Molecular epidemiology of Kaposi's sarcoma-associated herpesvirus in an endemic country

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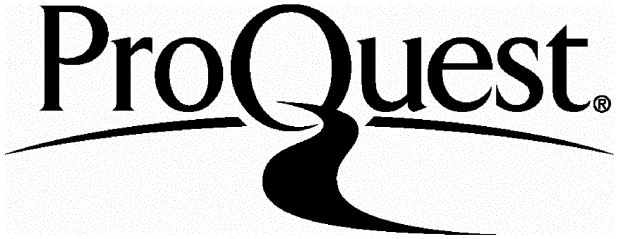

Abstract

The molecular epidemiology of Kaposi's sarcoma-associated herpesvirus (KSHV) was investigated in family groups in Malawi, a region of high KSHV endemicity. It was hypothesised that: KSHV transmission may not only occur along intra-familial routes, as seroepidemiologic studies suggest, but also extra-familially; and that a host living in an endemic region may carry multiple KSHV strains.

KSHV DNA in mouth rinse samples was frequently detected in study individuals (n=89 from 22 families) suggesting that saliva was a potential source of infection. Molecular sequences were compared between members of the same family by sequence analysis of hypervariable domains of opening reading frame (ORF) K1 PCR products generated from blood and oral samples. Phylogenetic analysis revealed that in some families (n=5), identical sequences in the variable region 1 were present. In others (n=4), dissimilar sequences were recovered (range of nucleotide sequence divergence: approximately 0.5% to 27%). While sequence similarity between family members is consistent with familial KSHV transmission, sequence dissimilarity and sequence clustering point to extra-familial transmission of closely related viral variants as having taken place.

A PCR-based restriction fragment length polymorphism (RFLP) procedure to screen for sequence variation in the internal repeat domain of ORF 73 was then adapted to complement the ORF K1 nucleotide sequencing studies. RFLP patterns were unique for each individual and could be compared between family members. The PCR-RFLP findings broadly corroborated with the sequencing data.

Intra-host KSHV sequence variation was investigated using denaturing gel gradient electrophoresis to screen multiple ORF K1 clones derived from oral samples. While intra-sample clonal diversity was observed, further investigation using KSHV infected cell lines revealed that such diversity could be attributed to *Taq* polymerase nucleotide misincorporation. Therefore, no firm evidence for KSHV viral diversity within single individuals could be found.

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Abbreviations

AIDS	acquired immunodeficiency syndrome
bp	base pair
bFGF	basic fibroblast growth factor
cdk	cyclin dependent kinase
CMV	cytomegalovirus
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxynuclotide triphosphate
EBV	Epstein-Barr virus
EDTA	disodium ethylenediaminetetra-acetate
ELISA	enzyme linked immunosorbent assay
FLICE	Fas-associated death domain-like interleukin-1 beta-converting enzyme
FLIP	FLICE inhibitory protein
GPCR	g-protein coupled receptor
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HHV8	human herpesvirus 8
HIV	human immunodeficiency virus
HMA	heteroduplex mobility assay
HVS	herpesvirus saimiri
IA	immunoblot assay
IFA	immunofluoresence assay
IFN	interferon
IL-6	interleukin 6
IRD	internal repeat domain
IRF	interferon regulatory factor
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma associated herpesvirus
kb	kilobase
LNA	latency associated nuclear antigen

MCD	multicentric Castleman's disease
mRNA	messenger RNA
MIP	macrophage inhibitory protein
ORF	opening reading frame
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PEL	primary effusion lymphoma
PKR	protein kinase
pRB	retinoblastoma tumour suppressor
PHYLIP	phylogeny inference package
RDA	representational difference analysis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
Rta	replication and transcription activator
SSCP	single-strand conformational polymorphism
TAE	tris acetate EDTA
Tat	transactivator protein
TBE	tris borate EDTA
TPA	12-*O*-tetradecanoylphorbol 13 acetate
TR	terminal repeat
UPGMA	unweighted pair group method with arithmetic means
UV	untraviolet

Chapter 1

Introduction

1.1 Kaposi's Sarcoma and the discovery of KSHV

Clinical features of the disease now known as Kaposi's sarcoma (KS) were described by the Hungarian dermatologist Moritz Kaposi in 1872 (Kaposi, 1872) involving five patients with "idiopathic multiple pigmented sarcomas of the skin". The disease Kaposi described, involving tumours of the stomach small intestine, colon, skin, and liver, appeared to be more aggressive than most human immunodeficiency virus (HIV) negative cases of KS now seen in Europe. Four epidemiological forms of KS based on clinical and epidemiological differences have been established: classic KS, endemic - HIV-negative KS found in Africa, iatrogenic KS, and HIV-associated or epidemic KS. All stages are identical in terms of histology and are recognised as different manifestations of a single disease. In 1994, Chang *et al.* identified fragments of herpesvirus-like DNA in AIDS-KS biopsy samples. Kaposi's sarcoma associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8) is now recognised as the aetiological agent for all forms of KS.

1.1.1 Histogenesis

KS is a complex multifocal vascular lesion: early "patch stage" lesions are made up of endothelial lined spaces surrounding normal blood vessels and there is often a degree of inflammatory infiltrate present. A "plaque" stage lesion is characterised by an increased growth of spindle shaped vascular processes in the dermis together with the formation of slit like vascular spaces filled with erythrocytes. The late stage "nodular" lesion is comprised primarily of spindle shaped cells arranged in large sheets, some of which are undergoing mitosis, with vascular slits interspersed.

Spindle shaped cells that form the majority of late stage tumours are of unknown origin, although they are likely to be derived from endothelial cells. These spindle cells express the endothelial cell markers CD31 and CD34 on their surfaces, as well as a vascular endothelial growth factor receptor 3 (VEGFR-3) (Dupin *et al.,* 1999). VEGFR-3 is commonly expressed by lymphatic endothelium but not by mature endothelial cells. Additional cell types have

been found in KS lesions including smooth muscle, dendritic cells and macrophages (Nickoloff and Griffiths, 1989; Sturzl *et al.,* 1992) indicating that KS tumours are of a mixed cell type. Spindle-like cells have been isolated and cultured from the peripheral blood of HIV-infected patients with KS and express markers for both macrophage and endothelial cells (Browning *et al.,* 1994).

Levy and Ziegler (1983) first proposed that KS, particularly in patients with AIDS, was caused by secreated cytokines that had an angiogenic effect. Since then many studies have found that KS is driven, in part, by cytokine production. Spindle cells and CD8+ infiltrating macrophages or lymphocytes can produce interleukin 6 (IL-6), basic fibroblast growth factor (bFGF), gamma interferon, and a variety of other cytokines in abundance (Ensoli *et al.,* 1989; Miles *et al.,* 1990; Sirianni *et al.,* 1998; Fiorelli *et al.,* 1998). AIDS-KS cell lines have been shown to secrete large amounts of biologically active IL-6, which can promote spindle cell proliferation (Miles *et al.,* 1990). Gamma-interferon can promote the formation of KS spindle cells with an angiogenic phenotype and transform endothelial cells to resemble spindle cells (Fiorelli *et al.,* 1998). bFGF is an angiogenic factor, highly expressed in early lesions that promotes spindle cell growth. Gamma-interferon can induce KS-like tumour formation in nude mice and these tumours in turn produce bFGF (Ensoli *et al.,* 1989).

HIV-1-associated KS has a more aggressive clinical course than the other types of KS. Therefore, it has been proposed that HIV-1 gene products may positively influence KS development (Ensoli *et al.,* 1994). The *tat* gene in HIV-1 codes for an early trans-activator protein (Tat) essential for HIV-1 viral replication (Fisher *et al.,* 1986). It is secreted into the extracellular fluid during acute HIV infection, can promote spindle cell growth in vitro and produces an angiogenic phenotype in nude mice (Vogel *et al.,* 1988; Ensoli *et al.,* 1993). Normal endothelial cells display a spindle cell like morphology when exposed to Tat in the presence of other cytokines (e.g. IL-1, tumor necrosis factor, and gamma interferon) in culture (Barillari *et al.,* 1992). Indeed, it has been proposed that these cytokines, whose production is upregulated in persons with an HIV-1 infection (Fuchs *et al.,* 1992), work together with Tat in KS development

(Barillari *et al.,* 1992). Recent studies have indicated that Tat may influence the specific tissue distribution of KS (nasal, oral and genital mucosa) observed in HIV-associated KS (Prakash *et al.,* 2000).

1.1.2 Clonality

It is not yet known whether KS is a true neoplasm or a reactive hyperplasia. Several factors cast doubt on KS being a true malignancy. Although the spindle shaped cells are likely to be the neoplastic tumour cell, this is not certain as they lack aneuploidy, a common feature of neoplasia (Delli *et al.,* 1986). Very few spindle like cells are present in early patch/plaque stage tumours where the majority of the cell population is made up of inflammatory cells. In addition, patients often display multiple tumours simultaneously in different anatomical locations, making a primary tumour impossible to define. Complete remission of disease can occur even in severely immunocompromised patients.

Clonality is often used to define a neoplasm, since a true malignancy originates from a single cell (Sidransky *et al.,* 1992). Using a technique called the X-linked inactivation assay, several groups have investigated clonality in KS lesions. This technique is based on the random inactivation of either the maternal or paternal X chromosome in a cell. This inactivation is determined during implantation of the female embryo and is copied in all daughter cells. Common polymorphism arising from a methyl-sensitive restriction digest (Vogelstein *et al.,* 1985) and PCR amplification of the androgen receptor gene determine different patterns of X chromosome expression. Using this technique, Rabkin *et al.* (1997) concluded that KS is a monoclonal cancer and that the dissemination of Kaposi's sarcoma in a patient arises from a monoclonal population of circulating neoplastic cells. Changes allowing these cells to become malignant have taken place before dissemination. However, this finding is disputed by Gill *et al.* (1998), who found multiple lesions in a single patient to be of different clonal origin and discovered some evidence of polyclonal inactivation patterns in other lesions. Early characterisation of the KSHV genome showed that the viral DNA was flanked by variable numbers of terminal repeat regions (TR) (Russo *et al.,* 1996) of approximately 800 bp each. These

TR regions were used as molecular markers to investigate clonality, similar to that used to demonstrate Epstein-Barr virus (EBV) clonality in other tumours (Raab-Traub and Flynn, 1986). Judde *et al.* (2000) demonstrated that KSHV was clonal in nodular lesions of KS, indicating that KSHV was present prior to tumour growth. However the number of terminal repeat units (16-75) was much greater than in EBV (1 to 9 units of 500 bp each) (Raab-Traub and Flynn, 1986) and rearrangements occurring in the KSHV TR may falsely indicate oligo- or polyclonality. Therefore, it has been proposed that KS in its early stages is a non-clonal proliferation of lymphatic endothelial cells that then evolve into a true clonal disease.

1.1.3 Epidemiological forms of KS

Classical KS occurs most frequently in elderly patients of Mediterranean, Jewish or Arabic decent and is more common in men than in women (Franceschi and Geddes, 1995). This is a relatively mild disease with little incidence of internal organ involvement. Nodules, firm and purple-blue or reddish-brown in appearance, occur on the legs or arms and increase slowly over years or decades. Treatment is by excision of the tumour, which limits the growth of lesions in most patients.

KS had affected parts of equatorial Africa for many decades preceding the HIV epidemic (Oettle, 1962). In Uganda KS accounted for 3 to 9 percent of cancers in 1971 (Taylor *et al.*, 1971). African or endemic KS is usually more aggressive than the classic form of KS (D'Oliveira and Torres, 1972). Both adults and children are affected and lymph node involvement is a common feature (Olweny *et al.*, 1976; Ziegler and Katongole-Mbidde, 1996; Sturzl *et al.*, 1999b). In the early 1980s, a rapid increase in KS cases took place, with many cases presenting with an atypical form of the disease that was difficult to treat, aggressive, and often involved internal organs (Bayley, 1984). When the diagnosis of AIDS became reliable, many of these atypical cases were classified as the epidemic form of KS.

The third form, iatrogenic KS, follows immunosuppression either through drug therapy or after organ transplantation. Among renal transplant recipients, the incidence of KS is 150 times greater than in healthy Western populations (Penn, 1983). Iatrogenic KS is directly related to drug induced immunosuppression; reduction or cessation of immunosuppressive drug therapy often leads to remission of KS disease (Besnard *et al.,* 1996). KS is the most common post-transplant tumour in geographical regions where KS is endemic, e.g. the Mediterranean and the Middle East (Qunibi *et al.,* 1988). People from certain ethnic backgrounds, including those of Mediterranean, Jewish or African origin are at increased risk of acquiring KS through organ transplantation (Shepherd *et al.,* 1997), and do continue to be at increased risk even if they are born or migrated to a region of lower incidence, e.g. North America or Europe (Franceschi and Serraino, 1995).

The fourth type of KS, the epidemic or AIDS-related form, is associated with immunosuppression due to HIV infection. This form of KS became prominent in the wake of the AIDS epidemic in previously non-endemic countries. Friedman-Kien *et al.* (1981) described this new and aggressive form of the disease affecting a large number of previously healthy young homosexual men in New York City. HIV-positive men who had sex with men (MSM) had a 50% greater lifetime risk of developing KS at the start of the AIDS epidemic than other HIV-positive persons (Katz *et al.,* 1994). KS is now less common in the HIV-positive MSM group, with the incidence of KS having declined since the 1980's (Beral, 1991), falling further after the introduction of antiretroviral therapy (Huang *et al.,* 1992). However, KS is still the most common neoplasm in patients with AIDS (Beral *et al.,* 1990; Rabkin *et al.,* 1995). In the West, AIDS-KS is most common in the MSM group, less common in HIV-positive heterosexuals and virtually non-existent in persons who have contracted HIV parenterally (Beral *et al.,* 1990). AIDS-KS is widespread in Africa, varying by geographic region and affecting men more often than women (Wabinga *et al.,* 1993; Bassett *et al.,* 1995; Gao *et al.,* 1996b; Koulibaly *et al.,* 1997).

Rather than following an indolent course characterised by the classical form of KS, epidemic or AIDS-associated KS can rapidly develop into a fatal illness. Multiple parts of the body and organ systems become involved including skin, mucosa and the lymphatic system (Friedman-Kien, 1981). The disease course is often short with patients surviving only weeks to months following the onset of symptoms. Progression of KS disease is dependent on the condition of the host immune system (Osman *et al.,* 1999) and highly antiretroviral therapy (HAART) can restore immune function in some cases (Winceslaus, 1998).

1.1.4 Discovery of an infectious agent for KS

An infectious cause suspected for KS was suspected as early as 1962 (Oettle, 1962). The possibility that KS may have a viral aetiology was investigated extensively by Giraldo *et al.* (1972). In this study, herpesvirus-like particles were found in KS lesions using electron microscopy (EM), which were later were found to be cytomegalovirus (CMV) (Giraldo *et al.,*1980). After KS appeared dramatically in the 1980's as one of the most recognisable sequelae of the AIDS epidemic, many more studies were undertaken to discover its cause. Several infectious agents were implicated including human papilloma virus, CMV, human herpesvirus 6, and BK virus but were detected in only a small fraction of KS lesions studied (Huang *et al.,* 1992; Kempf *et al.,* 1995; Monini *et al.,* 1996)

In 1990 and 1991, Beral *et al.* published pivotal findings on the epidemiology of KS among patients with AIDS. The study found that KS was at least 20,000 times more common in persons infected with HIV than in the general population. Furthermore, AIDS patients were 300 times more likely to be affected by KS than other groups of immunosuppressed individuals. Sexual transmission appeared to play an important part, with 21% of homosexual or bisexual HIV-positive men developing KS as opposed to 1% of male haemophiliacs who acquired HIV parenterally. US and Canadian homosexual men were more likely to acquire KS if they had sexual contact with men from the US AIDS epicenters (San Francisco, Los Angeles, and New York) (Beral,

1991; Archibald *et al.,* 1992). The evidence pointed to an infectious agent, other than HIV, that was responsible for the development of KS.

In 1994, Chang *et al.* reported the discovery of unique viral sequences in 90% of KS tissues studied using a molecular technique called representational difference analysis (RDA) (Lisitsyn *et al.,* 1993). In this technique oligonucleotide adapters are ligated to fragments of the unknown "tester" DNA. In this case, DNA from an AIDS-KS tumour was used. Background normal tissue DNA from the patient, or "driver" DNA was then added, and the entire mixture was denatured and re-annealed to produce both double stranded (ds) and single stranded (ss) DNA molecules. Only unique sequences found in the diseased tissue will contain both oligonucleotide adapters and will be preferentially amplified during PCR. Following three rounds of amplification, four discrete bands were visualised: KS330Bam, KS390Bam, KS480Bam, and KS631Bam. Following removal of the oligonucleotide adapter sequences by restriction enzyme digestion, the fragments were gel purified and hybridised with KS and non-KS human tissue on a Southern blot. KS390Bam and KS480Bam hybridised non-specifically to both types of DNA and were thus eliminated from further analysis. The remaining two bands, KS330 and KS631, were cloned and sequenced. Both segments have homology to two members of the *gammaherpesvirus* sub-family. KS330 Bam is 51% homologous to opening reading frame (ORF) 26 of *Herpesvirus saimiri* (HVS) and 39% homologous to ORF BDLF1 of Epstein-Barr virus (EBV). KS631 Bam is homologous to a tegument protein coded for by ORF 75 in HVS and ORF BNRF1 in EBV.

1.1.5 Establishing causation

Although an infectious cause for KS had been proposed, the prevailing theory just before the discovery of KSHV was that KS was a hyperplastic, cytokine-driven neoplasm initiated by HIV-1 *tat* protein expression (Ensoli *et al.,* 1990). Epidemiological evidence, however, supported the hypothesis that another agent, independent of HIV, was causing KS (Beral *et al.,* 1990). This agent was likely to be non-ubiquitous in the general populations of North America and Northern Europe, would not be easily transmitted parenterally or through the

blood, and would be linked to sexual practices among gay men. Based on this critical epidemiological data, Chang *et al.* (1994) used the molecular technique RDA, as described above, to isolate fragments of KSHV from an AIDS-KS tumour. Using case control methodology, KS330Bam and KS631Bam sequences were found in 25 of 27 AIDS-KS tissue samples (93%) (Chang *et al.*, 1994) by both PCR and Southern blot analysis. To fulfil Hill's criteria for causation (Fredericks and Relman, 1996), the following four further general observations were made, which together are sufficient to implicate KSHV as the aetiological agent of KS.

i) Molecular techniques were first used to test samples from many geographical areas and PCR amplification of the Bam330 fragment confirmed that KSHV DNA was detected in KS tumours and rarely in control tissues (Cesarman *et al.*, 1995). This finding was confirmed in studies from North America (Ambroziak *et al.*, 1995), Asia (Su *et al.*, 1995), Europe (Boshoff *et al.*, 1995b; Dupin *et al.*, 1995) and Africa (Schalling *et al.*, 1995) thereby linking the presence of KSHV DNA with all epidemiological forms of KS.

ii) The presence of KSHV DNA in the PBMC of HIV-positive individuals as determined by PCR predicted the subsequent development of KS. Moore *et al.* (1996c) studied paired PBMC samples taken before and after KS onset from 21 AIDS-KS patients, 23 homosexual high risk HIV-positive patients, and single PBMC samples from 19 low risk HIV-positive haemophiliacs. A sensitive nested PCR detected KSHV DNA in 52% of AIDS-KS patients prior to the onset of KS and in only 9-13% of members in the two control groups. Whitby *et al.* (1995) found that 6 of 11 HIV and KSHV positive patients without KS developed the disease within 30 months of follow up versus only 12 of 132 KSHV-seronegative/HIV-seropositive patients. Furthermore, the KSHV detection rate in patients with KS was improved as the patient became more immunosuppressed.

iii) Cesarman *et al.* (1995) found evidence of KS330Bam sequences in cases of AIDS-related body cavity based lymphomas, or primary effusion lymphomas

(PEL). PEL-derived cell lines permitted virus to be propagated in culture and immunological tests to be developed using PEL cell lines as antigen sources. PEL cell lines appeared to express the full repertoire of KSHV gene products during both lytic and latent viral replication. The expression of KSHV genes will be more fully discussed in Section 1.3.2. Seroprevalence studies (also to be discussed in more detail in Section 1.5.2) showed correlation between the prevalence of KS in a given population and KSHV seroprevalence.

iv) KSHV DNA could be detected in the spindle shaped cells of KS lesions using *in situ* hybridisation. KSHV DNA was detected in all nodular KS tumours using *in situ* PCR but not in the surrounding epidermis and dermis or in other control tissue (Boshoff *et al.,* 1995a). RNA transcripts have been detected in all plaque/patch and nodular stages of KS in the spindle shaped cells of the tumour but not in surrounding healthy tissue or control tissue from other proliferative disorders (Staskus *et al.,* 1997). Most cells in late KS tumours appear to be latently infected. Immunocytochemistry of KS lesions has shown that the latent nuclear antigen (LNA), coded for by ORF 73, is widely expressed in KS spindle cells (Kellam *et al.,* 1999).

1.2 Other diseases associated with KSHV

1.2.1 Primary effusion lymphoma

As well as being the causative agent for KS, KSHV has been linked to a number of other diseases. As previously mentioned, KSHV DNA has been found in PEL cells and cell lines. PEL is a subtype of non-Hodgkin's lymphoma (NHL) characterised by a malignant effusion of B-cells in the pleural or abdominal cavity. Studies have shown that, often, these malignant cells are dually infected with both KSHV and EBV (Cesarman *et al.,* 1995) but in rarer cases of PEL, cells are infected with KSHV only (Renne *et al.,* 1996). Most cases of PEL occur in HIV-infected patients and cells from these malignancies can be immortalised when grown in culture. PEL derived cell lines maintain a high viral copy number when grown in vivo and are useful for characterisation of individual strains of the virus (Cesarman *et al.,* 1995). As previously

mentioned, the successful propagation of several PEL cell lines infected with KSHV alone greatly aided in the development of antibody assays (Renne *et al.*, 1996).

1.2.2 Castleman's disease

Castleman disease (CD) is a lymphoproliferative disorder of unknown aetiology, which can lead to development of secondary tumours, including B cell lymphomas. Originally described by Castleman *et al.* (1956), CD is characterised by a benign localised mass of lymphoid tissue. Histologically, the CD contains large follicles separated by vascular lymphoid tissue containing lymphocytes; this is known as the hyaline vascular (HV) type of CD. When sheets of plasma cells are found in the interfollicular zone, this is described as the plasma cell variant. The plasma cell variant can often become multicentric. Multicentric CD (MCD) has a poorer prognosis and is associated with the concurrent development of lymphomas including NHL and plasmablastic B-cell lymphomas (Weisenburger *et al.*, 1985). Cases of MCD have been found in HIV-positive individuals, and Soulier *et al.* (1995) first reported the presence of KHSV DNA detected by PCR in MCD biopsy samples. The clinical course of KSHV positive MCD tends to be worse, particularly among HIV-infected individuals, and is not always improved through therapy with HAART (Dupin *et al.*, 1997; Dupin *et al.*, 2000). KSHV DNA has been localised in plasmablasts of B cell lineage in the mantle zone of the B-cell follicles in the tumour (Dupin *et al.*, 2000; Katano *et al.*, 2000). These plasmablasts show only λ chain restriction and high levels of cytoplasmic IgM, in contrast to KSHV-negative plasma cells located in the follicular region that are IgM negative and polytypic. The mechanism of KSHV pathogenesis in MCD is not clear; however, viral IL-6 (vIL-6) is highly expressed in a subset of plasmablasts (Parravicini *et al.*, 1997a; Du *et al.*, 2001} and may drive KSHV infected naïve B cells to become plasmablasts.

1.2.3 Other disorders

KSHV infection has been suggested in a number of diseases including multiple myeloma (Rettig *et al.*, 1997), some reactive lymphadenopathies (Luppi *et al.*,

1996), a variety of skin carcinomas and lymphomas, and sarcoidosis (Di Alberti *et al.,* 1997). Despite these associations, no conclusive evidence has been found to strongly link any of these diseases to infection with KSHV.

1.3 Organisation of the KSHV genome

Only two years after the discovery of less than 1 percent of the viral genome, the entire KSHV genome of 165 kilobases was sequenced (Russo *et al.,* 1996; Moore *et al.,* 1996b). The virus was assigned the trivial name, Kaposi's sarcoma associated herpesvirus and the proper name, human herpesvirus 8 (HHV-8), now used interchangeably in the literature. Of the three types of known herpesviruses (alpha-, beta-, and gamma-herpesvirus), only gamma-herpesviruses are known to infect lymphocytes (Roizmann *et al.,* 1992) and are often implicated in the development of lymphomas (Neipel *et al.,* 1998). The gamma-herpesvirus subfamily is further divided into two genera, *rhadinovirus* (gamma -2) and *lymphocryptovirus* (gamma -1). EBV is a gamma-1 herpesvirus and is the prototypic member of this group.

KSHV is the first known gamma-2 herpesvirus to infect humans (Russo *et al.,* 1996). In New World monkeys, HVS, the prototype rhadinovirus, infects squirrel monkeys (Albrecht *et al.,* 1992), and *Herpesvirus ateles* (HVA) infects spider monkeys (Albrecht, 2000). Extensive study has been done to find evidence of herpesvirus infection in primates and as a result many species of Old World primates have been found to be infected with multiple gamma-2 herpesviruses. Gamma-2 herpesviruses discovered to date in Old World primates are summarised in Table 1.1. Old World primate rhadinoviruses can be divided into two distinct lineages, RV1 and RV2. KSHV belongs to the RV1 lineage and its close phylogenetic association with primate rhadinoviruses indicates a common ancestral origin. The presence of RV1 and RV2 type rhadinoviruses in many Old World primates points to yet undiscovered rhadinoviruses of the RV2 lineage capable of infecting humans (Lacoste *et al.,* 2001).

Table 1.1. Summary of gamma-2 herpesviruses infecting non-human species of Old World primates. RFHV = *retroperitoneal fibromatosis herpesvirus*, RRV = *rhesus rhadinovirus*, RHV = *rhadino-herpesvirus*

Common name	Species	Virus	
		RV1	**RV2**
Rhesus macaque	*Macaca mulatta*	RFHVMm[1]	RRV[3,8]
Pig-tailed macaque	*Macaca nemestrina*	RFHVMn[1]	MneRV2[2]
African green monkey	*Chlorocebus aethiops*	ChRV1[4]	ChRV2[4]
Mandrill/drill	*Mandrillus sphinx Mandrillus leucophaeus*	MndRHV1[5]	MndRHV2[5]
Chimpanzee	*Pan troglodytes*	PanRHV1a[6] PanRHV1b[6]	PanRHV2[7]
Gorilla	*Gorilla gorilla*	GorRHV1[6]	

[1](Rose *et al.*, 1997), [2](Schultz *et al.*, 2000), [3](Searles *et al.*, 1999), [4](Greensill *et al.*, 2000), [5](Lacoste *et al.*, 2000c), [6](Lacoste *et al.*, 2000b), [7](Lacoste *et al.*, 2001), [8](Desrosiers *et al.*, 1997)

1.3.1 Size and structure

Shortly after its discovery, the full sequence of KSHV was reported of the viral genome derived from a PEL cell line (Russo *et al.*, 1996) and from a KS biopsy specimen (Neipel *et al.*, 1997b). All members of the rhadinovirus genus share a common genome structure characterised by an area of low GC content called L-DNA that is flanked by repetitive regions of high GC content, H-DNA (Bornkamm *et al.*, 1976). In the KSHV genome, numerous 801 bp tandem repeat regions of high GC content (84.5%) flank a 140.5 kb L-DNA segment of lower GC content (53.5%). All of the known KSHV ORFs, 87 in total, are coded for in the L-DNA region (Russo *et al.*, 1996; Neipel *et al.*, 1997b). The genome of KSHV has been compared to HVS, a well-characterised rhadinovirus prototype (Fleckenstein and Bornkamm, 1975), in a collinear fashion. KSHV ORFs are named according to their homology with HVS ORFs (Albrecht *et al.*, 1992) although the ORFs do not appear in the same order in each genome. Unique regions are numbered consecutively and given K as a prefix (e.g.

K1...K15), with newly discovered unique ORFs designated by the addition of a decimal point (e.g. K4.1).

As with other gamma-herpesviruses, essential ORFs that code for viral replication and assembly proteins are arranged in blocks and are referred to as the conserved genes. There are approximately 67 such genes conserved among members of herpesvirus subfamilies. In KSHV, these include: ORF 25, coding for the major structural protein; ORF 9, DNA polymerase; and ORF 17, a proteinase and assembly protein (Moore *et al.,* 1996b).

Non-conserved gene blocks in the KSHV genome lie between the conserved blocks and contain some ORFs that are found in other rhadinoviruses including homologues to mammalian proteins. These proteins appear to be more extensively coded for in KSHV than in other rhadinoviruses (Russo *et al.,* 1996) and are thought to have been pirated from the host genome during evolution (Neipel *et al.,* 1998). The KSHV genome has cellular homologues not found in other rhadinoviruses including a homologue to human IL-6, members of the CC chemokine family and interferon regulatory factors (IRFs). These gene products may contribute to the pathogenesis of KSHV and will be discussed in more detail in Section 1.3.2.

1.3.2 Gene expression

As do other herpesviruses, KSHV establishes latency in the host (Gao *et al.,* 1996a) and is under stringent latent replication control in PEL cell lines (Russo *et al.,* 1996). While a minority of cells in culture may express lytic cycle proteins at any given time, chemical induction with TPA is needed for the full expression of lytic gene products in PEL cells *in vitro*. Three broad categories of gene expression have been described (Sarid *et al.,* 1998) based on TPA induction of PEL cell lines. However this classification may not accurately represent KSHV gene transcription in tissue. For the purposes of this discussion, these broad categories of expression have been described with the caveat that in vivo expression in different tissue types may not be straightforward.

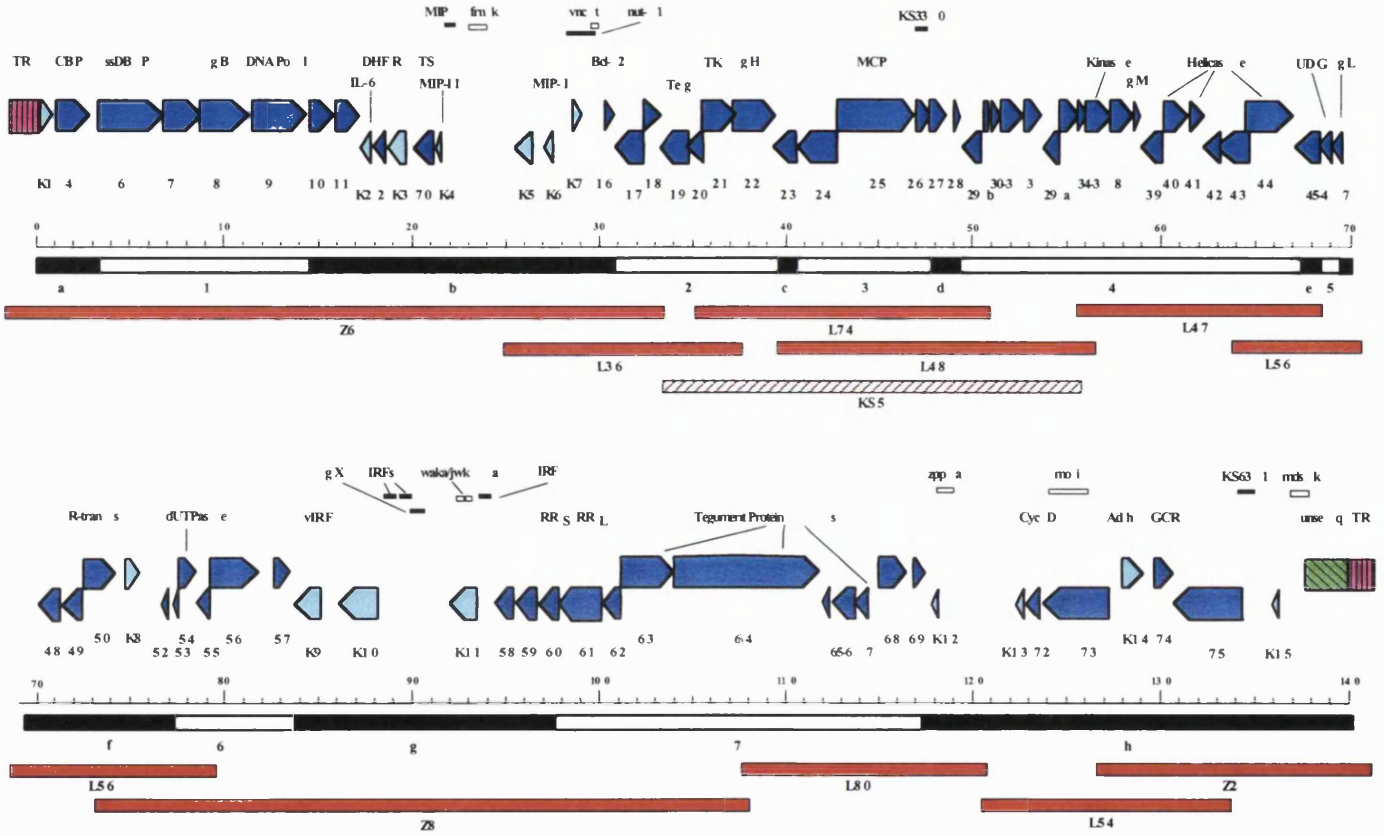

Figure 1.1 BC-1 KSHV genome reproduced with permission from: Russo *et al.,* (1996) Nucleotide sequence of the Kaposi's sarcoma-associated herpesvirus (HHV-8). PNAS 93, 14862-67. Copyright (1996) National Academy of Sciences, USA.

Class I transcripts

Sarid *et al.* (1998) established three basic classes of gene transcription based on studies of BC-1 cell lines. Class I (latent) mRNAs can be detected under standard growth conditions and are not upregulated by TPA. Three constitutively expressed, and latent transcripts are created by the polycistronic transcription of two overlapping genes LT1 and LT2 (Dittmer *et al.,* 1998; Kellam *et al.,* 1999; Sarid *et al.,* 1999).

The first of these, ORF 71, produces a homologue to cellular FLICE (Fas-associated death domain-like interleukin-1 beta-converting enzyme or caspase-8) inhibitory proteins (FLIP). vFLIPs found in other herpesviruses are known to inhibit fas-mediated apoptosis by preventing the recruitment of caspase-8 (FLICE) to the death-induced signalling complex (Thome *et al.,* 1997). vFLIP in KSHV has a similar function and can prevent apoptosis in latently infected cells (Djerbi *et al.,* 1999). ORF 71 is produced as a bi-or tri-cistronic transcript with either ORF 72 or ORFs 72 and 73, and utilises internal ribosome entry site (IRES)–mediated initiation to produce a functional protein (Bieleski and Talbot, 2001).

ORF 72 is a functional cyclin D homologue cell cycle regulator (Chang *et al.,* 1996). Cyclins regulate the cell cycle by controlling cyclin dependent protein kinases (cdk) (Hunter and Pines, 1991; Sherr, 1993). Viral cyclins are homologous to types D and E cellular cyclins and act by interrupting the cell cycle at the end of the G1 phase through the inactivation of the retinoblastoma tumour suppressor protein (pRB) via cdks (Meyerson and Harlow, 1994). The KSHV viral cyclin has been shown to associate primarily with cdk6 and to a lesser extent with cdk4 (Li *et al.,* 1997), both important cell cycle regulators. Viral cyclin association with cdk6 results in phosphorylation of the pRB (Godden-Kent *et al.,* 1997) thereby allowing progression through the cell cycle past this checkpoint.

Prominently expressed during latency, LNA, coded for by ORF 73, is a highly immunogenic protein. During latency, it is important for the virus to maintain its DNA as an episome in order to replicate effectively and produce new viruses. LNA assists in maintaining viral episomes by attaching viral DNA to the host chromatin through binding to the TR region of the KSHV genome (Cotter and Robertson, 1999; Ballestas and Kaye, 2001). This interaction creates the characteristic pattern of intra-nuclear dots observed under immunofluoresence. LNA can also bind to and inhibit p53 mediated apoptosis to allow KSHV infected cells to persist (Friborg, Jr. *et al.,* 1999).

Class II transcripts

A switch from the latent to the lytic phase gene expression is essential for virus replication and pathogenesis. In KSHV, this switch is initiated by ORF 50/Rta (replication and transcription activator) (Sun *et al.,* 1998; Lukac *et al.,* 1999). ORF 50/Rta is essential and sufficient to initiate and control the entire viral lytic cycle and can auto-stimulate its own expression (Gradoville *et al.,* 2000). The ORF 50/Rta promoter region is heavily methylated. Demethylation of the promoter by TPA induces lytic cycle replication; demethylation of the Rta promoter region is essential for expression of Rta (Chen *et al.,* 2001). Biopsy samples from patients with KSHV associated diseases show extensive demethylation of the Rta promoter while latently infected cells remain methylated (Chen *et al.,* 2001). This indicates that demethylation of Rta and induction of lytic cycle gene expression are necessary for the development of KSHV associated diseases.

Class II transcripts can often be detected at varying levels in resting cells but are upregulated in the presence of TPA. Two highly expressed RNAs, T0.7 (ORF K12, Kaposin) and T1.1 (nut-1), that are homologous to EBER of EBV and U-RNA of HVS, respectively, fall into this category (Renne *et al.,* 1996; Zhong and Ganem, 1997; Sarid *et al.,* 1998). As they are so highly expressed, even during latency, they have been exploited as targets for in situ hybridisation (Staskus *et al.,* 1997). T1.1 appears most abundantly when cells were induced

with TPA (Sarid *et al.,* 1998) but T1.1 nuclear RNA does not associate with polyribosomes and is not transcriptionally active (Sun *et al.,*1996). T0.7 has been detected in all types of KS tumour samples in the spindle shaped cells (Staskus *et al.,* 1997) and in PEL cell lines (Renne *et al.,* 1996). Of the three small opening reading frames in T0.7, one of these, ORF K12, or kaposin, has been shown to induce tumorigenic transformation and is expressed in the PEL cell lines BCBL-1 and KS-1 (Muralidhar *et al.,* 2000).

Also included in the group of class II transcripts are a variety of genes encoding viral cytokines and chemokines, signal transduction, and regulatory proteins that may contribute to viral pathogenesis. A number of these important genes are reviewed below.

i) Viral macrophage inhibitory proteins (vMIP)

The KSHV genome encodes several chemokines of the CC or β chemokine family (Moore *et al.,* 1996a), designated vMIP-I, vMIP-II and vMIP-III. vMIP-I and vMIP-II share 50% similarity at the amino acid level (Moore *et al.,* 1996a) while vMIP-III is less closely related. vMIP-I binds selectively to the CCR8 chemokine receptor, expressed predominately on Th2 type T cells, and acts as a CCR8 agonist (Dairaghi *et al.,* 1999; Endres *et al.,* 1999). In contrast, vMIP-II has a broad range of activity and can bind to a wide variety of CC and CXC type chemokine receptors. Maddon *et al.* (1986) demonstrated that the presence of CD4 on a cell was sufficient to bind HIV-1 but could not facilitate HIV-1 penetration or fusion with the host cell. It was ten years later that the co-receptor necessary to trigger viral entry was elucidated (Deng *et al.,* 1996; Dragic *et al.,* 1996). The beta chemokine receptor, CCR5, acts in synergy with CD4 to allow HIV-1 to infect the host cell, and is the most prevalent co-receptor. A variety of other co-receptors have been discovered, including CCR3 (Deng *et al.,* 1996) and CXCR4 (Feng *et al.,* 1996). vMIP-II acts as an antagonist for CCR5 and CXCR3 (Kledal *et al.,* 1997) and can block viral entry of HIV-1 through its interaction with co-receptors CCR5, CXCR4 and CCR3 (Kledal *et al.,* 1997; Boshoff *et al.,* 1997)

V-MIP-II has been reported to have a chemotactic effect on cells displaying CCR8 and CCR3 chemokine receptors (Boshoff *et al.,* 1997; Sozzani *et al.,* 1998), predominately Th2 type T cells. vMIP-III (ORF K4.1) acts as an agonist for cells with CCR4 chemokine receptors, again expressed by Th2 type T cells but cannot block the entry of HIV-1 (Stine *et al.,* 1999).

All three viral chemokines display angiogenic properties (Boshoff *et al.,* 1997; Stine *et al.,* 1999). It appears that these three chemokines preferentially target receptors found on Th2 type cells. This may skew the Th1/Th2 response in the host and create a unique microenvironment consisting of a mainly Th2 type inflammatory cell population. In this way, the virus may avoid a cytotoxic response caused by Th1 type cells, thereby successfully evading a host immune response (Dairaghi *et al.,* 1999; Stine *et al.,* 1999; Weber *et al.,* 2001).

(ii) G-protein couple receptor (GPCR)

ORF 74 of the KSHV genome encodes a G-protein-coupled receptor (GPCR) (Cesarman *et al.,* 1996) homologous to the GPCR of HVS (Nicholas *et al.,* 1992) and human interleukin 8 (IL-8) receptors (Holmes *et al.,* 1991; Murphy and Tiffany, 1991). KSHV GPCR is a constitutively expressed chemokine receptor exhibiting affinity for a wide range of chemokines and does not require activation through an agonist (Arvanitakis *et al.,* 1997). GPCRs that are constantly active have been shown to transform cells and act as oncogenes in some human cancers (Gutkind *et al.,* 1991; Bais *et al.,* 1998). KSHV GPCR activates nuclear factor kappa B (NF-kappa B), a transcriptional regulatory factor that in turn up-regulates the expression of a wide variety of inflammatory cytokines and adhesion molecules (Pati *et al.,* 2001). In a KS lesion, only a small number of cells are undergoing lytic replication, but all cells, particularly in later lesions, are infected and display the characteristic spindle cell like phenotype (Blasig *et al.,* 1997). It is possible that KSHV GPCR, by activating VEGF, NF-kappa B and in turn a variety of inflammatory cytokines, can indirectly affect uninfected or latently infected cells in an autocrine or paracrine manner (Pati *et al.,* 2001).

iii) Bcl-2 homologue

Bcl-2 is a potent cell death-suppressor that operates by inhibiting apoptosis (Reed, 1994). The KSHV genome contains a homologue to bcl-2 (KSbcl-2) with low sequence homology to other bcl-2 homologues (15-20%) (Cheng *et al.,* 1997). This homology is concentrated in the two bcl-2 conserved domains, designated BH1 and BH2. When over-expressed in cell culture, KSbcl-2 prevents apoptosis and prolongs cell life (Cheng *et al.,* 1997). By prolonging cell survival, genetic changes in cellular oncogenes can occur, thus promoting tumour progression (Strasser *et al.,* 1990; McDonnell and Korsmeyer, 1991).

iv) Interferon regulatory factors (vIRFs)

KSHV encodes four homologues to cellular interferon regulatory factors (IRFs), vIRF-1 to vIRF-4. vIRFs 1 to 3 have been characterised thus far. Interferons (IFN) are a group of cytokines with wide ranging effects including defence against viral infection and inhibition of cell growth. IRFs regulate IFN-responsive genes thereby modifying the effects brought about by IFN activity (Reis *et al.,* 1992; Taniguchi *et al.,* 1995).

The ORF K9 (vIRF-1) is homologous to cellular IRFs, inhibits cellular responses to IFNs (Gao *et al.,* 1997; Zimring *et al.,* 1998), and can down-regulate the transcriptional activation induced by IFNs α, β, and γ (Gao *et al.,* 1997; Li *et al.,* 1998). This activity suppresses the host's IFN mediated innate immune response to viruses. vIRF-1 also acts as an oncoprotein by transforming rodent fibroblasts *in vitro* and causing tumour formation in nude mice (Gao *et al.,* 1997; Li *et al.,* 1998). It has been demonstrated that vIRF-1 can bind to p53 (Nakamura *et al.,* 2001), a tumour suppressor gene involved in many types of human cancers (Vogelstein *et al.,* 2000). p53 responds to cellular stress, such as DNA damage or abnormal growth signals, to induce cell death, providing protection against viral infections and tumours (el Deiry *et al.,* 1993; Miyashita and Reed, 1995). By suppressing phosphorylation and acetylation of p53, vIRF-

1 inhibits its activity and prevents apoptosis in the infected cell (Nakamura *et al.*, 2001). VIRF-1 is not expressed in PEL cell lines or KS tissue but may be involved in the pathogenesis of CD (Katano *et al.*, 2000; Parravicini *et al.*, 2000).

ORF K11.1 (vIRF-2), although discussed here, is a latently expressed gene product in PEL cell lines (Burysek *et al.*, 1999). Some IRFs have the ability to bind nucleotides; while vIRF-1 has no such activity, vIRF-2 can bind oligonucleotides corresponding to Nf-kappaB binding sites (Burysek and Pitha, 2001). vIRF-2 is constitutively expressed in the nucleus of PEL cells and inhibits the effects of cellular IFN through its interaction with a ds RNA-activated protein kinase (PKR) (Burysek and Pitha, 2001). PKR is normally induced by IFN and mediates the anti-viral and anti-proliferative effects of IFN (Hovanessian, 1989; Sen and Ransohoff, 1993; Patel *et al.*, 1995; Clemens and Elia, 1997). By down-regulating the antiviral response caused by PKR, vIRF-2 may be important for maintenance of viral latency (Burysek and Pitha, 2001).

ORF K10.5 (LANA-2) has a degree of homology to IRF-4 and is latently expressed in a PEL cells and CD but not in KS lesions (Rivas *et al.*, 2001). LANA-2 is highly expressed in cultured B cells infected with KSHV. Reporter assays showed that LANA-2 inhibits p53 expression, like LANA-1, potentially contributing to the proliferation of infected B cells in CD (Rivas *et al.*, 2001).

v) Viral interleukin 6 (vIL-6)

Coded for by ORF K2, KSHV vIL-6 is approximately 25% homologous to human interleukin 6 (hIL-6) (Moore *et al.*, 1996a; Nicholas *et al.*, 1997; Neipel *et al.*, 1997a). VIL-6 is readily secreted by KSHV infected cells in PEL and CD during latency and to a lesser extent in KS lesions (Moore *et al.*, 1996a; Parravicini *et al.*, 1997a). Like its cellular counterpart, vIL-6 activates signal transduction pathways through its interaction with the gp130 receptor, but unlike hIL-6 it does not need to interact with the IL6 co-receptor (IL-6R) to activate signalling. However, interaction with both gp130 and IL-6R may increase

signalling efficiency (Wan *et al.*, 1999). Both hIL-6 and vIL-6 are able to inhibit apoptosis and induce B-cell proliferation (Moore *et al.*, 1996a; Nicholas *et al.*, 1997). They can also transform NIH3T3 cells and cause tumour formation (Aoki *et al.*, 1999). Although most cells in a PEL or CD do not secrete vIL-6, this cytokine has a paracrine effect on the surrounding tissue which could be important in KSHV related disease progression. For instance, in cases of KSHV infected CD, vIL-6 appears to cause a proliferation of uninfected B cells that comprise the majority of the tumour mass (Parravicini *et al.*, 1997a).

Class III transcripts

A number of genes involved in nucleic acid synthesis are coded for in the KSHV genome, in common with all herpesviruses (Roizman and Pellett, 2001). Members of the rhadinoviruses, including KSHV, encode a larger number of these proteins compared to other herpesviruses (Virgin *et al.*, 1997). Some of these proteins include DNA polymerase (ORF9), thymidine kinase (TK) (ORF 21), dihydrofolate reductates (DHFR) (ORF 2) and ribonucleotide reductase (ORF 60 and 61) (Russo *et al.*, 1996; Neipel *et al.*, 1997b; Nicholas *et al.*, 1998; Goudsmit *et al.*, 2000). This group of genes is expressed as class III products during the late lytic phase. Their function may be to replace or enhance cellular DNA biosynthesis to aid in virion production.

1.4 KSHV genotypes

Studies comparing KSHV genome sequences from both KS biopsies and PEL cell lines demonstrated that the genome is highly conserved, with only 0.1% nucleotide variation between sequences, excepting an area of increased polymorphism at the far ends of the genome (Russo *et al.*, 1996; Moore *et al.*, 1996b). In order to determine genotype, two small regions of the genome showing some diversity were originally investigated. An analysis of the ORF 26 and ORF 75 regions in several isolates made it possible to discriminate three subtypes (A, B and C) (Zong *et al.*, 1997). The degree of variability was low

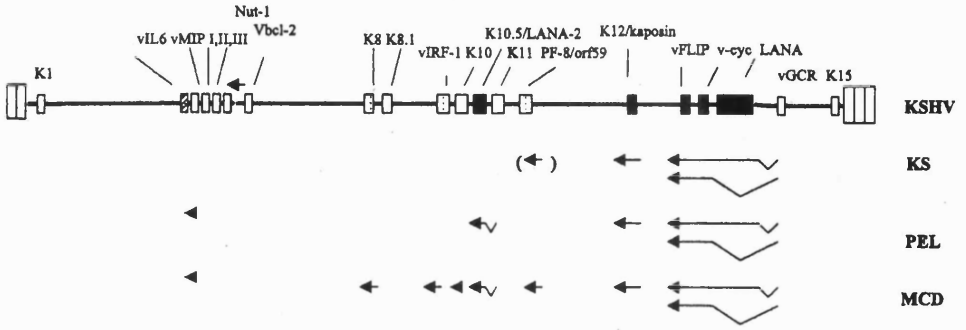

Figure 1.2 KSHV genome map showing viral genes possibly associated with KSHV pathogenesis. Arrows indicate transcriptional patterns in different types of KSHV associated malignancies. Reproduced from Virus Research, volume 82, Schulz *et al.*, "Kaposi's sarcoma associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8).", pages 115-126, copyright (2002), with permission from Elsevier Science.

with only 2% divergence over a 500 bp region of ORF 26 and very few substitutions resulting in a change at the amino acid level. Zong *et al.* (1997) amplified and sequenced a region of the ORF 75 gene to confirm the system of subtyping developed from ORF 26 sequences. Analysis of ORF 75 did confirm the three-letter typing system but the nucleotide variation within each group was less than 0.1%.

A region showing a high degree of polymorphism at the far left hand side (LHS) of the genome (Russo *et al.*, 1996), ORF K1, was analysed to complement genotyping based on ORF 26 and ORF 75. ORF K1 codes for a highly glycosylated integral membrane protein with similarities to the immunoglobulin receptor family (Lagunoff and Ganem, 1997; Lee *et al.*, 1998) and is expressed in the early lytic phase after induction by TPA in PEL cell lines. The transforming potential of the ORF K1 protein in vivo was reported by Lee *et al.* (1998) and while it has no homologues in other herpesviruses, it appears at an equivalent position in the HVS genome where a transforming protein is located.

Meng *et al.* (1999) found extensive polymorphism in the extracellular domain of ORF K1 over an 840 bp region coding for 270 amino acids. A pairwise comparison between two strains revealed a maximum variability of 16% at the nucleotide level and 32% at the amino acid level. ORF K1 is conserved at its N and C terminal ends and is variable between amino acid positions 20 and 226 (Meng *et al.,* 1999). Within this variable region are two blocks of greatest polymorphism. Between amino acids 51 and 92 lies the variable region 1 (VR1). Variable region 2 (VR2) is encompassed between residues 191 and 231. Certain segments within and connecting these two blocks are conserved presumably to maintain the tertiary structure of the protein.

ORF K1 sequences derived from samples could be assigned to one of four genotypes I-IV, based on phylogenetic analysis and a distinctive, small, in frame deletion in the VR2 region (Meng *et al.,* 1999). Genotype I could be further divided into four subsections, A-F. Zong *et al.* (1999) also identified four main genotypes, which they named A-D, from which 13 clades or subtypes could be discriminated based on amino acid differences of 5% or greater (i.e. A1...A5, C1 etc). A recent comprehensive study by this group involving 139 samples, from which at least VR1 sequence data were available, identifies 24 ORF K1 genotypic variants (Zong *et al.,* 2002) (summarised in Table 1.2).

Initial studies of the KSHV genome (Russo *et al.,* 1996) alluded to another area of variability at the far right hand side of the genome (RHS). This region initially proved difficult to clone and study due to its high G+C content and the presence of multiple splicing sites. Located within this region is ORF K15, which codes for an integral membrane protein, and exists as two highly divergent alleles, P (predominant) and M (minority) (Poole *et al.,* 1999). The K15 gene resembles the LMP2A coding region of the EBV genome in position as well as in splicing pattern and predicted protein structure. K15 P or M alleles can be distinguished using a three step PCR incorporating the adjacent K14.1 junction region (Poole *et al.,* 1999).

ORF K1 subtypes correlate with ethnicity and geography, as shown in Table 1.2. The B subtype is found predominately in sub-Saharan Africa or in persons of African heritage. B subtype sequences are divergent in ORF K1 but show little phylogenetic clustering, thus allowing only three subgroups to be distinguished (B1, B2 and B3) based on 4 amino acid changes in the VR2 regions (Zong *et al.*, 2002) and a deletion in the B3 variant (Treurnicht *et al.*, 2002). Less frequently, African samples carry A5 type ORF K1 sequences. The A and C subtypes are widely distributed throughout Europe, USA, Asia and the Middle East; AIDS KS patients in the US are normally infected with subtypes A1, A4 and C3 (Zong *et al.*, 1999). These predominant subtypes associated with the AIDS epidemic reflect reactivation of endogenous KSHV variants, originally introduced by immigrants from endemic Europe, the Middle East and Africa as well as horizontal transmission events occurring locally. Rare D subtypes have been isolated from persons of Pacific Island heritage only (Poole *et al.*, 1999). Another divergent subtype was reported by Biggar *et al.* (2000) among a group of Brazilian Amerindians. Two ORF K1 sequences were isolated from 35 blood samples differing by 25% to 30% from all previously identified sequences. While the two sequences group most closely with subtype D, they may belong to a more divergent subtype, E.

The wide geographical and ethnic distribution of ORF K1 subtypes suggests that KSHV is a very ancient virus, originating in sub-Saharan Africa in Palaeolithic times. It is speculated that human migration 60,000 years ago into Asia, Australia and the Pacific Islands founded the D subtype. Two major branches then migrated into Europe and North Asia 35,000 years ago to found the A and C subgroups. These two subtypes are very similar, having diverged most recently, while subtype B is the most divergent subtype, displaying a 30% overall amino acid difference from types A and C (Zong *et al.*, 1999), and is therefore the oldest subtype.

Geographically prevalent subtypes often display a high degree of sequence similarity, despite frequent population movements. This suggests that KSHV is transmitted in a familial fashion (between family members) and that multiple

infections and recombination events are uncommon. Other loci across the genome have been investigated to determine whether the clustering patterns as revealed by ORF K1 sequencing can be confirmed. Data from three major loci, ORF 26, T0.7/K12 and ORF 75 confirmed that a linkage exists across the genome with ORF K1 subtype patterns. In most instances, the four major ORF K1 subtypes can also be discriminated at these loci (Poole *et al.,* 1999). Nevertheless, in 20-30% of genomes studied, there is some evidence of recombination between internal loci. At the RHS of the genome, the divergent ORF K15 M allele is thought to be the result of a recombination event with an exogenous primate virus, followed by recombination events, first with subtype C viruses of the P form and then with A and B types (Poole *et al.,* 1999). Zong *et al.* (2002) demonstrated that 4 of 32 subtype A and 23 of 37 subtype C genomes studied had M alleles at their K14.1 and K15 loci. To date, only two cases of B subtype genomes have been found to carry M alleles, both of which were from patients in North America (Zong *et al.,* 2002).

The presence of chimeric KSHV DNA indicates that recombination and multiple infection occurred at some time in the evolutionary history of KSHV. These recombination events are unlikely to happen frequently and the presence of certain conserved chimeric combinations in populations (i.e. C6 type chimeras in Sephardi Jews or in B type American genomes) supports a slow rate of viral divergence.

1.5 Epidemiology of KSHV

1.5.1 Detection of KSHV infection

PCR based testing was initially developed to amplify KSHV DNA derived from its ORFs. However, only half of KS patients have detectable levels of KSHV DNA in peripheral-blood cells (Whitby *et al.,* 1995; Moore *et al.,* 1996d). Thus,

Table 1.2

Distribution of *ORF*-K1 variants

	A1	A1′	A2	A3	A4	A5	A6	A7	A8	A9	A10	C1	C2	C3	C3′	C4	C5	C6	D1	D2	B1	B2
Brazil (AKS)	1								1					1								
Scandinavia (CKS)				1									3	1								
Sicily (CKS)														3								
Israel (Ashkenazi)	2	7		1										1								
Israel (Sephardi)	1	2											5	3				4				
Saudi Arabia (RT)	1												4			1	1					
South Korea (CKS)													2		3							
Taiwan (Chinese)													1		7							
Taiwan (Hwalien)					1														2			
New Zealand		2																		2		
Africa (sub-Saharan)						8															12	3
USA classic KS (TX, TN, MD)				1			2	1			1		4	2								
USA AIDS KS (MD, NY, DC)	7		1		4							1		3							1	
USA AIDS KS (TX, TN, FL)		1		1	2		2					1		1							2	
USA PEL and MCD	1	1	1	1	2					1				3								
Totals (139)	13	13	2	5	9	8	4	1	1	1	1	2	19	18	11	1	1	4	5	2	15	3

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PCR based testing is not suitable for estimating prevalence of infection. For most immunocompetent patients who are infected with KSHV, clinical signs of KS are not present and DNA is even more difficult to detect in their PBMC.

Nested PCR increases the sensitivity of the PCR by 100 to 1000 times, compared to using the original $KS330_{233}$ primers alone (Moore *et al.*, 1996c). However, increased sensitivity of PCR can create problems with contamination, creating false PCR positive results and rendering sequence data unusable (Moore and Chang, 1998). Indeed, when a group reported detection of KSHV DNA in a large proportion of semen samples from healthy Italian blood donors (Monini *et al.*, 1996) this finding could not be corroborated by other groups and it was confirmed to be the result of PCR contamination (Howard *et al.*, 1997; Pellett *et al.*, 1999). However, PCR is needed to sequence any part of the KSHV genome. Important information regarding viral evolution and geographical genotype distribution has been obtained in this way (Zong *et al.*, 2002).

Serological assays have been developed to test for the presence of KSHV antibodies and to complement testing by PCR. Serological testing can give a better indication of KSHV infection in population groups. The sensitivity and specificity of serological testing vary depending on which assay is used. First generation assays were developed using KSHV infected PEL cell lines as sources of antigen in either an immunofluoresence assay (IFA) or in immunoblotting assay format (IA) (Lennette *et al.*, 1996; Gao *et al.*, 1996a; Moore *et al.*, 1996d). PEL cell lines express latent antigens the most prominent of which under normal culture conditions is LNA. Treatment of PEL cells with TPA induces lytic antigens to be expressed on the cell surface in a subset of PEL cells. Assays using lytic phase PEL cells are the most sensitive (Lennette *et al.*, 1996) and are widely used. However, they may suffer from problems associated with cross reactivity to other herpesvirus antibodies, particularly as no KSHV negative PEL cell lines are available to act as controls for non-specific reactions. Recent assays using recombinant KSHV antigen expressed be Semliki forest virus allow high levels of antigen to be expressed and appropriate negative controls to be included. This approach is reported to enhance both the

specificity and sensitivity of the IFA assay (Inoue *et al.,* 2000). In the assay developed by Inoue *et al.* (2000), the ORF K8.1 gene product was used as antigen, and as it is has no homologue to other herpesviruses, cross-reactivity does not occur.

To facilitate high throughput testing, enzyme-linked immunosorbent assays (ELISA) have been developed. Earlier assays used purified whole virion as antigen and have been made commercially available (Chatlynne *et al.,* 1998). To increase sensitivity and specificity, other ELISAs have been developed using, as antigens, recombinant proteins (Andre *et al.,* 1997; Davis *et al.,* 1997; Pau *et al.,* 1998) and oligopeptides. Antigens commonly utilised in this type of serological assay include products from ORF 65 (Pau *et al.,* 1998), ORF 73 (Gao *et al.,* 1996a) and K8.1.

Comparisons of PEL based IFA and ELISA tests have indicated that KSHV is readily detectable in classical and AIDS related cases of KS as well as in HIV-seropositive individuals, but with less frequency in healthy blood donors (Rabkin *et al.,* 1998). However, disagreement between assays is commonplace, particularly in the low seroprevalence blood donor group. Currently available assays have limited function in diagnosing individual cases of suspected KSHV infection in persons at low risk for KS, but are appropriate for use in epidemiological studies of KS and KSHV infection, particularly in endemic regions.

1.5.2 Seroprevalence

Serological assays show that KSHV seroprevalence is low among the general populations of Europe and North America, reflecting the prevalence of KS in these countries. In the United States and parts of Northern Europe, the prevalence of infection ranges from 0% to 5% among blood donors (Simpson *et al.,* 1996; Gao *et al.,* 1996a). An even lower percentage of the population is infected in countries such as Japan, where KSHV seroprevalence among blood donors is only 0.2% (Fujii *et al.,* 1999). Mediterranean and Middle Eastern countries have higher KSHV seroprevalences; studies in Italy report

approximately 10% KSHV seropositivity in blood donor or community groups (Gao *et al.,* 1996a; Perna *et al.,* 2000). Both the incidence of KS and KSHV seroprevalence vary geographically in Italy, being greater in southern areas of the country (Geddes *et al.,* 1995; Whitby *et al.,* 1998). KSHV seroprevalence is higher in Israeli Jews (9.9%) than in European and North American blood donors (Davidovici *et al.,* 2001). The incidence of classic KS is the highest of any developed country in Israel, more than 40 times that of the reported incidence in countries of Western Europe (Iscovich *et al.,* 1998). Important predictors for positive KSHV serostatus in Israel include the presence of infected family members (e.g. spouse, sibling or parent) and place of birth, the greatest number of seropositive individuals originating from North Africa (Davidovici *et al.,* 2001).

In African populations where the incidence of KS was high prior to the AIDS epidemic (de-The *et al.,* 1999), KSHV seroprevalence levels of 50% or more are commonly reported (Simpson *et al.,* 1996; Gao *et al.,* 1996a; Sitas *et al.,* 1999a; Rezza *et al.,* 2000). A study of black cancer patients in South Africa found that KSHV infection was associated with KS only and not other types of cancers (Sitas *et al.,* 1999a). Sitas *et al.* (1999a) also reported a rise in KSHV seroprevalence among children with increasing age, and a decrease in those adults with a higher level of education. KS has now been observed in young children from Uganda (Ziegler and Katongole-Mbidde, 1996). Prior to the AIDS epidemic, KS in children was rare in this region but is now increasingly common (Bouquety *et al.,* 1989; Athale *et al.,* 1995). Other studies have shown that KSHV seroprevalence in Africa steadily increases with age from early childhood (Mayama *et al.,* 1998; Gessain *et al.,* 1999; Rezza *et al.,* 2000) indicating that transmission of KSHV is likely to occur through familial routes.

Certain groups at increased risk for HIV infection and who have a greater incidence of KS also show a higher KSHV seroprevalence. In Europe and North America, HIV-positive homosexual men have the highest seroprevalence of any risk group. A study of men in the San Francisco area, from samples dating back to 1984, reported a seroprevalence of 47.7% among homosexual or bisexual men

who were HIV-positive but did not have KS (Martin *et al.,* 1998). A similar study conducted in Amsterdam between 1984 and 1996 reported a baseline KSHV incidence of 29.8% among homosexual men who were also HIV-positive but had not developed KS (Dukers *et al.,* 2000). Similar KSHV seroprevalence values for HIV-1-positive homosexual men (roughly 30%) have been described elsewhere in North America and Western Europe (Simpson *et al.,* 1996; Kedes *et al.,* 1996; Gao *et al.,* 1996b).

HIV-1-negative homosexual men consistently show a lower seroprevalence of KSHV infection than HIV-1-positive homosexual men, (17.3%-12%) (Kedes *et al.,* 1996; Martin *et al.,* 1998; Melbye *et al.,* 1998). However, these levels of seropositivity are higher than what is observed among blood donors in Europe and North America. Persons attending sexually transmitted disease clinics, including heterosexual men and women, show slightly increased seroprevalence when compared to the general population (Melbye *et al.,* 1998; Whitby *et al.,* 1999) suggesting a sexual route of KSHV transmission in the heterosexual population.

1.6 Transmission

1.6.1 Sexual transmission of KSHV

Seroprevalence studies, as mentioned above, indicated a link between sexual behaviour and KSHV seropositivity in Europe and North America. Martin *et al.* (1998) collected data regarding sexual practices from men participating in the San Francisco Men's Health Study. As mentioned above, the prevalence of KSHV infection was found to be higher in homosexual men, both with and without pre-existing HIV infection (Martin *et al.,* 1998) than in the general population. This increased seroprevalence correlates with the degree of homosexual activity reported by the participants in the past five years. The association between number of sexual partners and KSHV infection was supported in a study of young homosexual men recruited from the San Francisco Young Men's Health Study. In this study an increased number of sexual

partners was positively correlated with an increased risk for KSHV infection (Blackbourn *et al.,* 1999).

Certain sexual practices are associated with this increased risk for KSHV infection. Data from the Amsterdam Cohort study demonstrated that an increased risk of infection was associated with orogenital insertive and receptive sex. The study found that oral sex among homosexual men was a more important predictor of KSHV infection than the number of sexual partners (Dukers *et al.,* 2000). KSHV DNA has been detected in prostate tissue biopsies of HIV- positive men without KS (Staskus *et al.,* 1997; Diamond *et al.,* 1998) indicating that KSHV can be shed from the prostate into the semen. As was discussed in Section 1.5.1, a high KSHV DNA detection rate was reported in the semen samples of healthy donors (Monini *et al.,* 1996). However, subsequent studies reported only rare positive samples in the semen of patients with KS, and almost never in healthy semen donors (Boshoff and Weiss, 2001). Transmission of KSHV through semen therefore seems unlikely, and as Dukers *et al.* (2000) reported, oral sex may be the most significant practice associated with KSHV infection. Reports of KSHV detection in the saliva of HIV-positive individuals have been made (Koelle *et al.,* 1997). Thus, saliva may be a possible vehicle of transmission among gay men. However the exact mode of such transmission is not yet fully known.

1.6.2 Non-sexual transmission

While some correlation between number of sexual partners and KSHV infection has been found in African studies (Sitas *et al.,* 1999a), the presence of KSHV infection in children is indicative of a significant non-sexual route of transmission. Gessain *et al.* (1999) found that in Cameroon, KSHV infection was common among children. Of the 258 children and adolescents studied in this region, the overall seroprevalence by lytic and latent antibody IFA testing was 27.5%. Infants who received maternal IgG at birth reached a seroprevalence of 46% followed by a sharp drop after the fifth month of life. From the age of 4 years, seroprevalence steadily increased to 39% among 12-14

year olds and then to 48% in children over 15 years of age, approaching the level of KSHV infection reported in adults.

A Zambian study discovered that the pattern of KSHV infection was different from that of sexually transmitted HIV infection. During 1985 in Zambia, HIV infection was greatest in persons aged 20-29 years and was not observed in any person over 50 years of age. In contrast, KSHV infection was already high (47%) in adolescents between the ages of 14-19 and increased with age (Olsen *et al.*, 1998). This pattern of KSHV infection indicates non-sexual transmission with continued, possibly sexual, transmission occurring throughout adulthood.

Gessain *et al.* (1999) found that vertical transmission between mother and child was rare in Cameroon, and Calabro *et al.* (2000) reported that all infants born to KSHV-seropositive mothers who tested positive for KSHV antibodies at three months were negative by 24 months. Therefore, it is likely that children acquire infection through casual contact in the home and community rather than via vertical transmission events (Bourboulia *et al.*, 1998; Plancoulaine *et al.*, 2000).

Plancoulaine *et al.* (2001) speculated that horizontal transmission between mother to child and siblings occurs frequently in endemic populations. The study, based on a population of villagers of African origin in French Guiana, found high correlation in KSHV seroprevalence between mother and child and between siblings. No significant correlation in KSHV serostatus between spouses was identified. In South Africa, transmission between mothers and their children occur at a rate of 30% or more, and the probability of KSHV transmission is increased in mothers with a high KSHV antibody titre (Sitas *et al.*, 1999b). In the South African population, very few children of KSHV seronegative mothers studied by Sitas *et al.*, (1999b) under the age of 10 years were KSHV antibody positive, indicating that transmission is unlikely to occur from a source other than the mother.

In Mediterranean and Middle Eastern countries, evidence of KSHV infection in children also supports non-sexual transmission although the exact route of

transmission has not been known as precisely as for equatorial and Southern Africa. In Egyptian children, KSHV seroprevalence is as high as 44.7% in anti-lytic antibody tests (Andreoni *et al.,* 1999). In this study, KSHV seroprevalence increased steadily up to 10 years of age and then stabilised thereafter. There is now evidence demonstrating that KSHV primary infection may be associated with a febrile illness and skin rash in Egyptian children (Andreoni *et al.,* 2002). KSHV infection in Egyptian children has been positively associated with close contact with at least two other children in the community, pointing to saliva as an important route of paediatric infection (Andreoni *et al.,* 2002)

As discussed previously, a high incidence of KS and KSHV is found in Israeli adults. Moreover, a substantial proportion of children in Israel are infected with KSHV and seroprevalence rises with age. The most important risk factor associated with KSHV infection in children is having a KSHV seropositive mother (Davidovici *et al.,* 2001), a situation similar to that of Africa. Furthermore, Davidovici *et al.* (2001) found that KSHV infection in children of a seropositive mother was influenced by the antibody titre of the mother, but was unaffected by the presence of a KSHV infected father.

In Italian studies, 4.4% of Italian children tested were carrying KSHV antibodies, with no significant variation noted between children living in the North and South of Italy (Whitby *et al.,* 2000). Clustering of KSHV seroprevalence between spouses, children, and siblings was demonstrated in a Sardinian cohort, without an apparent mother-child predominance, indicating that non-sexual horizontal transmission of KSHV occurs broadly in the family (Angeloni *et al.,* 1998).

KSHV DNA has been detected in the saliva of HIV-infected/KS-positive, HIV infected/KS-negative, and HIV-uninfected/KS-positive persons (Koelle *et al.,* 1997). KSHV shedding into the oral cavity and in nasal secretions has been demonstrated (Blackbourn *et al.,* 1998) and the KSHV virus particles isolated from saliva are infectious (Koelle *et al.,* 1997). Viral DNA isolated from saliva was similar to DNA from virions and cell free saliva fractions from infected

individuals could infect 293 cells (Vieira *et al.*, 1997). While it possible that salivary shedding may be important in facilitating both sexual and non-sexual transmission of KHSV, it is unclear where in the mouth KSHV replicates. It is significant that Pauk *et al.* (2000) localised, in mucosal samples obtained from homosexual men with no apparent KS disease, KSHV DNA and RNA most frequently in the epithelial cells of the buccal mucosa and less frequently in samples from the genital tract. It is also significant that KSHV DNA was detected at higher titre in saliva than in other types of samples tested, and some men shed virus at consistently high titres despite showing no signs of clinical KS disease.

1.6.3 Organ transplantation

Organ transplantation poses a minor threat for KSHV infection. Small numbers of organ donation recipients develop KS in areas of low seroprevalence, increasing in areas of higher KS incidence. In most cases, transplant patients are infected with KSHV prior to transplantation (Parravicini *et al.*, 1997b) and KS develops as a result of viral reactivation due to drug induced immunosuppression. Although less common, allograft transmission of KSHV can also take place (Regamey *et al.*, 1999).

1.7 Phylogenetic analysis

Phylogenetic analysis has been used for many years to study all types of evolutionary relationships. In early studies, phylogenetic trees were constructed based on gene frequency data and morphological characteristics (Weiller *et al.*, 1995). Since the introduction of PCR amplification and sequencing, a large amount of DNA sequence data has become available. Phylogenetic analysis of amino acid or nucleotide sequence data can be used to track the spread of pathogens in populations, to infer evolutionary relationships in viruses and bacteria, and to facilitate typing.

It is important to note that a phylogenetic tree is only an estimate of the actual genetic relationship between sequences, and the quality of the tree depends on

the quality of the data entered. In order to produce a tree that most accurately estimates the true behaviour of sequences, homologous regions of similar length with common ends should be selected. Sequences of sufficient length and variability should also be included to ensure enough information is provided for a meaningful analysis.

Making an alignment of the nucleotide or amino acid sequences to be studied is the first step in any phylogenetic analysis. Algorithms to make an alignment of two sequences were first described in 1970 (Altschul *et al.,* 1997) but multiple alignments were not yet possible. Today, many algorithms are available to make multiple sequence alignments by individual pairwise comparisons. The Clustel W program (Thompson *et al.,* 1994) is one of the most commonly used multiple alignment programs. The alignment is built by way of progressive pairwise alignments where pairs of closely related sequences are compared to other such pairs until an optimal alignment is created. Clustel W introduces gap penalties to allow for insertions and deletions in the input sequences.

The three most commonly used methods for constructing a phylogenetic tree from a multiple alignment are briefly reviewed here:

i) Distance methods

The multiple alignment (nucleotide or amino acid) is first used to create a genetic distance matrix by pairwise comparisons of input sequences. The distance matrix is based on the number of nucleotide or amino acid substitutions between paired sequences and can be calculated using a number of algorithms. All algorithms have different features. Jukes-Cantor (JC) assumes that all substitutions are equally likely to occur and that base frequencies are equal (Jukes and Cantor, 1969). The commonly used Kimura-2 parameter algorithm takes into consideration transition and transversions ratios while assuming equal base frequencies (Kimura, 1980). Other distances methods assume unequal base frequencies and different rates of substitutions (Felsenstein, 1981; Hasegawa *et al.,* 1985; Li *et al.,* 1996).

The distance matrix is next used to create a phylogenetic tree by applying one of a number of clustering algorithms. The UPGMA (unweighted pair group method with arithmetic means) is a simple method used to estimate the phylogenetic tree from a distance matrix (Sneath and Sokal, 1973). In this method, sequences with the smallest distances are clustered and treated as one unit. They are then compared to all other sequences in the matrix, and the process is repeated until all sequences have been clustered. The Fitch and Margoliash method (Fitch and Margoliash, 1967) is based on the UPGMA clustering method but considers the standard deviation between the expected distance values in the matrix and the actual branch lengths. The tree with the smallest standard deviation value is chosen as the best tree. The commonly used neighbor-joining (NJ) method finds pairs of sequences or "neighbours" connected at a node and arranges them on a star shaped tree (Saitou and Nei, 1987). Branch lengths are minimised at each addition to create the most phylogenetically correct tree.

ii) Maximum parsimony

The maximum parsimony method was first described by Camin and Sokal in 1965and has since been developed to study nucleotide sequences (Williams and Fitch, 1990). In this method each tree topology is examined to find the minimum number of mutations necessary to explain each branch length. The tree with the least changes is accepted as the most parsimonious. Only sites that are phylogenetically informative are analysed. Standard maximum parsimony methods weigh all changes equally, do not assume that changes at conserved loci are more significant and do not weigh transitions and transversions differently. Algorithms allowing dynamic weighting of input sequences have been developed (Williams and Fitch, 1990).

iii) Maximum likelihood

This type of algorithm calculates the possibility that a nucleotide or amino acid occurs at a particular site in the alignment of all possible trees (Felsenstein,

1981). A log likelihood value is calculated for all possible trees and is used to select the most likely tree. While this method creates a tree that is statistically comparable to other trees, it requires a vast amount of computing power when large data sets are analysed.

Most of these algorithms can produce trees that have a true topology. However, certain errors can produce misleading or meaningless trees. Certain assumptions made by algorithms are not always reflected in the true data set. For example, the assumption that all sites are independent is often false when the structure of the nucleotide requires complementary segments to be present in the sequence. In addition, different types of mutations, i.e. transitions and transversions, can occur at unequal rates providing different phylogenetic information. Weighting options available in phylogenetic programs should be used to avoid these pitfalls. Statistical analysis of phylogenetic trees using bootstrapping or jack-knifing resampling methods can determine the robustness of tree (Felsenstein, 1988).

1.8 Aims of this study

The broad aim of this study was to investigate the transmission of KSHV in Malawian families using molecular epidemiological tools and to investigate intra-individual KSHV genomic variability.

1.8.1 Molecular epidemiology of KSHV transmission

It was hypothesised that KSHV transmission patterns in endemic countries may be more complex than what is being suggested by seroepidemiologic studies. Two regions of the hypervariable KSHV ORF K1 were amplified and sequenced from individuals with KS and their family members. The relatedness of ORF K1 sequences was compared between family members.

To complement the sequence based studies, a PCR based restriction fragment length polymorphism (RFLP) technique, which detects sequence differences in

the KSHV ORF 73, was modified to investigate KSHV strain variations within families.

1.8.2 Intra-individual variability of KSHV

Few studies have reported intra-individual KSHV variability, and all have used PCR-sequencing to study small numbers of clones. A high-throughput denaturing gradient gel electrophoresis (DGGE) technique was developed to screen multiple clones derived from KSHV PCR products from single individuals for sequence polymorphism. This method potentially allowed many clones to be analysed at one time thereby permitting intra-individual KSHV variability to be identified.

Chapter 2

Materials and Methods

All samples described and tested in this study were collected by Dr Tim Hodgson at the Queen Elizabeth Hospital, Blantyre, Malawi during June 1999. Ethical approval was obtained prior to commencing this study from Eastman Dental Institute for Oral Health Care Sciences, UCL, University of London (UK) and the University of Malawi.

2.1 Amplification of KSHV DNA

2.1.1 Preparation of whole blood

Peripheral blood from study individuals was collected in EDTA treated vacutainers and stored, if necessary, for not more than two days at 4° C. Plasma was separated from blood by centrifugation at 1200 RPM for 5 min with 1 ml of plasma removed. The remaining blood was resuspended. To separate different leukocyte fractions from the blood, a technique using Dynabeads (Dynal A.D., Oslo, Norway) was utilised. Dynabeads are small magnetically charged uniformly shaped beads coated with a specific monoclonal antibody. For this study Dynabeads coated with antibodies against CD45 and CD31 were used. To 1 ml of whole blood, 50 µl of Dynabeads was added to give an approximate final concentration of $2x10^7$ beads/ml. The whole blood and Dynabead mixture was incubated at 4°C for 20 min. Dynabeads were collected using a magnetic particle concentrator (MPC) (Dynal), washed three times with 1ml of PBS containing 2% foetal calf serum, and eluted into 250 µl of nuclease free water.

2.1.2 Preparation of saliva samples

Study participants were asked to rinse with PBS and spit into a universal centrifuge tube. The saliva was spun at 1200 rpm for 5 min and the supernatant was separated. The supernatant and residual saliva were stored separately at -20°C until needed.

2.1.3 DNA extraction from blood

Dynabead preparations were extracted using the Geneclean III DNA extraction kit (BIO 101, La Jolla, CA). Sodium iodide was added to the sample in the first step to lyse the cells and to inactivate any nuclease activity. A suspension of

silica particles (EZ-glassmilk) was introduced and with mechanical agitation, the DNA became bound to the silica particles. The DNA was eluted in 50 µl of nuclease free water.

2.1.4 DNA extraction from saliva

A 150 µl aliquot of the pellet portion of the mouth rinse sample was extracted using a guanidinium thiocyanate-silica method (Boom *et al.,* 1990). Buffers L6, L2 and the silica suspensions used in this method were purchased from Severn Biotech Ltd. (Kidderminster, UK). Samples were eluted in 50 µl of nuclease free water.

2.1.5 ORF 26 and ORF K1 PCR conditions

The primary PCR for KSHV ORF 26 was carried out in a 25 µl reaction mixture containing nuclease free water, 1X PCR buffer, 4.0 mM $MgCl_2$, 10 mM each dNTP, 1 unit Taq DNA polymerase (Invitrogen, Paisley, UK), 20 pmol each first round primer (sequence shown in Table 2.1) and 2 µl extracted DNA. The secondary PCR mix was identical except 20 pmol of each inner primer was used and 2 µl of the first round product was added as template.

First round PCR amplification of the first variable region of KSHV ORF K1 (K1/V1) was carried out in a 50 µl reaction mixture containing nuclease free water, 1X buffer number 8 (Stratagene Europe, Amsterdam, The Netherlands) containing 3.2 mM $MgCl_2$, 20 pmol each outer primer, 10 mM each dNTP and 1 unit Taq DNA polymerase (Invitrogen). To this mixture 5 µl of extracted DNA was added. The second round conditions were the same as the first with the addition of 20 pmol of each inner primer and 2 µl of the primary product as template.

Amplification of both regions was carried out under the same PCR thermocycling conditions. Samples were heated to 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min followed by a 5 min extension period at 72°C.

Primer sequences for ORF 26 and ORF K1 are shown in Table 2.1. The ORF K1 outer primers produced a 255 bp fragment from positions 568 to 823 in the BCBL-1 K1 sequence (GenBank accession number U86667). The inner primers produced a 247 bp fragment from positions 573 to 820. Outer ORF 26 primers produced a product 233 bp in length from position 355 to 588 and the inner primers yielded a 211 bp product from position 366 to 577. Nucleotide positions for the ORF 26 fragment are numbered according to the sequence deposited in GenBank under accession number U75698.

A selection of samples positive by K1/V1 PCR were amplified by hemi-nested PCR for the second variable region of KSHV ORF K1 (K1/V2). The primer sequences for K1/V2 inner and outer primers are shown in Table 2.1. First round PCR took place in a 50 µl reaction mixture containing nuclease free water, 1X buffer number 8 (Stratagene) containing 3.2 mM $MgCl_2$, 20 pmol each outer primer, 10 mM each dNTP, 1 unit *Taq* DNA polymerase (Invitrogen) and 5 µl extracted DNA. Second round K1/V2 PCR was carried out in a 50 µl reaction mixture of nuclease free water, 1X buffer number 7 (Stratagene) containing 3.5 mM $MgCl_2$, 20 pmol each inner primer, 10 mM each dNTP, 1 unit *Taq* DNA polymerase (Invitrogen) and 2 µl of first round PCR product. First round amplification was carried out by heating to 94° C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final extension period at 72°C for 5 min. Second round amplification conditions were similar to the first round conditions; however, the annealing temperature for each cycle was increased to 52°C for 1 min. The outer primers amplified the majority of the coding region of ORF K1 from positions 402 to 1466 to give a product of 1064 bp in length. The inner primers amplified the K1/V2 region from positions 891 to 1466 to give a product of 575 bp in length. All positions are numbered according to the BCBL-1 K1 sequence deposited in GenBank under accession number U86667.

2.1.6 ORF 73 and ORF K8.1 PCR conditions

Both primary and secondary PCR mixes for amplification of ORF K8.1 and ORF 73 were identical to those described for ORF K1 PCR (Section 2.1.5).

2.1.6 ORF 73 and ORF K8.1 PCR conditions

Both primary and secondary PCR mixes for amplification of ORF K8.1 and ORF 73 were identical to those described for ORF K1 PCR (Section 2.1.5). Amplification was carried out for ORF K8.1 and ORF 73 PCR using the same thermocycling conditions as described for ORF K1 PCR in both the first and second rounds (Section 2.1.5). The primer sequences for these regions are shown in Table 2.2. The ORF K8.1 outer primers produced a fragment of 795 bp in size and the inner ORF K8.1 primers amplified a fragment of 537 bp, as previously described (Raab *et al.*, 1998). The outer ORF 73 primers produced a 584 bp fragment and the inner primers produced a 547 bp product, as previously described (Dittmer *et al.*, 1998).

Table 2.1 Oligonucleotide primers used for the amplification of KSHV ORF 26 and ORF K1 fragments

Region	Position	Name	Sequence
ORF 26	Outer-sense	KS 1[§]	5'AGCCGAAAGGATTCCACCAT
	Outer-antisense	KS 2[§]	5'TCCGTGTTGTCTACGTCCAG
	Inner-sense	Ksinn1	5'TTCCACCATTGTGCTCGAAT
	Inner-antisense	Ksinn2	5'TACGTCCAGACGATATGTGC
ORF K1/V1	Outer-sense	K1inn5	5'CCCTGGAGTGATTTCAACGC
	Outer-antisense	K1inn6	5'ACATGCTGACCACAAGTGAC
	Inner-sense	K1-1	5'GAGTGATTTCAACGCCTTAC
	Inner-antisense	K1-N	5'TGCTGACCACAAGTGACTGT
ORF K1/V2	Outer-sense	LGH 2089*	5'GTTCTGCCAGGCATAGTC
	Inner-sense	K1/408/1[‡]	5'CCGTGTCACAAACTAAATAC
	Outer/Inner-antisense	LGH 2089*	5'AATAAGTATCCGACCTCAT

*(Cook *et al.*, 1999)

[‡] (Zong *et al.*, 1999)

[§] (Chang *et al.*, 1994)

Table 2.2 Oligonucleotide primers used for the amplification of KSHV ORF 73 and ORF K8.1 fragments

Region	Position	Name	Sequence
ORF 73	Outer-sense	7308*	5'GCATTCCCGGGGGCGCCATC
	Outer-antisense	7-out	5'AGCAGTCACGTCCCCAAGAG
	Inner-sense	7311*	5'TCCTCGGGAAATCTGGTCT
	Inner-antisense	7*	5'AGCAGCAGCTTGGTCCGGCTG
ORF K8.1	Outer-sense	K8.1-B1‡	5'TAACCATGAGTTCCACACAGATTC
	Outer-antisense	K8.1-X1‡	5'GGTTTTGTGTTACACTATGTAGG
	Inner-sense	K8.1-MBAM‡	5'AATTGTCCCACGTATCGTTC
	Inner-antisense	K8.1-HINDR‡	5'TGGGACACGGTTACTAGCACC

* (Dittmer *et al.*, 1998)

‡ (Raab *et al.*, 1998)

2.1.7 Visualisation of PCR products

PCR products were mixed with 2 µl Orange G loading dye and loaded onto a 2% agarose gel (SB fine gel, Severn Biotech Ltd.) along with 1 µg of 1 kb DNA ladder (Invitrogen) to estimate the size of the PCR product. Electrophoresis took place in a 1X tris-borate-EDTA buffer (TBE supplied as 10X stock, Invitrogen) after which the gel was stained in a solution of 5 µg/ml ethidium bromide in TBE buffer. The bands were visualised using short wave UV transillumination.

2.2 Amplification of EBV ORFs

2.2.1 Preparation of saliva samples

Samples were prepared as described in Section 2.1.2.

2.2.2 Extraction of DNA from mouth rinse samples

DNA was extracted from mouth rinse samples as described in Section 2.1.4

2.2.3 EBV PCR conditions

The EBV *Bam*HI K and EBV *Bam*HI N regions were amplified (Triantos *et al.*, 1998) using the following PCR conditions: each outer primer at 20 pmol, 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase (Invitrogen), each dNTP at 10 mM, 5 µl template DNA and nuclease free water to make a total reaction volume of 50 µl. For the second round of PCR, 2 µl of first round product was added to 48 µl of second round reaction mix that contained either the inner primers for *Bam*HI K or the outer sense and hemi-nested anti-sense primer for *Bam*HI N. Primer sequences and their relative positions in the genome of the EBV infected cell line, B958, are shown in Table 2.3.

Table 2.3 Oligonucleotide primers for the amplification of EBV DNA fragments

Primer	**Nucleotide Sequence**	**5' end***
*Bam*HI K region		
Outer, sense	TGATGGAGGCAGGCGCAAAAAAG	109311
Outer, anti-sense	GAAACCAGGGAGGCAAATCTACT	109780
Inner, sense	CGCAAAAAAGGAGGGTGGTTT	109324
Inner, anti-sense	CATCGTCAAAGCTGCACACAG	109756
*Bam*HI N region		
Sense	GAGAAGGAGAGCAAGGCCTA	169382
Anti-sense	CGCACTGCCTTTCCATTTCC	169779
Hemi-nested	AAGGCCTAGGGAAGAGGAGA	169394

2.2.4 Visualisation of PCR products

PCR products were visualised as described in Section 2.1.6.

2.3 DNA sequencing

2.3.1 Automated sequencing

All samples were sequenced using the Beckman CEQ2000 automated capillary array sequencer (Beckman Coulter, California, USA). To prepare the samples for sequencing they were first run on a 2% agarose gel and excised. The excised bands were then purified using a spin column based purification kit (Amersham Pharmacia Biotech, Little Chalfont, UK) following the manufacturer's instructions. To estimate how much DNA to add to the sequencing reaction, 2 µl of purified product was electrophoresed through an agarose gel along with 1 µg of 1 kb ladder (Invitrogen). Purified DNA was added to the PCR sequencing reaction consisting of the following components: purified DNA X µl, sterile water X µl, inner PCR primers (3pmol) 2 µl, and Beckman CS sequencing kit mix (Beckman) 12 µl to a total volume of 20 µl.

Sequencing PCR reactions were purified by ethanol precipitation. The 20 µl sequencing reaction was added to 5 µl of "stop solution" which consisted of sodium acetate (Sigma-Aldrich), 0.5M EDTA (Sigma-Aldrich) and glycogen (Beckman) in a ratio of 2:2:1 respectively. To this, 60 µl of ice-cold 95% molecular grade ethanol (Sigma-Aldrich) was added and the samples were spun at 13,000 rpm at 4°C for 25 min to precipitate the DNA. The DNA pellet was washed twice with 200 µl of ice-cold 70% molecular grade ethanol (Sigma-Aldrich) and vacuum dried for 10 min. DNA pellets were resuspended in 35 µl of deionized formamide and frozen at -20°C until they were loaded onto the automated sequencer.

The Beckman CEQ2000 uses a capillary system to electrophorese the sample through a polyacrylamide gel contained inside the capillary. In the sequencing PCR mix, chain terminating nucleotide bases labelled with different fluorescent tags are present and are incorporated into the DNA during PCR amplification. During electrophoresis a laser reads these fluorescent bases to determine the sequence of the sample.

2.3.2 Analysis of sequence data

Raw chromatograph data were analysed using SeqMan sequence analysis software and multiple alignments were made in Megalign, both programs from the LASARGENE sequence analysis package (DNAstar Inc., Madison, WI, USA). A Clustel V alignment was produced in Megaline to construct a guide tree using the UPGMA method of clustering analysis (Section 1.8). Genetic distances were expressed as a percent nucleotide divergence over the entire tree.

Further analysis was performed after all sequence data had been collected. Multiple alignments were made in Clustel W (Thompson *et al.,* 1994), which is part of the BioEdit software package (Hall, 1999). Pairwise alignments were made between nucleotide sequences to produce a guide tree estimating the final phylogenetic tree. Clustel W alignments were entered into the PHYLIP suite of programs (Felsenstein, 1993) where further analysis took place. The following programs were used to analyse sequence data in PHYLIP: SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE.

Alignments were first analysed using SEQBOOT to create 100 to 1000 multiple data sets, resampled from the input data set using random sampling methods with replacement. Trees generated in SEQBOOT were entered into the CONSENSE program and the branching patterns that occurred most frequently were reflected on the consensus tree. Values at each node indicate the percentage of trees containing each branching pattern and could be used to interpret the robustness of the resulting tree.

DNADIST creates a DNA matrix using one of three possible models of substitution; Jukes and Cantor (Jukes and Cantor, 1969), Kimura (Kimura, 1980) and a maximum likelihood model (Felsenstein, 1981). The Kimura "2-parameter" method is used most often and assumes that independent changes occur at all sites with equal probability and allows for differences in transition and transversions rates. The output format of DNADIST is a matrix where genetic distances between sequences are roughly equivalent to percent nucleotide divergence values created using Megalign. The genetic distance

values between species can be used in other programs to estimate branch lengths and draw trees. The Kimura "2-parameter" method was used to create distance matrices from sequence data in this study.

Trees were drawn using the NEIGHBOR program, which utilises either the neighbour-joining algorithm (Saitou and Nei, 1987) or the UPGMA method of clustering (Sneath and Sokal, 1973). NEIGHBOR creates successive clusters of closely related sequences and minimises branch lengths as the lineages join. NEIGHBOR does not assume an evolutionary clock and the resulting tree is unrooted. When the SEQBOOT programme is used, multiple trees are drawn from the NEIGHBOR program. CONSENSE then takes all these trees and creates a consensus tree to be viewed.

2.4 Cloning

Secondary ORF K1 PCR products were cloned prior to automated DNA sequencing using the TOPO TA cloning kit (Invitrogen). This cloning step was found to achieve better quality sequencing results and allowed multiple clones from one sample to be compared, thereby ensuring sequence fidelity. During PCR, *Taq* polymerase will add a single adenosine (A) to 3' end of the PCR product through its non-template dependent terminal transferase activity (Marchuk *et al.,* 1991). The PCR4–TOPO vector is supplied linearised with the addition of a 3' thymidine (T) overhang to complement the 3' terminal A of the PCR product. Ligation of vector and PCR product occurs through the activity of topoisomerase I, covalently bound to the vector (Shuman, 1994). Chemically competent *E .coli* are used to transform the cloning reaction and are grown overnight on L agar (Luria agar: 1.0% tryptone, 5.0% yeast extract, 1.0% 0.17 M NaCl, 1.5% agar, pH 7.0) containing 50 mg/ml ampicillin. PCR using second round primers was performed directly on the resulting colonies prior to sequencing. Samples were prepared for sequencing as previously described (Section 2.3.1)

2.5 KSHV and HIV-1/2 serology

Serum samples prepared as previously described (Section 2.1.1) were diluted 1:20 in nuclease free water and applied to the IgG IFA. (Advanced Biotechnologies Incorporated, USA) according to the manufacturer's instructions. IFA slides were read independently by two people using fluorescence microscopy and samples were assigned a rating based on the intensity of signal, ranging from 0 (neg) to 4+ (strong positive). HIV-1- and HIV-2-serostatus was determined from serum samples using an IgG capture particle adherence test (GACPAT) (Parry *et al.*, 1995).

2.6 Denaturing gradient gel electrophoresis (DGGE) screening

2.6.1 GC-clamp colony PCR

Using TOPO TA cloning as described in Section 2.4, 35-40 clones were generated for each PCR product amplified as described in Section 2.1.5. For KSHV-infected cell line controls, K1/V1 was amplified using the EXPAND system (Roche Diagnostics Ltd., Lewes, UK). The primary PCR mix contained 1X EXPAND buffer (which included 1.5 mM $MgCl_2$), 10 mM each dNTP, 0.7 units EXPAND high fidelity polymerase mix (Roche Diagnostics Ltd.), 20 pmoles each outer primer (Table 1), 5 µl extracted DNA and nuclease free water to a final volume of 50 µl. The second round PCR was carried out under identical conditions except for the addition of 2 µl of first round product in the second round mix and 20 pmol each inner K1/V1 primer (Table 2.1). PCR products generated from cell lines were cloned as previously described (Section 2.4). Colony PCR was carried out using bacterial colonies generated during TOPO TA cloning as template DNA and inner K1/V1 primers (Table 2.1). A special "clamped" primer was used in place of the inner sense primer. A 40-bp GC-clamp was attached to the 5' end of the sense primer giving a sequence as follows:

K1-1 clamp

5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGG

AGTGATTTCAACGC

The sequence for the inner sense primer is as described in Table 2.1. Colony PCR products were visualised on an agarose gel as described in Section 2.1.6.

2.6.2 Preparing denaturing gradient polyacrylamide gels

Stock solutions of each acrylamide mix were prepared beforehand and cooled to 4°C prior to use. For the K1/V1 PCR product, a gel gradient of 30% to 50% denaturants was necessary to achieve discrimination to a 1 base pair mutation. The 50% solution contained 10% acrylamide (National Diagnostics, Hull, UK), 0.6% TRIS-acetate-EDTA (TAE) buffer (Invitrogen), 20% formamide (BDH, Poole, UK) and 3.5 M urea (Sigma-Aldrich, Poole, UK). The 30% solution contained 10% acrylamide, 0.6X TAE, 12% formamide, and 2.1 M urea. Using a gravity driven gradient maker with pump drive (GRI, Braintree, UK), the gradient gel was poured using 30 ml of each solution. To allow the gel to polymerise 13 µl of N, N, N', N' -tetramethylethylenediamine (TEMED, Invitrogen) and 250 µl of 10% ammonium persulfate (APS, Sigma-Aldrich) per each 30 ml of stock solution were added prior to pouring. A 48 well comb was inserted and the gel was left to polymerise for at least 2 hr.

2.6.3 Loading and electrophoresis

Clamped colony PCR products (2 µl) generated as described in Section 2.5.1 were mixed with an equal volume of Orange G loading buffer. After all samples were loaded into the wells, electrophoresis in 0.6X TAE buffer at 60°C and 100 V took place over 18 hr. The electrophoresis apparatus allowed for continuous flow of buffer to maintain a constant temperature of 60°C throughout the run (Igeny PhorU, Goes, The Netherlands).

2.6.4 Staining of the gels

Gels were stained in a solution of 0.5X SYBR Green I (Flowgen, Ashby de la Zouch, UK) in 200 ml of 0.6X TAE buffer for 20 min to 1 hr. Bands were

visualised by ultraviolet transillumination and gel images were captured using a Kodak Digital Science DC40 camera.

2.7 Restriction fragment length polymorphism of PCR products (PCR-RFLP)

2.7.1 Amplification of ORF73 for RFLP PCR

The internal repeat domain (IRD) of ORF 73 was amplified from a selection of samples that were PCR positive for ORF K1 and/or ORF 26. The IRD of ORF 73 is a highly variable region and the size of the product varies between 1898 bp to 1350 bp in KSHV infected cell lines (Gao *et al.,* 1999). Either a single round PCR or nested PCR was performed depending on the quantity of single round product produced. The nucleotide sequences for first and second round primers are shown in Table 2.3. The PCR mixture for single round PCR contained 1.5 mM $MgCl_2$, 10 mM each dNTP, 1X PCRx enhancer solution (Invitrogen), 2 units platinum *Taq* DNA polymerase (Invitrogen), 50 pmol each inner primer, 2µl of extracted DNA and nuclease free water in a total reaction volume of 25µl. Amplification was carried out for 35 cycles of 94°C for 30s, 58°C for 30s and 68°C for 2 min, preceded by a 5 min denaturation step and ending with a final extension step of 5 min at 68°C.

For nested PCR, a primary PCR mixture was made to 25 µl total volume containing nuclease free water, 1.5 mM $MgCl_2$, 10 mM each dNTP, 2X PCRx enhancer solution (Invitrogen), 1.25 units platinum *Taq* DNA polymerase (Invitrogen), 50 pmol each outer primer, and 2 µl of extracted DNA. The second round PCR was carried out under the same conditions except for the addition of 2 µl primary product and 50 pmol each inner primer. Thermocycling conditions for both rounds of nested PCR were identical to those described for single round PCR. The second round of nested PCR produced the same product as the single round PCR.

Table 2.4 Oligonucleotide primers used for the amplification of the IRD of KSHV ORF 73.

Region	Position	Name	Primer Sequence
ORF 73 IRD	Outer-sense	IRD1-F	5'ACGCCAACCGCCTACATCT
	Outer-antisense	IRD1-R	5'TCATGTGTGCTAACAACAGG
	Inner-sense*	LNAII F	5'ATGGGGACAACGAGATTAGC
	Inner-antisense*	LNAII R	5'CGACCCGTGCAAGATTATG

*(Gao *et al.*, 1999)

2.7.2 Detection of PCR products

PCR products were visualised as described in 2.1.6 except samples were run on a 1% agarose gel (SB fine gel, Severn Biotech Ltd.) alongside 1 µg of a 1 kb ladder (Sigma-Aldrich).

2.7.3 Restriction digest of PCR products

Digests were carried out in a reaction mixture consisting of 5-7.9 µl of PCR product, 0.1 µl BSA, 1 µl Buffer C (Promega, Southampton, UK), 0.5 µl *Mbo*I and 0.5 µl *Ban*II (Promega) plus nuclease free water to 10 µl if necessary. Digests were heated to 37°C for 1 hr, the optimal temperature for enzyme activity, and at 85°C for 30 min to inactivate the enzymes. Digests were stored at 4°C until they could be visualised on a 2% agarose gel (SB fine gel, Severn Biotech Ltd.) alongside 1 µg of 100 bp ladder (Invitrogen) to estimate the size of resulting bands. Digital images of gels were captured using the Kodak Digital Science DC40 camera.

Chapter 3

PCR-sequencing analysis of KSHV transmission

3.1 Introduction

At the end of 2001, an estimated 40 million people globally were living with HIV. In sub-Saharan Africa alone a total 28.1 million people are now infected with HIV (UNAIDS, 2001). Malawi, in Southern Africa, has been hard hit by the HIV epidemic; an estimated 800,000 people are now infected with HIV including 40,000 children between 0-14 years of age. HIV seroprevalence in urban antenatal clinics has increased from 2% in 1985 to 30% in 1993, and in rural clinics from 6% in 1992 to 18% in 1998. More than 50% of STD clinic attendees in urban areas between 1989 and 1996 were infected with HIV (UNAIDS, 2000).

The HIV epidemic has impacted the incidence of certain cancers in Malawi including Kaposi's sarcoma. A cancer incidence survey in Malawi conducted between 1976 and 1980 reported a high male to female sex ratio (8.5:1) and age profile for KS similar to that observed in Malawi in the 1970s (O'Connell, 1977) indicating that HIV had then not yet influenced the epidemiology of KS. A more recent cancer survey was undertaken at the Queen Elizabeth Central Hospital (QECH), Blantyre, Malawi (Banda *et al.,* 2001). The QECH is the only facility providing cancer care and histological diagnosis of cancers in the country and is part of a network linked to smaller urban and rural health clinics. Among cancer cases reported between 1994 and 1998, KS accounted for 54% of all cancers in men and 27% of cancers in women. Although less common in children, KS was reported in 19 cases of childhood cancer. The sex ratio for KS incidence is approaching unity (2.5:1) and the average age of KS cases (34 yr in men and 28 yr in women) reflects the average age of HIV infection in sub-Saharan Africa and among women in urban Malawi (Taha *et al.,* 1998).

There are limited data supporting sexual transmission of KS in Africa (Sitas *et al.,* 1999a) and in some studies no link between sexual activity and KS has been found (Wawer *et al.,* 2001). Children were rarely diagnosed with KS prior to the HIV epidemic (Olweny *et al.,* 1976). However, a recent study involving 100 cases of KS in Ugandan children under 15 years of age reported a 40-fold

increase following the introduction of HIV (Ziegler and Katongole-Mbidde, 1996). Of 63 reported cases in this study, 78% were infected with HIV. As discussed in Chapter 1, KSHV seroprevalence in African children is high and increases with age (Olsen *et al.,* 1998; Mayama *et al.,* 1998). Non-sexual transmission of KSHV between family members has been postulated (Bourboulia *et al.,* 1998; Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000) and the predominant role of intra-familial infection strongly suggested.

Transmission studies undertaken thus far have relied on results from serological assays to seek correlations between the KSHV serostatus of parent and child, or between siblings. Molecular epidemiology has not yet been applied to study familial KSHV transmission. This chapter describes such an attempt. Two genomic regions of KSHV ORF K1, VR1 and VR2, were amplified and sequenced in family groups. The resulting sequences were compared between members of the same family to discover whether or not identical viruses were being transmitted in each family. The ORF K1 region of the KSHV genome was selected because of its highly variable nature (Russo *et al.,* 1996; Zong *et al.,* 1999) and the wealth of sequencing and genotyping data already available for it in the literature and nucleotide sequence databases.

3.2 Study group

Patients attending the Central Hospital, Blantyre, Malawi with presumptive cutaneous, oral and nodal KS were invited to join the study. All cases of patients with nodal KS were confirmed by histology. At presentation, all patients were offered palliative treatment with intravenous vincristine ($2mg/m^2$). Regardless of whether treatment was administered, patients were asked to return to the clinic within one week with all available members of their household. At the return visit, informed consent was obtained from all adults and from the parents of children involved in the study to collect body fluid samples, including venous blood and mouth rinses. KSHV antibody status was determined by an ABI IFA (Section 2.5). This IFA uses the KSHV infected cell line, KS-1, as a source of KSHV antigen and is specific and sensitive. Previous studies have

proven that the IFA is appropriate for use in epidemiological studies (Chatlynne *et al.,* 1998) and has been applied to serological studies in similar African populations (Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000). All KSHV-seropositive KS patients were included in the study as index cases. The original study group contained two patients, D_i and L_i, who were later found to have disease inconsistent with KS and who were also KSHV-seronegative. These two patients and their family members were excluded from the study group. The final group of index cases included 17 adults (age range 24-43 yrs) and 5 children (age range 2-8 yrs). The household members of the 22 index cases totalled 68. One child in family Y (Y4, a 3-year-old female) did not provide any blood or mouth rinse samples and was therefore not included in this study. The largest family groups were B (8 members), E and W (each with 7 members). Oral mucosal KS was present in 8 (47%) of the adult index cases. The palate was the most commonly affected intra-oral site (in 7 index cases). The characteristics of the entire study group are described in Table 3.1.

3.3 HIV-1 and KSHV serostatus

The HIV-1, HIV-2 and KSHV infection status for each study member was determined through serological testing as described in Section 2.5. The results of these assays are summarised in Table 3.1.

Among the 22 KS index cases, 20 (91%) were HIV-1 seropositive and all were KSHV-seropositive. Therefore, all but two cases can be considered to be affected by epidemic KS; the remaining two individuals, B_i (a 4-year-old boy) and Y_i (a 32-year-old male) were affected by endemic KS. Of the 67 family members available for study, 16 (24%) were HIV-1-seropositive and 46 (69%) were KSHV-seropositive. Overall, there was a positive association between KSHV and HIV-1 seropositivity in the total population of study participants ($p=0.001$) (χ^2 test). One KS index case, K_i, was antibody positive for both HIV-1 and HIV-2.

	Index case			Family members			
	Age/sex	Site of KS	Vincristine given?	Spouse (age)	Children/siblings (age/sex)	Mother (age)	Father (age)
A[a,c,d,e]	7y/F	Abdomen, limbs	No		A2(10y/M)[c,f], A3(7m/M)	A1(34y)[c,d,f]	
B[c]	4y/M	Cervical	Yes		B1(20y/F)[c,d], B2(13y/M)[c,f], B3(12y/M)[b,f], B4(10y/M)[c], B5(7y/M)[b,c,f], B6(11m/F)	B7(40y)[c]	
C[b,c,d,e,f]	8y/M	Abdomen, limbs	No		C2(14y/F)[c], C3(12y/M)[c]		C1(45y)[d]
E[a,c,d,e]	31y/F	Legs, palate	No		E1(5y/F)[c], E2(9y/F)[c,f], E3(6y/F)[c,f], E4(6y/F)[b,c,f], E5(13y/M)[b,c,f], E6(10y/M)[b,c,f]		
F[a,c,d,e]	2y/M	Foot	No		F2(7y/M)[d]	F1(35y)[c,d]	
G[c,d,e]	34y/F	Foot, leg, palate	No	G1(32y)[b,c,d,f]	G2(23y/M)[b,c,f], G3(19y/F)[c], G4(15y/M)[c]		
H[c,d]	35y/F	Leg	Yes		H1(13y/F)[c,f], H2(12y/M)[b,c,f], H3(9y/M)[c], H4(8y/M)		
I[c,d]	36y/F	Leg	Yes		I1(12y/M)[c], I2(11y/F)		
J[c,d,e]	28y/M	Leg	No	J1(22y)[c]	J2(3y/M)		
K[a,b,c,d,e,f]	30y/F	Leg	Yes		K1(12y/M)[b,e,f]		
M[c,d]	33y/M	Leg, foot	No	M4(32y)[c]	M1(3y/M), M2(7m/F), M3(8y/F)[c,d]		
N[c,d,f]	30y/F	Leg, palate	No		N1(18y/F)		
P[c,d,f]	30y/M	Leg, palate	Yes	P1(28y)[c,d]	P2(2y/M)		
Q[a,b,c,d,e,f]	24y/M	Foot	No	Q1(21y)[c,d]			
R[c,d]	41y/M	Leg	Yes	R1(25y)[c,d]	R2(12y/M)[c], R3(9y/M)[b,f]		
S[c,d]	40y/M	Leg	Yes	S1(37y)			
T[c,d]	33y/F	Leg, foot	Yes	T1(37y)[c,d,e,f]	T2(11y/M)[b,c,f], T3(9y/M)[b,c,f], T4(4y/M)[c,d,e]		
U[c,d,f]	32y/F	Leg, foot, gingiva	No		U1(14y/M)[c], U2(12y/F), U3(8y/F)		
W[c,d]	43y/F	Leg, palate	Yes	W1(36y)[b,c,d,f]	W2(20y/M)[b,c,f], W3(19y/M)[c], W4(13y/F)[b,c,f], W5(6y/M)[c,d], W6(7m/F)[c,d]		
X[c,d]	41y/F	Chest, palate	Yes	X1(44y)[b,c,d,f]	X2(18y/F)[b], X3(11m/M)		
Y[c]	32y/M	Arm	Yes	Y1(38y)[c]	Y2(8y/F), Y3(5y/F), Y4(3y/F)		
Z[b,c,d,e,f]	21m/M	Cervical	No		Z1(5y/F)[c]	Z2(22y)[b,c,f]	

[a]KSHV K1/V1 DNA amplified from blood (CD45 +); [b]KSHV K1/V1 DNA amplified from saliva; [c]KSHV-seropositive; [d]HIV-1-seropositive [e]KSHV ORF 26 DNA amplified from blood (CD45 +), [f]KSHV ORF 26 DNA amplified from saliva

Table 3.1 General characteristics and virological status of KS patients and their family members. KSHV seronegative/ KSHV DNA positive family members underscored.

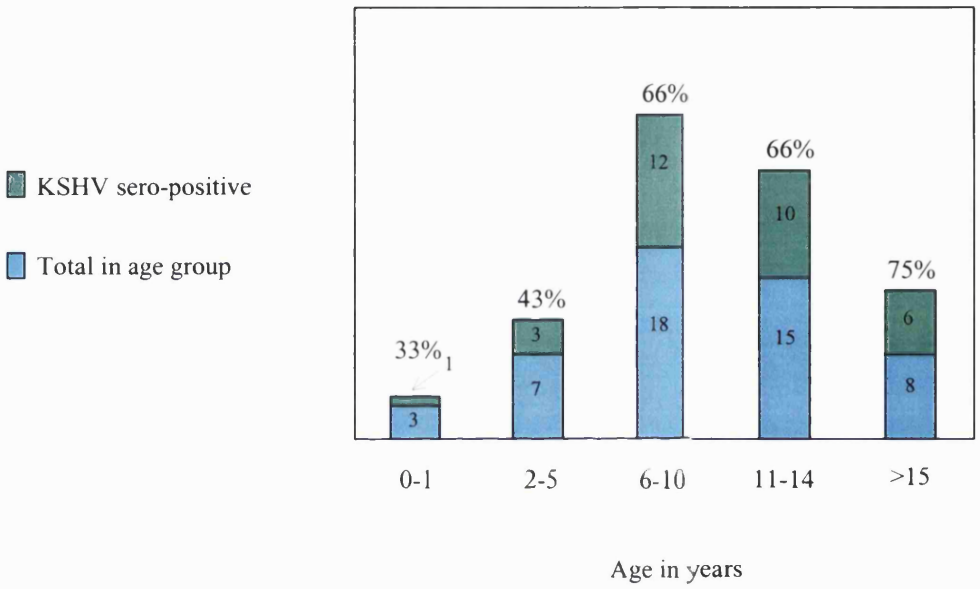
In order to examine the age distribution of KSHV infection in this group of study participants, children without KS were stratified into five age groupings. The overall percentage of KSHV-seropositive children in each age group was calculated. The results of this analysis, set out in Figure 3.1, showed an overall increase in KSHV seropositivity with age until 15 yr following which levels of KSHV infection became comparable to the overall seropositivity of the entire sample group. Of the 15 families for which the mother was available for study and was KSHV-seropositive, only four (J, K, N and X) had no KSHV-seropositive children. Therefore, the presence of a KSHV-seropositive mother is indicative of KSHV seropositivity in her children. In the largest families enrolled in the study (B, E, and W), KSHV serology revealed that the majority of siblings in each of these families were seropositive (Table 3.1). In all these families, the mother was KSHV-seropositive, pointing to transmission both between mother and child and between siblings.

3.4 Amplification of KSHV ORFs from blood samples

DNA from regions of KSHV ORF 26 and ORF K1 were initially amplified from all CD45+ leukocyte blood fraction samples as described in Section 2.1.5. Samples from which ORF 26 and/or ORF K1 DNA could be amplified are denoted in Table 3.1.

Of the 22 index cases, only 5 (23%) were positive for K1/V1 DNA and ORF 26 DNA could be amplified from 9 (41%) in the blood. In the CD45+ blood fraction, none of the 67 family members were positive for K1/V1 DNA and 4 family members (6%) were ORF 26 DNA positive. These results were unexpected since approximately 50% of patients with KS are expected to be positive for KSHV DNA in their PBMC samples (Moore and Chang, 1995) (Whitby *et al.*, 1995). Nested PCR, as used in this study, should show a further increase PCR sensitivity (Moore *et al.*, 1996c).

Figure 3.1 Percentage of KSHV seropositive children, stratified by age, among 51 siblings without KS.



However, previous studies have reported infrequent and intermittent detection of KSHV DNA in the PBMCs of KS patients and KSHV seropositive individuals (Kasolo *et al.,* 1998; Gessain *et al.,* 1999). Immunocompetent individuals may be able to control the KSHV viral load in their blood thus making KSHV DNA difficult to detect, even using highly sensitive nested PCR assays.

Both blood and saliva samples were processed within 4 hr after being collected from study participants and were stored at -20°C until DNA extraction was performed. CD45+ cells had been separated from whole blood using a biomagnetic cell separation technique and extracted using a simple commercially available DNA extraction kit (Section 2.1.3). This technique has been previously applied to successfully detect KSHV DNA in the CD45 + blood fraction of HIV-1 infected men (Leao *et al.,* 2000) and is not likely to have resulted in the low levels of ORF K1/V1 DNA detection in the blood samples of KS index cases.

All KS patients were offered chemotherapy with vincristine and those that underwent therapy are indicated in Table 3.1. Vincristine therapy was significantly associated with a decrease in K1/V1 DNA detection by PCR from CD45+ blood samples in those patients undergoing treatment. The difference in the ability to amplify K1/V1 DNA from the CD45+ blood fraction between those receiving vincristine treatment and those who were not, was statistically significant ($p=0.001$) (χ^2 test).

In developed countries, standard treatment for advanced systemic KS is by administration of a combination of chemotheraputic drugs: doxorubicin, bleomycin, and vincristine (ABV) or bleomycin and vincristine (BV). However, in Africa, combined drug therapy is not practical. The cost of ABV or BV treatment is prohibitive and side effects, including leucopenia and opportunistic infections are difficult to manage. Single drug treatment with vincristine was commonly used in the clinic attended by the study subjects because it provided effective palliative treatment with minimal costs and side effects.

Samples that were positive for either ORF K1, ORF 26 or for both ORFs were amplified for fragments of two additional KSHV ORFs, K8.1 and 73 (Section 2.1.6). The results of these PCRs for each study participant, over all four ORFs, are summarised in Table 3.2. Seven of the 10 KS index cases tested were positive for all 4 ORFs. Good correlation between PCR results was found in the 3 remaining index cases, from which KSHV DNA could be amplified in 3 of the 4 ORFs studied.

Owing to the low percentage of index cases PCR positive in the CD45+ blood fraction, a selected number of samples were tested for KSHV DNA in their CD31+ blood fraction. CD31, or platelet/endothelial cell adhesion molecule 1 (PECAM-1), is expressed on certain types of T cells, monocytes, platelets and neutrophils (Newman, 1997). PECAM-1 is present in large quantities at endothelial intercellular junctions and may be involved in recruitment of leukocytes in an inflammatory process (Muller *et al.,* 1993). Late stage KS tumours are made up of cells expressing endothelial markers, such as CD31/PECAM-1 (Dupin *et al.,* 1999). Dynabeads coated with anti CD31 antibody were used to investigate if KS patients and KHSV seropositive individuals had circulating KSHV infected endothelial cells in their blood. All index cases, whether positive for a KSHV ORF in their CD45+ fraction or not, and family members positive for one or more KSHV ORF in their CD45+ blood sample were tested for KSHV ORFs in their CD31+ blood fraction. The PCR results and HIV status for each patient are summarised in Table 3.3. Only 4 of 22 index cases (18%) and none of 8 family members tested were ORF 26 DNA positive. Only two samples tested (A_i and K_i), were positive for ORF K1/V1 DNA. Two additional samples (C_i and Q_i) were positive for 3 of the four ORFs tested. As so few index cases and family members were PCR-positive in their CD31+ blood fraction, no further testing was undertaken using these samples.

3.5 Amplification of KSHV DNA from oral samples

Oral mouth rinse samples from patients and family members were amplified for KSHV ORF 26 and ORF K1/V1 DNA. Extensive phylogenetic study of ORF 26 and ORF K1 sequence data has revealed distinctive genetic grouping patterns (Zong *et al.*, 1997; Zong *et al.*, 1999; Zong *et al.*, 2002). ORF K1 appears to be one of the most heterogeneous regions of the KSHV genome and can be divided into four general subtypes. The inter-individual sequence variability in ORF K1 is discussed in more detail in Chapter 1. Sequencing information from the hypervariable ORF K1 region would conceivably permit the most meaningful comparison between family member sequences. Therefore, for the purposes of this study, it was not necessary to amplify DNA from the other two KSHV ORFs (ORF K8.1 and ORF 73) from rinse samples. ORF 26 was amplified in parallel with ORF K1 as control.

Among the 22 index cases, 7 (32%) were ORF 26 DNA-positive and 4 (18%) were positive for K1/V1 DNA in their mouth rinse samples (Table 3.1). Of the 67 family members included in the study, 24 (36%) were positive for ORF 26 DNA and 18 (27%) for K1/V1 DNA in their mouth rinse (Table 3.1). Four family members who were PCR positive for K1/V1 DNA were seronegative for KSHV by IFA (B3, K1, R3, X2). This may reflect a recent infection and on-going seroconversion to a KSHV antibody-positive status.

Table 3.2. PCR amplification of selected CD45 + blood fraction

Case/family member	ORF 26	ORF K1	ORF K8.1	ORF 73
A_i	+	+	+	+
C_i	+	-	+	+
E_i	+	+	-	+
F_i	+	+	+	+
G_i	+	-	+	+
H_i	+	-	-	-
J_i	+	-	-	-
K_i	+	+	+	+
K1	+	-	-	-
Q_i	+	+	+	+
T1	+	-	+	-
T4	+	-	-	-
W6	-	+	-	-
Y2	-	+	-	-
Z_i	+	-	-	-

Table 3.3. PCR amplification of the CD31+ blood fraction samples

Case/family member	ORF 26	ORF K1	ORF K8.1	ORF 73	HIV-1 sero-status
A_i	-	+	+	-	+
B_i	-	-	-	-	-
C_i	+	-	+	+	+
E_i	-	-	-	-	+
E3	-	-	-	-	-
F_i	-	-	+	+	+
G_i	-	-	-	-	+
H_i	-	-	-	-	+
I_i	-	-	-	-	+
J_i	-	-	-	-	+
K_i	-	+	-	-	+
M_i	-	-	-	-	+
N_i	-	-	-	-	+
P_i	-	-	-	-	+
Q_i	+	-	+	+	+
R_i	-	-	-	+	+
R3	-	-	-	-	-
S_i	-	-	-	-	+
T_i	-	-	-	-	+
T1	-	-	-	-	+
T4	-	-	-	-	+
U_i	-	-	-	-	+
W_i	+	-	-	-	+
W6	-	-	-	-	+
X_i	-	-	-	-	+
Y_i	-	-	-	-	-
Y2	-	-	-	-	-
Z_i	+	-	+	-	+

3.6 Sequencing of KSHV ORF K1 and ORF 26

Sequencing of selected ORF 26 and ORF K1 PCR positive samples was carried out as described in Section 2.3. A simple dendrogram was produced in Megalign (DNAstar) to show the basic phylogenetic relationships between all K1/V1 sequences recovered from study participants (Figure 3.3).

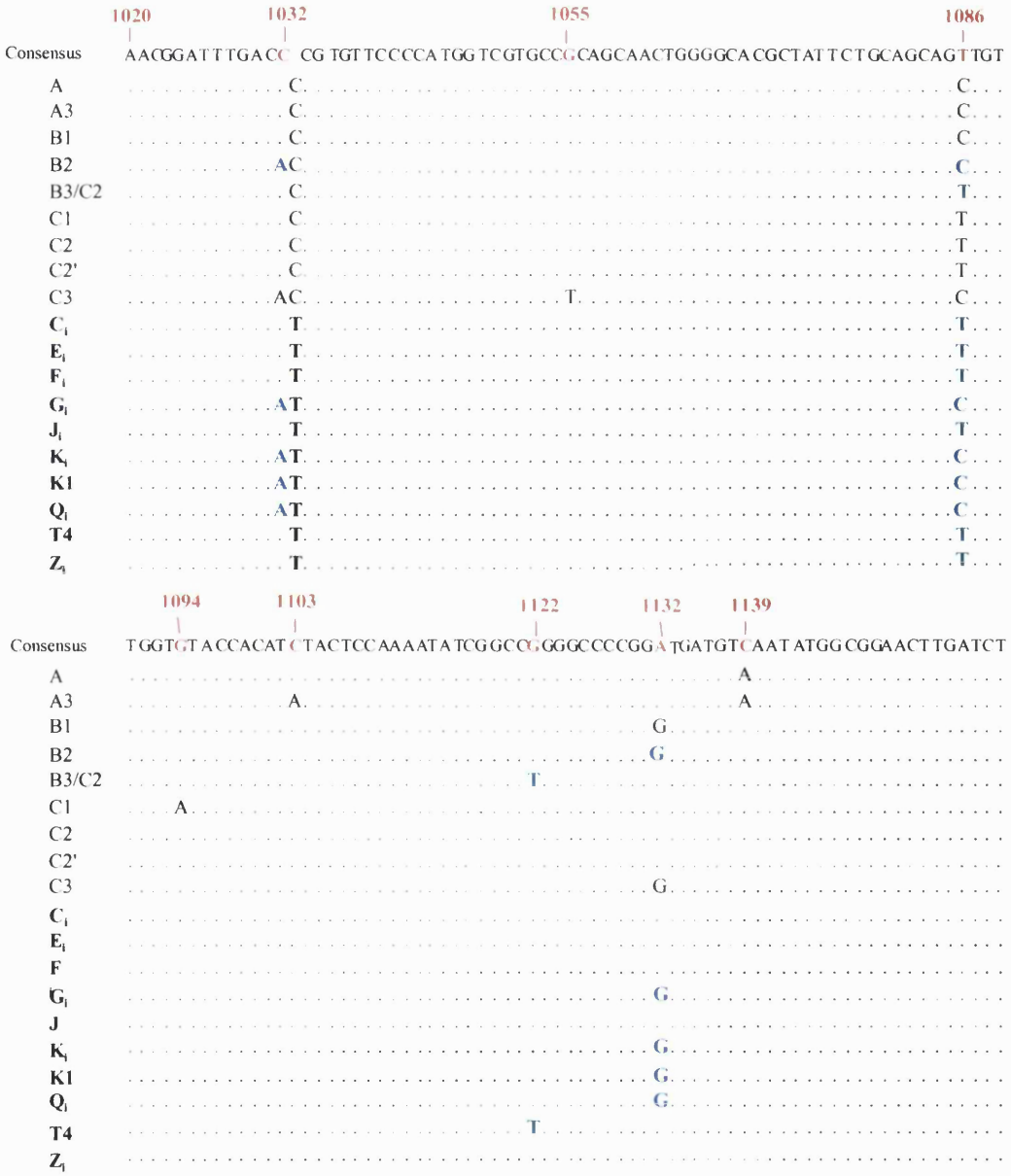
3.6.1 ORF 26 genotyping

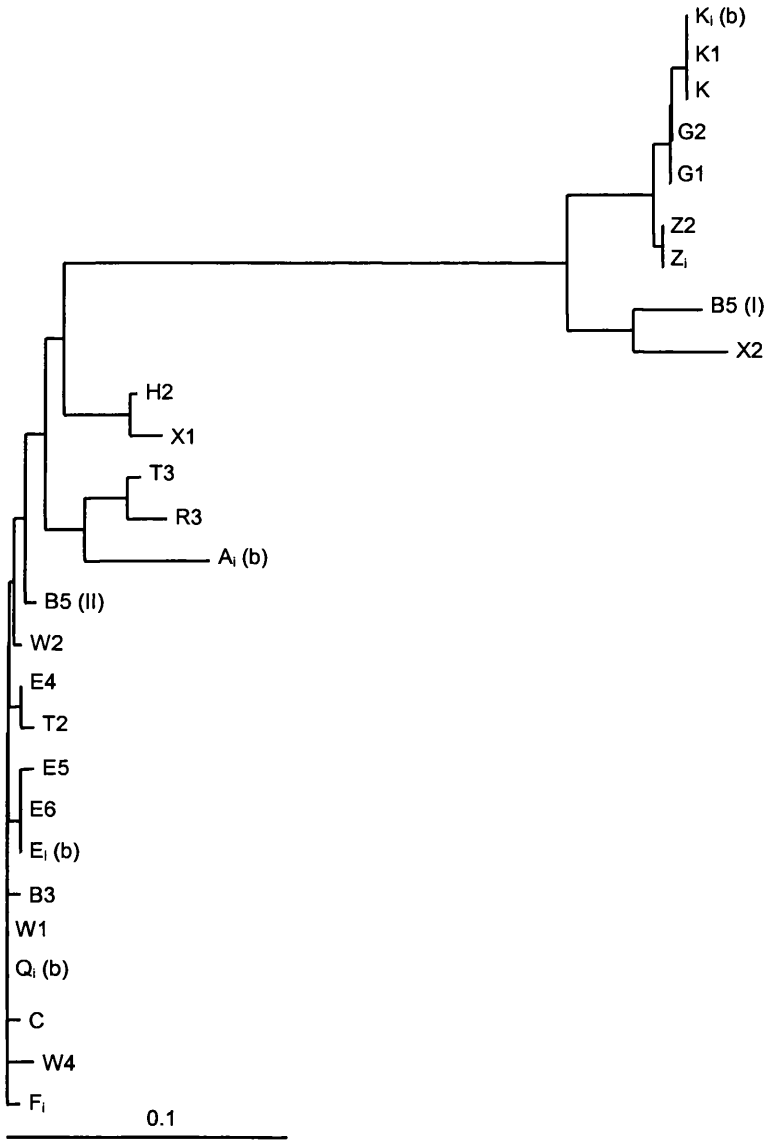
Distinct ORF 26 subtypes can be determined from sequence polymorphism at specific nucleotide positions in the ORF 26 sequence (Zong *et al.*, 1997; Zong *et al.*, 1999). A nucleotide alignment of all ORF 26 sequences recovered from samples in this study and prototype ORF 26 sequences is displayed in Figure 3.2. All samples in this study belonged to either the B3/C2 subtype (highlighted in green) or to the B2 subtype (highlighted in blue). Little polymorphism was observed over this region of ORF 26 and was therefore not considered appropriate for use in comparing between family member sequences. Only those samples positive for ORF 26 in their CD45+ leukocyte fraction were sequenced. Thus a comparison could be made between sequences in the K family (K_i and K1), which were identical.

3.6.2 ORF K1/V1 genotyping

As discussed in Chapter 1, ORF K1 displays a high level of sequence variability both between and within known subtypes. For this reason, ORF K1/V1 and K1/V2 PCR products were sequenced in order to compare nucleotide sequences derived from members of the same family. It was possible to compare ORF K1/V1 sequences between two or more members of 8 families (21 samples). ORF K1/V2 DNA was amplified from the 21 ORF K1/V1 DNA positive samples (Section 2.1.5) to make a more complete comparison over this larger region (575 bp) between family member sequences. A total of 6 families (B, E, T, G, K and W) had two or more family members from whom ORF K1/V2 DNA could be amplified. In 5 of these families, ORF K1/V2 sequence data could be compared (G1 sequence data could not be recovered).

Figure 3.2 Nucleotide alignment of ORF 26 PCR product sequences recovered from the CD45 + blood fraction of KS index cases and family members (in bold) with selected prototype sequences (Zong *et al.*, 1997). Nucleotide positions highlighted in red signify areas of known polymorphism that permit subtyping. Subtype B2 is highlighted in blue and subtype B3/C2 in green.





Proportion of nucleotides substituted for a given horizontal branch length

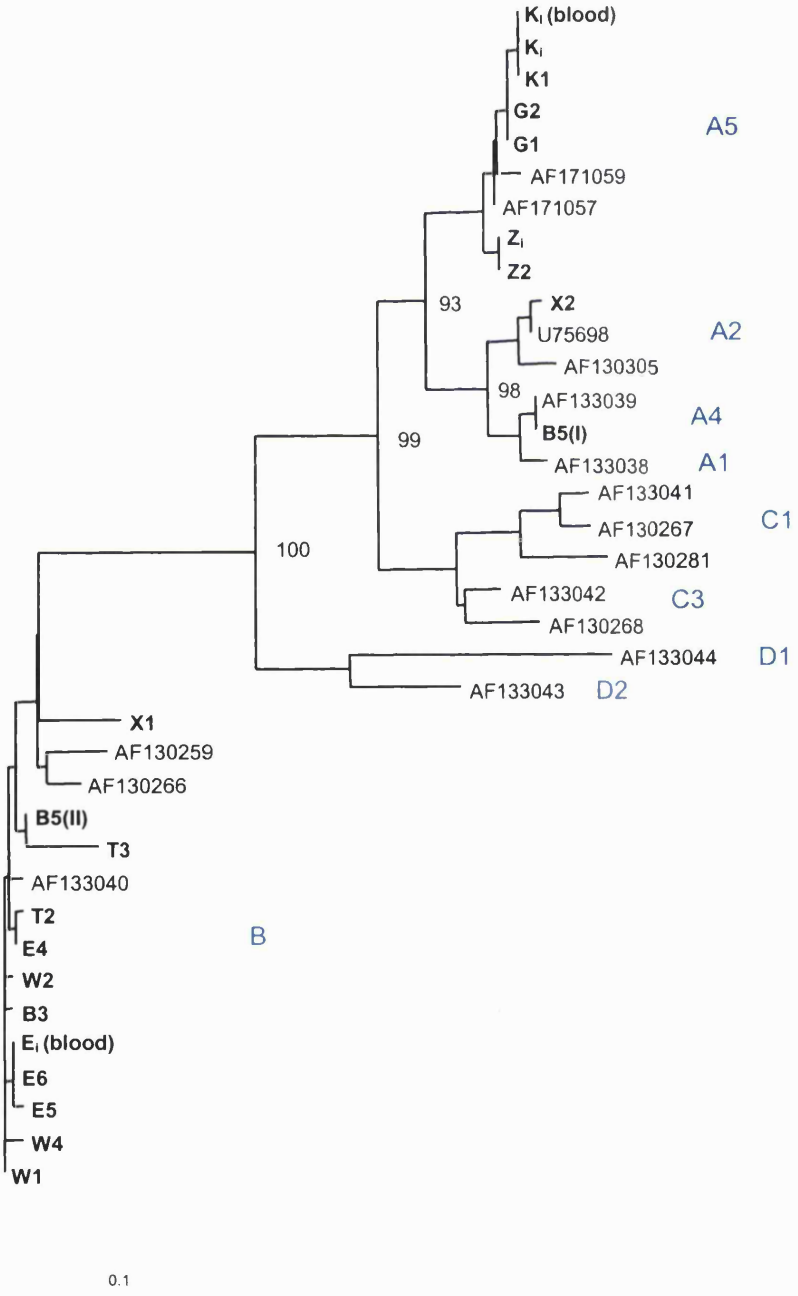
Figure 3.3 Phylogenetic tree produced from a Clustel W nucleotide alignment of all 27 ORF K1/V1 sequences recovered from KS index cases and family members. (b) indicates derived from CD45+ blood fraction

For all families in which ORF K1/V1 and K1/V2 sequence comparisons could be made, phylogenetic trees were constructed using the neighbor-joining method of analysis (Section 2.3.2). GenBank sequences representing major ORF K1 subtypes were included in each phylogenetic tree. Phylogenetic clustering identified subtype assignments for ORF K1/V1 and K1/V2 sequences recovered from the study participants (Figures 3.4a and 3.4b). The genetic distances between all 21 family members from whom ORF K1/V1 nucleotide sequences were available were calculated using DNADIST (Figure 3.5).

For all PCR products, 3-5 clones were sequenced and all ORF K1/V1 sequences were identical except in sample B5. In B5, two divergent sequences were recovered, designated B5(I) and B5 (II). ORF K1/V2 sequences from clones of the same sample differed by 1 to 2 nucleotides in some instances.

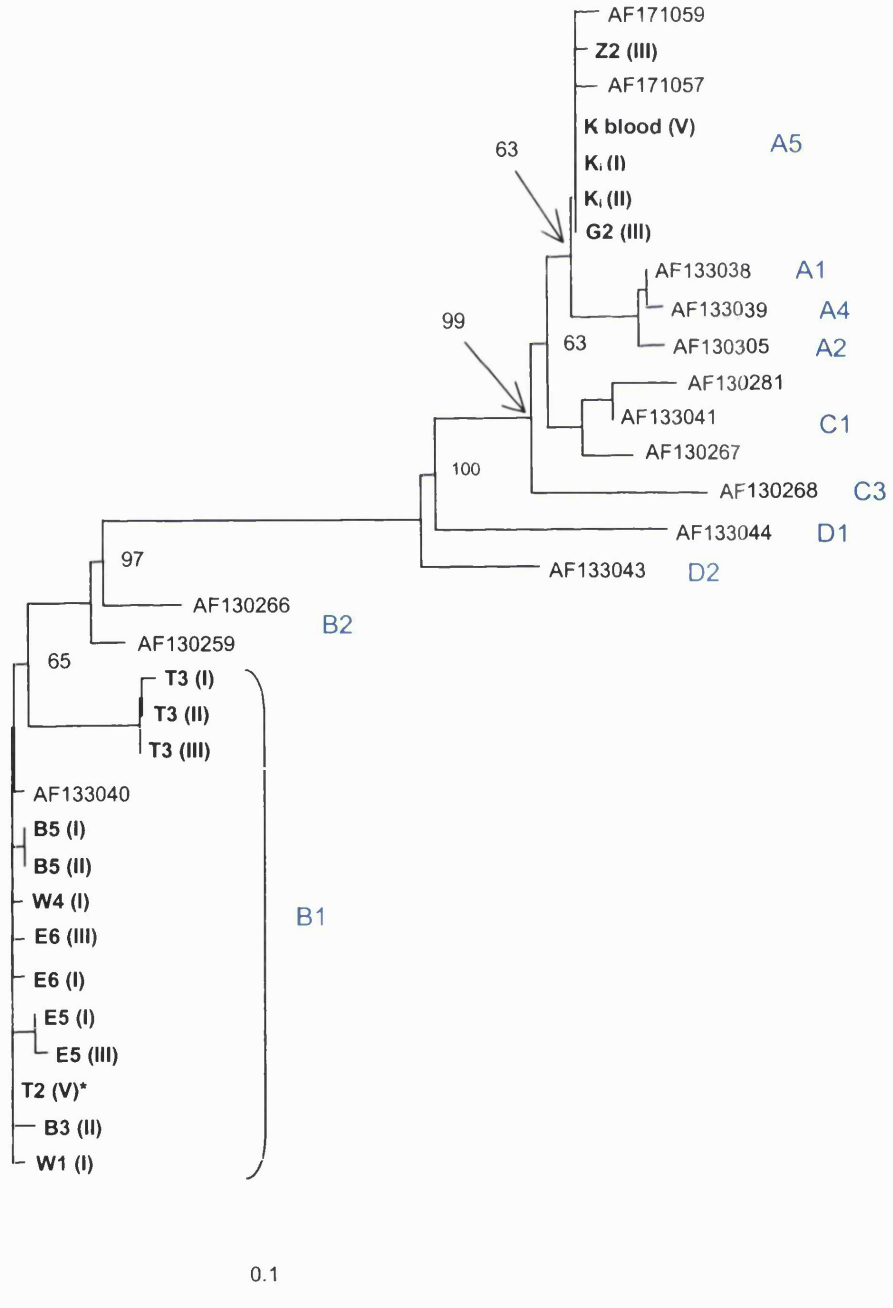
More specific determinations of ORF K1 subtype were made by examining the predicted amino acid composition of study samples and comparing with sequences of known subtypes over regions of increased polymorphism (Zong *et al.*, 1999; Zong *et al.*, 2002). Subtype A sequences could be differentiated using the amino acid sequence found in the ORF K1/V1 region (Figure 3.6a) and subtype B sequences were distinguished based on small changes in the ORF K1/V2 region (Figure 3.6b). The majority of samples in this study belonged to either the A5 or B1 subtypes. ORF K1/V2 DNA could not be amplified from sample X1, and since X1 belonged to ORF K1/V1 subtype B, it could not be further differentiated. The most commonly reported subtypes in sub-Saharan Africa are A5 and B (Meng *et al.*, 1999; Cook *et al.*, 1999; Lacoste *et al.*, 2000a; Kakoola *et al.*, 2001) and the samples in this study fall mainly into these two subtypes. Two samples, X2 and B5(I) belonged to subtypes A2 and A4 respectively based on their amino acid composition and relative positions in the phylogenetic tree.

Figure 3.4a Unrooted phylogenetic tree incorporating 21 ORF K1/V1 sequences from 8 families in which sequence comparisons could be made. Genbank sequences, indicated by accession numbers are included to represent major ORF K1 subtypes. Subtype assignments follow Zong *et al.* 1999 and Zong *et al.* 2002. The tree was constructed using the neighbor-joining algorithm in the NEIGHBOR program of PHYLIP. Selected bootstrapping values for 1000 replicates are shown at major branch points.



Proportion of nucleotides substituted for a given horizontal branch length

Figure 3.4b Unrooted phylogenetic tree incorporating the ORF K1/V2 sequences of selected clones for 13 individuals originally included in the ORF K1/V1 phylogenetic tree. Roman numeral numbers clones. GenBank sequences, indicated by accession number, are included to represent ORF K1 subtypes. Subtype assignments follow Zong *et al.* 1999 and Zong *et al.* 2002. The neighbor-joining algorithm in the NEIGHBOR program of PHYLIP was used to construct the tree. Selected bootstrapping values for 1000 replicates are shown at major branch points. *Clones B5(III), T2(VI), E4(II), W4(IV), W2(V), B3(V), E4(III), E4(I), B3(IV), B3(I), W4(V) have an identical sequence to T2(V).



Proportion of nucleotides substituted for a given horizontal branch length

Figure 3.5Genetic distances between family member ORF K1/V1 nucleotide sequences calculated using DNADIST.

	B3	B5(I)	B5(II)	E_i(bl)	E4	E5	E6	G1	G2	K_i (bl)	K_i	K1	T2	T3	W1	W2	W4	X1	X2	Z_i	Z2
B3	0	0.250	0.014	0.009	0.009	0.014	0.009	0.242	0.242	0.249	0.249	0.249	0.014	0.048	0.047	0.095	0.014	0.059	0.263	0.241	0.241
	B5(I)	0	0.251	0.244	0.258	0.244	0.244	0.091	0.091	0.091	0.091	0.091	0.251	0.283	0.250	0.249	0.265	0.278	0.059	0.097	0.097
		B5(II)	0	0.014	0.014	0.019	0.014	0.243	0.243	0.250	0.250	0.250	0.019	0.033	0.009	0.009	0.019	0.064	0.264	0.242	0.242
			E_i (bl)	0	0.009	0.004	0	0.235	0.235	0.243	0.243	0.243	0.014	0.048	0.004	0.009	0.014	0.059	0.256	0.234	0.234
				E4	0	0.014	0.009	0.249	0.249	0.257	0.257	0.257	0.004	0.049	0.004	0.009	0.014	0.054	0.270	0.242	0.242
					E5	0	0.004	0.235	0.235	0.243	0.243	0.243	0.019	0.054	0.009	0.014	0.019	0.064	0.256	0.234	0.234
						E6	0	0.235	0.235	0.243	0.243	0.243	0.014	0..048	0.004	0.009	0.014	0.059	0.256	0.234	0.234
							G1	0	0	0.004	0.004	0.004	0.243	0.281	0.242	0.241	0.256	0.247	0.085	0.009	0.009
								G2	0	0.004	0.004	0.004	0.243	0.281	0.242	0.241	0.256	0.247	0.085	0.009	0.009
									K_i (bl)	0	0	0	0.250	0.289	0.249	0.248	0.264	0.255	0.080	0.014	0.014
										K_i	0	0	0.250	0.289	0.249	0.248	0.264	0.255	0.080	0.014	0.014
											K1	0	0.250	0.289	0.249	0.248	0.264	0.255	0.080	0.014	0.014
												T2	0	0.054	0.009	0.014	0.019	0.049	0.264	0.235	0.235
													T3	0	0.043	0.044	0.054	0.096	0.298	0.280	0.280
														W1	0	0.004	0.009	0.054	0.263	0.241	0.241
															W2	0	0.014	0.059	0.262	0.243	0.243
																W4	0	0.064	0.277	0.255	0.255
																	X1	0	0.277	0.233	0.233
																		X2	0	0.091	0.091
																			Z_i	0	0
																				Z2	0

Figure 3.6a Deduced amino acid alignment of sequences recovered from study participants, together with sequences of known subtype from GenBank, between positions 41 to 93 in the ORF K1 gene. Areas of increased polymorphism in the prototype sequences are highlighted in pink. Sequences from study participants belonged to one of three subtypes; A5 (shown in green), A2 (shown in orange) and A4 (shown in blue). Reproduced in part from Journal of Clinical Virology, volume 23, Zong *et al.* "Genotypic analysis at multiple loci across Kaposi's sarcoma herpesvirus (KSHV) DNA molecules: clustering patterns, novel variants and chimerism", pages 119-148, with permission from Elsevier Science.

Figure 3.6b Deduced amino acid alignment, between residues 191 and 231 in the ORF K1/V2 region, of sequences recovered from the study participants (in bold), together with sequences from GenBank representing the three subtype B clades. Areas of increased polymorphism in the prototype sequences are noted in pink. All sequences from study participants belong to the B1 clade (in blue). Reproduced in part from Journal of Clinical Virology, volume 23, Zong *et al.* "Genotypic analysis at multiple loci across Kaposi's sarcoma herpesvirus (KSHV) DNA molecules: clustering patterns, novel variants and chimerism", pages 119-148, with permission from Elsevier Science.

3.6.3 Chimeric genomes

DNA sequencing of a number of conserved internal loci as well as the hypervariable ORF K1 and ORF K15, at the extreme left hand side (LHS) and right hand side (RHS) of the KSHV genome, identified chimeric viruses (Kakoola *et al.*, 2001; Zong *et al.*, 2002). In the predominately African ORF K1 B subtype, 5 main types of chimerism have been described; however, a predominant B subtype linkage is normally present across the conserved internal loci (Zong *et al.*, 2002). Only two KSHV ORFs were analysed in this study; ORF K1 and the conserved internal loci, ORF 26. Three types of linkage patterns were identified in those study samples from which both ORFs could be analysed: B2/A5, B3/A5 and B3/B (ORF 26/ORF K1). The two genes included in this study can only provide information about the LHS of the genome. More specific information from hypervariable loci at the RHS and from other internal loci would be needed to make any conclusions about the types of chimeric viruses present in this population.

3.7 Intra-familial comparison between ORF K1/V1 sequences

3.7.1 Patterns of sequence identity

In 4 of the 8 families from whom ORF K1/V1 DNA sequences could be compared, identical sequences were recovered between 2 or more family members. In family E, identity was found between the mother E_i (31 yr), and one of her sons, E6 (10 yr). In families K and Z, identical sequences were recovered between the mothers, K_i (30 yr) and Z2 (22 yr), and their sons K1 (12 yr) and Z_i (21 mo), and in family G between the father, G1 (32 yr) and son, G2 (Figure 3.4a).

3.7.2 Patterns of sequence non-identity

In five families (B, E, T, W and X), non-identical sequences were recovered from two or more family members. In family E, minor nucleotide differences were revealed in ORF K1/V1 sequences between a brother E5 (13 yr), sister E4 (6 yr) and their mother and brother (E_i/E6). However, only E4 carried a divergent amino acid sequence (Figure 3.7). In family T, brothers T2 (11 yr)

Figure 3.7 Deduced amino acid alignment of the entire 71 amino acid region of ORF K1/V1 amplified from study subjects in the five families where ORF K1/V1 nucleotide variations were revealed. Residues divergent from those of corresponding family members are shown in orange.

and T3 (9 yr) carried K1/V1 sequences with a genetic distance of 0.054 (i.e. approximately 5% divergent) from each other, indicating a more significant divergence than in family E (Figure 3.5). K1/V1 sequence differences among siblings W2 (20 yr), W4 (13 yr) and their father W1 (36 yr) were small, although some of these nucleotide changes resulted in polymorphism at the amino acid level (Figure 3.8). In family X, ORF K1/V1 sequences recovered from a father X1 (44 yr) and daughter X2 (18 yr) were significantly divergent and belonged to different ORF K1 subtypes (A2 and B, respectively) (Figure 3.4a).

3.8 EBV *Bam*HI N and *Bam*HI K variability

EBV is ubiquitous in the general population, infecting 90% of the adult population regardless of geographical region. Transmission of EBV is by close oral contact with an infectious person (Yao *et al.,* 1985) and in countries where levels of hygiene are low, primary infection is often asymptomatic, occurring in early childhood. In industrialised countries, primary infection often does not occur until adolescence and is more likely to cause a symptomatic infection, referred to as infectious mononucleosis (IM) (Steven *et al.,* 1996). IM is characterised by chronic fatigue, fever, sore throat and painfully enlarged lymph glands.

The *Bam*HI N and *Bam*HI K regions of the EBV genome, which have been reported to display increased levels of nucleotide variability (Triantos *et al.,* 1998) were selected to compare the molecular transmission patterns between this virus with KSHV. EBV is very closely related to KSHV, both viruses belonging to the gamma-herpesvirus subfamily. In addition, the transmission patterns of KSHV and EBV may be similar in an endemic environment, facilitated by close family and community contact and possible transmission through oral fluids.

Figure 3.8a Nucleotide alignment of EBV *Bam*HI K sequences amplified from ORF K1/V1 DNA positive mouth rinse samples. Nucleotide sequence from EBV infected cell line, B958, included as reference.

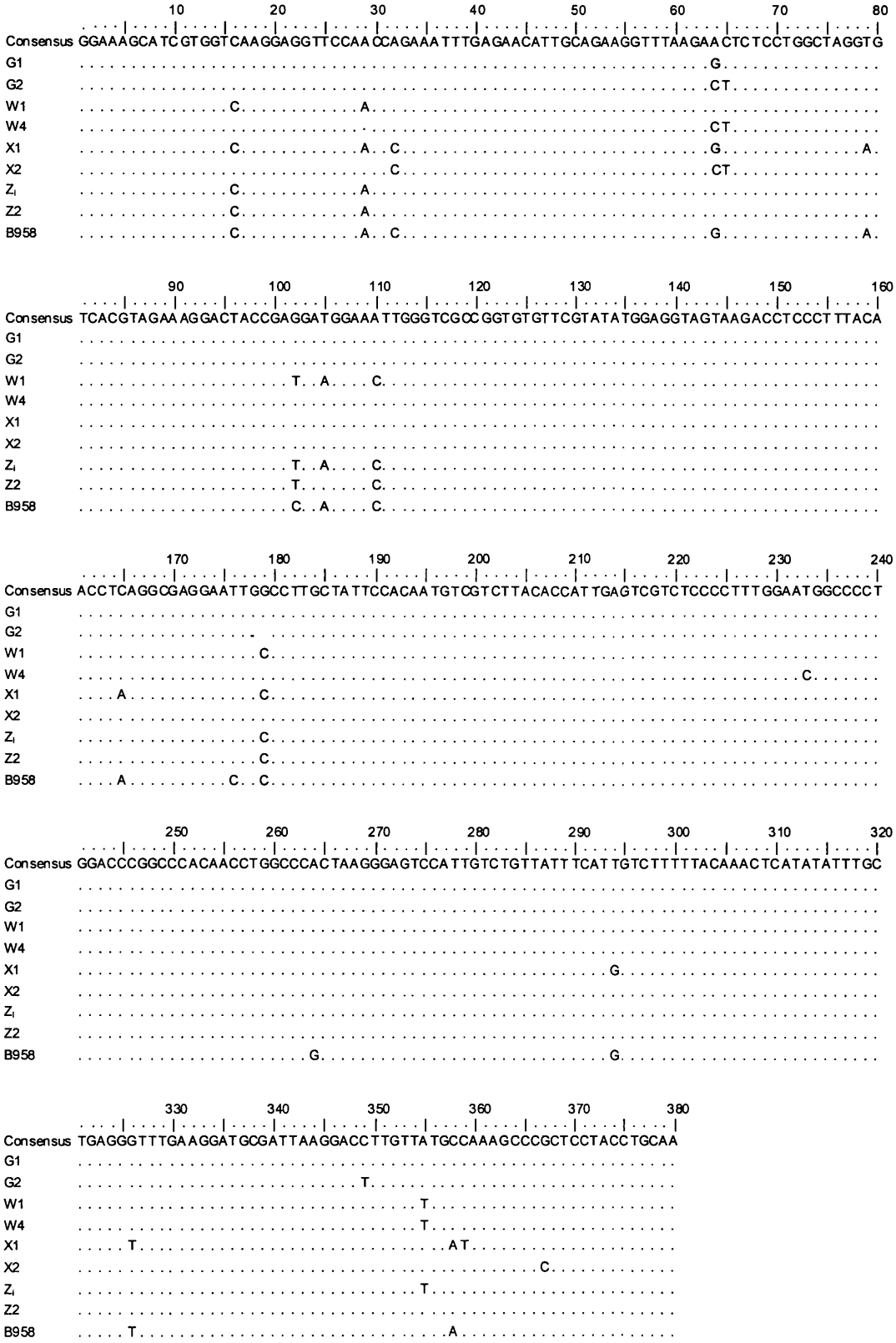
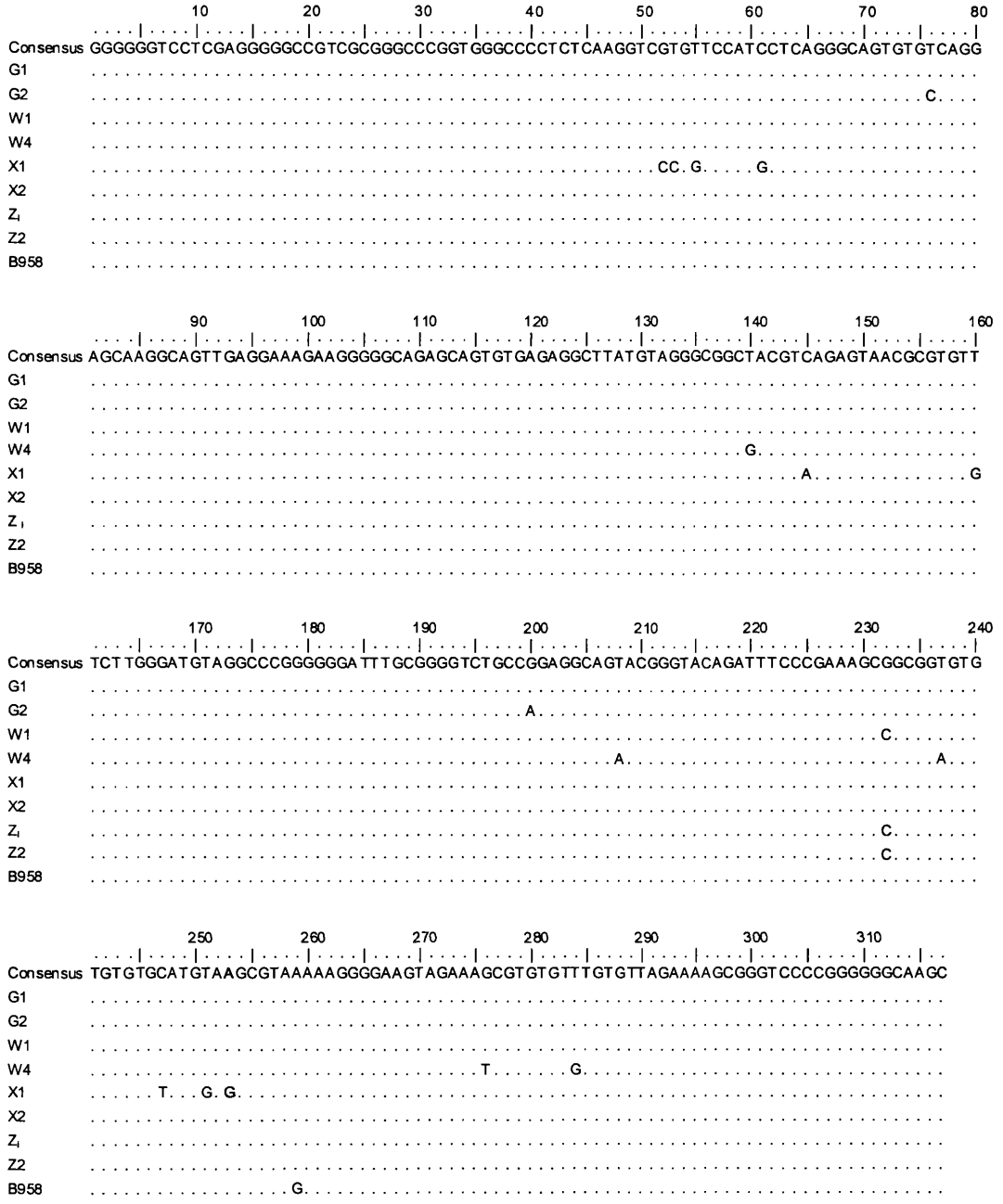


Figure 3.8b Nucleotide alignment of EBV *Bam*HI N sequences amplified from ORF K1/V1 DNA positive mouth rinse samples. Nucleotide sequence from EBV infected cell line, B958, included as reference



In the 21 samples from 8 families for which ORF K1/V1 sequences could be compared, EBV *Bam*HI N and *Bam*HI K DNA were amplified by hemi-nested or nested PCR (Section 2.2.3). Approximately 70% of the samples tested were positive for EBV DNA by PCR. From these samples, sequence comparisons could be made between two sequences in 4 families (G, W, X and Z). Overall variability was low for these regions of the EBV genome and was not comparable to the level of heterogeneity found in the KSHV ORF K1/V1 DNA fragment.

Despite this relatively low level of nucleotide variability, the alignments for each region (Figure 3.8a and 3.8b) do show some minor nucleotide differences in the sequences studied. In family Z, EBV *Bam*HI N sequences for the mother (Z2) and son (Z_i) were identical (as were their KSHV ORF K1/V1 sequences). In families W, X, and G, some nucleotide changes were revealed between the two family members. While for families W and X nucleotide divergences also existed in the KSHV ORF K1/V1 sequences, in family G, the ORF K1/V1 sequences between the father (G1) and son (G2) were the same. Over the *Bam*HI K region of the EBV genome, no two family member sequences were identical

3.9 Discussion

Epidemiological studies have thus far relied on serological data to estimate the prevalence of KSHV infection in populations and to study patterns of transmission. In North America and Europe, serological data point to a link between sexual behaviour and risk of KSHV infection (Martin *et al.*, 1998); (Dukers *et al.*, 2000). In sub-Saharan Africa, evidence for transmission between mother and child and between siblings is supported by serological correlation data (Plancoulaine *et al.*, 2000). In the current sample set, 60% of children 15 yr of age or less without KS were KSHV-seropositive. Stratification of the sample set indicated that KSHV seroprevalence increased with age in these children. However, the numbers of samples available for study were relatively few and the small numbers of subjects in each age group may have biased the results.

Nevertheless, it is evident from the high percentage of KSHV-seropositive children that non-sexual transmission is important in Malawi. The relatively high KHSV seroprevalence in this group of relatives of KS patients (69%) when compared to other reported levels of KSHV infection in East Africa (30-65%) (Simpson *et al.,* 1996) points to an increased risk of KSHV infection among family members of KS patients. Similar results were found in a Sardinian study, where KSHV infection was more prevalent among family members of KS patients (39%) than in the control group (11%) (Angeloni *et al.,* 1998).

To clarify the routes of KSHV transmission in Malawian families, two hypervariable regions of the KSHV genome (K1/V1 and K1/V2) were analysed and compared between members of the same family in which one member had KS. Direct evidence of KSHV transmission could be discovered and studied through this specific method of sequence comparison. Previous studies have documented intra-familial hepatitis B transmission in Gambian families using a similar phylogenetic analysis approach (Dumpis *et al.,* 2001). A conserved region of the HBV genome was amplified and sequenced from Gambian chronic carriers and their family members. Phylogenetic analysis revealed clustering between sequences in families and villages, but not in larger geographical regions, pointing to familial or community transmission of HBV.

Evidence obtained from this study of KSHV transmission in Malawi suggests that the mother–to-child and sibling-to-sibling modes of KSHV transmission may not fully describe the complexity of transmission events occurring in this endemic setting. In families E, G, K and Z, identical ORF K1/V1 sequences were recovered from a number of the family members. Identity was found between the mothers, E_i, K_i, and Z2, and their sons, E6, K1, and Z_i. In family G, sequence identity was revealed between the father (G1) and his son (G2). In families E, K, and Z, the sequence identity revealed supports the hypothesis that KSHV transmission frequently occurs between mothers and their children (Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000). In family G, a father and son shared an identical sequence. Transmission between fathers and their children has, however, not been supported in serological studies. Nevertheless, the

mother's sequence (G_i) was not available and the possibility that she was the source of infection for both her spouse and her son cannot be excluded.

Disparate ORF K1/V1 sequences were recovered from members of five families. In family X, the father (X1) and his daughter (X2) carried K1/V1 sequences belonging to different ORF K1 subtypes (A2 and B1, respectively) indicating no transmission linkage between each other. In family W, very little nucleotide divergence was revealed between sequences recovered from the father, W1, his daughter, W4, and his son, W2 (Figure 3.4a) although some amino acid divergence was present (Figure 3.7). As in family G, the mother's ORF K1/V1 sequence (W_i) was not available for analysis so she cannot be ruled out as a source of intra-familial transmission. ORF K1 sequences recovered from members of family E indicated very little nucleotide divergence between the mother (E_i) and her children, E4 and E5; only E4 carried a divergent amino acid sequence. In family E, transmission through the mother, via familial routes, cannot be disregarded given that the sequences recovered from this family are so similar. Siblings T2 and T3 provide the best evidence for nonsexual extra-familial transmission event(s) having taken place. Their K1/V1 and K1/V2 DNA sequences were sufficiently diverse to indicate that they were not infected from each other or the same source.

For all ORF K1/V1 PCR products included in this study, 3-5 clones were sequenced for each sample. The sequence for all clones was identical except for clones from the B5 PCR product. Two divergent sequences were isolated from this PCR product, B5(I) and B5(II). Their relative positions are shown in the phylogenetic tree (Figure 3.4a); B5(I) belonged to the A4 ORF K1 subtype and B5(II) to the B subtype. In terms of KSHV transmission, it unlikely that the B5(I) virus was acquired from the sibling, B3, who carried a subtype B sequence. However, B5(II) and B3 sequences differed by only three nucleotides and were identical at the amino acid level. Transmission of this variant between members of the B family cannot therefore be ruled out.

There has been little evidence for mixed infection in KSHV-positive patients although Zong *et al.* (2002) reported two divergent KSHV sequences isolated from a KS lesion in a single patient. Nucleotide misincorporation error associated with the use of *Taq* DNA polymerase and possible contamination of the sample from outside sources have made it difficult to determine whether or not these are true mixed infections. It may also be necessary to sequence many clones to get an accurate picture of the population of virus types infecting a single patient. This process can be expensive and time-consuming using traditional sequencing techniques. The existence of chimeric viruses circulating in the population, as discussed in Chapter 1, indicates that recombination events can occur between different KSHV viruses. Thus, it is possible for mixed infections to occur in a single person. B5 may be an example of a mixed infection involving a major, B5(II) and minor, B5 (I) KSHV variant infecting a single person. In the case of the B5(I) isolate, it was not possible to amplify consistently from the DNA extract, and possible contamination of this sample from an outside source cannot be excluded.

In families E and W, similar but not identical sequences were recovered from family members, making it difficult to draw conclusions about transmission patterns in these families. The ORF K1/V1 fragment originally amplified and sequenced was only 213 bp in length and did not include the majority of ORF K1 that was located in the second variable region (K1/V2). Thus, ORF K1/V2 sequences were amplified from samples that were PCR-positive for ORF K1/V1 to generate a broader set of sequence data in an attempt to discriminate between closely related sequences. However, as Figure 3.4b shows, even less sequence diversity was present between the ORF K1/V2 sequences. ORF K1/V2 sequencing of multiple clones from each PCR product resulted in sequences differing by one or two base pairs in some instances. While is possible that the majority of these minor nucleotide differences were due to *Taq* polymerase error, considering the length of the PCR product and the hemi-nested PCR procedure used, it is difficult to determine which of these clones represented the majority sequence without sequencing even more clones. Sequencing of ORF

K1/V2 PCR products did confirm that in family T, sequences recovered from siblings T2 and T3 were divergent over their ORF K1/V2 nucleotide sequences

Sequences in this study belonged to the A5 and B subtypes, were similar within each subtype and clustered together on the phylogenetic tree. While intra-familial transmission via the mother is likely in some families (G, K, and Z), the sequence divergence in other families was not great enough to make firm conclusions regarding routes of transmission. Although data regarding the village or geographical location where the study subjects were living was not collected, it is plausible that some family groups were living in proximity to one another. It is therefore possible that KSHV infection was acquired not only within family groups but also through contact with other infected persons in the immediate environment. In addition, KSHV antibody positive family members from whom ORF K1 DNA could not be amplified were not available for comparative study. Some of these family members could have acted as sources of infection in the past.

In Africa, children often acquire KSHV infection indicating that non-sexual routes of transmission are important in endemic settings (Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000). In an Ugandan study, children reached adult levels of KSHV seropositivity (~50%) before puberty (Mayama *et al.,* 1998). Mayama *et al.* (1998) found that KSHV seropositivity correlated with HBV positivity. Thus environmental conditions conducive to HBV transmission in Africa may also be conducive to KSHV transmission. HBV transmission in endemic countries normally occurs through close contact with siblings and other children early in life (Whittle *et al.,* 1983; Tabor *et al.,* 1985).

Patterns of KSHV transmission may follow those of other common gamma-herpesviruses such as EBV. In contrast to KSHV, EBV is ubiquitous in the general population worldwide and children are commonly infected by three years of age in underdeveloped countries. Saliva is the main source of transmission and viral shedding occurs even in asymptomatic carriers throughout their life (Yao *et al.,* 1985). However, the EBV *Bam*HI N and K

regions analysed in this study were not as divergent as the KSHV ORF K1/V1 region and could be amplified from only 8 of the study participants. Therefore, it was not possible to make a valid comparison between the molecular epidemiologies of KSHV and EBV transmission in this endemic setting. The sequence data recovered from the EBV positive samples revealed some heterogeneity between family member sequences. Members of families G, W and X carried divergent sequences in both of the EBV regions studied. In family Z, the two family members were infected with very similar EBV DNA sequences, identical in the *Bam*HI N region and differing by only one nucleotide in *Bam*HI K, indicating that intra-familial mother to child transmission of EBV is plausible in this family. Future studies involving larger sample sets may yet reveal common patterns of transmission between these two gamma-herpesviruses.

Among all KSHV antibody positive family members and index cases, 26 % were PCR positive for K1/V1 DNA in their mouth rinse samples. In contrast, only 5 of 22 (23%) index cases and no family members were K1/V1 DNA positive in their CD45+ samples. Some decline in K1/V1 detectability in blood may have been due to vincristine therapy in the KS index cases. Despite this, ORF K1/V1 DNA amplification in untreated KSHV-seropositive family members occurred only in the mouth rinse samples and with greater frequency than in KS index cases (30% in family members versus 18% in index cases). Unlike the CD45+ samples, no correlation was found between vincristine therapy and KSHV DNA detection in the mouth rinses of index cases. In this study group, some correlation between an HIV-1 seropositive status and K1/V1 DNA detection in the saliva was found, but this was not statistically significant.

Frequent detection of KSHV DNA in the saliva of homosexual men has been previously reported, and high titre of virus has been discovered in oral fluid and oral scrapings collected from these men (Koelle *et al.,* 1997; Pauk *et al.,* 2000). While there is no evidence to suggest that KSHV infects the salivary glands (Klussmann *et al.,* 2000), in situ hybridisation has localised KSHV DNA and RNA in the epithelial cells of the oral mucosa, pointing to a potential site of

viral replication. In the study reported by Pauk *et al.,* 2000 involving KSHV seropositive homosexual men without KS, 60 % of participants had at least one oral mucosal sample from which KSHV DNA or RNA could be isolated. Among the 92 HIV-1 seronegative study participants, a history of sexual contact with a partner with KS or deep kissing with an HIV-1-positive partner were risk factors for KSHV infection (Pauk *et al.,* 2000). The presence of infectious virus in the cell free fraction of saliva from HIV-1-positive persons with KS (Koelle *et al.,* 1997) further indicates that contact with saliva may be an important risk factor in acquiring KSHV infection among homosexual men.

In North America and Europe, the majority of KSHV transmission is principally sexual and is particularly associated with male homosexual practices. It is unclear how saliva can facilitate the transmission of KSHV in homosexual men, since heterosexual individuals commonly practice open-mouth kissing as well. Very few studies have been conducted to determine if immunocompetent individuals who are KSHV-seropositive are shedding KSHV in their saliva, although Pauk *et al.* (2000) suggested that KSHV shedding does occur. Likewise, little work has been done in highly endemic regions, such as Africa, to determine the frequency of KSHV DNA detection in the mouth. A study involving HIV-1 positive heterosexual women in Zimbabwe reported that KSHV DNA could be detected at a high frequency in oral samples from women with KS but not in KSHV-seropositive individuals without KS (Lampinen *et al.,* 2000). However, only 9 study participants without KS disease, who were also seropositive for KSHV, were studied.

Similar sequences identified between mothers and their children, between siblings, and even between family groups suggested that both intra-familial and possibly community wide transmission of common KSHV variants occur in Malawi. If KSHV transmission in endemic regions does follow the transmission patterns of other related human herpesviruses, such as EBV, then the extent of KSHV infection in the oral samples from children needs to be addressed. Frequent oral carriage of KSHV was found among the children involved in this study. In 37% of children under 15 years of age, KSHV DNA could be detected

in their mouth rinses, demonstrating asymptomatic KSHV shedding in the saliva. The presence of KSHV DNA in the saliva of immunocompetent individuals without KS, particularly during childhood, points to likely non-sexual transmission through close contact with the saliva of KSHV infected individuals. Transmission through the saliva may be more important in endemic regions, such as Africa, where levels of KSHV infection are already high and where standards of living are lower than in developed countries with low KSHV seroprevalence. Two other highly endemic viruses in Africa, EBV and HBV, are more common in childhood in this geographical region than in developed countries. Poorer standards of living and hygiene likely facilitate transmission of these viruses in early life.

Chapter 4

Molecular epidemiology of KSHV evaluated by restriction fragment length polymorphism analysis of ORF 73 PCR products

4.1 Introduction

Restriction fragment length polymorphism (RFLP) techniques have been frequently used in epidemiological studies to determine viral genotype and to study patterns of transmission. Thus, RFLP analysis were used to type human strains of CMV (Kilpatrick *et al.,* 1976), to track CMV transmission between mothers and babies (Huang *et al.,* 1980), and to investigate nosocomial outbreaks of CMV infection (Buchman *et al.,* 1978). Furthermore, associations between subtype and pathology have been made for adenoviruses through RFLP analysis (Li *et al.,* 1996) and RFLP studies in immunocompromised patients have facilitated genetic analysis of common adenoviruses (Wigand and Adrian, 1991).

The RFLP technique utilises restriction enzymes that recognise specific base sequences in DNA. Most RFLP methods rely on mutations present at these restriction sites that are specific to different viral types. If mutations appear at other sites in the DNA sequence, they are not recognised by RFLP. Because RFLP analysis requires DNA as the starting material, viruses with RNA genomes can only be analysed after RT-PCR. Thus, hepatitis C virus (HCV) has been analysed in this way to determine which subtypes are most common in England and Wales (Harris *et al.,* 1999).

A PCR-RFLP technique based on mutations in the internal repeat domain (IRD) region of ORF 73 has been described (Zhang *et al.,* 2000). ORF 73 encodes LNA, a highly immunodominant antigen whose function has been described in Chapter 1. Molecular mass polymorphisms were identified in LNA, which were correlated with DNA size polymorphisms in the IRD region of ORF 73 (Gao *et al.,* 1999). The ORF 73 IRD region is made up of multiple heterogeneous repeat domains separated by short non-repeated sequences (Russo *et al.,* 1996). Sequence deletions and insertions, particularly in the second of these repeat regions, account for the DNA size polymorphism observed in ORF 73 IRD PCR products (KVNAtype) and for distinct RFLP patterns (Gao *et al.,* 1999; Zhang *et al.,* 2000). The ORF 73 IRD DNA size was stable in cell lines passaged over

time, in multifocal tumour samples in a single patient, and during both lytic and latent replication (Gao *et al.*, 1999). PCR-RFLP patterns were found invariable in multifocal lesions from single patients, suggesting that a single or dominant KSHV genome was responsible for the development of KS in an individual (Zhang *et al.*, 2000).

The work of Gao *et al.* (1999) and Zhang *et al.* (2000) showed that a unique KVNAtype and RFLP pattern were specific to an individual. KVNAtyping and RFLP analysis may thus provide a rapid system of typing that is more specific than current genotyping methods based on DNA sequence analysis. The KVNAtyping and PCR-RFLP methods were adapted for this study and applied to samples from Malawian families, in which two or more members were DNA positive for KSHV ORF K1, ORF 26 or both. This chapter describes the evaluation of the suitability of these techniques for the study of KSHV epidemiology and transmission.

4.2 Materials and methods

4.2.1 Study group

Study participants whose blood or mouth rinses were PCR positive for KSHV DNA in ORF K1, ORF 26 (Sections 3.4 and 3.5), or both, were included. DNA from the ORF 73 IRD was amplified from each sample by single round or nested PCR and digested using restriction enzymes *Mbo*I and *Ban*II as previously described (Section 2.7). All 25 participants from whom ORF 73 DNA was available are denoted in Table 4.1 and include 2 or more members in 8 families, and 5 more individuals.

4.2.3 Nested ORF 73 IRD PCR

Although the single round PCR used to amplify ORF 73 IRD DNA (LNA PCR) was reported to be extremely sensitive, insufficient amounts of DNA were amplified from some samples using only one round of PCR. This may have been due to a lower KSHV viral load in mouth rinse samples than in KS lesions

Individual	KS (Yes/No)	Age/sex	ORF K1 genotype	PCR-RFLP subtype
A_i (blood)	Yes	7y/F	B	2
A1	No	34y/F	N/A*	2
A2	No	10y/M	N/A*	2
B3	No	12y/M	B1	2
B5	No	7y/M	B1	2
E4	No	6y/F	B1	3
E5	No	13y/M	B1	3
E6	No	10y/M	B1	3
E2	No	9y/F	N/A*	3
G1	No	32y/M	A5	2
G2	No	23y/M	A5	3
G_i (blood)	Yes	34y/F	N/A*	3
H1	No	13y/F	N/A*	3
H2	No	12y/M	N/A*	3
K_i, K_i(blood)	Yes	30y/F	A5	2
K1	No	12y/M	A5	2
T2	No	11y/M	B1	3
T3	No	9y/M	B1	2
W2	No	20y/M	B1	3
W4	No	13y/F	B1	3
C_i	Yes	8y/M	B1	3
D1	No	18/F	A5	2
P_i	Yes	30y/M	N/A*	2
X1	No	44y/M	B1	3
Z2	No	22y/F	A5	2

Table 4.1. Characteristics of study participants selected for RFLP analysis. All samples were mouth rinses unless otherwise noted. ORF K1 genotype assignments as determined in 3.6.3. *Indicates K1/V1 DNA could not be amplified from these samples

and PBMC of KS cases. In order to increase the sensitivity of the PCR, a set of nested primers was designed, located outside the original first round primers described by Zhang *et al.* (2000) (Section 2.1.7).

Initially, nested PCR was attempted using identical PCR conditions as described for single round LNA PCR but without adding the enhancing agent, PCRx, provided with Platinum *Taq* DNA polymerase (Invitrogen, UK). These conditions resulted in a specific first round PCR product of the expected size but the nested product appearing as a high molecular weight smear when visualised on an agarose gel (Figure 4.1). Various strategies were attempted to clarify the second round product, including reducing the primer concentration in both rounds, diluting the first round PCR product, reducing the number of thermocycling rounds, titrating the magnesium chloride concentration in the reaction mixture, and adjusting the annealing temperature. Clarification of the second round product was achieved by adding the PCRx enhancing reagent at a concentration of 2X in both rounds of PCR and maintaining all other PCR conditions as described for single round PCR. The nested PCR results using different concentrations of PCRx reagent are shown in Figure 4.1.

When study participant blood and mouth rinse samples were amplified by nested PCR using the optimised method, some smearing or non-specific bands were also observed in the second round product. This may have been due to inconsistent amounts of DNA being added to the first round, since DNA extracts were not quantified. Dilution of the first round PCR product ten-fold often resulted in a correct second round product being produced. Figure 4.2 shows the results of diluting a first round PCR product to avoid non-specific or smeared second round PCR products. The single round IRD PCR showed that sample H2 was visibly positive after only one round of amplification (Figure 4.2). It was therefore unnecessary to attempt nested PCR on this sample, as a clear second round product would not be obtained. Accordingly, all samples were first amplified using only the single round LNA PCR to determine if nested PCR was necessary.

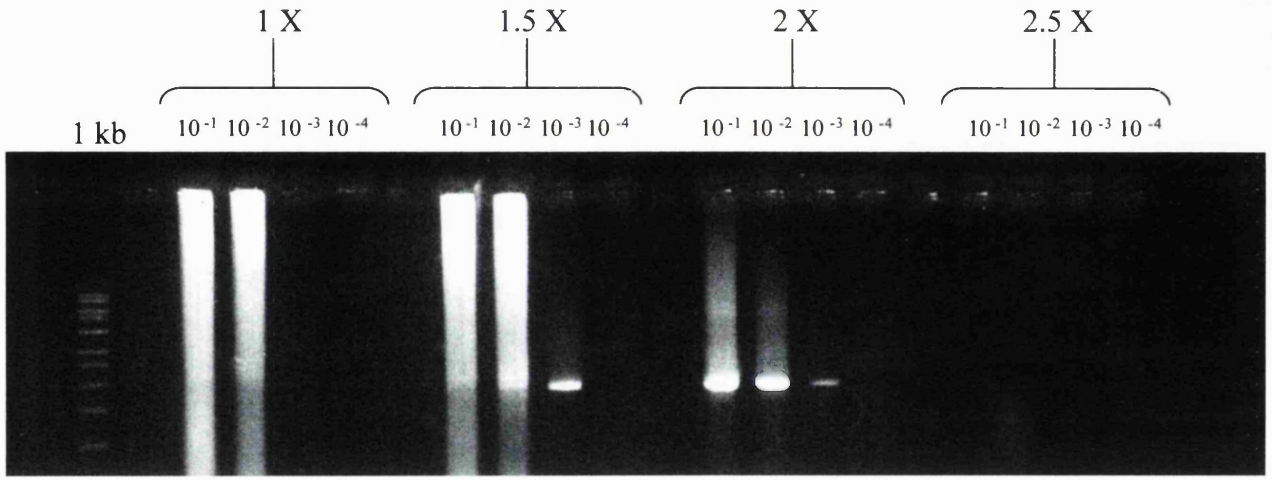


Figure 4.1 Optimisation of the nested ORF 73 IRD PCR using varying concentrations of the PCRx reagent supplied with Platinum *Taq* DNA polymerase. Ten fold dilutions of the KSHV infected cell line, BC-1, were used as a controls. A 1.5X concentration of PCRx in both rounds of PCR mix produced a specific product at a dilution of 10^{-3}. A 2X concentration produced a specific product for dilutions 10^{-1} to 10^{-3}.

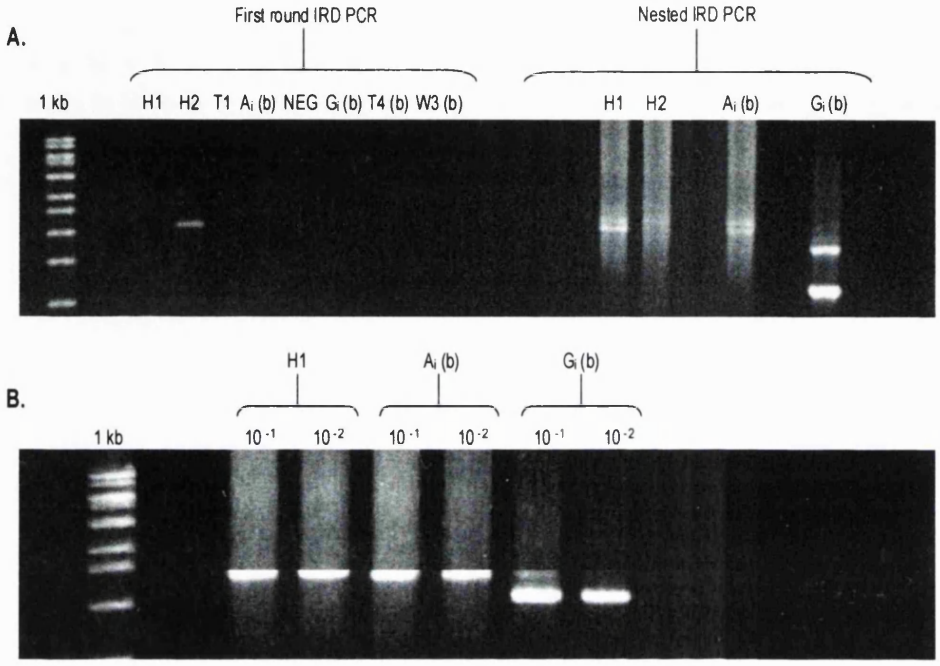


Figure 4.2 Single round and nested PCR to amplify the IRD region of KSHV ORF 73. First round PCR was performed using primers designed for this study (Section 2.7.1). As shown in panel A, additional samples were PCR positive after nested PCR, however non-specific products were also produced. Dilution of the first round product ten-fold produced a specific second round product (panel B). ORF 73 IRD DNA was amplified from sample H2 by single round PCR. (b) indicates blood samples

4.3 KVNAtyping

The results of either single round or nested PCR for the ORF 73 IRD region are shown in Figure 4.3. The typing system introduced by Gao *et al.* (1999) based on molecular polymorphism in the LNA region of KSHV, KVNAtyping, is applicable to these samples. Samples with PCR products of different sizes are considered to have a different KVNAtype. Based on this, it is seen that samples in families B (B5 and B3), G (G1, G2 and G) and W (W2 and W4) were infected with KSHV genomes of different KVNAtype. However, in families A, E, H, K, and T, ORF 73 IRD products appeared to be the same size indicating identical KVNAtypes were present between members of these families. Indeed, the sizes of ORF73 IRD PCR products were similar for many of the study participant samples. In family E, it appeared that some size differences were present between samples but they were too small to identify clearly. KVNAtypes between different families and individual samples were also difficult to discriminate between.

4.4 RFLP subtyping

RFLP analysis was able to provide further information about the sequence of the ORF 73 IRD region in study participant samples. Some samples with similar or identical KVNAtyping patterns were distinguishable by their RFLP patterns (Figure 4.4). In family E, small differences were now obvious between family member sequences although their RFLP patterns were still very similar. In family E, identity was found between E6 and E2 and non-identity between E4, E5 and E2/E6. Identity in RFLP patterns was present in 3 additional families, A, H and K. Patterns of non-identity were demonstrated in 4 families, B, G, T and W. Of the additional study subjects from whom RFLP patterns were analysed, all but one appeared to have unique RFLP patterns. RFLP patterns revealed from samples C and W4 were indistinguishable.

Based on sequencing data from KSHV infected BC-1 and PK-1 cell lines, Zhang *et al.* (2000) characterised four possible RFLP subtypes (1-4). Genetic variation

Figure 4.3 ORF 73 IRD nested and single round PCR results from (A) samples A1-H2 and (B) samples K_i-P_i. Lanes marked BL=blank.

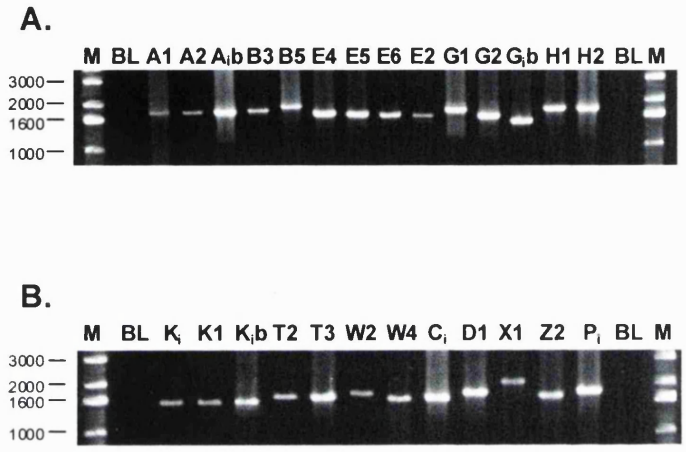
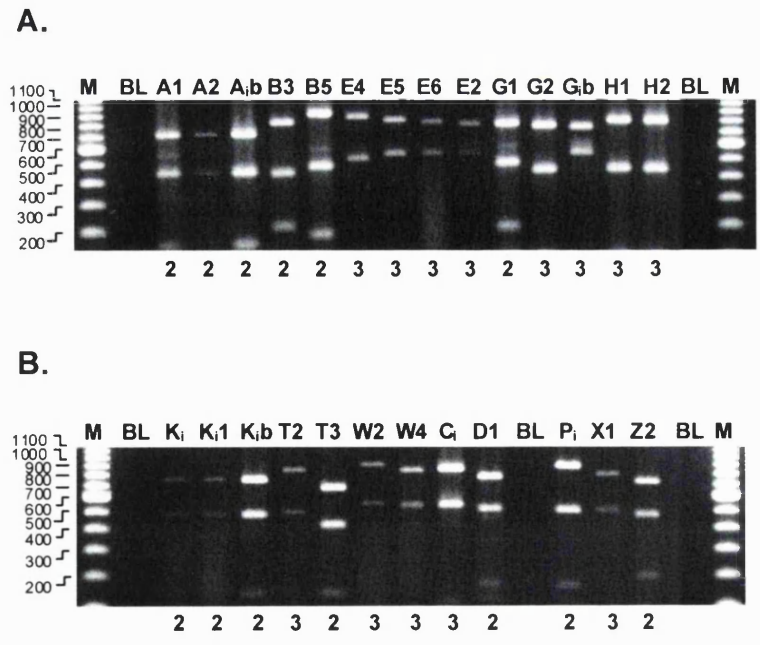


Figure 4.4 PCR-RFLP analysis of the ORF 73 IRD PCR products from (A) samples A1-H2 and (B) samples K_i-P_i. Lanes marked BL=blank. PCR-RFLP subtype assignments are indicated beneath each lane



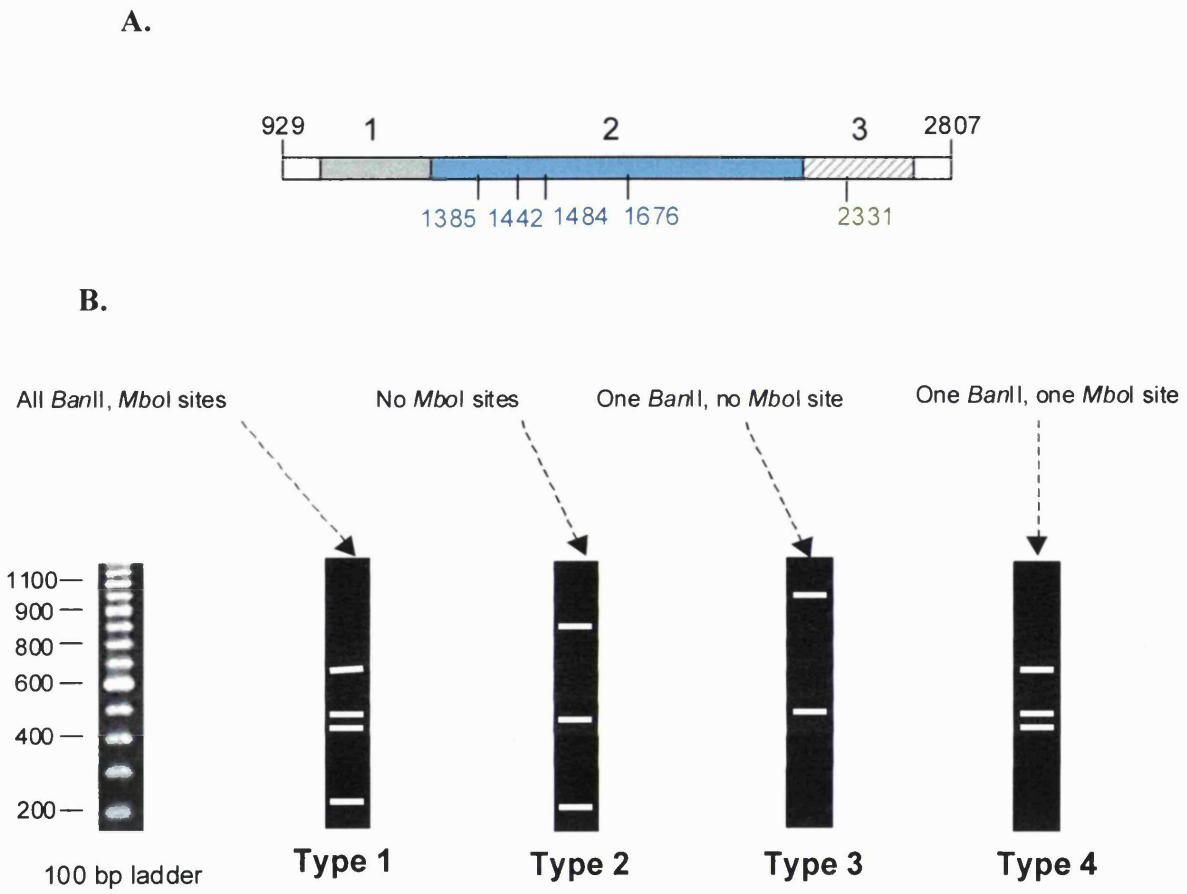


Figure 4.5 Location of the *Ban*II (blue) and *Mbo*I (green) restriction sites in the three repeat regions of the ORF 73 IRD (A). Base locations refer to the BC-1 KSHV sequence (Russo *et al.*, 1996). The four possible RFLP genotypes based on ORF 73 IRD sequence and presence or absence of restriction sites are illustrated in B (Zhang *et al.*, 2000).

in the IRD concentrated in the second repeat region resulted in the loss of expected restriction sites, creating the four different RFLP patterns (Figure 4.5). Thus, in the KSHV IRD of the BC-1 cell line, there are five possible *Ban*II restriction sites, which will produce four bands that can be visualised on an agarose gel, and one *Mbo*I restriction site creating two visible bands. Subtype 1 samples, such as BC-1, have an IRD with both *Ban*II and *Mbo*I restriction sites, resulting in bands of approximately 655, 476/457 and 192 bp in length. The bands at 476 bp and 457 bp cannot always be distinguished. Subtype 2 samples have at least 2 *Ban*II sites but no *Mbo*I site, resulting in three visible bands at approximately 1121, 457 and 192 bp. There is only one *Ban*II site in subtype 3 samples, resulting in two bands at 1131 and 457 bp while subtype 4 has one of each restriction site, resulting in three visible bands with the 192 bp band missing. All band sizes are approximate, since these will vary depending on the extent of sequence variation in individual IRD regions. A schematic illustration of PCR-RFLP patterns representing the 4 RFLP subtypes is shown in Figure 4.5.

The subtype assignments for all study participant samples are noted in Figure 4.4. In the study of Zhang *et al.* (2000), no correlation was found between the four KSHV ORF K1 subtypes and RFLP subtypes. Where possible, RFLP subtype assignments were compared with ORF K1 subtype assignments (Section 3.6.3) for individual study participants. No correlation was found between these two systems of subtyping in the Malawi sample set (Table 4.1).

4.5 Discussion

4.5.1 RFLP comparisons between family members

Distinct RFLP patterns were obtained from study participant samples allowing comparisons to be made between family members without the need for PCR-sequencing. RFLP identity, and therefore evidence of familial KSHV transmission, was present in 4 families (A, E, H and K). In families A and K, identity was found between mothers (A1 and K_i) and their children, sons (A2/K1), and daughter (A_i). Identical RFLP patterns were shared between siblings: brothers E5 and H2, and their corresponding sisters E2 and H1.

Non-identical RFLP patterns were recovered from members of 5 families (B, G, E, T and W), which suggested transmission of KSHV had occurred outside the family. In 2 families (T and G), subtypes 2 and 3 were found in each family, while in families B, E and W, all samples were of the same subtype but had RFLP patterns with differing band sizes. These results indicate that the IRD region amplified from each sample possesses different types of nucleotide insertions and deletions. While the presence of non-identical RLFP patterns between family members may implicate extra-familial transmission, as was inferred from the ORF K1 sequencing data (Section 3.7.2), ORF 73 could not be amplified from all family members. It is still possible that other family members may have acted as past sources of infection.

4.5.2 Comparison of RFLP patterns and ORF K1 sequencing

In families B, E, G, K, T and W, ORF K1/V1 sequencing results (Chapter 3) and ORF 73 IRD RFLP results were available. Siblings B3 and B5 yielded different RFLP patterns and different ORF K1/V1 sequences; however, these differed by only 3 synonymous nucleotide changes. The additional information provided by RFLP typing might further substantiate the nucleotide divergences that were revealed between these siblings. ORF K1/V1 sequencing revealed minor nucleotide divergences between siblings E4, E5 and E6; E2 was not available for ORF K1 analysis. RFLP patterns demonstrated some ORF 73 sequence differences between these siblings. However no comparison could be made with their mother, E_i, from whom no ORF 73 IRD PCR product was available. RFLP analysis also concurred with ORF K1/V1 sequence polymorphism data between siblings in family W (W2 and W4) and in family T (T2 and T3).

In family K, identity was found between a mother (K_i) and son (K1) in both RFLP patterns and in ORF K1/V1 nucleotide sequences. In family G, identity was found between a father and son (G1 and G2) when ORF K1/V1 sequences were compared, although the ORF K1/V1 sequence from the mother, (G_i), was not available for analysis. ORF 73 IRD PCR products could be amplified from all three members of this family to make comparisons between their RFLP patterns. This revealed differences between all three family members both in

RFLP subtype and in KVNAtype. It is therefore unlikely that these family members carried viruses with identical genomes.

4.5.3 Suitability of the method for epidemiological study

Four genotypes have now been distinguished by ORF 73 IRD RFLP analysis (Zhang *et al.,* 2000) based on sequence polymorphism and loss of certain restriction sites. A comparison between the RFLP subtypes recovered from study samples and subtypes determined by ORF K1 sequence data (Section 3) found no correlation (Table 4.1.). These four RFLP subtypes only describe broad categories of sequence polymorphism in the IRD region and do not reflect all insertions or deletions occurring between restriction sites. Among the samples included in this study, only two subtypes (2 and 3) were found. Nevertheless, greater diversity was present between samples belonging to the same subtype.

The discrepancies in RFLP patterns found between members of the same subtype, which are sometimes subtle, may make it difficult to compare results from one study to another unless accurate measurements of the RFLP band sizes are taken. Nevertheless, the variety of RFLP patterns recovered from these study samples indicated that the IRD region of the KSHV genome is highly polymorphic and potentially provides a more specific indication of sequence variability than ORF K1 sequence data alone. It was also possible to apply the RFLP method to a set of samples from individuals who were not affected by KS. Furthermore, the method provided a rapid comparison between samples derived from members of the same family. Therefore it was possible to confirm patterns of sequence similarity found in ORF K1 sequence data or to find negative correlation, as was the case in samples from family G. This RFLP method would be appropriate for use in epidemiological studies in which specific sequence information is required and where PCR sequencing would be too time consuming or impractical.

Chapter 5

Investigating intra-individual KSHV variability using denaturing gradient gel electrophoresis

5.1 Introduction

Nucleotide sequencing of PCR products is often necessary to accurately determine viral subtype. Nucleotide sequencing has been used to gain extensive knowledge about the evolutionary history of KSHV and to establish a system of subtyping, as discussed in Section 1.4. This information was gathered by analysing small loci spread across the entire length of the genome and through phylogenetic comparisons between sequences. Phylogenetic analysis methods have been discussed in Section 1.7. Molecular epidemiology is an important application of DNA sequencing when comparisons between sequences at the nucleotide level and subsequent phylogenetic analyses are necessary. In the study described in Chapters 3 and 4, DNA sequencing was used to track the transmission of KSHV among Malawian family members by studying segments of the hypervariable ORF K1 genomic region. While this technique can be extremely accurate it is also time-consuming, making analysis of many samples difficult. To study intra-individual genomic KSHV variability, many clones would need to be analysed. Consequently, a range of non-sequencing based methods was considered in order to more effectively identify mutations in multiple PCR clones.

5.1.1 Gel based mutation screening methods

A variety of gel based mutation detection methods have been developed to screen PCR products for mutations without sequencing, many are able to distinguish one base pair mutations. Mutation detection can be used to trace patterns of transmission, identify viral genotype, and assess intra-patient viral mutations (Arens, 1999).

Single strand conformation polymporphism (SSCP)

SSCP was developed to detect polymorphism in single stranded pieces of DNA by observing changes in the mobility of the DNA when run through a neutral polyacrylamide gel (Orita *et al.,* 1989). The secondary structure of DNA will affect its mobility, allowing single nucleotide substitutions to be detected by

relative positioning on the gel. DNA for this technique was originally derived from RFLP fragments but can now be generated using PCR. Radiolabelled bases are incorporated into the PCR product and can be visualised using autoradiography (Orita *et al.,* 1989). Non radioactive methods using silver staining (Ainsworth *et al.,* 1991) and automated DNA sequencers are now available, but radioactive methods are often preferred. Sensitivity of the technique has been reported to be as low as 35% (Sarkar *et al.,* 1992) and as high as 100% (Orita *et al.,* 1989), depending on the size of DNA fragment used. Optimal results are obtained using a fragment of 200 bp, an increase in fragment size leading to a decrease in sensitivity (Hayashi and Yandell, 1993; Sheffield *et al.,* 1993). SSCP analysis has been used to determine HCV genotype (Lareu *et al.,* 1997) and to investigate HBV disease outbreaks through genotyping (Hardie *et al.,* 1996).

RFLP

In this method, PCR products are cleaved in a sequence dependent manner using restriction endonucleases, resulting in distinct banding patterns visualised by agarose gel electrophoresis. As shown in the previous chapter, this technique is particularly useful for genotyping where a particular banding pattern represents a specific genotype. In general, a well-conserved region of the genome is selected for PCR amplification and mutations are detected only if they occur at a restriction site. The technique requires a large amount of sequence data to be generated before appropriate endonucleases can be selected.

Heteroduplex mobility assay –HMA

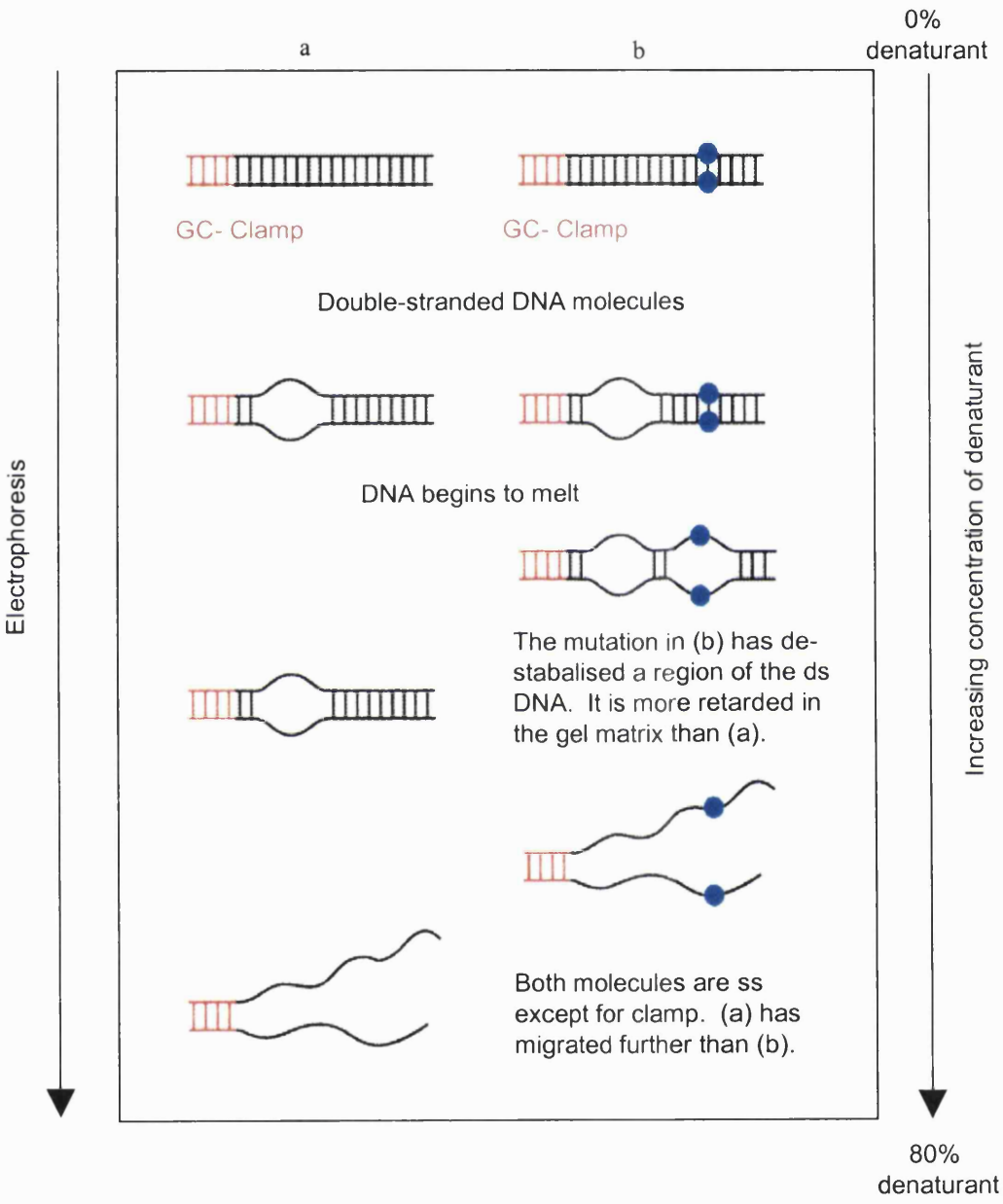
This technique was developed by studying the behaviour of synthetic DNA duplexes when electrophoresed through a cross-linked gel (Wang and Griffith, 1991) and is now used to study the genetic variability of viruses. Heteroduplexes are formed by denaturing and re-annealing a mixed population of PCR products; this will result in duplexes with the same sequence (homoduplex) and those with a sequence mismatch (heteroduplexes).

Heteroduplexes will migrate more slowly when electrophoresed through a non-denaturing gel than the homoduplexes, the relative retardation of the heteroduplex in relation to the homoduplex being associated with the number of mismatch base pairs (Barlow *et al.,* 2000). This technique has been used to genotype HIV when PCR products of known genotype are mixed with PCR products from the sample whose genotype is not known (Tatt *et al.,* 2000) but has not yet been applied to the study of KSHV.

Denaturing gel gradient electrophoresis – DGGE

Developed primarily for the detection of genetic mutations in human disease, DGGE can resolve single nucleotide differences between DNA fragments. The technique is based on variations in the electrophoretic mobility of double stranded DNA through a gradient of increasing concentrations of formamide or urea denaturants (Myers *et al.,* 1987). The DNA fragment will migrate through the gradient gel until it reaches a point where the concentration of denaturants is equal to the melting temperature, T_m, of its lowest melting domain. This will cause the fragment to unravel, thus retarding its progress through the gel. The T_m of any domain is dependent on its nucleotide composition and mutations in low melting point domains of as few as one nucleotide will affect the fragment's mobility and ultimate position in the gel. When the mutation occurs in the domain with the highest T_m, the strand will disassociate completely and the nucleotide change will not be detected. Use of a 'GC Clamp', a high GC rich domain introduced during PCR to one end of the fragment, increases sensitivity by preventing the DNA from becoming completely denatured under DGGE conditions (Sheffield *et al.,* 1989; Fodde and Losekoot, 1994). A schematic illustration of the DGGE procedure is included in Figure 5.1.

Figure 5.1 Schematic diagram illustrating the concept of DGGE.



5.1.2 Studying intra-individual KSHV variability

Nucleotide sequencing of multiple samples from the same individual, from multifocal KS lesions or from different sample types (e.g. PBMC and KS lesions), has revealed invariant KSHV sequences (Poole *et al.,* 1999; Stebbing *et al.,* 2001; Zong *et al.,* 2002).

RFLP analysis has confirmed these findings by recovering identical RFLP patterns from multifocal lesions and in different sample types from an individual (Zhang *et al.,* 2000). Analysing large numbers of cloned PCR products was not done in many of these studies, particularly in studies using PCR sequencing where such analysis would have been impractical. In other studies, multiple clones were analysed and some divergent sequences were recovered (Zong *et al.,* 2002). However, the number of clones was not large, making a meaningful analysis of the results difficult. Because direct PCR sequencing will not identify minor populations of the virus, it is necessary to study a large number of cloned PCR products to determine the extent of multiple KSHV infection in an individual. The denaturing gradient gel electrophoresis (DGGE) technique was selected to rapidly screen a large number of cloned PCR products derived from the K1/V1 region of KSHV in selected study participants. This region was chosen because of its hypervariable nature and because ORF K1/V1 sequence data was already available for each individual. Using this technique, mutations of as few as one base pair could be detected and up to 40 clones could be analysed from one gel. To determine if family members carried minor variants in common with each other, samples from two members in each selected family were analysed.

5.2 Materials and methods

5.2.1 Study participants

Three families were chosen for DGGE analysis, two in which divergent ORF K1/V1 sequences were recovered (T and X) and one in which an identical K1/V1 sequence was identified (G). Full details of these family members have

been previously described and are summarised in Table 3.1. ORF K1/V1 DNA was amplified from mouth rinse samples in all individuals.

5.2.2 Methods

ORF K1/V1 sequences were amplified as described in Section 2.1.5 and gel purified nested PCR products were cloned using TOPO TA cloning (Section 2.4). Thirty-five to 40 clones picked from L-agar plates were directly amplified by PCR using the inner ORF K1/V1 primers (Section 2.1.7). A 40-bp G-C rich "clamp" sequence was added to the 5' end of the forward inner primer that was then incorporated into the PCR product during amplification (Section 2.6.1). PCR amplified clones were visualised by agarose gel electrophoresis (Section 2.1.7). Colony PCR products were electrophoresed through a polyacrylamide gel containing a gradient of denaturant (formamide and urea) from 30% to 50% (Section 2.6.3) and visualised with SYBR Green (Section 2.6.4). The addition of the GC clamp directly to the colony PCR product allow all mutations, even those in the highest melting domain, to be identified by preventing the PCR product from becoming completely denatured. DNA sequencing of variant clones was carried out as previously described (Section 2.3) and compared with the majority sequence.

5.2.3 Optimisation of denaturant gradient

Additional ORF K1/V1 clones were produced and sequenced from study participants B3, B5, E4 and E5, identifying those that differed by one or more base pairs from the other clones. Four to 5 clones from each sample were included in the DGGE optimisation panel used to select the appropriate denaturant gradient for the K1/V1 PCR product. A multiple alignment of all clones included in the panel is shown in Figure 5.3. Colony PCR products from the optimisation panel were first electrophoresed through a 12% polyacrylamide gel with a gradient of 10% to 70% denaturant at 100 V for 16 hr. Owing to the presence of the G-C clamp region, the product will cease to migrate once the remainder of the PCR product is completely denatured. Thus, it was possible to run the gel overnight with no adverse effect on the results. The migratory position of all clones is shown in Figure 5.2 upper panel. The gradient was able

to detect 2 bp mutations between clones but was not able to differentiate those differing by only 1 bp. The colony PCR products comprising the optimisation panel were analysed using several narrower gradients containing different concentrations of polyacrylamide. The optimal conditions for DGGE analysis of the ORF K1/V1 PCR product was a gradient of 30% to 50% denaturant in a 10% polyacrylamide gel (Figure 5.2 lower panel). This gradient allowed single nucleotide mutations to be recognised. Those products differing by 1 bp from surrounding products are marked with a * in figure 5.2.

5.3 Results

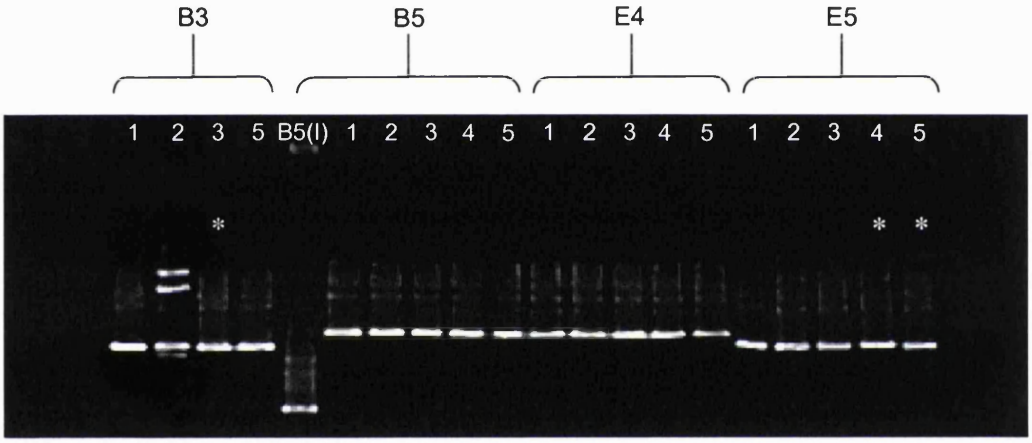
5.3.1 K1/V1 variation as studied by DGGE

The DGGE results for samples in family G are shown in Figure 5.4, for family T in Figure 5.7, and for family X in Figure 5.10. Those clones marked with a * had been sequenced. Sequencing of selected clones was necessary to determine both the sequence of the majority of clones isolated from a single individual and to investigate the level of intra-individual diversity present. Alignments of all sequenced clones from each family member are shown in Figures 5.5 and 5.6 (family G), Figures 5.8 and 5.9 (family T) and Figures 5.11 and 5.12 (family X).

In all family members, a majority sequence was identified by DGGE along with a variable number of clones differing by 1 or 2 bp from the majority. In most cases, the majority sequence was identical to the consensus ORF K1/V1 sequence from each sample. In X2, the consensus sequence differed by 1 bp from the majority sequence as determined by DGGE. In families T and X, where divergent ORF K1/V1 sequences were originally revealed (Section 3.7.2), no minority sequence was common to both individuals (Figures 5.8, 5.9 and 5.11, 5.12). In family G, the majority sequence was identical in both G1 and G2 (Figures 5.5 and 5.6), as the original consensus ORF K1/V1 sequence data suggested (Section 3.7.1).

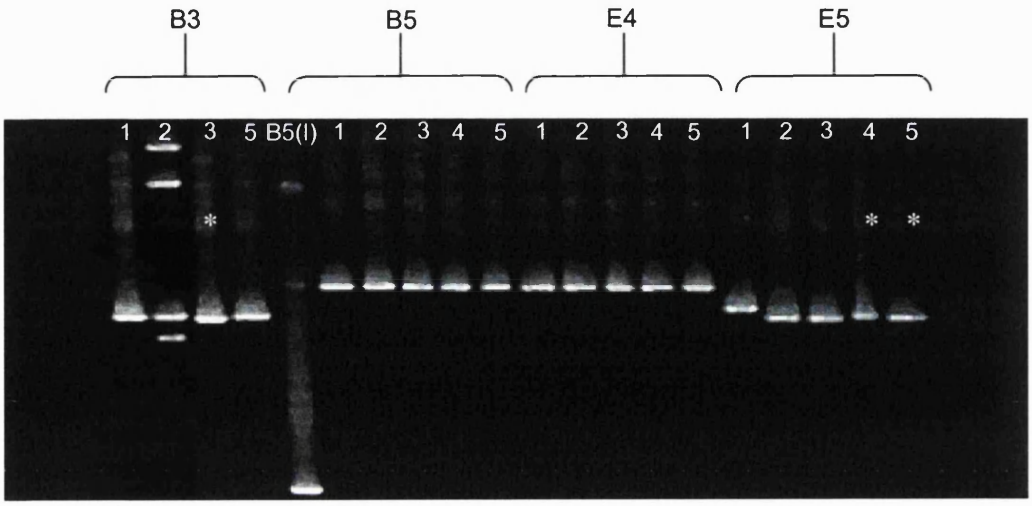
Figure 5.2 DGGE optimisation panel for ORF K1/V1 PCR products. Figure 5.3 shows an alignment of all clones. In A, a denaturant gradient of 70% to 10% was used. In B, a gradient of 50% to 30% denaturants was used in a 10% polyacrylamide gel allowing greater discrimination.

A.



70% to 10% denaturant, 12% polyacrylamide

B.



50% to 30% denaturant, 10% polyacrylamide

Figure 5.3 Clustel W multiple alignment of the ORF K1/V1 sequences of clones included in the optimisation panel (figure 5.2). The original variant B5 (I) sequence is designated by ‡

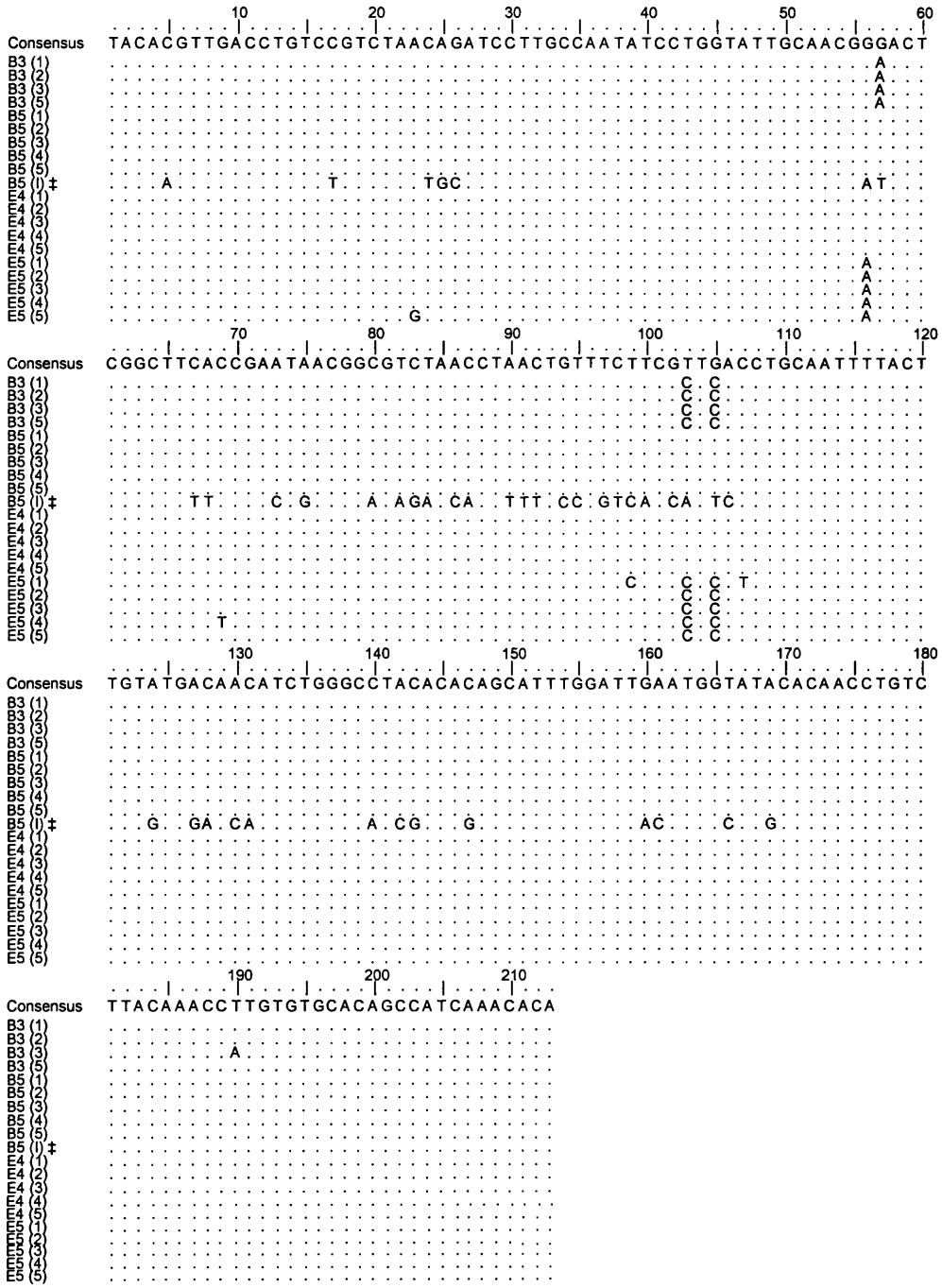
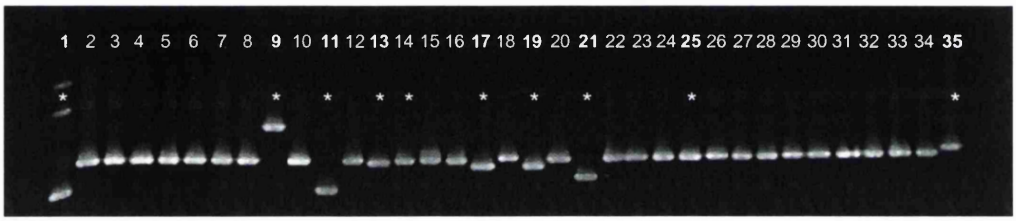


Figure 5.4 DGGE results from G1 and G2 ORF K1/V1 PCR products. Lanes marked with * indicate those samples that were sequenced. A sequence could not be recovered from clone G2 (lane 30).

G1



G2

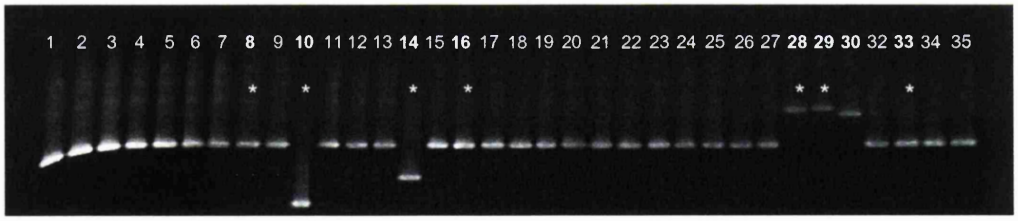


Figure 5.5 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from G1 PCR product clones designated by * on figure 5.4. § indicates the consensus G1 K1/V1 sequence. Samples denoted in blue are the majority sequence as determined by DGGE.

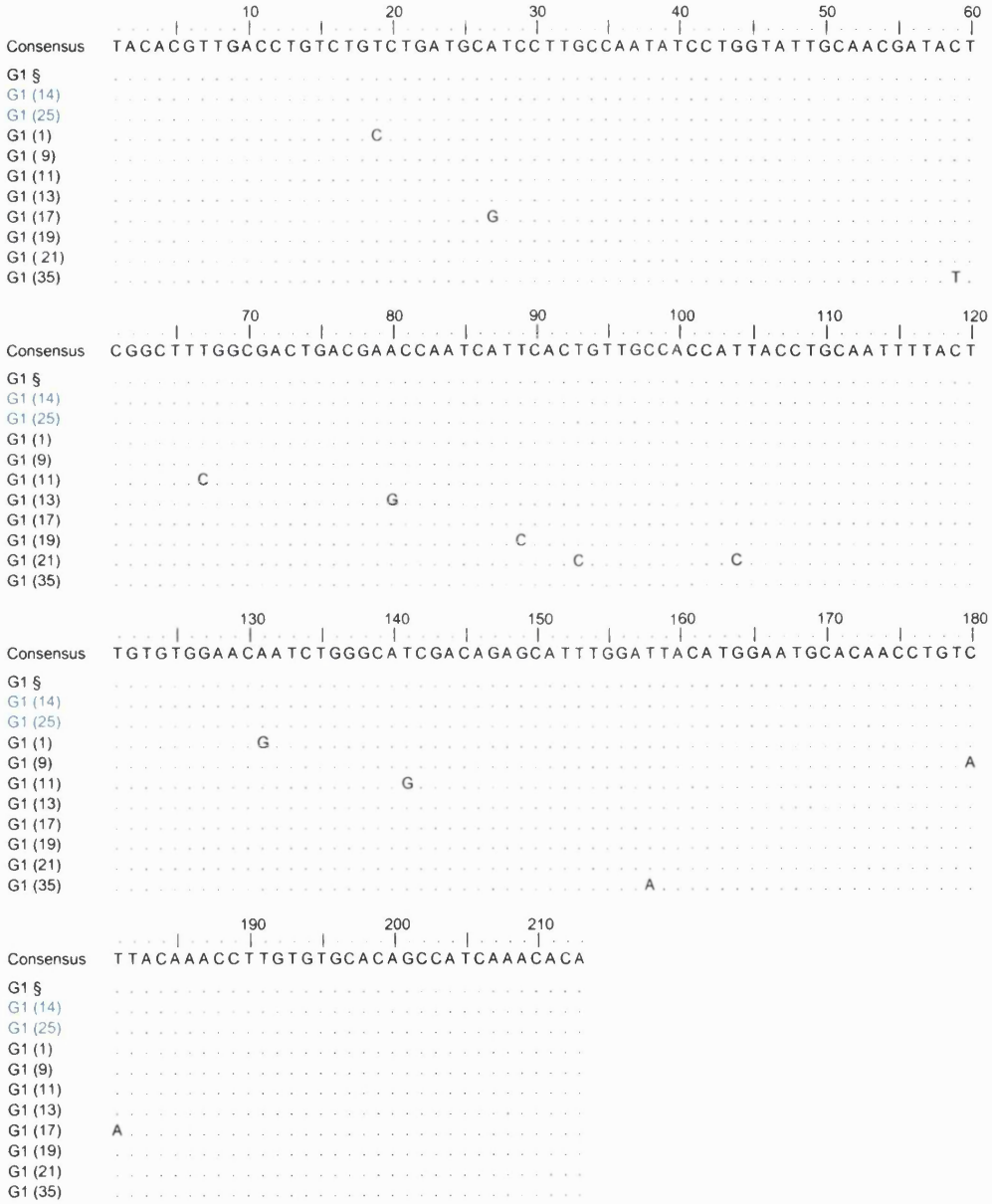


Figure 5.6 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from G2 PCR product clones designated by * on figure 5.4. § indicates the consensus G2 K1/V1 sequence. Samples denoted in blue are the majority sequence as determined by DGGE.

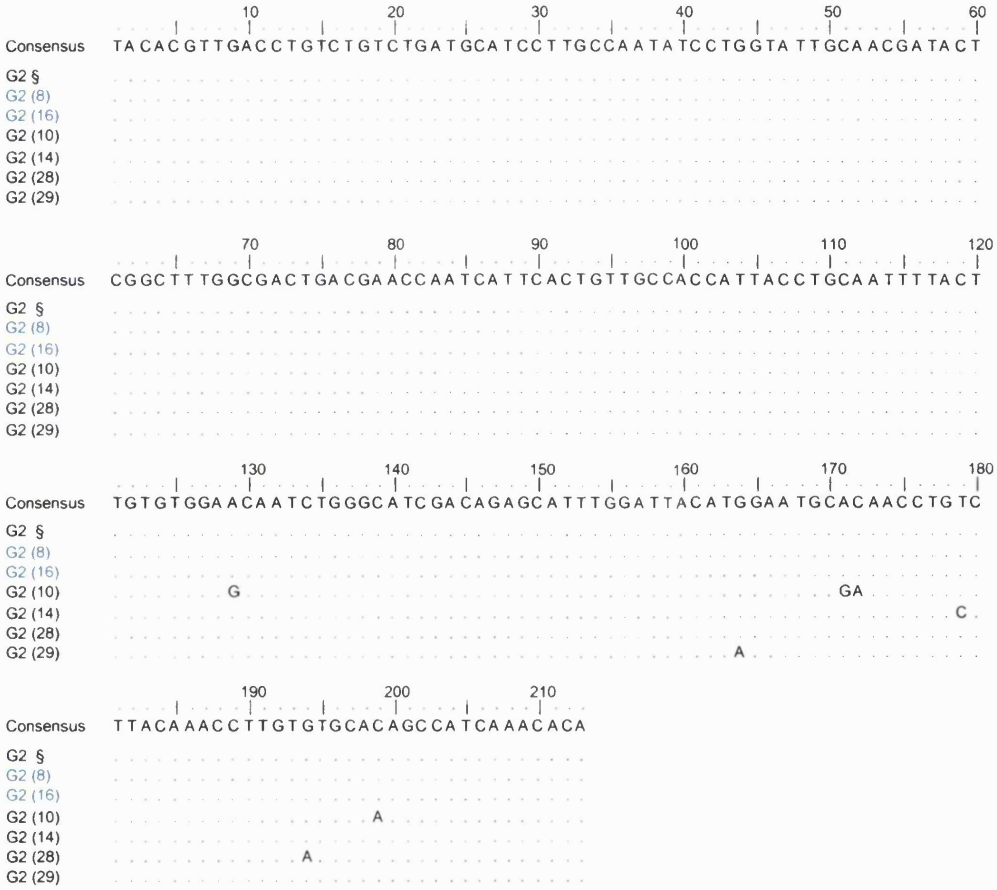


Figure 5.7 DGGE results from T2 and T3 ORF K1/V1 PCR products. Lanes marked with * indicate those samples that were sequenced.

T2

T3

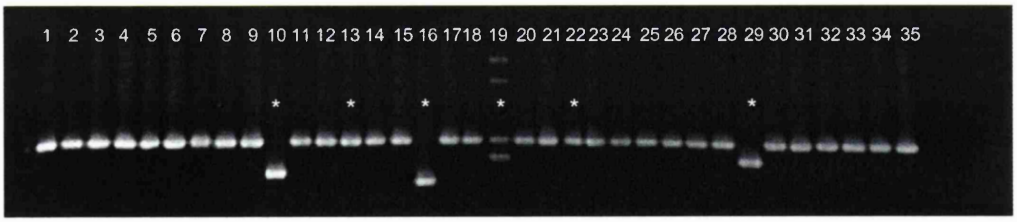


Figure 5.8 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from T2 PCR product clones designated by * on Figure 5.7. § indicates the consensus T2 K1/V1 sequence. Samples denoted in blue are the majority sequence as determined by DGGE.

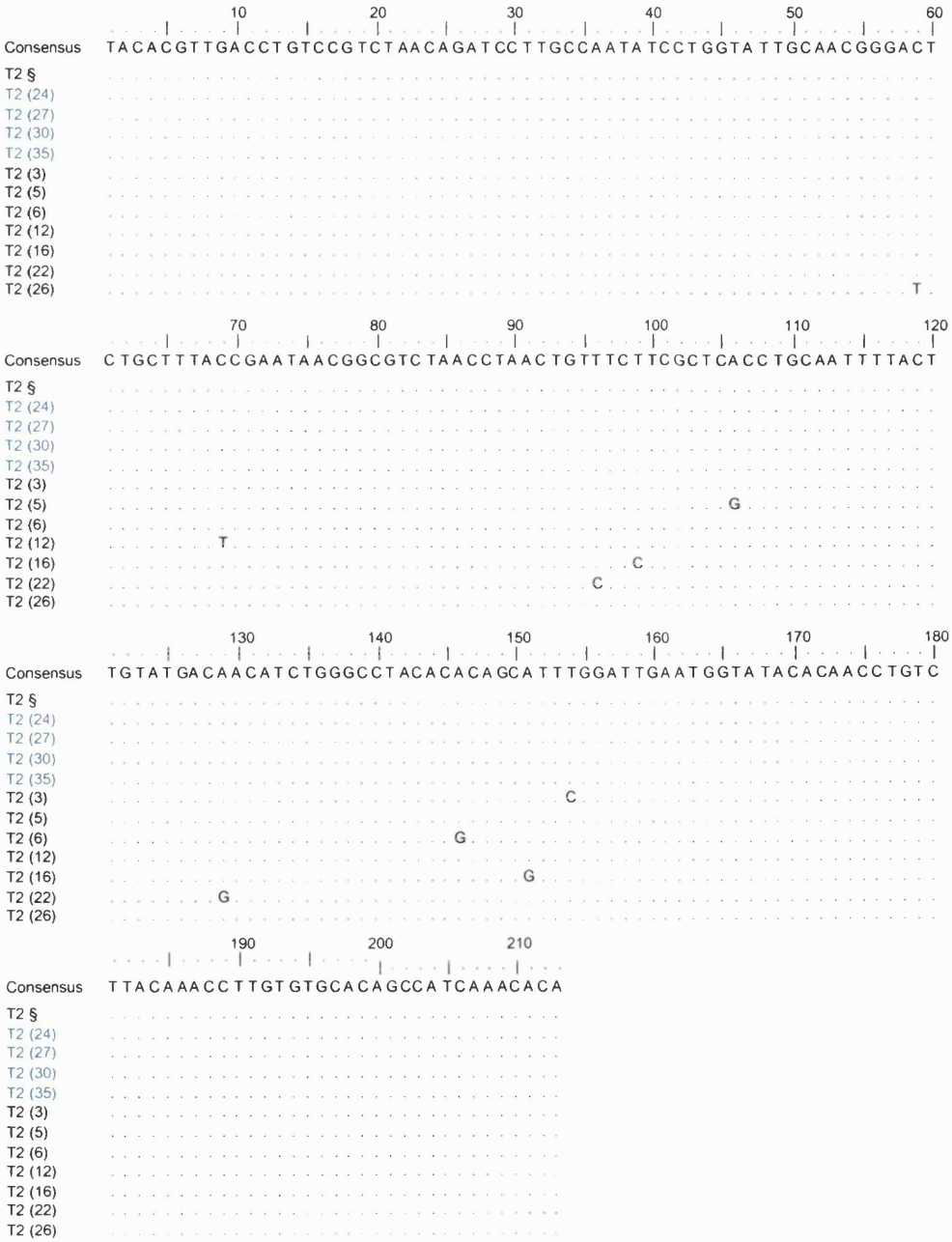


Figure 5.9 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from T3 PCR product clones designated by * on figure 5.7. § indicates the consensus T3 K1/V1 sequence. Samples denoted in blue are the majority sequence as determined by DGGE.

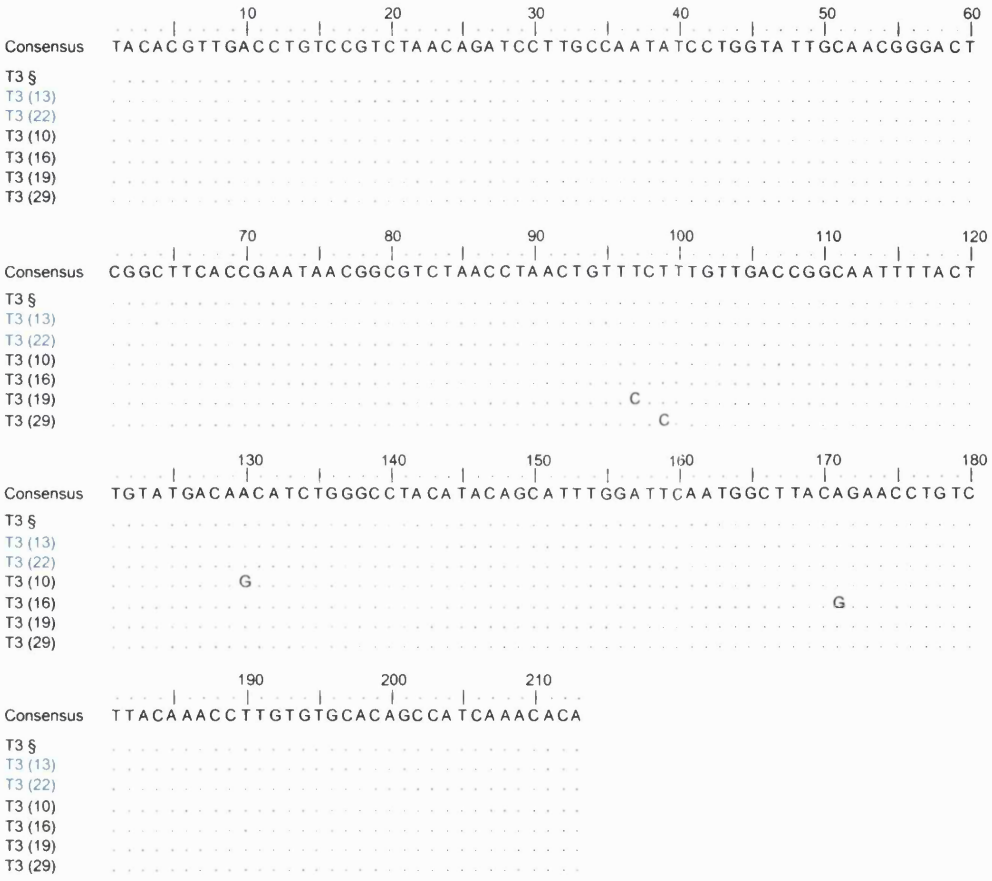
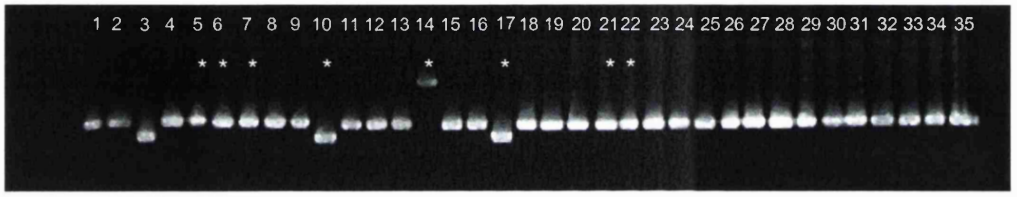


Figure 5.10 DGGE results from X1 and X2 ORF K1/V1 PCR products. Lanes marked with * indicate those samples that were sequenced. A sequence could not be recovered from clone X1 (lane 3).

X1



X2

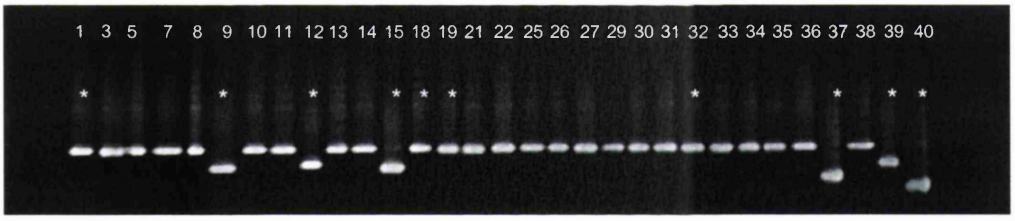


Figure 5.11 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from X1 PCR product clones designated by * on figure 5.10. § indicates the consensus X1 K1/V1 sequence. Samples denoted in blue are the majority sequence as determined by DGGE.

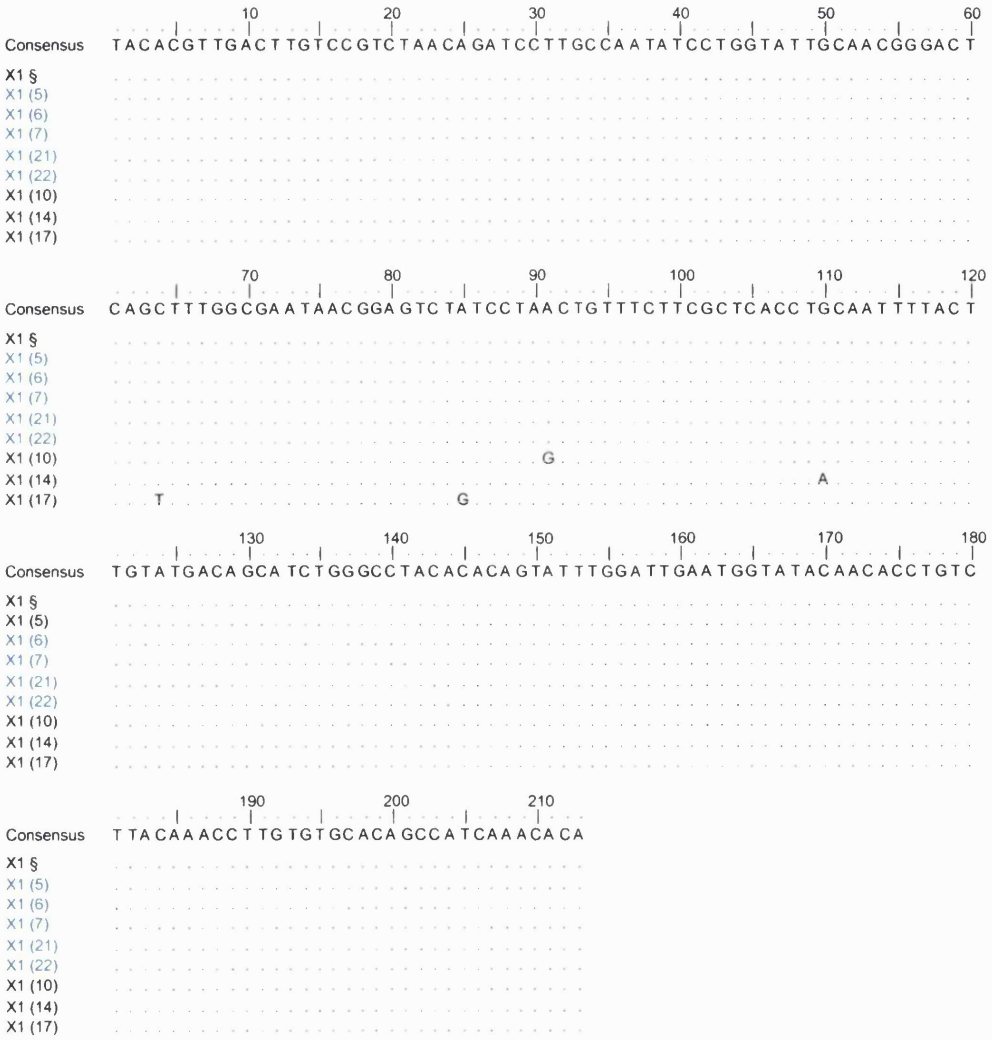
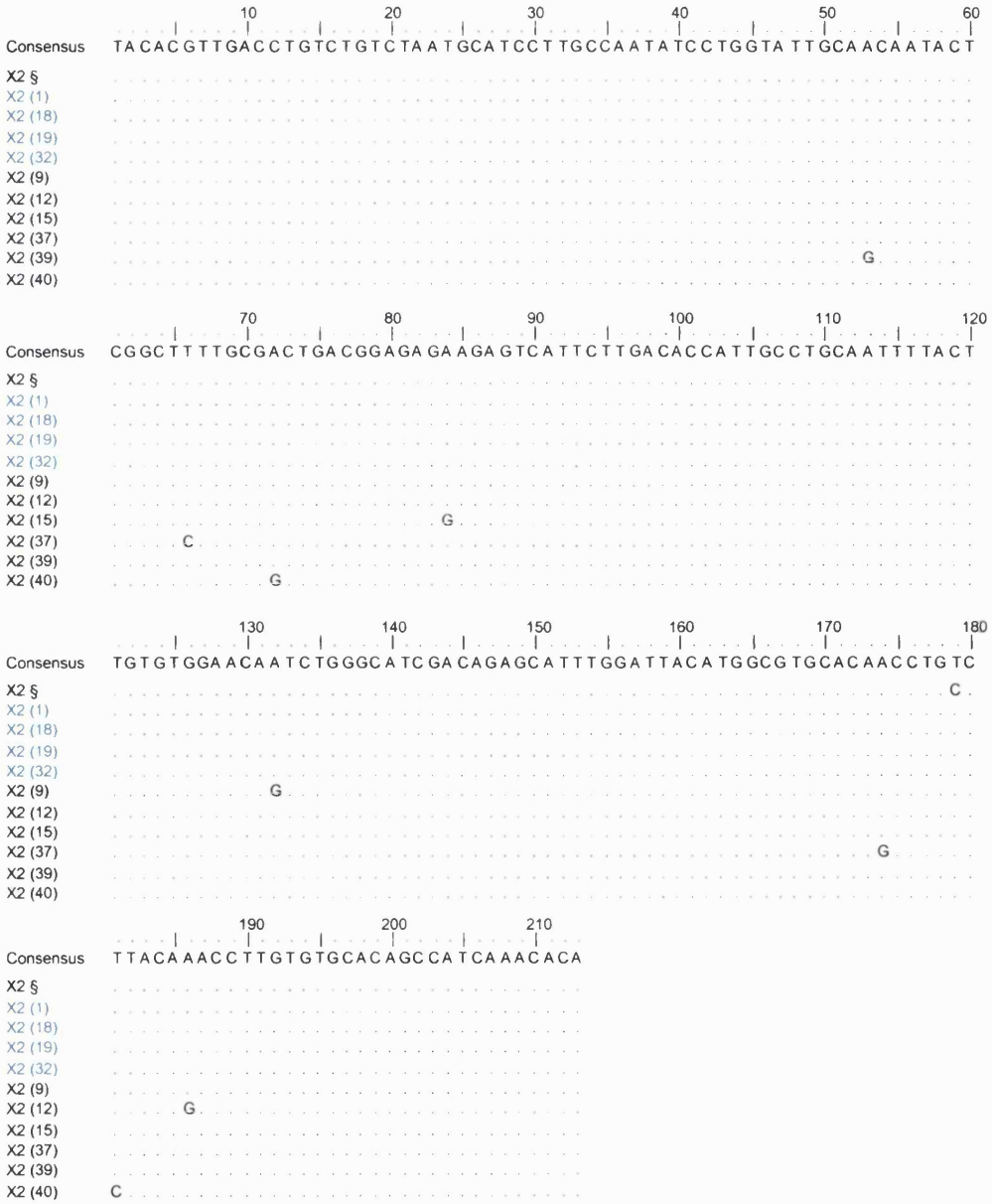


Figure 5.12 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from X2 PCR product clones designated by * on figure 5.10. § indicates the consensus X2 K1/V1 sequence. Samples denoted in blue are the majority sequence as determined by DGGE.



5.3.2 Significance of multiple DGGE band formation

For 2 of the clones, a pattern of 4 bands was visualised following DGGE (Figure 5.4, G1, lane 1 and Figure 5.7, T3, lane 19). There are several potential explanations for this observation. The lower two bands may represent two amplicons with different sequences. The upper two bands are a heteroduplex of these two sequences, formed in later cycles of PCR amplification. The heteroduplexes, with lower melting temperatures due to sequence mismatches, migrate more slowly through the gel than homoduplexes. Multiple sequences can be present in a single clone via transformation of competent *E. coli* with more than one plasmid, each containing an insert with a different sequence, during transformation and cloning. No evidence of multiple peaks (a characteristic sign of mixed infection) was found in chromatogram data recovered from samples with heteroduplex banding patterns. However, a 1 bp mutation will cause a change in the migratory distance of a PCR product on DGGE. Therefore, it is possible that two sequences differing by only 1 bp may not be readily recognisable from the chromatogram data. In addition, dimers between two fragments with different sequences can form prior to the cloning and transformation steps. The competent cells can then take up the double size insert and both sequences will amplified during colony PCR. This will result in the formation of multiple bands similar to the superinfection model described above. The multiple sequences present in a single colony PCR product may also be due to *Taq* polymerase misincorporation error. However, even if this error occurred in the first round of PCR it is unlikely that enough of the PCR product containing the artificial mutation would be produced to be visible on the polyacrylamide gel.

There are other possible explanations for multiple bands appearing on DGGE, including the presence of *E. coli* DNA. No purification step was included following colony PCR and it is possible that genomic bacterial DNA was present in the PCR sample. However, only 2 of the clones displayed unusual band patterns despite all clones being handled in a similar manner. Multiple bands on DGGE may reflect the presence of primer-dimers or non-specific bands. These fragments may not have been visible on an agarose gel but were

enhanced when run on a polyacrylamide gel stained with SYBR Green. This explanation is unlikely to be the cause of heteroduplex formation since the samples do not appear to be overloaded on the gel and more than this small number of clones would be affected. Therefore, transformation of competent cells with more than one plasmid or dimer formation between two closely related sequences most likely explains the presence of unusual bands in these samples.

5.3.3 Investigation of *Taq* polymerase induced misincorporation

The PCR products used to produce clones for DGGE analyses were originally amplified using *Taq* polymerase as previously described (Section 2.1.5). *Taq* polymerase is commonly used when the primary consideration of the researcher is efficiency and cost. In this study, 96 samples were tested by nested PCR for at least 2 KSHV ORFs. As this required large amounts of polymerase to be used during PCR reactions, *Taq* polymerase was chosen, being the most cost-effective enzyme available. However, *Taq* polymerase does not have 3' to 5' exonuclease activity and cannot therefore excise misincorporated nucleotides during polymerisation. This may lead to the presence of artificial mutation errors in a proportion of the PCR amplicons. It has been estimated that *Taq* polymerase will introduce an error at a rate of approximately 0.85×10^{-4} errors/base pair/cycle. During sequencing, *Taq* polymerase error can be minimised by either sequencing directly from a PCR product to obtain the consensus sequence of all amplicons or by taking the majority sequence from a number of cloned PCR products. In studies where viral diversity is to be investigated by examining the sequence of individual cloned amplicons, such as in DGGE, the effect of *Taq* polymerase error can be magnified and may over represent the extent of viral diversity.

From estimates of *Taq* polymerase error, it is expected that for a 200 bp fragment, 5% of amplicons will contain a *Taq* polymerase induced mutation after 20 effective cycles of amplification. The number of effective cycles refers to 60% of the total machine cycles after which point an excess of template impedes amplification in future cycles. Therefore, 5% of amplicons in the first

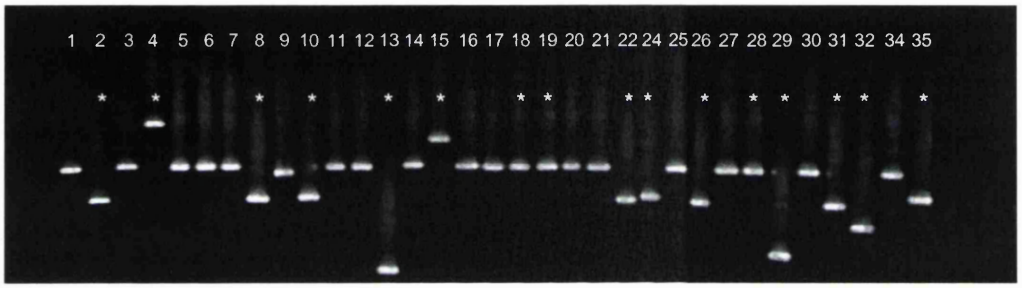
round of PCR alone would be expected to contain an artificial error due to *Taq* polymerase misincorporation. The aliquot of first round product that is added to the second round PCR mix will contain a mixture of both true sequences and amplicons containing an artificial mutation. In the second round of PCR these misrepresentative sequences are further amplified. In addition, *Taq* polymerase misincorporation error will create another 5% of amplicons with erroneous mutations. Thus, the artificial error can be significantly increased in the second round of a nested PCR. In the study described in this chapter, the percentage of clones showing a divergent sequence by DGGE ranged from 9% to 20% for each individual sample. While this level of diversity may be greater than what is expected for artificial mutations alone, the effect of *Taq* polymerase induced error cannot be ignored.

In order to estimate the impact of *Taq* polymerase induced mutations on the clonal diversity displayed by DGGE, 35 clones produced from an ORF K1/V1 PCR product, amplified from the KSHV infected cell line, BC-1, were analysed. Previous SSCP studies on BC-1 K1/V1 clones, amplified using the high fidelity EXPAND DNA polymerase system, had indicated no viral diversity was present in this cell line (C. Bez, personal communication). EXPAND contains a mixture of both *Taq* polymerase and *Pwo* polymerase. *Pwo* polymerase possesses 3' to 5' exonuclease proofreading activity and as a result the EXPAND system will produce artificial errors at a rate of only 8.5×10^{-6} errors/base pair/cycle. For a 200 bp PCR product, this is equivalent to 2% of amplicons containing an artificial mutation after 20 effective cycles of replication. DGGE of 35 *Taq* polymerase amplified BC-1 clones revealed that 37% had a sequence which was divergent from the majority sequence (Figure 5.13 top panel). Since this was an unexpected result, further controls were performed to examine the true extent of the viral diversity inferred from individual samples by DGGE using *Taq* polymerase.

The KSHV infected cell line, CRO-AP/3, was selected as a second study cell line because it had earlier shown little viral diversity following SSCP analysis (C.Bez, personal communication). ORF K1/V1 PCR was performed as

Figure 5.13 DGGE results from BC-1 ORF K1/V1 PCR products amplified using both *Taq* DNA polymerase and the EXPAND system. Lanes marked with a * indicate those samples that were sequenced.

BC-1 *Taq*



BC-1 Expand

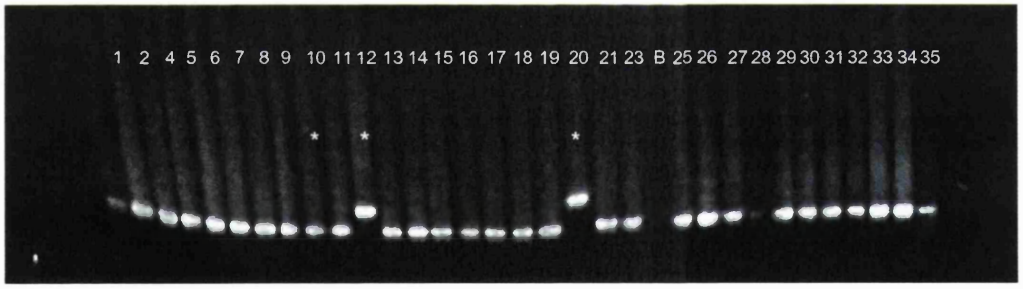


Figure 5.14 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from BC-1 PCR product clones amplified using *Taq* and designated by a * on figure 5.13. The BC-1 sequence U75698 was added as a reference Samples denoted in blue are the majority sequence as determined by DGGE.

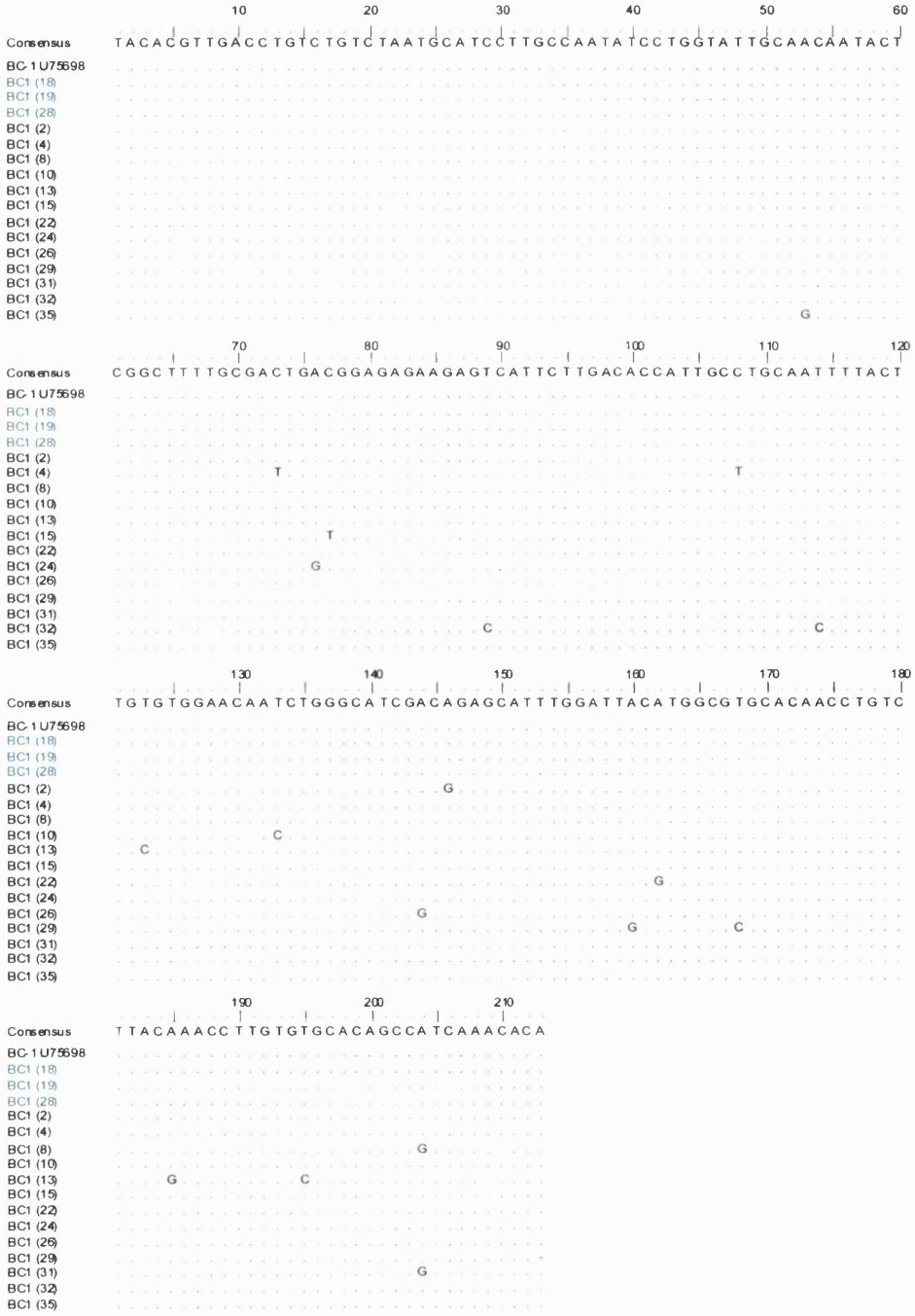


Figure 5.15 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from BC-1 PCR product clones amplified using EXPAND and designated by a * on figure 5.13. The BC-1 sequence U75698 was added as a reference. Samples denoted in blue are the majority sequence as determined by DGGE.

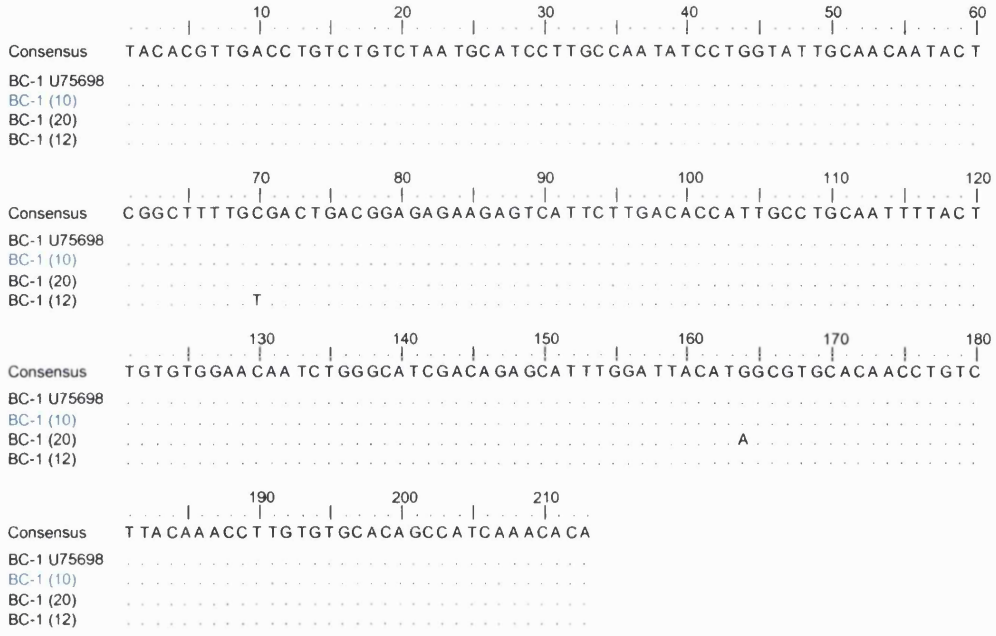
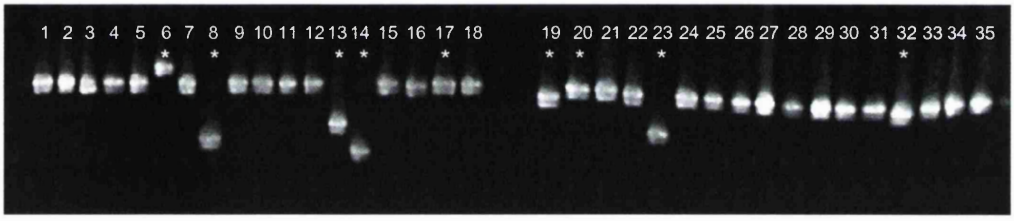


Figure 5.16 DGGE results from CRO-AP/3 ORF K1/V1 PCR products amplified using both *Taq* DNA polymerase and the EXPAND system. Lanes marked with a * indicate those samples that were sequenced.

CRO AP3 *Taq*



CRO AP3 EXPAND

Figure 5.17 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from CRO-AP/3 PCR product clones amplified using *Taq* and designated by a * on figure 5.16. There was no CRO-AP/3 GenBank sequence available for reference. Samples denoted in blue are the majority sequence as determined by DGGE.

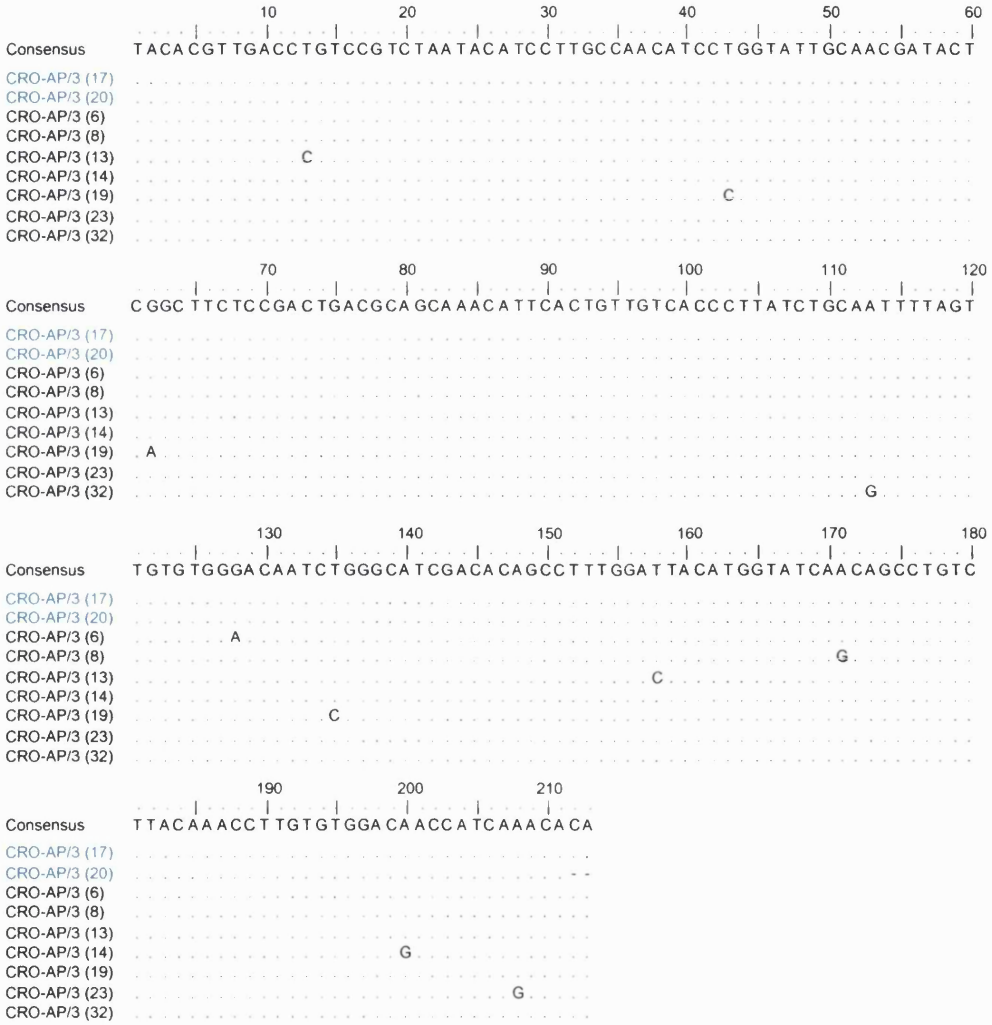
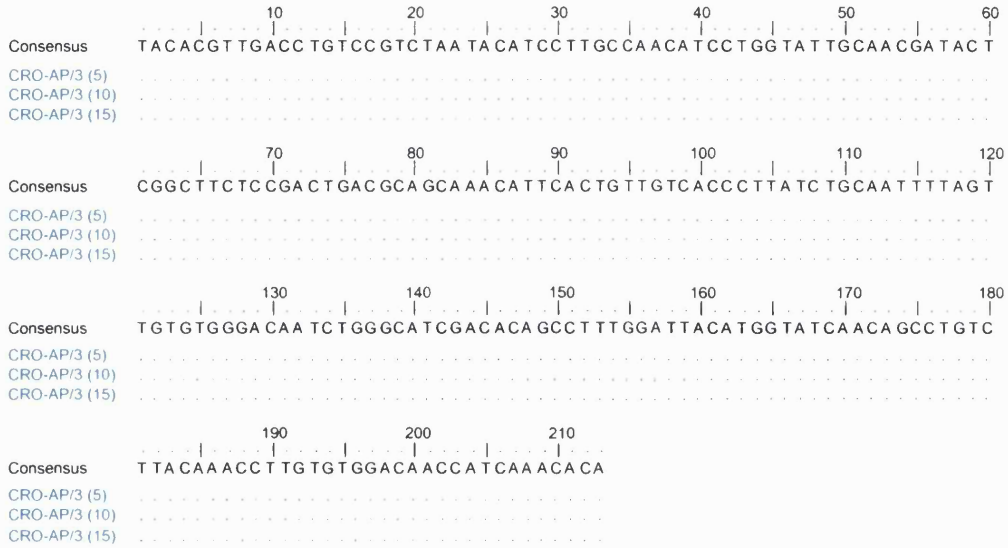


Figure 5.18 Clustel W multiple alignment of the ORF K1/V1 sequences recovered from CRO-AP/3 PCR product clones amplified using EXPAND and designated by a * on figure 5.16. There was no CRO-AP/3 GenBank sequence available for reference. Samples denoted in blue are the majority sequence as determined by DGGE.



previously described (Section 2.1.5) using both *Taq* polymerase and the EXPAND high fidelity PCR system. The ORF K1/V1 region was also amplified from BC-1 cell line extracts using EXPAND to compare with the results obtained using *Taq* polymerase. From each PCR product amplified using either *Taq* polymerase or the EXPAND system, 35-40 clones were produced and amplified in colony PCR. It is not necessary to consider polymerase error during the colony PCR step. Even if a misincorporation occurred early in the PCR no more than 50% of all amplicons would contain the error.

The DGGE results obtained from both *Taq* polymerase and EXPAND amplified clones for both BC-1 and CRO-AP/3 cell lines are shown in Figure 5.13 and 5.16. As mentioned earlier, DGGE analysis of *Taq* polymerase amplified BC-1 clones identified 13 variant clones out of 35 (37%) (Figure 5.13, top panel). All of these clones were verified by sequence analysis and an alignment of their sequences is shown in Figure 5.14. When BC-1 K1/V1 clones, amplified using EXPAND were run on a DGGE, much less clonal diversity was observed, with only 2 of 32 (6%) clones having a divergent sequence (Figure 5.13, bottom panel). Both of these variants were confirmed to be divergent by sequencing (Figure 5.15). DGGE analyses of CRO-AP/3 ORF K1/V1 revealed no variants present in the cell line

5.4 Discussion

The ORF K1 region, coding for an early lytic membrane signalling protein, exhibits a high degree of sequence diversity, facilitating subtyping. The biological advantage that this diversity brings to the virus is not yet known but may reflect a positive selection leading to immune selection or evasion (Zong *et al.*, 1999). It is also possible that ORF K1 is a more recently acquired gene that has yet to reach equilibrium. However, the closely related rhadinovirus, RRV, also expresses a highly divergent protein (R1) at a similar position in its genome (Searles *et al.*, 1999)

Immunocompetent individuals infected with multiple viral strains have been identified for CMV and EBV (Meyer-Konig *et al.,* 1998; Srivastava *et al.,* 2000). Multiple infection with HIV-1 and HIV-2 is also possible. For KSHV, no evidence for multiple infection in a given host has been found (Zong *et al.,* 1999; Stebbing *et al.,* 2001; Zong *et al.,* 2002). To investigate the possibility that minor variants may be present in a single person, but have not been detected due to the low number of clones screened, multiple clones were analysed by DGGE.

Initial DGGE results indicated that individuals were infected with 9% to 20% minority variants. The majority sequence, determined by DGGE, was identical to the sequence taken from the consensus of multiple clones, except in X2, where a 1 bp difference was found. In addition, in families where the original ORF K1/V1 sequences differed, no minority variant sequences recovered from an individual were identical to any sequence recovered from their family member. In order to evaluate the impact *Taq* polymerase error may have had on the apparent presence of variant KSHV genomes in an individual, clones amplified from the BC-1 and CRO-AP/3 cell lines were analysed by DGGE. Data that was gathered from SSCP analysis of clones derived from these cell lines indicated little to no variant genomes were present. Initially, clones were generated from BC-1 ORF K1/V1 PCR products generated using *Taq* polymerase, and DGGE analysis revealed 37% of clones to be carrying a sequence varying by 1-2 bp from the majority. Additional clones of ORF K1/V1 PCR products amplified from both BC-1 and CRO-AP/3 cell line extracts using both the EXPAND system, designed to introduce fewer polymerase induced errors, and *Taq* polymerase were analysed by DGGE. The results from these experiments suggested that the use of *Taq* polymerase had significantly contributed to the apparent viral diversity present in individuals. Using the EXPAND system, only 2 of 32 (6%) BC-1 clones had a sequence differing from the majority. When clones amplified from CRO-AP/3 DNA were analysed, no diversity was found using EXPAND. However 6 of 35 (17%) clones yielded divergent sequences when *Taq* polymerase was used.

It has been previously noted that the use of *Taq* polymerase for studies of viral diversity can significantly misrepresent the true level of diversity present (Smith *et al.,* 1997). *Taq* polymerase has been found to have an error rate 9.3 times higher than the error rate obtained using the high fidelity *Pfu* polymerase when amplifying DNA templates, and estimates of viral diversity based on the analysis of both *Taq* polymerase and *Pfu* polymerase amplified clones have also been observed to be dissimilar (Bracho *et al.,* 1998). *Taq* polymerase induced error can lead to incorrect conclusions regarding viral diversity and can distort evolutionary analysis following phylogenetic analysis. In particular, comparing sequences recovered from clones to those gathered by direct sequencing can indicate greater diversity was actually present in the cloned sequences as a result of polymerase error. Conserved templates are more prone to misincorporation error because there is less background sequence diversity to minimise the effect of incorrect mutations. As the clones used in this study originated from single individuals, lower diversity is expected in this situation than from a group of samples from separate individuals.

Various strategies exist to minimise the impact of *Taq* polymerase error. PCR conditions can affect the fidelity of *Taq* polymerase: there is an important relationship between the concentrations of $MgCl_2$ and dNTPs in the PCR mix (Eckert and Kunkel, 1990). *Taq* polymerase error can be reduced by minimising the number of PCR cycles, avoiding nested PCR, using a polymerase with proofreading capabilities, and eliminating the need for cloning by sequencing the PCR products directly. When clones are required to study variability, the most straightforward method of avoiding unnecessary errors is to use a high fidelity polymerase during PCR. The EXPAND system contributes an acceptable rate of polymerase error where less than 1 clone out of 35 would be expected to contain an artificial mutation. It is noteworthy to consider that an additional 0.5% -1% error rate can be attributed to the automated DNA sequencing procedure (Koop *et al.,* 1993). Analysing several clones to obtain a consensus sequence could significantly reduce this rate of error.

As DGGE experiments of cell lines in this study indicated, *Taq* polymerase can produce a significantly greater number of variant clones, potentially overestimating the extent of viral diversity. *Taq* polymerase mutations often occur sporadically (i.e. in only one clone) when multiple clones are studied, since a misincorporation error will represent only a small percentage of all amplicons. Mutations occurring in more than one clone are often a true polymorphism representing a genomic shift in the viral population (Smith *et al.*, 1997). Independent point mutations may only be identified if a large number of clones are screened; however, a non-sporadic segregating polymorphism may be identified from a smaller sample of clones.

Sporadic mutations often occur at a rate equivalent to *Taq* polymerase error and in some studies these errors have been falsely interpreted as true viral diversity (Smith *et al.*, 1997). The frequency of sporadic mutations in each individual studied by DGGE is indicated in Table 5.1. The range of sporadic mutations in these individuals varied from $0.13 \times 10^{-4} - 0.76 \times 10^{-5}$ mutations/base pair/cycle, falling in or just under the expected range of sporadic mutations caused by *Taq* polymerase incorporation error ($0.2 - 2 \times 10^{-4}$ mutations/base pair/cycle) (Lundberg *et al.*, 1991; Barnes, 1992). Therefore, it is likely that the sporadic mutations present in the individuals in this study are a result of *Taq* polymerase error and do not represent true viral diversity. In addition, 2/3 of *Taq* polymerase mutations result in non-synonymous amino acid substitutions, a proportion similar to that observed in a non-coding region or intron where protein function is not critical. In some instances, these erroneous mutations will lead to the amplification of replication incompetent genomes that can be identified and eliminated (Gunther *et al.*, 1998). The amino acid alignment of clones from individual G1 is shown in Figure 5.19. The percentage of non-synonymous mutations was 69%, indicative of *Taq* polymerase induced mutations having occurred.

The identification of chimeric genomes in several studies indicated that multiple infection and viral recombination are possible in an individual (Poole *et al.*, 1999; (Poole *et al.*, 1999; Kakoola *et al.*, 2001; Zong *et al.*, 2002). In this study,

Figure 5.19 Deduced amino acid alignment of divergent G1 ORF K1/V1 clones.

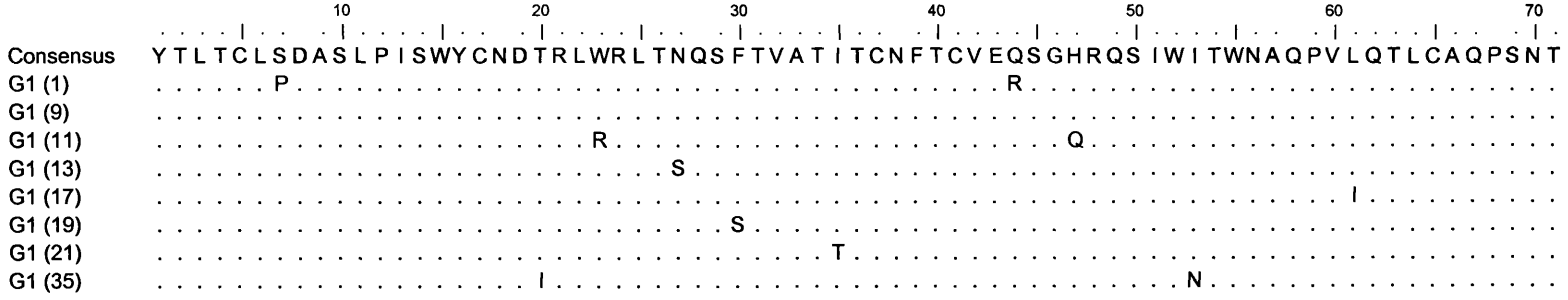


Table 5.1 Frequency of sporadic mutations in all samples amplified using *Taq*.

Sample	No. of clones	Length of product	No. of PCR cylces	No. of variants	No. of sporadic	Frequency* of sporadic
G1	35	213	70	13	13	0.24×10^{-4}
G2	35	213	70	7	7	0.13×10^{-4}
T2	35	213	70	9	9	0.17×10^{-4}
T3	35	213	70	4	4	0.76×10^{-5}
X1	35	213	70	4	4	0.76×10^{-5}
X2	31	213	70	8	8	0.17×10^{-4}
BC-1	35	213	70	17	16	0.31×10^{-4}
CRO-AP/3	35	213	70	17	10	0.19×10^{-4}

* Sporadic mutations/ base pair/PCR cycle

2 different strains were isolated from sample B5. However, only one sequence was consistently amplified from this sample. In addition, DGGE analysis of multiple ORF K1/V1 clones in the other study participants did not reveal a level of variability exceeding the mutation rate of *Taq* polymerase. It was anticipated that an individual would be multiply infected, as was observed for both EBV and CMV infection, even in immunocompetent individuals. From the epidemiological data concerning geographical subtype distribution, it may not be surprising to find so little evidence of intra-individual KSHV variability, even when potential immunosuppression is present. Subtypes found in certain ethnic groups and populations, such as the A1 subtype prevalent amoung Ashkenazi Jews, are highly conserved, indicating very low levels of mutation. Over all K1 subtypes, examples can be found of unrelated samples with common subtypes carrying nearly identical ORF K1 sequences (Zong *et al.,* 2002). It has been estimated that the greatest possible rate of viral divergence is only 3-5 amino acid changes over 20 years of multiple transmission events, even in the presence of host immunosuppression (Zong *et al.,* 2002). This extremely slow rate of mutation over long periods of time indicates that multiple KSHV infection with a population of rapidly divergent variants is unlikely. This is supported by the DGGE results in this study, revealing little to no real viral divergence in an individual.

Despite the failure of DGGE analysis to uncover intra-individual KSHV diversity, the technique may be useful in other areas of study. DGGE can reliably detect single base pair mutations and can be used to rapidly screen many PCR products or clones. In the context of this study, multiple ORF K1/V1 clones from members of the same family could be screened simultaneously to rapidly identify sequence similarity, eliminating the need for multiple sequencing analyses. This technique may also be useful in longitudinal studies. While it is unlikely that KSHV sequences will vary over time, the affects of drug treatment may have an unknown effect (i.e. increasing the rate of KSHV genomic evolution). ORF K1/V1 PCR products from various time point samples can be tested by DGGE and quickly screened for divergent sequences. Any PCR products with an apparent variable sequence can be verified by

sequencing, thus eliminating the need for time consuming multiple sequencing analysis. It is important to consider the impact of polymerase error on DGGE results to avoid erroneously describing viral diversity. This type of error can be significantly reduced by using a high fidelity enzyme, such as EXPAND, or by analysing the PCR product directly without creating multiple clones.

Chapter 6

Conclusions and suggestions to further work

The epidemiology of virus transmission can be inferred from serological data, but these data cannot provide specific information about routes of transmission. In this study, several molecular methods were utilised to investigate more precisely the epidemiology of KSHV transmission at the inter- and intra-individual levels.

6.1 Transmission of KSHV in Malawian families

Serological studies already conducted of children and family groups in several parts of Africa (Mayama *et al.,* 1998; Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000) point to significant intra-familial transmission between mothers and their children and between siblings. To date, no studies have been done using molecular techniques to confirm whether such transmission routes exist. In the present study, Malawian family groups in which one member had KS were selected to investigate modes of familial KSHV transmission.

To effect the study, two regions of the hypervariable ORF K1, designated K1/V1 and K1/V2, were chosen for amplification and sequencing. These regions were selected as their variable nature facilitates the best possible comparison between sequences. From a total of 22 families, 2 or more sequences could be compared in 8 families. In 4 families, identical K1/V1 sequences were recovered, and in 5 some diversity was present. Both similar and divergent sequences could be recovered from one family, E. These data suggest that in families E, G, K and Z, intra-familial transmission events have taken place. In families W, X, T, E and B non-identical sequences were revealed between family members suggesting extra-familial transmission events occurred. However, not all of the family members were infected with KSHV and their genomes were not available for sequence comparison. In some instances, the mother, who may be considered the most likely source of infection, was not available for study. Within the two predominant subtypes, A5 and B, sequences between family groups were very similar. K1 subtypes appear to be highly conserved within certain ethnic populations and across geographical

regions (Zong *et al.,* 2002), so while family groups may not live in the same locality, they may have carried closely related viral variants.

The degree of nucleotide variation in many families was less than 2%, although the same sequence for each sample was consistently detected in multiple clones. Nevertheless, a proofreading polymerase was not used to amplify ORF K1/V1 and K1/V2 from the samples. Multiple clones were taken and analysed from a single PCR reaction and if a polymerase induced error occurred early in PCR cycling, it would represent a significant percentage of the final pool of amplicons. Sequencing only three to five of these amplicons would not necessarily ensure that the true consensus sequence was obtained.

KSHV DNA could not be amplified from the blood of any KSHV antibody positive family member, but was amplified in a high percentage of their mouth rinse samples. As this study and others have shown, children are frequently infected with KSHV in endemic regions like Malawi (Angeloni *et al.,* 1998; Mayama *et al.,* 1998; Olsen *et al.,* 1998; Andreoni *et al.,* 1999; Gessain *et al.,* 1999; Plancoulaine *et al.,* 2000; Davidovici *et al.,* 2001). A nonsexual route of transmission, facilitated by close contact with the saliva of an infected individual, may explain why KSHV is acquired during childhood. KSHV transmission may thus be similar to EBV transmission in underdeveloped regions: EBV is principally acquired before 3 yrs of age through close contact with infected saliva (Yao *et al.,* 1985).

A previously described PCR RFLP method (Zhang *et al.,* 2000) was adapted to make further comparisons of KSHV genome relatedness between family members. For this, another region of the KSHV genome, the IRD of ORF 73 was examined. Using the PCR RFLP technique, 8 families, 6 of which had been analysed by ORF K1 sequencing, and 5 individual samples were studied. The RFLP method confirmed the data obtained by K1/V1 sequencing in 5 of these 6 families. Study individuals in family G revealed dissimilar RFLP patterns however the father G2 and son G1 carried identical ORF K1/V1 sequences. Samples from study subject G (mother of G1 and wife of G2) could not be

amplified for ORF K1/V1 but yielded a different RFLP pattern from both her son and husband. Previous studies suggest that RFLP patterns are unique to an individual and are invariant in cell lines passaged over time (Zhang *et al.,* 2000). Therefore, a comparison between two individual samples may be considered accurate and specific and members of family G can be said to carry different KSHV genomes based on the RFLP results. The polymorphic nature of the IRD region (Gao *et al.,* 1999) allowed sequence variation between samples to be readily identified, however it did not provide the specific sequence information gathered from PCR sequencing. A proofreading polymerase was not used and errors generated due to the high G+C content of the IRD region and areas of multiple repeated sequence may have created the apparent differences in RFLP pattern.

Further work: KSHV transmission is clearly very complex in Malawi, involving both familial and community wide routes of infection. In this study, 8 families could be studied by direct sequence comparison of KSHV genomic regions. It is apparent that routes of transmission other than from mother to child are involved. Further clarification of KSHV transmission routes may be achieved by studying a larger sample size than that described here. In addition, significant data have been gathered about chimeric genomes and K1 subtype linkage across the genome (Zong *et al.,* 2002). Gathering such information could allow more specific comparisons to be made between viral sequences recovered from various family members.

6.2 Intra-individual KSHV variability

DGGE was developed to screen multiple K1/V1 clones from oral samples. This method was applied to screen for PCR clones that had different sequences from the majority without needing to sequence all the clones. Initially, it was observed that individuals carried between 9% and 20% variant viral genomes in their mouth rinse samples based on DGGE clonal analysis. To evaluate the impact *Taq* polymerase misincorporation may have had on the apparent viral diversity these individuals, DNA was amplified and cloned from two KSHV

infected cell lines using EXPAND high fidelity polymerase. Previous studies indicated that the cell lines carried a homogeneous population of virus (C.Bez personal communication). Comparing *Taq* polymerase amplified clones with those amplified using EXPAND for these two cell lines suggested *Taq* polymerase errors had significantly over-represented the actual viral diversity present in these cell lines. The rate of sporadic mutations for all individual samples studied was less than or equal to the published range of sporadic errors due to *Taq* polymerase (Lundberg *et al.,* 1991; Barnes *et al.,* 1992) and no evidence of a segregating mutation was found. These mutations are likely to be due to *Taq* polymerase error alone. Therefore, it is unlikely that any genomic diversity or mixed infection was present in the mouth rinse samples of these KSHV infected individuals.

Further work:. The presence of chimeric viruses presupposes that recombination, facilitated by mixed infection, does occur. The results of this study, after taking into account the impact of *Taq* polymerase, suggest that mixed infection or intra-individual KSHV diversity do not occur to any significant extent. The use of a proofreading enzyme, such as EXPAND, may minimise the effect of *Taq* polymerase error. However, previous studies have shown a decreased PCR sensitivity using EXPAND (C. Bez personal communication). Nevertheless, the sample set was small and a follow-up study involving more individuals should be useful to confirm the rarity of mixed KSHV infection. Furthermore, none of the patients studied had AIDS although two were HIV seropositive; studying severely immunosuppressed patients might reveal a more varied KSHV viral population. In addition, different types of samples could be studied, e.g. blood, KS lesions, salivary glands, or samples taken over the course of drug therapy (e.g. HAART or other types of chemotherapy) may reveal evolving K1/V1 viral diversity. The DGGE technique shown in this study to be useful in rapidly identifying different sequences could be applied to such studies requiring sequence differences from multiple samples or clones to be screened.

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Cook, R.D., Hodgson, T.A., Waugh, A.C.W., Molyneux, E.M., Borgstein, E., Sherry, A., Teo, C.G., and Porter, S.R. (2002) Mixed patterns of transmission of human herpesvirus-8 (Kaposi's sarcoma-associated herpesvirus) in Malawian families. *Journal of General Virology* **83,** 1613-1619.

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Cook, R.D., Hodgson, T.A., Waugh, A.C.W., Molyneux, E.M., Borgstein, E., Sherry, A., Teo, C.G., and Porter, S.R. Molecular epidemiology of familial KSHV transmission in Malawi (poster presentation) Sixth International Conference on Malignancies in AIDS and other Immunodeficiencies, Bethesda, USA, 22nd –24th April 2002.

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Mixed patterns of transmission of human herpesvirus-8 (Kaposi's sarcoma-associated herpesvirus) in Malawian families

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To study transmission patterns of human herpesvirus-8 (HHV-8) (Kaposi's sarcoma-associated herpesvirus) in families in Malawi, nucleotide sequences derived from two hypervariable loci of the HHV-8 genome, the V1 and V2 regions of open reading frame K1 (K1/V1 and K1/V2, respectively), were amplified from blood and mouth rinse samples of 22 patients with treated and untreated Kaposi's sarcoma (KS) and their first-degree relatives (n = 67). In patients with KS, vincristine therapy was significantly associated with non-detectability of circulating, but not oral, K1/V1 DNA. Intra-familial K1/V1 phylogenetic comparisons of eight families were possible. Both identical and non-identical sequences were observed between family members, suggesting transmission of HHV-8 along both intra- and extra-familial transmission routes.

Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV-8), was first isolated from an AIDS-associated Kaposi's sarcoma (KS) biopsy sample (Chang *et al.*, 1994). HHV-8 is now considered to be causally associated with all epidemiological forms of KS (Boshoff & Weiss, 2001).

Serological studies have indicated that, unlike other human herpesviruses, HHV-8 is not ubiquitous. The prevalence of serologically determined HHV-8 infection is low in the USA and Europe, rising in Mediterranean countries and reaching levels of greater than 50% in some geographic regions of Africa (Gao *et al.*, 1996; Lenette *et al.*, 1996; Simpson *et al.*, 1996; Whitby *et al.*, 1995; Olsen *et al.*, 1998).

In North America and Europe, HHV-8 primary infection occurs mainly in adulthood, most notably among HIV-1-positive homosexual men (Martin *et al.*, 1998; Dukers *et al.*, 2000). Transmission of HHV-8 in homosexual men is likely to occur during sexual activity and HHV-8 seroprevalence in this group is linked to the number of sexual partners and to specific sexual practice (Martin *et al.*, 1998; Melbye *et al.*, 1998; Dukers *et al.*, 2000).

In African populations, KS was infrequently observed in children prior to the HIV epidemic; however, it is now increasingly prevalent (Athale *et al.*, 1995; Ziegler & Katongole-Mbidde, 1996). Studies of HHV-8 seroprevalence in African populations indicate that HHV-8 infection occurs largely before puberty through casual family and community contact (Mayama *et al.*, 1998; Gessain *et al.*, 1999). Evidence to support non-sexual transmission of HHV-8 between mother and child and between siblings has been documented in African serological studies (Plancoulaine *et al.*, 2000). Passive transmission of maternal HHV-8 antibodies to the infant has also been demonstrated. However, HHV-8 seroprevalence in African children under 2 years of age is low, indicating vertical transmission of HHV-8 is not significant (Gessain *et al.*, 1999; Lyall *et al.*, 1999).

As both KS and HIV-1 infection are highly endemic in Malawi (Thomas, 2001), we wanted to investigate the extent of non-sexual transmission of HHV-8 within family groups resident in this region. We describe here a molecular epidemiological study evaluating the transmission patterns of particular HHV-8 variants infecting families of known HHV-8-infected individuals. DNA sequences were amplified from two reported highly variable loci in the HHV-8 genome, the variable regions 1 and 2 (V1 and V2) of open reading frame (ORF) K1 (Cook *et al.*, 1999; Zong *et al.*, 1999).

Ethical approval was obtained prior to commencing this

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All ORF K1/V1 DNA sequences have been deposited in GenBank, accession numbers AF398120–AF398140 and AF451301–AF451321.

Table 1. Characteristics and virological status of Malawian KS patients and their family members

HHV-8-seronegative/HHV-8 DNA-positive family members are underlined.

	Index case				Family members		
	Age/sex	Site of KS	Vincristine given?	Spouse (age)	Children/siblings (age/sex)	Mother (age)	Father (age)
A_i*‡§	7 y/F	Abdomen, limbs	No		A2 (10 y/M)‡, A3 (7 m/M)	A1 (34 y)‡§	
B_i‡	4 y/M	Cervical	Yes		B1 (20 y/F)‡§, B2 (13 y/M)‡, B3 (12 y/M)†, B4 (10 y/M)‡, B5 (7 y/M)†‡, B6 (11 m/F)	B7 (40 y)‡	
C_i†‡§	8 y/M	Abdomen, limbs	No		C2 (14 y/F)‡, C3 (12 y/M)‡		C1 (45 y)§
E_i*‡§	31 y/F	Legs, palate	No		E1 (5 y/F)‡, E2 (9 y/F)‡, E3 (6 y/F)‡, E4 (6 y/F)†‡, E5 (13 y/M)†‡, E6 (10 y/M)†‡		
F_i*‡§	2 y/M	Foot	No		F2 (7 y/M)§	F1 (35 y)‡§	
G_i‡§	34 y/F	Foot, leg, palate	No	G1 (32 y)†‡§	G2 (23 y/M)†‡, G3 (19 y/F)‡, G4 (15 y/M)‡		
H_i‡§	35 y/F	Leg	Yes		H1 (13 y/F)‡, H2 (12 y/M)†‡, H3 (9 y/M)‡, H4 (8 y/M)		
I_i‡§	36 y/F	Leg	Yes		I1 (12 y/M)‡, I2 (11 y/F)		
J_i‡§	28 y/M	Leg	No	J1 (22 y)‡	J2 (3 y/M)		
K_i*†‡§	30 y/F	Leg	Yes		K1 (12 y/M)†		
M_i‡§	33 y/M	Leg, foot	No	M4 (32 y)‡	M1 (3 y/M), M2 (7 m/F), M3 (8 y/F)‡§		
N_i‡§	30 y/F	Leg, palate	No		N1 (18 y/F)		
P_i‡§	30 y/M	Leg, palate	Yes	P1 (28 y)‡§	P2 (2 y/M)		
Q_i*†‡§	24 y/M	Foot	No	Q1 (21 y)‡§			
R_i‡§	41 y/M	Leg	Yes	R1 (25 y)‡§	R2 (12 y/M)‡, R3 (9 y/M)†		
S_i‡§	40 y/M	Leg	Yes	S1 (37 y)			
T_i‡§	33 y/F	Leg, foot	Yes	T1 (37 y)‡§	T2 (11 y/M)†‡, T3 (9 y/M)†‡, T4 (4 y/M)‡§		
U_i‡§	32 y/F	Leg, foot, gingiva	No		U1 (14 y/M)‡, U2 (12 y/F), U3 (8 y/F)		
W_i‡§	43 y/F	Leg, palate	Yes	W1 (36 y)†‡§	W2 (20 y/M)†‡, W3 (19 y/M)‡, W4 (13 y/F)†‡, W5 (6 y/M)‡§, W6 (7 m/F)‡§		
X_i‡§	41 y/F	Chest, palate	Yes	X1 (44 y)†‡§	X2 (18 y/F)†, X3 (11 m/M)		
Y_i‡	32 y/M	Arm	Yes	Y1 (38 y)‡	Y2 (8 y/F), Y3 (5 y/F), Y4 (3 y/F)		
Z_i†‡§	21m/M	Cervical	No		Z1 (5 y/F)‡	Z2 (22 y)†‡	

* HHV-8 DNA (K1/V1 DNA) amplified from blood.
† HHV-8 DNA (K1/V1 DNA) amplified from saliva.
‡ HHV-8 seropositive.
§ HIV-1 seropositive.

study from the Eastman Dental Institute (UK), University College London (UK) and the University of Malawi. Consecutive patients of the Central Hospital, Blantyre, Malawi, with presumptive cutaneous, oral and histologically confirmed nodal KS were invited to join the study. At presentation, all patients were offered palliative treatment with intravenous vincristine (2 mg/m^2). Regardless of whether treatment was administered, patients were requested to return within a week with all available household members. During the return visit, blood and mouth rinse samples were taken from patients and from each family member.

HIV-1 seropositivity was confirmed by an IgG capture particle adherence test (Parry *et al.*, 1995), and HHV-8 seropositivity by the HHV-8 IgG IFA Kit (Advanced Biotechnologies Incorporated). This IFA utilizes the KS-1 cell line as a source of HHV-8, expressing both latent and lytic HHV-8 antigens on its cell surface. This highly specific and sensitive assay is appropriate for use in epidemiological studies (Chatlynne *et al.*, 1998).

DNA was extracted from the immunomagnetically selected CD45$^+$ leukocyte fraction of blood, as described previously (Leao *et al.*, 2000). Study participants were asked to rinse with

PBS and spit the rinse into a universal centrifuge tube. These samples were subsequently spun to separate the pellet from the cell-free fraction. DNA was extracted from 150 µl of mouth-rinse pellets by a guanidium thiocyanate–silica procedure (Boom *et al.*, 1990).

A 246 bp segment from the V1 region of ORF K1 (K1/V1; nt 573–819) (Zong *et al.*, 1999) was amplified by a nested PCR, using as first-round primers 5′ CCCTGGAGTGA-TTTCAACGC 3′ (sense) and 5′ ACATGCTGACCACAAG-TGAC 3′ (antisense), and as second-round primers 5′ GAGTG-ATTTAACGCCTTAC 3′ (sense) and 5′ TGCTGACCACAA-GTGACTGT 3′ (antisense). Negative controls were included during extraction and PCR to control for possible contamination resulting from the nested PCR. Selected K1/V1 DNA-positive extracts were processed to amplify a 575 bp segment from the V2 region of ORF K1 (K1/V2) by hemi-nested PCR using LGH2088 and LGH2089 (Cook *et al.*, 1999) as first-round primers, and LGH2088 and K1/408/1 (Zong *et al.*, 1999) as second-round primers.

All K1/V1 and K1/V2 products were cloned using the TOPO TA cloning kit (Invitrogen). DNA of three to five clones from each product was sequenced, using the CEQ 2000 dye terminator cycle sequencing kit and capillary array automated sequencing system (Beckman Coulter). Raw DNA sequence data were analysed using SeqMan software (DNASTAR). Phylogenetic trees were generated in PHYLIP (Felsenstein, 1993) using NEIGHBOR to construct a NEIGHBOR-joining tree from the DNA distance matrix generated in DNADIST. Bootstrapping for 1000 replicates is reported (as a percentage) at major branch points as a measure of confidence.

Twenty-two of the 24 patients with KS were HHV-8 seropositive. These 22 comprised the index cases (Table 1). Sixty-seven family members of the index cases were enrolled into the study. Characteristics of the complete study group are shown in Table 1.

Twenty (91%) of the 22 index cases were HIV-1 seropositive and all were HHV-8 seropositive. Of the relatives, 16 (23%) were HIV-1 seropositive and 46 (69%) were HHV-8 seropositive. Overall, there was a positive association between HHV-8 and HIV-1 seropositivity in the total number of study subjects ($P = 0{\cdot}004$) (χ^2 test).

Of the 22 index patients, 5 (23%) were K1/V1 DNA positive in blood and 4 (18%) in oral rinses (Table 1). HHV-8 DNA negativity in the blood in index cases was significantly associated with prior vincristine therapy ($P = 0{\cdot}001$) (χ^2 test). In the samples of 67 family members of patients with KS, K1/V1 DNA was positive in the oral rinse of 18 (27%) but not in any of the blood samples. ORF K1/V1 DNA was detected in the oral sample in the absence of HHV-8 seropositivity in four of the family members (B3, K1, R3 and X2).

This higher rate of HHV-8 detection in the mouth compared with blood substantiates previous observations of frequent oral carriage of HHV-8. In Zimbabwean women with KS, HHV-8 DNA could be detected in oral rinse samples as frequently as in blood (Lampinen *et al.*, 2000), and in homosexual men, infectious HHV-8 has been isolated at high titre in the saliva (Koelle *et al.*, 1997). RNA transcripts have been visualized in the buccal mucosa of homosexual men by *in situ* hybridization (Pauk *et al.*, 2000), suggesting active HHV-8 replication within the oral epithelium.

Oral and blood samples were extracted using different techniques and this may have negatively affected our ability to amplify ORF K1/V1 DNA from the blood. However, both sets of samples were processed within 4 h of collection and stored at −20 °C until extraction. Furthermore, immunomagnetically selected blood fraction samples are stable when stored and are readily amplified by PCR (Leao *et al.*, 2000). We suggest our inability to amplify ORF K1/V1 DNA from blood as opposed to mouth rinse samples was due to the increased frequency of carriage of HHV-8 in the oral compartment of HHV-8 antibody-positive persons without overt KS disease. In addition, we attribute the significant decrease in PCR detectability of ORF K1/V1 DNA in the blood of the index cases to vincristine therapy.

ORF K1/V1 DNA sequences could be compared in eight families (B, E, G, K, T, W, X and Z). Characteristic amino acid motifs in either the V1 or V2 regions of ORF K1 permitted subtyping (Zong *et al.*, 1999, 2002). In areas of sub-Saharan Africa, ORF K1 subtypes A5 and B are most common (Lacoste *et al.*, 2000), and these two subtypes were predominantly represented in our sample set (Fig. 1a).

Identical K1/V1 DNA sequences were recovered in families E, G, K and Z. Identical sequences were found in family E, between the mother (E_i) and one of her sons (E6), in families K and Z, between the mothers (K_i and Z2) and their sons (K1 and Z_i), and in family G between a father (G1) and son (G2). Common sequences recovered from these family members indicated that intra-familial transmission of HHV-8 may have occurred. Transmission between mothers and their children has been previously reported (Plancoulaine *et al.*, 2000), and this may have occurred in families E, K and Z. In family G, a father and son shared the same sequence. The mother's sequence (G_i) was not available for study and the possibility that she may have acted as a source of infection for both her spouse and her son cannot be excluded. In addition, sequences recovered from families G, K and Z, all ORF K1 A5 subtypes, were very similar and it is possible that a regional HHV-8 variant is circulating in this population.

Intra-family divergences of K1/V1 sequences were revealed in families B, E, T, W and X. Nucleotide divergence between family members ranged from 0·47% (between W1 and W2) to 27·7% (between X1 and X2). In all families except the B family, the sequences of all clones recovered from each sample were identical.

Within family B, there were two sequences recovered from B5 clones, designated B5(I) and B5(II). Subject B5 might have been dually infected with two HHV-8 variants, one A4 subtype virus [B5(I)] and one B1 subtype virus [B5(II)].

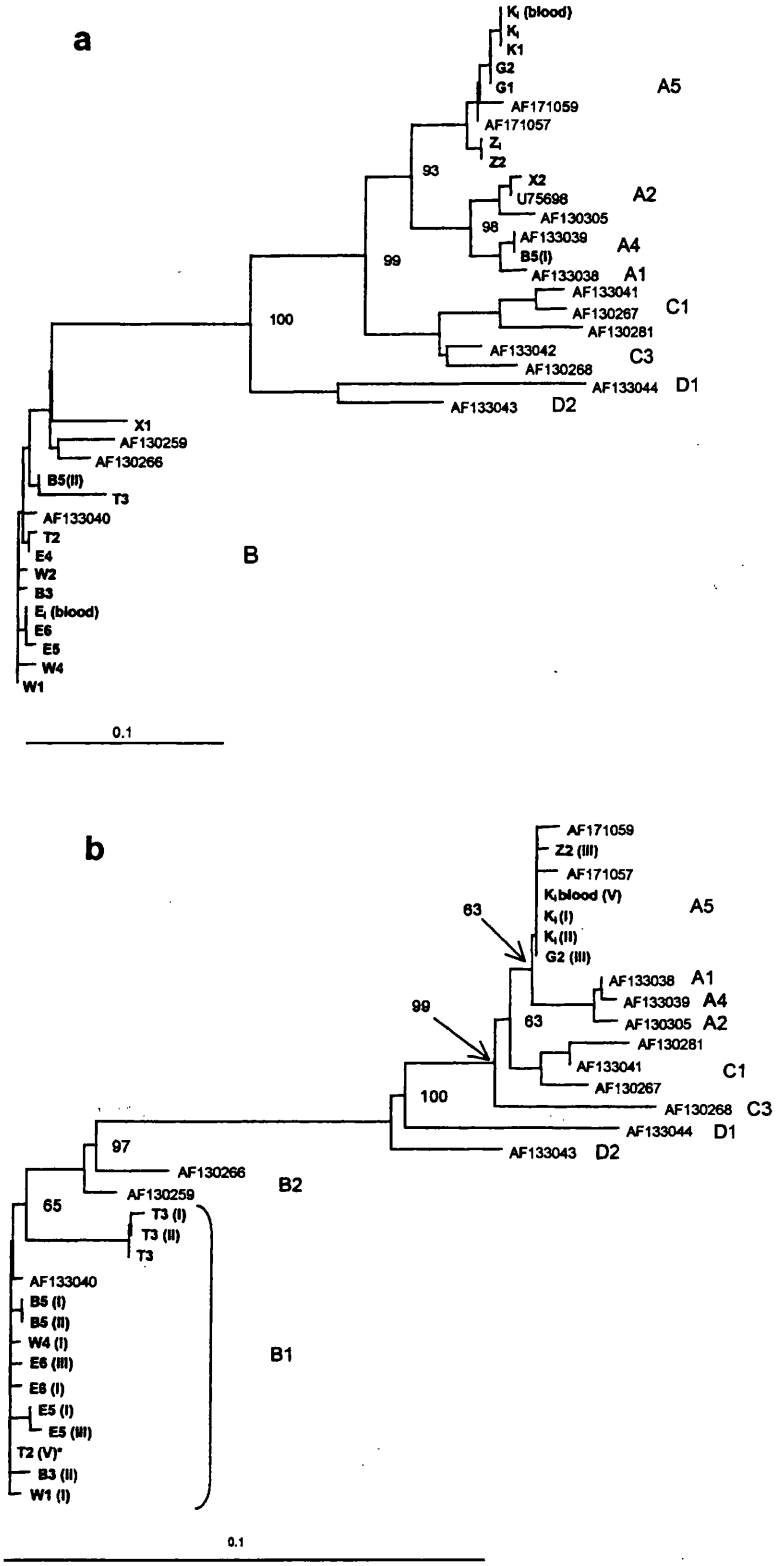

Fig. 1. Predicted phylogenetic distribution of a 213 bp segment of HHV-8 ORF K1/V1 (a) and a 462 bp segment of ORF K1/V2 (b) amplified from KS patients and their family members (in bold) in a background of GenBank sequences representing major ORF K1 subtypes. Sequences were recovered from oral samples unless otherwise indicated. Linear unrooted phylogenetic dendograms were generated using PHYLIP, DNADIST and NEIGHBOR programs. Bootstrapping for 1000 replicates is noted as a percentage at major branch points. The genetic distance size scale for 0·1 (10% divergence) is indicated. *Clones B5(III), T2(VI), E4(II), W4(IV), W2(V), B3(V), E4(III), E4(I), B3(IV), B3(I) and W4(V) have an identical sequence to T2(V).

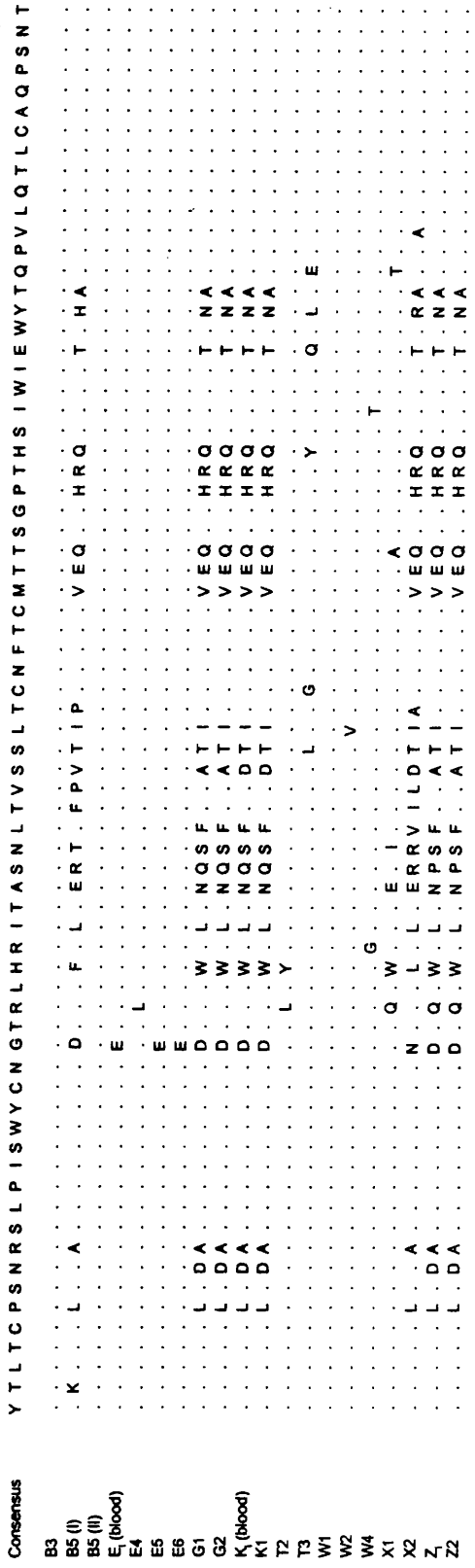

Fig. 2. CLUSTAL w alignment of HHV-8 K1/V1 amino acid sequences from families having more than two members positive for K1/V1 DNA.

Recombination in HHV-8 viral strains recovered from African patients suggests dual infection may sometimes occur (Zong *et al.*, 2002). We were not able to consistently amplify the B5(I) sequence, and contamination from an external source cannot be ruled out.

Of the eight families studied for K1/V1 sequence diversity, K1/V2 DNA could be amplified from two or more members in six families. Sequence data were available for clones from B, E, T, G, K and W families, and comparisons could be made for all families other than family G (in which G1 sequence data could not be recovered). Sequencing was carried out on at least three clones for each sample; one to two nucleotide substitutions were observed between some clones. All clones with available sequence data were phylogenetically analysed (Fig. 1b).

The disparate K1/V1 and K1/V2 sequences found between T2 and T3 demonstrated that these siblings were unlikely to have acquired HHV-8 infection from one another. ORF K1/V1 sequences recovered from X1 and his daughter, X2, belonged to different genotypes (A2 and B1, respectively) indicating no transmission linkage between each other. ORF K1/V1 sequences compared between W4, her father, W1, and brother, W2, differed by one or two amino acids. Nevertheless, intrafamilial HHV-8 transmission via the mother (W_i), whose ORF K1 sequence was not available, is possible. In family E, recovered ORF K1/V1 sequences revealed minor nucleotide variations between E4, E5 and E_i/E6. However, only E4 carried a divergent amino acid sequence (Fig. 2) and transmission from the mother, E_i, was possible in this family.

Non-identical sequences recovered in each of these families indicated possible extra-familial transmission. However, intrafamilial transmission through the seropositive/HHV-8 DNA-negative family members from whom ORF K1 sequences could not be recovered cannot be excluded. Very little sequence divergence was revealed between families E, B, W and T (ORF K1 B or B1 subtype), and it is possible that these individuals were infected with common subtype B variant present in the environment.

We found in our sample of Malawian families both patterns of HHV-8 sequence identity and non-identity among different members of the same family. Although our sample set was small, the data presented here indicated that patterns of transmission in endemic regions may be more complicated than that suggested by the mother-to-child model. Similar but not identical sequences were recovered both within and between families. Non-sexual transmission of prevailing HHV-8 variants within the population may result in the patterns we observed in this study.

These data also substantiate previous observations of early non-sexual HHV-8 acquisition in children living in endemic populations (Mayama *et al.*, 1998; Gessain *et al.*, 1999; Plancoulaine *et al.*, 2000). The higher rate of recovery of HHV-8 sequences from oral compared with blood samples suggests that oral secretions potentially act as vehicles of non-sexual horizontal spread. Thus, the control of HHV-8 infection

and the KS epidemic cannot rely on community-wide environmental sterilization and disinfection. Reducing the size of the reservoir of infection by anti-viral treatment of infected persons is also impractical. The optimal route to control of KS in endemic regions would be through vaccination.

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Tracking familial transmission of Kaposi's sarcoma-associated herpesvirus using restriction fragment length polymorphism analysis of latent nuclear antigen

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Abstract

Intra-familial transmission of Kaposi's sarcoma associated herpesvirus (KSHV) is likely to occur in geographical regions where KSHV infection is highly endemic. Transmission has been studied previously indirectly using serological techniques, however direct documentation of specific transmission routes has yet to be reported. The internal repeat domain (IRD) of the KSHV opening reading frame (ORF) 73 was shown previously to exhibit restriction-fragment length polymorphism (RFLP). Analysis of such polymorphism was undertaken using nested ORF 73 IRD PCR products derived from the blood and mouth rinse samples of individuals in Malawian family groups. The resulting RFLP patterns were unique to an individual and could be compared between family members. In three of eight families studied, identical RFLP patterns were recovered from family members; in the remaining five families, dissimilar RFLP patterns were revealed. Results from RFLP analysis were compared to sequencing data recovered from family members for the first variable region of the hypervariable KSHV ORF K1. Patterns of intra- and extra-familial transmission inferred from ORF K1 sequencing data were corroborated mainly using ORF 73 IRD RFLP analysis.

Keywords: Kaposi's sarcoma associated herpesvirus; Restriction fragment length polymorphism; Familial transmission; Oral

1. Introduction

Kaposi's sarcoma associated herpesvirus (KSHV) is linked causally to all epidemiological forms of Kaposi's sarcoma (KS) (Boshoff and Weiss, 2001). The prevalence of KSHV infection, as measured by serology, is low in North America

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and Europe, where 0–5% of blood donor groups are KSHV-antibody positive (Gao et al., 1996; Simpson et al., 1996). However, in areas of sub-Saharan Africa, KSHV sero-prevalence levels of 50% or greater have been reported previously (Gao et al., 1996; Sitas et al., 1999; Rezza et al., 2000).

Sexual transmission of KSHV is implicated among homosexual men in North America and Europe (Martin et al., 1998; Blackbourn et al., 1999; Dukers et al., 2000), in whom the incidence of Kaposi's sarcoma and the prevalence of KSHV infection is higher (Beral et al., 1990; Gao et al., 1996; Kedes et al., 1996; Simpson et al., 1996) than the general population. In Africa, where KSHV infection in children is common and where KSHV sero-prevalence increases with age (Olsen et al., 1998; Gessain et al., 1999; Rezza et al., 2000) non-sexual transmission of KSHV within the family is suspected to occur (Bourboulia et al., 1998; Gessain et al., 1999; Sitas et al., 1999; Plancoulaine et al., 2000). The vehicle for such non-sexual transmission is likely to be oral fluids as KSHV DNA has been detected in both the oral mucosa (Pauk et al., 2000) and saliva (Koelle et al., 1997).

Despite multiple serological studies, direct evidence of KSHV transmission has not been demonstrated. KSHV DNA sequence analysis can determine viral subtype (Zong et al., 2002), but the methods involved are time consuming. Recently, a rapid restriction fragment length polymorphism analysis of KSHV derived PCR products (PCR-RFLP) was reported (Zhang et al., 2000). PCR-RFLP targets the internal repeat domain (IRD) of KSHV opening reading frame (ORF) 73, a region of significant polymorphism which encodes the latent nuclear antigen (Rainbow et al., 1997). Numerous point mutations and deletions concentrated mainly in the second of three repeat domains in the ORF 73 IRD result in size and sequence polymorphism. The objective of this study was to analyse samples from patients with Kaposi's sarcoma and their family members from Malawi to determine whether PCR-RFLP subtyping could be applied to study familial transmission of KSHV.

2. Materials and methods

2.1. Study group

Twenty-two patients attending the Central Hospital, Blantyre, Malawi with cutaneous or oral mucosal Kaposi's sarcoma screened to be seropositive for KSHV by an immunofluorescence assay (IFA) (Advanced Biotechnologies Incorporated, MD) were selected as the index cases. A further 67 family members of the index cases without Kaposi's sarcoma were invited to join the study. Following informed consent from each member of the study group, venous blood and mouth rinses were obtained.

2.2. DNA extraction

DNA was extracted from the immunomagnetically selected CD45 + leukocyte fraction of blood samples as described previously (Leao et al., 2000). DNA was extracted from the mouth rinse samples using a guanidium thiocyanate-silica procedure (Boom et al., 1990).

2.3. KSHV IRD PCR

PCR amplification of the IRD region of ORF 73 was carried out as described previously (Zhang et al., 2000) with one minor variation. In each single round PCR reaction, 2 U of Platinum *Taq* DNA polymerase (Invitrogen, Paisley, UK) was used instead of 1 U to ensure a consistently positive PCR result.

Using the single round PCR described above, none or too little PCR product was occasionally generated from our samples. To increase the sensitivity of PCR detection, a nested PCR was developed, using as outer primers IRD1-F 5′ ACGCCAACCGCCTACATCT 3′ and IRD1-R 5′ TCATGTGTGCTAACAACAGG 3′ and the original single round primers described by Zhang et al. (2000) as second round primers. Both rounds of PCR were carried out in a 25 μl reaction mixture containing 2 μl extracted DNA, 1.25 U Platinum *Taq* DNA polymerase, 100 mM each dNTP, 50 pM each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl (pH 8.4) and 2 ×

PCRx enhancer solution (Invitrogen, UK). Thermocycling conditions were as previously described (Zhang et al., 2000). All samples negative after single round PCR were repeated using the nested PCR procedure.

2.4. Nested IRD PCR optimisation

Initially nested PCR using the same conditions as described for single round PCR resulted in high molecular weight smearing instead of the expected nested product. Various strategies to clarify the second round product included reduced primer concentration, 25 vs. 35 rounds of thermocycling in the second round of PCR, titration of key reagents and reduction in the amount of *Taq* polymerase used. A clear second round product could be visualised after titration of the PCRx enhancer solution supplied with Platinum *Taq* polymerase. Using this reagent at a 2 × concentration in both rounds of PCR yielded a specific product of the correct size when applied to a KSHV infected BC-1 cell line extract.

When nested PCR was applied to patient samples, a non-specific or smeared second round product was occasionally observed. Dilution of the first round product 10- to 20-fold resulted in a specific second round product in these cases. In some cases, reducing the number of thermocycling rounds to 25 improved second round PCR results.

2.5. RFLP

For the RFLP, PCR products generated by single or nested PCR were digested for 90 min at 37 °C with *Ban*II and *Mbo*I restriction enzymes followed by 30 min at 85 °C to inactivate the enzymes. RFLP products were visualised with ethidium bromide using UV transillumination after electrophoresis through a 1.5% agarose gel.

3. Results and discussion

Based on sequencing data from KSHV infected BC-1 and PK-1 cell lines, Zhang et al. (2000) characterised four possible RFLP subtypes; deletions in the IRD resulting in the loss of expected restriction sites created the different RFLP patterns. Thus, in the KSHV IRD of the BC-1 cell line, there are five possible *Ban*II restriction sites resulting in three bands that can be visualised and one *Mbo*I restriction site resulting in two visible bands. Subtype 1 samples have an IRD with both *Ban*II and *Mbo*I restriction sites, resulting in bands of 655, 476/457 and 192 bp in length. Subtype 2 samples have at least two *Ban*II sites but no *Mbo*I site, resulting in three visible bands at $\approx$1121, 457 and 192 bp. There is only one *Ban*II site in subtype 3 samples, resulting in two bands at 1131 and 457 bp, while subtype 4 has one of each restriction site, resulting in three visible bands with the 192-bp band missing.

Previously, no correlation has been demonstrated between these four RFLP subtypes and the previously described KSHV subtypes (A–C) based on sequencing of the KS330 fragment of ORF 26 (Zhang et al., 2000). ORF K1 sequencing revealed that the Malawian KSHV samples belong to either the A5 or B1 subtype (Cook et al., 2002). No correlation was found between these two ORF K1 subtypes and the RFLP subtypes 2 and 3 recovered in this sample group (Table 1).

Zhang et al. (2000) found that RFLP patterns from multifocal Kaposi's sarcoma lesions in a single patient were invariant. This study of Malawian individuals identified one patient with Kaposi's sarcoma, K_i, from whom KSHV DNA could be amplified in both blood and mouth rinse samples. In this patient, the two samples revealed an identical RFLP pattern, indicating that the same KSHV variant is carried in both the blood and the oral compartments. However, samples from only one subject were available for this analysis and the findings should be interpreted with caution.

It was possible to compare RFLP patterns between two or more family members in eight of the family groups. Five additional samples with no matching family members were also included in the RFLP analysis to determine the overall distribution of subtypes present in this population. The results of the single round or nested PCR to amplify the IRD region of ORF 73 are shown in Fig. 1. The previously described KSHV nuclear antigen typing (KVNA typing) procedure (Gao et

al., 1999) could be applied directly to these samples and we detected size polymorphism in the IRD between samples in families B, G and W. However, the IRD products for all other families appeared identical.

RFLP analysis of all samples is represented in Fig. 2. Distinct RFLP patterns could be differentiated in the samples from Malawian individuals, allowing us to make comparisons between family members. In four families (A, E, H, K) identical RFLP patterns were present suggesting familial transmission of KSHV had occurred in these family groups. In families A and K, identical RFLP results were found between a mother ($A1/K_i$) and her children, a son ($A2/K_1$) and daughter (A_i). Identity could also be found between siblings; brothers E6 and H2 carried the same RFLP patterns as their corresponding sisters, E2 and H1.

In families B, G, E, T and W, non-identical RFLP patterns were discovered. In two of these families (T and G), subtypes 2 and 3 existed in each family, while in families B, E and W, all samples were of the same subtype but with differing RFLP band sizes. Studied in family B were

Table 1
Characteristics of the patients with Kaposi's sarcoma and family members involved in the study

Individual	Kaposi's sarcoma (Yes/No)	Relationship	Age/sex	ORF K1 genotype	PCR-RFLP subtype
A_i (blood)	Yes	Daughter	7y/F	B	2
A1	No	Mother	34y/F	N/A*	2
A2	No	Son	10y/M	N/A*	2
B3	No	Brother	12y/M	B1	2
B5	No	Brother	7y/M	B1	2
E4	No	Sister	6y/F	B1	3
E5	No	Brother	13y/M	B1	3
E6	No	Brother	10y/M	B1	3
E2	No	Sister	9y/F	N/A*	3
G1	No	Father	32y/M	A5	2
G2	No	Son	23y/M	A5	3
G_i (blood)	Yes	Mother	34y/F	N/A*	3
H1	No	Sister	13y/F	N/A*	3
H2	No	Brother	12y/M	N/A*	3
K_i, K_i(blood)	Yes	Mother	30y/F	A5	2
K1	No	Son	12y/M	A5	2
T2	No	Brother	11y/M	B1	3
T3	No	Brother	9y/M	B1	2
W2	No	Brother	20y/M	B1	3
W4	No	Sister	13y/F	B1	3
C_i	Yes	Son	8y/M	B1	3
D1	No	Daughter	18/F	A5	2
P_i	Yes	Father	30y/M	N/A*	2
X1	No	Father	44y/M	B1	3
Z2	No	Mother	22y/F	A5	2

All samples are derived from mouth rinses unless otherwise noted. ORF K1 genotype assignments were determined by sequence alignment with sequences of known subtype deposited in GenBank and by the presence of characteristic amino acid motifs in the VR1 and VR2 region of ORF K1 (Zong et al., 1999, 2002). PCR-RFLP subtype assignment was as determined by Zhang et al. (2000).

* Denotes ORF K1 DNA could not be amplified from these samples (Cook et al., 2002).

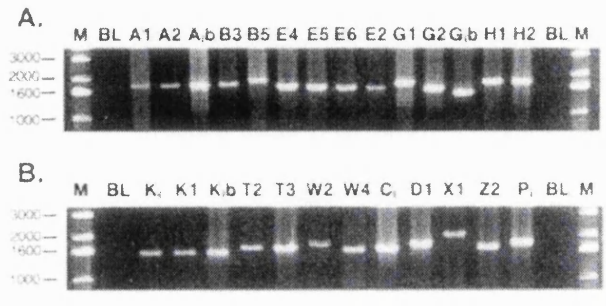

Fig. 1. ORF 73 IRD PCR results from (A) samples A1–H2 and (B) samples K_i–P_i. Lanes marked BL correspond to blank lanes. Samples were amplified using either single round or nested PCR and were visualised on a 1% agarose gel alongside a 1 Kb DNA marker.

brothers (B3 and B5), in family T, brothers (T2 and T3), in family E, a sister (E4) and her brothers (E2, E5 and E6), and in family G, a father (G1), a mother (G) and their son (G2). In family E, RFLP patterns from siblings E4 and E5 differed not only from each other, but also from those of their siblings E2 and E6.

The dissimilar RFLP patterns recovered from each of these family members indicates that they may not have acquired KSHV from each other. Nevertheless, as the subjects studied here were part of a larger immediate family from whom

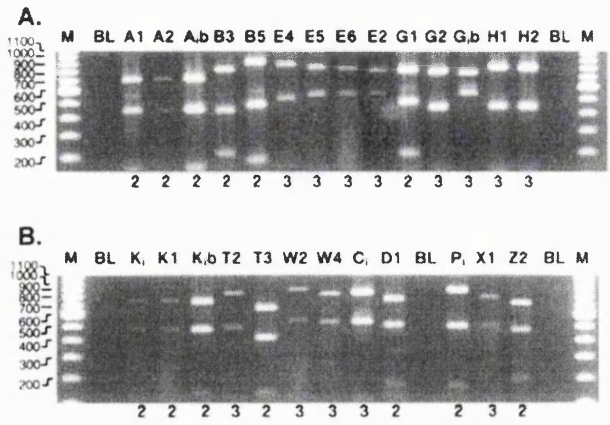

Fig. 2. PCR-RFLP analysis of the ORF 73 IRD PCR products from (A) samples A1–H2 and (B) samples K_i–P_i. PCR-RFLP subtype assignments are indicated beneath each lane. Lanes marked BL correspond to blank lanes. Samples were digested using *Mbo*I and *Ban*II restriction enzymes and visualised on a 1.5% agarose gel alongside a 100 bp ladder DNA marker to estimate fragment size.

KSHV DNA could not be amplified (Cook et al., 2002) it cannot be determined whether or not they were infected by other family members. However, a comparison could be made between previously obtained sequencing data (Cook et al., 2002) from the first variable region of the hypervariable ORF K1 and RFLP patterns for six of the eight families in this study (B, E, G, K, T, W). RFLP analysis corroborated with ORF K1 sequence identity found in families E and K and non-identity found in families B, E, T and W. Previous evidence of ORF K1 sequence identity was not confirmed in family G by RFLP (Fig. 2).

In all families except family G and W (W2), the children were 13 years of age or younger, consistent with KSHV acquisition through a non-sexual route. In Africa, where the predominant mode of KSHV transmission is non-sexual, saliva is likely to be an important vehicle. Saliva has been detected at high titre in American homosexual men with and without Kaposi's sarcoma (Koelle et al., 1997) and in mouth rinses from Zimbabwean women with Kaposi's sarcoma (Lampinen et al., 2000). Of the samples included in this study, 23 of 26 were mouth rinses and all but three of these were derived from family members without Kaposi's sarcoma. The PCR-RFLP assay was able to reveal size and sequence polymorphism in these mouth rinse samples from individuals without Kaposi's sarcoma. Subtyping through such a PCR-RFLP approach should enable KSHV transmission in other endemic populations to be similarly studied.

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