0.049	0.049	0.049	0.049	0.049
DS895	0.048	0.048	0.048	0.048	0.048	0.048	0.048
MEN21	0.045	0.045	0.045	0.045	0.045	0.045	0.045
RS820	0.045	0.045	0.045	0.045	0.045	0.045	0.045
LN2	0.045	0.045	0.045	0.045	0.045	0.045	0.045
LN4	0.045	0.045	0.045	0.045	0.045	0.045	0.045
KS89	0.046	0.046	0.046	0.046	0.046	0.046	0.046
KS33	0.049	0.049	0.049	0.049	0.049	0.049	0.049
Sum	0.372	0.372	0.372	0.372	0.372	0.372	0.372
Avg	0.047	0.047	0.047	0.047	0.047	0.047	0.047

Inter-strain variation between N and B subtypes

N\B	ST2	ST3	431KAP
KS78	0.044	0.044	0.044
DS895	0	0.041	0.041
MEN21	0.038	0.038	0.038
RS820	0.038	0.038	0.038
LN2	0.038	0.038	0.038
LN4	0.038	0.038	0.038
KS89	0.040	0.040	0.040
KS33	0.042	0.042	0.042
Sum	0.279	0.320	0.320
Avg	0.035	0.040	0.040
		Tot. Avg=	0.038
		% Avg=	3.80%

Tot. Avg= 0.047
% Avg= 4.70%

Inter-strain Variation between N and C subtypes

N\C	KSHV75	DS814	ASM72	ASM70	HBL-6
KS78	0.052	0.052	0.051	0.051	0.051
DS895	0.046	0.046	0.045	0.045	0.045
MEN21	0.044	0.044	0.042	0.042	0.042
RS820	0.044	0.044	0.042	0.042	0.042
LN2	0.044	0.044	0.042	0.042	0.042
LN4	0.044	0.044	0.042	0.042	0.042
KS89	0.045	0.045	0.044	0.044	0.044
KS33	0.048	0.048	0.046	0.046	0.046
Sum	0.366	0.366	0.355	0.355	0.355
Avg	0.046	0.046	0.044	0.044	0.044
				Tot. Avg=	0.045
				% Avg=	4.50%

From DNADIST in the PHYLIP programme, a distance matrix was obtained from which the nucleic acid differences between the novel subgroup (N), shown in the extreme left hand column, and the 3 reference subgroups was calculated.

Table 10: Intra-strain variation within the C and N HHV-8 subgroups

Intra-strain variation within the N subgroup

N\N	KS78ZA	DS895ZA	MEN21ZA	RS820ZA	LN2ZA	LN4ZA	KS89ZA	KS33ZA
KS78	0	0.01	0.008	0.008	0.008	0.008	0.009	0.014
DS895	0.010	0	0.003	0.003	0.003	0.003	0.004	0.009
MEN21	0.008	0.003	0	0	0	0	0.001	0.006
RS820	0.008	0.003	0	0	0	0	0.001	0.006
LN2	0.008	0.003	0	0	0	0	0.001	0.006
LN4	0.008	0.003	0	0	0	0	0.001	0.006
KS89	0.009	0.004	0.001	0.001	0.001	0.001	0	0.008
KS33	0.013	0.009	0.006	0.006	0.006	0.006	0.008	0
Sum	0.062	0.033	0.017	0.017	0.017	0.017	0.025	0.055
Avg	0.008	0.004	0.002	0.002	0.002	0.002	0.003	0.007
							Tot. Avg=	0.004
							% Avg=	0.40%

Intra-strain variation within the C subgroup

C/C	KSHV75	DS814ZA	ASM72	ASM70	HBL6	KS70ZA	KS91ZA
KSHV75	0	0.001	0.001	0.001	0.001	0.004	0.008
DS814ZA	0.001	0	0.003	0.003	0.003	0.005	0.009
ASM72ZA	0.001	0.003	0	0	0	0.005	0.010
ASM70ZA	0.001	0.003	0	0	0	0.005	0.010
HBL6	0.001	0.003	0	0	0	0.005	0.010
KS70ZA	0.004	0.005	0.005	0.005	0.005	0	0.010
KS91ZA	0.008	0.009	0.010	0.010	0.010	0.010	0
Sum	0.017	0.024	0.019	0.019	0.019	0.035	0.056
Avg	0.002	0.003	0.003	0.003	0.003	0.005	0.008
						Tot. Avg=	0.004
						% Avg=	0.40%

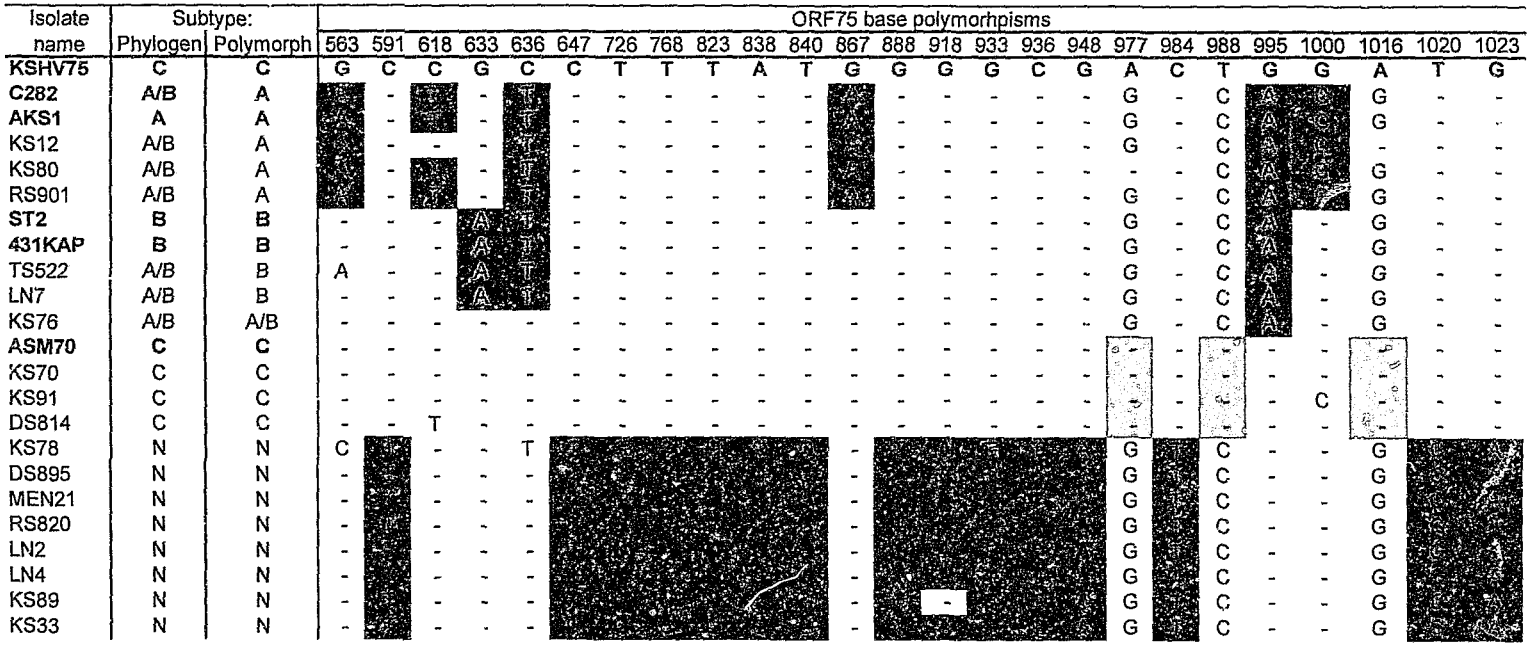
From DNADIST in the PHYLIP programme, a distance matrix was obtained from which the nucleic acid differences were calculated. There were no differences within the A/A and B/B subgroups (i.e. the reference subgroups; not shown).

4.2.3 Nucleic acid polymorphisms in the ORF75 gene

Three subgroup categories of HHV-8 have previously been described by Zong and co-workers based on 17 characteristic mutations at distinct loci (Zong *et al.*, 1997). Five of these

loci differentiated the A group, 1 the B group and 11 the C subgroup. We performed a similar analysis on a subset (n=17) of the South African isolates to determine if they contained these group-specific polymorphisms (Table 11). Most of the South African A/B strains showed mutations characteristic for either subgroup A (nucleotide positions: 563, 618, 867, 1000, 1085) or subgroup B (633) and, therefore, based on this analysis would be classified as such. However, they also showed an additional 4 mutations (636, 995, 1034, 1035) which were shared by all A/B variants. This supports the phylogenetic data indicating that South African A and B strains show significant overlap (Fig. 14 and 15). KS76ZA was unusual in that it had 3 of the 4 mutations in common with A/B variants, but none of the other loci which identified the A or B subgroup. This strain showed a similar pattern to ST1, an isolate from a Ugandan AIDS patient, which was considered to be a recombinant strain (Zong *et al.*, 1997). KS76ZA may therefore represent a similarly unusual strain but further analysis is needed. Six subgroup C loci were lost as a result of the addition of the South African group N samples. Thus nucleotide positions 636, 995,1034, 1035, 1274 and 1276 occurred in both C and N subgroups and were, therefore, no longer specific for the C subgroup, although, 4 of them now defined the A/B variants. In addition, a G at position 1028 was not found in South African C strains and was therefore not a characteristic mutation for this subgroup. However, four subgroup C loci were retained (977, 988, 1016, 1084) plus a new locus (1278) was identified for this subgroup. An additional 32 key diagnostic loci were identified for the N subgroup which were distinct and did not overlap with the 17 previously identified loci. This gives a total of 47 loci which defined the 4 subgroups. These subgroup-specific patterns are indicated as different colours in Table 10. Over a 804 bp region this translates to an overall variation of 5.9% (47/804) as opposed to 2.1% (17/804) calculated from the study by Zong and co-workers (Zong *et al.*, 1997).

Table 11 (following 2 pages): Nucleic acid polymorphisms in the ORF75 gene. KSHV75 is used as the comparative sequence from Genbank (accession number: U75698) and corresponds to a C clade isolate. The following reference sequences were used: C282 and AKS1 are subgroup A, ST2 and 431KAP are subgroup B and ASM70 is subgroup C (Zong *et al.*, 1997) and are highlighted in yellow. These references were compared to 17 South African HHV-8 isolates. Within the 47 diagnostic loci, 17 shown in yellow were those identified by Zong (Zong *et al.*, 1997). The subgroups of various strains, as determined by sequencing analysis (phylogen) and by characteristic polymorphisms (polymorph), are indicated. Sequence data shown in the following colours illustrate the characteristic positions delineating the different HHV-8 subgroups: pink = A subgroup; green = B subgroup; orange = C subgroup; blue = N subgroup and red = A/B subgroup.



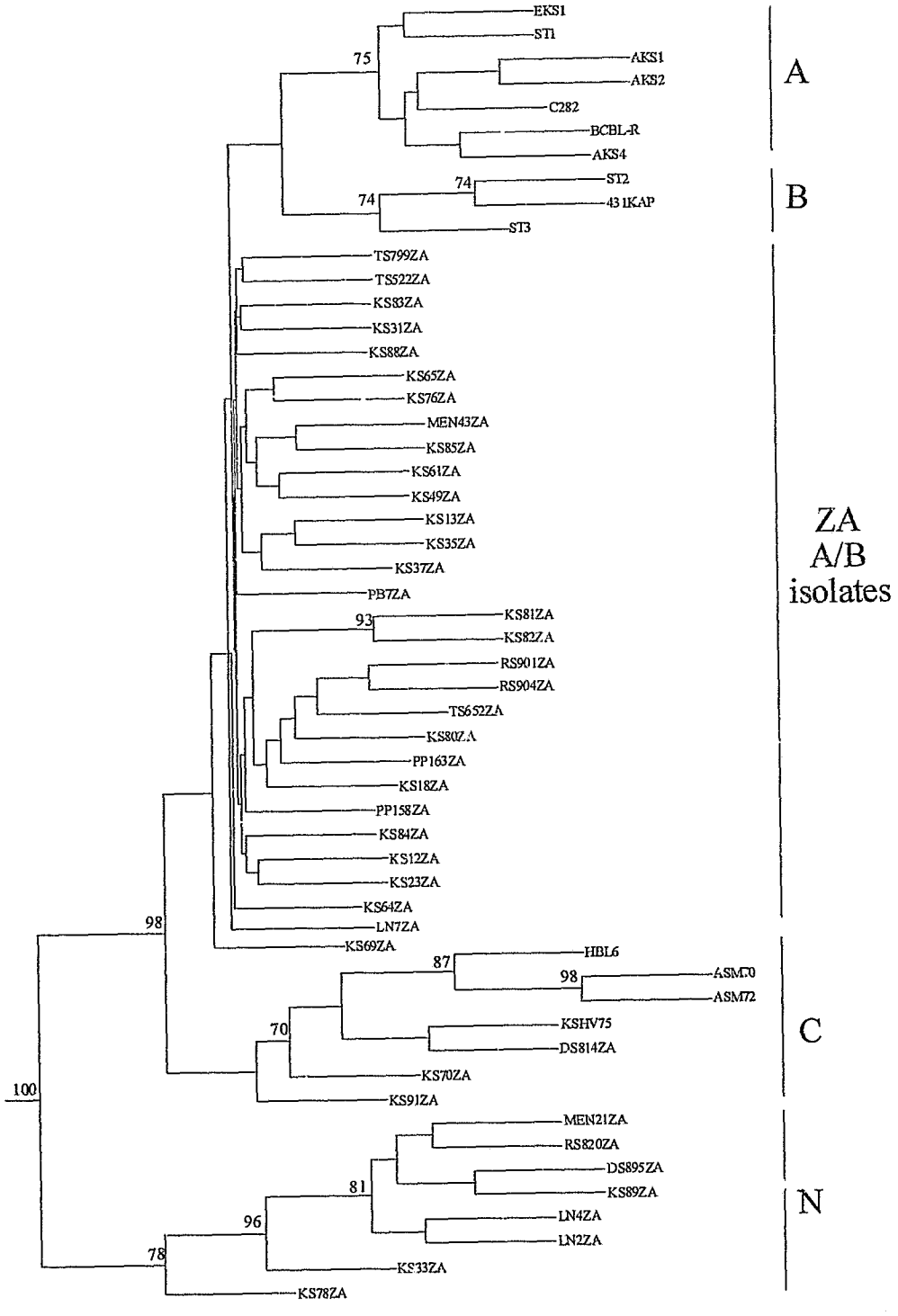
Isolate name	Subtype: Phylogen	Subtype: Polymorph	ORF75 base polymorhpisms																									
			563	591	618	633	636	647	726	768	823	838	840	867	888	918	933	936	948	977	984	988	995	1000	1016	1020	1023
KSHV75	**C**	**C**	G	C	C	G	C	C	T	T	T	A	T	G	G	G	G	C	G	A	C	T	G	G	A	T	G
C282	**A/B**	**A**		-		-		-	-	-	-	-	-		-	-	-	-	-	G	-	C			G	-	-
AKS1	**A**	**A**		-		-		-	-	-	-	-	-		-	-	-	-	-	G	-	C			G	-	-
KS12	A/B	A		-	-	-		-	-	-	-	-	-		-	-	-	-	-	G	-	C			-	-	-
KS80	A/B	A		-		-		-	-	-	-	-	-		-	-	-	-	-	-	-	C			G	-	-
RS901	A/B	A		-		-		-	-	-	-	-	-		-	-	-	-	-	G	-	C			G	-	-
ST2	**B**	**B**	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	G	-	C		-	G	-	-
431KAP	**B**	**B**	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	G	-	C		-	G	-	-
TS522	A/B	B	A	-	-			-	-	-	-	-	-	-	-	-	-	-	-	G	-	C		-	G	-	-
LN7	A/B	B	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	G	-	C		-	G	-	-
KS76	A/B	A/B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	C		-	G	-	-
ASM70	**C**	**C**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KS70	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KS91	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
DS814	C	C	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KS78	N	N	C		-	-	T							-						G		C	-	-	G		
DS895	N	N	-		-	-	-							-						G		C	-	-	G		
MEN21	N	N	-		-	-	-							-						G		C	-	-	G		
RS820	N	N	-		-	-	-							-						G		C	-	-	G		
LN2	N	N	-		-	-	-							-						G		C	-	-	G		
LN4	N	N	-		-	-	-							-						G		C	-	-	G		
KS89	N	N	-		-	-	-							-						G		C	-	-	G		
KS33	N	N	-		-	-	-							-						G		C	-	-	G		

Isolate name	Subtype: Phylogen	Subtype: Polymorph	ORF75 base polymorhpisms																									
			1028	1030	1034	1035	1036	1040	1050	1066	1084	1085	1086	1098	1146	1148	1158	1182	1214	1260	1265	1266	1272	1274	1276	1278	1322
KSHV75	**C**	**C**	G	T	G	C	A	T	G	C	A	C	A	G	T	T	C	A	G	A	T	T	A	A	T	C	G
C282	**A/B**	**A**	T	-			-	-	-	-	G		-	-	-	-	-	-	-	-	-	-	-	C	G	G	-
AKS1	**A**	**A**	T	-			-	-	-	-	G		-	-	-	-	-	-	-	-	-	-	-	C	G	G	-
KS12	A/B	A	T	-			-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	-
KS80	A/B	A	T	-			-	-	-	-	G		-	-	-	-	-	-	-	-	-	-	-	-	C	G	-
RS901	A/B	A	T	-			-	-	-	-	G		-	-	-	-	-	-	-	-	-	-	-	-	C	G	-
ST2	**B**	**B**	T	-			-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	C	C	G	-
431KAP	**B**	**B**	T	-			-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	C	G	G	-
TS522	A/B	B	T	-			-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	-
LN7	A/B	B	T	-			-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	C	G	-
KS76	A/B	A/B	T	-			-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	C	G	-
ASM70	**C**	**C**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	G	-
KS70	C	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
KS91	C	C	T	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DS814	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KS78	N	N	T		-	-					G	-												-	-	G	
DS895	N	N	T		-	-					G	-												-	-	G	
MEN21	N	N	T		-	-					G	-												-	-	G	
RS820	N	N	T		-	-					G	-												-	-	G	
LN2	N	N	T		-	-					G	-												-	-	G	
LN4	N	N	T		-	-					G	-												-	-	G	
KS89	N	N	T		-	-					G	-												-	-	G	
KS33	N	N	T		-	-					G	-												-	-	G	

4.2.4 Amino acid phylogenetic analysis

The phylogenetic relationships based on the predicated amino acid sequences showed a similar pattern to that of the nucleic acid sequences (Fig. 16). The South African A/B variants clustered with the A and B reference strains although only the reference strains were separated by high bootstrap values of >70%. The A/B and C variants were separated from each other by a 98% bootstrap value, while a bootstrap value of 100% separated the N subgroup from all other subgroups. Two strains (KS81ZA and KS82ZA) showed a tight cluster within the A/B group that was not evident in the nucleic acid analysis. This is due to the fact that 11 nucleic acid differences between these 2 isolates translated to only 5 amino acid changes. Both of these individuals resided in the Northern Transvaal and may have been infected with the same isolate. However, KS81ZA was HIV-seropositive, while KS82ZA was one of the few cases of HIV-negative associated KS.

Figure 16 (next page): Phylogenetic tree based on the 267 predicted amino acid sequences translated from the 804 bp sequenced. The best tree was constructed from 100 replicates after formulating a distance matrix using PROTDIST. The parameters were included into the KITSCH algorithm from the PHYLIP package to show the relationships between the different isolates. Bootstrap values of ≥70% are shown. The reference specimens are shown in red, the blue writing denotes the subgroups and HIV-negative individuals are highlighted in green.


EKS1
ST1
75
AKS1
AKS2
C282
BCBL-R
AKS4
A
74
ST2
74
431KAP
ST3
B
TS799ZA
TS522ZA
KS83ZA
KS31ZA
KS88ZA
KS65ZA
KS76ZA
MEN43ZA
KS85ZA
KS61ZA
KS49ZA
KS13ZA
KS35ZA
KS37ZA
PB7ZA
93
KS81ZA
KS82ZA
RS901ZA
RS904ZA
TS652ZA
KS80ZA
PP163ZA
KS18ZA
PP158ZA
KS84ZA
KS12ZA
KS23ZA
KS64ZA
98
LN7ZA
KS69ZA
ZA
A/B
isolates
87
HBL6
98
ASM70
ASM72
KSHV75
70
DS814ZA
KS70ZA
100
KS91ZA
C
MEN21ZA
RS820ZA
DS895ZA
81
KS89ZA
96
LN4ZA
LN2ZA
78
KS33ZA
KS78ZA
N

4.2.5 Amino acid polymorhisms in the ORF75 protein

To determine whether distinct amino acid mutations differentiated the 4 subgroups, 17 South African sequences were aligned and compared ro reference sequences (Table 12). Sixteen subgroup-specific polymorphisms were found, 2 were characteristic for the A subgroup (amino acids: 87, 115), 1 in the B subgroup (91), 3 in the C subgroup (87, 104 and 119) and 12 in the novel subgroup (27, 66, 93, 102, 103, 104, 105, 168, 169, 174, 192 and 233). All subgroup N and C strains had the designated mutations, whereas, only 4 of the 5 A strains and 2 of the 5 B strains shared these loci. Two loci were shared by both A and B strains (A/B variants) (103, 104). While KS76ZA contained both of these loci it did not have any other subgroup-specific mutations as was previously shown with nucleic acid analysis. Although amino acid mutations were less able to define subgroups compared to nucleic acid data, this analysis clearly delineated the N subgroup due to its highly polymorphic nature.

Isolate	Subtype:		Polymorphisms associated with ORF75 gene product																
name	Phylogen	Polymorph	23	24	27	66	87	91	93	102	103	104	105	115	119	168	169	174	192	233
KSHV75	**C**	**C**	N	F	S	I	V	S	R	N	M	H	H	A	Q	E	V	Q	I	A
C282	**A/B**	**A**			-	-		-	-	-					R	-	-	-	-	-
AKS1	**A**	**A**			-	-		-	-	-					R	-	-	-	-	-
KS12	A/B	A	S	-	-	-	A	-	-	-			-		R	-	-	-	-	-
KS80	A/B	A	S	-	-	-		-	-	-			-		R	-	-	-	-	-
RS901	A/B	A	S	-	-	-		-	-	-			-		R	-	-	-	-	-
ST2	**B**	**A/B**			-	-	A	-	-	-			-	-	R	-	-	-	-	-
431KAP	**B**	**A/B**			-	-	A	-	-	-			-	-	R	-	-	-	-	-
TS522	A/B	B	S	-	-	-	A		-	-			-	-	R	-	-	-	-	-
LN7	A/B	B	S	-	-	-	A		-	-			-	-	R	-	-	-	-	-
KS76	A/B	A/B	S	-	-	-	A	-	-	-			-	-	R	-	-	-	-	-
ASM70	**C**	**C**	S	I	-	-		-	-	-	-		-	-		-	-	-	-	-
KS70	C	C	-	-	-	-		-	-	-	V		-	-		-	-	-	-	-
KS91	C	C	-	-	-	-		-	-	-	L		-	R		-	-	-	-	-
DS814	C	C	-	-	-	-		-	-	-	-		-	-		-	-	-	-	-
KS78	N	N	-	-			A	-						-	R					
DS895	N	N	-	-			A	-						-	R					
MEN21	N	N	-	-			A	-						-	R					
RS820	N	N	-	-			A	-						-	R					
LN2	N	N	-	-			A	-						-	R					
LN4	N	N	-	-			A	-						-	R					
KS89	N	N	-	-			A	-						-	R					
KS33	N	N	-	-			A	-						-	R					

Table 12: Amino acid polymorphisms associated with ORF75. The predicted amino acid sequences of 17 South African HHV-8 isolates and 5 subgroup reference specimens are compared to KSHV75 (Genbank accession number: U75698). The polymorphisms represented are all the mutations determined by comparison with KSHV75. Gene expression of the sequenced region occurs in a reverse direction to transcription due to the orientation of the gene as shown in Fig. 2. The amino acids are shown from amino to carboxyl terminus. Codon 1 represents the first translatable amino acid of the ORF75 gene product. Characteristic phenotypes for each subgroup are represented as follows: pink = A strains, green = B strains, orange = C strains, blue = N strains and red = both A/ B strains.

4.3 Discussion

Phylogenetic analysis of the ORF75 gene of HHV-8 revealed that subgroups A/B, C and a novel (N) strain are present in South Africa. Most individuals were infected with A/B variants, while a small but significant number were infected with the novel subgroup. The significance of these findings as yet remain unclear as there is no apparent differences in the clinical presentation or demographic features of the individuals infected with these different subgroups. There was also no genetic differences between HHV-8 DNA from individuals who had asymptomatic infection or those who had clinically confirmed KS. Three isolates from individuals originating from neighbouring countries did not show any difference to the South African subgroups although it is not known where these individuals acquired their HHV-8 infection. All three were asymptomatic for KS and may have recently been infected with HHV-8. Thus, while KS has only recently gained prominence in South Africa, the high level of genetic heterogeneity of HHV-8 suggests that this virus may have been circulating in this region for a long period of time.

Subgroup determination was originally based on polymorphic differences within the ORF75 gene (Zong *et al.*, 1997). Based on 17 characteristic loci, three distinct genetic subgroupings (A, B and C) were identified. According to the geographical location from which the samples were obtained, it was suggested that the A strains predominated in regions associated with classical KS, whereas, the B and C strains prevail in Africa, although these associations are tenuous (Cook *et al.*, 1999, Zong *et al.*, 1997). The AIDS epidemic may have had a role to play in the variability presented in HHV-8 as the majority of AIDS patients in the US are infected with HHV-8 variants presenting little heterogeneity. The implication is that these variants originated from a single common isolate (Zong *et al.*, 1997). All 3 subgroups were found to occur in South Africa although subgroup C was only rarely found (3/40, 8%). In addition, a fourth newly defined novel (N) subgroup was identified. As a consequence of this new subgroup an additional 33 new loci, of which 32 were unique for the N subgroup have been characterised. Of the original 17 loci, 14 were retained giving a total of 47 key diagnostic polymorphism within the 804 bp region which defined the 4 subgroups in South Africa. A similar analysis carried out using the predicted amino acid sequences in the ORF75 gene product, recorded 16 polymorphisms which were characteristic for the 4 variants. The majority of mutations delineated the N subgroup with only 1-3 point mutations descriptive for the other

3 subgroups. Subgroup designation based on polymorphism is therefore limited using amino acids compared to nucleic acids, although, both methods can be used for subgrouping, particularly for identifying the N subgroup.

Based on the analysis of specific polymorphisms it may be concluded that distinct A and B subgroups exist in South Africa. However this analysis is based on only 6% (47/804) of the ORF75 gene. More detailed and statistically accurate analysis of this region based on phylogenetic relationships has shown that the A and B subgroups were not supported by strong bootstrap values and hence were termed A/B variants. Closer analysis of the polymorphic changes revealed that the A/B variants shared 4 specific loci. These loci were masked in the previous analysis as they identified the C subgroup which were subsequently lost with the addition of the N group (Zong *et al.*, 1997). Thus, while South African A and B isolates show certain subgroup-specific pattern they also share common mutations and this prevents their being segregated as distinct subgroups on a phylogenetic tree. The A and B subgroups are also less distinct in other regions of the genome lying just outside of ORF75 where only 1 bp difference between the A and B strains was found (Zong *et al.*, 1997). Since the A and B isolates are closely related, it is possible that these subgroups may have arisen from a common progenitor or that one of these subgroups may have been the original isolate. It is possible that the A/B variants defined by ORF75 are precursors to the A and B subgroups identified in the USA and other parts of Africa.

The C and N subgroups remain singular clusters supported by strong bootstrap values and characteristic loci. Within the N subgroup there was limited variation between the 8 sequences analysed (intra-strain variation). However, when compared to sequences from other subgroups (inter-strain variation) there was a high level of genetic diversity (3.8 - 4.7%). Previous analysis of this region using subgroups A, B and C showed only 1.5% variation (Zong *et al.*, 1997). Thus, the addition of the N group has revealed that ORF75 is a more divergent region than first realised. It is likely that the N group also shows unique antigenic differences from other subgroups as analysis of predicted amino acids showed that the N group was distinct. A total of 12 loci of which 10 were unique for the N subgroup were identified over a 267 amino acid region. Since the N group has never been reported before, it could be unique to this region. It may have been present for a long period of time only coming to the fore as a result of the HIV epidemic, or alternatively it could be a new strain evolving due to continuous reactivation of

HHV-8 in AIDS-infected individuals, especially in developing countries where HIV antiviral therapy is not used. Studies in other parts of Africa on both the ORF75 and the K1 regions have not revealed the presence of the N group (Cook *et al.*, 1999). The N subgroup has so far only be identified in South Africa and may represent a new subgroup or a vestige of modern day HHV-8 subgroups. This is suggested by studies on the ORF26 and T0.7/K12 of 2 N isolates (LN2ZA and LN4ZA) which also showed sequences which did not align with previously defined subgroups (Dr G. Hayward, personal communication). Further studies need to be done to determine if the N group is unique to this region and whether this subgroup also shows distinct differences in other regions of the genome.

The first open reading frame, K1, shows a higher level of variation compared to ORF75 and has been proposed as a suitable region to define subgroup nomenclature. Four major subgroups and at least 13 clades within these subgroups have been defined, although not all are supported by high bootstrap values (Zong *et al.*, 1999). The four subgroups A, B, C and D which do not correspond to those identified using ORF75, differ by 8-15 % at the nucleic acid level and 15-30% at the amino acid level. This high degree of non-synonymous mutations contrasts with that of ORF75 which display many silent mutations. In addition there appears to be some association between the viral subgroups based on ORFK1, the geographic distribution and the types of KS. Subgroups B were found almost exclusively in Africa, the C2 subgroups in Saudi Arabia, C3' in Taiwan and the recently discovered subgroup D in the Pacific Islands. Based on these distribution patterns, it has been hypothesized that HHV-8 is an ancient virus associated with the migration patterns of human populations out of Africa over the last 100,000 years.

There are some indications that HHV-8 subgroups may be associated with distinct pathologies similar to other herpesviruses. The two subgroups of EBV, A and B, have different transforming potentials in primary B-lymphocytes while the A, B and C subgroups of HVS differ in the region of the genome responsible for T-cell transformation (Zong *et al.*, 1997). HHV-8 subgroup A has been found more frequently in mucosal and visceral lesions which display more aggressive characteristics of pathogenicity. Some B and C variants of HHV-8 have been found in lymphoproliferative disorders, other than BCBL, which were not completely malignant (Luppi *et al.*, 1997). However, some C strains in HHV-8 have been found in aggressive disseminated disease in Africa. Although these findings remain to be verified, determining which HHV-8 subgroups exist and which ones predominate in South Africa may

have important implications for the future burden of clinical KS. In particular more studies on the newly identified subgroup, N, need to be done to determine its distribution, transmission, genetic diversity and pathogenic potential.

5. SCREENING METHODS FOR NOVEL HHV-8 SUBGROUPS

Current techniques used to identify HHV-8 subgroups are based on genetic analysis which are time consuming and sophisticated and require the use of specialised and expensive equipment. Simplified methods based on detecting genetic variants relative to wild-type or reference strains have been developed and used successfully with other viruses (Chezzi & Scoub, 1996, Delwart *et al.*, 1993, Kreis & Whistler, 1997, Lin *et al.*, 1998). Since genetic variation in HHV-8 is minimal, methods of detecting polymorphisms should be sufficiently sensitive to recognise minor changes based on strand mobility in a gel. HMA and SSCP were therefore selected to assess whether such minor differences could be detected.

5.1 Heteroduplex mobility assay (HMA)

An HMA is able to distinguish different genotypes based on sequence variation of the isolate relative to a reference fragment. Due to unpaired nucleotides the DNA hybrid is retarded and this can be visualised on a gel. This technique has been successfully used to distinguish genomes with high variability such as HIV (Delwart *et al.*, 1995). The ORF75 fragment was used to determine if different groups of HHV-8 could be distinguished from each other. Specimens of known subgroup, as determined by sequencing analysis, were annealed to a BCP-1 DNA reference fragment. This was done by both slow annealing and quick snap cooling methods. Rapid cooling promotes the formation of stable heteroduplexes in highly divergent strains. However, both slow and quick snap annealing procedures failed to resolve the heteroduplexes and were thus unable to differentiate the subgroups. Annealing buffer was also added to the DNA in order to stabilise heteroduplex formation but had little effect. Different gel formulations were also tried including 5% polyacrylamide and 0.5-1 x MDE (mutation detection enhancement) polymer. An example of a 1x MDE gel with specimens from the 4 subgroups hybridized to a BCP-1 reference fragment is shown in Fig. 17. No clear heteroduplex formation was evident between the subgroups. MDE and polyacrylamide gels were therefore run with specimens annealed to different subgroups. These specimens were slow and snap annealed with and without annealing buffer. No heteroduplexes were observed on MDE gels although feint bands were visible using 5% polyacrylamide gels with poor resolution of fragments (not shown).

Gel matrices containing 5-15% urea with and without 2% glycerol were also used to destabilise heteroduplexes in order to retard their mobility and improve resolution, but also proved to be ineffective.

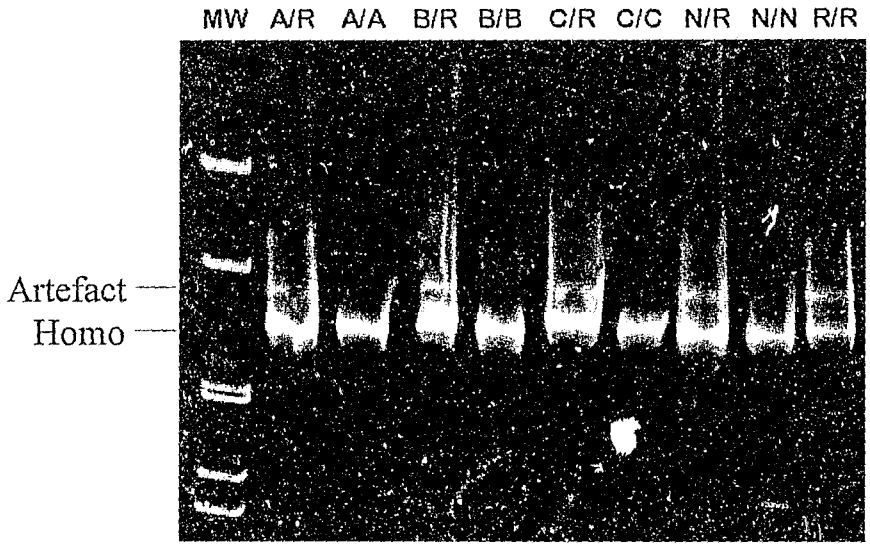


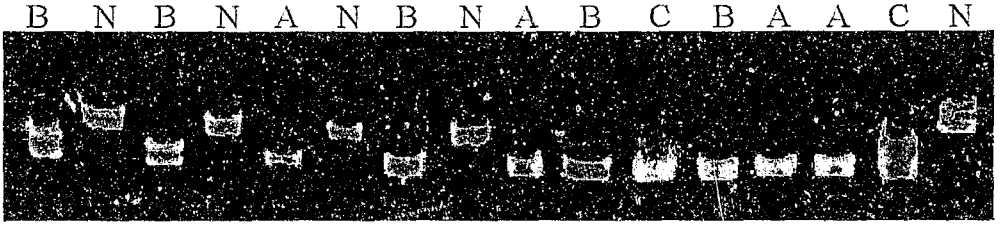
Figure 17: HMA of 4 HHV-8 subgroups. Amplicons from the 4 subgroups (A, B, C and N) were slow annealed in the presence of annealing buffer against a reference fragment (R) and against themselves and run on 1x MDE gel in 1x TBE for 16 hours at 100V. The A or B subgroups were taken as outliers of the A and B reference strains. No heteroduplex bands were evident in any of the lanes. The artefactual band is present in all lanes with the R fragment.

5.2 Single strand conformation polymorphism (SSCP)

SSCP can be used to identify DNA polymorphisms or mutations as a result of the primary folded structure of a single DNA strand (Orita *et al.*, 1989). SSCP was performed on the 804 bp amplified fragments generated from the ORF75 gene of the 41 South African specimens. For convenience, these were labelled based on their similarities to A and B reference isolates. Sixteen samples (A = 4, B = 5, C = 2 and N = 5) are shown in Fig 18. The migration of single-stranded DNA specimens from the A, B or C groups were indistinguishable from one another. However, an electrophoretic shift was visible in all five N specimens although this was

not identical for all samples. A variety of other migration patterns are shown but none conform to a single A, B or C subgroup. Some of the A and B strains showed similar migration patterns confirming their high degree of genetic overlap. All 3 C strains showed different patterns.

Figure 18: SSCP of South African HHV-8 subgroups. 5% polyacrylamide non-denaturing gel



showing 16 KS2000/2034 fragments. Sequencing analysis has was used to determine subgroup designation. The A, B and C subgroups show no distinct migration pattern. The slower migration patterns of the novel DNA fragments distinguishing this group from the others.

5.3 Discussion

An easy to use, cost-effective method for detecting the N subgroup of HHV-8 has been developed. The SSCP was able to distinguish the N subgroup from other subgroups, although it was unable to discern the A, B (A/B) or C groups. These latter subgroups probably contain a less complex tertiary structure and are not retarded in the gel. The fragment length used for SSCP subgroup analysis was 804 bp. It is possible that shorter fragments may be more useful to differentiate between all groups as mutations of a few bases are often adequate to separate variants, but only within short fragments of DNA, less than 200 bp (Lin *et al.*, 1995a). An HMA appears to be unsuitable for differentiating HHV-8 subgroups in the ORF75 gene. Previous studies have shown that between 3% - 20% nucleotide mismatches are needed between the two strands of the heteroduplex before adequate separation (Delwart *et al.*, 1995). Although there is a 3.8 - 4.7% variation between the N and other subgroups this was not sufficient to distinguish the different subgroups of HHV-8 using an HMA. Our findings concur with those of others which find that SSCP is often able to discern minor differences compared to HMA [Lin, 1998 #2221][Orita, 1989 #2217][Delwart, 1993 #2197]. Although the N subgroup accounts for 20% of the HHV-8 subgroups in South Africa, this rapid screening SSCP method will allow faster identification of these unique variants.

6. CONCLUDING REMARKS

Recently there has been considerable interest in HHV-8 and KS in South Africa. Three studies have defined the sero-prevalence of HHV-8 in different cohorts [Sitas, 1999 #2207][Bourboulia, 1998 #2158] (Wilkinson *et al.*, 1999) and two studies examined the presence of HHV-8 DNA in KS patients (Engelbrecht *et al.*, 1997, Sitas *et al.*, 1997). This study is the first to examine the presence of HHV-8 DNA in large number of HIV-infected individuals. It confirms the association between HHV-8 and KS and also documents the presence of HHV-8 DNA in the blood of individuals without KS. It is also the first to analyse the genetic subgroups of HHV-8 and to describe the presence of a novel HHV-8 subgroup in South Africa. Collectively these data indicate that there is a significant background prevalence of HHV-8 in South Africa, further study of which may yield clues as to the evolutionary history of this ancient virus.

What do these figures predict about the future incidence of KS in South Africa? The burden of asymptomatic HHV-8 infection suggests that there may be a significant increase in the number of KS cases in the near future. The continued expansion of the HIV epidemic in South Africa is likely to exacerbate this situation. There are currently 3.5 million HIV-infected individuals in South Africa and the current estimate indicates that 16 - 32% have antibodies to HHV-8 while 4.2% have HHV-8 DNA in blood. Studies done elsewhere have shown that approximately half of HIV-infected individuals with HHV-8 DNA in blood develop KS [Whitby, 1995 #735]. Extrapolation of these data suggests that in the absence of any interventions 73,500 individuals in South Africa have the potential to develop KS within the next 3-5 years. These emerging cases of KS need to be carefully monitored. Surveillance of HHV-8 in the HIV-infected population in South Africa may become important if KS is shown to be transmitted through occupational exposure or through blood transfusions. For this, better diagnostic tests need to be developed in order to monitor the presence of this pathogen. Less subjective methods of detection based on ELISA technology would be more suitable than the current IFA tests. Further studies need to be done to elucidate the transmission patterns of HHV-8 in developing countries in order to develop appropriate interventions and treatment. While the incidence of KS in developed countries has declined as a consequence of anti-retroviral drug therapies, their use remains unfordable in developing countries, further indicating that KS is likely to become a major cancer in South Africa.

Genetic studies of HHV-8 have revealed evolutionary patterns which may have emerged as a result of selective forces of the immune system or due to founder effects in different regions. Given that herpesviruses evolve slowly, the finding of a new subgroup in South Africa is significant. HHV-8 was first thought to have arisen in Africa over 100,000 years ago as the B subgroup (Hayward, 1999). A significant divergence between the B and N subgroups in the ORF75 region suggests that the N subgroup has a different evolutionary history. It is possible that the N variants may be a new subgroup or a vestige of newer subgroups which has become manifest as a result of co-infection with HIV. While there is some suggestion that genetic variants correlate with pathogenesis the evidence for this is limited and requires further study. It thus remains to be determined what biological significance and evolutionary phylogeny of this novel subgroup is and whether it will expand in what is likely to become a significant cause of morbidity and mortality in South Africa.

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8. APPENDIX

8.1 List of reagents and equipment used in this project and their suppliers

Reagents

	Product	Supplier
1	Cy5TM AutoReadTM Sequencing kit	Pharmacia Biotech, Upsala, Sweden
2	Cy5TM-dATP Labelling Mix	Pharmacia Biotech, Upsala, Sweden
3	dRhodamine cycle sequencing ready reaction kit	P.E Applied Biosystems, Warrington, UK
4	Ficoll-Hypaque	Sigma Chem. Co., St. Louis, Mo
5	High Pure Plasmid Isolation Kit	Boehringer Mannheim, Germany
6	Luria Broth	GibcoBRL, Life Technologies
7	MDE gel	FMC BioProducts, Rockland, USA
8	Mouse anti-human IgG-FITC conjugate	Zymed Labs Inc, California, USA
9	pGEM®-T vector kit	Promega, Madison, USA
10	pMOSBlue T-vector kit	Amersham International, Buckinghamshire, England, UK
11	Proteinase K	Boehringer Mannheim, Germany
12	Sabax water	Adcock Ingram LTD, Aeroton, Johannesburg
13	Sequenase PCR product sequencing kit	Amersham Life Science, Arlington Heights. IL
14	Supertherm Taq DNA polymerase	Advanced Biotechnologies Ltd., Surry, UK
15	Taq polymerase	Boehringer Mannheim, Mannheim, Germany
16	Thermosequenase cycle sequencing kit	Amersham International, Buckinghamshire, England, UK
17	Cy5 labeled sequencing primer synthesis	MWG-Biotech, Munchen, Germany
18	PCR primer synthesis	University of Cape Town,Cape Town, South Africa

Equipment/Consumables

	Product	Supplier
1	0.2 ml 96 well polycarbonate OmniPlates	Hybaid Ltd., Teddington, Middlesex, U.K
2	2400 or 9600 GeneAmp PCR system	P.E Applied Biosystems, Warrington, UK
3	ABI 377 sequencer	P.E Applied Biosystems, CA, USA
4	ALFexpress DNA sequencer	Pharmacia Biotech AB, Upsala, Sweden
5	Biometra Trio- thermablock TB-1 cycler	Gottingen, Germany
6	Bio-Rad chamber	Protean II xi, Hercules, CA
7	Centri-Sep columns	Princeton separations Inc., Adelphia, New Jersey
8	HTC super cured slides	Erie Scientific Co., Portsmouth, New Hampshire, USA
9	Hybaid touchdown thermal cycler	Hybaid Ltd., Teddington, Middlesex, U.K
10	Microflex HFX-II fluorescent microscope	Nikon, Japan

8.2 Ethics Clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Alagiozoglou

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 970801

PROJECT Characterization of HHV-8 in South Africa

INVESTIGATORS Mr P Alagiozoglou

DEPARTMENT Virology,
National Institute for Virology

DATE CONSIDERED 970829

DECISION OF THE COMMITTEE

Approved unconditionally

DATE 970910

CHAIRMAN. .....(Professor P E Cleaton-Jones)

c c Supervisor: Dr D J Martin
Dept of Virology, NIH

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE..10/9/97.....SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES